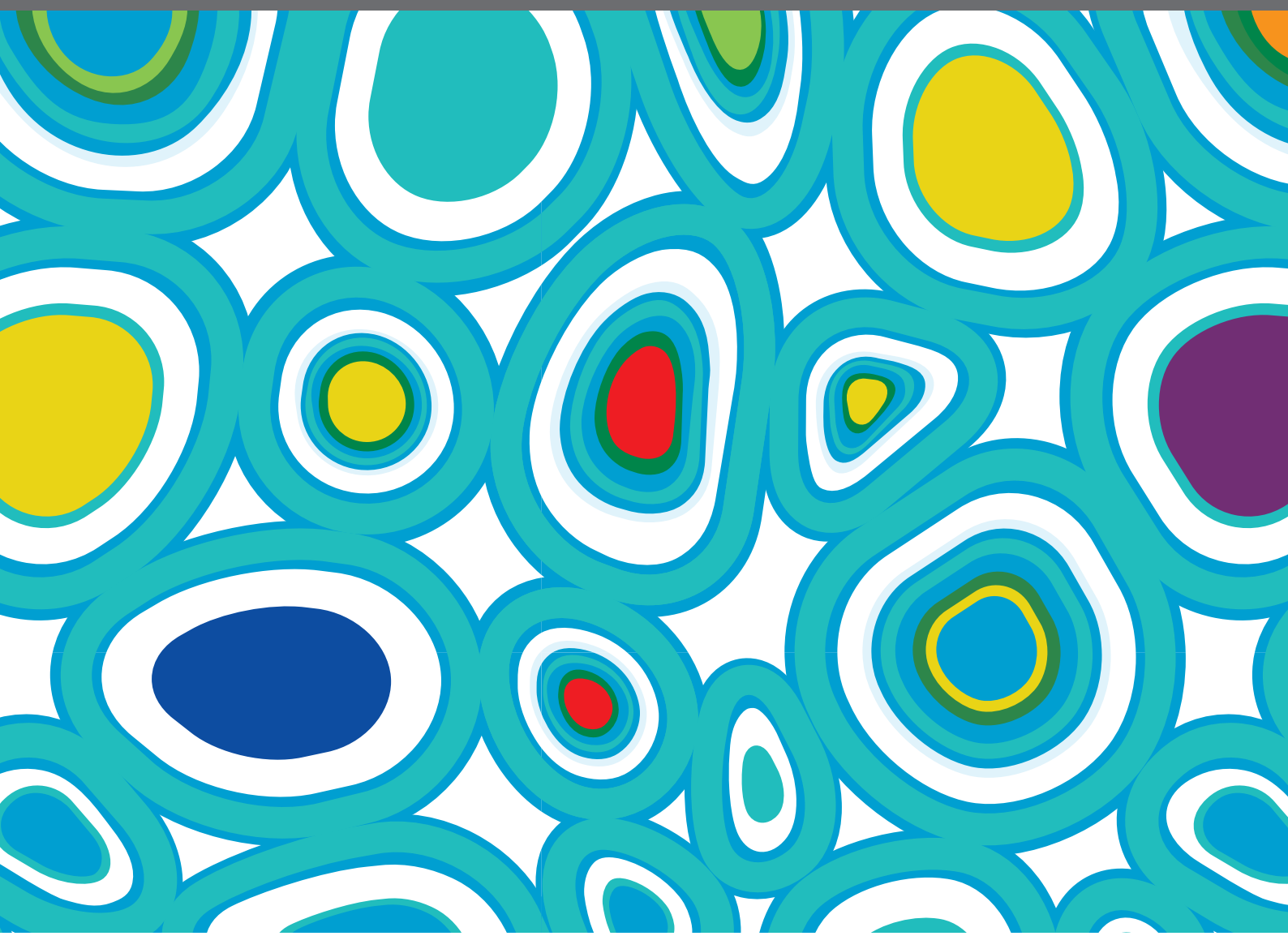
TUMOR VESSELS AS DIRECTORS OF THE TUMOR MICROENVIRONMENT: NEW FINDINGS, CURRENT CHALLENGES & PERSPECTIVES

EDITED BY: Lucas Treps, Ann Ager, Kyoko Hida and Kairbaan Hodivala-Dilke

PUBLISHED IN: Frontiers in Cell and Developmental Biology and Frontiers in Oncology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-966-9
DOI 10.3389/978-2-88974-966-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

TUMOR VESSELS AS DIRECTORS OF THE TUMOR MICROENVIRONMENT: NEW FINDINGS, CURRENT CHALLENGES & PERSPECTIVES

Topic Editors:
Lucas Treps, INSERM U1232 Centre de Recherche en Cancérologie et Immunologie Nantes Angers (CRCINA), France
Ann Ager, Cardiff University, United Kingdom
Kyoko Hida, Hokkaido University, Japan
Kairbaan Hodivala-Dilke, Queen Mary University of London, United Kingdom

Citation: Treps, L., Ager, A., Hida, K., Hodivala-Dilke, K., eds. (2022). Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges & Perspectives. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-966-9

Table of Contents

frontiers in Cell and Developmental Biology

EDITORIAL
published: 29 March 2022
doi: 10.3389/fcell.2022.885670

Editorial: Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges & Perspectives

Lucas Treps[1]*, *Ann Ager*[2] *and Kyoko Hida*[3]

[1]Nantes Université, Inserm UMR 1307, CNRS UMR 6075, Université d'Angers, CRCI2NA, Nantes, France, [2]Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom, [3]Vascular Biology and Molecular Biology, Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan

Keywords: tumor endothelial cell, angiogenesis, tumor microenvironment, extracellular vesicles, anti-tumor immunity, immunotherapy, anti-angiogenic therapies

Editorial on the Research Topic

Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges

INTRODUCTION

Half a century ago, Judah Folkman postulated that solid tumors rely on the formation of new blood vessels (angiogenesis) from pre-existing established vessels for appropriate nutrient and oxygen supply (Folkman, 1971). Accordingly, tumor spheroids infiltrated with new capillaries would exponentially grow, while avascular tumors remain dormant. This original premise was the cornerstone for the development of anti-angiogenic therapies, aiming at starving and asphyxiating the tumor to death by destroying tumor blood vessels. Since that time, intense research has been conducted to identify angiogenic factors controlling tumor angiogenesis. Several pro-angiogenic cues were discovered, including the vascular endothelial growth factor (VEGF) first characterized by the Dvorak laboratory (Senger et al., 1983). VEGF is probably one of the most notable angiogenic factors, and in the late 90's, nearly a decade after its discovery, a humanized monoclonal neutralizing antibody was developed by the team of Napoleone Ferrara which shows promising anti-tumor effects (Presta et al., 1997). This was a real breakthrough and paved the way for the development of numerous anti-angiogenic strategies that are now approved in clinics for eye diseases (i.e., wet age-related macular degeneration) and anti-cancer treatments. Even though such therapies improved patients' survival, they are still facing resistance mechanisms that partly results from our incomplete understanding of the molecular mechanisms and signaling pathways that govern tumor angiogenesis. Additionally, far from being incapable of genetic plasticity (as initially suggested by Folkman) tumor endothelial cells are heterogeneous by essence and harbor a plethora of phenotypes with varying sensitivity to anti-angiogenic therapies (Goveia et al., 2020). This complexity in tumor vessels also implies a richer variety of cell-cell interactions within the tumor microenvironment than anticipated.

This special research topic of "*Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges & Perspectives*" comprises seven original research articles and eleven review articles for a total of 18 original contributions. These articles cover many topics articulated around tumor vessels and scrutinize mechanisms of resistance to anti-angiogenic therapies, molecular signaling driving and regulating pathological angiogenesis, and recent approaches

OPEN ACCESS

Edited and reviewed by:
Shyamala Maheswaran, Massachusetts General Hospital and Harvard Medical School, United States

***Correspondence:**
Lucas Treps
lucas.treps@univ-nantes.fr

Specialty section:
This article was submitted to Cancer Cell Biology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 February 2022*
Accepted: *15 March 2022*
Published: *29 March 2022*

Citation:
Treps L, Ager A and Hida K (2022) Editorial: Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges & Perspectives. Front. Cell Dev. Biol. 10:885670. doi: 10.3389/fcell.2022.885670

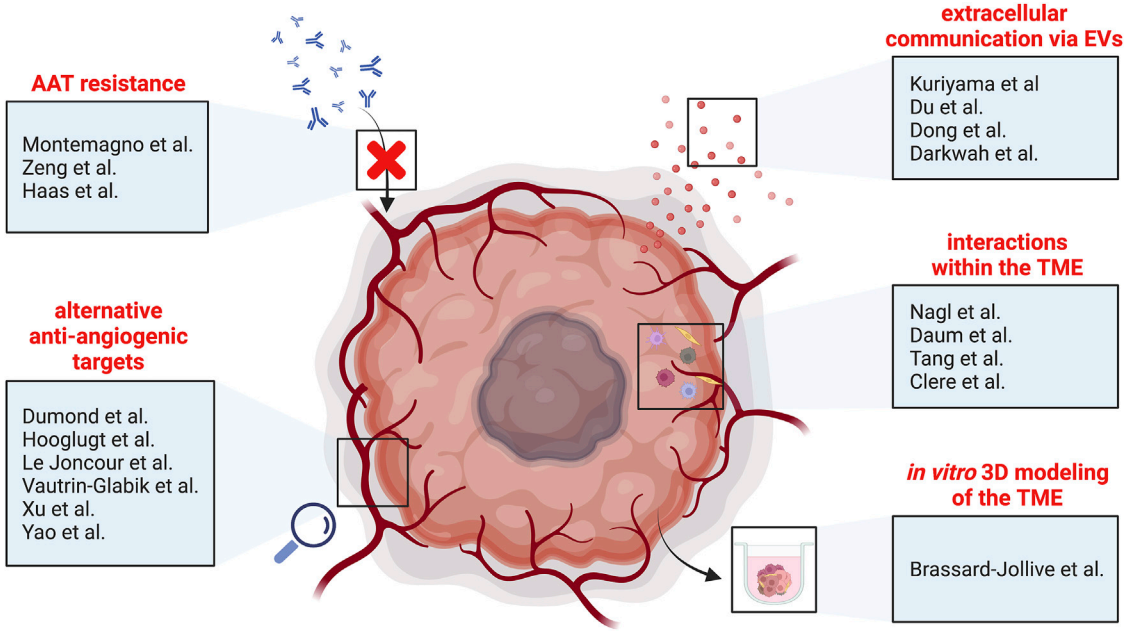


FIGURE 1 | Schematic overview of the different topics covered by this special research topic of "*Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges & Perspectives*". AAT, anti-angiogenic therapy; EVs, extracellular vesicles; TME, tumor microenvironment.

modulating tumor vessels and their micro-environment (**Figure 1**). We will discuss these aspects in this editorial and hope to provide insights into the complexity of tumor vessel biology.

THE WIDESPREAD DISSIMILARITIES BETWEEN NORMAL AND TUMOR ENDOTHELIAL CELLS

A striking difference between normal and tumor vessels is morphology - the former exhibit a smooth lining of endothelial cells (ECs) that are mature and hierarchically organized, while the latter show irregular and tortuous endothelium, with higher permeability and impaired perfusion (**Figure 1**). In addition, it has been reported that tumor endothelial cells (TECs) are different from normal endothelial cells (NECs) in many aspects such as morphology, molecular, transcriptomic, metabolic profiles, and cytogenetically. Technological breakthroughs from the last decade with single cell transcriptomics (scRNA-seq), has unveiled the wide diversity of cellular and molecular composition of the tumor microenvironment (TME). As such, recent studies have shown that TECs are composed of heterogeneous populations/phenotypes and adapt their traits in response to the TME (e.g., hypoxia or reactive oxygen species). Also, surrounding tumor cells, cancer-associated fibroblasts (CAFs) and immune cells (to name a few) affect TECs. Vice versa, TEC may regulate and shape the TME by releasing angiocrine factors (Maishi et al., 2019). TECs foster tumor progression and metastasis, and even induce therapy resistance by alteration of their molecular signature during tumor progression or anti-cancer treatments (anti-angiogenic, immunotherapy). Nagl et al. provide an overview of the studies on TECs and their interactions within the TME. They focus on the role of TECs as immune regulators within their cellular habitat. Indeed, TECs can affect: 1) immune cell migration and priming, 2) T cell activation and apoptosis (e.g., by upregulating FAS-L), and 3) antigen presentation. For instance, ECs in non-cancerous tissue can express major histocompatibility complex (MHC) class I and II molecules, enabling them to present processed antigens to T cells. However, TECs downregulate genes that are responsible for MHC expression and show suppressed antigen-presenting functions (Goveia et al., 2020; Lambrechts et al., 2018; Li et al., 2021). Thus, TECs may contribute to tumor immune evasion and may influence responses to immunotherapies.

RESISTANCE TO ANTI-ANGIOGENIC THERAPIES

As a spearhead into the therapeutic armory, bevacizumab was the first anti-angiogenic treatment (AAT) approved for metastatic colorectal cancer as a first line treatment combined with chemotherapy (Hurwitz et al., 2004). Thereafter, an armamentarium of molecules was developed to target pro-angiogenic cues (e.g., VEGF, FGF, PDGF) and their cognate receptors. Although some tumors display sensitivity to AATs, several cancer entities remain resilient to such treatments and demonstrated limited impact on overall survival. Intrinsic and acquired resistance are deemed to be responsible for such failures in therapeutic response. Montemagno et al. provide an interesting

update on AAT resistance, by distinguishing early-stage resistance, mostly involving tumor cells with, for example, the upregulation of genes involved in angiogenic redundancy, to late-stage resistance related to the adaptation of the tumor microenvironment. In this regard, Daum et al. put forward a comprehensive overview about how highly adaptive the tumor microenvironment can be in non-small cell lung cancer. Along with these new insights, they present combinational approaches including chemotherapy, anti-angiogenic and immunotherapy which could be developed to yield a more target-oriented anti-tumor treatment. Once the tumor has disseminated throughout the body, micro-metastases can remain dormant and thus unresponsive to anti-angiogenic therapy. In their mini review article, Zeng et al. provide a concise overview of the resistance mechanisms developed by the tumor to counteract AAT. Haas et al. review another means by which micro-metastases stay beyond the reach of these drugs, namely vessel co-option whereby cancer cells hijack pre-existing abundant vasculature of the host organ (e.g., liver, lung) without the need for angiogenesis. Unexpectedly, recent pre-clinical evidence highlights the fact that co-opted vessels share transcriptomic similarities with quiescent ECs in healthy vessels and are nearly devoid of angiogenic tip and proliferating ECs, thus potentially explaining unresponsiveness to AAT (Teuwen et al., 2021).

ALTERNATIVE APPROACHES AND NEW TARGETS TO ATTACK TUMOR VESSELS

In 2001, Rakesh Jain hypothesized that AATs could be used to normalize, rather than prune and destroy tumor blood vessels (Jain, 2001). The rationale behind this paradigm was to heal tumor blood vessels, in order to improve oxygenation for successful tumor treatment and thereby: 1) breaking the vicious cycle of hypoxia-mediated tumor progression; 2) improving drug delivery, radiation- and immunotherapy efficacy (Fukumura et al., 2018); and 3) recruiting more immune cells to the tumor. One way to achieve tumor vessel normalization is by fine-tuning the dose and timing of anti-VEGF/R AATs during the window of normalization. Other promising targets include the Neuropilins, which act as receptors for the semaphorins but also as VEGF co-receptors, and are important for EC function (Treps et al., 2013), to modulate tumor permeability (Treps et al., 2016) and vessel normalization (Casazza et al., 2011). Dumond et al. overview the function of Neuropilins and their multifaceted contributions to the TME, and highlight their relevance in anti-tumor targeting. Among others, YAP/TAZ are critical regulators of developmental angiogenesis. Hooglugt et al. present an appealing review on the role of YAP/TAZ signaling as another pathway amenable to induce tumor vessel formation and cancer cell growth via oncogenic activation. In that regard, the STAT3/YAP/TAZ signaling was recently described to be critical in promoting tumor vascularization in human colorectal carcinomas and skin melanoma (Shen et al., 2021).

A better comprehension of the mechanisms driving angiogenesis is a pre-requisite to improve AATs. As such, this research topic contributed to this never-ending goal with Le Joncour et al. who investigated the role of the vasoactive peptide urotensin II and its receptor urotensin on glioblastoma angiogenesis. Interestingly, in preclinical models of glioblastoma, urotensin antagonist and biased urotensin ligand were able to significantly delay tumor growth, and evoke strong angiogenic activity through targeting integrin activation. Vautrin-Glabik et al. demonstrated the anti-angiogenic activity of a 13 amino acid sequence (dubbed QS-13) derived from the type IV collagen, presumably through its binding to α5β1 integrin onto the surface of ECs. Long non-coding RNA (LncRNA) can function as a sponge to counteract endogenous RNA including micro-RNA (miR). As such, Xu et al. described the function of the LncRNA NEAT1 in the inhibition of the miR-17-5p/TGFβR2 axis in gastric cancer lines, thereby leading to the secretion of pro-angiogenic factors in the milieu. Yao et al. showed that miR-9-induced angiogenesis occurred via targeting the sphingosine-1-phosphate receptor.

TUMOR VESSELS AND THEIR CELLULAR MICROENVIRONMENT

Tumor vessels reside within a complex mixture of immune cells, fibroblasts and matrix molecules which together constitute the TME. The exact composition and structural organization of the TME depends on type and stage of cancer and is regulated either directly or indirectly by TECs. The recent breakthrough in cancer therapy using immune checkpoint blockade inhibitors has revealed the dominant role of immune escape in supporting cancer progression. Moreover, the success of immunotherapy has been linked to a pre-existing $CD8^+$ T-cell rich and/or $Foxp3^+$ Treg poor TME as well as the presence of tertiary lymphoid structures (Bruni et al., 2020). As mentioned above, TECs regulate the recruitment of distinct immune cell subsets and therefore shape the immune cell signature of the TME. Chemokine synthesis by tumor cells will also control immune cell infiltration of the TME. In this collection Tang et al. reveal a role for the transcription factor Twist1 in regulating CCL2 generation by colorectal cancer cells in the recruitment of macrophages. Interestingly, the transcription factor Forkhead Box Q1 (FOXQ1) controlled Twist1 expression by colorectal cancer cells. FOXQ1 expression by colorectal cancer cells also controlled the balance of angiogenic and angiostatic factors generated thereby directly impacting TECs in the TME.

CAFs are a heterogeneous, poorly understood cell population in the TME with a range of pro-tumoral functions. Evidence that CAFs can be generated from TECs and the signaling pathways involved in this endothelial-to-mesenchymal transition (EndMT) are reviewed by Clere et al. Interestingly, EndMT is accompanied by the development of multiple therapeutic resistance mechanisms and other pro-tumoral functions such as cancer cell extravasation and sprouting angiogenesis. Studying angiogenesis, vasculogenesis, EndMT and the functional properties of tumor blood vessels in the complexity of the TME is very challenging. Brassard-Jollive et al. comprehensively review the limitations of established 3D *in vitro* models of tube formation. New approaches using EC

grown on beads and co-cultured with defined components of the TME such as tumor cells and fibroblasts within 3D hydrogels containing defined ECM components are described. However, significant challenges remain to develop 3D models of tumor blood vessels within a TME for high throughput screening required for drug testing. Another limitation is the use of non-tumor sources of EC due to the difficulties of working with isolated TECs, which could potentially preclude clinical translation.

TUMOR VESSELS EXTRACELLULAR COMMUNICATION

Since their discovery 2 decades ago, the generic term of extracellular vesicles (EVs) has been acknowledged and encompasses a large variety of appellations such as exosomes, microvesicles, microparticles, etc. Driven by a vibrant research community (International Society for Extracellular Vesicles, ISEV), it is now well established that EVs are involved at various steps of tumor development, cell-cell communication with tumor microenvironment, and anti-cancer therapy resistance. Kuriyama et al. present a comprehensive review on the involvement of EVs in tumor vascular-related cancer progression and their contribution to AAT resistance mediated by vasculogenic mimicry. Vasculogenic mimicry is a process whereby aggressive cancer cells form *de novo* vascular networks that are associated with malignant phenotype (Treps et al., 2021). Du et al. analyzed the expression of 5-methylcytosine regulators and DNA methylation-driven genes in EVs and tissue samples in order to identify a risk signature for colon cancer. Interestingly, Dong et al. identified by micro-array analysis of EVs miR-3682-3p as repressed in highly metastatic hepatocellular carcinoma cells and shown to impair angiogenesis by targeting ANGPT1. It is worth mentioning that EVs should not always be presented as the villains during cancer. Indeed, Darkwah et al. presented muscle derived-EVs and point to their potentially protective roles in cancer progression and metastasis.

SUMMARY AND PROSPECTIVE

Initially uncovered upon characterization of ECs from different vascular beds, endothelial heterogeneity is now acknowledged in a broad variety of pathologies including cancers, whereby various phenotypes of TECs have a myriad of possible cell-cell interactions. The complexity and dynamics of these interplays are challenging to study in the patient either using resections or biopsies. However, the advent of scRNA-seq, multiplexed imaging, spatially resolved sequencing technologies and computational strategies inferring cell–cell interactions have opened novel avenues in deciphering communications occurring within the TME.

The immunological properties of ECs is a topic under intense investigations and may offer enthralling discoveries that could be used to tailor existing anti-cancer treatments. For example, a recent pre-clinical study has shown that the secreted leucine-rich α-2-glycoprotein 1 (LRG1) is induced in TECs from lung and melanoma cancer mouse models, but also in particular human biopsies, and that LRG1 function-blocking antibody treatment results in tumor vessel normalization. Moreover, LRG1-blocking antibody combined with cisplatin chemotherapy, adoptive T cell therapy, or anti-PD1 immune checkpoint inhibition monotherapy could enhance the efficacy of these therapies (O'Connor et al., 2021). Seemingly, combination approaches using AAT and anti-PD-L1 therapy showed improved efficiency by counteracting therapy-induced adaptive immunosuppressive pathways, by enhancing the tumor infiltration of T cell via promoting high endothelial venule (HEV) formation (Allen et al., 2017). Indeed, tumor-associated HEVs represent the main site of lymphocytes extravasation upon combined anti-PD-1/anti-CTLA-4 immunotherapy, and it was recently uncovered that LTßR-mediated HEV maturation is crucial to improve the efficacy of these immunotherapies (Asrir et al., 2022). Different targeted approaches meant to foster tumor HEV generation led to promising reinforced efficacy of combination therapy in several cancer entities including pancreatic neuroendocrine tumors (Johansson-Percival et al., 2017), glioblastoma (He et al., 2018), lung metastases (He et al., 2020) but would now require clinical translation. Although, the heterogeneity of HEV from adult mouse peripheral lymph nodes has been characterized in homeostasis and inflammation (Veerman et al., 2019), the tumor HEV heterogeneity calls for in depth scrutiny to better understand how these structures are formed, and how they could be reliably induced to fight anti-tumor immunosuppression and therapy resistance mechanisms.

AUTHOR CONTRIBUTIONS

LT wrote the editorial. KH and AA made substantial contribution to the writing of this editorial. The authors have approved it for publication.

FUNDING

LT has been supported by the I-SITE NExT Junior Talent, and the fonds européen de développement régional (FEDER). AA has been supported by Cancer Research United Kingdom grant C42412/A24416 and Health and Care Research Wales grant CA05.

ACKNOWLEDGMENTS

We want to thank all reviewers for their insightful comments on manuscripts in this Research Topic. We also compliment all the authors for their work and contributions with high quality manuscripts. Figure created with BioRender.com.

REFERENCES

Allen, E., Jabouille, A., Rivera, L. B., Lodewijckx, I., Missiaen, R., Steri, V., et al. (2017). Combined Antiangiogenic and Anti-PD-L1 Therapy Stimulates Tumor Immunity through HEV Formation. *Sci. Transl. Med.* 9 (385). doi:10.1126/scitranslmed.aak9679

Asrir, A., Tardiveau, C., Coudert, J., Laffont, R., Blanchard, L., Bellard, E., et al. (2022). Tumor-associated High Endothelial Venules Mediate Lymphocyte Entry into Tumors and Predict Response to PD-1 Plus CTLA-4 Combination Immunotherapy. *Cancer Cell* 40, 318–334. doi:10.1016/j.ccell.2022.01.002

Bruni, D., Angell, H. K., and Galon, J. (2020). The Immune Contexture and Immunoscore in Cancer Prognosis and Therapeutic Efficacy. *Nat. Rev. Cancer* 20 (11), 662–680. doi:10.1038/s41568-020-0285-7

Casazza, A., Fu, X., Johansson, I., Capparuccia, L., Andersson, F., Giustacchini, A., et al. (2011). Systemic and Targeted Delivery of Semaphorin 3A Inhibits Tumor Angiogenesis and Progression in Mouse Tumor Models. *Arterioscler Thromb. Vasc. Biol.* 31 (4), 741–749. doi:10.1161/ATVBAHA.110.211920

Fukumura, D., Kloepper, J., Amoozgar, Z., Duda, D. G., and Jain, R. K. (2018). Enhancing Cancer Immunotherapy Using Antiangiogenics: Opportunities and Challenges. *Nat. Rev. Clin. Oncol.* 15 (5), 325–340. doi:10.1038/nrclinonc.2018.29

Goveia, J., Rohlenova, K., Taverna, F., Treps, L., Conradi, L.-C., Pircher, A., et al. (2020). An Integrated Gene Expression Landscape Profiling Approach to Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates. *Cancer Cell* 37 (1), 21–36. e13. doi:10.1016/j.ccell.2019.12.001

He, B., Jabouille, A., Steri, V., Johansson-Percival, A., Michael, I. P., Kotamraju, V. R., et al. (2018). Vascular Targeting of LIGHT Normalizes Blood Vessels in Primary Brain Cancer and Induces Intratumoural High Endothelial Venules. *J. Pathol.* 245 (2), 209–221. doi:10.1002/path.5080

He, B., Johansson-Percival, A., Backhouse, J., Li, J., Lee, G. Y. F., Hamzah, J., et al. (2020). Remodeling of Metastatic Vasculature Reduces Lung Colonization and Sensitizes Overt Metastases to Immunotherapy. *Cell Rep.* 30 (3), 714–724. e715. doi:10.1016/j.celrep.2019.12.013

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., et al. (2004). Bevacizumab Plus Irinotecan, Fluorouracil, and Leucovorin for Metastatic Colorectal Cancer. *N. Engl. J. Med.* 350 (23), 2335–2342. doi:10.1056/NEJMoa032691

Jain, R. K. (2001). Normalizing Tumor Vasculature with Anti-angiogenic Therapy: a New Paradigm for Combination Therapy. *Nat. Med.* 7 (9), 987–989. doi:10.1038/nm0901-987

Johansson-Percival, A., He, B., Li, Z.-J., Kjellén, A., Russell, K., Li, J., et al. (2017). De Novo induction of Intratumoral Lymphoid Structures and Vessel Normalization Enhances Immunotherapy in Resistant Tumors. *Nat. Immunol.* 18 (11), 1207–1217. doi:10.1038/ni.3836

Lambrechts, D., Wauters, E., Boeckx, B., Aibar, S., Nittner, D., Burton, O., et al. (2018). Phenotype Molding of Stromal Cells in the Lung Tumor Microenvironment. *Nat. Med.* 24 (8), 1277–1289. doi:10.1038/s41591-018-0096-5

Li, C., Guo, L., Li, S., and Hua, K. (2021). Single-cell Transcriptomics Reveals the Landscape of Intra-tumoral Heterogeneity and Transcriptional Activities of ECs in CC. *Mol. Ther. - Nucleic Acids* 24, 682–694. doi:10.1016/j.omtn.2021.03.017

Maishi, N., Annan, D. A., Kikuchi, H., Hida, Y., and Hida, K. (2019). Tumor Endothelial Heterogeneity in Cancer Progression. *Cancers* 11 (10), 1511. doi:10.3390/cancers11101511

O'Connor, M. N., Kallenberg, D. M., Camilli, C., Pilotti, C., Dritsoula, A., Jackstadt, R., et al. (2021). LRG1 Destabilizes Tumor Vessels and Restricts Immunotherapeutic Potency. *Med* 2 (11), 1231–1252. e1210. doi:10.1016/j.medj.2021.10.002

Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., et al. (1997). Humanization of an Anti-vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors and Other Disorders. *Cancer Res.* 57 (20), 4593–4599.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983). Tumor Cells Secrete a Vascular Permeability Factor that Promotes Accumulation of Ascites Fluid. *Science* 219 (4587), 983–985. doi:10.1126/science.6823562

Shen, Y., Wang, X., Liu, Y., Singhal, M., Gürkaşlar, C., Valls, A. F., et al. (2021). STAT3-YAP/TAZ Signaling in Endothelial Cells Promotes Tumor Angiogenesis. *Sci. Signal.* 14 (712), eabj8393. doi:10.1126/scisignal.abj8393

Sherwood, L. M., Parris, E. E., and Folkman, J. (1971). Tumor Angiogenesis: Therapeutic Implications. *N. Engl. J. Med.* 285 (21), 1182–1186. doi:10.1056/NEJM197111182852108

Teuwen, L.-A., De Rooij, L. P. M. H., Cuypers, A., Rohlenova, K., Dumas, S. J., García-Caballero, M., et al. (2021). Tumor Vessel Co-option Probed by Single-Cell Analysis. *Cell Rep.* 35 (11), 109253. doi:10.1016/j.celrep.2021.109253

Treps, L., Clere, N., and Faure, S. (2021). Vasculogenic Mimicry, a Complex and Devious Process Favor-Ing Tumorigenesis – Interest in Making it a Therapeutic Target. *Pharmacol. Ther.* 223, 107805. doi:10.1016/j.pharmthera.2021.107805

Treps, L., Edmond, S., Harford-Wright, E., Galan-Moya, E. M., Schmitt, A., Azzi, S., et al. (2016). Extracellular Vesicle-Transported Semaphorin3A Promotes Vascular Permeability in Glioblastoma. *Oncogene* 35 (20), 2615–2623. doi:10.1038/onc.2015.317

Treps, L., Le Guelte, A., and Gavard, J. (2013). Emerging Roles of Semaphorins in the Regulation of Epithelial and Endothelial Junctions. *Tissue Barriers* 1 (1), e23272. doi:10.4161/tisb.23272

Veerman, K., Tardiveau, C., Martins, F., Coudert, J., and Girard, J.-P. (2019). Single-Cell Analysis Reveals Heterogeneity of High Endothelial Venules and Different Regulation of Genes Controlling Lymphocyte Entry to Lymph Nodes. *Cell Rep.* 26 (11), 3116–3131. e3115. doi:10.1016/j.celrep.2019.02.042

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 07 July 2020
doi: 10.3389/fcell.2020.00584

Resistance to Anti-angiogenic Therapies: A Mechanism Depending on the Time of Exposure to the Drugs

Christopher Montemagno[1,2,3]* *and Gilles Pagès*[1,2,3]*

[1] *Département de Biologie Médicale, Centre Scientifique de Monaco, Monaco, Monaco,* [2] *CNRS UMR 7284, Institute for Research on Cancer and Aging of Nice, Université Côte d'Azur, Nice, France,* [3] *INSERM U1081, Centre Antoine Lacassagne, Nice, France*

Angiogenesis, the formation of new blood vessels from preexisting one, represents a critical process for oxygen and nutrient supply to proliferating cells, therefore promoting tumor growth and metastasis. The Vascular Endothelial Growth Factor (VEGF) pathway is one of the key mediators of angiogenesis in cancer. Therefore, several therapies including monoclonal antibodies or tyrosine kinase inhibitors target this axis. Although preclinical studies demonstrated strong antitumor activity, clinical studies were disappointing. Antiangiogenic drugs, used to treat metastatic patients suffering of different types of cancers, prolonged survival to different extents but are not curative. In this review, we focused on different mechanisms involved in resistance to antiangiogenic therapies from early stage resistance involving mainly tumor cells to late stages related to the adaptation of the microenvironment.

Keywords: VEGFA, anti-angiogenic treatments, resistance, tumor microenvironment, combined therapies

OPEN ACCESS

Edited by:
Lucas Treps,
VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Ali Ibrahim Shamseddine,
American University of Beirut, Lebanon
Andreas Pircher,
Innsbruck Medical University, Austria

***Correspondence:**
Christopher Montemagno
cmontemagno@centrescientifique.mc
Gilles Pagès
gpages@unice.fr

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *17 April 2020*
Accepted: *16 June 2020*
Published: *07 July 2020*

Citation:
Montemagno C and Pagès G (2020) Resistance to Anti-angiogenic Therapies: A Mechanism Depending on the Time of Exposure to the Drugs. Front. Cell Dev. Biol. 8:584. doi: 10.3389/fcell.2020.00584

INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing ones (Hanahan and Folkman, 1996). It is a crucial physiological process that occurs throughout the life time, from the embryo to establish an adequate vasculature for growing and developing organs, to adults during wound healing or ovarian cycle (Folkman and Shing, 1992; Wilting and Christ, 1996; Hazzard and Stouffer, 2000; Tonnesen et al., 2000). Angiogenesis is tightly regulated and disruption of any part of this process induces various disorders, such as psoriasis, diabetic retinopathy, and cancer (Nishida et al., 2006; Walsh, 2007; Crawford et al., 2009; Heidenreich et al., 2009). Angiogenesis involves migration, proliferation and differentiation of endothelial cells (ECs). During the angiogenic cascade, stable vessels undergovascular permeability and a basement membrane degradation by the matrix-metalloproteases (MMPs) liberating extracellular matrix-sequestred growth factors. In response to these growth factors, ECs proliferate and migrate to assemble as lumen-bearing cords with branching structure (**Figure 1**; Bryan and D'Amore, 2007). Angiogenesis is a tightly balanced mechanism regulated by both pro-angiogenic and anti-angiogenic factors. In tumors, this balance shift toward pro-angiogenic factors sustaining angiogenesis.

One of the first relationships between angiogenesis and cancer was introduced 55 years ago when Ehrmann and Knoth (1968); Greenblatt and Shubi (1968), P highlighted for the first time, that tumors secrete substances targeting ECs that stimulate angiogenesis. Three years later, Judah Folkman observed that the growth of solid tumors relies on this process (Folkman, 1971, 1972). The newly formed vascular network supplies tumor with oxygen, nutrients and growth factors. Based

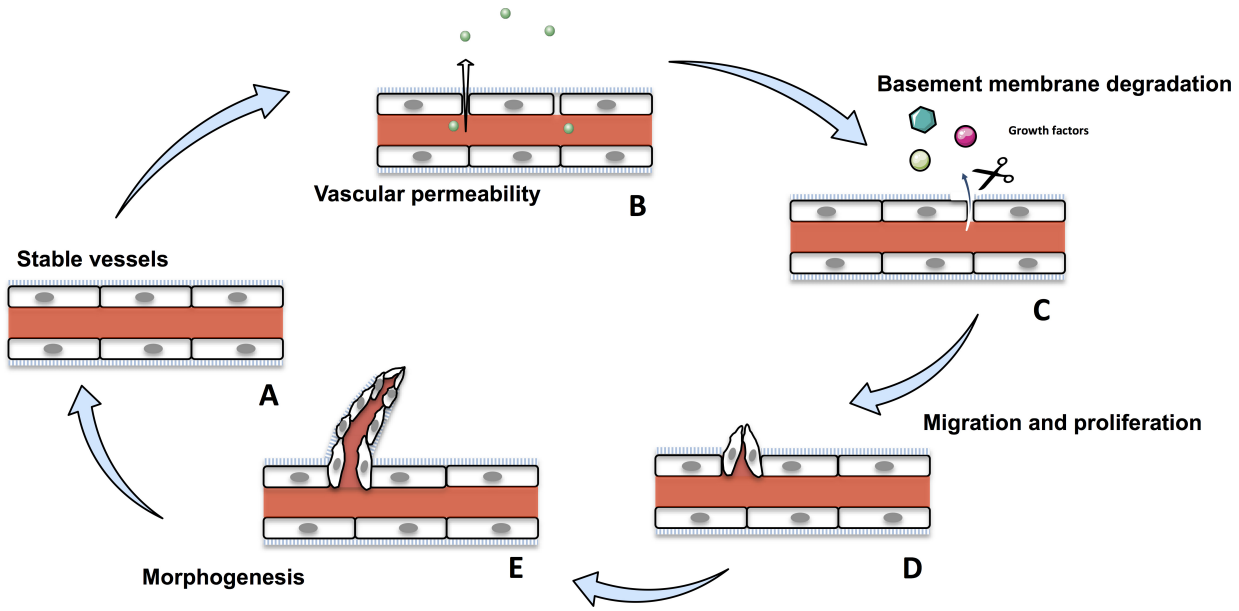


FIGURE 1 | The physiological angiogenic cascade. Stable vessels **(A)** undergo a vascular permeability allowing extravasation of plasma proteins **(B)**. MMPs degrate the extracellular matrix liberating growth factors **(C)**. ECs proliferate and migrate **(D)** and undergo morphogenesis and assemble as lumen-bearing cords **(E)**.

on these observations, Folkman proposed that inhibiting angiogenesis through ECs inhibition should constitute a promising anti-cancer treatment, by preventing nutrients supply and oxygen to tumors. This original concept stipulated that ECs are normal cells incapable of genetic plasticity as compared to tumor cells. Therefore, destruction of the blood vessel should have lead to tumor cell asphyxia and thereafter complete tumor regression. This concept was confirmed by the discovery of several angiogenic factors, such as transforming growth factor-α and β (TGF-α and TGF-β), angiopoietin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)A (Schreiber et al., 1986; Ferrara and Henzel, 1989; Levéen et al., 1994; van Cruijsen et al., 2005; van Meeteren et al., 2011; Fagiani and Christofori, 2013).

In 1989, the discovery of VEGFA, one of the most important angiogenic factors, by independent teams was a real breakthrough in understanding the mechanisms of angiogenesis (Keck et al., 1989; Leung et al., 1989; Guyot and Pagès, 2015). Four years later, the first monoclonal neutralizing antibody directed against VEGFA was described by the team of N. Ferrara, the winner of the Lasker Award few years laters for the use of these antibodies in eye pathologies especially wet age-related macular degeneration (Kim et al., 1993). This antibody inhibited the growth of experimental models of rhabdomyosarcoma, glioblastoma and colorectal and prostate cancers (Kim et al., 1993; Asano et al., 1995; Warren et al., 1995; Borgström et al., 1998). These promising anti-tumoral effects led to the development of bevacizumab (Avastin®), a humanized anti-VEGFA monoclonal antibody (Presta et al., 1997). Bevacizumab by specifically inhibiting the binding of VEGFA to its receptor VEGFR2 present on ECs, blocks signaling pathways involved in ECs proliferation and subsequently tumor angiogenesis (Shih and Lindley, 2006). In 2004, bevacizumab was approved by the Food and Drug Administration (FDA) as part of combination therapy for metastatic colorectal cancers (Hurwitz et al., 2004). Since 2008, bevacizumab was approved for the treatment of non-small-cell lung, breast, kidney and ovarian cancers in combination with standard chemotherapy (Sandler et al., 2006; Harshman and Srinivas, 2010; Russo et al., 2017). The strong competition in the field led to the development of alternative strategies to inhibit angiogenesis. Since VEGF receptors possess a tyrosine kinase domain, several companies developed small ATP mimetics to inhibit the activity of tyrosine kinases receptors involved in angiogenesis (Qin et al., 2019). Another strategy was designed to inhibit the activity of mTOR, a kinase activated in response to the stimulation of receptors (Faes et al., 2017). The inhibitor molecules sorafenib (Nexavar®), sunitinib (Sutent®), everolimus (Afinitor®), temsirolimus (Torisel®), have for instance been extensively studied in several metastatic cancers (Ebos and Kerbel, 2011; Motzer et al., 2013; Patel et al., 2016; Faes et al., 2017). Sorafenib and sunitinib were the first multikinase inhibitors to be approved, in the therapeutic arsenal for metastatic renal cell carcinoma (RCC) and advanced hepatocellular carcinoma management on the base of increased progression free survival (PFS). However, the impact of these treatment on overall survival (OS) was limited (Yang et al., 2003; Sandler et al., 2006; Miller et al., 2007; Kerbel, 2008; Escudier et al., 2010). Moreover, they induced detrimental side effects such increased blood pressure and hand and foot syndrome (Motzer et al., 2013).

Renal cell carcinoma became a paradigm for the development of more efficient and less toxic agents. Hence, axitinib whose affinity for targets equivalent to those of sunitinib was higher, presented equivalent therapeutic effects with reduced toxicity

(Motzer et al., 2013). New drugs were also developed for the treatment of RCC including pazopanib (Votrient®), vandetanib (Caprelsa®) or lenvatinib (Lenvima®) (Llovet et al., 2008; Escudier et al., 2014; Motzer et al., 2015; Rizzo and Porta, 2017). Lenvatinib exploited the inhibition of Fibroblast Growth Factor receptors that are key in endothelial cell proliferation.

Several tumor cells aberrantly expressed VEGFRs and exhibit exacerbated genetic plasticity following anti-angiogenic therapies that is highlighted by several mechanisms of adaptation/resistance. The crosstalk between tumor and stromal cells allows escape mechanisms counteracting the effects of anti-angiogenic therapies.

The objective of this review is to present an overview of the different resistance mechanisms to angiogenic therapies, from the earliest to the late ones, including tumor and stromal cells adaptation. The different mechanisms were divided into "immediate early" resistance mainly reffering to adaptation of tumor cells following exposure to the drug for few minutes/hours; into "early" resistance reffering to days/weeks after treatment exposure and into "late" one occuring several months/years after the treatment and depending on metastasis (**Figure 2**). Understanding the different spatiotemporal mechanisms leading to such resistance is essential to propose innovative therapeutic strategies for patients presenting innate or acquired resistances.

"IMMEDIATE-EARLY" RESISTANCE TO ANTI-ANGIOGENIC

Redundant Angiogenic Pathways and Hypoxia

Angiogenic redundancy is one of the earliest mechanisms leading to refractoriness or acquired resistance to anti-angiogenic therapies mainly targeting VEGFA and its receptors. Although VEGFA is the best known angiostimulatory protein, angiogenesis can be triggered by several growth factors including angiopoietins (ANGs), epidermal growth factor (EGFs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), transforming growth factors (TGFs), placental growth factor (PlGF) or stromal cell-derived factor 1 (SDF1) (Xin et al., 2001; Bergers and Hanahan, 2008; Pardali et al., 2010; Brooks et al., 2012; Fagiani and Christofori, 2013). Except PlGF, which binds to VEGF receptors, all these angiogenic factors signal through different receptors expressed at the membrane of ECs (van Beijnum et al., 2015). This diversity of growth factors extents the toolbox of tumors to create blood vessels. Breast or pancreatic cancers for example rely on these angiogenic factors rather than on VEGFA and are poor responders to bevacizumab (Casanovas et al., 2005). Moreover, preclinical and clinical studies showed that anti-VEGFA antibodies and tyrosine-kinase inhibitors of VEGF receptors stimulate the production of these different growth factors (van Beijnum et al., 2015; Falcon et al., 2016; Haibe et al., 2020).

Preclinical studies for instance demonstrated an increase of SDF1 and PlGF in mice treated with anti-VEGFR2 compounds (Ebos et al., 2007; Fischer et al., 2007). The prolonged use of anti-VEGFR2 antibodies in transgenic mice models of pancreatic cancer stimulated the expression of ANG1 and FGFs. This increase correlated with a shorter survival (Casanovas et al., 2005). Comparable results were reported in head and neck squamous cell carcinoma (HNSCC) xenografts models. Indeed, microarray analysis, showed increased levels of FGF2 and its receptor (FGFR3) in bevacizumab-resistant tumors (Gyanchandani et al., 2013). These results were extended to lung cancer models resistant to angiogenesis-inhibitors, overexpressing EGFRs and FGFRs (Cascone et al., 2011). Clinical studies conducted on bevacizumab-treated colorectal cancer patients evidenced an increase of circulating PlGF, SDF1 and HGF levels (Willett et al., 2005; Kopetz et al., 2010). Equivalent results were obtained for glioblastoma patients treated with cediranib/Recentin®, a tyrosine kinase inhibitor of the VEGFR1, VEGFR2 and VEGFR3 (Batchelor et al., 2007, 2010). FGF and SDF1 increased expression was correlated with tumor relapse in cediranib-treated glioblastoma patients.

Angiopoietin-2

Angiopoietins belong to a family of protein controlling vascular maturation during developmental and pathophysiological angiogenesis (Stratmann et al., 1998; Jeansson et al., 2011; Thurston and Daly, 2012). The predominant angiopoietins are ANG1 and ANG2. ANG1 mediates migration and survival of endothelial cells through binding to Tie2 receptor found on ECs of blood vessels and monocytes, whereas ANG2 promotes cell death and vascular regression (Hanahan, 1997; Maisonpierre et al., 1997). VEGFA and ANG2 promote neovascularization and ANG2 plays a key role in tumor relapse following anti-VEGFA treatment (Asahara et al., 1998). In preclinical models of anti-VEGFR-treated tumors, upregulation of ANG2 stimulates vascular remodeling and sprouting (Crawford and Ferrara, 2009). This observation was supported by clinical studies showing that patients suffering of colorectal cancers who are poor responders to bevacizumab, exhibit high serum levels of ANG2 (Ogawa et al., 2004; Goede et al., 2010). Equivalent results were obtained for melanoma and breast cancer patients treated with anti-angiogenic therapies. Increased serum level of ANG2 correlated with disease progression (Sfiligoi et al., 2003; Helfrich et al., 2009). Preclinical studies recently showed that simultaneous blockade of VEGFA and ANG2 inhibits angiogenesis and tumor growth (Brown et al., 2010; Kienast et al., 2013; Schmittnaegel et al., 2017; Wolf and Langmann, 2019). Clinical trials using such combination are ongoing for the treatment of metastatic colorectal cancers (NCT01688206, NCT02141295). Recently, the vanucizumab, a bispecific anti-ANG2/anti-VEGFA antibody has been evaluated in a phase I study. Vanucizumab displayed acceptable safety profile and encouraging anti-tumor activity (Hidalgo et al., 2018).

Fibroblast-Growth Factors

Fibroblast-growth factors belongs to a family of 22 cell-signaling proteins involved in a broad variety of processes. FGF binds to tyrosine kinase receptors (FGFRs), expressed on tumor and stromal cells including endothelial cells, cancer-associated-fibroblasts or myeloid cells infiltrating tumors

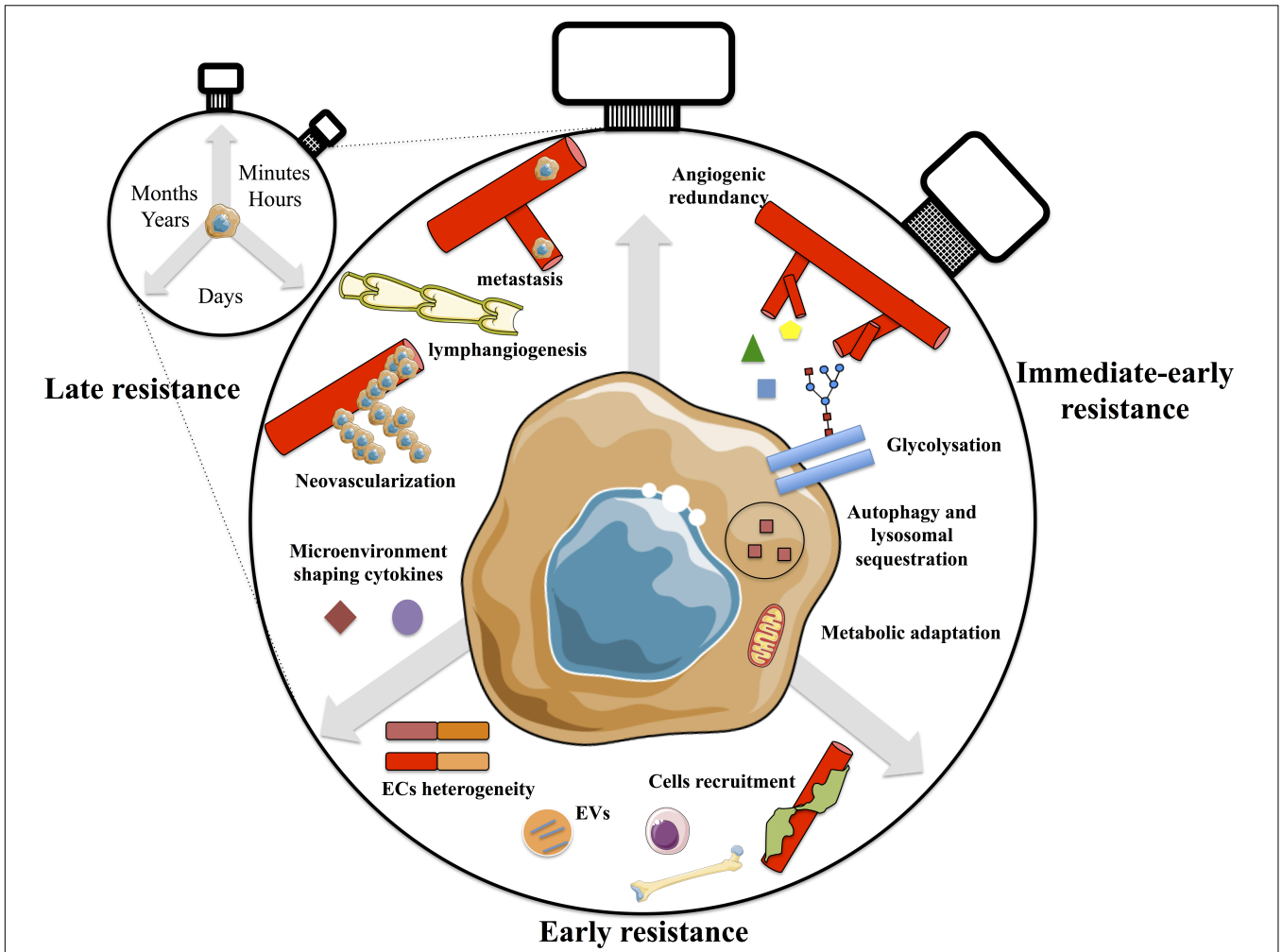


FIGURE 2 | The adaptation to anti-angiogenic therapies; a clock ticking mechanism. Immediate-early resistance occuring within minutes to few hours following anti-angiogenic treatment involved angiogenic redundancy, glycosylation of VEGFR2, metabolic adaptation and sequestration of drugs in lysosomes inducing incomplete autophagy. Early resistance occurs during the following days after treatment and involved BMDCs, stromal cells recruitment into the tumor mass and ECs. The heterogeneity of ECs mediates resistance. Finally, within months following therapy, tumors adopt neovascularization strategies and increased lymphangiogenesis triggering metastasis and poor outcome in patients.

(Beenken and Mohammadi, 2009; Ornitz and Itoh, 2015). The FGF pathway promotes cancer progression and angiogenesis by activating RAS/RAF/MEK//ERK and PI3K/AKT/mTOR pathways (LaVallee et al., 1998; Hart et al., 2000). FGFs and FGFRs up-regulation, are involved in mechanisms of resistance to anti-VEGFA therapy (Casanovas et al., 2005; Kopetz et al., 2010). VEGFR2 inhibitors induce FGF2 expression and accelerate the growth murine pancreatic neuroendocrine tumors (Casanovas et al., 2005). Clinical studies on glioblastoma furthers confirmed this observation (Batchelor et al., 2007; Lee et al., 2019).

The proangiogenic role of FGF and its involvement in resistance to VEGFA inhibitors constitute a strong rationale for the development of inhibitors targeting the FGF and VEGFA pathways. The combined inhibition of FGF2 and VEGFA was highly efficient in preclinical models of head and neck carcinoma or pancreatic tumors (Casanovas et al., 2005; Gyanchandani et al., 2013). FGFR inhibitors notably restore the sensibility to bevacizumab in experimental models in mice suggesting a promising therapeutic combination (Gyanchandani et al., 2013). However, clinical investigations failed to demonstrate the relevance of this association (Norden et al., 2015; Semrad et al., 2017). Lenvatinib, a multiple receptor tyrosine kinase inhibiting the VEGFRs, FGFRs, and PDGFRs has shown promising therapeutic effects against various solid tumors and should be considered for counteracting resistance to anti-angiogenic agents (Suyama and Iwase, 2018).

Plateled-Derived-Growth Factor

In the 1970's several groups demonstrated the existence of growth factors for fibroblasts and smooth muscle cells derived from platelets (Paul et al., 1971; Bowen-Pope and Ross, 1982). These factors were named platelet-derived-growth factors (PDGF) and were one of the first growth factors to be

characterized. By binding to their receptors PDGFRs, PDGFs are major mitogens for many cell types and actively participate in angiogenesis (Papadopoulos and Lennartsson, 2018). In cancer, PDGFs exert autocrine loops that stimulate tumor cell proliferation, and paracrine signaling for angiogenesis (Liu et al., 2011; Manzat Saplacan et al., 2017). Upregulation of PDGF was evidenced in glioblastoma patients following anti-angiogenic therapy (Liu T. et al., 2018). The blockade of PDGFR pathway increases the sensibility to VEGFA-neutralizing treatment, giving the rationale for new therapeutic opportunities. However, imatinib, a PDGFR inhibitor, in combination with bevacizumab, failed to demonstrate efficacy in renal cell carcinoma (RCC) patients (Hainsworth et al., 2007; Rock et al., 2007). Despite an increase of PFS, the VEGFRs and PDGFR inhibitor sunitinib is not curative for RCC patients (Motzer et al., 2009).

Based on the redundancy in angiogenic pathways, limited benefits to patients were observed by targeting a single angiogenic growth factor or its receptor. This redundancy is at the origin of innate or acquired resistance, by activation of alternative proliferation/survival pathways. Inhibition of ANG2-, FGF- or PlGF-mediated signaling pathways with those of VEGFA overcomes aspects of resistance to VEGFA blockade, but a sustained inhibition remains to be demonstrated.

Transforming Growth Factor-β

The Transforming Growth factor-β (TGF-β) family regulates cell proliferation, differentiation and apoptosis (Massagué, 2000). In tumors, the role of TGF-β is ambivalent with tumor suppressive effects in early stage, thereafter switching toward tumor progression at later stages (Derynck et al., 2001). TGF-β induces the production of extracellular matrix and stimulates tube formation by ECs therefore inducing angiogenesis (Ferrari et al., 2009). Upregulation of TGF-β expression was reported in mice models of glioma resistant to anti-VEGF therapy (Park et al., 2016). Inhibition of TGF-β in hepatocellular carcinoma (HCC) and glioblastoma revealed anti-angiogenic benefit offering the rational to combine anti-TGF-β agents with anti-VEGF (Fransvea et al., 2009; Comunanza and Bussolino, 2017). The combination of galunisertib, a small inhibitor of TGF-β with sorafinib led to durable response in mice models of breast cancer (Holmgaard et al., 2018). TGF-β is also a major inducer of cancer associated fibroblast (CAF) development and fibrosis that are determinant in tumor aggressiveness. Targeting two hallmarks of cancer with one molecule probably explain the therapeutic response.

Combining anti-VEGF/VEGFR therapies to inhibitors of alternative angiogenic pathways appears relevant. However, the toxicity of such approach is an important issue. Treatment targeting concomitantly VEGFR and receptors involved in relapse is another option. One of the best example was the approval of cabozantinib (Cabometyx®) an inhibitor of VEGFR but also of cMET and AXL to actors involved in relapses after sunitinib treatment. Cabozantinib was approved as a second line treatment for RCC patient experiencing progression on sunitinib (Choueiri et al., 2016). It showed also a better efficacy as compared to sunitinib for RCC patients with poor or intermediate risk (Choueiri et al., 2017).

Hypoxia, a Key Mediator of Angiogenic Redundancy

Hypoxia arises from the combination of high proliferative and metabolic rates with abberant tumor vascularisation with poor oxygen delivery (Semenza, 2014). Beside redundant pro-angiogenic pathways, tumor hypoxia is considered as an "immediate early" response to anti-angiogenic therapy. Although anti-angiogenic therapies reduce and normalize tumor vasculature, limiting tumor hypoxia, alternative theory defends an increased intra-tumor hypoxia (Kerbel and Folkman, 2002; Jain, 2005). Hypoxia plays an important role in resistance to conventional therapies leading to the selection of more aggressive stem cells and a shorter survival (Harris, 2002; Wilson and Hay, 2011; Chen et al., 2018). Indeed, anti-angiogenic agents induce intra-tumoral hypoxia and a concomitant stabilization of the hypoxia-inducible factors 1 and 2 alpha (HIF1/2α). HIF1 is considered as a tumor suppressor whereas HIF2 is considered as an oncogene. HIF1α is a major transcriptional regulator of angiogenic factors. It transactivates hundreds of pro-angiogenic genes, including growth factors (VEGFA, PlFG, FGF-2, PDGF) and their receptors (VEGFRs) (Hirota and Semenza, 2006; Rapisarda and Melillo, 2009). Moreover, HIF1 inhibits the production of anti-angiogenic factors, exacerbating angiogenesis (Hanahan and Folkman, 1996; Laderoute et al., 2000). Hence, HIF1 exerts also potent transcriptional inhibition especially following a long exposure in hypoxic conditions (Hantelys et al., 2019).

Hypoxia and HIF1 activation also trigger EMT and metastasis by regulating the expression of key genes such as *c-MET*, *CXCR4*, and lysyl oxidase (*LOX)*, events occurring later as discussed above (Joseph et al., 2018). Moreover, the hypoxic microenvironment generated following anti-angiogenic therapy stimulates β1-integrin expression, a well-known marker of resistance to cancer treatments (Foubert and Varner, 2012) which is consistent with its upregulation in clinical specimens of bevacizumab-resistant glioblastoma (Cordes and Park, 2007). Preclinical studies in mice models of glioblastoma demonstrated also the implication of β1-integrin in resistance to angiogenic therapies (Sidorov et al., 2016). The tumor microenvironment is hypoxic and the active metabolism of tumor cells induces the release of CO2 and lactate (Parks et al., 2013). The effect of hypoxia on tumor metabolism is detailled in the tumor metabolic adaptation part below.

The important role played by HIF in tumor aggressiveness stimulated the development of HIF inhibitors especially HIF2 that has oncogenic properties. Such treatments dissociate the HIF2α HIF1β dimer and consequently inhibit the transcriptional activation of HIF2. This treatment was successfully used in a multi-treated RCC patients (Chen W. et al., 2016). Hence, this treatment combined with classical anti-angiogenic drugs or immunotherapies (see below) is promising and should be further validated (Martínez-Sáez et al., 2017).

Autophagy and Lysosomal Sequestration

Autophagy is a physiological process involving the sequestration of unnecessary or dysfunctional cell components and their degradation in lysosomes (Mizushima, 2007; Janku et al., 2011;

David, 2012). In pathophysiological conditions, autophagy is an adaptative response to stress. In cancer, autophagy acts as a double-edged sword by serving as a pro-survival or pro-death process (Mathew et al., 2007). Autophagy plays an important role in enabling tumor cells to overcome harsh conditions arising from the microenvironment following treatment (Chandra et al., 2019). By enhancing the survival of tumor cells, it is indeed now considered as an important mechanism of resistance to cancer drugs (Li et al., 2017; Desantis et al., 2018). Hypoxia-induced autophagy favor the survival of hypoxic tumor cells (Brahimi-Horn et al., 2011). Two mechanisms drive hypoxia-dependent autophagy; the *non-selective* and the *selective* autophagy extensively reviewed (Chandra et al., 2019).

A cytoprotective role of autophagy was supported by several preclinical studies using radiation or imatinib as anti-cancer strategies (Miyazawa et al., 2010; Gewirtz, 2014). Resistance to sorafenib in hepatocellular carcinoma was attributed to increased activation of mTOR or Akt pathway triggering autophagy and cell survival (Zhai et al., 2014; Luan et al., 2019). The pro-tumoral role of autophagic processes in mediating resistance to anti-cancer treatments in HCC was highlighted by combining sorafenib to autophagy inhibitors (Shimizu et al., 2012; Lin et al., 2013; Hwang et al., 2015). These preclinical studies gave the proof of concept to initiate clinical trials combining inhibitors of autophagy to sorafenib.

In addition to tumor cells, stromal cells use autophagy as a mechanism of resistance to anti-angiogenic drugs. ECs, the direct targets on anti-angiogenic therapies, are inevitably exposed to drugs via the blood stream. Hence, resistance to sunitinib depends at least, on autophagy processes in ECs (Wu et al., 2020). Sunitinib-resistant RCC display an increased number of lysosomes allowing an enhanced sequestration of the drug which limits its therapeutic activity by isolating the drug from its cytoplasmic targets (Giuliano et al., 2015). The basic pKa of sunitinib induces its lysosomal sequestration., It prevents its accessibility to the tyrosine kinase domains of the receptors targeted by the drug (VEGFR1, 2, 3, PDGFR, CSF1R and cKIT), limiting the efficacy of the treatment.

Tumor Metabolic Adaptation

The updated "Hallmarks of cancer: The Next Generation" includes the deregulation of cellular energetics as a key actor of tumor progression (Hanahan and Weinberg, 2011). Over the last decades, tumor hypoxia, by shaping cell metabolism was demonstrated as a key actor of tumor adaptation to anti-angiogenic therapies. Tumor cell metabolism and angiogenesis are tightly regulated by hypoxia (Semenza, 2014). Several genes involved in glycolysis are under HIF1 control, such as *GLUT1*, *GLUT3*, *PDK1* or *LDHA* (Favaro et al., 2011). The more hypoxic the cell, the more glycolysis is used, leading to pyruvate production. Instead of entering the tricarboxylic acid cycle, most of pyruvate is converted to lactate. This excess of lactate diffuses in the extracellular environment and is picked up by oxygenated cells, that revert the lactate to pyruvate and enhance their oxidative phosphorylation (Cassim et al., 2020; Parks et al., 2020). Consequently, their need for glucose decreased, and more glucose is available for the more hypoxic area of tumors (Nakajima and Van Houten, 2013). Following sunitinib treatment, the establishment of this symbiotic loop allows the proliferation of the remaining viable cells despite the dramatic increase of hypoxia following angiogenesis inhibition (Pisarsky et al., 2016).

In addition to low oxygen, increased acidification is also a hallmark of hypoxic tumors. It plays a key role in resistance to anti-cancer therapy (Erra Díaz et al., 2018). While mammalian cells protect their cytosol from acidification through expression of membrane transporters and exchangers such as the Na^+/H^+ exchanger (L'Allemain et al., 1985) and the monocarboxylate transporter 1 (Halestrap and Price, 1999), hypoxic tumors have developed additional mechanisms to regulate their pH. In solid tumors, the transcription of carbonic anhydrase (CA) IX is controled by HIF1. CAIX catalyzes the hydration of carbon dioxide (CO_2) into H^+ and bicarbonate ($HCO3^-$) which is rapidly uptaken into cell by Na^+-$HCO3^-$ transporters sustaining alkaline pHi compatible with cell survival (Parks et al., 2013). In bevacizumab-resistant glioblastomas, increased levels of CAIX and of c-MET were observed (Jahangiri et al., 2013). Analysis of bevacizumab-resistant glioblastoma further revealed modifications in the expression of genes regulating cell metabolism, with (i) an increase of glycolysis-involved genes and (ii) a decrease of genes regulating oxidative phosphorylation (Kumar et al., 2013). Soluble CAIX is also correlated with a poor response to bevacizumab in breast cancers (Janning et al., 2019). Moreover, hypoxia leads to AMPK activation, inducing the metabolic switch from glycolysis to oxidative phosphorylation (McIntyre and Harris, 2015). Following anti-angiogenic therapy, tumor metabolism shifts from glycolysis to lipid consumption allowing tumor relapse (Sounni et al., 2014). Several clinical trials combining metabolism-targeting or hypoxia-targeting drugs with anti-angiogenics are ongoing (McIntyre and Harris, 2015). Recently, exciting novel concepts involving dual blockade of angiogenesis and metabolic adaptation have emerged and could revert the resistance to anti-angiogenic drugs (Jiménez-Valerio and Casanovas, 2017).

Recent findings demonstrated that metabolic reprogramming also occurs in TECs. TECs display upregulation of anabolic pathways in comparison to normal ECs. Unbiased meta-analysis revealed that *Aldh18a1* and *Sqle* were consistently induced in TECs raising the possibility to identify specific targetable TECs markers (Rohlenova et al., 2020).

Glycosylation–Mediated Resistance

Activation of angiogenic receptors also occurs independently of ligand binding, therefore constituting another mechanism of insensitivity to cancer therapies. This process depends in part on galectins. They belong to a family of carbohydrate-binding proteins displaying high affinity for beta-galactoside (Camby et al., 2006). Galectin-1 is overexpressed in tumors and its expression correlated with metastatic dissemination and immune-escape (Hsu et al., 2013). Tumors refractory to anti-VEGFA treatments exhibit enhanced angiogenesis. Anti-VEGFA treatment and hypoxia increased galectin-1 production. Galectin-1 binds to N-glycans glycoproteins on endothelial cells, including VEGFR2. This binding prolongs the presence of VEGFR2 at the

cell surface and promotes angiogenesis without VEGFA binding (Croci et al., 2014; Stanley, 2014). Further clinical investigations are needed to consider galectins as relevant targets for anti-angiogenic therapies.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) play a key role in angiogenesis and in tumor progression (Deryugina and Quigley, 2010). MMPs can be pro- or anti-angiogenic depending of their categories. On the one hand, MMP-3 and MMP-7 display anti-angiogenic properties (Deryugina and Quigley, 2010). On the other hand, MMP-2 and MMP-9 promote the release of VEGFA from the ECM sustaining angiogenesis (Bergers et al., 2000). MMP-1 induces matrix remodeling and migration of ECs (Chun et al., 2004). Hence, it is now established that MMP inhibitors can induce tumor progression by favoring tumor angiogenesis. Therefore, MMP inhibitors combined to inhibitors of angiogenesis should be considered as a therapeutic option (Winer et al., 2018).

Tumor Stroma

Tumors are a complex association of cancer cells as well as a stromal compartment with cellular and noncellular components. Tumor stroma plays crucial roles in tumor progression and in resistance to treatments. The dense tumor stroma can limit the access of therapeutic agents to their target due to fibrosis, high interstitial pressure and degradation of drugs by stromal enzymes (Valkenburg et al., 2018). The rigid extracellular matrix can reduce blood vessel density, creating a barrier that drugs cannot perfuse (Olive et al., 2009). In parallell, the high interstitial pressure in tumor microenvironment affects drug delivery (Provenzano and Hingorani, 2013). Beside these effects, the cytochrome P450, expressed by fibroblasts, metabolizes toxic molecules including therapeutic drugs contributing to agressive behaviors of tumors (Hirth et al., 2000). In mice models of lung cancer, treatment with bevacizumab led to acquired resistance via upregulation of VEGFA, FGF2 and its receptor FGFR2 and PDGFR in stromal cells (Mitsuhashi et al., 2015). It now becomes evident that cancer therapies should include strategies to target and constrain the tumor stroma. Some agents targeting CXCR4, TGF-β or hyaluronic acid are currently under clinical consideration (Valkenburg et al., 2018).

EARLY RESISTANCE TO ANTI-ANGIOGENIC THERAPIES

Recruitment of Local Stromal Cells

Cells constituting the tumor environment play a key role in the resistance to angiogenesis inhibitors, especially cancer-associated fibroblasts (CAFs) and pericytes.

Cancer-Associated Fibroblasts

Cancer-associated fibroblasts are the principal component of the stroma within the tumor microenvironment. They exhibit diverse functions including matrix remodeling, crosstalk with tumor, endothelial or immune cells, promoting tumorigenesis. CAFs notably allow the recruitment of endothelial progenitors cells (EPCs) and bone-marrow-derived cells (BMDCs) through SDF1 expression and stimulation of its receptor CXCR4 on EPCs (Orimo et al., 2005). The role of EPCs and BMDCs are discussed in the next part. CAFs also promotes angiogenesis through the expression of galectin-1, VEGFA, FGF, HGF or PDGF (Tang et al., 2016; Wang et al., 2019). In tumor cells deficient for VEGFA, CAFs produce VEGFA to sustain angiogenic processes (Dong et al., 2004). CAFs isolated from anti-VEGFA resistant tumors, exhibit high levels of ANG2, and PDGF promoting tumor growth (Crawford and Ferrara, 2009). The pro-angiogenic and pro-invasive role of CAFs in resistance to antiangiogenic drugs can also arise from metalloproteinases (MMPs) production (Sternlicht et al., 1999; Boire et al., 2005).

Blocking the pro-angiogenic role of CAFs with an anti-FGF-2 ^{125}I-radiolabeled antibody resulted in the inhibition of HCC tumor growth and decreased angiogenesis (Wang et al., 2012; Hu et al., 2016). Lenvatinib (Lenvima®), which inhibits VEGFRs has a potent anti-angiogenic effect and inhibits also FGF receptors involved in anti-angiogenic resistance. It is now used in the therapeutic arsenal against kidney tumors (Motzer et al., 2016). Brivanib from Bristol Myers Squibb, an anti-VEGFR and FGFR, increased the PFS of 43 patients with recurrent endometrial cancers in a phase II clinical trial (Powell et al., 2014; Hosaka et al., 2018).

Pericytes

Blood vessels are composed of two interacting cell types: the ECs, forming the inner face of vessels, and perivascular cells, called pericytes. Pericytes are peri-endothelial cells that directly interact with ECs, regulating vessel diameter, permeability and therefore the blood flow (Bergers and Song, 2005). Recruitment of pericytes by ECs relies, at least, on the PDGF-PDGFR signaling (Abramsson et al., 2003). Pericytes negatively regulate the proliferation of ECs promoting maturation of neo-vessels (Orlidge and D'Amore, 1987). In preclinical models of glioma or RCC, an increased tumor blood vessel coverage by pericytes following sunitinib or bevacizumab treatments was observed (Norden et al., 2009; Cao et al., 2013; Pinto et al., 2016). Residual tumor vessels, in a preclinical model of colorectal cancer, were heavily covered by pericytes following treatment with Anti-ANG2 antibodies (Thomas et al., 2013). Moreover, the number of vessels covered by pericytes following sunitinib was correlated to aggressiveness of RCC (Cao et al., 2013). Pericyte coverage enhances tumor resistance to these therapies through limited ECs proliferation and through the availability of survival signals (Orlidge and D'Amore, 1987). These different mechanisms highlight the role of pericytes in the resistance to anti-angiogenic treatments observed in the clinic. Therefore, inhibiting blood vessels maturation by targeting blood vessel coverage by pericytes is a relevant strategy to overcome the resistance to anti-angiogenic therapies. Inhibition of PDGFR by imatinib and sunitinib in combination with anti-VEGFR showed anti-tumor effects on experimental tumors in mice (Pietras and Hanahan, 2005). FGF2/FGFR2 signaling and PDGF/PDGFR signaling crosstalk to enhance pericyte proliferation and recruitment (Hosaka et al., 2018). PDGF stimulates the

pericytes-fibroblast transition, which contributes to metastatic processes (Hosaka et al., 2016). Therefore, inhibition of PDGF-mediated recruitment of pericytes showed potent anti-tumor effects (Thijssen et al., 2018). Hence, disrupting pericytes support, by using an anti-PDGFR and destabilizing pre-existing tumor vasculature with an anti-VEGFR, is an attractive strategy to overcome tumor refractoriness to conventional anti-angiogenic therapies.

Recruitment of Bone-Marrow Derived-Cells (BMDCS)

Anti-angiogenic therapies normalize vessels but also increase intra-tumoral hypoxia leading to the recruitment of bone marrow-derived cells (BMDCs) (Jain and Duda, 2003). Infiltration of BMDCs in tumors has been linked to tumor progression and angiogenesis for several years (Jain and Duda, 2003). As above-mentioned, anti-angiogenic therapies stimulate the production of pro-angiogenic factors (VEGFA, Angiopoietins, FGFs). However, the stress induced by the treatment stimulates inflammatory pathways involved in the production of cytokines such as SDF1, IL-8 or granulocyte colony-stimulating factor (G-CSF). These cytokines trigger the recruitment of BMDCs that exhibit high plasticity and pro-angiogenic potential limiting the efficacy of anti-angiogenic drugs (van Beijnum et al., 2015).

$CD11b^+$ $Gr1^+$ Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs), also known as $CD11b^+$ $Gr1^+$ cells are composed of a mixed population including neutrophils, macrophages and dendritic cells displaying immunosuppressive and pro-tumorigenic capacities (Yang et al., 2004; Marigo et al., 2008; Crawford and Ferrara, 2009). Preclinical and clinical studies evidenced an increased number of MDSCs in cancers, promoting tumorigenesis and angiogenesis (Yang et al., 2004; Serafini et al., 2006; Diaz-Montero et al., 2009). The infiltration of tumors by MDSCs is therefore correlated with a poor outcome in patients. It participates in mechanisms of resistance to anti-angiogenic therapies (Shojaei and Ferrara, 2008). Indeed, tumors resistant to anti-VEGFA-treatments presented increased infiltration of MDSCs in comparison to anti-VEGFA sensitive tumors (Shojaei et al., 2007a). The presence of tumor infiltrating Th-17 cells induces the expression of G-CSF by CAFs and increased production of IL-6 and SDF1 by CAFs and tumor cells, allow the recruitment of MDSCs (Shojaei and Ferrara, 2008; Shojaei et al., 2009). Upregulation of G-CSF by resistant tumors triggers prokinectin-2 (Bv8) overexpression in the bone marrow (BM). Bv8 induces the migration of progenitor cells from the BM to the tumor. Anti-Bv8 antibodies reduce MDSCs recruitment and inhibit tumor growth and angiogenesis, suggesting a role of Bv8 in relapses following anti-VEGFA treatment (Shojaei et al., 2007a,b). Moreover, hypoxia induces resistance to sunitinib in glioblastoma, breast and metastatic RCC by increasing the recruitment of MDSCs to the tumor niche (Finke et al., 2011; Piao et al., 2012). In agreement with these observations, depletion of MDSCs sensitized tumors to anti-angiogenic therapies, highlighting their pivotal role in resistance (Holmgaard et al., 2016).

Among the MDSCs population, increased tumor-infiltration by neutrophils promote resistance to bevacizumab. Neutrophils induce Bv8-dependent tumor angiogenesis independently from the VEGFA signaling. Preclinical studies demonstrated that blockade of Bv8 decreases the recruitment of MDSC and angiogenesis (Shojaei et al., 2007b; Piao et al., 2012).

Macrophages, specialized phagocytic cells, also display plasticity and shape their phenotype in response to environmental conditions, making them a relevant candidate for treatment resistance (Ruffell and Coussens, 2015; Sarode et al., 2020). The first relationship between macrophages and angiogenesis was proposed in Knighton et al. (1983). Depending on their localization and on their polarization profiles, macrophages are pro- or anti-tumoral actors (Cheng et al., 2019). Recruitment of macrophages in tumors is induced by several cytokines including VEGFA or M-CSF. Macrophages secrete growth factors such as VEGFA or EGF triggering angiogenesis (van Beijnum et al., 2015). They also secrete matrix metalloproteases, and physically associate with ECs, promoting angiogenesis. In several preclinical studies, anti-VEGFA therapies reduced macrophage infiltration (Salnikov et al., 2006; Dineen et al., 2008). Nevertheless, specific macrophages with immunoglobin-like and EGF-like domains, the Tie-2-expressing macrophages (TEM), are recruited in hypoxic zones and by ANG2 (Murdoch et al., 2007). TEM also promote angiogenesis and tumor progression in hypoxic environment, through upregulation of HIF1α (De Palma and Naldini, 2011). Therefore, macrophages contribute to anti-angiogenic resistance. Although ANG2 inhibitors do not prevent the recruitment of TEM, they decrease their activity, illustrated by a downregulation of growth factors production and a decrease of their physical association with blood vessels (Mazzieri et al., 2011).

These results suggest that BMDCs are therapeutic targets for counteracting tumor refractoriness to anti-angiogenic therapies. Inhibition of the SDF1 pathway notably prevents BMDCs tumor infiltration and overcomes such resistance (Liu et al., 2010). Equivalent results were obtained with anti-Bv8 antibodies (Hasnis et al., 2014). Clinical studies recently demonstrated that plasma TEM are predictive markers of anti-angiogenic treatment failure in colorectal and ovarian cancers (Jayson et al., 2018). However, clinical investigations consisting in preventing tumor-infiltration of TEM are needed to further consider this therapeutic perspective.

Endothelial Progenitors Cells

The discovery of endothelial progenitor cells (EPCs) in adults and their putative vascular-promoting properties has generated debate in the field of vascular biology (Pasquier and Dias, 2010). EPCs were first isolated in 1997 by Asahara et al. (1997) EPCs are subtypes of stem cells that originate from the bone marrow. A controversy concerning their origin, their isolation and their functioning still exists. EPCs have high proliferative potential, capable of differentiation into mature ECs, therefore contributing to neovascularization and angiogenesis (Asahara et al., 1997; Reale et al., 2016). Several surface markers (CD133, CD34,

and VEGFR2) characterize bone marrow derived-EPCs. They acquired CD31 and CD146 expression during their transport to the blood. They become mature ECs in the target tissues where they expressed VEGFR2, CD31, CD136, VE-cadherin, eNOS and von Willebrand factor (Puente et al., 2013). EPCs have a dual role in promoting angiogenesis into the tumor tissue; they regulate the angiogenic process through the production of growth factors and provide structural function in sprouting nascent vessels (Puente et al., 2013). The main chemo-attractants for EPCs in tumor tissue are VEGFA and SDF1, released by ECs, cancer cells and CAFs (Orimo et al., 2005; Grunewald et al., 2006). When recruited, EPCs promote angiogenesis by differentiating in ECs and by incorporating newly formed blood vessels (Puente et al., 2013). Anti-angiogenics, through hypoxia and HIF1α activation lead to the production of VEGFA and SDF1 by tumor cells triggering mobilization and recruitment of EPCs (Ceradini et al., 2004). Activated EPCs secrete pro-angiogenic factors leading to limited effects of anti-angiogenic therapies. Although the precise mechanism of EPCs-induced neovascularization remains poorly understood, recent studies in non-small-cell lung carcinoma (NSCLC) demonstrated a key role of histone deacetylase 7 (HDAC7) in the regulation of angiogenic genes (Wei et al., 2018). Nevertheless, the therapeutic implication of EPCs still remains to be elucitated.

Heterogeneity of Tumor Endothelial Cells

Heterogeneity of tumor endothelial cells (TECs) contributes to resistance to anti-angiogenic therapy (Maishi et al., 2019). TECs cover the inner surfaces of tumor blood vessels and are consequently directly exposed to anti-angiogenic drugs. TECs differ in several points from normal ECs. They display cytogenetic abnormalities, upregulation of pro-angiogenic factors and expression of stemness genes leading to drug resistance (Hida et al., 2004; Maishi et al., 2019). TECs express high levels of VEGFR1, VEGFR2, VEGFR3, and Tie-2 leading to strong responses to their respective angiogenic ligands (Alessandri et al., 1999; Bussolati et al., 2003). Moreover, TECs produce nonconventional growth factors such as biglycan, LOX and pentraxin, sustaining angiogenesis processes (Maishi et al., 2019). These observations led to the development of LOX inhibitors. Inhibition of LOX and biglycan reduces tumor metastasis suggesting the relevance of LOX targeting (Yamamoto et al., 2012; Osawa et al., 2013).

The hypoxic tumor microenvironment stimulates the expression of stemness genes in TECs, such as stem cell antigen 1 (Sca-1), MDR-1 and aldehyde lactate deshydrogenase (ALDH), leading to resistance to paclitaxel and to fluorouracil (5-FU) (Xiong et al., 2009). The vascular stem cells, that constitute a minor population in tumors, were suggested to contribute to tumor resistance to conventional chemotherapy and to anti-angiogenic treatments. Indeed, TECs derived from HCC are also more resistant to sorafenib, in comparison to human umbilical vein endothelial cells (HuVECs) (Xiong et al., 2009).

Transforming growth factors can originate from dedifferentiation of tumor cells, monocytes or from EPCs contributing to high heterogeneity and to resistance to anti-angiogenic treatments. Although cancer cells acquired drug resistance is well documented, the heterogeneity of TECs must be considered as a major actor. Recent single cell RNA-sequencing studies revealed endothelial cell heterogeneity following anti-VEGF therapy (Zhao et al., 2018). TECs can be classified into tip-like, transition and stalk-like cells. The sequencing of 56,771 endothelial cells from human/mouse (peri)-tumoral lung cells revealed different phenotypes following anti-angiogenic treatment. Tip-like signatures correlated with patient survival and tip-like TECs were most sensitive to anti-VEGF therapies (Goveia et al., 2020).

Among TECs-targeting therapies, inhibitors of CXCR4 were scrutinized since TECs are CXCR4-enriched populations is associated with a poor outcome in HCC. Inhibition of CXCR4 induces promising anti-tumor response mainly by preventing recruitment of BMDCs in the tumor mass and must be considered as a future therapeutic option (Kioi et al., 2010).

Extracellular Vesicles

Metastatic dissemination of cancer cells relies on several parameters and notably on the bi-directional communication between primary tumor and future metastatic tissues. This crosstalk essentially involves the production of particles by cancer or stromal cells. These particles are known as Extracellular vesicles (EVs). EVs carry onco peptides, RNA species or lipids from donor to recipient cells, triggering phenotypic changes of the future pre-metastatic niches (Xu et al., 2018). EV stimulate angiogenesis by transporting growth factors (VEGFA, PDGF, FGF-2), transcription factors (STAT3 and STAT5) or micro-RNAs (Todorova et al., 2017).

Recently, the emergence of EVs as a novel player of drug resistance has gained interest. EVs transfer drugs from resistant to sensitive cells triggering cell resistance (Maacha et al., 2019). VEGFA contained in EVs correlates with disease progression in bevacizumab treated patients, raising the possibility that resistance to bevacizumab relies on this process (Ko et al., 2019). Moreover, bevacizumab could be shed and exported by EVs leading to therapeutic escape (Simon et al., 2018).

LATE RESISTANCE

The Angiogenic-Dormancy as an Intrinsic Resistance Mechanism

Metastases can remain for months or years in a quiescent, dormant state, in the tissue they colonized. These micro-metastases constitute a residual disease characterized by the persistence of tumor cells, undetectable by conventional diagnostic techniques. The tumor dormancy can be defined as the lag in tumor growth occurring between primary tumor formation and the appearance of clinically detectable metastases (Yadav et al., 2018). The presence of disseminated tumor cells (DTCs) in bone marrow of prostate-cancer and breast-cancer patients have been reported before the development of overt metastases (Banys et al., 2012; Lam et al., 2014). Three molecular mechanisms characterize tumor dormancy: mitotic arrest, immunological and angiogenic dormancy (Senft and Ronai, 2016). The angiogenic dormancy may explain the reasons why

angiogenic therapies simply delay tumor progression. More than 20 years ago, pharmacological inhibition of angiogenesis was found to induce dormancy in several mouse models (Holmgren et al., 1995; O'Reilly et al., 1996). The supposed but unproven "angiogenic switch" is supposed to play a key role in the maintenance of the dormancy, since dormant cells upregulate angiogenesis inhibitors such as thrombospondin-1 (TSP-1) (Senft and Ronai, 2016). Despite the lack of clinical evidences, the "angiogenic switch" of dormant cells has to be considered in cancer relapse following treatment arrest.

Induction of Cancer Stem Cells

Cancer stem cells (CSCs) constitute a small population of cells within tumor exhibiting abilities of self-renewal, differentiation and high tumorigenicity potential. They play a key role in the initiation of cancer and in the metastatic cascade. In 2003, CSCs were first identified in human breast and brain cancers (Al-Hajj et al., 2003; Singh et al., 2003). CSCs express CD44, CD24, CD29, CD90, CD133 and aldehyde deshydrogenase (ALDH1) allowing their identification (Yu et al., 2012). CSCs drive angiogenesis in hypoxia and HIF mediates CSCs proliferation and self-renewal (Tong et al., 2018). CSCs was suggested to give rise to endothelial cells and thus neovascularization processes (Fujita and Akita, 2017). Moreover, CSCs can differentiate in pericytes, supporting tumor vessel function (Cheng et al., 2013).

Their tumor initiating properties and their metastatic potential suggest that CSCs are involved in resistance to therapies. Conventional treatments including chemo- and radiation therapies generate the production of CSCs promoting tumor escape (Chen X. et al., 2016; Li et al., 2016; Liu L. et al., 2018). CSCs are actors of anti-angiogenic resistance. Preclinical studies on experimental models of breast cancers showed that sunitinib and bevacizumab increase the CSCs populations through HIF1 activation (Conley et al., 2012). These results indicate that administration of anti-angiogenic agents accelerate tumor growth by increasing CSCs population. Several CSCs-targeting therapies are currently under development. Inhibition of ALDH1 prevents CSCs enrichment and reduces tumor formation of experimental triple-negative breast cancer and NSCLC in mice (Schech et al., 2015; MacDonagh et al., 2017). Evaluation of CD44, CD133 or Hedgehog inhibitors are currently under considerations for further clinical developments (Shibata and Hoque, 2019).

Induction of Epithelial-Mesenchymal Transition and Invasion

Epithelial-mesenchymal transition (EMT) defines the acquisition of characteristics of invasive mesenchymal cells by epithelial cells. EMT is implicated in tumor invasion and metastasis and correlates with poor clinical outcome in several solid tumors (Mittal, 2018). During the EMT process, epithelial cells lose their phenotypes, with a downregulation of E-cadherin and α-catenin and acquire mesenchymal markers (N-cadherin, vimentin, fibronectin) leading to cell mobility and invasiveness (Zeisberg and Neilson, 2009). Several signaling pathways induce EMT (TGF-β, Wnt, Notch), by controlling the transcription factors Snail, Slug, ZEB1/2 and Twist (Garg, 2013). Hypoxia and HIF1α are also well-known drivers of EMT. The expression of Twist and Snail, the downregulation of E-cadherin and the induction of vimentin promoting tumor invasiveness, have been reported following anti-angiogenic treatments (Cooke et al., 2012; Maione et al., 2012). Similarly, enhanced invasiveness and growth capacity of glioblastoma and RCC cells have been demonstrated following VEGFA inhibition (Grepin et al., 2012; Lu et al., 2012). This enhanced invasion abilities led to metastatic dissemination, a later step discussed in part 3.

Several studies highlighted the role of the tyrosine kinase receptor c-MET in promoting tumor invasiveness and metastasis in response to anti-angiogenic therapies (Pàez-Ribes et al., 2009; Lu et al., 2012; Sennino et al., 2012).

Although sunitinib and anti-VEGFA decreased tumor volume, invasiveness, hypoxia and EMT markers are increased (Ebos et al., 2009; Mizumoto et al., 2015). In addition, c-MET and the phosphorylated active forms of c-MET also increased as a consequence of treatment-induced hypoxia. The c-MET pathway is one of the most investigated pathways in the field of resistance to anti-angiogenic therapies. Its stimulation through HGF binding, triggers the activation of the RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, and STAT3 pathways promoting tumor growth and invasiveness (Jeon and Lee, 2017). Bevacizumab-treated glioblastoma patients have increased relapse in comparison to bevacizumab-untreated patients. This clinical observation was recently linked to the upregulation of c-MET and phospho-c-MET (Jahangiri et al., 2013). Hence, c-MET is a robust actor of anti-angiogenic resistance by promoting EMT-like phenotype and invasiveness in glioblastoma. This observation has subsequently led to development of c-MET inhibitors. Cabozantinib, a promising multi-kinase inhibitor of c-MET, VEGFR2, and AXL, improves overall survival of RCC patients with bone metastases (Motzer et al., 2018).

Lymphangiogenesis Induction

Historically, lymphatic vessels were considered as passive participants in metastatic dissemination, only acting as channels for tumor cells transit. Nowadays, it becomes evident that lymphatic vessels have an active role in promoting metastasis. The first pro-lymphangiogenic factors identified more than 20 years ago were the VEGFC and VEGFD that bind to VEGFR3 expressed on lymphatic endothelial cells triggering lymphangiogenesis (Joukov et al., 1996; Yamada et al., 1997). Overexpression of VEGFC and VEGFD increases the number of tumor-associated lymphatic vessels and the incidence of lymph node metastases (Christiansen and Detmar, 2011). Moreover, overexpression of VEGFC and VEGFD is correlated to intra-tumoral lymphatic vessel density, lymph node metastasis and poor outcome in patients with melanoma and breast cancers (Mohammed et al., 2007; van der Schaft et al., 2007). More recently, HGF, c-MET, Tie-2, PDGF and FGF were also identified as pro-lymphangiogenic factors (Christiansen and Detmar, 2011). Immunohistochemical analysis of tumor samples showed that lymphatic vessel invasion (LVI) correlated with lymph node metastasis (Christiansen and Detmar, 2011). Moreover, tumor cells through the expression of chemokine receptors exploit

the lymphatic network to form metastases. Indeed, CXCR4 and CCR7 expressed on human breast cancer cells promote metastasis to organs expressing their respective ligands, SDF1 and CCL21 (Müller et al., 2001). CXCR4 is upregulated by hypoxia. Since dissemination to distant organs is governed by the SDF1 gradient, CXCR4/SDF1 antagonists inhibited lymph nodes spreading of cancer cells in experimental tumors in mice (Müller et al., 2001).

Drugs destroying blood vessels stimulate the development of tumor lymphatic vessels contributing to treatment failure. Tumors from sunitinib-treated RCC patients in a neoadjuvant setting exhibit increased lymphatic vessels and increased lymph node invasion. This detrimental effect is explained at least by the stimulation of VEGFC expression following sunitinib administration (Dufies et al., 2017a). Indeed, sunitinib stimulate *vegfc* gene transcription, mRNA stability and protein production and the subsequent VEGFC-dependent development of lymphatic vessels. Moreover, hypoxia upregulated VEGFC expression (Morfoisse et al., 2014; Ndiaye et al., 2019). Lymphangiogenesis participates in treatment failure and its targeting can be considered in the therapeutic arsenal but only for advanced tumors.

Microenvironment Shaping by Cytokines

The central role played by VEGFA plus ELR+CXCL cytokines and especially CXCXL8/IL-8 was first documented by Sparmann and Bar-Sagi (2004) in colon cancers. The role of ELR+CXCL and their receptors-CXCR1/2 on tumor cell proliferation, angiogenesis and microenvironment adaptation following anti-angiogenic therapies was highly documented (Vandercappellen et al., 2008). The pro-inflammatory interleukin (IL-1 β stimulates CXCL7 production in RCC models resulting in tumor growth (Grepin et al., 2012; Grépin et al., 2014). CXCL7 is a predictive marker of sunitinib efficacy in RCC (Dufies et al., 2017b). CXCL5 in response to lysosomal sequestration of anti-angiogenic drugs plays also a key role in resistance to anti-angiogenic in renal and breast cancers (Giuliano et al., 2019). Inhibitors of CXCR1 and CXCR2 efficiently inhibit the growth of experimental HNSCC and RCC by decreasing tumor cell proliferation, angiogenesis and inflammation (Dufies et al., 2019).

Novel Neovascularization Modalities

Beside angiogenesis, new vascular networks are generated by the attraction of endothelial progenitor cells, intussusceptive angiogenesis, vessel co-option and vasculogenic mimicry.

Vessel Co-option

Tumor can use alternative ways to obtain blood supply, and therefore counteracting the effects of anti-angiogenic therapies. Tumor cells can hijack pre-existing blood vessels of the surrounding non-tumoral tissue and migrate along these vessels. This process, which occurs in the absence of angiogenic growth factors, is called vessel co-option (Kuczynski et al., 2019). Basically, the cancer cells migrate along the surface of pre-existing vessels leading to their incorporation in the tumor mass. Vessel co-option has been extensively reported in histopathological specimens of lung, liver and brain cancers (Nakashima et al., 1995; Pezzella et al., 1997; Offersen et al., 2001; Winkler et al., 2009; Yao et al., 2018). This process sustains the growth of brain metastases emerging from melanomas, liver and breast cancers (Leenders et al., 2004; Kuczynski et al., 2016, 2019).

A major question is whether vascular co-option constitutes an intrinsic resistance or does it occur as an acquired resistance mechanism following therapy. Inhibition of VEGFA promotes cancer invasion, inducing vessel co-option *in vivo*. Mechanistic studies identified the actin-related protein, Arp2/3, c-MET, ZEB2- and WNT- EMT dependent signaling as promoters of cell motility and vessel co-option (Navis et al., 2013; Depner et al., 2016; Frentzas et al., 2016). Simultaneous blockade of VEGFA and ARP2/3, VEGFA and c-MET or VEGFA and ZEB2 suppresses tumor invasion (Sennino et al., 2012; Depner et al., 2016; Frentzas et al., 2016). Other therapeutic approaches include the blockade of cell-adhesion receptors, since tumor cells adhere to endothelial cells during co-option. Hence, a β1-integrin inhibitor combined with bevacizumab induced sustained anti-tumor response in bevacizumab-resistant glioma xenografts (Carbonell et al., 2013; Jahangiri et al., 2014).

The prognostic value of vessel co-option in cancer patients remains to be elucidated. Bevacizumab-treated colorectal cancer patients with liver metastases demonstrated a limited response due to vessel co-option (Frentzas et al., 2016). Combining cell-motility or cell-adhesion inhibitors with anti-angiogenic compounds deserves to be considered as a therapeutic alternative.

Vasculo Mimicry

The vasculo-mimicry is defined as the formation of vascular-like structures by non-vascular cells. In 1999, it was first reported that tumor can dedifferentiate and form vascular-like structure (Maniotis et al., 1999). Later, vasculo mimicry has been described in several tumor types such as breast, ovarian cancers or Ewing sarcoma (Sood et al., 2001; Shirakawa et al., 2002; van der Schaft et al., 2005). This dedifferentiation is accompanied by the acquisition of endothelial features such as VE-cadherin or Tie-2 expression (Maniotis et al., 1999). In addition, HIF1α is an important regulator in the process of vasculo-mimicry (Delgado-Bellido et al., 2017). Despite this dedifferentiation, tumors remain refractory to anti-angiogenic therapy (van der Schaft et al., 2004). Bevacizumab elicits vasculo-mimicry of tumors leading to tumor escape and metastasis (Xu et al., 2012). Sunitinib stimulates vasculo-mimicry by differentiating tumor cells to endothelial-like cells (Serova et al., 2016; Sun et al., 2017). Nevertheless, further studies are needed to clarify the correlation between vasculo-mimicry and resistance to anti-angiogenic therapies.

Increased Metastasis Rate

The ultimate consequence of the resistance of anti-angiogenic therapies is the increased rate of metastasis. As developed in the previous parts, anti-angiogenesis therapies lead to (i) intrinsic reprogramming of tumor cells with upregulation of alternative pro-angiogenic pathways, increased of lymphangiogenesis-related genes and processes and initiation of EMT (ii) conditioning the microenvironment, with the recruitment of local- and bone marrow-derived cells or used novel neovascularization modalities. All of these mechanisms lead

to increased metastatic rate. Ten years ago, Ebos et al. (2009); Pàez-Ribes et al. (2009) were the first to describe the association between anti-angiogenesis drugs and increased distant metastases. Preclinical models of breast cancers showed that sunitinib enhance lung and liver metastasis (Ebos et al., 2009). Anti-angiogenic treatments can make the host more permissive for metastatic seeding. Sunitinib-treated mice exhibit vascular changes such as reduced pericyte coverage and increased leakiness of normal vessels (Chung et al., 2012; Maione et al., 2012; Singh et al., 2012). Therefore, these systemic actions facilitate the creation of a metastatic niche at distance from the primary tumor.

Increased metastasis rate following anti-angiogenic therapies are highly variable and depends on several parameters such as the type of treatment, the dose and the schedule. Singh et al. (2012) showed that sunitinib enhanced the agressiveness of tumor cells whereas the use of an anti-VEGF antibody did not. Chung et al. (2012) further demonstrated that inhibition of VEGF signaling by antibodies does not promote metastasis, in contrast to small molecule RTK inhibitors at elevated-therapeutic drug dosages. Dosing and scheduling of anti-angiogenic administration can also induce resistance. Short-term and high dose of sunitinib increased growth of breast cancer and enhance liver and lung metastasis (Ebos et al., 2009). In contrast, treatment with low dose of sunitinib did not induce metastasis (Welti et al., 2012).

CONCLUSION AND FUTURE OF ANTI-ANGIOGENIC THERAPIES

Angiogenesis processes, through the establishment of a new vascular network, are an important contributor to tumor development and metastatic dissemination. Once the tumor has reached 1-2 mm^2, the core of tumors become hypoxic and tumor cells counteract hypoxia by the production of angiogenic growth factors. Among them, VEGFA is one of the most important. Targeting the VEGFA/VEGFRs represented a great breakthrough in the therapeutic management of cancer patients. Unfortunately, complete responses are rare, and tumors counteract this inhibition through different processes. The molecular mechanisms of resistance are not fully understood and deciphering them has gained interest. It is now evident that several mechanisms exist. They involve a wide range of processes; (i) the earliest, with the upregulation of genes involved in angiogenic redundancy, EMT or the lysosomal sequestration of drugs, to the latest (ii) with an adaptation of the tumor microenvironment, reflected by the recruitment of progenitors cells, lymphangiogenesis, and adapted neovascularization modalities. All these mechanisms allow tumor metastasis and serve as limitations to anti-angiogenic drug efficacy.

Hence, combining treatments targeting tumor cells and cells of the tumor microenvironment should limit resistance and should improve patients' survival. One of the first and of the obvious way of resistance involves angiogenic redundancy by multiple growth factors as suggested by the anti-tumoral effects of FGF inhibition and bevacizumab (Casanovas et al., 2005; Gyanchandani et al., 2013). Although combining anti-angiogenic therapies may improve benefit, the other alternative pathways lead to resistance. Moreover, the balance between therapeutic efficacy and toxicity must be evaluated before administration to patients. Another therapeutic strategy consists in targeting BMDCs or pericytes and CAFs in addition to tumor cells. This approach seems relevant since BMDCs and local stromal cells blockade leads to an impairment of tumor growth (Bergers and Hanahan, 2008; Crawford and Ferrara, 2009; Liu et al., 2010). The treatment of patients with diffuse-type giant tumor cells with a CSF-1 antibody elicits objective response (Ries et al., 2014). This result raises the possibility to combine this antibody to anti-angiogenic agents.

Another promising therapeutic strategy consists in targeting lymphangiogenesis and angiogenesis. Lymphangiogenesis induced by anti-angiogenesis dedicated compounds gives rise to node metastasis, leading at term to an increased metastatic rate and poor outcome in patients (Dufies et al., 2017a). Moreover, these lymphatic vessels play a key role in the cancer-induced immune tolerance. Indeed, tumor associated lymphatic vessels upregulated Program-Death Ligand 1 (PDL1) inhibiting T cell activation and therefore anti-tumor response (Dieterich et al., 2017). Recently, the anti-PDL1 antibody, avelumab combined with axitinib was compared to sunitinib for advanced RCC. The progression free survival was 13.8 months and significantly higher than sunitinib alone (8.4 months) (Motzer et al., 2019). A phase III study comparing the anti-PDL1 antibody, atezolizumab, plus bevacizumab versus sunitinib was assessed in metastatic RCC and confirmed these results (Rini et al., 2019b, 3). Among the tested combinations, the anti-PD1, pembrolizumab plus axitinib combo improved the PFS but also the OS of RCC patients (Rini et al., 2019a).

Nevertheless, despite their effects on PFS and OS, these combinations are not curative. The development of animal models mimicking the tumor microenvironment as well as preclinical evaluations of combo therapies are urgently needed to improve patients PFS and OS. To reach the "Golden Age" of tumor treatment as defined by Hsieh et al. (2017) new treatment options are needed either to improve the therapeutic effects of anti-angiogenics and immunotherapies or by inhibiting new relevant pathways involved in innate refractoriness or acquired resistance. The current anti-cancer strategies are based on the inhibition of a specific target playing a key role in tumor development [example: EGFR (lung cancers); HER2 (breast cancers); BRAF (melanoma)]. Because of relapses on these strategies, combinations with conventional chemotherapy [taxanes (breast) platin salts (lung)] or other targeted therapies like anti-angiogenics or immunotherapies have entered in the therapeutic arsenal. However, the second strategy often combined different toxicities and cannot be administered at long terms, limiting the therapeutic index. However, the "magic bullet" does not exist because cancers integrate several mechanisms of evasion to one treatment. Hence, instead of inhibiting several targets with several drugs, the ideal strategy relies on the use of one inhibitor targeting multiple hallmarks of cancers, i.e., tumor cell proliferation/stemness, angiogenesis, chronic inflammation, and immune tolerance.

By destroying the vascular network, antiangiogenic therapies efficacy should have cause vascular network destruction leading to tumor cells asphyxia and nutrient starvation. Moreover,

antiangiogenic treatments should have targeted only normal endothelial cells that cannot undergo genetic plasticity, a specific property of tumor cell adaptation to treatments. However, aberrant expression, by tumor cells, of receptors inhibited by antiangiogenic drugs stimulated the genetic adaptation of tumor cells mainly through epigenetic modifications. For example, EZH2 a specific histone methyl transferase is a driver of sunitinib resistance in kidney cancers (Adelaiye-Ogala et al., 2017). In addition to tumor cells, tumor endothelial cells undergo epigenetic modifications crucial for adaptation to the antiangiogenic therapies (Ciesielski et al., 2020).

The correlation between the efficacy of antiangiogenic drugs and tumor grade was also a neglected parameter. Controversial results emerged from their efficacy in non-metastatic versus metastatic kidney cancers. Whereas they are the standard of cancer for metastatic tumors their efficacy as an adjuvant therapy gave conflicting results. The ASSURE trial (NCT00326898) showed no survival benefit relative to placebo whereas the S-TRAC trial showed that sunitinib in an adjuvant setting prolonged the disease free survival for more than 1 year (Haas et al., 2016; Ravaud et al., 2016). These complex features supposed that anti-angiogenic drugs affect other cells than ECs. Hence, the drugs indirectly affect immune cells. Sunitinib for example reverses immune suppression (Finke et al., 2008). In this process, myeloid derived suppressor cells are one of the main targets of sunitinib (Ko et al., 2009). Moreover, inhibition of VEGFA or VEGFR decreased the expression of immune checkpoints involved in immune tolerance, by T cells (Voron et al., 2015). Hence, the crosstalk between angiogenesis and immune cells explain the efficacy of combining antiangiogenic drug to immune checkpoint inhibitors (Motzer et al., 2019; Rini et al., 2019b). Immunetolerance is most of the time encountered in advanced tumors in which angiogenesis is key for metastatic spreading. The relevance of inhibiting angiogenesis was based on these extreme cases. However, blood or lymphatic vessels vehiculate active cytotoxic immune cells to prevent the development of low-grade tumors that did not undergo immune tolerance. Hence, favoring the development of lymphatic vessels through injection of VEGFC decreased the growth of experimental glioblastoma by enabling immunosurveillance (Song et al., 2020). Hence, these experiments completely revisited the notion that angiogenesis is systematically detrimental. The hypothesis that vessels must be normalized in cancer had emerged during the last decade (Goel et al., 2011). This hypothesis stipulates that normalization of tumor vessels will shape the tumor microenvironment leading to the control of tumor progression and to the improvement of the therapeutic response (Martin et al., 2019).

With the advent of the immunotherapy, the blockage of angiogenesis should be reconsidered and the "blasting missil" must be discovered.

It is now evident that targeting only one mechanism involved in cancer development is insufficient. The cancer Hallmarks described by Hanahan and Weimberg probably shape the future treatments to increase the percentage of complete remissions. What is the ideal strategy? Targeting at the same time different Hallmarks with already approved therapies or to find targets that drive concomitantly the different Hallmarks? If these targets exist, a specific inhibitor will serve as a "blasting missile" to destroy the tumor. The reality is probably an intermediate option.

AUTHOR CONTRIBUTIONS

CM and GP are equally responsible for all parts of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The authors acknowledge funding from the Fondation Xavier-Mora, The Fondation de France and La Ligue contre le Cancer-Equipe labellisée 2019.

REFERENCES

Abramsson, A., Lindblom, P., and Betsholtz, C. (2003). Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J. Clin. Invest.* 112, 1142–1151. doi: 10.1172/JCI18549

Adelaiye-Ogala, R., Budka, J., Damayanti, N. P., Arrington, J., Ferris, M., Hsu, C.-C., et al. (2017). EZH2 modifies sunitinib resistance in renal cell carcinoma by kinome reprogramming. *Cancer Res.* 77, 6651–6666. doi: 10.1158/0008-5472.CAN-17-0899

Alessandri, G., Chirivi, R. G., Fiorentini, S., Dossi, R., Bonardelli, S., Giulini, S. M., et al. (1999). Phenotypic and functional characteristics of tumour-derived microvascular endothelial cells. *Clin. Exp. Metastasis* 17, 655–662. doi: 10.1023/a:1006738901839

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3983–3988. doi: 10.1073/pnas.0530291100

Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., et al. (1998). Tie2 receptor ligands, Angiopoietin-1 and Angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ. Res.* 83, 233–240. doi: 10.1161/01.RES.83.3.233

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., et al. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964–967. doi: 10.1126/science.275.5302.964

Asano, M., Yukita, A., Matsumoto, T., Kondo, S., and Suzuki, H. (1995). Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor121. *Cancer Res.* 55, 5296–5301.

Banys, M., Hartkopf, A. D., Krawczyk, N., Kaiser, T., Meier-Stiegen, F., Fehm, T., et al. (2012). Dormancy in breast cancer. *Breast Cancer Targets Ther.* 4, 183–191. doi: 10.2147/BCTT.S26431

Batchelor, T. T., Duda, D. G., di Tomaso, E., Ancukiewicz, M., Plotkin, S. R., Gerstner, E., et al. (2010). Phase II study of cediranib, an oral pan-vascular endothelial growth factor receptor tyrosine kinase inhibitor, in patients with recurrent glioblastoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 28, 2817–2823. doi: 10.1200/JCO.2009.26.3988

Batchelor, T. T., Sorensen, A. G., di Tomaso, E., Zhang, W.-T., Duda, D. G., Cohen, K. S., et al. (2007). AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell* 11, 83–95. doi: 10.1016/j.ccr.2006.11.021

Beenken, A., and Mohammadi, M. (2009). The FGF family: biology, pathophysiology and therapy. *Nat. Rev. Drug Discov.* 8, 235–253. doi: 10.1038/nrd2792

Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., et al. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat. Cell Biol.* 2, 737–744. doi: 10.1038/35036374

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Bergers, G., and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol.* 7, 452–464. doi: 10.1215/S1152851705000232

Boire, A., Covic, L., Agarwal, A., Jacques, S., Sherifi, S., and Kuliopulos, A. (2005). PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* 120, 303–313. doi: 10.1016/j.cell.2004.12.018

Borgström, P., Bourdon, M. A., Hillan, K. J., Sriramarao, P., and Ferrara, N. (1998). Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. *Prostate* 35, 1–10. doi: 10.1002/(sici)1097-0045(19980401)35:1<1::aid-pros1<3.0.co;2-o

Bowen-Pope, D. F., and Ross, R. (1982). Platelet-derived growth factor. II. Specific binding to cultured cells. *J. Biol. Chem.* 257, 5161–5171.

Brahimi-Horn, M. C., Bellot, G., and Pouysségur, J. (2011). Hypoxia and energetic tumour metabolism. *Curr. Opin. Genet. Dev.* 21, 67–72. doi: 10.1016/j.gde.2010.10.006

Brooks, A. N., Kilgour, E., and Smith, P. D. (2012). Molecular pathways: fibroblast growth factor signaling: a new therapeutic opportunity in cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 18, 1855–1862. doi: 10.1158/1078-0432.CCR-11-0699

Brown, J. L., Cao, Z. A., Pinzon-Ortiz, M., Kendrew, J., Reimer, C., Wen, S., et al. (2010). A human monoclonal anti-ANG2 antibody leads to broad antitumor activity in combination with VEGF inhibitors and chemotherapy agents in preclinical models. *Mol. Cancer Ther.* 9, 145–156. doi: 10.1158/1535-7163.MCT-09-0554

Bryan, B. A., and D'Amore, P. A. (2007). What tangled webs they weave: Rho-GTPase control of angiogenesis. *Cell. Mol. Life Sci. CMLS* 64, 2053–2065. doi: 10.1007/s00018-007-7008-z

Bussolati, B., Deambrosis, I., Russo, S., Deregibus, M. C., and Camussi, G. (2003). Altered angiogenesis and survival in human tumor-derived endothelial cells. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 17, 1159–1161. doi: 10.1096/fj.02-0557fje

Camby, I., Le Mercier, M., Lefranc, F., and Kiss, R. (2006). Galectin-1: a small protein with major functions. *Glycobiology* 16, 137R–157R. doi: 10.1093/glycob/cwl025

Cao, Z., Shang, B., Zhang, G., Miele, L., Sarkar, F. H., Wang, Z., et al. (2013). Tumor cell-mediated neovascularization and lymphangiogenesis contrive tumor progression and cancer metastasis. *Biochim. Biophys. Acta* 1836, 273–286. doi: 10.1016/j.bbcan.2013.08.001

Carbonell, W. S., DeLay, M., Jahangiri, A., Park, C. C., and Aghi, M. K. (2013). β1 integrin targeting potentiates antiangiogenic therapy and inhibits the growth of bevacizumab-resistant glioblastoma. *Cancer Res.* 73, 3145–3154. doi: 10.1158/0008-5472.CAN-13-0011

Casanovas, O., Hicklin, D. J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 8, 299–309. doi: 10.1016/j.ccr.2005.09.005

Cascone, T., Herynk, M. H., Xu, L., Du, Z., Kadara, H., Nilsson, M. B., et al. (2011). Upregulated stromal EGFR and vascular remodeling in mouse xenograft models of angiogenesis inhibitor-resistant human lung adenocarcinoma. *J. Clin. Invest.* 121, 1313–1328. doi: 10.1172/JCI42405

Cassim, S., Vučetiæ, M., Žďralevíæ, M., and Pouyssegur, J. (2020). Warburg and beyond: the power of mitochondrial metabolism to collaborate or replace fermentative glycolysis in cancer. *Cancers* 12:1119. doi: 10.3390/cancers12051119

Ceradini, D. J., Kulkarni, A. R., Callaghan, M. J., Tepper, O. M., Bastidas, N., Kleinman, M. E., et al. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat. Med.* 10, 858–864. doi: 10.1038/nm1075

Chandra, A., Rick, J., Yagnik, G., and Aghi, M. K. (2019). Autophagy as a mechanism for anti-angiogenic therapy resistance. *Semin. Cancer Biol.* doi: 10.1016/j.semcancer.2019.08.031

Chen, A., Sceneay, J., Gödde, N., Kinwel, T., Ham, S., Thompson, E. W., et al. (2018). Intermittent hypoxia induces a metastatic phenotype in breast cancer. *Oncogene* 37, 4214–4225. doi: 10.1038/s41388-018-0259-3

Chen, W., Hill, H., Christie, A., Kim, M. S., Holloman, E., Pavia-Jimenez, A., et al. (2016). Targeting renal cell carcinoma with a HIF-2 antagonist. *Nature* 539, 112–117. doi: 10.1038/nature19796

Chen, X., Liao, R., Li, D., and Sun, J. (2016). Induced cancer stem cells generated by radiochemotherapy and their therapeutic implications. *Oncotarget* 8, 17301–17312. doi: 10.18632/oncotarget.14230

Cheng, H., Wang, Z., Fu, L., and Xu, T. (2019). Macrophage polarization in the development and progression of ovarian cancers: an overview. *Front. Oncol.* 9:421. doi: 10.3389/fonc.2019.00421

Cheng, L., Huang, Z., Zhou, W., Wu, Q., Donnola, S., Liu, J. K., et al. (2013). Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. *Cell* 153, 139–152. doi: 10.1016/j.cell.2013.02.021

Choueiri, T. K., Escudier, B., Powles, T., Tannir, N. M., Mainwaring, P. N., Rini, B. I., et al. (2016). Cabozantinib versus everolimus in advanced renal cell carcinoma (METEOR): final results from a randomised, open-label, phase 3 trial. *Lancet Oncol.* 17, 917–927. doi: 10.1016/S1470-2045(16)30107-3

Choueiri, T. K., Halabi, S., Sanford, B. L., Hahn, O., Michaelson, M. D., Walsh, M. K., et al. (2017). Cabozantinib versus sunitinib as initial targeted therapy for patients with metastatic renal cell carcinoma of poor or intermediate risk: the alliance A031203 CABOSUN trial. *J. Clin. Oncol.* 35, 591–597. doi: 10.1200/JCO.2016.70.7398

Christiansen, A., and Detmar, M. (2011). Lymphangiogenesis and cancer. *Genes Cancer* 2, 1146–1158. doi: 10.1177/1947601911423028

Chun, T.-H., Sabeh, F., Ota, I., Murphy, H., McDonagh, K. T., Holmbeck, K., et al. (2004). MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. *J. Cell Biol.* 167, 757–767. doi: 10.1083/jcb.200405001

Chung, A. S., Kowanetz, M., Wu, X., Zhuang, G., Ngu, H., Finkle, D., et al. (2012). Differential drug class-specific metastatic effects following treatment with a panel of angiogenesis inhibitors. *J. Pathol.* 227, 404–416. doi: 10.1002/path.4052

Ciesielski, O., Biesiekierska, M., Panthu, B., Vialichka, V., Pirola, L., and Balcerczyk, A. (2020). The epigenetic profile of tumor endothelial cells. effects of combined therapy with antiangiogenic and epigenetic drugs on cancer progression. *Int. J. Mol. Sci.* 21:2606. doi: 10.3390/ijms21072606

Comunanza, V., and Bussolino, F. (2017). Therapy for cancer: strategy of combining anti-angiogenic and target therapies. *Front. Cell Dev. Biol.* 5:101. doi: 10.3389/fcell.2017.00101

Conley, S. J., Gheordunescu, E., Kakarala, P., Newman, B., Korkaya, H., Heath, A. N., et al. (2012). Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2784–2789. doi: 10.1073/pnas.1018866109

Cooke, V. G., LeBleu, V. S., Keskin, D., Khan, Z., O'Connell, J. T., Teng, Y., et al. (2012). Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell* 21, 66–81. doi: 10.1016/j.ccr.2011.11.024

Cordes, N., and Park, C. C. (2007). beta1 integrin as a molecular therapeutic target. *Int. J. Radiat. Biol.* 83, 753–760. doi: 10.1080/09553000701639694

Crawford, T. N., Alfaro, D. V., Kerrison, J. B., and Jablon, E. P. (2009). Diabetic retinopathy and angiogenesis. *Curr. Diabetes Rev.* 5, 8–13. doi: 10.2174/157339909787314149

Crawford, Y., and Ferrara, N. (2009). Tumor and stromal pathways mediating refractoriness/resistance to anti-angiogenic therapies. *Trends Pharmacol. Sci.* 30, 624–630. doi: 10.1016/j.tips.2009.09.004

Croci, D. O., Cerliani, J. P., Dalotto-Moreno, T., Méndez-Huergo, S. P., Mascanfroni, I. D., Dergan-Dylon, S., et al. (2014). Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. *Cell* 156, 744–758. doi: 10.1016/j.cell.2014.01.043

David, R. (2012). Metabolism: keeping fit with autophagy. *Nat. Rev. Mol. Cell Biol.* 13:136. doi: 10.1038/nrm3287

De Palma, M., and Naldini, L. (2011). Angiopoietin-2 TIEs up macrophages in tumor angiogenesis. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 17, 5226–5232. doi: 10.1158/1078-0432.CCR-10-0171

Delgado-Bellido, D., Serrano-Saenz, S., Fernández-Cortés, M., and Oliver, F. J. (2017). Vasculogenic mimicry signaling revisited: focus on non-vascular VE-cadherin. *Mol. Cancer* 16:65. doi: 10.1186/s12943-017-0631-x

Depner, C., Zum Buttel, H., Böğürcü, N., Cuesta, A. M., Aburto, M. R., Seidel, S., et al. (2016). EphrinB2 repression through ZEB2 mediates tumour invasion and anti-angiogenic resistance. *Nat. Commun.* 7:12329. doi: 10.1038/ncomms12329

Derynck, R., Akhurst, R. J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nat. Genet.* 29, 117–129. doi: 10.1038/ng1001-117

Deryugina, E. I., and Quigley, J. P. (2010). Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: contrasting, overlapping and compensatory functions. *Biochim. Biophys. Acta* 1803, 103–120. doi: 10.1016/j.bbamcr.2009.09.017

Desantis, V., Saltarella, I., Lamanuzzi, A., Mariggiò, M. A., Racanelli, V., Vacca, A., et al. (2018). Autophagy: a new mechanism of prosurvival and drug resistance in multiple myeloma. *Transl. Oncol.* 11, 1350–1357. doi: 10.1016/j.tranon.2018.08.014

Diaz-Montero, C. M., Salem, M. L., Nishimura, M. I., Garrett-Mayer, E., Cole, D. J., and Montero, A. J. (2009). Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol. Immunother. CII* 58, 49–59. doi: 10.1007/s00262-008-0523-4

Dieterich, L. C., Ikenberg, K., Cetintas, T., Kapaklikaya, K., Hutmacher, C., and Detmar, M. (2017). Tumor-associated lymphatic vessels upregulate PDL1 to inhibit T-Cell activation. *Front. Immunol.* 8:66. doi: 10.3389/fimmu.2017.00066

Dineen, S. P., Lynn, K. D., Holloway, S. E., Miller, A. F., Sullivan, J. P., Shames, D. S., et al. (2008). Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. *Cancer Res.* 68, 4340–4346. doi: 10.1158/0008-5472.CAN-07-6705

Dong, J., Grunstein, J., Tejada, M., Peale, F., Frantz, G., Liang, W.-C., et al. (2004). VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. *EMBO J.* 23, 2800–2810. doi: 10.1038/sj.emboj.7600289

Dufies, M., Giuliano, S., Ambrosetti, D., Claren, A., Ndiaye, P. D., Mastri, M., et al. (2017a). Sunitinib Stimulates expression of VEGFC by tumor cells and promotes lymphangiogenesis in clear cell renal cell carcinomas. *Cancer Res.* 77, 1212–1226. doi: 10.1158/0008-5472.CAN-16-3088

Dufies, M., Giuliano, S., Viotti, J., Borchiellini, D., Cooley, L. S., Ambrosetti, D., et al. (2017b). CXCL7 is a predictive marker of sunitinib efficacy in clear cell renal cell carcinomas. *Br. J. Cancer* 117, 947–953. doi: 10.1038/bjc.2017.276

Dufies, M., Grytsai, O., Ronco, C., Camara, O., Ambrosetti, D., Hagege, A., et al. (2019). New CXCR1/CXCR2 inhibitors represent an effective treatment for kidney or head and neck cancers sensitive or refractory to reference treatments. *Theranostics* 9, 5332–5346. doi: 10.7150/thno.34681

Ebos, J. M. L., and Kerbel, R. S. (2011). Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nat. Rev. Clin. Oncol.* 8, 210–221. doi: 10.1038/nrclinonc.2011.21

Ebos, J. M. L., Lee, C. R., Christensen, J. G., Mutsaers, A. J., and Kerbel, R. S. (2007). Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17069–17074. doi: 10.1073/pnas.0708148104

Ebos, J. M. L., Lee, C. R., Cruz-Munoz, W., Bjarnason, G. A., Christensen, J. G., and Kerbel, R. S. (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15, 232–239. doi: 10.1016/j.ccr.2009.01.021

Ehrmann, R. L., and Knoth, M. (1968). Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. *J. Natl. Cancer Inst.* 41, 1329–1341.

Erra Díaz, F., Dantas, E., and Geffner, J. (2018). Unravelling the interplay between extracellular acidosis and immune cells. *Mediators Inflamm.* 2018:1218297. doi: 10.1155/2018/1218297

Escudier, B., Bellmunt, J., Négrier, S., Bajetta, E., Melichar, B., Bracarda, S., et al. (2010). Phase III trial of bevacizumab plus interferon alfa-2a in patients with metastatic renal cell carcinoma (AVOREN): final analysis of overall survival. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 28, 2144–2150. doi: 10.1200/JCO.2009.26.7849

Escudier, B., Porta, C., Bono, P., Powles, T., Eisen, T., Sternberg, C. N., et al. (2014). Randomized, controlled, double-blind, cross-over trial assessing treatment preference for pazopanib versus sunitinib in patients with metastatic renal cell carcinoma: PISCES Study. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 32, 1412–1418. doi: 10.1200/JCO.2013.50.8267

Faes, S., Santoro, T., Demartines, N., and Dormond, O. (2017). Evolving significance and future relevance of anti-angiogenic activity of mTOR inhibitors in cancer therapy. *Cancers* 9:152. doi: 10.3390/cancers9110152

Fagiani, E., and Christofori, G. (2013). Angiopoietins in angiogenesis. *Cancer Lett.* 328, 18–26. doi: 10.1016/j.canlet.2012.08.018

Falcon, B. L., Chintharlapalli, S., Uhlik, M. T., and Pytowski, B. (2016). Antagonist antibodies to vascular endothelial growth factor receptor 2 (VEGFR-2) as anti-angiogenic agents. *Pharmacol. Ther.* 164, 204–225. doi: 10.1016/j.pharmthera.2016.06.001

Favaro, E., Lord, S., Harris, A. L., and Buffa, F. M. (2011). Gene expression and hypoxia in breast cancer. *Genome Med.* 3:55. doi: 10.1186/gm271

Ferrara, N., and Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161, 851–858. doi: 10.1016/0006-291x(89)92678-8

Ferrari, G., Cook, B. D., Terushkin, V., Pintucci, G., and Mignatti, P. (2009). Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. *J. Cell. Physiol.* 219, 449–458. doi: 10.1002/jcp.21706

Finke, J., Ko, J., Rini, B., Rayman, P., Ireland, J., and Cohen, P. (2011). MDSC as a mechanism of tumor escape from sunitinib mediated anti-angiogenic therapy. *Int. Immunopharmacol.* 11, 856–861. doi: 10.1016/j.intimp.2011.01.030

Finke, J. H., Rini, B., Ireland, J., Rayman, P., Richmond, A., Golshayan, A., et al. (2008). Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 14, 6674–6682. doi: 10.1158/1078-0432.CCR-07-5212

Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., et al. (2007). Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 131, 463–475. doi: 10.1016/j.cell.2007.08.038

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285, 1182–1186. doi: 10.1056/NEJM197111182852108

Folkman, J. (1972). Anti-angiogenesis: new concept for therapy of solid tumors. *Ann. Surg.* 175, 409–416. doi: 10.1097/00000658-197203000-00014

Folkman, J., and Shing, Y. (1992). Angiogenesis. *J. Biol. Chem.* 267, 10931–10934.

Foubert, P., and Varner, J. A. (2012). Integrins in tumor angiogenesis and lymphangiogenesis. *Methods Mol. Biol. Clifton. NJ* 757, 471–486. doi: 10.1007/978-1-61779-166-6_27

Fransvea, E., Mazzocca, A., Antonaci, S., and Giannelli, G. (2009). Targeting transforming growth factor (TGF)-βRI inhibits activation of β1 integrin and blocks vascular invasion in hepatocellular carcinoma. *Hepatology* 49, 839–850. doi: 10.1002/hep.22731

Frentzas, S., Simoneau, E., Bridgeman, V. L., Vermeulen, P. B., Foo, S., Kostaras, E., et al. (2016). Vessel co-option mediates resistance to anti-angiogenic therapy in liver metastases. *Nat. Med.* 22, 1294–1302. doi: 10.1038/nm.4197

Fujita, K., and Akita, M. (2017). Tumor angiogenesis: a focus on the role of cancer stem cells. physiol. pathol. *Angiogenesis Signal. Mech. Target. Ther.* 2017:215. doi: 10.5772/66402

Garg, M. (2013). Epithelial-mesenchymal transition - activating transcription factors - multifunctional regulators in cancer. *World J. Stem Cells* 5, 188–195. doi: 10.4252/wjsc.v5.i4.188

Gewirtz, D. A. (2014). The autophagic response to radiation: relevance for radiation sensitization in cancer therapy. *Radiat. Res.* 182, 363–367. doi: 10.1667/RR13774.1

Giuliano, S., Cormerais, Y., Dufies, M., Grépin, R., Colosetti, P., Belaid, A., et al. (2015). Resistance to sunitinib in renal clear cell carcinoma results from sequestration in lysosomes and inhibition of the autophagic flux. *Autophagy* 11, 1891–1904. doi: 10.1080/15548627.2015.1085742

Giuliano, S., Dufies, M., Ndiaye, P. D., Viotti, J., Borchiellini, D., Parola, J., et al. (2019). Resistance to lysosomotropic drugs used to treat kidney and breast cancers involves autophagy and inflammation and converges in inducing CXCL5. *Theranostics* 9, 1181–1199. doi: 10.7150/thno.29093

Goede, V., Coutelle, O., Neuneier, J., Reinacher-Schick, A., Schnell, R., Koslowsky, T. C., et al. (2010). Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy. *Br. J. Cancer* 103, 1407–1414. doi: 10.1038/sj.bjc.6605925

Goel, S., Duda, D. G., Xu, L., Munn, L. L., Boucher, Y., Fukumura, D., et al. (2011). Normalization of the vasculature for treatment of cancer and other diseases. *Physiol. Rev.* 91, 1071–1121. doi: 10.1152/physrev.00038.2010

Goveia, J., Rholenova, K., Taverna, F., Treps, L., Conradi, L.-C., Pircher, A., et al. (2020). An integrated gene expression landscape profiling approach to identify

lung tumor endothelial cell heterogeneity and angiogenic candidates. *Cancer Cell* 37, 21–36. doi: 10.1016/j.ccell.2019.12.001

Greenblatt, M., and Shubi, P. (1968). Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. *J. Natl. Cancer Inst.* 41, 111–124.

Grépin, R., Guyot, M., Giuliano, S., Boncompagni, M., Ambrosetti, D., Chamorey, E., et al. (2014). The CXCL7/CXCR1/2 axis is a key driver in the growth of clear cell renal cell carcinoma. *Cancer Res.* 74, 873–883. doi: 10.1158/0008-5472.CAN-13-1267

Grepin, R., Guyot, M., Jacquin, M., Durivault, J., Chamorey, E., Sudaka, A., et al. (2012). Acceleration of clear cell renal cell carcinoma growth in mice following bevacizumab/Avastin treatment: the role of CXCL cytokines. *Oncogene* 31, 1683–1694. doi: 10.1038/onc.2011.360

Grunewald, M., Avraham, I., Dor, Y., Bachar-Lustig, E., Itin, A., Jung, S., et al. (2006). VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 124, 175–189. doi: 10.1016/j.cell.2005.10.036

Guyot, M., and Pagès, G. (2015). "VEGF splicing and the role of VEGF splice variants: from physiological-pathological conditions to specific Pre-mRNA splicing," in *VEGF Signaling Methods in Molecular Biology*, ed. L. Fiedler (New York, NY: Springer New York), 3–23. doi: 10.1007/978-1-4939-2917-7_1

Gyanchandani, R., Ortega Alves, M. V., Myers, J. N., and Kim, S. (2013). A proangiogenic signature is revealed in FGF-mediated bevacizumab-resistant head and neck squamous cell carcinoma. *Mol. Cancer Res. MCR* 11, 1585–1596. doi: 10.1158/1541-7786.MCR-13-0358

Haas, N. B., Manola, J., Uzzo, R. G., Flaherty, K. T., Wood, C. G., Kane, C., et al. (2016). Adjuvant sunitinib or sorafenib for high-risk, non-metastatic renal-cell carcinoma (ECOG-ACRIN E2805): a double-blind, placebo-controlled, randomised, phase 3 trial. *Lancet Lond. Engl.* 387, 2008–2016. doi: 10.1016/S0140-6736(16)00559-6

Haibe, Y., Kreidieh, M., El Hajj, H., Khalifeh, I., Mukherji, D., Temraz, S., et al. (2020). Resistance mechanisms to anti-angiogenic therapies in cancer. *Front. Oncol.* 10:221. doi: 10.3389/fonc.2020.00221

Hainsworth, J. D., Spigel, D. R., Sosman, J. A., Burris, H. A., Farley, C., Cucullu, H., et al. (2007). Treatment of advanced renal cell carcinoma with the combination bevacizumab/erlotinib/imatinib: a phase I/II trial. *Clin. Genitourin. Cancer* 5, 427–432. doi: 10.3816/CGC.2007.n.030

Halestrap, A. P., and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem. J.* 343(Pt 2), 281–299.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. *Science* 277, 48–50. doi: 10.1126/science.277.5322.48

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364. doi: 10.1016/s0092-8674(00)80108-7

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hantelys, F., Godet, A.-C., David, F., Tatin, F., Renaud-Gabardos, E., Pujol, F., et al. (2019). Vasohibin1, a new mouse cardiomyocyte IRES trans-acting factor that regulates translation in early hypoxia. *eLife* 8:e50094. doi: 10.7554/eLife.50094

Harris, A. L. (2002). Hypoxia — a key regulatory factor in tumour growth. *Nat. Rev. Cancer* 2, 38–47. doi: 10.1038/nrc704

Harshman, L. C., and Srinivas, S. (2010). The bevacizumab experience in advanced renal cell carcinoma. *OncoTargets Ther.* 3, 179–189.

Hart, K. C., Robertson, S. C., Kanemitsu, M. Y., Meyer, A. N., Tynan, J. A., and Donoghue, D. J. (2000). Transformation and Stat activation by derivatives of FGFR1. *FGFR3, and FGFR4. Oncogene* 19, 3309–3320. doi: 10.1038/sj.onc.1203650

Hasnis, E., Alishekevitz, D., Gingis-Veltski, S., Bril, R., Fremder, E., Voloshin, T., et al. (2014). Anti-Bv8 antibody and metronomic gemcitabine improve pancreatic adenocarcinoma treatment outcome following weekly gemcitabine therapy. *Neoplasia N. Y. N.* 16, 501–510. doi: 10.1016/j.neo.2014.05.011

Hazzard, T. M., and Stouffer, R. L. (2000). Angiogenesis in ovarian follicular and luteal development. *Best Pract. Res. Clin. Obstet. Gynaecol.* 14, 883–900. doi: 10.1053/beog.2000.0133

Heidenreich, R., Röcken, M., and Ghoreschi, K. (2009). Angiogenesis drives psoriasis pathogenesis. *Int. J. Exp. Pathol.* 90, 232–248. doi: 10.1111/j.1365-2613.2009.00669.x

Helfrich, I., Edler, L., Sucker, A., Thomas, M., Christian, S., Schadendorf, D., et al. (2009). Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 15, 1384–1392. doi: 10.1158/1078-0432.CCR-08-1615

Hida, K., Hida, Y., Amin, D. N., Flint, A. F., Panigrahy, D., Morton, C. C., et al. (2004). Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* 64, 8249–8255. doi: 10.1158/0008-5472.CAN-04-1567

Hidalgo, M., Martinez-Garcia, M., Le Tourneau, C., Massard, C., Garralda, E., Boni, V., et al. (2018). First-in-human phase I study of single-agent vanucizumab, a first-in-class bispecific anti-Angiopoietin-2/Anti-VEGF-A antibody, in adult patients with advanced solid tumors. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 24, 1536–1545. doi: 10.1158/1078-0432.CCR-17-1588

Hirota, K., and Semenza, G. L. (2006). Regulation of angiogenesis by hypoxia-inducible factor 1. *Crit. Rev. Oncol. Hematol.* 59, 15–26. doi: 10.1016/j.critrevonc.2005.12.003

Hirth, J., Watkins, P. B., Strawderman, M., Schott, A., Bruno, R., and Baker, L. H. (2000). The effect of an individual's cytochrome CYP3A4 activity on docetaxel clearance. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 6, 1255–1258.

Holmgaard, R. B., Schaer, D. A., Li, Y., Castaneda, S. P., Murphy, M. Y., Xu, X., et al. (2018). Targeting the TGFβ pathway with galunisertib, a TGFβRI small molecule inhibitor, promotes anti-tumor immunity leading to durable, complete responses, as monotherapy and in combination with checkpoint blockade. *J. Immunother. Cancer* 6:47. doi: 10.1186/s40425-018-0356-4

Holmgaard, R. B., Zamarin, D., Lesokhin, A., Merghoub, T., and Wolchok, J. D. (2016). Targeting myeloid-derived suppressor cells with colony stimulating factor-1 receptor blockade can reverse immune resistance to immunotherapy in indoleamine 2,3-dioxygenase-expressing tumors. *EBioMedicine* 6, 50–58. doi: 10.1016/j.ebiom.2016.02.024

Holmgren, L., O'Reilly, M. S., and Folkman, J. (1995). Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat. Med.* 1, 149–153. doi: 10.1038/nm0295-149

Hosaka, K., Yang, Y., Nakamura, M., Andersson, P., Yang, X., Zhang, Y., et al. (2018). Dual roles of endothelial FGF-2–FGFR1–PDGF-BB and perivascular FGF-2–FGFR2–PDGFRβ signaling pathways in tumor vascular remodeling. *Cell Discov.* 4:3. doi: 10.1038/s41421-017-0002-1

Hosaka, K., Yang, Y., Seki, T., Fischer, C., Dubey, O., Fredlund, E., et al. (2016). Pericyte–fibroblast transition promotes tumor growth and metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 113, E5618–E5627. doi: 10.1073/pnas.1608384113

Hsieh, J. J., Purdue, M. P., Signoretti, S., Swanton, C., Albiges, L., Schmidinger, M., et al. (2017). Renal cell carcinoma. *Nat. Rev. Dis. Primer* 3:17009. doi: 10.1038/nrdp.2017.9

Hsu, Y.-L., Wu, C.-Y., Hung, J.-Y., Lin, Y.-S., Huang, M.-S., and Kuo, P.-L. (2013). Galectin-1 promotes lung cancer tumor metastasis by potentiating integrin α6β4 and Notch1/Jagged2 signaling pathway. *Carcinogenesis* 34, 1370–1381. doi: 10.1093/carcin/bgt040

Hu, P.-H., Pan, L.-H., Wong, P. T.-Y., Chen, W.-H., Yang, Y.-Q., Wang, H., et al. (2016). 125I-labeled anti-bFGF monoclonal antibody inhibits growth of hepatocellular carcinoma. *World J. Gastroenterol.* 22, 5033–5041. doi: 10.3748/wjg.v22.i21.5033

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., et al. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* 350, 2335–2342. doi: 10.1056/NEJMoa032691

Hwang, K.-E., Kim, Y.-S., Jung, J.-W., Kwon, S.-J., Park, D.-S., Cha, B.-K., et al. (2015). Inhibition of autophagy potentiates pemetrexed and simvastatin-induced apoptotic cell death in malignant mesothelioma and non-small cell lung cancer cells. *Oncotarget* 6, 29482–29496.

Jahangiri, A., Aghi, M. K., and Carbonell, W. S. (2014). β1 integrin: critical path to antiangiogenic therapy resistance and beyond. *Cancer Res.* 74, 3–7. doi: 10.1158/0008-5472.CAN-13-1742

Jahangiri, A., De Lay, M., Miller, L. M., Carbonell, W. S., Hu, Y.-L., Lu, K., et al. (2013). Gene expression profile identifies tyrosine kinase c-Met as a targetable mediator of antiangiogenic therapy resistance. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 19, 1773–1783. doi: 10.1158/1078-0432.CCR-12-1281

Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307, 58–62. doi: 10.1126/science.1104819

Jain, R. K., and Duda, D. G. (2003). Role of bone marrow-derived cells in tumor angiogenesis and treatment. *Cancer Cell* 3, 515–516. doi: 10.1016/S1535-6108(03)00138-7

Janku, F., McConkey, D. J., Hong, D. S., and Kurzrock, R. (2011). Autophagy as a target for anticancer therapy. *Nat. Rev. Clin. Oncol.* 8, 528–539. doi: 10.1038/nrclinonc.2011.71

Janning, M., Müller, V., Vettorazzi, E., Cubas-Cordova, M., Gensch, V., Ben-Batalla, I., et al. (2019). Evaluation of soluble carbonic anhydrase IX as predictive marker for efficacy of bevacizumab: a biomarker analysis from the geparquinto phase III neoadjuvant breast cancer trial. *Int. J. Cancer* 145, 857–868. doi: 10.1002/ijc.32163

Jayson, G. C., Zhou, C., Backen, A., Horsley, L., Marti-Marti, K., Shaw, D., et al. (2018). Plasma Tie2 is a tumor vascular response biomarker for VEGF inhibitors in metastatic colorectal cancer. *Nat. Commun.* 9 :4672. doi: 10.1038/s41467-018-07174-1

Jeansson, M., Gawlik, A., Anderson, G., Li, C., Kerjaschki, D., Henkelman, M., et al. (2011). Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. *J. Clin. Invest.* 121, 2278–2289. doi: 10.1172/JCI46322

Jeon, H.-M., and Lee, J. (2017). MET: roles in epithelial-mesenchymal transition and cancer stemness. *Ann. Transl. Med.* 5:5. doi: 10.21037/atm.2016.12.67

Jiménez-Valerio, G., and Casanovas, O. (2017). Angiogenesis and metabolism: entwined for therapy resistance. *Trends Cancer* 3, 10–18. doi: 10.1016/j.trecan.2016.11.007

Joseph, J. P., Harishankar, M. K., Pillai, A. A., and Devi, A. (2018). Hypoxia induced EMT: a review on the mechanism of tumor progression and metastasis in OSCC. *Oral Oncol.* 80, 23–32. doi: 10.1016/j.oraloncology.2018.03.004

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., et al. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* 15, 290–298.

Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., et al. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246, 1309–1312. doi: 10.1126/science.2479987

Kerbel, R., and Folkman, J. (2002). Clinical translation of angiogenesis inhibitors. *Nat. Rev. Cancer* 2, 727–739. doi: 10.1038/nrc905

Kerbel, R. S. (2008). Tumor angiogenesis. *N. Engl. J. Med.* 358, 2039–2049. doi: 10.1056/NEJMra0706596

Kienast, Y., Klein, C., Scheuer, W., Raemsch, R., Lorenzon, E., Bernicke, D., et al. (2013). Ang-2-VEGF-A CrossMab, a novel bispecific human IgG1 antibody blocking VEGF-A and Ang-2 functions simultaneously, mediates potent antitumor, antiangiogenic, and antimetastatic efficacy. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 19, 6730–6740. doi: 10.1158/1078-0432.CCR-13-0081

Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., et al. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362, 841–844. doi: 10.1038/362841a0

Kioi, M., Vogel, H., Schultz, G., Hoffman, R. M., Harsh, G. R., and Brown, J. M. (2010). Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J. Clin. Invest.* 120, 694–705. doi: 10.1172/JCI40283

Knighton, D. R., Hunt, T. K., Scheuenstuhl, H., Halliday, B. J., Werb, Z., and Banda, M. J. (1983). Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science* 221, 1283–1285. doi: 10.1126/science.6612342

Ko, J. S., Zea, A. H., Rini, B. I., Ireland, J. L., Elson, P., Cohen, P., et al. (2009). Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 15, 2148–2157. doi: 10.1158/1078-0432.CCR-08-1332

Ko, S. Y., Lee, W., Kenny, H. A., Dang, L. H., Ellis, L. M., Jonasch, E., et al. (2019). Cancer-derived small extracellular vesicles promote angiogenesis by heparin-bound, bevacizumab-insensitive VEGF, independent of vesicle uptake. *Commun. Biol.* 2, 1–17. doi: 10.1038/s42003-019-0609-x

Kopetz, S., Hoff, P. M., Morris, J. S., Wolff, R. A., Eng, C., Glover, K. Y., et al. (2010). Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 28, 453–459. doi: 10.1200/JCO.2009.24.8252

Kuczynski, E. A., Vermeulen, P. B., Pezzella, F., Kerbel, R. S., and Reynolds, A. R. (2019). Vessel co-option in cancer. *Nat. Rev. Clin. Oncol.* 16, 469–493. doi: 10.1038/s41571-019-0181-9

Kuczynski, E. A., Yin, M., Bar-Zion, A., Lee, C. R., Butz, H., Man, S., et al. (2016). Co-option of liver vessels and not sprouting angiogenesis drives acquired sorafenib resistance in hepatocellular carcinoma. *JNCI J. Natl. Cancer Inst.* 108:djw030. doi: 10.1093/jnci/djw030

Kumar, K., Wigfield, S., Gee, H. E., Devlin, C. M., Singleton, D., Li, J.-L., et al. (2013). Dichloroacetate reverses the hypoxic adaptation to bevacizumab and enhances its antitumor effects in mouse xenografts. *J. Mol. Med. Berl. Ger.* 91, 749–758. doi: 10.1007/s00109-013-0996-2

Laderoute, K. R., Alarcon, R. M., Brody, M. D., Calaoagan, J. M., Chen, E. Y., Knapp, A. M., et al. (2000). Opposing effects of hypoxia on expression of the angiogenic inhibitor thrombospondin 1 and the angiogenic inducer vascular endothelial growth factor. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 6, 2941–2950.

L'Allemain, G., Paris, S., and Pouysségur, J. (1985). Role of a Na+-dependent Cl-/HCO3- exchange in regulation of intracellular pH in fibroblasts. *J. Biol. Chem.* 260, 4877–4883.

Lam, H.-M., Vessella, R. L., and Morrissey, C. (2014). The role of the microenvironment – dormant prostate disseminated tumor cells in the bone marrow. *Drug Discov. Today Technol.* 11, 41–47. doi: 10.1016/j.ddtec.2014.02.002

LaVallee, T. M., Prudovsky, I. A., McMahon, G. A., Hu, X., and Maciag, T. (1998). Activation of the MAP kinase pathway by FGF-1 correlates with cell proliferation induction while activation of the Src pathway correlates with migration. *J. Cell Biol.* 141, 1647–1658. doi: 10.1083/jcb.141.7.1647

Lee, E. Q., Muzikansky, A., Duda, D. G., Gaffey, S., Dietrich, J., Nayak, L., et al. (2019). Phase II trial of ponatinib in patients with bevacizumab-refractory glioblastoma. *Cancer Med.* 8, 5988–5994. doi: 10.1002/cam4.2505

Leenders, W. P. J., Küsters, B., Verrijp, K., Maass, C., Wesseling, P., Heerschap, A., et al. (2004). Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 10, 6222–6230. doi: 10.1158/1078-0432.CCR-04-0823

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306–1309. doi: 10.1126/science.2479986

Levéen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E., and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* 8, 1875–1887. doi: 10.1101/gad.8.16.1875

Li, F., Zhou, K., Gao, L., Zhang, B., Li, W., Yan, W., et al. (2016). Radiation induces the generation of cancer stem cells: a novel mechanism for cancer radioresistance. *Oncol. Lett.* 12, 3059–3065. doi: 10.3892/ol.2016.5124

Li, Y.-J., Lei, Y.-H., Yao, N., Wang, C.-R., Hu, N., Ye, W.-C., et al. (2017). Autophagy and multidrug resistance in cancer. *Chin. J. Cancer* 36:52. doi: 10.1186/s40880-017-0219-2

Lin, J., Sampath, D., Nannini, M. A., Lee, B. B., Degtyarev, M., Oeh, J., et al. (2013). Targeting activated Akt with GDC-0068, a novel selective Akt inhibitor that is efficacious in multiple tumor models. *Clin. Cancer Res.* 19, 1760–1772. doi: 10.1158/1078-0432.CCR-12-3072

Liu, B. Y., Soloviev, I., Chang, P., Lee, J., Huang, X., Zhong, C., et al. (2010). Stromal cell-derived factor-1/CXCL12 contributes to MMTV-Wnt1 tumor growth involving Gr1+CD11b+ cells. *PLoS One* 5:e8611. doi: 10.1371/journal.pone.0008611

Liu, K.-W., Hu, B., and Cheng, S.-Y. (2011). Platelet-derived growth factor signaling in human malignancies. *Chin. J. Cancer* 30, 581–584. doi: 10.5732/cjc.011.10300

Liu, L., Yang, L., Yan, W., Zhai, J., Pizzo, D. P., Chu, P., et al. (2018). Chemotherapy induces breast cancer stemness in association with dysregulated monocytosis. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 24, 2370–2382. doi: 10.1158/1078-0432.CCR-17-2545

Liu, T., Ma, W., Xu, H., Huang, M., Zhang, D., He, Z., et al. (2018). PDGF-mediated mesenchymal transformation renders endothelial resistance to anti-VEGF treatment in glioblastoma. *Nat. Commun.* 9, 1–13. doi: 10.1038/s41467-018-05982-z

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.-F., et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med.* 359, 378–390. doi: 10.1056/NEJMoa0708857

Lu, K. V., Chang, J. P., Parachoniak, C. A., Pandika, M. M., Aghi, M. K., Meyronet, D., et al. (2012). VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. *Cancer Cell* 22, 21–35. doi: 10.1016/j.ccr.2012.05.037

Luan, W., Pang, Y., Li, R., Wei, X., Jiao, X., Shi, J., et al. (2019). Akt/mTOR-mediated autophagy confers resistance to Bet inhibitor JQ1 In ovarian cancer. *OncoTargets Ther.* 12, 8063–8074. doi: 10.2147/OTT.S220267

Maacha, S., Bhat, A. A., Jimenez, L., Raza, A., Haris, M., Uddin, S., et al. (2019). Extracellular vesicles-mediated intercellular communication: roles in the tumor microenvironment and anti-cancer drug resistance. *Mol. Cancer* 18:55. doi: 10.1186/s12943-019-0965-7

MacDonagh, L., Gallagher, M. F., Ffrench, B., Gasch, C., Breen, E., Gray, S. G., et al. (2017). Targeting the cancer stem cell marker, aldehyde dehydrogenase 1, to circumvent cisplatin resistance in NSCLC. *Oncotarget* 8, 72544–72563. doi: 10.18632/oncotarget.19881

Maione, F., Capano, S., Regano, D., Zentilin, L., Giacca, M., Casanovas, O., et al. (2012). Semaphorin 3A overcomes cancer hypoxia and metastatic dissemination induced by antiangiogenic treatment in mice. *J. Clin. Invest.* 122, 1832–1848. doi: 10.1172/JCI58976

Maishi, N., Annan, D. A., Kikuchi, H., Hida, Y., and Hida, K. (2019). Tumor endothelial heterogeneity in cancer progression. *Cancers* 11:1511. doi: 10.3390/cancers11101511

Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., et al. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277, 55–60. doi: 10.1126/science.277.5322.55

Maniotis, A. J., Folberg, R., Hess, A., Seftor, E. A., Gardner, L. M., Pe'er, J., et al. (1999). Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am. J. Pathol.* 155, 739–752. doi: 10.1016/S0002-9440(10)65173-5

Manzat Saplacan, R. M., Balacescu, L., Gherman, C., Chira, R. I., Craiu, A., Mircea, P. A., et al. (2017). The role of PDGFs and PDGFRs in colorectal cancer. *Mediators Inflamm.* 2017:e4708076. doi: 10.1155/2017/4708076

Marigo, I., Dolcetti, L., Serafini, P., Zanovello, P., and Bronte, V. (2008). Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. *Immunol. Rev.* 222, 162–179. doi: 10.1111/j.1600-065X.2008.00602.x

Martin, J. D., Seano, G., and Jain, R. K. (2019). Normalizing function of tumor vessels: progress. opportunities, and challenges. *Annu. Rev. Physiol.* 81, 505–534. doi: 10.1146/annurev-physiol-020518-114700

Martínez-Sáez, O., Gajate Borau, P., Alonso-Gordoa, T., Molina-Cerrillo, J., and Grande, E. (2017). Targeting HIF-2 α in clear cell renal cell carcinoma: a promising therapeutic strategy. *Crit. Rev. Oncol. Hematol.* 111, 117–123. doi: 10.1016/j.critrevonc.2017.01.013

Massagué, J. (2000). How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* 1, 169–178. doi: 10.1038/35043051

Mathew, R., Karantza-Wadsworth, V., and White, E. (2007). Role of autophagy in cancer. *Nat. Rev. Cancer* 7, 961–967. doi: 10.1038/nrc2254

Mazzieri, R., Pucci, F., Moi, D., Zonari, E., Ranghetti, A., Berti, A., et al. (2011). Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell* 19, 512–526. doi: 10.1016/j.ccr.2011.02.005

McIntyre, A., and Harris, A. L. (2015). Metabolic and hypoxic adaptation to anti-angiogenic therapy: a target for induced essentiality. *EMBO Mol. Med.* 7, 368–379. doi: 10.15252/emmm.201404271

Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E. A., et al. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N. Engl. J. Med.* 357, 2666–2676. doi: 10.1056/NEJMoa072113

Mitsuhashi, A., Goto, H., Saijo, A., Trung, V. T., Aono, Y., Ogino, H., et al. (2015). Fibrocyte-like cells mediate acquired resistance to anti-angiogenic therapy with bevacizumab. *Nat. Commun.* 6:8792. doi: 10.1038/ncomms9792

Mittal, V. (2018). Epithelial mesenchymal transition in tumor metastasis. *Annu. Rev. Pathol.* 13, 395–412. doi: 10.1146/annurev-pathol-020117-043854

Miyazawa, K., Ohtomo, T., Moriya, S., Naito, M., Kuroda, M., Itoh, M., et al. (2010). Cytoprotective autophagy induction by imatinib mesylate in Non-BCR-ABL expressing cells. *Blood* 116, 4937–4937. doi: 10.1182/blood.V116.21.4937.4937

Mizumoto, A., Yamamoto, K., Nakayama, Y., Takara, K., Nakagawa, T., Hirano, T., et al. (2015). Induction of epithelial-mesenchymal transition via activation of epidermal growth factor receptor contributes to sunitinib resistance in human renal cell carcinoma cell lines. *J. Pharmacol. Exp. Ther.* 355, 152–158. doi: 10.1124/jpet.115.226639

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev.* 21, 2861–2873. doi: 10.1101/gad.1599207

Mohammed, R. A. A., Green, A., El-Shikh, S., Paish, E. C., Ellis, I. O., and Martin, S. G. (2007). Prognostic significance of vascular endothelial cell growth factors -A, -C and -D in breast cancer and their relationship with angio- and lymphangiogenesis. *Br. J. Cancer* 96, 1092–1100. doi: 10.1038/sj.bjc.6603678

Morfoisse, F., Kuchnio, A., Frainay, C., Gomez-Brouchet, A., Delisle, M.-B., Marzi, S., et al. (2014). Hypoxia induces VEGF-C expression in metastatic tumor cells via a HIF-1α-independent translation-mediated mechanism. *Cell Rep.* 6, 155–167. doi: 10.1016/j.celrep.2013.12.011

Motzer, R. J., Escudier, B., Powles, T., Scheffold, C., and Choueiri, T. K. (2018). Long-term follow-up of overall survival for cabozantinib versus everolimus in advanced renal cell carcinoma. *Br. J. Cancer* 118, 1176–1178. doi: 10.1038/s41416-018-0061-6

Motzer, R. J., Escudier, B., Tomczak, P., Hutson, T. E., Michaelson, M. D., Negrier, S., et al. (2013). Axitinib versus sorafenib as second-line treatment for advanced renal cell carcinoma: overall survival analysis and updated results from a randomised phase 3 trial. *Lancet Oncol.* 14, 552–562. doi: 10.1016/S1470-2045(13)70093-7

Motzer, R. J., Hutson, T. E., Glen, H., Michaelson, M. D., Molina, A., Eisen, T., et al. (2015). Lenvatinib, everolimus, and the combination in patients with metastatic renal cell carcinoma: a randomised, phase 2, open-label, multicentre trial. *Lancet Oncol.* 16, 1473–1482. doi: 10.1016/S1470-2045(15)00290-9

Motzer, R. J., Hutson, T. E., Ren, M., Dutcus, C., and Larkin, J. (2016). Independent assessment of lenvatinib plus everolimus in patients with metastatic renal cell carcinoma. *Lancet Oncol.* 17, e4–e5. doi: 10.1016/S1470-2045(15)00543-4

Motzer, R. J., Hutson, T. E., Tomczak, P., Michaelson, M. D., Bukowski, R. M., Oudard, S., et al. (2009). Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 27, 3584–3590. doi: 10.1200/JCO.2008.20.1293

Motzer, R. J., Penkov, K., Haanen, J., Rini, B., Albiges, L., Campbell, M. T., et al. (2019). Avelumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. *N. Engl. J. Med.* 380, 1103–1115. doi: 10.1056/NEJMoa1816047

Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410, 50–56. doi: 10.1038/35065016

Murdoch, C., Tazzyman, S., Webster, S., and Lewis, C. E. (2007). Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J. Immunol. Baltim. Md* 1950, 7405–7411. doi: 10.4049/jimmunol.178.11.7405

Nakajima, E. C., and Van Houten, B. (2013). Metabolic symbiosis in cancer: refocusing the Warburg lens. *Mol. Carcinog.* 52, 329–337. doi: 10.1002/mc.21863

Nakashima, O., Sugihara, S., Kage, M., and Kojiro, M. (1995). Pathomorphologic characteristics of small hepatocellular carcinoma: a special reference to small hepatocellular carcinoma with indistinct margins. *Hepatol. Baltim. Md* 22, 101–105.

Navis, A. C., Bourgonje, A., Wesseling, P., Wright, A., Hendriks, W., Verrijp, K., et al. (2013). Effects of dual targeting of tumor cells and stroma in human glioblastoma xenografts with a tyrosine kinase inhibitor against c-MET and VEGFR2. *PLoS One* 8:e58262. doi: 10.1371/journal.pone.0058262

Ndiaye, P. D., Dufies, M., Giuliano, S., Douguet, L., Grépin, R., Durivault, J., et al. (2019). VEGFC acts as a double-edged sword in renal cell carcinoma aggressiveness. *Theranostics* 9, 661–675. doi: 10.7150/thno.27794

Nishida, N., Yano, H., Nishida, T., Kamura, T., and Kojiro, M. (2006). Angiogenesis in Cancer. *Vasc. Health Risk Manag.* 2, 213–219.

Norden, A. D., Drappatz, J., and Wen, P. Y. (2009). Antiangiogenic therapies for high-grade glioma. *Nat. Rev. Neurol.* 5, 610–620. doi: 10.1038/nrneurol.2009.159

Norden, A. D., Schiff, D., Ahluwalia, M. S., Lesser, G. J., Nayak, L., Lee, E. Q., et al. (2015). Phase II trial of triple tyrosine kinase receptor inhibitor nintedanib in recurrent high-grade gliomas. *J. Neurooncol.* 121, 297–302. doi: 10.1007/s11060-014-1631-y

Offersen, B. V., Pfeiffer, P., Hamilton-Dutoit, S., and Overgaard, J. (2001). Patterns of angiogenesis in nonsmall-cell lung carcinoma. *Cancer* 91, 1500–1509.

Ogawa, M., Yamamoto, H., Nagano, H., Miyake, Y., Sugita, Y., Hata, T., et al. (2004). Hepatic expression of ANG2 RNA in metastatic colorectal cancer. *Hepatol. Baltim. Md* 39, 528–539. doi: 10.1002/hep.20048

Olive, K. P., Jacobetz, M. A., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., et al. (2009). Inhibition of hedgehog signaling enhances delivery of

chemotherapy in a mouse model of pancreatic cancer. *Science* 324, 1457–1461. doi: 10.1126/science.1171362

O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. (1996). Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* 2, 689–692. doi: 10.1038/nm0696-689

Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., et al. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121, 335–348. doi: 10.1016/j.cell.2005.02.034

Orlidge, A., and D'Amore, P. A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J. Cell Biol.* 105, 1455–1462. doi: 10.1083/jcb.105.3.1455

Ornitz, D. M., and Itoh, N. (2015). The fibroblast growth factor signaling pathway. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 215–266. doi: 10.1002/wdev.176

Osawa, T., Ohga, N., Akiyama, K., Hida, Y., Kitayama, K., Kawamoto, T., et al. (2013). Lysyl oxidase secreted by tumour endothelial cells promotes angiogenesis and metastasis. *Br. J. Cancer* 109, 2237–2247. doi: 10.1038/bjc.2013.535

Pàez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Viñals, F., et al. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15, 220–231. doi: 10.1016/j.ccr.2009.01.027

Papadopoulos, N., and Lennartsson, J. (2018). The PDGF/PDGFR pathway as a drug target. *Mol. Aspects Med.* 62, 75–88. doi: 10.1016/j.mam.2017.11.007

Pardali, E., Goumans, M.-J., and ten Dijke, P. (2010). Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol.* 20, 556–567. doi: 10.1016/j.tcb.2010.06.006

Park, S. Y., Piao, Y., Jeong, K. J., Dong, J., and de Groot, J. F. (2016). Periostin (POSTN) Regulates tumor resistance to antiangiogenic therapy in glioma models. *Mol. Cancer Ther.* 15, 2187–2197. doi: 10.1158/1535-7163.MCT-15-0427

Parks, S. K., Mazure, N. M., Counillon, L., and Pouysségur, J. (2013). Hypoxia promotes tumor cell survival in acidic conditions by preserving ATP levels. *J. Cell. Physiol.* 228, 1854–1862. doi: 10.1002/jcp.24346

Parks, S. K., Mueller-Klieser, W., and Pouysségur, J. (2020). Lactate and acidity in the cancer microenvironment. *Annu. Rev. Cancer Biol.* 4, 141–158. doi: 10.1146/annurev-cancerbio-030419-033556

Pasquier, E., and Dias, S. (2010). Endothelial progenitor cells: hope beyond controversy. *Curr. Cancer Drug Targets* 10, 914–921. doi: 10.2174/15680091079 3358041

Patel, S. B., Stenehjem, D. D., Gill, D. M., Tantravahi, S. K., Agarwal, A. M., Hsu, J., et al. (2016). Everolimus versus temsirolimus in metastatic renal cell carcinoma after progression with previous systemic therapies. *Clin. Genitourin. Cancer* 14, 153–159. doi: 10.1016/j.clgc.2015.12.011

Paul, D., Lipton, A., and Klinger, I. (1971). Serum factor requirements of normal and simian virus 40-transformed 3T3 mouse fibroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 68, 645–652. doi: 10.1073/pnas.68.3.645

Pezzella, F., Pastorino, U., Tagliabue, E., Andreola, S., Sozzi, G., Gasparini, G., et al. (1997). Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. *Am. J. Pathol.* 151, 1417–1423.

Piao, Y., Liang, J., Holmes, L., Zurita, A. J., Henry, V., Heymach, J. V., et al. (2012). Glioblastoma resistance to anti-VEGF therapy is associated with myeloid cell infiltration, stem cell accumulation, and a mesenchymal phenotype. *Neuro Oncol.* 14, 1379–1392. doi: 10.1093/neuonc/nos158

Pietras, K., and Hanahan, D. (2005). A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 23, 939–952. doi: 10.1200/JCO.2005.07.093

Pinto, M. P., Sotomayor, P., Carrasco-Avino, G., Corvalan, A. H., and Owen, G. I. (2016). Escaping antiangiogenic therapy: strategies employed by cancer cells. *Int. J. Mol. Sci.* 17:1489. doi: 10.3390/ijms17091489

Pisarsky, L., Bill, R., Fagiani, E., Dimeloe, S., Goosen, R. W., Hagmann, J., et al. (2016). Targeting Metabolic symbiosis to overcome resistance to anti-angiogenic therapy. *Cell Rep.* 15, 1161–1174. doi: 10.1016/j.celrep.2016.04.028

Powell, M. A., Sill, M. W., Goodfellow, P. J., Benbrook, D. M., Lankes, H. A., Leslie, K. K., et al. (2014). A phase II trial of brivanib in recurrent or persistent endometrial cancer: an NRG Oncology/Gynecologic oncology group study. *Gynecol. Oncol.* 135, 38–43. doi: 10.1016/j.ygyno.2014.07.083

Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., et al. (1997). Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res.* 57, 4593–4599.

Provenzano, P. P., and Hingorani, S. R. (2013). Hyaluronan, fluid pressure, and stromal resistance in pancreas cancer. *Br. J. Cancer* 108, 1–8. doi: 10.1038/bjc.2012.569

Puente, P., de la Muz, B., Azab, F., and Azab, A. K. (2013). Cell trafficking of endothelial progenitor cells in tumor progression. *Clin. Cancer Res.* 19, 3360–3368. doi: 10.1158/1078-0432.CCR-13-0462

Qin, S., Li, A., Yi, M., Yu, S., Zhang, M., and Wu, K. (2019). Recent advances on anti-angiogenesis receptor tyrosine kinase inhibitors in cancer therapy. *J. Hematol. Oncol.* 12:27. doi: 10.1186/s13045-019-0718-5

Rapisarda, A., and Melillo, G. (2009). Role of the hypoxic tumor microenvironment in the resistance to anti-angiogenic therapies. *Drug Resist. Updat. Rev. Comment. Antimicrob. Anticancer Chemother.* 12, 74–80. doi: 10.1016/j.drup.2009.03.002

Ravaud, A., Motzer, R. J., Pandha, H. S., George, D. J., Pantuck, A. J., Patel, A., et al. (2016). Adjuvant sunitinib in high-risk renal-cell carcinoma after nephrectomy. *N. Engl. J. Med.* 375, 2246–2254. doi: 10.1056/NEJMoa1611406

Reale, A., Melaccio, A., Lamanuzzi, A., Saltarella, I., Dammacco, F., Vacca, A., et al. (2016). Functional and biological role of endothelial precursor cells in tumour progression: a new potential therapeutic target in haematological malignancies. *Stem Cells Int.* 2016:11. doi: 10.1155/2016/7954580

Ries, C. H., Cannarile, M. A., Hoves, S., Benz, J., Wartha, K., Runza, V., et al. (2014). Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. *Cancer Cell* 25, 846–859. doi: 10.1016/j.ccr.2014.05.016

Rini, B. I., Plimack, E. R., Stus, V., Gafanov, R., Hawkins, R., Nosov, D., et al. (2019a). Pembrolizumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. *N. Engl. J. Med.* 380, 1116–1127. doi: 10.1056/NEJMoa1816714

Rini, B. I., Powles, T., Atkins, M. B., Escudier, B., McDermott, D. F., Suarez, C., et al. (2019b). Atezolizumab plus bevacizumab versus sunitinib in patients with previously untreated metastatic renal cell carcinoma (IMmotion151): a multicentre, open-label, phase 3, randomised controlled trial. *Lancet* 393, 2404–2415. doi: 10.1016/S0140-6736(19)30723-8

Rizzo, M., and Porta, C. (2017). Sunitinib in the treatment of renal cell carcinoma: an update on recent evidence. *Ther. Adv. Urol.* 9, 195–207. doi: 10.1177/1756287217713902

Rock, E. P., Goodman, V., Jiang, J. X., Mahjoob, K., Verbois, S. L., Morse, D., et al. (2007). Food and drug administration drug approval summary: sunitinib malate for the treatment of gastrointestinal stromal tumor and advanced renal cell carcinoma. *Oncologist* 12, 107–113. doi: 10.1634/theoncologist.12-1-107

Rohlenova, K., Goveia, J., Garcia-Caballero, M., Subramanian, A., Kalucka, J., Treps, L., et al. (2020). Single-Cell RNA sequencing maps endothelialmetabolic plasticity in pathological angiogenesis. *Cell Metab.* 31, 862–877. doi: 10.1016/j.cmet.2020.03.009

Ruffell, B., and Coussens, L. M. (2015). Macrophages and therapeutic resistance in cancer. *Cancer Cell* 27, 462–472. doi: 10.1016/j.ccell.2015.02.015

Russo, A. E., Priolo, D., Antonelli, G., Libra, M., McCubrey, J. A., and Ferraù, F. (2017). Bevacizumab in the treatment of NSCLC: patient selection and perspectives. *Lung Cancer Targets Ther.* 8, 259–269. doi: 10.2147/LCTT.S110306

Salnikov, A. V., Heldin, N.-E., Stuhr, L. B., Wiig, H., Gerber, H., Reed, R. K., et al. (2006). Inhibition of carcinoma cell-derived VEGF reduces inflammatory characteristics in xenograft carcinoma. *Int. J. Cancer* 119, 2795–2802. doi: 10.1002/ijc.22217

Sandler, A., Gray, R., Perry, M. C., Brahmer, J., Schiller, J. H., Dowlati, A., et al. (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N. Engl. J. Med.* 355, 2542–2550. doi: 10.1056/NEJMoa061884

Sarode, P., Schaefer, M. B., Grimminger, F., Seeger, W., and Savai, R. (2020). Macrophage and tumor cell cross-talk is fundamental for lung tumor progression: we need to talk. *Front. Oncol.* 10:324. doi: 10.3389/fonc.2020.00324

Schech, A., Kazi, A., Yu, S., Shah, P., and Sabnis, G. (2015). Histone deacetylase inhibitor entinostat inhibits tumor-initiating cells in triple-negative breast cancer cells. *Mol. Cancer Ther.* 14, 1848–1857. doi: 10.1158/1535-7163.MCT-14-0778

Schmittnaegel, M., Rigamonti, N., Kadioglu, E., Cassará, A., Wyser Rmili, C., Kiialainen, A., et al. (2017). Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci. Transl. Med.* 9:eaak9670. doi: 10.1126/scitranslmed.aak9670

Schreiber, A. B., Winkler, M. E., and Derynck, R. (1986). Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. *Science* 232, 1250–1253. doi: 10.1126/science.2422759

Semenza, G. L. (2014). Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu. Rev. Pathol.* 9, 47–71. doi: 10.1146/annurev-pathol-012513-104720

Semrad, T. J., Kim, E. J., Tanaka, M. S., Sands, J., Roberts, C., Burich, R. A., et al. (2017). Phase II study of Dovitinib in patients progressing on anti-vascular endothelial growth factor therapy. *Cancer Treat. Res. Commun.* 10, 21–26. doi: 10.1016/j.ctarc.2016.12.002

Senft, D., and Ronai, Z. A. (2016). Immunogenic, cellular, and angiogenic drivers of tumor dormancy-a melanoma view. *Pigment Cell Melanoma Res.* 29, 27–42. doi: 10.1111/pcmr.12432

Sennino, B., Ishiguro-Oonuma, T., Wei, Y., Naylor, R. M., Williamson, C. W., Bhagwandin, V., et al. (2012). Suppression of tumor invasion and metastasis by concurrent inhibition of c-Met and VEGF signaling in pancreatic neuroendocrine tumors. *Cancer Discov.* 2, 270–287. doi: 10.1158/2159-8290.CD-11-0240

Serafini, P., Borrello, I., and Bronte, V. (2006). Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin. Cancer Biol.* 16, 53–65. doi: 10.1016/j.semcancer.2005.07.005

Serova, M., Tijeras-Raballand, A., Santos, C. D., Martinet, M., Neuzillet, C., Lopez, A., et al. (2016). Everolimus affects vasculogenic mimicry in renal carcinoma resistant to sunitinib. *Oncotarget* 7, 38467–38486. doi: 10.18632/oncotarget.9542

Sfiligoi, C., de Luca, A., Cascone, I., Sorbello, V., Fuso, L., Ponzone, R., et al. (2003). Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. *Int. J. Cancer* 103, 466–474. doi: 10.1002/ijc.10851

Shibata, M., and Hoque, M. O. (2019). Targeting cancer stem cells: a strategy for effective eradication of cancer. *Cancers* 11:732. doi: 10.3390/cancers11050732

Shih, T., and Lindley, C. (2006). Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. *Clin. Ther.* 28, 1779–1802. doi: 10.1016/j.clinthera.2006.11.015

Shimizu, S., Takehara, T., Hikita, H., Kodama, T., Tsunematsu, H., Miyagi, T., et al. (2012). Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma. *Int. J. Cancer* 131, 548–557. doi: 10.1002/ijc.26374

Shirakawa, K., Kobayashi, H., Heike, Y., Kawamoto, S., Brechbiel, M. W., Kasumi, F., et al. (2002). Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenograft. *Cancer Res.* 62, 560–566.

Shojaei, F., and Ferrara, N. (2008). Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. *Cancer Res.* 68, 5501–5504. doi: 10.1158/0008-5472.CAN-08-0925

Shojaei, F., Wu, X., Malik, A. K., Zhong, C., Baldwin, M. E., Schanz, S., et al. (2007a). Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat. Biotechnol.* 25, 911–920. doi: 10.1038/nbt1323

Shojaei, F., Wu, X., Qu, X., Kowanetz, M., Yu, L., Tan, M., et al. (2009). G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6742–6747. doi: 10.1073/pnas.0902280106

Shojaei, F., Wu, X., Zhong, C., Yu, L., Liang, X.-H., Yao, J., et al. (2007b). Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450, 825–831. doi: 10.1038/nature06348

Sidorov, M., Jahangiri, A., Han, S.-W., De Lay, M., Wagner, J., Castro, B., et al. (2016). 340 c-Met/β1 integrin: a receptor complex driving invasive glioblastoma resistance to antiangiogenic therapy. *Neurosurgery* 63, 199–200. doi: 10.1227/01.neu.0000489829.38251.85

Simon, T., Pinioti, S., Schellenberger, P., Rajeeve, V., Wendler, F., Cutillas, P. R., et al. (2018). Shedding of bevacizumab in tumour cells-derived extracellular vesicles as a new therapeutic escape mechanism in glioblastoma. *Mol. Cancer* 17:132. doi: 10.1186/s12943-018-0878-x

Singh, M., Couto, S. S., Forrest, W. F., Lima, A., Cheng, J. H., Molina, R., et al. (2012). Anti-VEGF antibody therapy does not promote metastasis in genetically engineered mouse tumour models. *J. Pathol.* 227, 417–430. doi: 10.1002/path.4053

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., et al. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res.* 63, 5821–5828.

Song, E., Mao, T., Dong, H., Boisserand, L. S. B., Antila, S., Bosenberg, M., et al. (2020). VEGF-C-driven lymphatic drainage enables immunosurveillance of brain tumours. *Nature* 577, 689–694. doi: 10.1038/s41586-019-1912-x

Sood, A. K., Seftor, E. A., Fletcher, M. S., Gardner, L. M., Heidger, P. M., Buller, R. E., et al. (2001). Molecular determinants of ovarian cancer plasticity. *Am. J. Pathol.* 158, 1279–1288. doi: 10.1016/S0002-9440(10)64079-5

Sounni, N. E., Cimino, J., Blacher, S., Primac, I., Truong, A., Mazzucchelli, G., et al. (2014). Blocking lipid synthesis overcomes tumor regrowth and metastasis after antiangiogenic therapy withdrawal. *Cell Metab.* 20, 280–294. doi: 10.1016/j.cmet.2014.05.022

Sparmann, A., and Bar-Sagi, D. (2004). Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 6, 447–458. doi: 10.1016/j.ccr.2004.09.028

Stanley, P. (2014). Galectin-1 pulls the strings on VEGFR2. *Cell* 156, 625–626. doi: 10.1016/j.cell.2014.01.059

Sternlicht, M. D., Lochter, A., Sympson, C. J., Huey, B., Rougier, J. P., Gray, J. W., et al. (1999). The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98, 137–146. doi: 10.1016/s0092-8674(00)81009-0

Stratmann, A., Risau, W., and Plate, K. H. (1998). Cell type-specific expression of Angiopoietin-1 and Angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am. J. Pathol.* 153, 1459–1466.

Sun, H., Zhang, D., Yao, Z., Lin, X., Liu, J., Gu, Q., et al. (2017). Anti-angiogenic treatment promotes triple-negative breast cancer invasion via vasculogenic mimicry. *Cancer Biol. Ther.* 18, 205–213. doi: 10.1080/15384047.2017.1294288

Suyama, K., and Iwase, H. (2018). Lenvatinib: a promising molecular targeted agent for multiple cancers. *Cancer Control J. Moffitt Cancer Cent.* 25:1073274818789361. doi: 10.1177/1073274818789361

Tang, D., Gao, J., Wang, S., Ye, N., Chong, Y., Huang, Y., et al. (2016). Cancer-associated fibroblasts promote angiogenesis in gastric cancer through galectin-1 expression. *Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med.* 37, 1889–1899. doi: 10.1007/s13277-015-3942-9

Thijssen, V. L., Paulis, Y. W., Nowak-Sliwinska, P., Deumelandt, K. L., Hosaka, K., Soetekouw, P. M., et al. (2018). Targeting PDGF-mediated recruitment of pericytes blocks vascular mimicry and tumor growth. *J. Pathol.* 246, 447–458. doi: 10.1002/path.5152

Thomas, M., Kienast, Y., Scheuer, W., Bähner, M., Kaluza, K., Gassner, C., et al. (2013). A novel angiopoietin-2 selective fully human antibody with potent anti-tumoral and anti-angiogenic efficacy and superior side effect profile compared to Pan-Angiopoietin-1/-2 inhibitors. *PLoS One* 8:e54923. doi: 10.1371/journal.pone.0054923

Thurston, G., and Daly, C. (2012). The Complex Role of Angiopoietin-2 in the Angiopoietin–Tie Signaling Pathway. *Cold Spring Harb. Perspect. Med.* 2:a006550. doi: 10.1101/cshperspect.a006650

Todorova, D., Simoncini, S., Lacroix, R., Sabatier, F., and Dignat-George, F. (2017). Extracellular vesicles in angiogenesis. *Circ. Res.* 120, 1658–1673. doi: 10.1161/CIRCRESAHA.117.309681

Tong, W.-W., Tong, G.-H., and Liu, Y. (2018). Cancer stem cells and hypoxia-inducible factors (Review). *Int. J. Oncol.* 53, 469–476. doi: 10.3892/ijo.2018.4417

Tonnesen, M. G., Feng, X., and Clark, R. A. (2000). Angiogenesis in wound healing. *J. Investig. Dermatol. Symp. Proc.* 5, 40–46. doi: 10.1046/j.1087-0024.2000.00014.x

Valkenburg, K. C., de Groot, A. E., and Pienta, K. J. (2018). Targeting the tumour stroma to improve cancer therapy. *Nat. Rev. Clin. Oncol.* 15, 366–381. doi: 10.1038/s41571-018-0007-1

van Beijnum, J. R., Nowak-Sliwinska, P., Huijbers, E. J. M., Thijssen, V. L., and Griffioen, A. W. (2015). The great escape; the hallmarks of resistance to antiangiogenic therapy. *Pharmacol. Rev.* 67, 441–461. doi: 10.1124/pr.114.010215

van Cruijsen, H., Giaccone, G., and Hoekman, K. (2005). Epidermal growth factor receptor and angiogenesis: opportunities for combined anticancer strategies. *Int. J. Cancer* 117, 883–888. doi: 10.1002/ijc.21479

van der Schaft, D. W. J., Hillen, F., Pauwels, P., Kirschmann, D. A., Castermans, K., Egbrink, M. G. A. O., et al. (2005). Tumor cell plasticity in Ewing sarcoma, an alternative circulatory system stimulated by hypoxia. *Cancer Res.* 65, 11520–11528. doi: 10.1158/0008-5472.CAN-05-2468

van der Schaft, D. W. J., Pauwels, P., Hulsmans, S., Zimmermann, M., van de Poll-Franse, L. V., and Griffioen, A. W. (2007). Absence of lymphangiogenesis in ductal breast cancer at the primary tumor site. *Cancer Lett.* 254, 128–136. doi: 10.1016/j.canlet.2007.03.001

van der Schaft, D. W. J., Seftor, R. E. B., Seftor, E. A., Hess, A. R., Gruman, L. M., Kirschmann, D. A., et al. (2004). Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells. *J. Natl. Cancer Inst.* 96, 1473–1477. doi: 10.1093/jnci/djh267

van Meeteren, L. A., Goumans, M.-J., and ten Dijke, P. (2011). TGF-β receptor signaling pathways in angiogenesis; emerging targets for anti-angiogenesis therapy. *Curr. Pharm. Biotechnol.* 12, 2108–2120. doi: 10.2174/138920111798808338

Vandercappellen, J., Van Damme, J., and Struyf, S. (2008). The role of CXC chemokines and their receptors in cancer. *Cancer Lett.* 267, 226–244. doi: 10.1016/j.canlet.2008.04.050

Voron, T., Colussi, O., Marcheteau, E., Pernot, S., Nizard, M., Pointet, A.-L., et al. (2015). VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J. Exp. Med.* 212, 139–148. doi: 10.1084/jem.20140559

Walsh, D. A. (2007). Pathophysiological mechanisms of angiogenesis. *Adv. Clin. Chem.* 44, 187–221. doi: 10.1016/s0065-2423(07)44006-9

Wang, F.-T., Sun, W., Zhang, J.-T., and Fan, Y.-Z. (2019). Cancer-associated fibroblast regulation of tumor neo-angiogenesis as a therapeutic target in cancer. *Oncol. Lett.* 17, 3055–3065. doi: 10.3892/ol.2019.9973

Wang, L., Park, H., Chhim, S., Ding, Y., Jiang, W., Queen, C., et al. (2012). A novel monoclonal antibody to fibroblast growth factor 2 effectively inhibits growth of hepatocellular carcinoma xenografts. *Mol. Cancer Ther.* 11, 864–872. doi: 10.1158/1535-7163.MCT-11-0813

Warren, R. S., Yuan, H., Matli, M. R., Gillett, N. A., and Ferrara, N. (1995). Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J. Clin. Invest.* 95, 1789–1797. doi: 10.1172/JCI117857

Wei, Y., Zhou, F., Zhou, H., Huang, J., Yu, D., and Wu, G. (2018). Endothelial progenitor cells contribute to neovascularization of non-small cell lung cancer via histone deacetylase 7-mediated cytoskeleton regulation and angiogenic genes transcription. *Int. J. Cancer* 143, 657–667. doi: 10.1002/ijc.31349

Welti, J. C., Powles, T., Foo, S., Gourlaouen, M., Preece, N., and Foster, J. (2012). Contrasting effects of sunitinib within *in vivo* models of metastasis. *Angiogenesis* 15, 623–641. doi: 10.1007/s10456-012-9291-z

Willett, C. G., Boucher, Y., Duda, D. G., di Tomaso, E., Munn, L. L., Tong, R. T., et al. (2005). Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* 23, 8136–8139. doi: 10.1200/JCO.2005.02.5635

Wilson, W. R., and Hay, M. P. (2011). Targeting hypoxia in cancer therapy. *Nat. Rev. Cancer* 11, 393–410. doi: 10.1038/nrc3064

Wilting, J., and Christ, B. (1996). Embryonic angiogenesis: a review. *Naturwissenschaften* 83, 153–164. doi: 10.1007/bf01143056

Winer, A., Adams, S., and Mignatti, P. (2018). Matrix metalloproteinase inhibitors in cancer therapy: turning past failures into future successes. *Mol. Cancer Ther.* 17, 1147–1155. doi: 10.1158/1535-7163.MCT-17-0646

Winkler, F., Kienast, Y., Fuhrmann, M., Von Baumgarten, L., Burgold, S., Mitteregger, G., et al. (2009). Imaging glioma cell invasion in vivo reveals mechanisms of dissemination and peritumoral angiogenesis. *Glia* 57, 1306–1315. doi: 10.1002/glia.20850

Wolf, A., and Langmann, T. (2019). Anti−VEGF−A/ANG2 combotherapy limits pathological angiogenesis in the eye: a replication study. *EMBO Mol. Med.* 11:e10362. doi: 10.15252/emmm.201910362

Wu, S., Huang, L., Shen, R., Bernard-Cacciarella, M., Zhou, P., Hu, C., et al. (2020). Drug resistance-related sunitinib sequestration in autophagolysosomes of endothelial cells. *Int. J. Oncol.* 56, 113–122. doi: 10.3892/ijo.2019.4924

Xin, X., Yang, S., Ingle, G., Zlot, C., Rangell, L., Kowalski, J., et al. (2001). Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. *Am. J. Pathol.* 158, 1111–1120.

Xiong, Y.-Q., Sun, H.-C., Zhang, W., Zhu, X.-D., Zhuang, P.-Y., Zhang, J.-B., et al. (2009). Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 15, 4838–4846. doi: 10.1158/1078-0432.CCR-08-2780

Xu, R., Rai, A., Chen, M., Suwakulsiri, W., Greening, D. W., and Simpson, R. J. (2018). Extracellular vesicles in cancer - implications for future improvements in cancer care. *Nat. Rev. Clin. Oncol.* 15, 617–638. doi: 10.1038/s41571-018-0036-9

Xu, Y., Li, Q., Li, X.-Y., Yang, Q.-Y., Xu, W.-W., and Liu, G.-L. (2012). Short-term anti-vascular endothelial growth factor treatment elicits vasculogenic mimicry formation of tumors to accelerate metastasis. *J. Exp. Clin. Cancer Res. CR* 31:16. doi: 10.1186/1756-9966-31-16

Yadav, A. S., Pandey, P. R., Butti, R., Radharani, N. N. V., Roy, S., Bhalara, S. R., et al. (2018). The biology and therapeutic implications of tumor dormancy and reactivation. *Front. Oncol.* 8:72. doi: 10.3389/fonc.2018.00072

Yamada, Y., Nezu, J., Shimane, M., and Hirata, Y. (1997). Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* 42, 483–488. doi: 10.1006/geno.1997.4774

Yamamoto, K., Ohga, N., Hida, Y., Maishi, N., Kawamoto, T., Kitayama, K., et al. (2012). Biglycan is a specific marker and an autocrine angiogenic factor of tumour endothelial cells. *Br. J. Cancer* 106, 1214–1223. doi: 10.1038/bjc.2012.59

Yang, J. C., Haworth, L., Sherry, R. M., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., et al. (2003). A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N. Engl. J. Med.* 349, 427–434. doi: 10.1056/NEJMoa021491

Yang, L., DeBusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., et al. (2004). Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 6, 409–421. doi: 10.1016/j.ccr.2004.08.031

Yao, H., Price, T. T., Cantelli, G., Ngo, B., Warner, M. J., Olivere, L., et al. (2018). Leukaemia hijacks a neural mechanism to invade the central nervous system. *Nature* 560, 55–60. doi: 10.1038/s41586-018-0342-5

Yu, Z., Pestell, T. G., Lisanti, M. P., and Pestell, R. G. (2012). Cancer stem cells. *Int. J. Biochem. Cell Biol.* 44, 2144–2151. doi: 10.1016/j.biocel.2012.08.022

Zeisberg, M., and Neilson, E. G. (2009). Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* 119, 1429–1437. doi: 10.1172/JCI36183

Zhai, B., Hu, F., Jiang, X., Xu, J., Zhao, D., Liu, B., et al. (2014). Inhibition of Akt reverses the acquired resistance to sorafenib by switching protective autophagy to autophagic cell death in hepatocellular carcinoma. *Mol. Cancer Ther.* 13, 1589–1598. doi: 10.1158/1535-7163.MCT-13-1043

Zhao, Q., Eichten, A., Parveen, A., Adler, C., Huang, Y., Wang, W., et al. (2018). Single-cell transcriptome analyses revealendothelial cell heterogeneity in tumors and changes following antiangiogenic treatment. *Cancer Res.* 78, 2370–2382. doi: 10.1158/0008-5472

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 17 July 2020
doi: 10.3389/fcell.2020.00662

Neuropilins, as Relevant Oncology Target: Their Role in the Tumoral Microenvironment

Aurore Dumond[1]* *and Gilles Pagès*[1,2]*

[1] Medical Biology Department, Centre Scientifique de Monaco, Monaco, Monaco, [2] Inserm U1081, CNRS UMR 7284, Centre Antoine Lacassagne, Institut de Recherche sur le Cancer et le Vieillissement de Nice, Université Côte d'Azur, Nice, France

Angiogenesis is one of the key mechanisms involved in tumor growth and metastatic dissemination. The vascular endothelial growth factor (VEGF) and its receptors (VEGFR) represent one of the major signaling pathways which mediates angiogenesis. The VEGF/VEGFR axis was intensively targeted by monoclonal antibodies or by tyrosine kinase inhibitors to destroy the tumor vascular network. By inhibiting oxygen and nutrient supply, this strategy was supposed to cure cancers. However, despite a lengthening of the progression free survival in several types of tumors including colon, lung, breast, kidney, and ovarian cancers, modest improvements in overall survival were reported. Anti-angiogenic therapies targeting VEGF/VEGFR are still used in colon and ovarian cancer and remain reference treatments for renal cell carcinoma. Although the concept of inhibiting angiogenesis remains relevant, new targets need to be discovered to improve the therapeutic index of anti-VEGF/VEGFR. Neuropilin 1 and 2 (NRP1/2), initially described as neuronal receptors, stimulate angiogenesis, lymphangiogenesis and immune tolerance. Moreover, overexpression of NRPs in several tumors is synonymous of patients' shorter survival. This article aims to overview the different roles of NRPs in cells constituting the tumor microenvironment to highlight the therapeutic relevance of their targeting.

Keywords: neuropilins, tumor microenvironment, oncology, immunology, cancers

OPEN ACCESS

Edited by:
Lucas Treps, VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Luca Tamagnone, Institute for Cancer Research and Treatment (IRCC), Italy
Andrea Casazza, CoBioRes Nv Biotech, Belgium

***Correspondence:**
Aurore Dumond
adumond@centrescientifique.mc
Gilles Pagès
gpages@unice.fr;
gilles.pages@unice.fr

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *13 May 2020*
Accepted: *01 July 2020*
Published: *17 July 2020*

Citation:
Dumond A and Pagès G (2020) Neuropilins, as Relevant Oncology Target: Their Role in the Tumoral Microenvironment. Front. Cell Dev. Biol. 8:662. doi: 10.3389/fcell.2020.00662

GENERALITIES ON THE NEUROPILINS

Genomic Organization and Protein Structure

The Neuropilins are type-1 membrane glycoproteins of 130–140 kDa. Two proteins of the same family, Neuropilin 1 and 2 (NRP1 and NRP2), coded by two different genes on independent chromosomes (10p12 for NRP1 and 2q34 for NRP2), share 44% of sequence homology. They are composed of a N-terminal extracellular domain, a transmembrane domain and a cytoplasmic domain of 43–44 amino acids. The extracellular domain comprises five subdomains: a1, a2, b1, b2, and c. The cytoplasmic part does not contain a signaling domain but has a PDZ domain and a triplet of amino acids "serine, glutamic acid, alanine (SEA)." The PDZ domain enables the formation and the stimulation of signaling complexes. The membrane and cytoplasmic parts are implicated in the receptors' dimerization. Soluble forms of NRP1 and NRP2 (sNRP1, sNRP2) without transmembrane and without cytoplasmic domain and an isoform of NRP2 without the SEA amino acid triplet are formed after alternative splicing.

The Phenotype of Knock-Out Mice

NRP1 gene invalidation (KO) induces defects in vascular, nervous, and cardiac network and leads to an embryonic lethality between 10 and 12.5 days (Kawasaki et al., 1999). The overexpression of NRP1 is lethal for embryos of about 12.5 days with cardiac defects (Kitsukawa et al., 1995).

NRP2 KO is not lethal but a diminution of lymphatic vessels and some abnormalities during the neural development are observed (Yuan et al., 2002).

Mice with a double NRP1 and NRP2 KO present more severe vascular abnormalities and embryos die at 8.5 days (Takashima et al., 2002) with the presence of important avascular zones and of some gaps between the blood vessels.

NRP Ligands

The NRPs bind to specific ligands and form heterodimers with five families of receptors. The dimerized ligands bind to the NRP homo- or heterodimers and to partner receptors dimers to form a complex which induces a specific intracellular signal. The sNRP are competitive forms for the binding of vascular endothelial growth factor (VEGF) to the membrane NRP1.

SEMA3/Plexin

The NRPs were first described as neuronal receptors binding the semaphorins (SEMA, seven classes described) which constitute a family of proteins that guide axons growth and are involved in cell apoptosis, migration and tumor suppression. SEMA3C is involved in endothelial cell apoptosis, it inhibits pathological angiogenesis and it promotes invasion and metastasis in cancers. SEMA3A is an angiogenesis inhibitor, that is less expressed during tumor development. Indeed, it controls pericytes recruitment to vessels (Niland and Eble, 2019). Neuropilins form a complex with SEMA receptors, the plexins. The binding of the SEMA on NRP is established through the a1, a2, b1, and b2 domains (Roy et al., 2017). The ternary complex between NRPs, SEMAs and the plexins enhances signal transduction during development, axon guidance and immunity. NRP1 binds preferentially to SEMA3A and NRP2 to SEMA3C or 3F (Roy et al., 2017). SEMA3E/PlexinD1 pathway is involved in the initial development of axon tracts in the forebrain and in the establishment of functional neuronal networks. Some axons expressed plexinD1 but not NRP1, in this case SEMA3E acts as a repellant. When neurons express plexinD1 and NRP1, SEMA3E is an attractant (Chauvet et al., 2007). The extracellular part of NRP1 is sufficient in inducing the attractive axonal guidance. PlexinD1 is necessary for SEMA3E's effects on axonal guidance. However, NRP1 is necessary to control the gating response of SEMA3E to induce a repulsive or attractive axon growth (Chauvet et al., 2007). According to the major role played by the NRP1/SEMA3E signaling in neurodevelopment, any defect may be related to neural disorder as it was suggested in a mouse model of schizophrenia (Daoust et al., 2014).

VEGF/VEGFR

The VEGF gene is composed of eight exons. Exons 1–5 are implicated in the binding to vascular endothelial growth factor receptors (VEGFR) and exons 7 and 8 in the binding to NRP1 and NRP2 (Guyot and Pages, 2015). The differential splicings of exon 6, 7 and 8 induce two distinct families of isoforms. Isoforms with the exon 8a are pro-angiogenic and isoforms with exon 8b are anti-angiogenic (Harper and Bates, 2008). Four predominant forms of VEGF exist: VEGF121, VEGF189, VEGF206 and the more abundant and active in many cancers, the VEGF165. The VEGF165 binds preferentially to NRP1 (Kd = 0.2 nM) as compared to NRP2 (Kd = 5 nM).

In healthy people, VEGFs are involved in wound healing and vascular homeostasis. However, VEGFs promote tumor angiogenesis and lymphangiogenesis and high levels of VEGFs expression are synonymous of poor prognosis in cancers. NRP1 binds the VEGF165 and the receptors VEGFR1 and 2. VEGF binding stimulates this pathway leading to increased angiogenesis. NRP2 binds the VEGF165 and VEGFC, the main lymphangiogenic factor, and forms a complex with the receptors VEGFR2 or VEGFR3 to stimulate angiogenesis and lymphangiogenesis. The binding occurs through the NRPs' b1 and b2 domains. VEGFR activation by the VEGF does not require the NRP. However, in some tumors, VEGFRs are absent and NRP1 induce cell migration and angiogenesis in a VEGFR-independent manner. VEGF binding to NRP1, independently of VEGFR, activates RhoA and Ras, two effectors of different signaling pathways (Niland and Eble, 2019).

Thus, the stimulation of NRP by the VEGF is highly relevant in a therapeutic context.

PlGF/VEGFR

Placenta growth factor (PlGF) belongs to the VEGFs family and binds to VEGFR1 but not to VEGFR2. It was initially described as a placenta produced homodimeric protein. Three isoforms are initiated from alternative splicing: PlGF1, PlGF2, and PlGF3. PlGF2 is the only form containing exon 6, which codes for an heparin binding domain (Migdal et al., 1998). PlGF2 binds to NRP1 through amino acids encoded by exon 6 and exon 7 and PlGF1 through amino acids encoded by exon 7 (Migdal et al., 1998). In breast cancer, PlGF1 and NRP1 overexpression is correlated to a poor prognosis and PlGF2 is overexpressed in cancer tissues as compared to normal tissue (Escudero-Esparza et al., 2010). The PlGF/NRP pathway is implicated in tumor growth, angiogenesis, migration, and metastasis for melanoma cancers even in the absence of VEGFRs (Pagani et al., 2016). PlGF is also a relevant target in retinal diseases resistant to anti-VEGF therapies (Van Bergen et al., 2019). In the Sonic Hedgehog subgroup of medulloblastoma, PlGF binds to NRP1 leading to mitogen activated protein kinase (MAPK) signaling activation, tumor growth and dissemination (Snuderl et al., 2013). Moreover, the PlGF/NRP signaling pathway plays a key role in resistance to anti-angiogenic therapies (Pagani et al., 2016).

HGF/cMET

The signaling pathway induced by the hepatocyte growth factor (HGF) and its receptor (cMET) regulates endothelial cell survival, proliferation and migration. HGF/cMET complex plays an important role in tumor progression. NRP1, by binding to cMET, induces tumor invasion. As HGF/cMET inhibits apoptosis and promotes immune tolerance by interacting with the programmed

death ligand 1 (PD-L1) (Balan et al., 2015), the stimulation of this signaling pathway by NRP1 promotes tumor growth by inhibiting the antitumor immunity.

TGFβ1/TGFβRs

TGFβ1/TβRs stimulates the SMAD2/3 signaling pathway, which is involved in physiological development, host immunity, inflammation and in tumor progression, and invasion. TGFβ also promotes cancer progression and metastasis (Chaudhary et al., 2014). TGFβ binds to NRP1 via its b1 domain and forms a complex with TGFβ receptors I–III. Activation of this signaling pathway stimulates angiogenesis in a VEGFR2-independent manner. NRP1/TGFβ/TGFβR also promotes T regulatory lymphocytes activity and immune tolerance.

PDGF/PDGFR

The increased expression of PDGF and its receptors on tumor vasculature promotes pathological angiogenesis (Chaudhary et al., 2014). This signaling pathway also induces cell proliferation, differentiation, and epithelial to mesenchymal transition (Niland and Eble, 2019). Four PDGF variant exist: PDGFA, B, C, and D. These ligands bind to the tyrosine-kinase receptors PDGRFα or β. Depending on the ligand, the receptors will homo- or hetero-dimerize giving three possible combinations: αα, αβ, or ββ. PDGF-stimulated PDGFRs activate MAPK and PI3K signaling pathways. NRP1 forms a complex with PDGF and PDGFR amplifying their respective downstream signaling pathways.

FGF/FGFR2

FGF/FGFR2 complex induces cell migration and proliferation. This axis is key for endothelial cell proliferation and subsequent angiogenesis. By forming a complex with the FGFR2, the NRPs play a key role in amplifying its signaling pathways and consequently these biological phenomena.

Galectins

Galectins, part of the family of β-galactoside-binding proteins, are involved in cell-cell and cell-matrix interactions. Galectin-1 (Gal-1) induces tumor-associated HuVEC proliferation and migration, by enhancing VEGFA effects, and HuVEC adhesion. Gal-1 exerts these effects through VEGFR2 phosphorylation enhanced by Gal-1/NRP1 binding (Hsieh et al., 2008). The activation of NRP1/VEGFR1-dependent AKT signal by Gal-1 decreases endothelial-cadherin cell-cell junctions and increases the vascular permeability (Wu et al., 2014).

EGF/EGFR

Epidermal growth factor receptor (EGFR) is a monomeric transmembrane protein. EGFR mutations were described in several forms of cancers, such as breast or lung cancers and it is overexpressed in numerous tumors. EGFR activation stimulates AKT signaling. NRP1 extracellular domain is necessary for EGFR-endocytosis and AKT-dependent cancer cell viability and tumor growth. Hence, reduced expression of NRP1 limits EGFR endocytosis (Rizzolio et al., 2012). Furthermore, NRP2 is required, through WDFY1 (WD-repeat and FYVE-domain-containing protein 1), to activate EGFR endocytosis in cancer cells and to maintain EGFR activities (Dutta et al., 2016).

Hedgehog Signaling Pathway

This pathway is involved in embryogenesis and in adult's tissue healing. Its activation induces cell proliferation and differentiation. Its overexpression or downregulation induces cancer development and the epithelial-mesenchymal transition (EMT). NRPs are major regulators of the Hedgehog signaling pathway. A feedback loop exists between NRP1 and Hedgehog; Hedgehog signaling induces NRP1 expression, which promotes activation of Hedgehog targeted gene (Niland and Eble, 2019). A down-regulation of NRP1 by shRNA in ccRCC cell lines reduces sonic hedgehog (SHH) and its activator Gli1 expressions. SHH signaling pathway inhibition promotes tumor cell differentiation (Cao et al., 2008).

Integrins

NRPs also interacts with integrins. The intercellular interaction between integrins α5β1 and α9β1 expressed on endothelial cells and NRP2 expressed on tumor and endothelial cells increases tumor spreading and metastasis through and integrin-dependent mechanism (Cao et al., 2013; Alghamdi et al., 2020).

THE ROLE OF NEUROPILINS IN THE IMMUNE SYSTEM (FIGURE 1)

Dendritic Cells

They are recruited to the tumor site. After their contact with the antigen, they are maturated, which enables them to migrate to the lymphoid organs to activate naïve T cells and to induce the primary immune response. Two types of dendritic cells (DCs) exists: (i) myeloid DCs (mDCs) that present the antigen to T cells; (ii) plasmacytoid DCs (pDCs), generally involved in immune suppression. Activated pDCs have an antigen presenting capacity, they also activate T cells but to a lesser extent as compared to mDCs.

NRP1 is expressed on mature DC and on naïve T cells. This enables NRP1/NRP1 homophilic interaction and the formation of an immunological synapse between these two cell types. Thus, NRP1 mediates the primary immune response activation by promoting antigen presentation by DCs through this synapse (Sarris et al., 2008; Akkaya et al., 2019). NRP1 regulate cytoskeleton rearrangements allowing their transmigration to the lymphatics and lymphoid tissues to activate T cells. However, at a late stage of T cell activation, SEMA3A is secreted. By its interaction with NRP1 expressed on T cells, it disrupts the formation of the immunological synapse with the DC resulting in reduced T cell activation and immune tolerance (Lepelletier et al., 2006).

NRP2 expression increases during the differentiation from monocytes to dendritic cells (Schellenburg et al., 2017). Its sialylation protects DC during their migration to lymph nodes. In the lymph nodes, the polysialic acid is eliminated of NRP2 and DC activate T cells (Curreli et al., 2007; Rey-Gallardo et al., 2011).

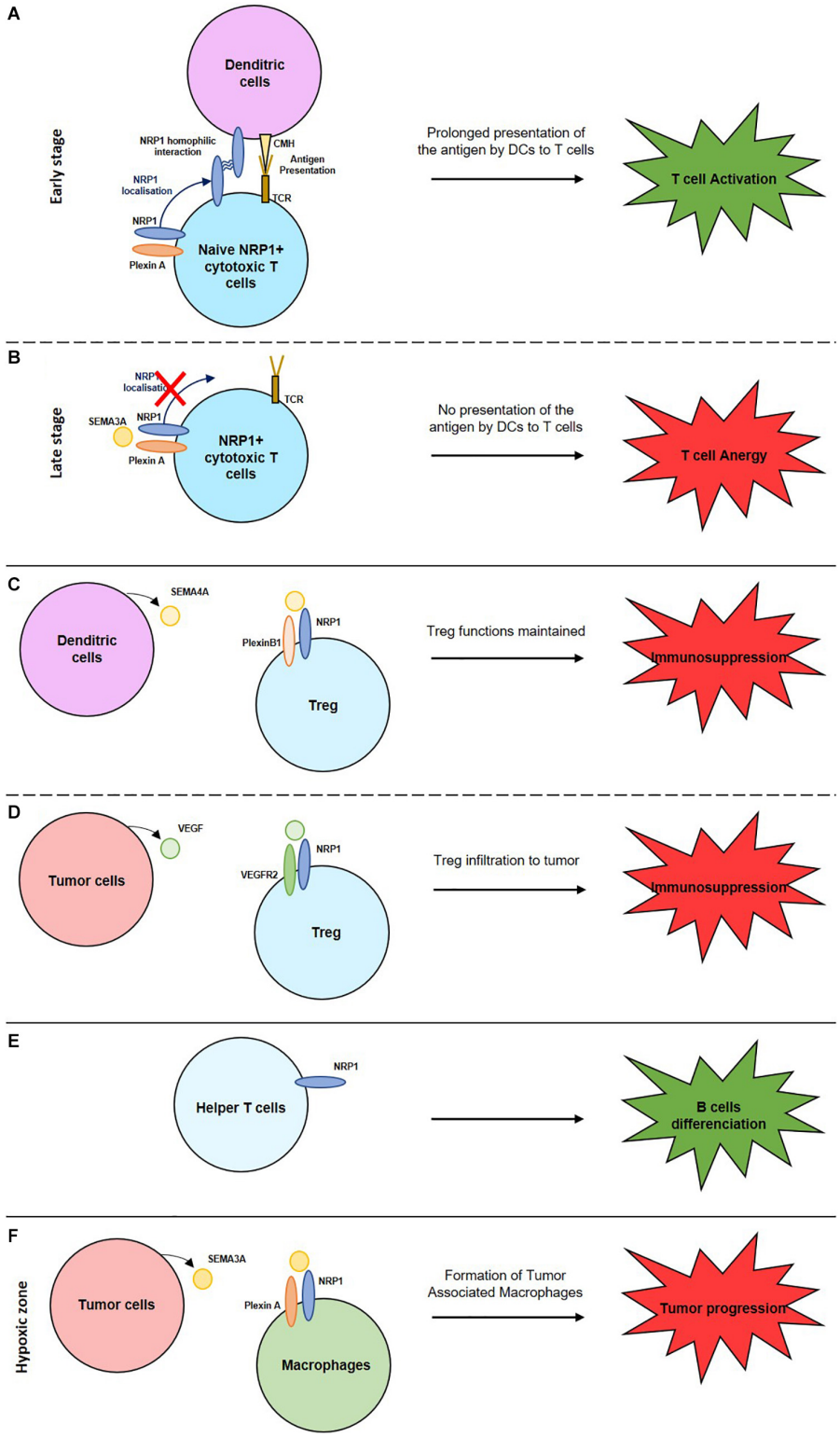


FIGURE 1 | Role of NRPs in the activation or suppression of the immune system. **(A)** NRP1 homophilic interaction enhances the interaction between naive cytotoxic T cells and dendritic cells inducing a prolonged antigen presentation and so T cell activation. **(B)** Expression of SEMA3A by mature cytotoxic T cells inhibits NRP1 localisation and induces T cell anergy. **(C)** Interaction between NRP1, expressed by Treg cells, with SEMA4A, expressed by dendritic cells maintain Treg functions. **(D)** Interaction between NRP1, expressed by Treg cells, with VEGF, expressed by tumor cells, enable Treg cells infiltration into the tumor and induce an immunosuppression. **(E)** NRP1+ helper T cells induce B cells differentiation to activate their immune response. **(F)** Interaction between NRP1, expressed by macrophages, with SEMA3A, expressed by tumor cells, induce the formation of tumor associated macrophages (TAM) and so a tumor progression.

Macrophages

They play a key role in immune surveillance, in cellular debris elimination and in antigen presentation. M1 macrophages are pro-inflammatory and M2 are pro-angiogenic, immunosuppressive, thus pro-tumoral particularly in hypoxic zones. Hypoxia induces SEMA3A expression on tumoral cells. It interacts with NRP1, and their receptors plexin A1 and A4, expressed on macrophages. Tumor-associated macrophages (TAM) reside in the hypoxic zones where they exert their pro-tumoral role. If NRP1 expression decreases, TAM remain in the normoxic peripheric zones of the tumor resulting in the suppression of their pro-tumoral role (Casazza et al., 2013; Chen et al., 2019). In the microglia, NRP1 plays an immune suppressive role by inducing a M2 phenotype. A NRP1/NRP1 homophilic interaction with the helper T cells induces immune suppression. NRP1 expression on glioma-associated macrophages (GAM) induces a pro-tumoral response. NRP1 inhibition reduces tumor growth and a macrophages polarization to an anti-tumoral role (Cherry et al., 2014; Caponegro et al., 2018).

NRP2 expression increases during the differentiation of monocytes to macrophages (Schellenburg et al., 2017) next to inflammatory zones to induce phagocytosis. NRP2 sialylation reduces phagocytosis capacity of the macrophages (Stamatos et al., 2014; Roy et al., 2018), thus NRP2+ M2 macrophages promote tumor progression (Niland and Eble, 2019).

T Cells

They are responsible of the adaptative immune response required for the control and the elimination of pathogenic agents and of tumor cells. Any dysfunctions in their development or activation induce auto-immune diseases and cancers. NRP1 is upregulated on active T cells (Chaudhary et al., 2014). Four types of T cells exist.

Cytotoxic T Cells (T CD8+)

They destroy the infected cells presenting the specific antigen through the class I major histocompatibility complex (MHC). The NRP1 expression is increased on CD8+ effective and memory T cells and promotes the antigen recognition (Roy et al., 2017). However, the exact NRP1 role in this context is unknown. NRP1 expression also correlates with PD1 expression on CD8+ T cells. Thus, NRP1 might represent a relevant biomarker to determine the efficacy of anti-PD1 immunotherapies. Indeed, patients with non-small cell lung cancer invaded with PD1-positive CD8+ T cells are highly responsive to anti-PD1 immunotherapies and present a longer survival (Leclerc et al., 2019).

Helper T Cells (T CD4+)

They are not cytotoxic but produce interleukin 2 and interferon gamma. These cytokines stimulate T and B cell proliferation. NRP1 is expressed on CD4+ T cells and induces B cells differentiation (Roy et al., 2017). Induction of NRP1 on regulatory T cells (Bruder et al., 2004) and on CD4+ T cells (Campos-Mora et al., 2019) induces immunosuppressive functions *in vivo*.

NKT Cells

They constitute a link between innate and adaptative immunity. Once activated, they lyse the targets and produce anti- and pro-inflammatory cytokines. NRP1 role on these NKT cells is unknown (Roy et al., 2017).

Regulatory T Cells (Treg)

Tregs play a role in immune homeostasis, allergic responses, auto-immune diseases, tumor immunity, and graft rejection. Their accumulation in tumors induces cancer progression and immune suppression (Sakaguchi et al., 1995). NRP1 is overexpressed by activated Tregs and promote their immunosuppressive role. NRP1 expression maintains the Tregs functions through the binding to SEMA4A, expressed by dendritic cells. NRP1/SEMA4A binding stabilizes the Treg by recruiting PTEN (Phosphatase and tensin homolog) and by inhibiting AKT phosphorylation. NRP1 expression on Treg induces their migration to the tumors where they play an immune-suppressive role (Hansen et al., 2012) by secreting IL-10 and IL-35, an anti-inflammatory cytokine. NRP1 expressed by Tregs are also attracted to tumors expressing VEGF where NRP1 acts as a VEGF co-receptor. The stimulation by VEGF, enhances T regs infiltration to tumors and an immunosuppressive response (Hansen et al., 2012).

CD4+/CD8+ T cells over-express NRP2 but NRP2 expression is lower on T cells expressing only CD8 or only CD4. The interaction between NRP2, SEMA3F and plexinA1 inhibits immature T cell migration.

Thus, the NRP have different roles in the immune system either in cell migration, cell-cell interaction or in the regulation of the immune response.

ROLES IN CANCER

Neuropilins expression level correlates with tumor growth, invasiveness, angiogenesis, and poor prognosis. NRPs over-expression is often observed in carcinoma, melanoma, glioblastoma, leukemia, and lymphoma in which NRPs exert diverse functions.

Functions of Neuropilins in Cancer

To grow over a few millimeters tumors turn into a pro-angiogenic environment that induces the formation of new blood vessels from the existing vascular network. This new vascular network surrounding the tumor, supplies oxygen and nutrients needed for tumors growth. Tumor cells, cells from the microenvironment and NRPs expressed on both cell types influence tumor angiogenesis (Niland and Eble, 2019). The roles of NRP1 in the growth and invasiveness of prostate, colorectal, kidney, lung, breast, ... human cancers have been confirmed with animal studies showing that exacerbated angiogenesis and a poor prognosis is correlated with NRP1 expression (Ellis, 2006). Only in pancreatic cancers, a high expression of NRP1 correlates with reduced vascularized areas, decreased tumor growth, and improved survival (Morin et al., 2018).

Expression of NRP2 is mostly correlated to tumor progression. In most cancers, the co-expression of NRP1 and NRP2 stimulates tumor growth and invasiveness (Rizzolio and Tamagnone, 2011). SEMA3C, which binds to NRP1 and NRP2 with equivalent affinity, inhibits tumor lymphangiogenesis by targeting immature vessels sprouting. However, its cleaved form, p65-SEMA3C, stimulates tumor lymphangiogenesis and metastatic dissemination of cancer cells expressing NRP2 (Mumblat et al., 2015).

Neuropilin 1 expression on tumor cells enhances cell viability, proliferation, migration, metastasis and favors cancer cell stemness. Since NRP1 promotes EMT through different pathways (TGF-β, Hedgehog, HGF. . .), which explains NRP1's pro-tumoral role.

Neuropilin 1 is expressed on breast cancer cells, and its interaction with VEGF165 inhibits apoptosis. Such inhibition is counteracted by SEMA3B (Ellis, 2006). SEMA3F competes with VEGF in binding to the NRPs and blocks breast cancer cell migration. However, SEMA3F decreases membrane E-cadherin, which promotes cell metastasis (Ellis, 2006). SEMA3A expressed on endothelial cells, antagonizes VEGF effects and correlates with a good prognosis (Niland and Eble, 2020). It is generally lost during tumor progression (Niland and Eble, 2019). In a VEGFA+/SEMA3A+ environment, NRP1 binds preferentially SEMA3A (Palodetto et al., 2017). Cells with a higher VEGF expression as compared to SEMA3A expression have promigratory characteristics.

In colon cancer, NRP1 expression correlates with increased vessel number and poor prognosis, while NRP2 over-expression stimulates tumor progression and the down-regulation of NRP2 expression inhibits tumorigenesis and increases apoptosis (Gray et al., 2008). In prostate cancer, elevated NRP1 levels stimulated by VEGF inhibit tumor cell apoptosis and angiogenesis and are synonymous of shorter survival. In ccRCC, NRP1 down-regulation reduces migration, invasion, and tumorigenesis (Cao et al., 2008), and NRP2 down-regulation decreases cell extravasation in the lymphatic network and the metastatic spread (Cao et al., 2013). NRP1 expression down-regulation in experimental model of lung cancer reduces cell migration, invasion, and metastasis (Hong et al., 2007).

Role in Cancer Stem Cells

A tumor is composed of cells differing in their morphology, their capacity to proliferate and to form metastasis and in their resistance to therapeutic agents. Among these different cells, only cancer stem cells (CSCs) are able to initiate a new primary tumor or metastasis. CSCs are cells that self-renew and that induce the heterogeneous aspect of the tumors. CSCs are resistant to chemo-and radiotherapy. As NRPs are less expressed in epithelial tissues compared to carcinomas, NRPs might play a role in stemness.

The role of the VEGFs/NRPs pathways have been studied in the triple negative breast cancer cell line MDA-MB-231 and the hormone sensitive MCF-7 cell line. While MDA-MB-231 have stemness characteristics MCF-7 cells have low stemness properties. In these cells, the level of stemness was correlated to the expression of VEGF and NRP1 (Zhang et al., 2017). Down-regulation of VEGF and NRP1 in MDA-MB-231 cells and overexpression of VEGF and NRP1 in MCF-7 cells confirmed that the VEGF/NRP1 signaling pathway is instrumental in driving stemness properties of breast cancer cells (Zhang et al., 2017). The VEGFC/NRP2 pathway is also involved in breast cancer stemness (Wang et al., 2014). The VEGF/NRP2 pathways stimulates stemness through activation of the YAP/TAZ signaling (Elaimy and Mercurio, 2018). This pathway also mediates homologous recombination by stimulating Rad51 expression leading to resistance to platinum chemotherapy in triple negative breast cancers (Elaimy et al., 2019). The NRP2/α6β1 integrin interaction activates the focal adhesion kinase (FAK) involved in tumorigenesis and associated to aggressive tumors (Goel et al., 2013). Furthermore, the VEGF/NRP1 pathway induces CSCs in breast cancers by activating the Wnt/β-catenin pathway (Zhang et al., 2017), which is involved in the induction of CSCs. The implication of VEGF/NRP1 pathway was also highlighted in glioma stem cells (Hamerlik et al., 2012) and in medulloblastoma stem cells (Gong et al., 2018).

Role in Cancer-Associated Fibroblasts

Fibroblasts are part of the tumor microenvironment and become myofibroblasts (normal activated fibroblasts) under tumoral conditions. By interacting with fibronectin, myofibroblasts promote fibronectin fibril assembly, and tumor growth through α5β1 integrin (Yaqoob et al., 2012). Fibronectin fibril assembly is regulated determinant of matrix stiffness involved in tumor progression. NRP1 induces integrin function by binding to fibronectin and by activating the intracellular kinase c-Abl (Yaqoob et al., 2012). Indeed, NRP1 intracellular domain stimulates c-Abl that activates small GTPases (Rac or Rho). These GTPases promote α5β1 integrin function and so increase fibronectin binding and assembly (Yaqoob et al., 2012). The NRP1 extracellular domain is O-linked glycosylated via the serine 612 residue, which increases NRP1 binding to fibronectin resulting in enhanced fibronectin and α5β1 integrin interaction (Yaqoob et al., 2012). Thus, NRP1 intra- and extracellular domains, through the activation of c-Abl and α5β1 integrin, increase fibronectin fibril assembly contributing to matrix stiffness and tumor progression and invasiveness. Furthermore, cancer-associated fibroblasts (CAFs) are one of the most expressed cells in the tumor microenvironment, and the principal source of TGFβ1. NRP1/TGFβ1 interaction stimulates endothelial-mesenchymal transition (EndMT), an important source of CAFs (Matkar et al., 2016). Finally, CAFs also promotes tumor migration and invasion by inducing EMT of cancers cells (Shan et al., 2017). This EMT induction is carried out through Hedgehog signaling. As above described, NRP1 is a major regulator of Hedgehog signaling. Thus, NRP1 expressed on CAF might also stimulate EMT which increases tumor cell migration and invasion worse prognosis.

Prognostic Role of NRP1 and NRP2 Pathways

Neuropilins correlate with poor prognosis in many cancers. Here are some examples. NRP1 is overexpressed in bladder cancer and correlates with poor prognosis (Cheng et al., 2014). In osteosarcoma, NRP1 is a prognostic factor of shorter progression-free (PFS) and overall survival (OS) (Zhu et al., 2014). NRP2 contributes to laryngeal squamous cell carcinoma progression and could serve as a new therapeutic target for this type of cancer (Yin et al., 2020). In prostate adenocarcinoma, NRP2 is a marker of bad prognosis (Borkowetz et al., 2020). Some activator of the NRP2 pathway including VEGFC were described as markers of good prognosis in non-metastatic kidney cancers but of poor prognosis in metastatic kidney cancers (Ndiaye et al., 2019). Thus, the level of expression of NRP2 and their partners, has to be determined to adapt a specific therapeutic strategy in tumors at different steps of their development.

Role in the Therapeutic Response

Resistances to targeted therapies are often related to the activation of alternative tyrosine-kinase receptors-mediated signaling pathways. As above described, NRPs interact with several tyrosine kinase receptors and enhance their activity.

Resistance to Chemo- and Radiotherapies

Radio- and chemotherapy are widely used to treat cancers.

A high expression of NRP1 in non-small cell lung cancer cells increases radio-resistance through an ABL-1-mediated up-regulation of RAD51 expression (Hu et al., 2018). In pancreatic cancer, NRP1 increases resistance to gemcitabine and 5-fluorouracil by activating the MAPK signaling pathway (Wey et al., 2005).

The NRP2/VEGFC pathway activates autophagy through the inhibition of mTOR complex 1 activity which helps cancer cells to survive following treatment (Stanton et al., 2013). NRP2 overexpression, induced by SEMA3F in adenocarcinoma, decreases integrin αvβ3 and enhances cell sensitivity to chemotherapy (Zheng et al., 2009).

In some cancers, NRP targeted drug decreases resistance to chemo/radiotherapies.

Resistance to Targeted Therapies

In pancreatic ductal adenocarcinoma (PDAC), an increase of active integrin β1 activates AKT signaling and resistance to cetuximab, an anti-EGFR monoclonal antibody (Kim et al., 2017). NRP1-dependent JNK signaling leads to the overexpression of EGFR and IGF1R, which induces resistance to BRAF (melanoma targeted therapy), HER2 (breast cancer targeted therapy) and MET (stomach and lung carcinomas therapy) inhibitors (Rizzolio et al., 2018b).

Neuropilin 2 overexpression decreases EGFR expression and resistance to MET-targeted therapies (Rizzolio et al., 2018a).

Thus, NRPs have become interesting biomarkers to determine the patients' responsiveness to radio- or chemotherapies or to targeted therapies. Indeed, patients with low NRP1 expression present a better OS than patients with high level of NRP1 (Van Cutsem et al., 2012; Napolitano and Tamagnone, 2019).

Again, combination of targeted therapies to NRP1 inhibitors increase the effects of therapies and reduces resistance.

CONCLUSION

Angiogenesis is one of the key mechanisms involved in cancer growth and dissemination. Anti VEGF were approved in combination with standard chemotherapies. Despite an improvement of progression free survival in several types of tumors by anti VEGF treatments, increases in OS were reported. The elevated expression in tumor, endothelial, and immune cells, makes NRP1 and 2 new relevant oncology targets to improve the treatment of cancers. This review describes the different roles and the expression level of NRPs in the different cells constituting the tumor microenvironment. NRPs form holoreceptors with many different receptors and, thus, are involved in many biological phenomena: angiogenesis, lymphangiogenesis, cell proliferation, migration, invasion, and tumor growth. Moreover, NRPs are expressed by several immune cells, in which they exert an activating or inhibiting role on the immune response. In many cancers, NRPs over-expression is synonymous of poor prognosis. This review highlights the implication of NRPs in several hallmarks of cancer and the relevance of targeting the NRPs for the treatment of cancers. Several molecules targeting NRPs are in development: (i) anti-NRP1 antibodies such as the MNRP1685A that has to be optimized to improve the therapeutic window and to decrease its toxic effects; (ii) cyclic, rigid or pseudo-peptides developed by optimizing the sequence ATWLPPR, mimicking the VEGF C-terminal domain interacting with NRP1; (iii) non-peptidic inhibitors such as NRPa-308 that exerts anti-cancer effects in triple negative breast cancer (Liu et al., 2018) and which is currently tested in ccRCC.

Despite these different therapeutic pathways, NRPs targeting must be improved to fight cancers that can benefit the most of these treatments. The antagonist role of NRPs as beneficial or detrimental markers depending on tumor stage suggests cautiousness before administration of anti NRPs treatments.

AUTHOR CONTRIBUTIONS

AD and GP were equally responsible for all parts of the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

The authors acknowledge funding from Helsinn Company. This work was supported by the Fondation de France, the Ligue Nationale contre le Cancer (Equipe Labellisée 2019), the National Institute of Cancer (INCA, SUNITRES), the National Agency for Research (ANR, TARMAC), and the FX Mora and Flavien Foundations.

REFERENCES

Akkaya, B., Oya, Y., Akkaya, M., Al Souz, J., Holstein, A. H., Kamenyeva, O., et al. (2019). Regulatory T cells mediate specific suppression by depleting peptide-MHC class II from dendritic cells. *Nat. Immunol.* 20, 218–231. doi: 10.1038/s41590-018-0280-2

Alghamdi, A. A. A., Benwell, C. J., Atkinson, S. J., Lambert, J., Johnson, R. T., and Robinson, S. D. (2020). NRP2 as an emerging angiogenic player; promoting endothelial cell adhesion and migration by regulating recycling of alpha5 integrin. *Front. Cell Dev. Biol.* 8:395. doi: 10.3389/fcell.2020.00395

Balan, M., Teran, M. Y. E., Waaga-Gasser, A. M., Gasser, M., Choueiri, T. K., Freeman, G., et al. (2015). Novel roles of c-Met in the survival of renal cancer cells through the regulation of HO-1 and PD-L1 expression. *J. Biol. Chem.* 290, 8110–8120. doi: 10.1074/jbc.m114.612689

Borkowetz, A., Froehner, M., Rauner, M., Conrad, S., Erdmann, K., Mayr, T., et al. (2020). Neuropilin-2 is an independent prognostic factor for shorter cancer-specific survival in patients with acinar adenocarcinoma of the prostate. *Int. J. Cancer* 146, 2619–2627. doi: 10.1002/ijc.32679

Bruder, D., Probst-Kepper, M., Westendorf, A. M., Geffers, R., Beissert, S., Loser, K., et al. (2004). Neuropilin-1: a surface marker of regulatory T cells. *Eur. J. Immunol.* 34, 623–630. doi: 10.1002/eji.200324799

Campos-Mora, M., Contreras-Kallens, P., Galvez-Jiron, F., Rojas, M., Rojas, C., Refisch, A., et al. (2019). CD4+Foxp3+T regulatory cells promote transplantation tolerance by modulating effector CD4+ T Cells in a neuropilin-1-dependent manner. *Front. Immunol.* 10:882. doi: 10.3389/fimmu.2019.00882

Cao, Y., Hoeppner, L. H., Bach, S., Guangqi, E., Guo, Y., Wang, E., et al. (2013). Neuropilin-2 promotes extravasation and metastasis by interacting with endothelial alpha5 integrin. *Cancer Res.* 73, 4579–4590. doi: 10.1158/0008-5472.can-13-0529

Cao, Y., Wang, L., Nandy, D., Zhang, Y., Basu, A., Radisky, D., et al. (2008). Neuropilin-1 upholds dedifferentiation and propagation phenotypes of renal cell carcinoma cells by activating Akt and sonic hedgehog axes. *Cancer Res.* 68, 8667–8672. doi: 10.1158/0008-5472.can-08-2614

Caponegro, M. D., Moffitt, R. A., and Tsirka, S. E. (2018). Expression of neuropilin-1 is linked to glioma associated microglia and macrophages and correlates with unfavorable prognosis in high grade gliomas. *Oncotarget* 9, 35655–35665. doi: 10.18632/oncotarget.26273

Casazza, A., Laoui, D., Wenes, M., Rizzolio, S., Bassani, N., Mambretti, M., et al. (2013). Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell* 24, 695–709. doi: 10.1016/j.ccr.2013.11.007

Chaudhary, B., Khaled, Y. S., Ammori, B. J., and Elkord, E. (2014). Neuropilin 1: function and therapeutic potential in cancer. *Cancer Immunol. Immunother.* 63, 81–99. doi: 10.1007/s00262-013-1500-0

Chauvet, S., Cohen, S., Yoshida, Y., Fekrane, L., Livet, J., Gayet, O., et al. (2007). Gating of Sema3E/PlexinD1 signaling by neuropilin-1 switches axonal repulsion to attraction during brain development. *Neuron* 56, 807–822. doi: 10.1016/j.neuron.2007.10.019

Chen, X. J., Wu, S., Yan, R. M., Fan, L. S., Yu, L., Zhang, Y. M., et al. (2019). The role of the hypoxia-Nrp-1 axis in the activation of M2-like tumor-associated macrophages in the tumor microenvironment of cervical cancer. *Mol. Carcinog.* 58, 388–397. doi: 10.1002/mc.22936

Cheng, W., Fu, D., Wei, Z. F., Xu, F., Xu, X. F., Liu, Y. H., et al. (2014). NRP-1 expression in bladder cancer and its implications for tumor progression. *Tumour Biol.* 35, 6089–6094. doi: 10.1007/s13277-014-1806-3

Cherry, J. D., Olschowka, J. A., and O'banion, M. K. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J. Neuroinflam.* 11:98. doi: 10.1186/1742-2094-11-98

Curreli, S., Arany, Z., Gerardy-Schahn, R., Mann, D., and Stamatos, N. M. (2007). Polysialylated neuropilin-2 is expressed on the surface of human dendritic cells and modulates dendritic cell-T lymphocyte interactions. *J. Biol. Chem.* 282, 30346–30356. doi: 10.1074/jbc.m702965200

Daoust, A., Bohic, S., Saoudi, Y., Debacker, C., Gory-Faure, S., Andrieux, A., et al. (2014). Neuronal transport defects of the MAP6 KO mouse - a model of schizophrenia - and alleviation by Epothilone D treatment, as observed using MEMRI. *Neuroimage* 96, 133–142. doi: 10.1016/j.neuroimage.2014.03.071

Dutta, S., Roy, S., Polavaram, N. S., Stanton, M. J., Zhang, H., Bhola, T., et al. (2016). Neuropilin-2 Regulates Endosome Maturation and EGFR trafficking to support cancer cell pathobiology. *Cancer Res.* 76, 418–428. doi: 10.1158/0008-5472.can-15-1488

Elaimy, A. L., Amante, J. J., Zhu, L. J., Wang, M., Walmsley, C. S., Fitzgerald, T. J., et al. (2019). The VEGF receptor neuropilin 2 promotes homologous recombination by stimulating YAP/TAZ-mediated Rad51 expression. *Proc. Natl. Acad. Sci. U.S.A.* 116, 14174–14180. doi: 10.1073/pnas.1821194116

Elaimy, A. L., and Mercurio, A. M. (2018). Convergence of VEGF and YAP/TAZ signaling: Implications for angiogenesis and cancer biology. *Sci. Signal.* 11:eaau1165. doi: 10.1126/scisignal.aau1165

Ellis, L. M. (2006). The role of neuropilins in cancer. *Mol. Cancer Ther.* 5, 1099–1107. doi: 10.1158/1535-7163.mct-05-0538

Escudero-Esparza, A., Martin, T. A., Douglas-Jones, A., Mansel, R. E., and Jiang, W. G. (2010). PGF isoforms, PLGF-1 and PGF-2 and the PGF receptor, neuropilin, in human breast cancer: prognostic significance. *Oncol. Rep.* 23, 537–544.

Goel, H. L., Pursell, B., Chang, C., Shaw, L. M., Mao, J., Simin, K., et al. (2013). GLI1 regulates a novel neuropilin-2/alpha6beta1 integrin based autocrine pathway that contributes to breast cancer initiation. *EMBO Mol. Med.* 5, 488–508. doi: 10.1002/emmm.201202078

Gong, C., Valduga, J., Chateau, A., Richard, M., Pellegrini-Moise, N., Barberi-Heyob, M., et al. (2018). Stimulation of medulloblastoma stem cells differentiation by a peptidomimetic targeting neuropilin-1. *Oncotarget* 9, 15312–15325. doi: 10.18632/oncotarget.24521

Gray, M. J., Van Buren, G., Dallas, N. A., Xia, L., Wang, X., Yang, A. D., et al. (2008). Therapeutic targeting of neuropilin-2 on colorectal carcinoma cells implanted in the murine liver. *J. Natl. Cancer Inst.* 100, 109–120. doi: 10.1093/jnci/djm279

Guyot, M., and Pages, G. (2015). VEGF splicing and the role of VEGF splice variants: from physiological-pathological conditions to specific Pre-mRNA splicing. *Methods Mol. Biol.* 1332, 3–23. doi: 10.1007/978-1-4939-2917-7_1

Hamerlik, P., Lathia, J. D., Rasmussen, R., Wu, Q., Bartkova, J., Lee, M., et al. (2012). Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth. *J. Exp. Med.* 209, 507–520. doi: 10.1084/jem.20111424

Hansen, W., Hutzler, M., Abel, S., Alter, C., Stockmann, C., Kliche, S., et al. (2012). Neuropilin 1 deficiency on CD4+Foxp3+ regulatory T cells impairs mouse melanoma growth. *J. Exp. Med.* 209, 2001–2016. doi: 10.1084/jem.20111497

Harper, S. J., and Bates, D. O. (2008). VEGF-A splicing: the key to anti-angiogenic therapeutics? *Nat. Rev. Cancer* 8, 880–887. doi: 10.1038/nrc2505

Hong, T. M., Chen, Y. L., Wu, Y. Y., Yuan, A., Chao, Y. C., Chung, Y. C., et al. (2007). Targeting neuropilin 1 as an antitumor strategy in lung cancer. *Clin. Cancer Res.* 13, 4759–4768. doi: 10.1158/1078-0432.ccr-07-0001

Hsieh, S. H., Ying, N. W., Wu, M. H., Chiang, W. F., Hsu, C. L., Wong, T. Y., et al. (2008). Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene* 27, 3746–3753. doi: 10.1038/sj.onc.1211029

Hu, C., Zhu, P., Xia, Y., Hui, K., Wang, M., and Jiang, X. (2018). Role of the NRP-1-mediated VEGFR2-independent pathway on radiation sensitivity of non-small cell lung cancer cells. *J. Cancer Res. Clin. Oncol.* 144, 1329–1337. doi: 10.1007/s00432-018-2667-8

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., et al. (1999). A requirement for neuropilin-1 in embryonic vessel formation. *Development* 126, 4895–4902.

Kim, Y. J., Jung, K., Baek, D. S., Hong, S. S., and Kim, Y. S. (2017). Co-targeting of EGF receptor and neuropilin-1 overcomes cetuximab resistance in pancreatic ductal adenocarcinoma with integrin beta1-driven Src-Akt bypass signaling. *Oncogene* 36, 2543–2552. doi: 10.1038/onc.2016.407

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* 121, 4309–4318.

Leclerc, M., Voilin, E., Gros, G., Corgnac, S., De Montpreville, V., Validire, P., et al. (2019). Regulation of antitumour CD8 T-cell immunity and checkpoint blockade immunotherapy by Neuropilin-1. *Nat. Commun.* 10:3345.

Lepelletier, Y., Moura, I. C., Hadj-Slimane, R., Renand, A., Fiorentino, S., Baude, C., et al. (2006). Immunosuppressive role of semaphorin-3A on T cell proliferation is mediated by inhibition of actin cytoskeleton reorganization. *Eur. J. Immunol.* 36, 1782–1793. doi: 10.1002/eji.200535601

Liu, W. Q., Lepelletier, Y., Montes, M., Borriello, L., Jarray, R., Grepin, R., et al. (2018). NRPa-308, a new neuropilin-1 antagonist, exerts in vitro anti-angiogenic and anti-proliferative effects and in vivo anti-cancer effects in a mouse xenograft model. *Cancer Lett.* 414, 88–98. doi: 10.1016/j.canlet.2017.10.039

Matkar, P. N., Singh, K. K., Rudenko, D., Kim, Y. J., Kuliszewski, M. A., Prud'homme, G. J., et al. (2016). Novel regulatory role of neuropilin-1 in endothelial-to-mesenchymal transition and fibrosis in pancreatic ductal adenocarcinoma. *Oncotarget* 7, 69489–69506. doi: 10.18632/oncotarget.11060

Migdal, M., Huppertz, B., Tessler, S., Comforti, A., Shibuya, M., Reich, R., et al. (1998). Neuropilin-1 is a placenta growth factor-2 receptor. *J. Biol. Chem.* 273, 22272–22278.

Morin, E., Sjoberg, E., Tjomsland, V., Testini, C., Lindskog, C., Franklin, O., et al. (2018). VEGF receptor-2/neuropilin 1 trans-complex formation between endothelial and tumor cells is an independent predictor of pancreatic cancer survival. *J. Pathol.* 246, 311–322. doi: 10.1002/path.5141

Mumblat, Y., Kessler, O., Ilan, N., and Neufeld, G. (2015). Full-Length Semaphorin-3C Is an Inhibitor of Tumor Lymphangiogenesis and Metastasis. *Cancer Res.* 75, 2177–2186. doi: 10.1158/0008-5472.can-14-2464

Napolitano, V., and Tamagnone, L. (2019). Neuropilins controlling cancer therapy responsiveness. *Int. J. Mol. Sci.* 20:2049. doi: 10.3390/ijms20082049

Ndiaye, P. D., Dufies, M., Giuliano, S., Douguet, L., Grepin, R., Durivault, J., et al. (2019). VEGFC acts as a double-edged sword in renal cell carcinoma aggressiveness. *Theranostics* 9, 661–675. doi: 10.7150/thno.27794

Niland, S., and Eble, J. A. (2019). Neuropilins in the context of tumor vasculature. *Int. J. Mol. Sci.* 20:639. doi: 10.3390/ijms20030639

Niland, S., and Eble, J. A. (2020). Neuropilin: handyman and power broker in the tumor microenvironment. *Adv. Exp. Med. Biol.* 1223, 31–67. doi: 10.1007/978-3-030-35582-1_3

Pagani, E., Ruffini, F., Antonini Cappellini, G. C., Scoppola, A., Fortes, C., Marchetti, P., et al. (2016). Placenta growth factor and neuropilin-1 collaborate in promoting melanoma aggressiveness. *Int. J. Oncol.* 48, 1581–1589. doi: 10.3892/ijo.2016.3362

Palodetto, B., Da Silva Santos Duarte, A., Rodrigues Lopes, M., Adolfo Corrocher, F., Marconi Roversi, F., Soares Niemann, F., et al. (2017). SEMA3A partially reverses VEGF effects through binding to neuropilin-1. *Stem Cell Res.* 22, 70–78. doi: 10.1016/j.scr.2017.05.012

Rey-Gallardo, A., Delgado-Martin, C., Gerardy-Schahn, R., Rodriguez-Fernandez, J. L., and Vega, M. A. (2011). Polysialic acid is required for neuropilin-2a/b-mediated control of CCL21-driven chemotaxis of mature dendritic cells and for their migration in vivo. *Glycobiology* 21, 655–662. doi: 10.1093/glycob/cw\break{}q216

Rizzolio, S., Battistini, C., Cagnoni, G., Apicella, M., Vella, V., Giordano, S., et al. (2018a). Downregulating Neuropilin-2 Triggers a Novel Mechanism Enabling EGFR-Dependent Resistance to Oncogene-Targeted Therapies. *Cancer Res.* 78, 1058–1068. doi: 10.1158/0008-5472.can-17-2020

Rizzolio, S., Cagnoni, G., Battistini, C., Bonelli, S., Isella, C., Van Ginderachter, J. A., et al. (2018b). Neuropilin-1 upregulation elicits adaptive resistance to oncogene-targeted therapies. *J. Clin. Invest.* 128, 3976–3990. doi: 10.1172/jci99257

Rizzolio, S., Rabinowicz, N., Rainero, E., Lanzetti, L., Serini, G., Norman, J., et al. (2012). Neuropilin-1-dependent regulation of EGF-receptor signaling. *Cancer Res.* 72, 5801–5811. doi: 10.1158/0008-5472.can-12-0995

Rizzolio, S., and Tamagnone, L. (2011). Multifaceted role of neuropilins in cancer. *Curr. Med. Chem.* 18, 3563–3575. doi: 10.2174/092986711796642544

Roy, S., Bag, A. K., Dutta, S., Polavaram, N. S., Islam, R., Schellenburg, S., et al. (2018). Macrophage-derived neuropilin-2 exhibits novel tumor-promoting functions. *Cancer Res.* 78, 5600–5617. doi: 10.1158/0008-5472.can-18-0562

Roy, S., Bag, A. K., Singh, R. K., Talmadge, J. E., Batra, S. K., and Datta, K. (2017). Multifaceted role of neuropilins in the immune system: potential targets for immunotherapy. *Front. Immunol.* 8:1228. doi: 10.3389/fimmu.2017.01228

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164.

Sarris, M., Andersen, K. G., Randow, F., Mayr, L., and Betz, A. G. (2008). Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* 28, 402–413. doi: 10.1016/j.immuni.2008.01.012

Schellenburg, S., Schulz, A., Poitz, D. M., and Muders, M. H. (2017). Role of neuropilin-2 in the immune system. *Mol. Immunol.* 90, 239–244. doi: 10.1016/j.molimm.2017.08.010

Shan, T., Chen, S., Chen, X., Lin, W. R., Li, W., Ma, J., et al. (2017). Prometastatic mechanisms of CAF-mediated EMT regulation in pancreatic cancer cells. *Int. J. Oncol.* 50, 121–128. doi: 10.3892/ijo.2016.3779

Snuderl, M., Batista, A., Kirkpatrick, N. D., Ruiz De Almodovar, C., Riedemann, L., Walsh, E. C., et al. (2013). Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. *Cell* 152, 1065–1076. doi: 10.1016/j.cell.2013.01.036

Stamatos, N. M., Zhang, L., Jokilammi, A., Finne, J., Chen, W. H., El-Maarouf, A., et al. (2014). Changes in polysialic acid expression on myeloid cells during differentiation and recruitment to sites of inflammation: role in phagocytosis. *Glycobiology* 24, 864–879. doi: 10.1093/glycob/cwu050

Stanton, M. J., Dutta, S., Zhang, H., Polavaram, N. S., Leontovich, A. A., Honscheid, P., et al. (2013). Autophagy control by the VEGF-C/NRP-2 axis in cancer and its implication for treatment resistance. *Cancer Res.* 73, 160–171. doi: 10.1158/0008-5472.can-11-3635

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., et al. (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3657–3662. doi: 10.1073/pnas.022017899

Van Bergen, T., Etienne, I., Cunningham, F., Moons, L., Schlingemann, R. O., Feyen, J. H. M., et al. (2019). The role of placental growth factor (PlGF) and its receptor system in retinal vascular diseases. *Prog. Retin. Eye Res.* 69, 116–136. doi: 10.1016/j.preteyeres.2018.10.006

Van Cutsem, E., De Haas, S., Kang, Y. K., Ohtsu, A., Tebbutt, N. C., Ming Xu, J., et al. (2012). Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. *J. Clin. Oncol.* 30, 2119–2127. doi: 10.1200/jco.2011.39.9824

Wang, C. A., Harrell, J. C., Iwanaga, R., Jedlicka, P., and Ford, H. L. (2014). Vascular endothelial growth factor C promotes breast cancer progression via a novel antioxidant mechanism that involves regulation of superoxide dismutase 3. *Breast Cancer Res.* 16:462.

Wey, J. S., Gray, M. J., Fan, F., Belcheva, A., Mccarty, M. F., Stoeltzing, O., et al. (2005). Overexpression of neuropilin-1 promotes constitutive MAPK signalling and chemoresistance in pancreatic cancer cells. *Br. J. Cancer* 93, 233–241. doi: 10.1038/sj.bjc.6602663

Wu, M. H., Ying, N. W., Hong, T. M., Chiang, W. F., Lin, Y. T., and Chen, Y. L. (2014). Galectin-1 induces vascular permeability through the neuropilin-1/vascular endothelial growth factor receptor-1 complex. *Angiogenesis* 17, 839–849. doi: 10.1007/s10456-014-9431-8

Yaqoob, U., Cao, S., Shergill, U., Jagavelu, K., Geng, Z., Yin, M., et al. (2012). Neuropilin-1 stimulates tumor growth by increasing fibronectin fibril assembly in the tumor microenvironment. *Cancer Res.* 72, 4047–4059. doi: 10.1158/0008-5472.can-11-3907

Yin, D., Guo, L., Li, S., Tuerdi, A., Yang, X., Tang, Q., et al. (2020). Clinical significance of neuropilin-2 expression in laryngeal squamous cell carcinoma. *Am. J. Otolaryngol.* 41, 102540.

Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M. J., Alitalo, K., et al. (2002). Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 129, 4797–4806.

Zhang, L., Wang, H., Li, C., Zhao, Y., Wu, L., Du, X., et al. (2017). VEGF-A/neuropilin 1 pathway confers cancer stemness via activating

wnt/beta-catenin axis in breast cancer cells. *Cell Physiol. Biochem.* 44, 1251–1262. doi: 10.1159/000485455

Zheng, C., Zhou, Q., Wu, F., Peng, Q., Tang, A., Liang, H., et al. (2009). Semaphorin3F down-regulates the expression of integrin alpha(v)beta3 and sensitizes multicellular tumor spheroids to chemotherapy via the neuropilin-2 receptor in vitro. *Chemotherapy* 55, 344–352. doi: 10.1159/000232449

Zhu, H., Cai, H., Tang, M., and Tang, J. (2014). Neuropilin-1 is overexpressed in osteosarcoma and contributes to tumor progression and poor prognosis. *Clin. Transl. Oncol.* 16, 732–738. doi: 10.1007/s12094-013-1141-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 07 August 2020
doi: 10.3389/fcell.2020.00755

MiR-9 Promotes Angiogenesis via Targeting on Sphingosine-1-Phosphate Receptor 1

Xinghong Yao[1,2†], Linshen Xie[3†] and Ye Zeng[1‡]*

[1] Institute of Biomedical Engineering, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, Chengdu, China, [2] Department of Radiation Oncology, Sichuan Cancer Hospital & Institute, School of Medicine of University of Electronic Science and Technology of China, Chengdu, China, [3] West China School of Public Health and West China Fourth Hospital, Sichuan University, Chengdu, China

OPEN ACCESS

Edited by:
Lucas Treps,
VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Jiwei Guo,
Binzhou Medical University, China
Delphine Fradin,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Ye Zeng
ye@scu.edu.cn;
yeqgzeng@Gmail.com

[†] These authors have contributed equally to this work

[‡] **ORCID:**
Ye Zeng
orcid.org/0000-0001-5440-6194

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 24 May 2020
Accepted: 20 July 2020
Published: 07 August 2020

Citation:
Yao X, Xie L and Zeng Y (2020) MiR-9 Promotes Angiogenesis via Targeting on Sphingosine-1-Phosphate Receptor 1. Front. Cell Dev. Biol. 8:755. doi: 10.3389/fcell.2020.00755

We previously demonstrated that vascular endothelial cells released VEGF-enriched exosomes to promote the tumor vasculogenesis and progression after anti-angiogenic therapies (AATs). To clarify how microRNA (miR)-9 promoted the angiogenesis of tumor-associated endothelial cells, in the present study, we investigated the association between miR-9 and sphingosine-1-phosphate (S1P) receptors in angiogenesis. The levels of miR-9 and S1P receptors in normal and tumor endothelial cells were compared with EndoDB database and their correlations were analyzed. The levels of $S1P_1$, $S1P_2$, and $S1P_3$ were detected in miR-9 overexpressing endothelial cells by qRT-PCR and western blot. The binding sites of miR-9 on $S1P_1$ and $S1P_3$ were predicted and tested by dual-luciferase reporter assays. Then, angiogenesis in endothelial cells overexpressing both $S1P_1$ and miR-9 was detected. The results showed that miR-9 is overexpressed in ECs from medulloblastoma and glioblastoma xenograft, which is negatively associated with $S1P_1$ and $S1P_3$. Overexpression of miR-9 significantly inhibited $S1P_1$ and $S1P_3$ in both mRNA and protein levels. We predicted that binding sites exist between miR-9 and $S1P_1$, $S1P_3$, but only $S1P_1$ was directly targeted by miR-9. Overexpression of $S1P_1$ significantly suppressed the miR-9-induced angiogenesis. Therefore, miR-9 induces angiogenesis via targeting on $S1P_1$.

Keywords: sphingosine-1-phosphate receptor, miR-9, angiogenesis, endothelial cell, microRNA

INTRODUCTION

Angiogenesis is an important progress during physiological and pathophysiological development. Angiogenesis is a complex process of vessel growth but in the strictest sense denotes vessels sprouting from pre-existing ones (Potente et al., 2011). Inadequate angiogenesis causes ischemia in myocardial infarction, stroke, and neurodegenerative or obesity-associated disorders, whereas excessive angiogenesis promotes many ailments including cancer, inflammatory disorders such as atherosclerosis, and eye diseases. Anti-angiogenic therapies (AATs) have been developed to combat tumor metastasis (Hosein et al., 2020). Nowadays, AATs strategies include blood vessel pruning,

disruption or normalization of the tumor vasculature, and tumor immunosensitization but did not yield satisfactory efficacy (Cully, 2017; Cloughesy et al., 2020). Bevacizumab antagonizes vascular endothelial growth factor (VEGF) to induce vascular normalization and therefore reduce edema. Vascular disruptive agents such as VB-111 disrupts the angiogenic vasculature via promoting tumor starvation and enhancing the vascular permeability in the tumor environment to increase edema and recruit the immune cells (Cloughesy et al., 2020). AATs and immune checkpoint inhibitors were combined to acquire promising outcomes of cancer patients (Yi et al., 2019). However, how and by which mechanism does the intratumoral vessel form remains unclear.

The angiogenetic process is rather complex involving localized breakdown of the basement membrane and extracellular matrix of a pre-existing vessel, proliferation, and migration of capillary endothelial cells (ECs) into the surrounding tissue, and new vessel formation. Stimulating by the proangiogenic signals such as hypoxia (Cantelmo et al., 2017) and cytokines (e.g., VEGF; Gerhardt et al., 2003), ECs become motile and invasive. MicroRNAs (miRNAs) have recently been shown to regulate gene expression associated with tumorigenesis and angiogenesis (Zhuang et al., 2012; Zeng et al., 2019). Oncogenic miR-9 is significantly elevated in breast cancer cells (Ma et al., 2010), hepatocellular carcinoma (Zhuang et al., 2012; Drakaki et al., 2015), squamous cell carcinomas (White et al., 2013), lung and colorectal carcinoma (Ma et al., 2010), and ovarian cancer (Laios et al., 2008). MiR-9 is significantly increased in ECs upon *in vitro* tumor-ECs co-cultures. We recently demonstrated that miR-9 promotes angiogenesis via activating the autophagy pathway (Zeng et al., 2019). MiR-9 also promotes the angiogenesis of endothelial progenitor cells via activating the autophagy pathway (Zhou et al., 2020). However, the molecular mechanism involved in miR-9-induced angiogenesis in ECs has not been fully explored.

G protein-coupled sphingosine-1-phosphate (S1P) receptors including $S1P_1$ (formerly endothelial differentiation gene-1, EDG-1), $S1P_2$ (EDG-5), and $S1P_3$ (EDG-3) are abundant in ECs. During vascular development *in vivo*, $S1P_1$ is required in ECs (Gaengel et al., 2012). Decrease of $S1P_1$ increases angiogenic sprouting and destabilization of the endothelium, while the activation of $S1P_1$ signaling inhibits angiogenic sprouting and enhances cell-to-cell adhesion (Gaengel et al., 2012; Cartier and Hla, 2019). It was reported that $S1P_2$ inhibited tumor angiogenesis in ECs in mouse models (Du et al., 2010). $S1P_3$ promotes EC migration and plays a critical role in developmental and pathological angiogenesis (Jin et al., 2018). Therefore, we hypothesis that miR-9 induces angiogenesis in ECs via targeting S1P receptors.

In the present study, we aimed to investigate which S1P receptor is regulated by miR-9 and aberrant expressed in ECs during angiogenesis. The miR-9 was transfected into the ECs to simulate the tumor-associated ECs as previously described (Zeng et al., 2019). The mRNA and protein levels of $S1P_1$, $S1P_2$, and $S1P_3$ were detected. Then, miR-9 binding sites of the S1P receptors were predicted and verified by Dual-Luciferase Reporter Assay. The role of putative S1P receptor in cell migration, invasion, and angiogenesis was explored. A rescue assay was performed to validate that the putative S1P receptor is a bona fide antigenic target regulated by miR-9 in ECs.

MATERIALS AND METHODS

Expression of miR-9 and S1P Receptors in Normal ECs and Tumor ECs

Databases E-GEOD-73753 [normal hindbrain ECs, $n = 8$; Sonic hedgehog (Shh)-driven medulloblastoma ECs, $n = 8$; Wnt-driven medulloblastoma ECs, $n = 8$], E-MTAB-3949 (normal ECs, $n = 2$; angiogenic glioblastoma xenograft ECs, $n = 2$), GSE-77199 [colorectal cancer ECs (colon), $n = 4$; colon ECs, $n = 3$; ECs from colorectal metastasis to the liver, $n = 4$; liver ECs, $n = 4$; renal cell carcinoma ECs, $n = 4$; renal ECs, $n = 4$], E-GEOD-51401 (CD105+ ECs from hepatocellular carcinoma, $n = 7$; CD105+ normal ECs, $n = 8$; CD31+ ECs from hepatocellular carcinoma, $n = 8$; CD31+ normal ECs, $n = 8$) and E-GEOD-41614 (vessels isolated from bladder cancer tissues, $n = 5$; vessels isolated from bladder normal tissues, $n = 5$) in EndoDB[1] from Khan et al. (2019) were obtained. In the human genome, three independent miR-9 loci including miR-9-1, miR-9-2, miR-9-3 reside at chromosomes 1, 5, and 15, respectively, which share an identical mature miR-9 sequence. The expression of miR-9-1, miR-9-2, miR-9-3, $S1P_1$, $S1P_2$, and $S1P_3$ in those normal ECs and tumor ECs were compared. The relationships between the significant differentially expressed miR-9 loci and specific S1P receptors were analyzed.

Cell Culture

HUVECs (Allcells, Shanghai, China) were cultured in HUVEC medium (HUVEC-004; Allcells) as previously described (Zeng et al., 2019). Cells (passages three to six) were maintained at 37°C with 5% CO_2 in a humidified incubator. Lentivirus infections of miR-9 mimic (5′-TCTTTGGTTATCTAGCTGTATGA-3′) and negative control (NC; 5′-UUGUACUACACAAAAGUACUG-3′; B04001; GenePharma, Shanghai, China) were performed as previously described (Zeng et al., 2019). cDNAs for $S1P_1$ were ligated into plasmid pcDNA3.1. For overexpression of $S1P_1$, cells were transfected with pcDNA3.1 vector expressing $S1P_1$ using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States). Transfected cells after 48 h were used for the subsequent experiments. Recombinant human VEGF 165 Protein (50 ng/mL; hVEGF; 293-VE, R&D system, Minneapolis, MN, United States) was used to test the migration, invasion and *in vitro* angiogenesis of cells overexpressing $S1P_1$.

Real-Time Quantitative PCR Analysis

RNA was extracted from cells using TRIzol (Invitrogen) and qRT-PCR assays were performed using a SYBR Premix Ex Taq kit (TaKaRa, Shiga, Japan). The primer sequences were as follows: *S1P*$_1$, 5′-TTTCTGCGGGAAGGGAGTATGT-3′ (forward) and 5′- GCAGGAAGAGGCGGAAGTTATT-3′

[1]https://endotheliomics.shinyapps.io/endodb/

(reverse); *S1P*$_2$, 5′-CTGTATGGCAGCGACAAGAGC-3′ (forward) and 5′-GAGGCAGGACAGTGGAGCAG-3′ (reverse); *S1P*$_3$, 5′-TACGCACGCATCTACTTCCTGG-3′ (forward) and 5′-GCTCCGAGTTGTTGTGGTTGG-3′ (reverse); and *GAPDH*, 5′-TGTTCGTCATGGGTGTGAAC-3′ (forward) and 5′-ATGGCATGGACTGTGGTCAT-3′ (reverse). All primers and probes were obtained from TaKaRa. Gene expression was normalized to that of GAPDH using the $2^{-\Delta\Delta CT}$ method, and data are presented as expression relative to the indicated controls. The stem-loop primers and probes for mature miR-9 and U6 small nuclear (sn)RNA were as follows: hsa-miR-9-5p, 5′-ACACTCCAGCTGGGTCTTTGGTTATCTAG-3′ (forward) and 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGT CATACAG-3′ (reverse); and U6 snRNA 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse). The relative expression level of miR-9 was normalized to that of U6 snRNA and shown as a ratio relative to the expression level in the control. Data are representative of three independent experiments.

Western Blot Analysis

Proteins were extracted from cells using radioimmunoprecipitation assay lysis buffer containing a protease inhibitor cocktail (Beyotime, Beijing, China). After determination of protein concentration using a protein determination kit (Cayman Chemical Company, Ann Arbor, MI, United States), equal amounts (20–30 μg) of protein samples were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred onto a polyvinylidene fluoride membrane (Millipore, United States), blocked with 5% non-fat milk in PBS, and hybridized with antibodies against $S1P_1$ (TA311878; Origene, Rockville, MD, United States), $S1P_2$ (TA311287; Origene), and $S1P_3$ (TA321693; Origene) at 4°C overnight. A 1:1000 dilution of each antibody was used for detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The blots were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; Beyotime), and enhanced chemiluminescence was performed using an Immobilon Western Chemiluminescent HRP Substrate (WBKLS0050; Millipore, Billerica, MA, United States) to visualize the bands. Densitometric quantification was performed using ImageJ software (version 1.52u; National Institutes of Health, Bethesda, MD, United States).

Dual-Luciferase Reporter Assay

The wild-type (WT) and the corresponding mutation (MUT) sites at 3′-untranslated regions (3′UTRs) of $S1P_1$ and $S1P_3$ were cloned into the psiCHECK-2 vector (Promega, Madison, WI, United States), respectively. The recombinant reporter plasmids were validated by DNA sequencing and then transfected into the miR-9 overexpressing cells and NC cells using Lipofectamine 2000 reagent. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (E1910, Promega). Renilla luciferase activity was normalized to Firefly luciferase activity.

In vitro Angiogenesis Assay

The *in vitro* angiogenesis assay was performed as previously described (Zeng et al., 2019). The 24-well plates were coated with Matrigel (300 μL/well; BD Biosciences, San Jose, CA, United States) without introducing air bubbles. After gelling of the Matrigel, 5×10^4 cells were plated into each Matrigel-coated well along with 200 μL of HUVEC basal medium containing 10% FBS. After 6 h incubation with or without 50 ng/mL hVEGF at 37°C with 5% CO_2 in a humidified incubator, the medium was gently aspirated from each well and incubated with Diff-Quick fixative (Dade Behring, Deerfield, IL, United States) for 30 s and subsequently stained with solution II for 2 min. Tube structures were observed and imaged by microscopy. The pseudo-vascular organization of cells was analyzed by ImageJ software (version 1.52u) using the Angiogenesis Analyzer plugin[2].

Cell Migration and Invasion Assay

Cells were digested with 0.25% EDTA trypsin and resuspended in HUVEC basal medium (HUVEC-004B; Allcells) with or without 50 ng/mL hVEGF. Cells were seeded at a density of 1×10^5 cells per Transwell (BD Biosciences), and HUVEC basal medium containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare, Logan, UT, United States) was added to the lower chamber. The Transwell membrane was precoated with Matrigel for invasion and not precoated for migration. After 48 h, cells that had migrated or invaded through the membrane were quantified as previously described (Zeng et al., 2019).

Statistical Analysis

Statistical significance was determined by Student's *t*-test or one-way analysis of variance with either the least significant difference test or Tamhane's T2 test (depending on Levene's statistic for homogeneity of variance) using SPSS software (v25.0; IBM, Armonk, NY, United States). The relationships between miR-9 and S1P receptors were measured using Pearson correlation methods. Data were presented as Mean ± SEM. $P < 0.05$ was considered statistically significant.

RESULTS

MiR-9 Is Overexpressed in Tumor ECs, Which Is Negatively Associated With $S1P_1$

The levels of miR-9 and S1P receptors in normal ECs and tumor ECs in multiple tumors were compared (**Figure 1**). As shown in **Figure 1A**, databases E-GEOD-73753 and E-MTAB-3949 show miR-9-1, miR-9-2., miR-9-3, $S1P_1$, $S1P_2$, and $S1P_3$ levels in Shh-medulloblastoma ECs, Wnt-medulloblastoma ECs, and ECs from angiogenic glioblastoma xenograft. Databases GSE-77199, E-GEDO-51401, and E-GEOD-41614 show levels of S1P receptors in colorectal cancer ECs, renal cell carcinoma ECs, hepatocellular carcinoma ECs, and bladder cancer ECs. Specifically, miR-9-1 was significantly upregulated

[2]https://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt

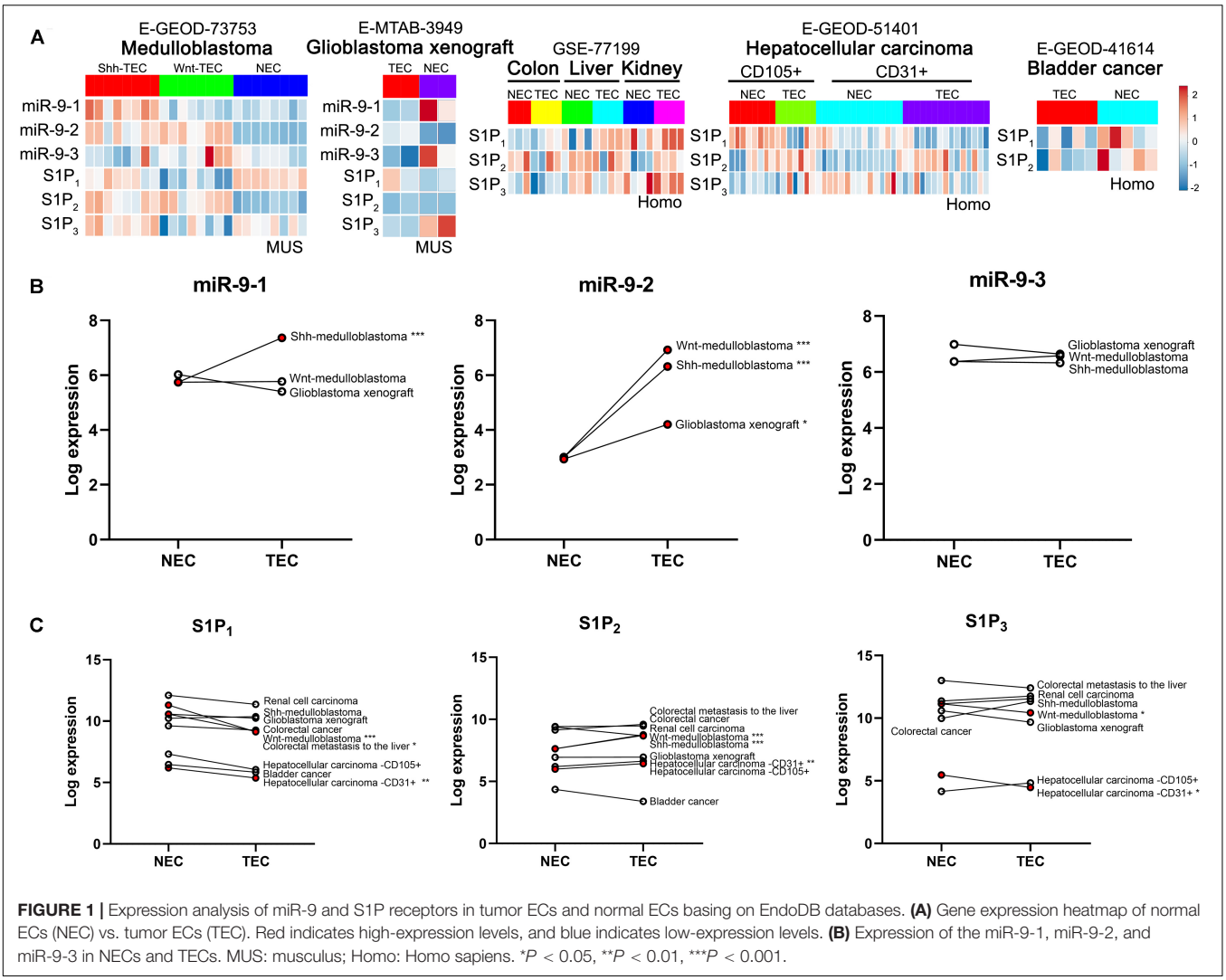


FIGURE 1 | Expression analysis of miR-9 and S1P receptors in tumor ECs and normal ECs basing on EndoDB databases. **(A)** Gene expression heatmap of normal ECs (NEC) vs. tumor ECs (TEC). Red indicates high-expression levels, and blue indicates low-expression levels. **(B)** Expression of the miR-9-1, miR-9-2, and miR-9-3 in NECs and TECs. MUS: musculus; Homo: Homo sapiens. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

in Shh-medulloblastoma ECs (**Figure 2B**). miR-9-2 was significantly upregulated in Wnt-medulloblastoma ECs, Shh-medulloblastoma ECs, and ECs from glioblastoma xenograft. miR-9-3 was not significantly changed between normal ECs and those tumor ECs. $S1P_1$ was significantly downregulated in Wnt-medulloblastoma ECs, ECs from colorectal metastasis to liver, and hepatocellular carcinoma ECs (**Figure 2C**). $S1P_2$ was significantly upregulated in medulloblastoma and hepatocellular carcinoma ECs. $S1P_3$ was significantly downregulated in Wnt-medulloblastoma and hepatocellular carcinoma ECs. Taken together, miR-9 was significantly upregulated in multiple tumor ECs. It was accompanied by significant downregulation of $S1P_1$ and $S1P_3$.

Furthermore, the correlations between miR-9-2 and $S1P_1$, $S1P_2$, and $S1P_3$ in normal ECs and medulloblastoma ECs were analyzed (**Figures 2A,B**). Results have shown miR-9-2 negatively correlated to $S1P_1$ and $S1P_3$ in Shh-medulloblastoma ECs, but there was no significant correlation in normal ECs. There was no significant correlation between miR-9-2 and $S1P_2$ in both Shh- and Wnt-medulloblastoma ECs (**Figures 2A,B**). Thus, the upregulation of miR-9 in tumor ECs is significantly associated with the downregulation of $S1P_1$ and $S1P_3$.

MiR-9 Negatively Regulates $S1P_1$ and $S1P_3$

Upregulation of miR-9 in tumor-associated ECs was simulated by the transfection of miR-9-expressing lentivirus into HUVECs. A 94.3-fold increase in miR-9 was shown in the ECs overexpressing miR-9, compared with NC (**Figure 2C**). The mRNA levels of $S1P_1$, $S1P_2$ and $S1P_3$ in the ECs overexpressing miR-9 were significantly downregulated (0.18 ± 0.04 vs. 1.01 ± 0.07 for $S1P_1$, $P < 0.001$; 0.58 ± 0.02 vs. 1.01 ± 0.02 for $S1P_2$, $P < 0.01$; 0.52 ± 0.05 vs. 1.00 ± 0.03 for $S1P_3$, $P < 0.01$, **Figure 2C**). Levels of $S1P_1$, $S1P_2$, and $S1P_3$ in the ECs overexpressing miR-9 were also detected by Western Blot assay (**Figures 3A,B**). There were no significant changes in levels of $S1P_1$, $S1P_2$, and $S1P_3$ between normal and NC cells, while miR-9 overexpression significantly downregulated the levels of $S1P_1$ (0.30 ± 0.11 vs. 1.20 ± 0.07, miR-9 vs. NC, $P < 0.001$) and $S1P_3$

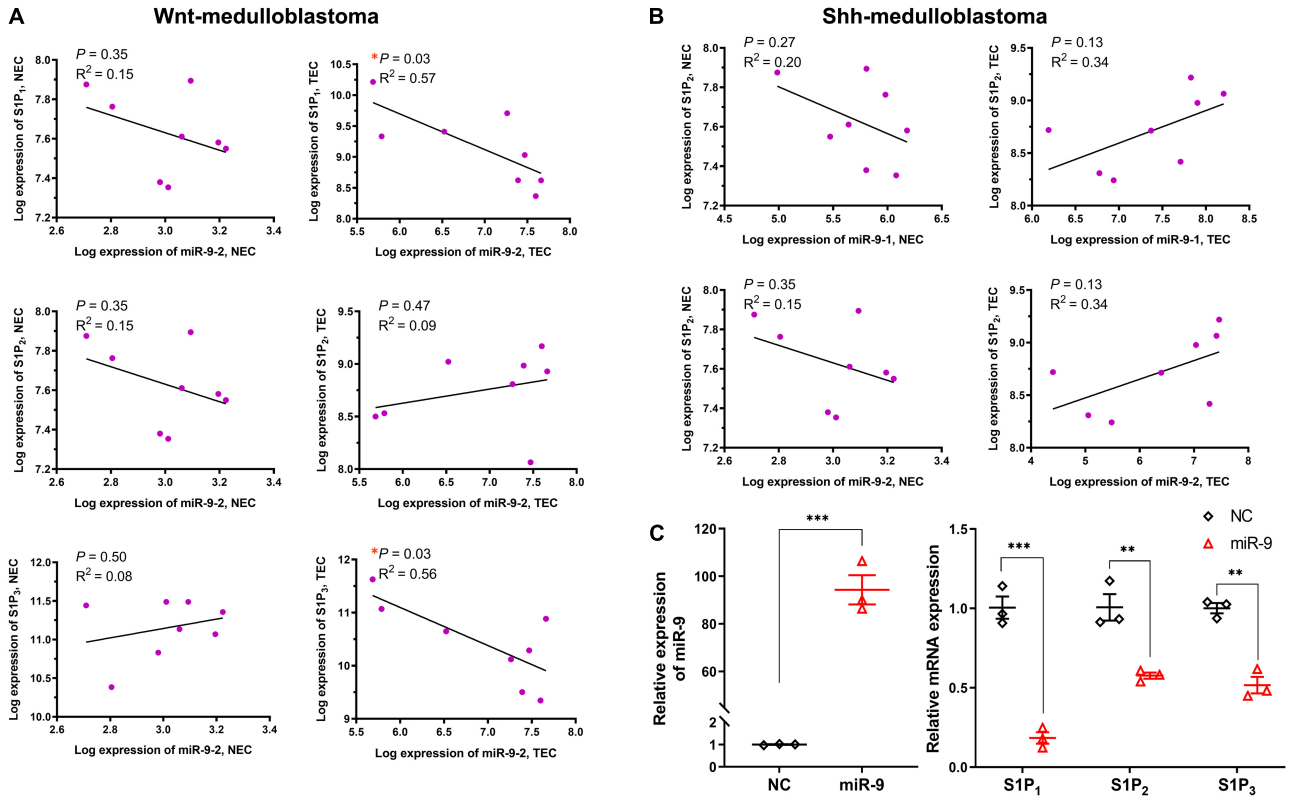


FIGURE 2 | Relationship between miR-9 and S1P receptors. The correlation between the significant differentially expressed miR-9 loci and specific S1P receptors in normal ECs (NEC) and tumor ECs (TEC) were performed. **(A)** Correlation between miR-9-2 and S1P receptors in Wnt-medulloblastoma; **(B)** correlation between miR-9-1, miR-9-2, and $S1P_2$ in Shh-medulloblastoma. Then, the levels of S1P receptors in HUVECs upon miR-9 mimics transfection were detected by qRT-PCR. **(C)** miR-9 levels in HUVECs overexpressing miR-9 (left) and mRNA expression of $S1P_1$, $S1P_2$, and $S1P_3$ (right). NC: miR-9 mimics negative control. Mean ± SEM. $n = 4$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

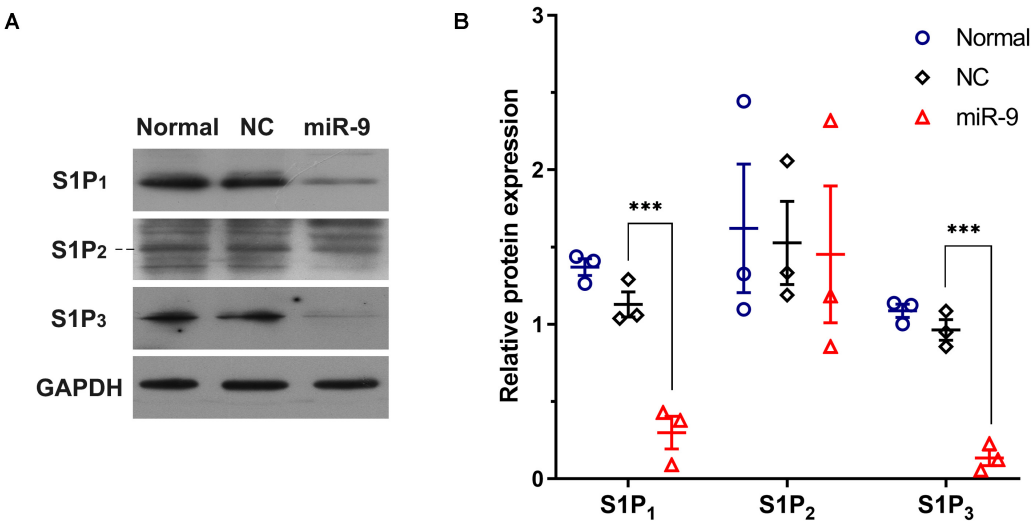


FIGURE 3 | Western blot detection of $S1P_1$, $S1P_2$, and $S1P_3$ in HUVECs overexpressing miR-9. Cell lysates were collected from HUVECs with or without NC or miR-9 transfection, and then 20 μg of protein samples were subjected to western blot analysis. **(A)** Western blot of cell lysates using anti-S1P1, anti-S1P2, and anti-S1P3. **(B)** The quantification was performed with ImageJ. The protein expression was normalized to the GAPDH level. NC: miR-9 mimics negative control. Mean ± SEM. $n = 3$, $^{***}P < 0.001$.

(0.13 ± 0.05 vs. 0.96 ± 0.07, miR-9 vs. NC, $P < 0.001$) in the ECs. The expression of $S1P_2$ was not significantly changed in HUVECs overexpressing miR-9 (1.45 ± 0.44 vs. 1.53 ± 0.27, miR-9 vs. NC, $P > 0.05$). These results suggest that miR-9 significantly inhibited $S1P_1$ and $S1P_3$ in both mRNA and protein levels.

MiR-9 Directly Downregulates the $S1P_1$ but Does Not Directly Target the $S1P_3$

The binding sites between $S1P_1$, $S1P_3$, and miR-9 were predicted using TargetScan (Release 7.1[3], Whitehead Institute for Biomedical Research, Cambridge, MA, United States) and microRNA.org (Release August 2010[4], the Computational Biology Center at Memorial Sloan-Kettering Cancer Center, Rockville, MD, United States). There was a predicted miR-9 binding site (7mer-A1) at position 2380–2386 of $S1P_1$ 3′UTR and two predicted miR-9 binding sites at position 1587–1593 (7mer-m8) of $S1P_3$ 3′UTR and 816-822 (7mer-A1) (**Figures 4A,B**). However, the miR-9 binding site at 816-822 is within the coding region of $S1P_3$ (NM_005226.4). Therefore, the luciferase reporter plasmids containing the WT and the corresponding MUT sites at 3′UTR of $S1P_1$ (position 2380–2386) and $S1P_3$ (position 1587–1593) were generated. The luciferase activity of cells with WT-3′UTR of $S1P_1$ was significantly inhibited by miR-9, while the luciferase activity of cells with MUT-3′UTR of $S1P_1$ was not significantly inhibited by miR-9 (**Figure 4A**). It was not observed a significant decrease in luciferase activity of cells with WT-3′UTR of $S1P_3$ by miR-9, indicating that miR-9 was not directly targeted the 3′UTR of $S1P_3$ (**Figure 4B**). These results suggest that $S1P_1$ but not $S1P_3$ is a bona fide miR-9 target.

[3]http://www.targetscan.org/

[4]http://www.microrna.org/

Overexpression of $S1P_1$ Inhibits the VEGF-Induced Angiogenesis

To investigate the role of $S1P_1$ in angiogenesis, $S1P_1$ was significantly overexpressed in ECs (2.17 ± 0.11 vs. 0.89 ± 0.03, $S1P_1$ overexpression vs. vector, $P < 0.05$, **Figure 5A**). Overexpression of $S1P_1$ significantly suppressed 50 ng/mL VEGF-induced migration and invasion (**Figures 5B–D**). As a consequence, the overexpression of $S1P_1$ significantly inhibited the VEGF-induced tube formation. The tube length and number of junctions in the network were significantly reduced by $S1P_1$ overexpression (**Figures 5E–G**). These results

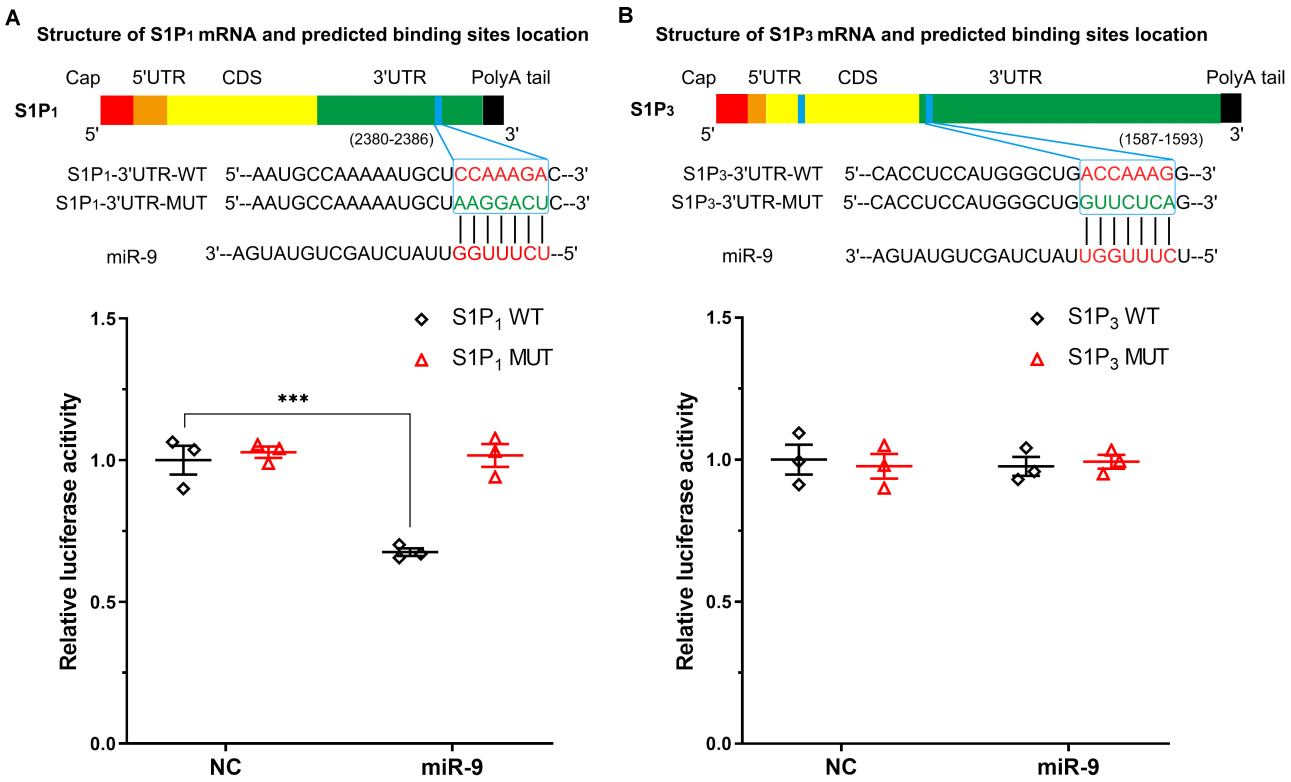


FIGURE 4 | Associations between $S1P_1$, $S1P_3$, and miR-9 were detected by dual-luciferase reporter assay. The structures of $S1P_1$ mRNA (transcript variant 1 M_001400.5, and transcript variant 2 NM_001320730.1; the variant 2 differs in the 5′UTR compared to variant 1, both variants 1 and 2 encode the same protein) and $S1P_3$ mRNA (NM_005226.4) including coding DNA sequence (CDS) and untranslated regions were depicted. The blue band indicated the putative miR-9 binding sequence in the target mRNA molecules. The binding sites located at 3′UTR were screened for Dual-Luciferase Reporter Assay analysis. **(A)** Putative wild-type (WT) binding sequence and its mutation (MUT) sequence in the 3′UTR of $S1P_1$. Luciferase activity was measured. **(B)** Putative WT binding sequence and its MUT sequence in the 3′UTR of $S1P_3$. Luciferase activity was measured. NC: miR-9 mimics negative control. Mean ± SEM. $n = 3$, $^{***}P < 0.001$.

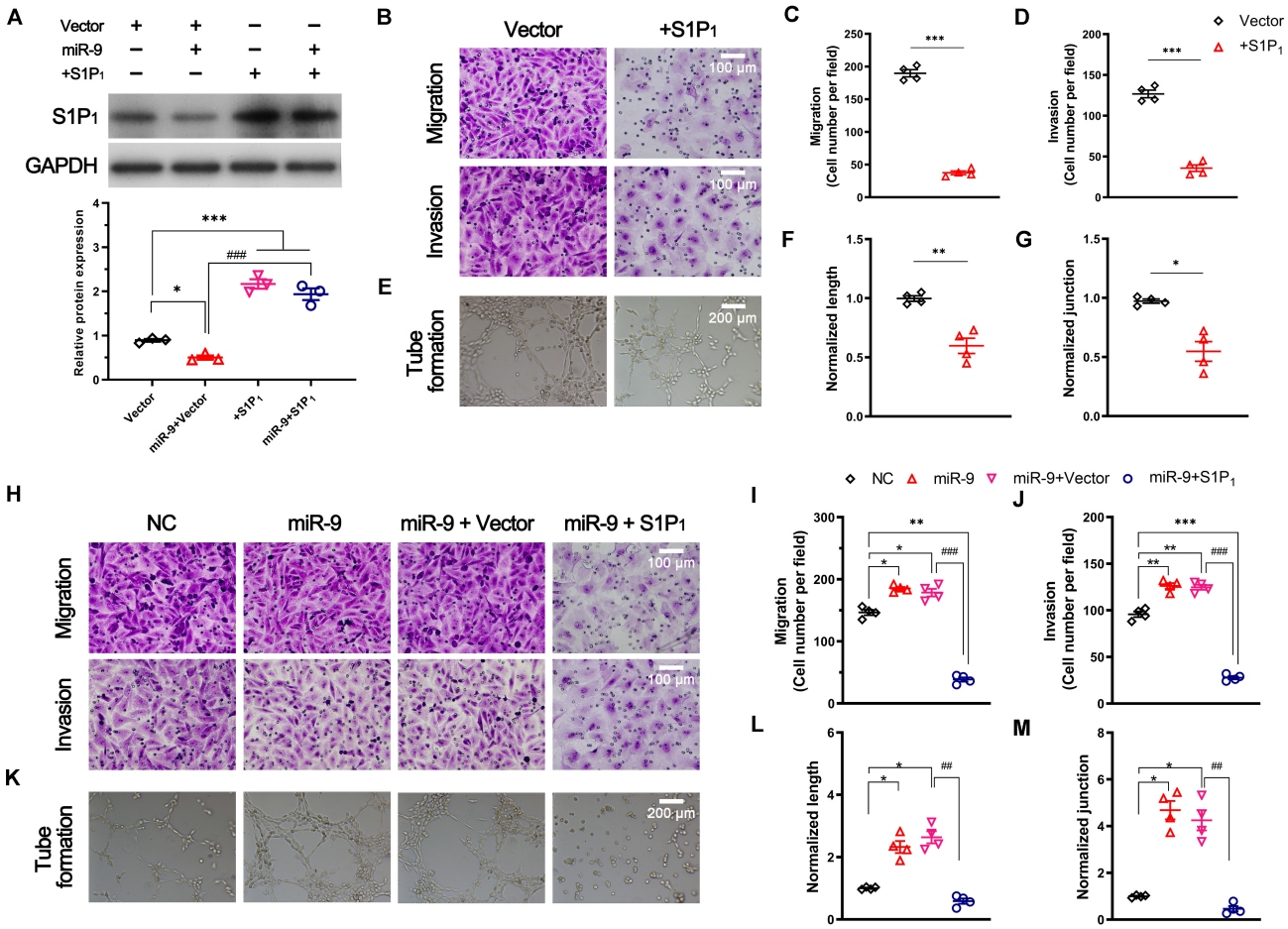


FIGURE 5 | Effect of $S1P_1$ on angiogenesis. Cells were transfected with pcDNA3.1 vector expressing $S1P_1$ (+$S1P_1$) or pcDNA3.1 vector (vector). For the rescue assay, cells overexpressing miR-9 were transfected with pcDNA3.1 vector expressing $S1P_1$ (miR-9 + $S1P_1$) or pcDNA3.1 vector (miR-9 + vector). After transfection for 48 h, the protein level, migration, invasion and *in vitro* angiogenesis were performed. **(A)** Western blot of $S1P_1$ and quantification. Mean ± SEM. $n = 3$, $^{*}P < 0.05$, $^{***}P < 0.001$. $^{\#\#\#}P < 0.001$. **(B–D)** Transwell assays **(B)** of migration (top) and invasion (bottom) of HUVECs overexpressing $S1P_1$ in response to 50 ng/mL hVEGF. Mean ± SEM, $n = 4$. $^{***}P < 0.001$. **(E–G)** Tube formation **(E)**, normalized tube length **(F)**, and number of junctions **(J)** formed by HUVECs overexpressing $S1P_1$ in response to 50 ng/mL hVEGF. Mean ± SEM, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$. **(H–J)** Transwell assays **(H)** of migration (top) and invasion (bottom) of HUVECs overexpressing miR-9 and $S1P_1$. NC: miR-9 mimics negative control. Mean ± SEM, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $^{\#\#\#}P < 0.001$. **(K–M)** Tube formation **(K)**, normalized tube length **(L)**, and the number of junctions **(M)** formed by HUVECs overexpressing miR-9 and $S1P_1$. NC: miR-9 mimics negative control. Mean ± SEM, $n = 4$. $^{*}P < 0.05$; $^{\#\#}P < 0.01$.

suggest that overexpression of $S1P_1$ inhibits the VEGF-induced angiogenesis in HUVECs.

MiR-9 Promotes Angiogenesis via Downregulation of $S1P_1$

We then investigated the role of $S1P_1$ in miR-9-induced angiogenesis. A rescue assay demonstrated the significant upregulation of $S1P_1$ in ECs overexpressing miR-9 (1.94 ± 0.23 vs. 0.50 ± 0.08, miR-9 + $S1P_1$ vs. miR-9 + vector, $P < 0.001$, **Figure 5A**). The restoration of $S1P_1$ expression significantly reversed the miR-9-induced migration, invasion, and angiogenesis in HUVECs (**Figures 5H–M**). The tube formation, tube length, and the number of junctions in the network induced by miR-9 were significantly reduced by the restoration of $S1P_1$ (**Figures 5K–M**). These results suggest that miR-9 promotes angiogenesis via direct downregulation of $S1P_1$.

DISCUSSION

Angiogenesis is considered one of the critical pathophysiological events in multiple disorders including atherosclerotic plaque rupture (Perrotta et al., 2020), cancer, and so on (Hosein et al., 2020). MiR-9 is elevated in atherosclerosis and many cancers such as breast cancer, hepatocellular carcinoma, squamous cell carcinomas, lung and colorectal carcinoma, and ovarian cancer (Laios et al., 2008; Ma et al., 2010; Zhuang et al., 2012; White et al., 2013; Drakaki et al., 2015). Our previous study has shown miR-9 induced angiogenesis via activating of the autophagy

pathway (Zeng et al., 2019). Our new findings in the present study demonstrate that upregulation of miR-9-2 locus in tumor ECs is significantly associated with downregulation of $S1P_1$ and $S1P_3$, and further validate that $S1P_1$ acts as a bona fide target for miR-9, and the downregulation of $S1P_1$ contributes to EC migration, invasion, and angiogenesis.

The role of miR-9 in angiogenesis is a promising therapy for many diseases. MiR-9 could promote migration, invasion, and angiogenesis of endothelial progenitor cells via downregulating transient receptor potential melastatin 7 (TRPM7) and activating PI3K/Akt/autophagy pathway, which might facilitate thrombi recanalization in deep vein thrombosis (Zhou et al., 2020). Translocation of miR-9 from bone marrow-derived mesenchymal stem cells into vascular ECs could induce angiogenesis via activating PI3K/AKT pathway to repair the severe acute pancreatitis (Qian et al., 2018). Moreover, miR-9 promotes angiogenesis via the downregulation of CXC chemokine receptor-4 (CXCR4) and inhibition of PI3K/AKT/mammalian target of rapamycin (mTOR) pathway in HUVECs, thereby suppressing the high glucose-induced injury in HUVECs (Yi and Gao, 2019). It is unclear why both activation and inhibition of PI3K/AKT regulated by miR-9, but both of them involved in promoting angiogenesis in HUVECs. PI3K/AKT/mTOR pathway is an important regulator of autophagy. We previously described that miR-9 activated autophagy in HUVECs and reviewed that miR-9 may induce autophagy via targeted suppression of FOXO1, CUL4A, CK1α, GSK3β, Notch2, cyclin D1, and MCPIP1 (Zeng et al., 2019). In the present study, we verified that miR-9 directly bound to 3′UTR of $S1P_1$. The overexpression of $S1P_1$ inhibited the VEGF-induced angiogenesis. A rescue assay also demonstrated that restoration of $S1P_1$ inhibited the miR-9 induced angiogenesis. For the rescue assay, the transfection efficacy of the pcDNA3.1 vector in EC is more than 80%. After transfected with pcDNA3.1 vector, $S1P_1$ expression in ECs was reduced less by miR-9 compared with that without pcDNA3.1 vector transfection, without significant difference (by 43.4 ± 7.7% miR-9/NC vs. 74.3 ± 8.9% miR-9 + vector/vector, Mean ± SEM, $P > 0.05$, **Figures 2**, **4**), suggesting the change in endogenous $S1P_1$ could be disregarded. The slight change in reduction of $S1P_1$ might be caused by too many vectors transfection into ECs overexpressing miR-9 or miR-9 trapped somewhere else by other cellular elements such as long non-coding RNAs.

Excessive tumor vessels were present in mice with endothelial cell-specific knockout of $S1P_1$ (Cartier et al., 2020). Loss of $S1P_1$ induced angiogenesis might be associated with increased VEGFR2 activity (Gaengel et al., 2012). Inhibition of $S1P_1$ enhanced VEGFR2 activation in murine ECs upon murine VEGF injection (Fischl et al., 2019). The CD34 labeled vessels tended to increase with $S1P_1$ inhibition, but the combined inhibition of $S1P_1$ and VEGF pathways reduced blood flow in tumor, increased tumor cell apoptosis, and inhibited the tumor CD34 positive vessels in clear cell renal cell carcinoma tumor models (Fischl et al., 2019). We previously demonstrated that activation of $S1P_1$ protects ECs against glycocalyx shedding and promotes the glycocalyx synthesis via activating PI3K signaling (Zeng et al., 2014, 2015). The association among $S1P_1$, PI3K/AKT/mTOR and autophagy will be investigated in the future. In atherosclerosis, miR-9 upregulation is associated with inhibition of intracellular lipid accumulation and macrophage foam cell formation (Shao et al., 2020). Whether miR-9 contributed to the angiogenesis in atherosclerotic plaque rupture remains to be clarified by *in vivo* experiments.

Many biological functions of $S1P_1$ on the vasculature and its expression are regulated by S1P. S1P acts primarily as an extracellular signaling molecule activated G protein-coupled S1P receptors including $S1P_1$, $S1P_2$, and $S1P_3$ in ECs. $S1P_1$ is the main S1P receptor expressed by ECs (Panetti, 2002). S1P induces migration of various ECs via $S1P_1$ and $S1P_3$ but does not induce the migration of nonendothelial cells (Panetti, 2002). It was demonstrated that S1P induces angiogenesis only in the presence of low levels of VEGF, providing a major caveat as an angiogenic factor (Panetti, 2002). $S1P/S1P_1$ signaling activation inhibited VEGF-induced angiogenic responses (Jozefczuk et al., 2020). More than 50% of S1P is carried by high-density lipoprotein (HDL). HDL promotes angiogenesis via $S1P/S1P_3$ mediated VEGFR2 activation (Jin et al., 2018). In the present study, we found that $S1P_3$ was downregulated by miR-9. However, we failed to validate the predicted miR-9 binding sites within $S1P_3$ 3′UTR. There might be an unknown intermediate element such as a transcription factor in the miR-9/$S1P_3$ pathway. In the future, it will be worth to find the intermediate element and test how is $S1P_3$ regulated by miR-9 and associated with angiogenesis. S1P is significantly reduced in cardiovascular diseases (Jozefczuk et al., 2020), and significantly evaluated in hepatocellular carcinoma tissues (Zeng et al., 2016). The relationship between miR-9 and S1P in a special environment should be carefully considered. Although the $S1P_2$ mRNA was downregulated by miR-9, the protein level of $S1P_2$ was only slightly decreased without significant difference. This might be due to the poor sensitivity of the commercially available $S1P_2$ antibody. Moreover, the miR-9 binding site was not predicted within the $S1P_2$ 3′UTR using the TargetScan and microRNA.org.

It was believed that most known miRNA target sites (miRNA seed) have 7 nt Watson-Crick seed matches (seed matches) (Lewis et al., 2003). The complete UTR sequence is most critical for mRNA recognition. After changing one or more base pairings between miRNAs and mRNAs, the target UTRs would not identify by miRNAs. We mutated 6 nt and had not tested other constructs with different mutations for $S1P_1$. Moreover, it was demonstrated that the miRNA response elements might locate within the luciferase coding region (Campos-Melo et al., 2014). We predicted whether miR-9 targeting the mRNA sequence of Renilla luciferase gene (hRluc) or firefly luciferase gene (hluc+) by using custom prediction tools of miRbase (Release 22.1[5]; managed by the Griffiths-Jones lab at the Faculty of Biology, Medicine and Health, University of Manchester, United Kingdom) and miRDB (Xiaowei Wang's lab[6] at the Department of Radiation Oncology, Washington University School of Medicine in St. Louis, United States) databases. The

[5]http://www.mirbase.org

[6]http://mirdb.org/

predicted miRNAs do not include miR-9, suggesting luciferase could not modify our results.

In HUVECs, an about 94-folds overexpression of miR-9 was achieved artificially. Nevertheless, the luminescence in the miR-9 cells with WT-3′UTR of $S1P_1$ was decreased by only about 40%. Bioluminescence is a chemical process in which an enzyme such as luciferase breaks down a substrate such as luciferin and one of the by-products of this reaction is light. The transfection efficacy of the psiCHECK-2 vector and conversion efficiency of luciferin to oxyluciferin might limit the diminution of the luminescence. It was worth noting that the psiCHECK-2 vector contains both renilla and firefly luciferase genes. As Renilla transcript expressed with the 3′UTR sequence of interest gene, Renilla luciferase activity is used as a measure of the effect of the 3′UTR on transcript stability and translation efficiency. Firefly luciferase is used to normalize transfections and eliminates the need to transfect a second vector control. The transfection efficacy of the psiCHECK-2 vector was not detected in EC.

In summary, building on previous findings that miR-9 overexpression in HUVECs promotes angiogenesis, and AATs triggers VEGF-enriched exosomes to promote tumor vasculogenesis, we present data to show that $S1P_1$ acts as a bona fide target for miR-9 to regulate the migration, invasion, and angiogenesis. The expressions of $S1P_1$/$S1P_2$/$S1P_3$ between normal-associated ECs and tumor-associated ECs should be carried out by *in vivo* and *in vitro* experiments such as normal cells/tumor cells and ECs crosstalk assays in the future. The investigation on the molecular mechanism whereby miR-9 promotes angiogenesis in HUVECs might provide a critical cue to reveal the AATs triggered exosomes release and further to control the tumor vasculogenesis and progression following AATs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

YZ contributed to the conception and design of the work. YZ, XY, and LX performed the acquisition, analysis, and interpretation of data for the work. YZ and XY drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant nos. 11932014, 11402153).

REFERENCES

Campos-Melo, D., Droppelmann, C. A., Volkening, K., and Strong, M. J. (2014). Comprehensive luciferase-based reporter gene assay reveals previously masked up-regulatory effects of miRNAs. *Int. J. Mol. Sci.* 15, 15592–15602. doi: 10.3390/ijms150915592

Cantelmo, A. R., Pircher, A., Kalucka, J., and Carmeliet, P. (2017). Vessel pruning or healing: endothelial metabolism as a novel target? *Expert Opin. Ther. Targets* 21, 239–247. doi: 10.1080/14728222.2017.1282465

Cartier, A., and Hla, T. (2019). Sphingosine 1-phosphate: lipid signaling in pathology and therapy. *Science* 366:6463. doi: 10.1126/science.aar5551

Cartier, A., Leigh, T., Liu, C. H., and Hla, T. (2020). Endothelial sphingosine 1-phosphate receptors promote vascular normalization and antitumor therapy. *Proc. Natl. Acad. Sci. U.S.A.* 117, 3157–3166. doi: 10.1073/pnas.1906246117

Cloughesy, T. F., Brenner, A., de Groot, J. F., Butowski, N. A., Zach, L., Campian, J. L., et al. (2020). A randomized controlled phase III study of VB-111 combined with bevacizumab vs bevacizumab monotherapy in patients with recurrent glioblastoma (GLOBE). *Neuro Oncol.* 22, 705–717. doi: 10.1093/neuonc/noz232

Cully, M. (2017). Cancer: tumour vessel normalization takes centre stage. *Nat. Rev. Drug Discov.* 16:87. doi: 10.1038/nrd.2017.4

Drakaki, A., Hatziapostolou, M., Polytarchou, C., Vorvis, C., Poultsides, G. A., Souglakos, J., et al. (2015). Functional microRNA high throughput screening reveals miR-9 as a central regulator of liver oncogenesis by affecting the PPARA-CDH1 pathway. *BMC Cancer* 15:542. doi: 10.1186/s12885-015-1562-1569

Du, W., Takuwa, N., Yoshioka, K., Okamoto, Y., Gonda, K., Sugihara, K., et al. (2010). S1P(2), the G protein-coupled receptor for sphingosine-1-phosphate, negatively regulates tumor angiogenesis and tumor growth in vivo in mice. *Cancer Res.* 70, 772–781. doi: 10.1158/0008-5472.Can-09-2722

Fischl, A. S., Wang, X., Falcon, B. L., Almonte-Baldonado, R., Bodenmiller, D., Evans, G., et al. (2019). Inhibition of sphingosine phosphate receptor 1 signaling enhances the efficacy of VEGF receptor inhibition. *Mol. Cancer Ther.* 18, 856–867. doi: 10.1158/1535-7163.Mct-18-0548

Gaengel, K., Niaudet, C., Hagikura, K., Lavina, B., Muhl, L., Hofmann, J. J., et al. (2012). The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. *Dev. Cell.* 23, 587–599. doi: 10.1016/j.devcel.2012.08.005

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell. Biol.* 161, 1163–1177. doi: 10.1083/jcb.200302047

Hosein, A. N., Brekken, R. A., and Maitra, A. (2020). Pancreatic cancer stroma: an update on therapeutic targeting strategies. *Nat. Rev. Gastroenterol. Hepatol.* [Epub ahead of print]. doi: 10.1038/s41575-020-0300-1

Jin, F., Hagemann, N., Sun, L., Wu, J., Doeppner, T. R., Dai, Y., et al. (2018). High-density lipoprotein (HDL) promotes angiogenesis via S1P3-dependent VEGFR2 activation. *Angiogenesis* 21, 381–394. doi: 10.1007/s10456-018-9603-z

Jozefczuk, E., Guzik, T. J., and Siedlinski, M. (2020). Significance of sphingosine-1-phosphate in cardiovascular physiology and pathology. *Pharmacol. Res.* 156:104793. doi: 10.1016/j.phrs.2020.104793

Khan, S., Taverna, F., Rohlenova, K., Treps, L., Geldhof, V., de Rooij, L., et al. (2019). EndoDB: a database of endothelial cell transcriptomics data. *Nucleic Acids Res.* 47, D736–D744. doi: 10.1093/nar/gky997

Laios, A., O'Toole, S., Flavin, R., Martin, C., Kelly, L., Ring, M., et al. (2008). Potential role of miR-9 and miR-223 in recurrent ovarian cancer. *Mol. Cancer* 7:35. doi: 10.1186/1476-4598-7-35

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell* 115, 787–798. doi: 10.1016/s0092-8674(03)01018-3

Ma, L., Young, J., Prabhala, H., Pan, E., Mestdagh, P., Muth, D., et al. (2010). miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat. Cell Biol.* 12, 247–256. doi: 10.1038/ncb2024

Panetti, T. S. (2002). Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. *Biochim. Biophys. Acta* 1582, 190–196. doi: 10.1016/s1388-1981(02)00155-5

Perrotta, P., Van der Veken, B., Van Der Veken, P., Pintelon, I., Roosens, L., Adriaenssens, E., et al. (2020). Partial inhibition of glycolysis reduces atherogenesis independent of intraplaque neovascularization in mice.

Arterioscler. Thromb. Vasc. Biol. 40, 1168–1181. doi: 10.1161/atvbaha.119.313692

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887. doi: 10.1016/j.cell.2011.08.039

Qian, D., Song, G., Ma, Z., Wang, G., Jin, L., Hu, M., et al. (2018). MicroRNA-9 modified bone marrow-derived mesenchymal stem cells (BMSCs) repair severe acute pancreatitis (SAP) via inducing angiogenesis in rats. *Stem Cell Res. Ther.* 9:282. doi: 10.1186/s13287-018-1022-y

Shao, D., Di, Y., Lian, Z., Zhu, B., Xu, X., Guo, D., et al. (2020). Grape seed proanthocyanidins suppressed macrophage foam cell formation by miRNA-9 via targeting ACAT1 in THP-1 cells. *Food Funct.* 11, 1258–1269. doi: 10.1039/c9fo02352f

White, R. A., Neiman, J. M., Reddi, A., Han, G., Birlea, S., Mitra, D., et al. (2013). Epithelial stem cell mutations that promote squamous cell carcinoma metastasis. *J. Clin. Invest.* 123, 4390–4404. doi: 10.1172/jci65856

Yi, J., and Gao, Z. F. (2019). MicroRNA-9-5p promotes angiogenesis but inhibits apoptosis and inflammation of high glucose-induced injury in human umbilical vascular endothelial cells by targeting CXCR4. *Int. J. Biol. Macromol.* 130, 1–9. doi: 10.1016/j.ijbiomac.2019.02.003

Yi, M., Jiao, D., Qin, S., Chu, Q., Wu, K., and Li, A. (2019). Synergistic effect of immune checkpoint blockade and anti-angiogenesis in cancer treatment. *Mol. Cancer* 18:60. doi: 10.1186/s12943-019-0974-6

Zeng, Y., Adamson, R. H., Curry, F. R., and Tarbell, J. M. (2014). Sphingosine-1-phosphate protects endothelial glycocalyx by inhibiting syndecan-1 shedding. *Am. J. Physiol. Heart Circ. Physiol.* 306, H363–H372. doi: 10.1152/ajpheart.00687.2013

Zeng, Y., Liu, X. H., Tarbell, J., and Fu, B. (2015). Sphingosine 1-phosphate induced synthesis of glycocalyx on endothelial cells. *Exp. Cell Res.* 339, 90–95. doi: 10.1016/j.yexcr.2015.08.013

Zeng, Y., Yao, X., Chen, L., Yan, Z., Liu, J., Zhang, Y., et al. (2016). Sphingosine-1-phosphate induced epithelial-mesenchymal transition of hepatocellular carcinoma via an MMP-7/ syndecan-1/TGF-beta autocrine loop. *Oncotarget* 7, 63324–63337. doi: 10.18632/oncotarget.11450

Zeng, Y., Yao, X., Liu, X., He, X., Li, L., Liu, X., et al. (2019). Anti-angiogenesis triggers exosomes release from endothelial cells to promote tumor vasculogenesis. *J. Extracell. Vesicles* 8:1629865. doi: 10.1080/20013078.2019.1629865

Zhou, D. M., Sun, L. L., Zhu, J., Chen, B., Li, X. Q., and Li, W. D. (2020). MiR-9 promotes angiogenesis of endothelial progenitor cell to facilitate thrombi recanalization via targeting TRPM7 through PI3K/Akt/autophagy pathway. *J. Cell. Mol. Med.* 24, 4624–4632. doi: 10.1111/jcmm.15124

Zhuang, G., Wu, X., Jiang, Z., Kasman, I., Yao, J., Guan, Y., et al. (2012). Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *EMBO J.* 31, 3513–3523. doi: 10.1038/emboj.2012.183

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 11 August 2020
doi: 10.3389/fcell.2020.00775

Angiogenesis Inhibition by a Short 13 Amino Acid Peptide Sequence of Tetrastatin, the α4(IV) NC1 Domain of Collagen IV

*Alexia Vautrin-Glabik[1,2], Jérôme Devy[1,2], Camille Bour[1,2], Stéphanie Baud[1,2,3], Laurence Choulier[4], Anthony Hoarau[1,2†], Aurélie Dupont-Deshorgue[1,2], Christèle Sellier[1,2], Bertrand Brassart[1,2], Jean-Baptiste Oudart[1,2,5], Laurent Ramont[1,2,5], Jean Claude Monboisse[1,2,5] and Sylvie Brassart-Pasco[1,2]**

[1] Laboratoire de Biochimie, Université de Reims Champagne-Ardenne (URCA), Reims, France, [2] CNRS UMR 7369, Matrice Extracellulaire et Dynamique Cellulaire (MEDyC), Reims, France, [3] Plateau de Modélisation Moléculaire Multi-échelle, URCA, Reims, France, [4] CNRS UMR 7021, Laboratoire de Bioimagerie et Pathologies, Université de Strasbourg, Illkirch, France, [5] CHU Reims, Service Biochimie-Pharmacologie-Toxicologie, Reims, France

OPEN ACCESS

Edited by:
Lucas Treps,
VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Stephen Robinson,
University of East Anglia, United Kingdom
Parvez Khan,
University of Nebraska Medical Center, United States

***Correspondence:**
Sylvie Brassart-Pasco
sylvie.brassart-pasco@univ-reims.fr

†Present address:
Anthony Hoarau,
Inserm UMR-S 1250, Pathologies Pulmonaires et Plasticité Cellulaire, Reims, France

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 19 May 2020
Accepted: 23 July 2020
Published: 11 August 2020

Citation:
Vautrin-Glabik A, Devy J, Bour C, Baud S, Choulier L, Hoarau A, Dupont-Deshorgue A, Sellier C, Brassart B, Oudart J-B, Ramont L, Monboisse JC and Brassart-Pasco S (2020) Angiogenesis Inhibition by a Short 13 Amino Acid Peptide Sequence of Tetrastatin, the α4(IV) NC1 Domain of Collagen IV. Front. Cell Dev. Biol. 8:775. doi: 10.3389/fcell.2020.00775

Angiogenesis is defined as the formation of new capillaries by sprouting from the pre-existing microvasculature. It occurs in physiological and pathological processes particularly in tumor growth and metastasis. α1, α2, α3, and α6 NC1 domains from type IV collagen were reported to inhibit tumor angiogenesis. We previously demonstrated that the α4 NC1 domain from type IV collagen, named Tetrastatin, inhibited tumor growth in a mouse melanoma model. The inhibitory activity was located in a 13 amino acid sequence named QS-13. In the present paper, we demonstrate that QS-13 decreases VEGF-induced-angiogenesis *in vivo* using the Matrigel plug model. Fluorescence molecular tomography allows the measurement of a 65% decrease in Matrigel plug angiogenesis following QS-13 administration. The results are confirmed by CD31 microvessel density analysis on Matrigel plug slices. QS-13 peptide decreases Human Umbilical Vein Endothelial Cells (HUVEC) migration and pseudotube formation *in vitro*. Relevant QS-13 conformations were obtained from molecular dynamics simulations and docking. A putative interaction of QS-13 with $\alpha_5\beta_1$ integrin was investigated. The interaction was confirmed by affinity chromatography, solid phase assay, and surface plasmon resonance. QS-13 binding site on $\alpha_5\beta_1$ integrin is located in close vicinity to the RGD binding site, as demonstrated by competition assays. Collectively, our results suggest that QS-13 exhibits a mighty anti-angiogenic activity that could be used in cancer treatment and other pathologies with excessive angiogenesis such as hemangioma, psoriasis or diabetes.

Keywords: angiogenesis, matrikine, Tetrastatin, integrin, alpha 5 beta 1 collagen IV

INTRODUCTION

Angiogenesis is the formation of new blood vessels and capillaries from sprouting of pre-existing blood vessels. It normally occurs during wound healing and embryonic development, but it is also required for tumor growth and metastasis. This process is regulated by growth factors, such as vascular endothelial growth factors (VEGFs), which bind to their receptors on the normal

endothelial cell surface, induce transduction pathways and promote proliferation and migration of endothelial cells and vascular tube formation (Teleanu et al., 2019).

In recent years, the basement membrane (BM), a specialized extracellular matrix (ECM), has been recognized as a key regulator of cell behavior, and not only as an architectural support. BM is an important structural and functional component of blood vessels (Silva et al., 2018). Several BM components were reported to largely participate in the regulation of tumor angiogenesis (Kalluri, 2003).

Basement membrane are composed of type IV collagen in association with other minor collagens, such as collagens XVIII or XIX, laminins, nidogens, and perlecan. Type IV collagen is composed of three α(IV) chains, out of six possible [α1(IV)-α6(IV)]. Each α(IV) chain comprises a 7S N-terminal domain, a long interrupted triple helical domain and a globular C-terminal non-collagenous (NC1) domain (Wu and Ge, 2019). The α1(IV) NC1 domain (Arresten), the α2(IV) NC1 domain (Canstatin), the α3(IV) NC1 domain (Tumstatin), and the a6(IV) NC1 domain were reported to inhibit angiogenesis (Petitclerc et al., 2000; Wietecha et al., 2013; Monboisse et al., 2014). The α4(IV) NC1 domain was reported to slightly decrease bFGF-induced angiogenesis while α5(IV) NC1 domain has no effect (Petitclerc et al., 2000). Karagiannis and Popel reported the inhibitory effects of Pentastatin-1 and -2, two α5(IV) NC1 domain-derived peptides, on Human Umbilical Vein Endothelial Cells (HUVEC) proliferation (Karagiannis and Popel, 2008). They also pointed out the inhibitory effects of Tetrastatin-2 and Pentastatin-3, two peptides from the α4(IV) and α5(IV) NC1 domains, respectively, on VEGF-induced HUVEC migration We previously demonstrated the potent anti-tumor activity of the α4(IV) NC1 domain, named Tetrastatin (Brassart-Pasco et al., 2012). We recently identified the minimal active sequence (QKISRCQVCVKYS: QS-13) of Tetrastatin that reproduced whole Tetrastatin anti-tumor properties *in vivo* and *in vitro* on melanoma cell proliferation, migration, and invasion (Lambert et al., 2018).

In the present article, we investigated the inhibitory effects of QS-13 on angiogenesis *in vivo* in a Matrigel plug assay and *in vitro* on HUVEC proliferation, migration and pseudotube formation.

MATERIALS AND METHODS

Peptide Synthesis

QS-13 was purchased from Proteogenix® (Schiltigheim, France). It was obtained by solid-phase synthesis using a FMOC [N-(9-fluorenyl) methoxy-carbonyl] derivative procedure. It was then purified by reverse phase high performance liquid chromatography using a C18 column, eluted by a gradient of acetonitrile in trifluoroacetic acid and lyophilized. Its purity (>98%) was assessed by HPLC and mass spectroscopy.

Matrigel Plug Angiogenesis Assay

Eight-week-old albinos female B6(C)Rj -Tyr c/c mice were purchased from Janvier Laboratories (Saint-Berthevin, France). The animals were fed *ad libitum* with a chlorophyll-free diet 10 days before and during imaging experiments. The study was performed in compliance with "The French Animal Welfare Act" and following "The French Board for Animal Experiments." Experiments were conducted under approval of the French "Ministère de l'Enseignement Supérieur et de la Recherche" (Ethics Committees Nos. C2EA-56 and C2EA-75) in compliance with the "Directive 2010/63/UE". Protocol no. 4373_V1 APAFIS (07/09/2016).

Four hundred μL of Matrigel mix composed of growth factor-reduced Matrigel (Dutscher, Brumath, France) supplemented with 100 ng/mL recombinant mouse VEGF (R&D System-Bio-Techne, Lille, France), 350 ng/mL of recombinant mouse bFGF (R&D System-Bio-Techne, Lille, France) and 25 UI/mL of Heparin (R&D System-Bio-Techne, Lille, France) were injected into the left flank of each mouse. Mice were divided into two groups of 8 mice: positive control (Matrigel mix), QS-13-treated mice (Matrigel mix + 40 μM QS-13). Positive control mice received PBS and QS-13 treated mice received QS-13 (10 mg/kg) intraperitoneally at days 3, 7, and 11.

In vivo Fluorescence Imaging

At day 13, 100 μL of AngioSense680^{TM} (PerkinElmer, Inc., United States) were injected into the right orbital plexus of mice. At day 14, mice were anesthetized with 2% isoflurane and images were obtained with a fluorescence molecular tomographic (FMT®) imaging system (FMT4000, PerkinElmer, Inc., United States). Then, mice were sacrificed. Matrigel plug were removed and placed into 4% formaldehyde for histological analyses. 3D reconstruction and image analysis were performed using TrueQuant^{TM} software (PerkinElmer, Inc., United States).

CD31 Immunostaining

CD31 immunostaining were performed on 4 μm thick Matrigel plug sections. After deparaffinization, sections were incubated with a Tris–EDTA buffer, pH 8.4 for 20 min at 97°C, washed with distilled water and then incubated with hydrogen peroxide blocking solution (Abcam, Paris, France) for 10 min at room temperature and washed with PBS. They were incubated with Protein Block (Abcam) for 10 min at room temperature, washed with PBS, and incubated overnight at 4°C with an anti-CD31 rabbit monoclonal antibody diluted 1/1000 (ab 28364 from Abcam) and washed again with PBS. The first antibody was detected using the Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam) according to the manufacturer's instructions. Sections were counterstained with hematoxylin (Novocastra). To evaluate MicroVessel Density (MVD), three sections per plug were performed at three different depth levels of the plug and three different fields were acquired under an inverted microscope. Each positive endothelial cell cluster of immunoreactivity in contact with the selected field was counted as an individual vessel in addition to the morphologically identifiable vessels with a lumen, according to Weidner's method (Weidner, 1995).

Cell Culture

Human Umbilical Vein Endothelial Cells were purchased from Promocell (Heidelberg, Germany). Cells were grown

in Endothelial Cell Growth Medium (ECGM, Promocell, Heidelberg, Germany) at 37°C in a humid atmosphere with 5% CO_2 in air. At 70–90% confluency, cells were subcultured according to Promocell subcultivation protocol, using the Detach Kit (Hepes BSS, Trypsin/EDTA, Trypsin Neutralization Solution). They were used before passage 5.

Proliferation Assay

For cell proliferation measurement, 2,000 HUVECs were seeded in 96-well plates and cultivated in ECGM supplemented with 10 ng/mL of Vascular Endothelial Growth Factor (VEGF-165, Promocell) with or without 40 μM QS-13. After 24, 48, 72, and 96 h, cell proliferation was measured using the WST-1 cell proliferation reagent (Sigma, Saint-Quentin-Fallavier, France), according to the manufacturer's instructions. Absorbance was read at 450 nm using a Biochrom Asys UVM 340 microplate reader (Biochrom, Yvelines, France).

Scratch Wound Assay

Human Umbilical Vein Endothelial Cells were seeded in 24-well plates and cultivated to confluence in ECGM at 37°C in a humid atmosphere (5% CO_2, 95% air). At confluence, a homogenous wound was created in each well with a sterile 1,000 μL pipet tip. After washing, cells were incubated with fresh ECGM supplemented with 10 ng/mL VEGF with or without 40 μM QS-13 for 24 h. The wound area was measured at the beginning (T0) and end of the experiment (T24h) using the ImageJ analysis program (NIH, Bethesda, MD, United States) and the percentage of wound closure after 24 h was calculated.

Pseudotube Formation on Matrigel

The ability of HUVECs to form capillary tube structures was evaluated on Matrigel (Dutscher, Brumath, France). Matrigel (200 μL/well of a 10 mg/mL solution) was allowed to polymerize at 37°C for 30 min. After 30 min, 50,000 cells were suspended in ECGM supplemented with 10 ng/mL of VEGF with or without 40 μM QS-13 and seeded into each well. Plates were incubated at 37°C in a humid atmosphere (5% CO_2, 95% air) for 6 h. Capillary tube formation was imaged after 6 h under an inverted microscope. Quantitative evaluation of the capillary tubes was performed with ImageJ software using the Angiogenesis Analyzer tool. The number of master junctions, master segments, meshes, and the total mesh area of the capillary tube structures were determined.

Adhesion Assays

Cells were detached with 50 mM Hepes, 125 mM NaCl, 5 mM KCl, and 1 mM EDTA, washed three times with ECGM, pre-incubated for 30 min with effectors [mouse anti-human $\alpha_5\beta_1$, catalog number 555614 from BD Pharmingen (10 μg/mL), irrelevant IgG (10 μg/mL) or RGDS peptide 20 mg/mL (Sigma)]. 10,000 cells were seeded per well of a 96 well-plate previously coated with QS-13 and saturated with 1% BSA. After 60 min, cells were washed three times with HEPES buffered Balanced Salt Solution, fixed with 1.1% glutaraldehyde solution and stained with 1% crystal violet solution. Staining was extracted with 10% acetic acid and absorbance was read at 560 nm.

Affinity Chromatography

Human Umbilical Vein Endothelial Cells were cultured in 150 cm^2 culture flasks until 70% confluence and cell layer was scrapped in RIPA buffer (Sigma, St Quentin Fallavier, France) supplemented with a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific, Illkirch, France). Cell lysate was incubated for 30 min at 4°C and centrifuged at 10,000 *g* for 10 min at 4°C to remove insoluble debris. Concentration of soluble proteins was quantified using Biorad Protein Assay (BioRad, Marnes-La-Coquette, France) according to the manufacturer's instructions. Chromatography was performed on a HiTrap NHS-activated Sepharose High Performance column (GE Healthcare, Orsay, France) functionalized with QS-13 according to the manufacturer's instructions. Protein extract was chromatographed at 4°C. Unbound proteins were removed with 30 mL of washing buffer [10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6 supplemented with PIC (ProteoBlock Protease Inhibitor Cocktail, Fermentas, Illkirch, France; w/v) and 0.1% (w/v) octylglucoside]. Proteins bound to the affinity column were eluted with a buffer containing 10 mM Tris, pH 7.6, 0.1% (w/v) octylglucoside and PIC, supplemented with increasing concentrations of NaCl (0.15, 0.6, and 1 M). SDS sample buffer containing 10 mM DTT was added to eluted proteins; samples were incubated for 30 min at 37°C, denatured for 5 min at 95°C and electrophoresed in a 0.1% SDS, 10% polyacrylamide gel. They were then transferred onto Immobilon-P membranes (Millipore, St Quentin en Yvelines, France). Membranes were blocked with 5% non-fat dry milk, 0.1% Tween 20 in TBS for 2 h at room temperature, incubated overnight at 4°C with a rabbit anti-β_1 integrin polyclonal antibody (AB1952P, Merck Millipore) or a rabbit anti-α_5 integrin polyclonal antibody (#4705, Cell Signaling) diluted 1/1000 in 1% non-fat dry milk, 0.1% Tween 20 in TBS) and then for 1 h at room temperature with a second peroxidase-conjugated anti-IgG antibody. Immune complexes were visualized with the ECL chemiluminescence detection kit (GE Healthcare, Orsay, France).

Solid Phase Assay for Studying QS-13/Integrin Interaction

Wells of a 96-well plate were coated with 25 nM $\alpha_5\beta_1$ integrin (3230-A5-050; R&D Systems, Lille, France) in amounts overnight at room temperature. The coating was then blocked with TBS containing 5% dry milk, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ for 2 h at room temperature. After washing three times with washing buffer (0.1% dry milk, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in TBS), the plate was incubated for 90 min at room temperature with 100 μL per well of ranging from 1.25 to 20.10^{-11} moles/well biotinylated-QS-13 diluted in washing buffer. After 3 washes, 100 μL of streptavidin-peroxidase diluted 1/20000 in washing buffer were added to each well and incubated for 15 min at room temperature. After 4 washes, 100 μL per well of tetramethylbenzidine (TMB), a peroxidase substrate, were added and incubated in the dark for 15 min. The enzymatic reaction was stopped by adding 50 μL per well of 0.5 M H_2SO_4. The intensity of the yellow coloration was measured at 450 nm with a Biochrom Asys UVM 340 microplate reader.

For competition experiments, wells of a 96-well plate were coated with 25 nM $\alpha_5\beta_1$ integrin and then blocked as described above. Wells were then incubated for 90 min at room temperature with biotinylated QS-13 with or without unbiotinylated QS-13 (molar ratio ranging from 1/1 to 250/1) diluted in washing buffer. The following steps are as described above.

Surface Plasmon Resonance Analysis

All experiments were performed on a Biacore T200 instrument (GE Healthcare) at 25°C. Sensor surfaces and other Biacore consumables were purchased from GE Healthcare. Integrin $\alpha_5\beta_1$ was from R&D Systems. The running buffer, HEPES buffered saline (HBS, composed of 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA) was filtered through a 0.22 μm membrane and supplemented with 0.05% P20. Biotinylated peptides were captured on streptavidin-coated sensor chips. Briefly, CM5 sensor chips (GE Healthcare) were preconditioned by duplicate injections of 10 mM HCl, 50 mM NaOH, each for 10 s, and water for 20 s. Before covalent immobilization of streptavidin, traces of biotinylated products that could remain in the flow system were neutralized by injecting a streptavidin solution (0.1 mg/mL in running buffer) for 5 min through all flow cells (Baltzinger et al., 2013). Streptavidin was then stably immobilized using standard amine-coupling methods. The flow rate was 10 μL/min. Surfaces were activated by injection of a 1:1 mix of 0.2 M N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 0.05 M N-hydroxysuccinimide (NHS) for 10 min, followed by a 5 min injection of streptavidin at 200 μg/mL in 10 mM sodium acetate (pH 5) and then deactivated with ethanolamine-HCl (pH 8.5) for 10 min. The surface was then subjected to four pulses (100 μL) of 50 mM NaOH at a flow rate 50 μL/min to wash out all non-covalently bound streptavidin. Biotinylated QS-13 (QKISRCQVCVKYSK-biot) was injected onto streptavidin at 5 μg/mL in running buffer, for 10 s at a flow rate of 100 μL/min. Responses were stabilized by five pulse injections of 50 mM NaOH at a flow rate 50 μL/min. Reference surfaces (Fc1) were treated similarly except that an irrelevant biotinylated peptide was injected. Eleven different concentrations of $\alpha_5\beta_1$ integrin (0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 130 nM) were injected into the flow cells at 30 μL/min for 300 s. Dissociation was followed for 600 s. Binding curves were double-reference substracted from buffer blank and reference flow cell (Fc 1). The equilibrium response (Req) was recorded 5 s before the end of integrin injection. The K_D was determined by fitting the equilibrium response versus the [integrin] curve to a simple 1:1 interaction model with the Biacore T200 evaluation software (GE Healthcare).

Docking Experiments

Docking of QS-13 onto $\alpha_5\beta_1$ integrin (RCSB Protein Data Bank 3VI3) was performed using Autodock software (version 4.2; Morris et al., 2009). The docking parameters were as previously described (Lambert et al., 2018). The software was used with a fixed integrin and semi-flexible QS-13 ligand (the backbone was frozen as well as the amide links and guanidinium groups). Because the integrin is a large molecule, we performed several independent dockings targeting different subvolumes of the protein; we considered 125 overlapping boxes with a volume of 47.25 Å × 47.25 Å × 47.25 Å. Each box was divided along the three directions, and the distance between the nodes was equal to 0.375 Å. The Lamarckian genetic algorithm was used, and for each ligand, 150 dockings were performed with the default parameters of Autodock except for the population size (150), number of energy evaluations (5×10^6), and maximum number of generations (30,000; Lambert et al., 2018). Molecular models were derived from the preliminary study. Molecular models were graphed with VMD software, which is available online.

Statistical Analyses

For *in vitro* experiments, results are expressed as the mean ± SD and statistical significance were determined using Student's *t*-test. For *in vivo* experiments, the non-parametric Mann–Whitney test was performed.

RESULTS

QS-13 Decreases *in vivo* Matrigel Plug Angiogenesis

The subcutaneous Matrigel plug assay in mouse is a gold standard *in vivo* assay to screen pro- or anti-angiogenic molecules. Administration of a fluorescent imaging agent (AngioSense680^{TM}) and quantitative analyses with a fluorescence molecular tomographic (FMT) imaging system revealed a significant decrease (−57%) of angiogenesis in Matrigel plug of QS-13-treated mice compared to control mice at day 13 (**Figure 1A**). The Matrigel plugs were then excised and 4 μm thick sections were performed and stained using an anti-CD31 antibody as endothelial cell marker. The MVD analysis of the different sections confirmed that QS-13 inhibits *in vivo* angiogenesis by 61% (**Figure 1B**).

QS-13 Decreases *in vitro* Endothelial Cell Migration and Pseudotube Formation Without Affecting Cell Proliferation

In order to decipher the anti-angiogenic effect of QS-13, we performed *in vitro* studies.

As proliferation of endothelial cells plays an essential role in angiogenesis, HUVEC proliferation under the influence of QS13 was assessed at 24, 48, 72, and 96 h using the WST-1 method. QS-13 does not significantly alter HUVEC proliferation up to 96 h incubation (**Figure 2A**).

Human Umbilical Vein Endothelial Cell migration, a key step in angiogenesis, was studied using the scratch wound healing model. Scratch wounds were photographed at 0 and 24 h (**Figure 2B**). Compared to control, QS-13 decreased wound closure by 28% (**Figure 2C**).

Human Umbilical Vein Endothelial Cell pseudotube-formation assay is a well-established *in vitro* angiogenesis assay based on the ability of endothelial cells to form three-dimensional capillary-like tubular structures. We demonstrated that QS-13 strongly altered pseudotube formation (**Figure 2D**): it decreased the number of master segments by 47% (**Figure 2E**), the number

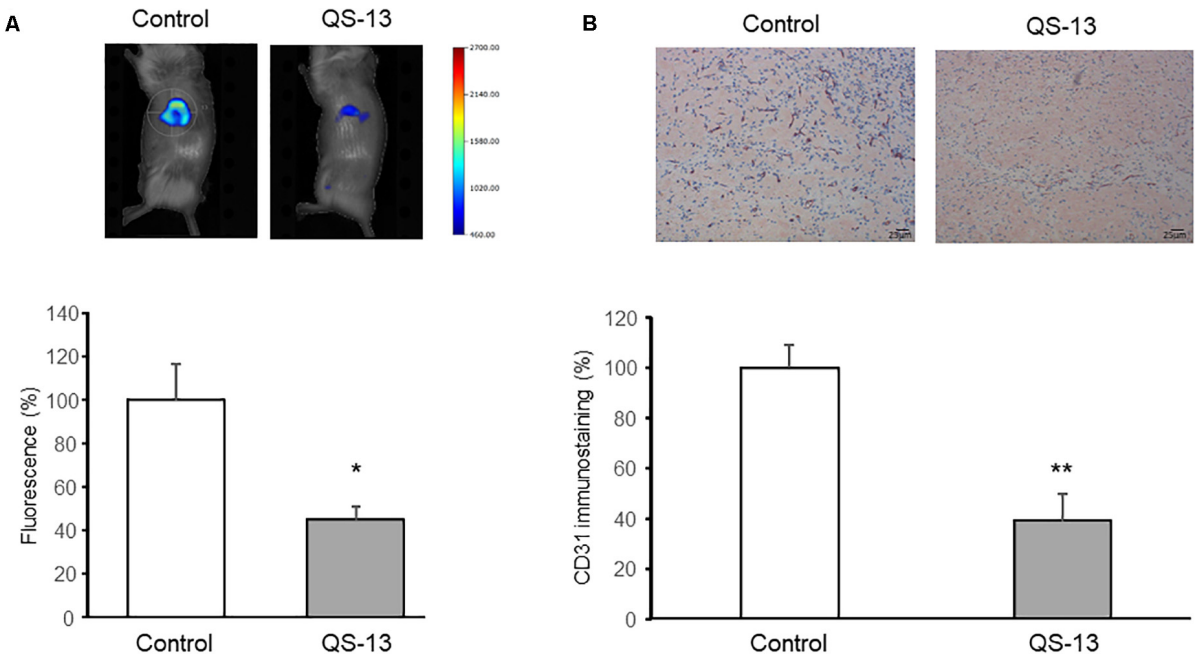


FIGURE 1 | QS-13 decreases *in vivo* angiogenesis in a Matrigel plug model. Matrigel (400 μL) was subcutaneously injected into mice. After 13 days of treatment with VEGF alone or with VEGF and QS-13, a fluorescent imaging agent was administrated. **(A)** At day 14, anesthetized mice were imaged using molecular fluorescence tomography and FMT signal intensities were measured. **(B)** Matrigel plugs were then excised, CD31-immunostaining was performed on Matrigel plug sections and microvessel density (MVD) was evaluated. Two independent experiments ($N = 2$) were carried out with 4 mice in each group ($n = 4$). The histograms represent the means of the two experiments ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

of master junction by 44% (**Figure 2F**), the number of meshes by 56% (**Figure 2G**) and the total mesh area by 87% (**Figure 2H**).

Identification of $\alpha_5\beta_1$ Integrin as a QS-13 Receptor on HUVECs

In silico Studies

$\alpha_5\beta_1$ integrin was previously reported to be largely expressed in HUVECs. The integrin mediates cell adhesion to ECM (Short et al., 1998; Zeng et al., 2009) and cell migration through a VEGFR-dependent mechanism (Orecchia et al., 2003). Thereby, we investigated a putative interaction of QS-13 with the $\alpha_5\beta_1$ integrin. We previously performed molecular dynamics simulations on isolated QS-13 (Lambert et al., 2018) and observed that a disulfide bond locked the CQVC sequence in a conformation that exposed the glutamine (Q) side chain. The presence of the disulfide bond was confirmed by MALDI-ToF MS analyses. Molecular docking experiments were carried out to test the hypothesis of a QS-13/$\alpha_5\beta_1$ integrin interaction (**Figure 3**). For the two considered conformations of QS-13 peptide (extracted from molecular dynamics simulations), the best 180 results (from the energy point of view) were used in order to cluster the solutions and to identify the main interaction areas. For each conformation, the most populated cluster (32.2% and 19.4% for conformation 1 and 2, respectively) contained the pose associated to the best free energy of binding (-5.71 kcal/mol and -7.25 kcal/mol for conformation 1 and 2, respectively). The visualization of these poses (**Figure 3A**) demonstrated that both conformations share the same interaction area with the $\alpha_5\beta_1$ integrin at the interface between the two integrin subunits. Despite the fact that the two QS-13 shapes were rather different [one could be considered "folded" (**Figure 3B**) and the second "elongated" (**Figure 3C**)], the contacts they made with the integrin were very similar. Indeed, the key interactions established by QS-13 with the $\alpha_5\beta_1$ integrin mainly involved the following residues: the first lysine (K2) with S134 and D137 of the β_1 integrin subunit; the arginine (R5) with D227 of the α_5 integrin subunit and E320 of the β_1 integrin subunit; the glutamine (Q7) exposed by the disulfide bond with Q189, Q221, and D227 of the β_1 integrin subunit. The number of inter-molecule hydrogen bonds is comparable from one pose to the other, and two pairs of interaction are conserved, namely S134/K2 and D227/Q7.

In vitro Experiments

To determine whether QS-13 binds to HUVECs through $\alpha_5\beta_1$ integrin, we measured HUVEC adhesion on QS-13 in the presence or absence of an anti-$\alpha_5\beta_1$ integrin blocking antibody (10 μg/mL). The preincubation of HUVECs with the blocking anti-$\alpha_5\beta_1$ antibody significantly inhibited (71%) cell adhesion on QS-13, whereas an irrelevant IgG had no effect (**Figure 4A**), suggesting a putative interaction between QS-13 and $\alpha_5\beta_1$ integrin. To verify this hypothesis, we performed affinity chromatography. HUVEC extracts were loaded onto a QS-13-functionalized affinity column. Proteins bound to the affinity column were eluted with increasing concentrations of NaCl (0.15, 0.6, and 1.0 M; **Supplementary Figure S1**). Eluted samples,

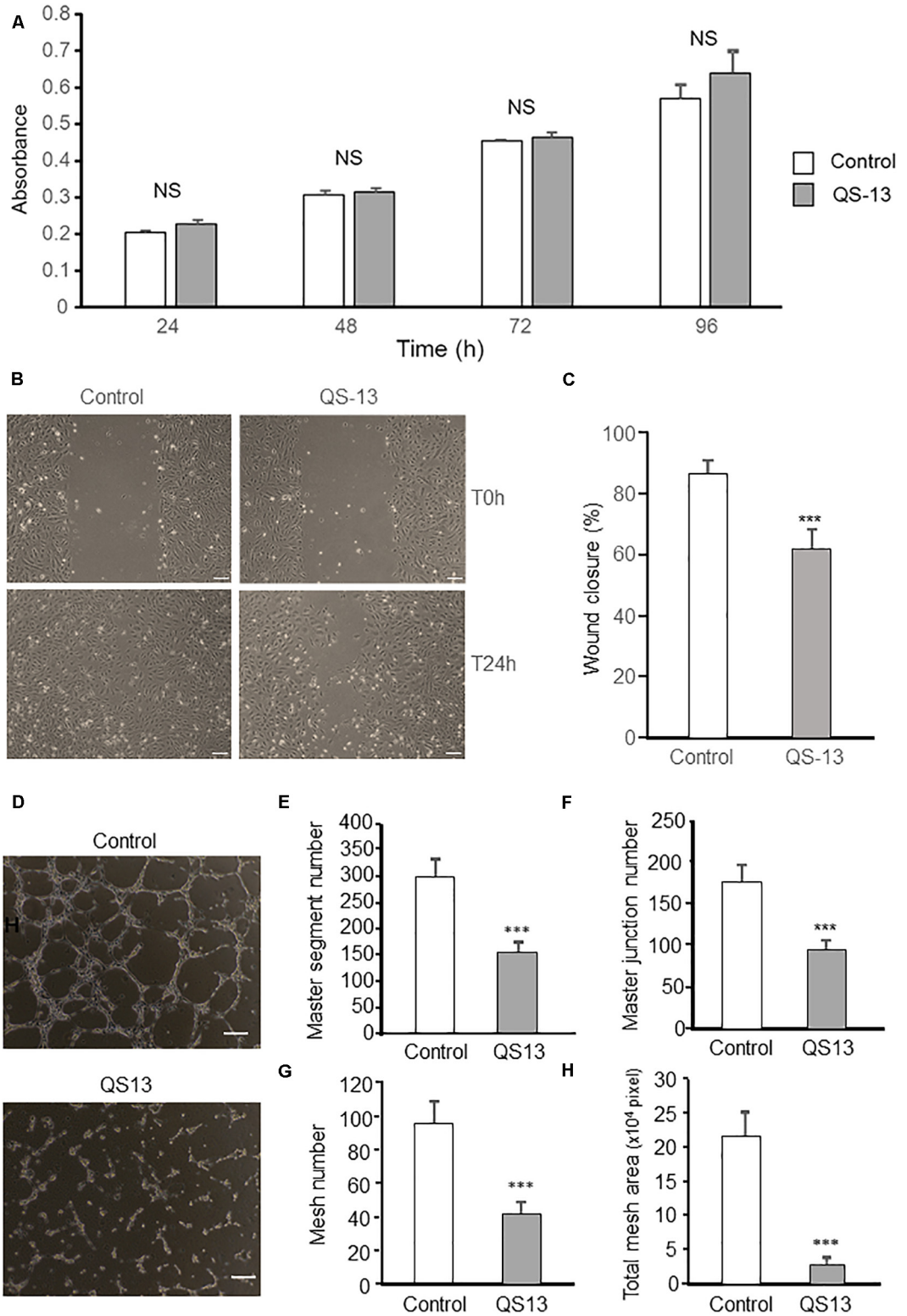


FIGURE 2 | QS-13 decreases *in vitro* endothelial cell migration and pseudotube formation without affecting cell proliferation. To study cell proliferation, HUVECs were incubated for 24, 48, 72, and 96 h with or without 40 μM QS-13. **(A)** Proliferation was measured using the WST-1 reagent and absorbance was read at 450 nm (n = 8). Three independent experiments were carried out. The histogram represents the means ± SD of the more representative one. NS, not significant. To study

(Continued)

FIGURE 2 | Continued
endothelial cell migration, a scratch wound was performed using a pipet tip at confluence. HUVECs were then incubated with or without 40 μM QS-13 for 24 h at 37°C. **(B)** Wounds were microphotographed after 24 h of incubation. Scale bar: 50 nm. **(C)** Wound closure was measured using ImageJ software. Three independent experiments were carried out. The histogram represents the means ± SD of the more representative one. $n = 8$, $^{***}p < 0.001$. To study pseudotube formation, HUVECs were seeded on Matrigel coated well and incubated with or without 40 μM QS-13. **(D)** Pseudotube formation was observed under an inverted microscope after 6 h and photographed. Scale bar: 200 nm. **(E)** The number of master segments, **(F)** the number of master junction, **(G)** the number of meshes and **(H)** the total mesh area of pseudotube were determined with ImageJ software. Three independent experiments were carried out. The histogram represents the means ± SD of the more representative one. $n = 8$, $^{***}p < 0.001$.

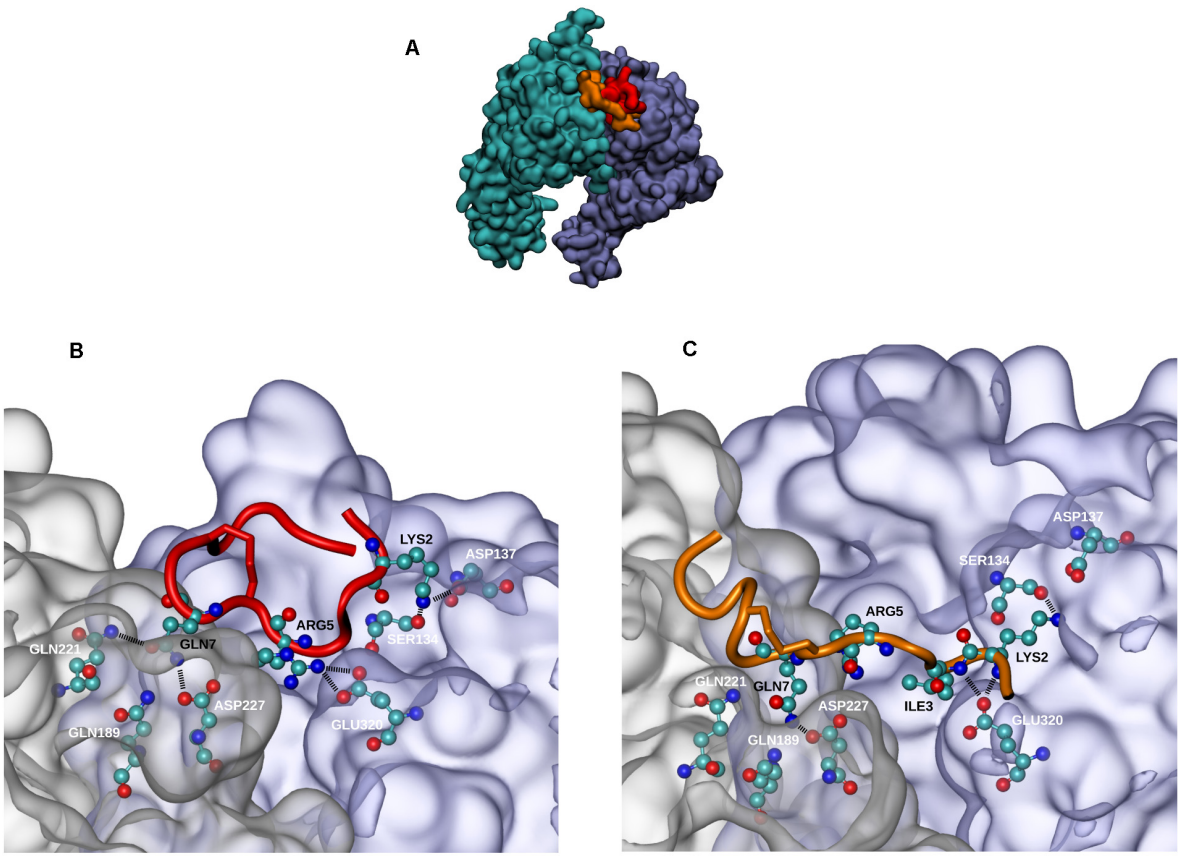


FIGURE 3 | Docking experiments of QS-13 on $\alpha_5\beta_1$ integrin. The best results of QS-13 docking experiment display the same interaction area with the $\alpha_5\beta_1$ integrin at the interface between the two integrin subunits. **(A)** Integrin subunits and QS-13 are represented using the Quick Surface scheme and colored according to the nature of the chain (cyan for α_5 and magenta for β_1) or the conformation of QS-13 (first conformation extracted from the MD in red and second conformation extracted from the MD in orange). **(B,C)** Zoomed-in representations of the best energy conformation 1 and 2 of QS-13, respectively. The New Cartoon scheme is used to represent QS-13 and the Quick Surface scheme used for integrin is set to transparent. Residues making contacts between integrin and QS-13 (a contact is defined as a distance between two atoms lower than 3 Å) are represented with the CPK mode. Color labels are adopted in order to distinguish integrin (white) and QS-13 (black) residues. Hydrogen bonds are highlighted with thick black dashed lines.

analyzed by western blot, showed the presence of α_5 and β_1 integrin subunits in the 0.6 M eluted fraction (**Figure 4B**). The existence of a direct interaction between $\alpha_5\beta_1$ integrin and QS-13 was investigated using two different methods: solid phase assay and surface plasmon resonance (SPR). For this purpose, QS-13 was biotinylated at its C-terminus. In the solid phase assay, we demonstrated that biotinylated-QS-13 binds to $\alpha_5\beta_1$ integrin in a ligand concentration dependent manner (**Figure 4C**). Biotinylated QS-13 was covalently immobilized on a streptavidin-coated CM5 sensor chip. $\alpha_5\beta_1$ integrin was then injected at eleven concentrations ranging from 0.12 to 130 nM. Moreover, a competitive assay was performed to confirm the specificity of the interaction. Increasing amounts of unbiotinylated QS-13 decreased biotinylated-QS-13 binding to $\alpha_5\beta_1$ integrin (**Figure 4D**). In SPR experiments, biotinylated-QS-13 was covalently immobilized on a streptavidin-coated CM5 sensor chip. $\alpha_5\beta_1$ integrin was then injected at eleven concentrations ranging from 0.12 to 130 nM. **Figure 4E** presents the sensorgrams obtained after double referencing (subtraction of reference channel and buffer injection). $\alpha_5\beta_1$ integrin binds to Q-S13 in a dose-dependent manner. The double referenced equilibrium responses recorded 5 s before the end of integrin

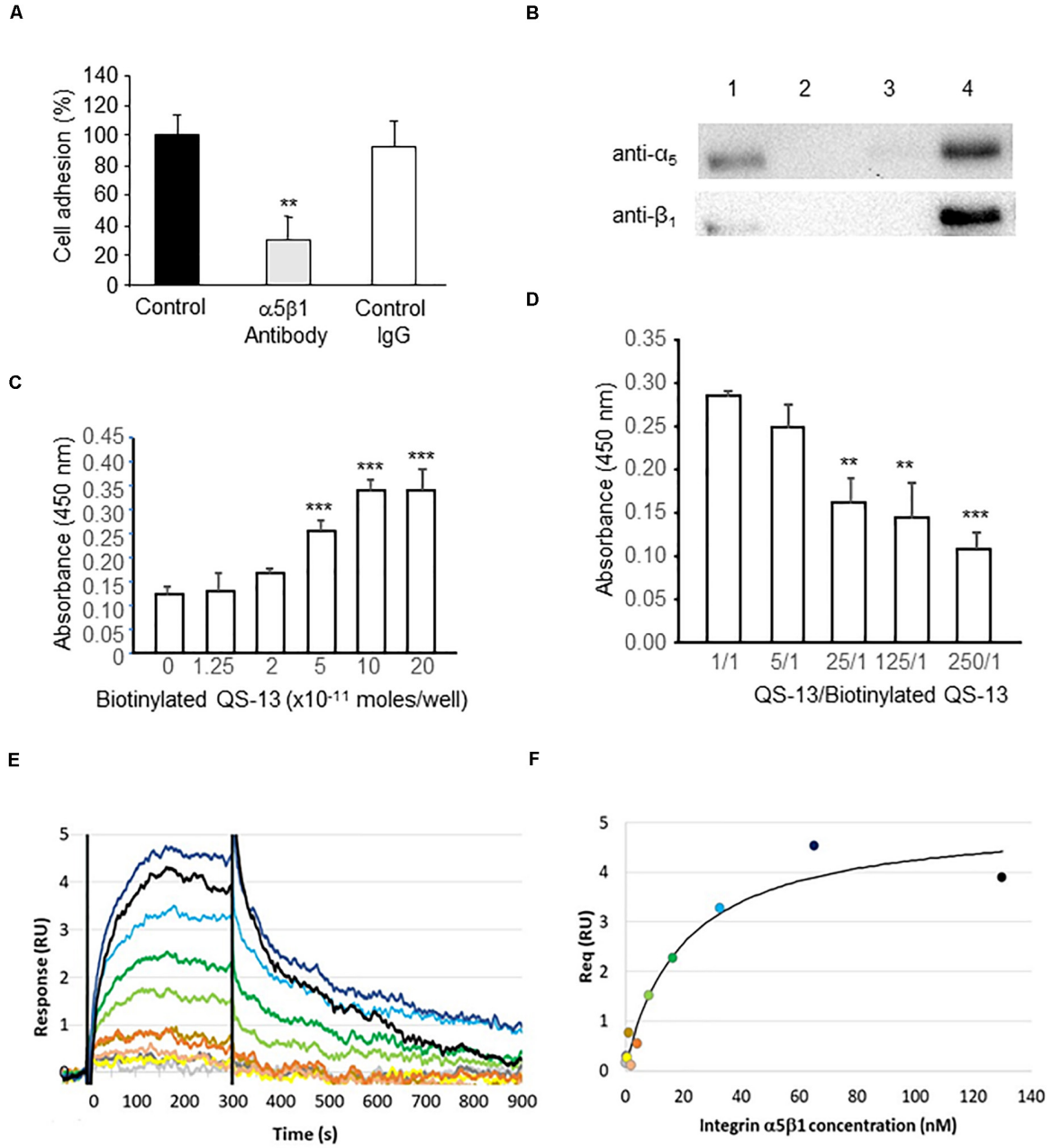


FIGURE 4 | QS-13 peptide binds to $\alpha_5\beta_1$ integrin. **(A)** HUVECs were pre-incubated with ECGM alone, ECGM containing an anti-$\alpha_5\beta_1$ blocking antibody or an irrelevant antibody (10 μg/mL). HUVEC adhesion was measured as described in the section "Materials and Methods." Three independent experiments were carried out. The histogram represents the means ± SD of the more representative one ($n = 8$), $^{**}p < 0.01$. **(B)** HUVEC extracts were submitted to affinity chromatography on a QS-13 -bound column. Lane 1: total cell extracts; lane 2: unbound proteins; lane 3: 0.15 M NaCl eluted fraction; lane 4: 0.6 M NaCl eluted fraction. **(C)** Direct interaction between $\alpha_5\beta_1$ integrin at concentrations ranging from 0.25 to 2.10^{-10} mole/well and QS-13 was studied using solid phase assay as described in the "Materials and Methods" section. Two independent experiments were carried out ($n = 4$), $^{***}p < 0.001$. **(D)** A competition assay was performed using increasing concentrations of unbiotinylated-QS-13 while biotinylated QS-13 and $\alpha_5\beta_1$ integrin concentrations were kept constant Two independent experiments were carried out ($n = 4$), $^{**}p < 0.01$, $^{***}p < 0.001$. **(E,F)** The equilibrium affinity of the $\alpha_5\beta_1$ integrin-QS-13 interaction was analyzed by SPR. **(E)** Concentration-dependent responses of integrin $\alpha_5\beta_1$ (0.12 to 130 nM range) to the streptavidin-captured biotinylated QS-13. **(F)** Fit of the equilibrium response (Req) versus [$\alpha_5\beta_1$ integrin] to a 1:1 binding indicates a K_D of 20.3 ± 7.5 nM.

injections were called Req. **Figure 4F** shows the plotted responses of Req as a function of $\alpha_5\beta_1$ integrin concentration. The equilibrium affinity parameter (K_D) was determined by fitting the Req versus the [$\alpha_5\beta_1$ integrin] curve to a simple 1:1 interaction model. We measured a K_D of 20.3 ± 7.5 nM for the QS-13/$\alpha_5\beta_1$ integrin interaction.

Taken together, the results confirm that QS-13 directly binds to $\alpha_5\beta_1$ integrin.

RGDS Peptide Competes With QS-13 for $\alpha_5\beta_1$ Integrin Binding on HUVECs

As specified in the "Materials and Methods" section, docking experiments were carried out using the structure associated with PDB ID 3VI3: this crystallographic data corresponded to the ligand-free form of $\alpha_5\beta_1$ integrin. The structure associated with PBD ID 3VI4 was related to $\alpha_5\beta_1$ integrin headpiece in complex with RGD peptide: the visualization and comparison of most likely QS-13 conformations with this experimental ligand-associated form (**Figure 5A** and **Supplementary Figure S2**) evidenced that the QS-13 binding site was in close vicinity to the RGD peptide binding site. In the case of the RGD peptide, the residues implicated in the interactions were the arginine (R) with S134 of the β_1 integrin sub-unit and the aspartic acid (D) with Q189, Q221, and D227 of the α_5 integrin sub-unit. Even though the sizes of the RGD peptide and of QS-13 were very different, the same residues are involved in both interactions with the integrin subunits.

To test this hypothesis, competition binding assay were performed. HUVECs were pre-incubated with medium alone or medium supplemented with either QS-13 or RGDS peptide and seeded on QS-13 coating. Cell pre-incubation with RGDS inhibited cell adhesion the same way as QS-13 itself (-60%) (**Figure 5B**).

DISCUSSION

Angiogenesis plays critical roles in human physiological processes. This is a complex phenomenon regulated in a spatial and temporal manner that depends on the cooperation between angiogenic factors, ECM components, and endothelial cells. Uncontrolled angiogenesis may lead to several angiogenic disorders and to vascular overgrowth (hemangiomas, psoriasis, vascularized tumors. . .). Numerous pro-angiogenic drivers have been identified such as VEGF. To date, the most common approaches to the inhibition of the VEGF axis include the blockade of VEGF receptors (VEGFRs) or ligands by neutralizing antibodies, as well as the inhibition of receptor tyrosine kinases (RTK). Unfortunately, this type of inhibitors leads to numerous side effects as well as resistance phenomena (Haibe et al., 2020).

In the present article, we demonstrate that the Tetrastatin QKISRCQVCVKYS peptide sequence (QS-13) inhibits *in vivo* angiogenesis in the Matrigel plug model. *In vitro*, QS-13 does not affect cell proliferation but decreases cell migration and pseudotube organization on Matrigel. The preincubation of HUVECs with a blocking anti-$\alpha_5\beta_1$ antibody significantly inhibited cell adhesion on QS-13, whereas an irrelevant IgG had no effect, suggesting an interaction between QS-13 and the $\alpha_5\beta_1$ integrin.

The NC1 domains of the different α(IV) collagen chains were reported to exert anti-tumor or anti-angiogenic effects (Mundel and Kalluri, 2007; Monboisse et al., 2014; Brassart-Pasco et al., 2020). We and others demonstrated that the anti-tumor activities of Tumstatin or Tetrastatin were mediated through binding to $\alpha_v\beta_3$ integrin at the tumor cell surface (Pasco et al., 2000; Brassart-Pasco et al., 2012; Lambert et al., 2018). It was also demonstrated that Arresten, Canstatin or Tumstatin also exerted anti-angiogenic activities mediated through binding to $\alpha_1\beta_1$, $\alpha_1\beta_1/\alpha_v\beta_3/\alpha_v\beta_5$, and $\alpha_v\beta_5/\alpha_v\beta_3$ integrin binding, respectively (Brassart-Pasco et al., 2020). Furthermore, several integrins, including $\alpha_5\beta_1$, and $\alpha_v\beta_3/\beta_5$, have been described to play an important role in tumor angiogenesis. Their overexpression on tumor neo-vessels suggest new anti-angiogenic therapies (Schaffner et al., 2013).

The anti-$\alpha_5\beta_1$ integrin antibody M200/volociximab was reported to inhibit angiogenesis and to suppress tumor growth and metastasis in mice (Almokadem and Belani, 2012). It showed preliminary evidence of efficacy in advanced NSCLC (Besse et al., 2013).

Peptides also emerged as important therapeutic agents in angiogenesis-dependent diseases due to their low toxicity and high specificity (Rosca et al., 2011). The $\alpha_5\beta_1$-blocking peptide ATN-161, derived from the synergy region of fibronectin, showed preclinical anti-cancer activities (Stoeltzing et al., 2003; Khalili et al., 2006). It entered clinical testing but failed to provide therapeutic benefits (Cianfrocca et al., 2006).

Another matrikine, a 20-kDa C-terminal fragment of type XVIII collagen, endostatin, was also reported to inhibit $\alpha_5\beta_1$ integrin (Sudhakar et al., 2003). It was tested in clinical studies in combination with chemotherapies and radiotherapies but the results were inconsistent, probably due to recombinant protein production (Alday-Parejo et al., 2019).

We also demonstrated that the inhibitory effects of Tetrastatin are conformation-dependent with a crucial role of the presence of a disulfide bond in QS-13 (Lambert et al., 2018). Because of the influence and importance of QS-13 disulfide bond, the present *in silico* investigation of the interaction between $\alpha_5\beta_1$ integrin and QS-13 was designed using peptide conformations displaying the presence of the disulfide bond. The results of the docking experiments demonstrate that QS-13 is able to bind to $\alpha_5\beta_1$ integrin in a stable mode since the evaluated free energy of binding of the best solutions is below the threshold of -3.00 kcal/mol. In addition to the energy aspect, the statistical analysis of the best molecular docking results indicates that the interaction areas with $\alpha_5\beta_1$ integrin are not randomly distributed. Indeed, the clustering of the best poses leads to the identification of a region gathering the highest number of results and corresponds to an interface region between the two integrin subunits. This region overlaps with the binding site of the RGD sequence, a well-known integrin recognition sequence. Despite the size difference between RGD and QS-13, the residues involved in the interaction at the protein level show a strong overlap and are either polar residues or residues with charged side chains prone to hydrogen-bonding. It should also be noted that in the case of QS-13, the glutamine residue exposed by the presence of the disulfide bond (Lambert et al., 2018), is one of the three residues in contact with the integrin.

The interaction between QS-13 and $\alpha_5\beta_1$ integrin is confirmed by affinity chromatography, solid phase binding assay and SPR. As the RGD binding site was shown to overlap QS-13 theoretical

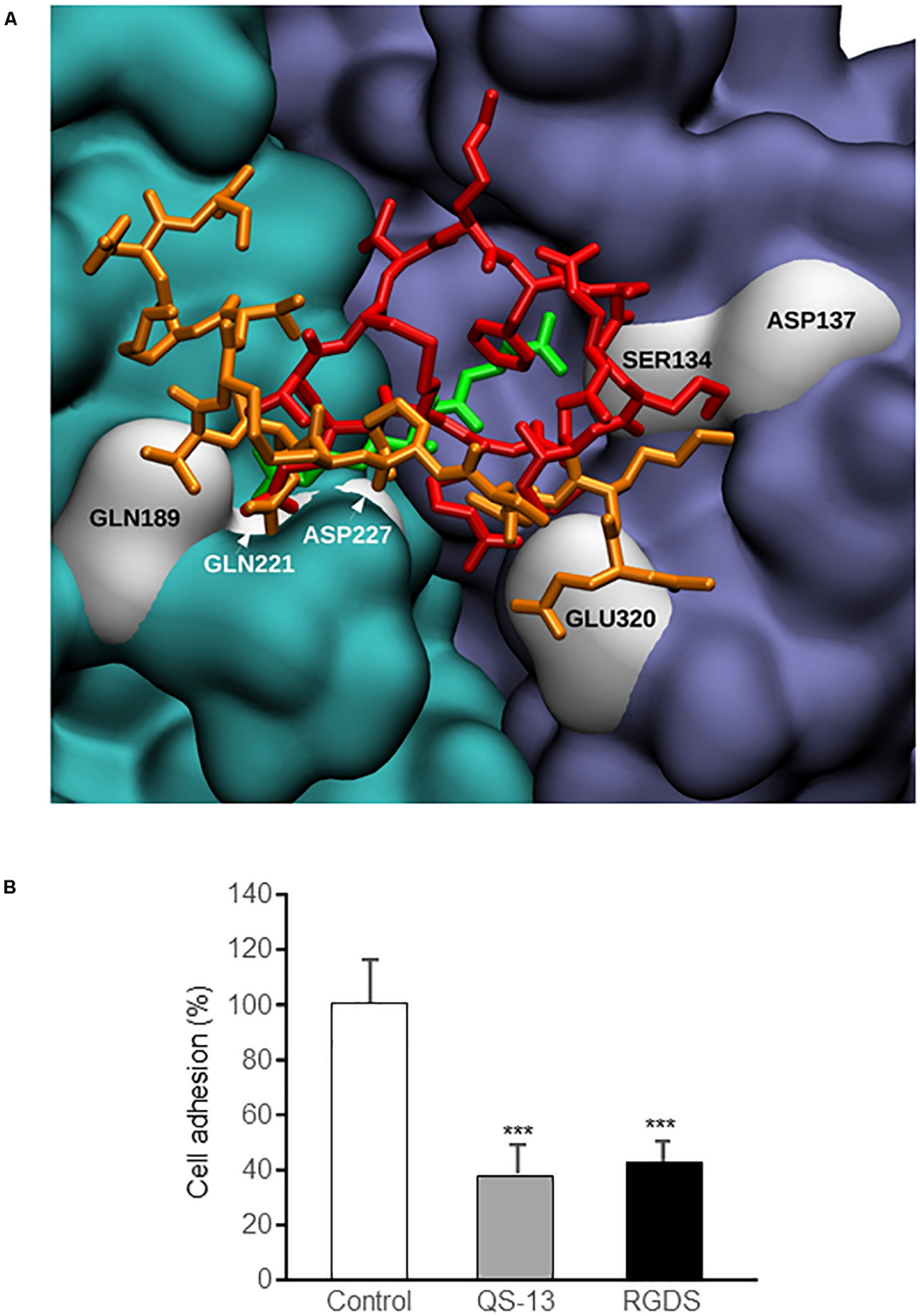


FIGURE 5 | Comparison of QS-13 and RGD theoretical binding sites on $\alpha_5\beta_1$ integrin and *in vitro* confirmation. **(A)** $\alpha_5\beta_1$ integrin is represented using the Quick Surface scheme and a color code related to the nature of the subunit (cyan for α_5 and magenta for β_1). The best docking poses of the two QS-13 conformations are superimposed with the conformation of the RDG peptide (from the experimental complex corresponding to the PDB ID 3VI4 solved with X-ray experiments) and depicted with a color coded (first QS-13 conformation extracted from the MD in red, the second in orange and RGD peptide in green) Licorice representation. Residues from the integrin making contacts with QS-13 (as evidenced in **Figure 3**) are highlighted with white surface representation and labeled. **(B)** HUVECs were pre-incubated for 30 min with culture medium alone (control), culture medium supplemented with RGDS peptide (20 μg/mL) or with QS-13 (20 μg/mL) and adhesion was measured. Three independent experiments were carried out. The histogram represents the means ± SD of the more representative one. $n = 8$, $^{***}p < 0.001$.

binding sites determined by molecular docking, a competition experiment was performed *in vitro*. Cell pre-incubation with RGDS inhibited cell adhesion to the QS-13 by about 60%, confirming that QS-13 binding site was in close vicinity to RGD binding site on $\alpha_5\beta_1$ integrin.

Taken together, our results demonstrate that QS-13 binds to $\alpha_5\beta_1$ integrin and inhibits endothelial cell migration and angiogenesis and is a potent anti-angiogenic agent. Since the disulfide bond forms spontaneously in solution, QS-13 may be protected from protease degradation *in vivo*. It offers new therapeutic strategies in hemangiomas and psoriasis treatment alone or in combination with anti-microbial peptides (Morizane and Gallo, 2012).

In tumors, $\alpha_5\beta_1$ integrin is overexpressed and represents an interesting target for the administration of anti-cancer agents *in situ* (Schaffner et al., 2013; Blandin et al., 2015; Alday-Parejo et al., 2019). In addition to its inhibitory effects on endothelial cell migration and angiogenesis, QS-13 also decreases cancer progression by inhibiting tumor cell migration and invasion and *in vivo* tumor growth (Lambert et al., 2018), It would be of interest to propose new therapeutic strategies based for example on QS-13 grafting on the surface of nanoparticles loaded with cytotoxic agents to a specific targeting and drug delivery to the tumor, allowing a decrease in the drug side effects. The patient will benefit from a targeted delivery of therapeutic agents, as well as the anti-angiogenic and anti-tumor activity of QS-13.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the French "Ministère de l'Enseignement Supérieur et de la Recherche" (Ethics Committees Nos. C2EA-56 and C2EA-75) in compliance with the "Directive 2010/63/UE". Protocol no. 4373_V1 APAFIS (07/09/2016).

AUTHOR CONTRIBUTIONS

AV-G, JD, CB, SB, LC, AH, AD-D, CS, and SB-P carried out the experiment. JD, SB, LC, BB, J-BO, LR, JM, and SB-P contributed to the interpretation of the results. SB-P took the lead in writing the manuscript. All authors provided critical feedback and helped to improve the manuscript.

FUNDING

This work was supported by the Centre National de la Recherche Scientifique (UMR 7369), the University of Reims Champagne-Ardenne, and the Conférence de Coordination Interrégionale de la Ligue Contre le Cancer du Grand Est (CCIR-GE).

ACKNOWLEDGMENTS

The authors thank the HPC-Regional Center ROMEO, the Multiscale Molecular Modeling Platform (P3M) of the University of Reims Champagne-Ardenne (France) for providing time and support. The authors also thank Caroline Fichel for her technical assistance in CD31 immunostaining.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00775/full#supplementary-material

REFERENCES

Alday-Parejo, B., Stupp, R., and Rüegg, C. (2019). Are integrins still practicable targets for anti-cancer therapy? *Cancers* 11:E978. doi: 10.3390/cancers11070978

Almokadem, S., and Belani, C. P. (2012). Volociximab in cancer. *Expert. Opin. Biol. Ther.* 12, 251–257. doi: 10.1517/14712598.2012.646985

Baltzinger, M., Sharma, K. K., Mély, Y., and Altschuh, D. (2013). Dissecting the oligonucleotide binding properties of a disordered chaperone protein using surface plasmon resonance. *Nucleic Acids Res.* 41, 10414–10425. doi: 10.1093/nar/gkt792

Besse, B., Tsao, L. C., Chao, D. T., Fang, Y., Soria, J.-C., Almokadem, S., et al. (2013). Phase Ib safety and pharmacokinetic study of volociximab, an anti-$\alpha_5\beta_1$ integrin antibody, in combination with carboplatin and paclitaxel in advanced non-small-cell lung cancer. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* 24, 90–96. doi: 10.1093/annonc/mds281

Blandin, A.-F., Renner, G., Lehmann, M., Lelong-Rebel, I., Martin, S., and Dontenwill, M. (2015). β1 integrins as therapeutic targets to disrupt hallmarks of cancer. *Front. Pharmacol.* 6:279. doi: 10.3389/fphar.2015.00279

Brassart-Pasco, S., Brezillon, S., Brassart, B., Ramont, L., Oudart, J. B., and Monboisse, J. C. (2020). Tumor microenvironment: extracellular matrix alterations influence tumor progression. *Front. Oncol.* 10:397. doi: 10.3389/fphar.2015.00397

Brassart-Pasco, S., Sénéchal, K., Thevenard, J., Ramont, L., Devy, J., Di Stefano, L., et al. (2012). Tetrastatin, the NC1 domain of the α4(IV) collagen chain: a novel potent anti-tumor matrikine. *PLoS One* 7:e29587. doi: 10.1371/journal.pone.0029587

Cianfrocca, M. E., Kimmel, K. A., Gallo, J., Cardoso, T., Brown, M. M., Hudes, G., et al. (2006). Phase 1 trial of the antiangiogenic peptide ATN-161 (Ac-PHSCN-NH(2)), a beta integrin antagonist, in patients with solid tumours. *Br. J. Cancer* 94, 1621–1626. doi: 10.1038/sj.bjc.6603171

Haibe, Y., Kreidieh, M., El Hajj, H., Khalifeh, I., Mukherji, D., Temraz, S., et al. (2020). Resistance mechanisms to anti-angiogenic therapies in cancer. *Front. Oncol.* 10:221. doi: 10.3389/fphar.2015.00221

Kalluri, R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat. Rev. Cancer* 3, 422–433. doi: 10.1038/nrc1094

Karagiannis, E. D., and Popel, A. S. (2008). A systematic methodology for proteome-wide identification of peptides inhibiting the proliferation and migration of endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13775–13780. doi: 10.1073/pnas.0803241105

Khalili, P., Arakelian, A., Chen, G., Plunkett, M. L., Beck, I., Parry, G. C., et al. (2006). A non-RGD-based integrin binding peptide (ATN-161) blocks breast

cancer growth and metastasis in vivo. *Mol. Cancer Ther.* 5, 2271–2280. doi: 10.1158/1535-7163.mct-06-0100

Lambert, E., Fuselier, E., Ramont, L., Brassart, B., Dukic, S., Oudart, J.-B., et al. (2018). Conformation-dependent binding of a Tetrastatin peptide to αvβ3 integrin decreases melanoma progression through FAK/PI3K/Akt pathway inhibition. *Sci. Rep.* 8:9837.

Monboisse, J. C., Oudart, J. B., Ramont, L., Brassart-Pasco, S., and Maquart, F. X. (2014). Matrikines from basement membrane collagens: a new anti-cancer strategy. *Biochim. Biophys. Acta* 1840, 2589–2598. doi: 10.1016/j.bbagen.2013.12.029

Morizane, S., and Gallo, R. L. (2012). Antimicrobial peptides in the pathogenesis of psoriasis. *J. Dermatol.* 39, 225–230. doi: 10.1111/j.1346-8138.2011.01483.x

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., et al. (2009). AutoDock4 and autodocktools4: automated docking with selective receptor flexibility. *J. Comput. Chem.* 30, 2785–2791. doi: 10.1002/jcc.21256

Mundel, T. M., and Kalluri, R. (2007). Type IV collagen-derived angiogenesis inhibitors. *Microvasc. Res.* 74, 85–89. doi: 10.1016/j.mvr.2007.05.005

Orecchia, A., Lacal, P. M., Schietroma, C., Morea, V., Zambruno, G., and Failla, C. M. (2003). Vascular endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the alpha 5 beta 1 integrin. *J. Cell Sci.* 116, 3479–3489. doi: 10.1242/jcs.00673

Pasco, S., Monboisse, J. C., and Kieffer, N. (2000). The alpha 3(IV)185-206 peptide from noncollagenous domain 1 of type IV collagen interacts with a novel binding site on the beta 3 subunit of integrin alpha Vbeta 3 and stimulates focal adhesion kinase and phosphatidylinositol 3-kinase phosphorylation. *J. Biol. Chem.* 275, 32999–33007. doi: 10.1074/jbc.m005235200

Petitclerc, E., Boutaud, A., Prestayko, A., Xu, J., Sado, Y., Ninomiya, Y., et al. (2000). New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. *J. Biol. Chem.* 275, 8051–8061. doi: 10.1074/jbc.275.11.8051

Rosca, E. V., Koskimaki, J. E., Rivera, C. G., Pandey, N. B., Tamiz, A. P., and Popel, A. S. (2011). Anti-angiogenic peptides for cancer therapeutics. *Curr. Pharm. Biotechnol.* 12, 1101–1116. doi: 10.2174/138920111796117300

Schaffner, F., Ray, A. M., and Dontenwill, M. (2013). Integrin $\alpha_5\beta_1$, the fibronectin receptor, as a pertinent therapeutic target in solid tumors. *Cancers* 5, 27–47. doi: 10.3390/cancers5010027

Short, S. M., Talbott, G. A., and Juliano, R. L. (1998). Integrin-mediated signaling events in human endothelial cells. *Mol. Biol. Cell* 9, 1969–1980. doi: 10.1091/mbc.9.8.1969

Silva, J. D., Lameiras, P., Beljebbar, A., Berquand, A., Villemin, M., Ramont, L., et al. (2018). Structural characterization and in vivo pro-tumor properties of a highly conserved matrikine. *Oncotarget* 9, 17839–17857. doi: 10.18632/oncotarget.24894

Stoeltzing, O., Liu, W., Reinmuth, N., Fan, F., Parry, G. C., Parikh, A. A., et al. (2003). Inhibition of integrin alpha5beta1 function with a small peptide (ATN-161) plus continuous 5-FU infusion reduces colorectal liver metastases and improves survival in mice. *Int. J. Cancer* 104, 496–503.

Sudhakar, A., Sugimoto, H., Yang, C., Lively, J., Zeisberg, M., and Kalluri, R. (2003). Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. *Proc. Natl. Acad. Sci. U.S.A.* 100, 4766–4771. doi: 10.1073/pnas.0730882100

Teleanu, R. I., Chircov, C., Grumezescu, A. M., and Teleanu, D. M. (2019). Tumor angiogenesis and anti-angiogenic strategies for cancer treatment. *J. Clin. Med.* 9:84. doi: 10.3390/jcm9010084

Weidner, N. (1995). Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. *Breast Cancer Res. Treat.* 36, 169–180. doi: 10.1007/bf00666038

Wietecha, M. S., Cerny, W. L., and DiPietro, L. A. (2013). Mechanisms of vessel regression: toward an understanding of the resolution of angiogenesis. *Curr. Top. Microbiol. Immunol.* 367, 3–32. doi: 10.1007/82_2012_287

Wu, Y., and Ge, G. (2019). Complexity of type IV collagens: from network assembly to function. *Biol. Chem.* 400, 565–574. doi: 10.1515/hsz-2018-0317

Zeng, Z.-Z., Yao, H., Staszewski, E. D., Rockwood, K. F., Markwart, S. M., Fay, K. S., et al. (2009). alpha(5)beta(1) integrin ligand PHSRN induces invasion and alpha(5) mRNA in endothelial cells to stimulate angiogenesis. *Transl. Oncol.* 2, 8–20. doi: 10.1593/tlo.08187

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 14 August 2020
doi: 10.3389/fcell.2020.00747

Endothelial-to-Mesenchymal Transition in Cancer

*Nicolas Clere[1], Sarah Renault[2] and Isabelle Corre[2]**

[1] *Micro and Nanomédecines Translationnelles, Université d'Angers, INSERM UMR U1066, CNRS 6021, Angers, France,*
[2] *Sarcomes Osseux et Remodelage des Tissus Calcifiés, Université de Nantes, INSERM UMR U1238, Nantes, France*

Cancer is one of the most important causes of morbidity and mortality worldwide. Tumor cells grow in a complex microenvironment constituted of immune, stromal, and vascular cells that supports growth, angiogenesis, and metastasis. Endothelial cells (ECs) are major components of the vascular microenvironment. These cells have been described for their plasticity and potential to transdifferentiate into mesenchymal cells through a process known as endothelial-to-mesenchymal transition (EndMT). This complex process is controlled by various factors, by which ECs convert into a phenotype characterized by mesenchymal protein expression and motile, contractile morphology. Initially described in normal heart development, EndMT is now identified in several pathologies, and especially in cancer. In this review, we highlight the process of EndMT in the context of cancer and we discuss it as an important adaptive process of the tumor microenvironment that favors tumor growth and dissemination but also resistance to treatment. Thus, we underline targeting of EndMT as a potential therapeutic strategy.

Keywords: endothelial, mesenchymal, plasticity, cancer, CAF, metastasis, resistance

OPEN ACCESS

Edited by:
Lucas Treps,
VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Giorgio Stassi,
University of Palermo, Italy
Tze-Sing Huang,
National Health Research Institutes, Taiwan

****Correspondence:***
Isabelle Corre
isabelle.corre@univ-nantes.fr

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *05 June 2020*
Accepted: *17 July 2020*
Published: *14 August 2020*

Citation:
Clere N, Renault S and Corre I (2020) Endothelial-to-Mesenchymal Transition in Cancer. Front. Cell Dev. Biol. 8:747. doi: 10.3389/fcell.2020.00747

INTRODUCTION

The tumor microenvironment (TME) is a complex network of stromal fibroblastic, immune, and endothelial cells (ECs), embedded in a supportive extracellular matrix. It is now considered as an essential player in cancer biology, participating to tumor progression, metastatic dissemination, and immune surveillance of cancer cells but also to responses to therapies (Maman and Witz, 2018). Furthermore, in response to environmental and oncogenic signals that evolved continuously while tumor growths, the TME appears highly dynamic and plastic (Quail and Joyce, 2013). In this environment, ECs that line microvessels are the key masters of angiogenesis. Indeed, in response to tumor environmental cues, ECs promote the formation of new vessels through proliferation, migration, adhesion, and matrix digestion to support tumor progression and dissemination (Potente et al., 2011). But beside their role in angiogenesis, ECs have been characterized the last decade as capable of an important phenotypic plasticity (Dejana et al., 2017), illustrated by their ability to modify their endothelial phenotype toward a mesenchymal profile. This plasticity, named the endothelial-to-mesenchymal transition (EndMT), was initially described in cardiac embryonic development but has been also highlighted in several postnatal pathologies such as cardiac fibrosis, atherosclerosis, pulmonary hypertension, and vascular calcification (Li et al., 2018a). Endothelial-to-mesenchymal transition appears as essential in these inflammation-associated disorders and may be a key link between endothelial dysfunction and inflammation (Cho et al., 2018). In the context of cancer, the process of EndMT was identified in 2007 in melanoma and pancreatic tumor mouse model (Zeisberg et al., 2007) and critically involved in tumor progression. Since then, increasing

evidence has reinforced the relevance of endothelial plasticity through EndMT in cancer biology. This review aims to describe the main features and roles of EndMT in cancer and to highlight its importance in shaping a tumor supportive microenvironment that favors tumor growth, metastatic dissemination, and resistance to treatment. Therefore, targeting EndMT as therapeutic strategy will also be discussed.

DESCRIPTION

The EndMT process is characterized as a transdifferentiation program where ECs lose their endothelial characteristics and gain mesenchymal features. This phenotypic switch is characterized by profound morphological, functional and molecular changes. Endothelial cells lose their cellular adhesion and delaminate, reorganize their cytoskeleton that converts their apico-basal polarity toward a front-end back polarity to form spindle-shape cells with enhanced properties of migration. This transition is accompanied with a marked decrease in endothelial markers such as VE-cadherin, CD31, Tie-1, Tie-2, and vWF conjugated to an increased expression of mesenchymal biomarkers such as α-SMA, SM22a, CD44, N-Cadherin, vimentin, COL I/III, and FSP-1 (S100A4) (Piera-Velazquez and Jimenez, 2019) (**Figure 1**). As described by Dejana et al. (2017), EndMT markers evolve from an early step with partial downregulation of endothelial markers and up-regulation of some early mesenchymal markers (α-SMA, SM22a, and FSP-1) to a later step, characterized by a decline of endothelial markers and up-regulation of mesenchymal markers such as matrix proteins and matrix metalloproteases (MMPs) (Fibronectin, COL I, and MMPs). Initially considered as a complete process of differentiation, EndMT might also be partial in pathophysiological context and in particular in cancer. Intermediate stages of differentiation in tumors-derived ECs have been identified (Xiao et al., 2015) and these tumor ECs display heterogeneity in their potentiality to undergo EndMT. This is consistent with the phenotypic heterogeneity of ECs in tumors (Maishi et al., 2019; Goveia et al., 2020), combined with the diversity of signals emanating from TME, as fine tuning of EndMT appears to be under the control of several factors such as transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF) (Xiao and Dudley, 2017). Moreover, partial EndMT has been assimilated as an initial step of endothelial sprouting in angiogenesis process (Welch-Reardon et al., 2015; Wang et al., 2017). Reversibility of EndMT is barely described in cancer, but a mesenchymal-to-endothelial transition was recently suggested as contributing to Kaposi sarcoma (Li et al., 2018b). Few *in vitro* studies suggest that EndMT reversibility may occur for a short period of time upon exposure to EndMT inducers (Rieder et al., 2011), while prolonged exposure forces mesenchymal differentiated cells to reach a no-return point toward endothelial features (Xiao et al., 2015).

Identifying the process of EndMT in living animals remains challenging. This process is inherently transitional and progressive, rendering detection of mesenchymal cells originating from endothelium difficult unless cells have not terminated their full transition. Therefore, detecting EndMT may be quite often underestimated *in vivo*. Nevertheless, exploration of EndMT has been rendered possible *in vivo* through fate mapping approach in transgenic mice harboring EC-specific lineage tracing, with the limitation that endothelial promoters are rarely totally exclusive to endothelial lineage (Li et al., 2018a). The first *in vivo* evidence of EndMT in a tumor context was shown in subcutaneous melanoma model in Tie-2-Cre x R26TRosa Lox-stop-LacZ-Lox crossed mice (Zeisberg et al., 2007). Since then, fate-mapping tracing has allowed to characterize EndMT in models of lung (Choi et al., 2018), pancreas (Anderberg et al., 2013), and brain (Huang et al., 2016). More importantly, EndMT has now been discovered in tumor specimen of human patients in a number of cancers: colorectal carcinoma (Fan et al., 2018), pancreatic ductal adenocarcinoma (Fan et al., 2019), lung cancer (Choi et al., 2018), or glioblastoma (GBM) (Huang et al., 2016; **Table 1**).

REGULATION OF EndMT

Major Inducers in Cancer

Members of TGF-β family of proteins are considered today as the major inducers of EndMT, both in physiologic cardiac development (Garside et al., 2013) but also in cancer, as TGF-βs are soluble factors often found overexpressed in tumors (Principe et al., 2014). Therefore, TGF-β1 produced by melanoma tumor cells leads to EndMT (Krizbai et al., 2015). TGF-β2 secreted by tumor in synergy with Interleukin-1β was shown essential to induce EndMT in esophageal carcinoma (Nie et al., 2014). TGF-β2 is also required to promote EndMT in the context of invasive colon carcinoma, *via* a tubulin-β3 dependent mechanism (Wawro et al., 2018). Nevertheless, additional signals to TGF-βs may be required, as highlighted in a 3D spheroid model of lung cancer where EndMT does not rely on TGF-β (Kim et al., 2019). In fact, TGF-β signaling appears insufficient in promoting EndMT in GBM but requires the Notch signaling pathway (Marin-Ramos et al., 2019). Such a crosstalk between Notch and TGF-β participates to the formation of a transient mesenchymal/endothelial niche in a murine breast cancer xenograft model (Ghiabi et al., 2015). In cancer studies, additional soluble factors have been depicted as EndMT inducers. In brain tumor, Hepatocyte growth factor (HGF) (Huang et al., 2016) and platelet-derived growth factor (PDGF) (Liu T. et al., 2018), two growth factors secreted by tumor cells, appear critical to drive EndMT. The bone matrix protein, osteopontin (OPN), through its binding to integrin αvβ3, promotes EndMT through an epigenetic controlled-repression of VE-cadherin in colorectal cancer (Fan et al., 2018). The Wnt signaling pathway through β-catenin/LEF signaling has also be shown to promote EndMT in oral squamous carcinoma (Wang et al., 2017) and very recently in GBM (Huang et al., 2020).

Hypoxia is an important hallmark of the TME and plays a major role in promoting angiogenesis. Nevertheless, hypoxia has also been described as a potent inducer of EndMT, especially in pulmonary arterial hypertension (Suzuki et al., 2018) and in radiation-induced fibrosis (Choi et al., 2015), through hypoxia-inducible factor (HIF)-dependent pathways (Tang et al., 2018;

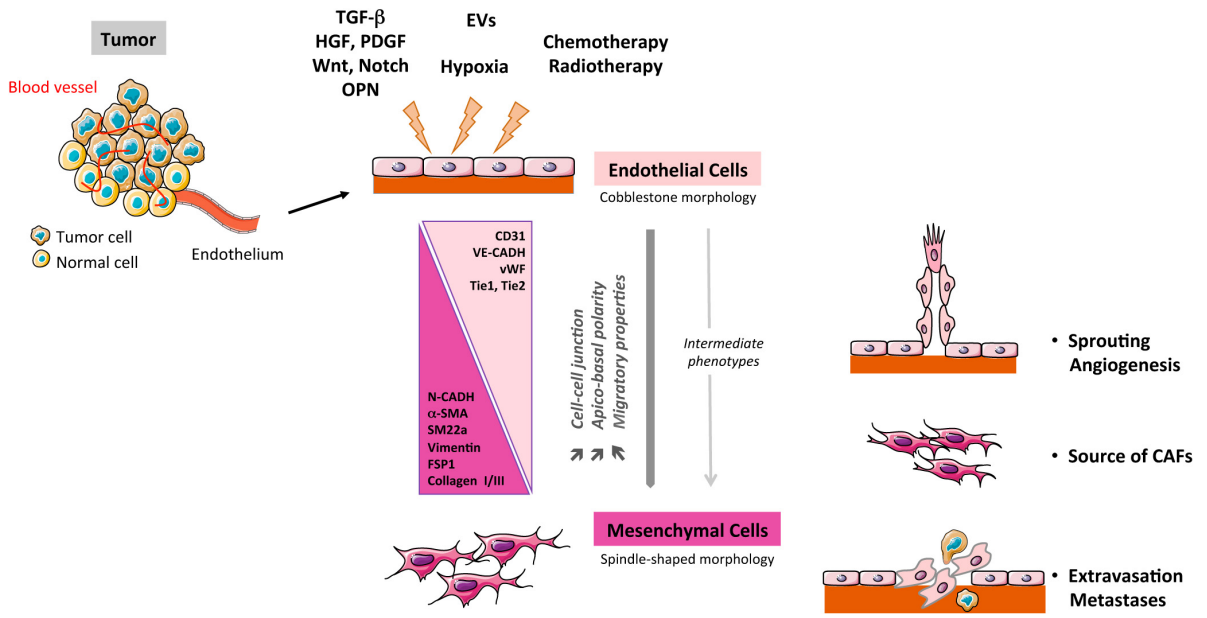


FIGURE 1 | Schematic representation of the EndMT process in cancer. TGF-β, transforming growth factor-β; PDGF, platelet derived growth factor; HGF, hepatocyte growth factor; CAFs, cancer-associated fibroblasts; OPN, osteopontin; EVs: extracellular vesicles.

TABLE 1 | Inducers and Roles of EndMT in different types of cancer.

Type of cancer	Inducers	Role	Models (Ref)
Melanoma	*nd*	Source of CAFs	Murine model (Zeisberg et al., 2007)
	Tumor-derived TGF-β	Metastasis	Cellular model (Krizbai et al., 2015)
Colorectal cancer	TGF-β1/2	Source of CAFs	Cellular model (Wawro et al., 2018)
	OPN	Source of CAFs	Patients biopsies, cellular model (Fan et al., 2018)
	EVs (mir-92a)	Angiogenesis	Cellular model (Yamada et al., 2019)
Pancreatic cancer	*nd*	Source of CAFs	Patient biopsies, murine model (Zeisberg et al., 2007; Fan et al., 2019)
	Endoglin deficiency	Metastasis	Murine model (Anderberg et al., 2013)
Lung cancer	Radiation	Resistance to radiotherapy	Patients biopsies, murine model (Choi et al., 2018)
	Tumor secretome	Resistance to chemotherapy	Cellular model (Kim et al., 2019)
Glioblastoma	HGF/β-catenin	Resistance to chemotherapy	Patients biopsies, murine model (Huang et al., 2016, 2020)
	PDGF	Resistance to anti-angiogenic agents	Murine model (Liu T. et al., 2018)
Oral squamous carcinoma	Wnt-5b	Lymphangiogenesis	Cellular model (Wang et al., 2017)
Esophageal cancer	TGF-β2-IL1β	Source of CAFs	Patients biopsies, cellular model (Nie et al., 2014)

OPN, osteopontin; EV, extracellular vesicles; CAFs, cancer-associated fibroblasts; TGF-β, transforming growth factor-β; PDGF, platelet derived growth factor; nd, not determined.

Toullec et al., 2018). Alternatively, hypoxia-induced EndMT in primary and immortalized ECs was shown to involve RhoJ leading to HIF-dependent and trimethylated histone H3K4-dependent pathways (Liu L. et al., 2018). Furthermore, expression of TGF-β, the master inducer of EndMT, may also be regulated by hypoxia (Nishi et al., 2004; Hung et al., 2013). Endothelial-to-mesenchymal transition-relied transcription factors Snail (Xu et al., 2015) and Twist-1 (Mammoto et al., 2018) are also identified as targets of hypoxia. Although particularly relevant in tumors, hypoxia, and HIF signaling remain to be deeper explored in EndMT in cancer.

Responses to cancer treatment have important issues, leading to efficiency but often to resistance. Cancer therapies such as conventional radiotherapies (Mintet et al., 2015; Choi et al., 2018), high-dose radiation therapies (Banerjee et al., 2020) but also chemotherapies (Murugavel et al., 2018) are also currently identified as promoting EndMT in diverse type of tumors, with consequences in tumor response as described below.

Main Intracellular Signaling Modulators of EndMT

Transcription Factors

As mentioned, TGF-β induces EndMT in cancer and involves the Snail family of transcription factors such as Snail, Slug, Twist, and ZEB. These factors are potent transcriptional repressors, notably

of endothelial markers VE-cadherin and CD31 (Piera-Velazquez and Jimenez, 2019). A direct regulation of Snail by TGF-β2 has been highlighted in mouse embryonic stem cell-derived ECs (Kokudo et al., 2008) and Medici et al. (2011) described Snail as a master transcription factor of EndMT, induced both by Smad-dependent and PI3K/p38 MAPK-dependent signaling pathways. Importantly, Snail has been identified as a direct target of HIF-1α in hypoxia-induced EndMT (Xu et al., 2015). Furthermore, hypoxia-induced EndMT involves TGF-β-induced phosphorylation of transcription factor Twist-1 (Mammoto et al., 2018) but also increased expression and nuclear translocation of Snail, Slug and Zeb1 (Doerr et al., 2016). TGF-β2 drives also EndMT through a Smad-dependent activation of the myocardin-related transcription factor-A (MRTF-A), in pancreatic MS-1 ECs (Mihira et al., 2012) while in an invasive colorectal cancer microenvironment, TGF-β2-induced EndMT involves MRTF-A and B transcription factors through Smad-independent RhoA pathway (Ciszewski et al., 2017).

Epigenetic Regulation

With EndMT characterized as a cellular transdifferentation where gene expression changes are necessary, epigenetics is now considered as a strong regulator of this process (Hulshoff et al., 2018). Furthermore, epigenetic regulation is also determinant for cellular differentiation during cancer pathologies (Petronis, 2010). The global histones methylation of lysine residues has been reported to be associated with EndMT. Thus, EZH2 (enhancer of zeste homolog 2), the major histone methyltransferase that processes the transcriptional repressive trimethylation on lysine 27 of histone H3 (H3K27me3), is down-expressed during EndMT induced by IL-1β and TGF-β2 (Maleszewska et al., 2015). This is associated with reduced H3K27me3 repressive marks at the promoter of the mesenchymal gene *transgelin/SM22α*. Recently, in colorectal cancer, EZH2 was identified as interacting with the transcription factor TCF12, helping this latter to transcriptionally repress *VE-cadherin* gene and thus facilitating EndMT (Fan et al., 2018). Additionally, Jumonji domain-containing protein 2B, JMJD2B, a demethylase of the repressive histone mark H3K9me3, is induced by EndMT-promoting stimuli and is associated to de-repression of genes in EndMT-related genes and pathways (Glaser et al., 2020). Description of EndMT in melanoma intratumoral ECs has recently shed the light on two transcription factors from the ETS family, ERG and Fli-1 (Nagai et al., 2018). These pivotal transcription factors promote expression of EC-specific genes but indirectly repress mesenchymal-related genes through epigenetic regulation by modifying histones methylation/acetylation marks. The dowregulation of ERG and Fli1 has been described in tumor-induced EndMT both *in vitro* and in clinical cancer patients (Nagai et al., 2018), pointing the alleviation of mesenchymal genes repression.

Accumulative evidence highlights that non-coding RNAs including miRNAs and lncRNAs are important epigenetic regulators of EndMT [for review, Hulshoff et al. (2018)]. Depending on their gene targets that mainly belonged to TGF signaling pathways, miRNAs may promote or repress the process of EndMT. The list of miRNAs regulating EndMT is constantly increasing in pathologies (cardiac, fibrosis, and pulmonary hypertension) (Kim, 2018). In malignant disorders, overexpression of miR-302c suppresses EndMT, therefore inhibiting hepatocarcinoma tumor growth (Zhu et al., 2014). A few of lncRNAs have been associated with EndMT. Metastasis-associated lung adenocarcinoma transcript 1 MALAT-1 facilitates EndMT through down-regulation of *Smad3*, *TGFBR2*-targeting miR-145 (Xiang et al., 2017) and recently, a combination of low expression of three lncRNAs (LOC340340, LOC101927256, MNX1-AS1) was proposed as an EndMT index in pancreas adenocarcinoma (Fan et al., 2019).

Metabolic Regulation

Activation of the EndMT program has been recently associated with profound metabolic alterations in ECs. Fatty acid oxidation (FAO) was shown to contribute to vessels sprouting by promoting, in angiogenic ECs, a *de novo* nucleotide synthesis for DNA replication for proliferation (Schoors et al., 2015; Eelen et al., 2018). Xiong and colleagues now demonstrate that inhibition of FAO, marked by a decreased expression of mitochondrial enzyme carnitine *O*-palmitoyltransferase 1 (CPT1) and by a fall of acetyl-CoA levels, is critical for TGF-β-induced EndMT (Xiong et al., 2018). Therefore, metabolic plasticity and metabolic transcriptome heterogeneity among normal and tumor ECs, as recently described (Rohlenova et al., 2020), have to be considered as important indicators of endothelial fate, with engagement to EndMT associated with a lower energy-producing metabolism of FAO inhibition.

ROLES OF EndMT IN CANCER

Angiogenesis as a Partial EndMT

Angiogenesis is required for an optimal tumor progression (Folkman, 1971) and is initiated by the sprouting of ECs from existent vessels. Sprouting-leader endothelial tip cells lose their apical-basal polarity, weaken their cell-cell interaction, degrade extracellular matrix to become more motile, but never complete their transition as they do maintain some cell-to-cell interactions. Overall, this sprouting resumes EndMT features and may be compared to a partial EndMT (Potenta et al., 2008; Welch-Reardon et al., 2015). Moreover, the master EndMT transcription factors Slug and Snail are found expressed in tumor ECs in several carcinoma (Welch-Reardon et al., 2015), with Slug expression recently associated with angiogenesis and tumor metastasis (Gu et al., 2017). Recently, colon cancer cells, *via* extracellular vesicles, have been shown to induce a partial EndMT associated with angiogenesis, in a miR-92a-dependent inhibition of junctional Claudin-11 and up-regulation of Snail, and vimentin (Yamada et al., 2019). Partial EndMT, stimulated by tumor-secreted Wnt ligand, has also been involved in oral cancer lymphangiogenesis (Wang et al., 2017).

EndMT as a Source of Cancer-Associated Fibroblasts (CAFs)

CAFs are major components of the TME, contributing to tumorigenesis in multiple ways: cancer cell proliferation, migration, invasion, stemness, mainly through the secretion

of cytokines, chemokines, extracellular vesicles, and matrix remodeling [for review, Bu et al. (2019)]. CAFs may originate from several cell types, namely resident fibroblasts, epithelial cells, mesenchymal stromal stem cell from bone marrow, and adipocytes. However, myofibroblastic/mesenchymal cells issued from ECs through EndMT represent also a unique source of CAFs, as initially described in mouse model (Zeisberg et al., 2007). The actual contribution of EndMT in CAFs production is still not fully characterized. Nevertheless, several studies indicate that EndMT-derived cells share functions with CAF-like cells, such as adhesion to collagen and wound healing (Wawro et al., 2018), production of VEGF in esophageal cancer (Nie et al., 2014) but also promotion of angiogenesis, tumor growth and stemness in colorectal cancer (Fan et al., 2018) or driving monocyte M2 polarization in pancreatic cancer (Fan et al., 2019).

EndMT and Metastasis

One major hallmark of EndMT is endothelial cytoskeletal reorganization through the Rho/ROCK signaling pathway (Mihira et al., 2012; Ciszewski et al., 2017), coupled to the loss of endothelial adhesion molecules (VE-cadherin, claudins). Therefore, EndMT contributes to disruption of the endothelial barrier that may favor the intra- and extravasation tumor cells. Indeed, melanoma cells increase their transendothelial migration when EndMT is induced by tumor secretome (Krizbai et al., 2015). To the same extend, hepatic and lung metastatic seeding is increased in pancreatic cancer mouse model upon endoglin deficiency-induced EndMT (Anderberg et al., 2013). Hence, EndMT by facilitating tumor transendothelial migration can be considered as an essential process for an optimal metastatic dissemination (Gasparics et al., 2016).

EndMT and Therapeutic Resistance

Vasculature is a double-edge sword in tumor, as it promotes tumor progression through angiogenesis but at the same time it assures delivery of drugs to tumor. Therefore, plasticity and dynamics of the endothelial compartment may impact response to therapies. The role of EndMT in resistance to therapies emerges as a novel field, both in the context of chemo- and radiotherapy. In fact, EndMT confers tumor radioresistance and promotes colorectal tumor growth tumor, by awaking cancer stem cells and stimulating polarization of M2 tumor-associated macrophages TAM (Choi et al., 2018). Resistance to chemotherapies cisplatin and gefitinib in a multicellular lung tumor spheroid model is alleviated when EndMT is reversed, implying EndMT as a resistance factor (Kim et al., 2019). Recently, a robust EndMT induced by HGF/c-Met was described in GBM, resulting in aberrant vasculature and in an increase of tumor cells chemoresistant to temozolomide (Huang et al., 2016), undoubtedly linked to drug delivery failure and hypoxia. The same group documented the induction of resistance of EndMT-issued cells to anti-angiogenic chemotherapies in this brain tumor. Indeed, PDGF-induced EndMT is associated with a decreased expression of VEGFR2, resulting in resistance to anti-VEGF treatment (Liu T. et al., 2018) whereas HGF/β-catenin-induced-EndMT leads to endothelial temozolomide resistance through up-regulation of drug pumping and efflux proteins ABBC1/Multidrug resistance associated protein (MRP-1; Huang et al., 2020). Furthermore, EndMT induces abnormal recruitment of pericytes (Choi et al., 2018) and generates pericyte-like cells abnormally covering the tumor vasculature (Anderberg et al., 2013). Such an aberrant coverage of pericytes is described as a signature of resistance to anti-VEGF anti-angiogenic therapy in two different cancer models [pancreas (Helfrich et al., 2010) and melanoma (Franco et al., 2011)]. Hence, EndMT appears to participate to establish a perivascular niche supportive for resistance in many ways: promotion of hypoxia, failure of drug delivery, vanishing of drug target (VEGFR2), and recruitment of pro-tumoral immune cells. Overall, EndMT has to be considered as a relevant factor of resistance.

TARGETING OF EndMT

In light of the overall pro-tumoral effect of EndMT, targeting of this process could represent a therapeutic avenue to treat cancer. Currently, several drugs have been tested as potential EndMT inhibitors. The mTOR inhibitor rapamycin is able to block EndMT, either by preventing EC migration and matrix degradation (Gao et al., 2011) or by lowering TGF-β, TNF-α and VEGF levels (Gonzalez-Mateo et al., 2015). Spironolactone, an aldosterone receptor-blocker, abrogates EndMT in a model of fibrosis, by blocking TGF-β and Notch signaling (Chen et al., 2015). Notably, a derived-form of temozolomide, the standard chemotherapy in GBM, can suppress EndMT in tumor-associated ECs via the same mechanisms of TGF-β and Notch signaling inhibition (Marin-Ramos et al., 2019), supporting the clinical value of this molecule.

An innovative adjuvant strategy could use molecules targeting metabolic pathways. As inhibition of fatty acid oxidation is described to potentiate EndMT (Xiong et al., 2018), one may consider activation of FAO to limit the EndMT process. Indeed, pharmacological approaches to increase FAO are available, mainly through the use of peroxisome proliferator-activated receptor PPAR agonists (Djouadi et al., 2005) or fatty acid synthase FASN inhibitors (Thupari et al., 2004) but are not explored yet in cancer.

Inhibiting kinase signaling engaged by recently identified EndMT-inducing factors such as PDGF or HGF (Huang et al., 2016; Liu T. et al., 2018) would be of interest. For example, Nintedanib, a tyrosine kinase inhibitor of PDGF, FGF, and VEGF, has been shown to ameliorate pulmonary hypertension by blocking EndMT (Tsutsumi et al., 2019) and inhibitors of the HGF receptor c-Met (e.g., carbozantinib) are at present intensively explored in Ewing sarcoma and osteosarcoma clinical trials (Italiano et al., 2020).

As TGF-β is currently considered as potent inducer of EndMT in cancer, targeting TGF-β could represent a potential therapeutic approach. Several TGF-β inhibitors have emerged, among which the small molecule inhibitor galunisertib (LY2157299) appears as one of the most advanced and promising molecules tested in two phase-II trials in pancreatic cancer (NCT01373164) or in hepatocellular carcinoma (NCT01246986). Despite not tested on tumor EndMT yet, galunisertib harbors a very safe toxicity

profile, with no cardiac toxicity that was a limitation for the use of the first generation of TGF-β inhibitors tested in clinic (Kovacs et al., 2015). Monoclonal antibodies against TGF-β are now also available and tested in different pathologies. As such, fresolimumab, initially developed for the treatment of idiopathic pulmonary fibrosis, has been investigated in phase-II trials in renal cell carcinoma and melanoma, but also metastatic breast cancer (NCT01401062) (Formenti et al., 2019) where TGF-β blocking was also associated with an increase of the immune anti-tumor response. If the beneficial properties of fresolimumab were confirmed in impairing the process of EndMT, inhibiting the TGF-β pathway could be particularly efficient for the treatment of aggressive tumors with the targeting of two essential components of the TME.

CONCLUSION

Despite its beneficial role in embryo development, the process of EndMT may be also detrimental, notably in cancer where it contributes to tumor development. This induced plasticity of the endothelium has an important role in shaping the TME. Endothelial-to-mesenchymal transition participates to initial angiogenesis sprouting, reinforces the stromal fibroblastic microenvironment and remodels the vasculature to support tumor cell dissemination and metastasis. Moreover, it contributes to the resistance to cancer treatment, by affecting directly or indirectly the microenvironment. Thus, the process of EndMT regulates different stages of tumorigenesis from initiation, dissemination to response to treatments.

Exploring EndMT remains very challenging mainly due to its inherent dynamics and to the complexity of its mechanisms. Nevertheless, its full understanding still requires deep exploration. Importantly, the role of EndMT transitional populations still need to be grasped, as these intermediate populations could also be relevant in tumor biology.

In conclusion, EndMT can be considered as an important process that reinforces the TME plasticity and devotion to cancer cells. Therefore, modulation of EndMT could be envisaged as a complementary therapeutic arm to counteract tumor progression. Currently, several strategies to inhibit EndMT are considered and deserve exploration in cancer context.

AUTHOR CONTRIBUTIONS

NC, SR, and IC contributed to the writing and editing of the manuscript. SR and IC elaborated the figure and table. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by "Région Pays de la Loire" for the TRENDOMOS project («2019-Pari Scientifique» Program), INSERM, CNRS and by the association La Ligue contre le cancer (Comities 22, 29, 37, 44, 49, and 56).

REFERENCES

Anderberg, C., Cunha, S. I., Zhai, Z., Cortez, E., Pardali, E., Johnson, J. R., et al. (2013). Deficiency for endoglin in tumor vasculature weakens the endothelial barrier to metastatic dissemination. *J. Exp. Med.* 210, 563–579. doi: 10.1084/jem.20120662

Banerjee, D., Barton, S. M., Grabham, P. W., Rumeld, A. L., Okochi, S., Street, C., et al. (2020). High-dose radiation increases notch1 in tumor vasculature. *Int. J. Radiat. Oncol. Biol. Phys.* 106, 857–866. doi: 10.1016/j.ijrobp.2019.11.010

Bu, L., Baba, H., Yoshida, N., Miyake, K., Yasuda, T., Uchihara, T., et al. (2019). Biological heterogeneity and versatility of cancer-associated fibroblasts in the tumor microenvironment. *Oncogene* 38, 4887–4901. doi: 10.1038/s41388-019-0765-y

Chen, X., Cai, J., Zhou, X., Chen, L., Gong, Y., Gao, Z., et al. (2015). Protective effect of spironolactone on endothelial-to-mesenchymal transition in HUVECs via notch pathway. *Cell Physiol. Biochem.* 36, 191–200. doi: 10.1159/000374063

Cho, J. G., Lee, A., Chang, W., Lee, M. S., and Kim, J. (2018). Endothelial to mesenchymal transition represents a key link in the interaction between inflammation and endothelial dysfunction. *Front. Immunol.* 9:294. doi: 10.3389/fimmu.2018.00294

Choi, S. H., Hong, Z. Y., Nam, J. K., Lee, H. J., Jang, J., Yoo, R. J., et al. (2015). A Hypoxia-induced vascular endothelial-to-mesenchymal transition in development of radiation-induced pulmonary fibrosis. *Clin. Cancer Res.* 21, 3716–3726. doi: 10.1158/1078-0432.CCR-14-3193

Choi, S. H., Kim, A. R., Nam, J. K., Kim, J. M., Kim, J. Y., Seo, H. R., et al. (2018). Tumour-vasculature development via endothelial-to-mesenchymal transition after radiotherapy controls CD44v6(+) cancer cell and macrophage polarization. *Nat. Commun.* 9:5108. doi: 10.1038/s41467-018-07470-w

Ciszewski, W. M., Sobierajska, K., Wawro, M. E., Klopocka, W., Chefczynska, N., Muzyczuk, A., et al. (2017). The ILK-MMP9-MRTF axis is crucial for EndMT differentiation of endothelial cells in a tumor microenvironment. *Biochim. Biophys. Acta Mol. Cell Res.* 1864, 2283–2296. doi: 10.1016/j.bbamcr.2017.09.004

Dejana, E., Hirschi, K. K., and Simons, M. (2017). The molecular basis of endothelial cell plasticity. *Nat. Commun.* 8:14361. doi: 10.1038/ncomms14361

Djouadi, F., Aubey, F., Schlemmer, D., Ruiter, J. P., Wanders, R. J., Strauss, A. W., et al. (2005). Bezafibrate increases very-long-chain acyl-CoA dehydrogenase protein and mRNA expression in deficient fibroblasts and is a potential therapy for fatty acid oxidation disorders. *Hum. Mol. Genet.* 14, 2695–2703. doi: 10.1093/hmg/ddi303

Doerr, M., Morrison, J., Bergeron, L., Coomber, B. L., and Viloria-Petit, A. (2016). Differential effect of hypoxia on early endothelial-mesenchymal transition response to transforming growth beta isoforms 1 and 2. *Microvasc. Res.* 108, 48–63. doi: 10.1016/j.mvr.2016.08.001

Eelen, G., de Zeeuw, P., Treps, L., Harjes, U., Wong, B. W., and Carmeliet, P. (2018). Endothelial cell metabolism. *Physiol. Rev.* 98, 3–58. doi: 10.1152/physrev.00001.2017

Fan, C. S., Chen, L. L., Hsu, T. A., Chen, C. C., Chua, K. V., Li, C. P., et al. (2019). Endothelial-mesenchymal transition harnesses HSP90alpha-secreting M2-macrophages to exacerbate pancreatic ductal adenocarcinoma. *J. Hematol. Oncol.* 12:138. doi: 10.1186/s13045-019-0826-2

Fan, C. S., Chen, W. S., Chen, L. L., Chen, C. C., Hsu, Y. T., Chua, K. V., et al. (2018). Osteopontin-integrin engagement induces HIF-1alpha-TCF12-mediated endothelial-mesenchymal transition to exacerbate colorectal cancer. *Oncotarget* 9, 4998–5015. doi: 10.18632/oncotarget.23578

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285, 1182–1186. doi: 10.1056/NEJM197111182852108

Formenti, S. C., Hawtin, R. E., Dixit, N., Evensen, E., Lee, P., Goldberg, J. D., et al. (2019). Baseline T cell dysfunction by single cell network profiling in metastatic breast cancer patients. *J. Immunother. Cancer* 7:177. doi: 10.1186/s40425-019-0633-x

Franco, M., Roswall, P., Cortez, E., Hanahan, D., and Pietras, K. (2011). Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. *Blood* 118, 2906–2917. doi: 10.1182/blood-2011-01-331694

Gao, H., Zhang, J., Liu, T., and Shi, W. (2011). Rapamycin prevents endothelial cell migration by inhibiting the endothelial-to-mesenchymal transition and matrix metalloproteinase-2 and -9: an in vitro study. *Mol. Vis.* 17, 3406–3414.

Garside, V. C., Chang, A. C., Karsan, A., and Hoodless, P. A. (2013). Co-ordinating Notch, BMP, and TGF-beta signaling during heart valve development. *Cell Mol. Life Sci.* 70, 2899–2917. doi: 10.1007/s00018-012-1197-9

Gasparics, A., Rosivall, L., Krizbai, I. A., and Sebe, A. (2016). When the endothelium scores an own goal: endothelial cells actively augment metastatic extravasation through endothelial-mesenchymal transition. *Am. J. Physiol. Heart Circ. Physiol.* 310, H1055–H1063. doi: 10.1152/ajpheart.00042.2016

Ghiabi, P., Jiang, J., Pasquier, J., Maleki, M., Abu-Kaoud, N., Halabi, N., et al. (2015). Breast cancer cells promote a notch-dependent mesenchymal phenotype in endothelial cells participating to a pro-tumoral niche. *J. Transl. Med.* 13:27. doi: 10.1186/s12967-015-0386-3

Glaser, S. F., Heumuller, A. W., Tombor, L., Hofmann, P., Muhly-Reinholz, M., Fischer, A., et al. (2020). The histone demethylase JMJD2B regulates endothelial-to-mesenchymal transition. *Proc. Natl. Acad. Sci. U.S.A.* 117, 4180–4187. doi: 10.1073/pnas.1913481117

Gonzalez-Mateo, G. T., Aguirre, A. R., Loureiro, J., Abensur, H., Sandoval, P., Sanchez-Tomero, J. A., et al. (2015). Rapamycin protects from type-i peritoneal membrane failure inhibiting the angiogenesis, lymphangiogenesis, and Endo-MT. *Biomed. Res. Int.* 2015, 989560. doi: 10.1155/2015/989560

Goveia, J., Rohlenova, K., Taverna, F., Treps, L., Conradi, L. C., Pircher, A., et al. (2020). An Integrated Gene expression landscape profiling approach to identify lung tumor endothelial cell heterogeneity and angiogenic candidates. *Cancer Cell* 37, 21–36. doi: 10.1016/j.ccell.2019.12.001

Gu, A., Jie, Y., Yao, Q., Zhang, Y., and Mingyan, E. (2017). Slug is associated with tumor metastasis and angiogenesis in ovarian cancer. *Reprod. Sci.* 24, 291–299. doi: 10.1177/1933719116654989

Helfrich, I., Scheffrahn, I., Bartling, S., Weis, J., von Felbert, V., Middleton, M., et al. (2010). Resistance to antiangiogenic therapy is directed by vascular phenotype, vessel stabilization, and maturation in malignant melanoma. *J. Exp. Med.* 207, 491–503. doi: 10.1084/jem.20091846

Huang, M., Liu, T., Ma, P., Mitteer, R. A. Jr., and Zhang, Z. (2016). c-Met-mediated endothelial plasticity drives aberrant vascularization and chemoresistance in glioblastoma. *J. Clin. Invest.* 126, 1801–1814. doi: 10.1172/JCI84876

Huang, M., Zhang, D., Wu, J. Y., Xing, K., Yeo, E., Li, C., et al. (2020). Wnt-mediated endothelial transformation into mesenchymal stem cell-like cells induces chemoresistance in glioblastoma. *Sci. Transl. Med.* 12:532. doi: 10.1126/scitranslmed.aay7522

Hulshoff, M. S., Xu, X., Krenning, G., and Zeisberg, E. M. (2018). Epigenetic regulation of endothelial-to-mesenchymal transition in chronic heart Disease. *Arterioscler. Thromb. Vasc. Biol.* 38, 1986–1996. doi: 10.1161/ATVBAHA.118.311276

Hung, S. P., Yang, M. H., Tseng, K. F., and Lee, O. K. (2013). Hypoxia-induced secretion of TGF-beta1 in mesenchymal stem cell promotes breast cancer cell progression. *Cell Transplant.* 22, 1869–1882. doi: 10.3727/096368912X657954

Italiano, A., Mir, O., Mathoulin-Pelissier, S., Penel, N., Piperno-Neumann, S., Bompas, E., et al. (2020). Cabozantinib in patients with advanced Ewing sarcoma or osteosarcoma (CABONE): a multicentre, single-arm, phase 2 trial. *Lancet Oncol.* 21, 446–455. doi: 10.1016/S1470-2045(19)30825-3

Kim, J. (2018). MicroRNAs as critical regulators of the endothelial to mesenchymal transition in vascular biology. *BMB Rep.* 51, 65–72. doi: 10.5483/bmbrep.2018.51.2.011

Kim, S. H., Song, Y., and Seo, H. R. (2019). GSK-3beta regulates the endothelial-to-mesenchymal transition via reciprocal crosstalk between NSCLC cells and HUVECs in multicellular tumor spheroid models. *J. Exp. Clin. Cancer Res.* 38:46. doi: 10.1186/s13046-019-1050-1

Kokudo, T., Suzuki, Y., Yoshimatsu, Y., Yamazaki, T., Watabe, T., and Miyazono, K. (2008). Snail is required for TGFbeta-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells. *J. Cell Sci.* 121(Pt 20), 3317–3324. doi: 10.1242/jcs.028282

Kovacs, R. J., Maldonado, G., Azaro, A., Fernandez, M. S., Romero, F. L., Sepulveda-Sanchez, J. M., et al. (2015). Cardiac safety of TGF-beta receptor i kinase inhibitor LY2157299 monohydrate in cancer patients in a first-in-human dose study. *Cardiovasc. Toxicol.* 15, 309–323. doi: 10.1007/s12012-014-9297-4

Krizbai, I. A., Gasparics, A., Nagyoszi, P., Fazakas, C., Molnar, J., Wilhelm, I., et al. (2015). Endothelial-mesenchymal transition of brain endothelial cells: possible role during metastatic extravasation. *PLoS One* 10:e0119655. doi: 10.1371/journal.pone.0119655

Li, Y., Lui, K. O., and Zhou, B. (2018a). Reassessing endothelial-to-mesenchymal transition in cardiovascular diseases. *Nat. Rev. Cardiol.* 15, 445–456. doi: 10.1038/s41569-018-0023-y

Li, Y., Zhong, C., Liu, D., Yu, W., Chen, W., Wang, Y., et al. (2018b). Evidence for kaposi sarcoma originating from mesenchymal stem cell through KSHV-induced mesenchymal-to-endothelial transition. *Cancer Res.* 78, 230–245. doi: 10.1158/0008-5472.CAN-17-1961

Liu, L., Chen, J., Sun, L., and Xu, Y. (2018). RhoJ promotes hypoxia induced endothelial-to-mesenchymal transition by activating WDR5 expression. *J. Cell Biochem.* 119, 3384–3393. doi: 10.1002/jcb.26505

Liu, T., Ma, W., Xu, H., Huang, M., Zhang, D., He, Z., et al. (2018). PDGF-mediated mesenchymal transformation renders endothelial resistance to anti-VEGF treatment in glioblastoma. *Nat. Commun.* 9:3439. doi: 10.1038/s41467-018-05982-z

Maishi, N., Annan, D. A., Kikuchi, H., Hida, Y., and Hida, K. (2019). Tumor endothelial heterogeneity in cancer progression. *Cancers* 11:1511. doi: 10.3390/cancers11101511

Maleszewska, M., Gjaltema, R. A., Krenning, G., and Harmsen, M. C. (2015). Enhancer of zeste homolog-2 (EZH2) methyltransferase regulates transgelin/smooth muscle-22alpha expression in endothelial cells in response to interleukin-1beta and transforming growth factor-beta2. *Cell Signal.* 27, 1589–1596. doi: 10.1016/j.cellsig.2015.04.008

Maman, S., and Witz, I. P. (2018). A history of exploring cancer in context. *Nat. Rev. Cancer* 18, 359–376. doi: 10.1038/s41568-018-0006-7

Mammoto, T., Muyleart, M., Konduri, G. G., and Mammoto, A. (2018). Twist1 in hypoxia-induced pulmonary hypertension through transforming growth factor-beta-smad signaling. *Am. J. Respir. Cell Mol. Biol.* 58, 194–207. doi: 10.1165/rcmb.2016-0323OC

Marin-Ramos, N. I., Jhaveri, N., Thein, T. Z., Fayngor, R. A., Chen, T. C., and Hofman, F. M. (2019). NEO212, a conjugate of temozolomide and perillyl alcohol, blocks the endothelial-to-mesenchymal transition in tumor-associated brain endothelial cells in glioblastoma. *Cancer Lett.* 442, 170–180. doi: 10.1016/j.canlet.2018.10.034

Medici, D., Potenta, S., and Kalluri, R. (2011). Transforming growth factor-beta2 promotes Snail-mediated endothelial-mesenchymal transition through convergence of Smad-dependent and Smad-independent signalling. *Biochem. J.* 437, 515–520. doi: 10.1042/BJ20101500

Mihira, H., Suzuki, H. I., Akatsu, Y., Yoshimatsu, Y., Igarashi, T., Miyazono, K., et al. (2012). TGF-beta-induced mesenchymal transition of MS-1 endothelial cells requires Smad-dependent cooperative activation of Rho signals and MRTF-A. *J. Biochem.* 151, 145–156. doi: 10.1093/jb/mvr121

Mintet, E., Rannou, E., Buard, V., West, G., Guipaud, O., Tarlet, G., et al. (2015). identification of endothelial-to-mesenchymal transition as a potential participant in radiation Proctitis. *Am. J. Pathol.* 185, 2550–2562. doi: 10.1016/j.ajpath.2015.04.028

Murugavel, S., Bugyei-Twum, A., Matkar, P. N., Al-Mubarak, H., Chen, H. H., Adam, M., et al. (2018). Valproic acid induces endothelial-to-mesenchymal transition-like phenotypic switching. *Front. Pharmacol.* 9:737. doi: 10.3389/fphar.2018.00737

Nagai, N., Ohguchi, H., Nakaki, R., Matsumura, Y., Kanki, Y., Sakai, J., et al. (2018). Downregulation of ERG and FLI1 expression in endothelial cells triggers endothelial-to-mesenchymal transition. *PLoS Genet.* 14:e1007826. doi: 10.1371/journal.pgen.1007826

Nie, L., Lyros, O., Medda, R., Jovanovic, N., Schmidt, J. L., Otterson, M. F., et al. (2014). Endothelial-mesenchymal transition in normal human esophageal endothelial cells cocultured with esophageal adenocarcinoma cells: role of IL-1beta and TGF-beta2. *Am. J. Physiol. Cell Physiol.* 307, C859–C877. doi: 10.1152/ajpcell.00081.2014

Nishi, H., Nakada, T., Hokamura, M., Osakabe, Y., Itokazu, O., Huang, L. E., et al. (2004). Hypoxia-inducible factor-1 transactivates transforming growth factor-beta3 in trophoblast. *Endocrinology* 145, 4113–4118. doi: 10.1210/en.2003-1639

Petronis, A. (2010). Epigenetics as a unifying principle in the aetiology of complex traits and diseases. *Nature* 465, 721–727. doi: 10.1038/nature09230

Piera-Velazquez, S., and Jimenez, S. A. (2019). Endothelial to mesenchymal transition: role in physiology and in the pathogenesis of human diseases. *Physiol. Rev.* 99, 1281–1324. doi: 10.1152/physrev.00021.2018

Potenta, S., Zeisberg, E., and Kalluri, R. (2008). The role of endothelial-to-mesenchymal transition in cancer progression. *Br. J. Cancer* 99, 1375–1379. doi: 10.1038/sj.bjc.6604662

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887. doi: 10.1016/j.cell.2011.08.039

Principe, D. R., Doll, J. A., Bauer, J., Jung, B., Munshi, H. G., Bartholin, L., et al. (2014). TGF-beta: duality of function between tumor prevention and carcinogenesis. *J. Natl. Cancer Inst.* 106:djt369. doi: 10.1093/jnci/djt369

Quail, D. F., and Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* 19, 1423–1437. doi: 10.1038/nm.3394

Rieder, F., Kessler, S. P., West, G. A., Bhilocha, S., de la Motte, C., Sadler, T. M., et al. (2011). Inflammation-induced endothelial-to-mesenchymal transition: a novel mechanism of intestinal fibrosis. *Am. J. Pathol.* 179, 2660–2673. doi: 10.1016/j.ajpath.2011.07.042

Rohlenova, K., Goveia, J., Garcia-Caballero, M., Subramanian, A., Kalucka, J., Treps, L., et al. (2020). Single-Cell RNA sequencing maps endothelial metabolic plasticity in pathological angiogenesis. *Cell Metab.* 31, 862–877. doi: 10.1016/j.cmet.2020.03.009

Schoors, S., Bruning, U., Missiaen, R., Queiroz, K. C., Borgers, G., Elia, I., et al. (2015). Fatty acid carbon is essential for dNTP synthesis in endothelial cells. *Nature* 520, 192–197. doi: 10.1038/nature14362

Suzuki, T., Carrier, E. J., Talati, M. H., Rathinasabapathy, A., Chen, X., Nishimura, R., et al. (2018). Isolation and characterization of endothelial-to-mesenchymal transition cells in pulmonary arterial hypertension. *Am. J. Physiol. Lung Cell Mol. Physiol.* 314, L118–L126. doi: 10.1152/ajplung.00296.2017

Tang, H., Babicheva, A., McDermott, K. M., Gu, Y., Ayon, R. J., Song, S., et al. (2018). Endothelial HIF-2alpha contributes to severe pulmonary hypertension due to endothelial-to-mesenchymal transition. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 314, L256–L275. doi: 10.1152/ajplung.00096.2017

Thupari, J. N., Kim, E. K., Moran, T. H., Ronnett, G. V., and Kuhajda, F. P. (2004). Chronic C75 treatment of diet-induced obese mice increases fat oxidation and reduces food intake to reduce adipose mass. *Am. J. Physiol. Endocrinol. Metab.* 287, E97–E104. doi: 10.1152/ajpendo.00261.2003

Toullec, A., Buard, V., Rannou, E., Tarlet, G., Guipaud, O., Robine, S., et al. (2018). HIF-1alpha deletion in the endothelium, but not in the epithelium, protects from radiation-induced enteritis. *Cell Mol. Gastroenterol. Hepatol.* 5, 15–30. doi: 10.1016/j.jcmgh.2017.08.001

Tsutsumi, T., Nagaoka, T., Yoshida, T., Wang, L., Kuriyama, S., Suzuki, Y., et al. (2019). Nintedanib ameliorates experimental pulmonary arterial hypertension via inhibition of endothelial mesenchymal transition and smooth muscle cell proliferation. *PLoS One* 14:e0214697. doi: 10.1371/journal.pone.0214697

Wang, S. H., Chang, J. S., Hsiao, J. R., Yen, Y. C., Jiang, S. S., Liu, S. H., et al. (2017). Tumour cell-derived WNT5B modulates in vitro lymphangiogenesis via induction of partial endothelial-mesenchymal transition of lymphatic endothelial cells. *Oncogene* 36, 1503–1515. doi: 10.1038/onc.2016.317

Wawro, M. E., Chojnacka, K., Wieczorek-Szukala, K., Sobierajska, K., and Niewiarowska, J. (2018). Invasive colon cancer cells induce transdifferentiation of endothelium to cancer-associated fibroblasts through microtubules enriched in tubulin-beta3. *Int. J. Mol. Sci.* 20:53. doi: 10.3390/ijms20010053

Welch-Reardon, K. M., Wu, N., and Hughes, C. C. (2015). A role for partial endothelial-mesenchymal transitions in angiogenesis? *Arterioscler. Thromb. Vasc. Biol.* 35, 303–308. doi: 10.1161/ATVBAHA.114.303220

Xiang, Y., Zhang, Y., Tang, Y., and Li, Q. (2017). MALAT1 modulates tgf-beta1-induced endothelial-to-mesenchymal transition through downregulation of miR-145. *Cell Physiol. Biochem.* 42, 357–372. doi: 10.1159/000477479

Xiao, L., and Dudley, A. C. (2017). Fine-tuning vascular fate during endothelial-mesenchymal transition. *J. Pathol.* 241, 25–35. doi: 10.1002/path.4814

Xiao, L., Kim, D. J., Davis, C. L., McCann, J. V., Dunleavey, J. M., Vanderlinden, A. K., et al. (2015). Tumor endothelial cells with distinct patterns of TGFbeta-driven endothelial-to-mesenchymal transition. *Cancer Res.* 75, 1244–1254. doi: 10.1158/0008-5472.CAN-14-1616

Xiong, J., Kawagishi, H., Yan, Y., Liu, J., Wells, Q. S., Edmunds, L. R., et al. (2018). A metabolic basis for endothelial-to-mesenchymal transition. *Mol. Cell* 69, 689–698. doi: 10.1016/j.molcel.2018.01.010

Xu, X., Tan, X., Tampe, B., Sanchez, E., Zeisberg, M., and Zeisberg, E. M. (2015). Snail is a direct target of hypoxia-inducible factor 1alpha (HIF1alpha) in hypoxia-induced endothelial to mesenchymal transition of human coronary endothelial cells. *J. Biol. Chem.* 290, 16653–16664. doi: 10.1074/jbc.M115.636944

Yamada, N. O., Heishima, K., Akao, Y., and Senda, T. (2019). Extracellular vesicles containing microRNA-92a-3p facilitate partial endothelial-mesenchymal transition and angiogenesis in endothelial cells. *Int. J. Mol. Sci.* 20:406. doi: 10.3390/ijms20184406

Zeisberg, E. M., Potenta, S., Xie, L., Zeisberg, M., and Kalluri, R. (2007). Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res.* 67, 10123–10128. doi: 10.1158/0008-5472.CAN-07-3127

Zhu, K., Pan, Q., Jia, L. Q., Dai, Z., Ke, A. W., Zeng, H. Y., et al. (2014). MiR-302c inhibits tumor growth of hepatocellular carcinoma by suppressing the endothelial-mesenchymal transition of endothelial cells. *Sci. Rep.* 4:5524. doi: 10.1038/srep05524

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 19 August 2020
doi: 10.3389/fcell.2020.00766

Tumor Endothelial Cells (TECs) as Potential Immune Directors of the Tumor Microenvironment – New Findings and Future Perspectives

*Laurenz Nagl[1], Lena Horvath[1], Andreas Pircher[1] and Dominik Wolf[1,2,3]**

[1] Department of Internal Medicine V (Haematology and Oncology), Medical University of Innsbruck, Innsbruck, Austria, [2] Tyrolean Cancer Research Institute (TKFI), Innsbruck, Austria, [3] Department of Oncology, Hematology, Rheumatology and Immunoncology, University Hospital Bonn (UKB), Bonn, Germany

OPEN ACCESS

Edited by:
Lucas Treps, VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Gabriele Multhoff, Technical University of Munich, Germany
Kyoko Hida, Hokkaido University, Japan

***Correspondence:**
Dominik Wolf
dominik.wolf@i-med.ac.at

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *11 May 2020*
Accepted: *21 July 2020*
Published: *19 August 2020*

Citation:
Nagl L, Horvath L, Pircher A and Wolf D (2020) Tumor Endothelial Cells (TECs) as Potential Immune Directors of the Tumor Microenvironment – New Findings and Future Perspectives. Front. Cell Dev. Biol. 8:766. doi: 10.3389/fcell.2020.00766

The tumor microenvironment (TME) plays a central role in cancer development and progression. It represents a complex network of cancer cell (sub-)clones and a variety of stromal cell types. Recently, new technology platforms shed light on the cellular composition of the TME at very high resolution and identified a complex landscape of multi-lineage immune cells (e.g., T and B lymphocytes, myeloid cells, and dendritic cells), cancer associated fibroblasts (CAF) and tumor endothelial cells (TECs). A growing body of evidence suggests that metabolically, genetically and on their transcriptomic profile TECs exhibit unique phenotypic and functional characteristics when compared to normal endothelial cells (NECs). Furthermore, the functional role of TECs is multifaceted as they are not only relevant for promoting tumor angiogenesis but have also evolved as key mediators of immune regulation in the TME. Regulatory mechanisms are complex and profoundly impact peripheral immune cell trafficking into the tumor compartment by acting as major gatekeepers of cellular transmigration. Moreover, TECs are associated with T cell priming, activation and proliferation by acting as antigen-presenting cells themselves. TECs are also essential for the formation of tertiary lymphoid structures (TLS) within the tumor, which have recently been associated with treatment response to checkpoint antibody therapy. Further essential characteristics of TECs compared to NECs are their high proliferative potential as well as greatly altered gene expression profile (e.g., upregulation of pro-angiogenic, extracellular matrix remodeling, and stemness genes), which results in enhanced secretion of immunomodulatory cytokines and altered cell-surface receptors [e.g., major histocompatibility complex (MHC) and immune checkpoints]. The TEC phenotype may be rooted in an aggressive tumor micro-milieu based on cellular stress *via* hypoxia and reactive oxygen species (ROS). *Vice versa* TECs might modulate TME immunogenicity thereby fostering cancer-associated immune suppression. This review aims to elucidate the currently emergent pathophysiological aspects of TECs with a particular focus

on their potential role as regulators of immune cell function in the TME. It is a main future challenge to deeply characterize the phenotypic and functional profile of TECs to illuminate their complex role within the TME. The ultimate goal is the identification of TEC-specific drug targets to improve cancer (immuno-)therapy.

Keywords: tumor microenvironment, tumor endothelial cells, immunoregulation, immunotherapy, antiangiogenic therapy

INTRODUCTION

Cancer cells are tightly embedded within the tumor microenvironment (TME) and are in constant interaction with surrounding stromal cells, encompassing multi-lineage immune cells such as antigen-presenting cells (APC), T and B lymphocytes and myeloid cells, cancer-associated fibroblasts (CAFs) and endothelial cells (ECs). The TME is a rapidly growing field of interest and recent technological advances like single cell RNA sequencing (scRNAseq) were able to elucidate the TME heterogeneity in high resolution, defining cellular sub-clusters of stromal cell types by their transcriptomic profile, and further functional investigations are ongoing (Lambrechts et al., 2018). The TME exerts a direct effect on cancer cells and impacts cancer development and progression (Goveia et al., 2020). From a clinical perspective, a better understanding of the structural and, more importantly, the functional characteristics of the TME is essential, as many novel therapy approaches target the TME as immunotherapies as well as anti-angiogenic therapies. A central therapeutic focus lies upon reversing the intrinsic cancer-associated immune escape, which involves numerous innate and adaptive immune responses as well as tumor neo-angiogenesis. The latter is regulated not only by the cancer cell itself, but also by the surrounding cellular components of the TME (Galon and Bruni, 2020).

Under physiological conditions, the critical functions of ECs in immune surveillance and angiogenesis are well known and specified (Ley et al., 2007; Potente et al., 2011). Contrarily, characterization of tumor endothelial cells (TECs) has long been lacking behind due to technical difficulties in TEC isolation and limited options to single cell profile cell populations in depth. These hurdles have been overcome based on novel cell isolation protocols from primary human material as well as the advent of novel technology platforms, such as scRNAseq analysis including bioinformatics (Lambrechts et al., 2018; Goveia et al., 2020). Notably, TECs have a markedly altered morphologic and genetic phenotype including structural chromosomal changes and mutations when compared to normal endothelial cells (NECs).They exhibit stem cell-like origin thus being key to orchestrate tumor neo-angiogenesis (St Croix et al., 2000; Maishi et al., 2019). TECs serve as major gatekeepers for TME infiltrating immune cells and are actively involved in priming, activation or down-regulation of effector immune cells, thereby directly impacting anti-cancer immune responses (Buckanovich et al., 2008; Kambayashi and Laufer, 2014; Goveia et al., 2020). TECs also form a barrier to immune-stimulatory cells promoting the loss of protective anti-cancer immunity, a process referred to as "endothelial anergy" (De Sanctis et al., 2018). Furthermore, EC subpopulations contribute to the formation of tertiary lymphoid structures (TLS), which are essential for mounting an effective anti-tumor immunity (Sautès-Fridman et al., 2019). Moreover, lymphatic endothelial cells (LECs) are associated with lymphatic metastasis and represent an additional target to anti-angiogenic therapies (Hsu et al., 2019). A growing body of evidence suggests that TECs may be involved in tumor progression and metastasis (Maishi et al., 2019) implicating their possible prognostic and predictive potential.

The knowledge on TEC phenotypes is rapidly growing, however, their functional role in cancer-associated immune escape including response to established immune and anti-angiogenic therapies still needs to be specified in detail. In this review we will discuss the function of TECs within the TME and the tumor vasculature, with a particular focus on their regulatory and immune-modulatory properties from a pathophysiological perspective. A better understanding of TEC-associated deregulation of molecular pathways and alterations will potentially help to find novel therapeutic targets to evade TEC-mediated cancer immune escape and to enhance efficacy of already established (immune-activating) drugs. Key characteristics and important molecular factors associated with TECs are summarized in **Tables 1**, **2**.

TUMOR VESSELS: EVOLUTION AND PHENOTYPE

Early on in tumorigenesis the sole diffusion of oxygen and nutrients from the surrounding tissue is not sufficient in supporting tumor cell growth. As cancer cells become hypoxic, they express angiogenic factors such as hypoxia inducible factor (HIF), vascular endothelial growth factor A (VEGFA), platelet-derived growth factor (PDGF) or angiopoietin 2 (ANGPT2) as well as proangiogenic chemokines and receptors to initiate neo-angiogenesis (a process termed "angiogenic switch") (Carmeliet and Jain, 2000; Potente et al., 2011). Angiogenesis is the process of vessel formation from pre-existing vascular beds and is primarily guided by the proliferation of ECs. Binding of VEGFA to VEGF receptor 2 (VEGFR2) on ECs triggers the evolution of highly invasive navigating tip ECs and proliferating stalk ECs that lead to the formation and elongation of novel vascular sprouts by following the VEGFA gradient (Potente et al., 2011; De Palma et al., 2017). Fully formed tumor vessels express an immature phenotype, consisting of an instable vessel wall (inconsistent coverage of smooth muscle and pericytes), a discontinuous basement membrane and an irregular endothelial lining. The vessel architecture itself is

TABLE 1 | Main characteristics of tumor endothelial cells (TEC) and tumor vessels.

Tumor vessels morphology	Tumor vessels represent with excessively branched and disorganized architecture. They are morphologically characterized by an irregular multi-layered endothelial lining, a discontinuous basement membrane and an inconsistent smooth muscle and pericyte sheath. These features result in an instable vessel wall promoting leakiness (fluid extravasation into the TME) and consequently higher interstitial pressures ultimately fostering tissue hypoxia and neo-angiogenesis (Klein, 2018; Tilki et al., 2007).
TEC morphology	TEC present with irregular cell surfaces and fenestrated cell walls. The intercellular junctions to neighboring cells, e.g., pericytes are loose and inconsistent (Morikawa et al., 2002). They may form multiple layered endothelia as compared to single layered NEC (Aird, 2012).
Metabolism	TEC are highly transcriptionally active and express higher RNA content compared to NEC (up to fourfold higher) (Lambrechts et al., 2018). Myc-target synthesis is highly enriched (Lin et al., 2012). TEC are hyper-glycolytic and largely use aerobic glycolysis for supplying their energy demands. Compared to NECS, they depict increased potential of self-renewal and are highly proliferative (De Palma et al., 2017).
Cytogenetics	TEC are characterized by chromosomal instability and abnormality, including aneuploic karyotypes, deletions, translocations or supernumerary centrosomes. These alterations might result in response to TME hypoxia and redox alterations, as well as epigenetic factors (e.g., methylations) (Hida et al., 2004; Deeb et al., 2011).
Molecular genetics	TEC show high intra-tumoral heterogeneity (St Croix et al., 2000) in terms of their gene expression profiles. Based on scRNAseq data, numerous TEC subtypes can be distinguished, such as tip and stalk EC (playing a major role in neo-angiogenesis) or postcapillary venous and activated postcapillary ECs (displaying immunoregulatory functions) (Lambrechts et al., 2018; Goveia et al., 2020). TEC are less responsive to pro-inflammatory stimulation (EC anergy). They can actively down regulate genes involved in immune cell homing (e.g., ICAM1 and VCAM1), chemostaxis (e.g., CXCL10 and CXCL11) as well as antigen presentation (e.g., HLA-DQB1 and HLA-DQA1) (Goveia et al., 2020). Furthermore, TEC may upregulate PD-L1 and PD-L2 to inhibit T cell activation via inhibitory immune checkpoints (Georganaki et al., 2018), express IDO 1 to promote local immunosuppression (Georganaki et al., 2020) and T cell apoptosis and upregulate Fas ligand expression inducing CD8+ T cell apoptosis (Motz et al., 2014).

TME, tumor microenvironment; NEC, normal endothelial cell; scRNAseq, single cell RNA sequencing; PD-L1/2, programmed death receptor ligand 1/2; IDO 1, indoleamine-pyrrole 2,3-dioxygenase.

excessively branched, chaotic and leaky (Tilki et al., 2007; Klein, 2018). These structural aberrations alter physiologic vessel functionality. Leakiness results in high intra-tumoral interstitial pressure (ITP) leading to poor perfusion and inconsistent blood flow in certain regions of the tumor,

TABLE 2 | Factors associated with tumor endothelial cells (TEC) and tumor vessels.

Molecule	Function
Growth factors	
VEGF superfamily	central angiogenic factors of tumor angiogenesis (Carmeliet and Jain, 2000; Potente et al., 2011). Induce genetic reprogramming of TECs, changes mode of immune cell interaction and adversely programs the TME (Albini et al., 2018; Maishi et al., 2019). Inhibits the up-regulation of leukocyte adhesion molecules (Griffioen et al., 1999; Dirkx et al., 2003; Flati et al., 2006).
VEGF-C	Central factor of lymphangiognesis (Hsu et al., 2019). Essential for HEV formation (Furtado et al., 2007). Overexpression fosters lymphatic metastasis (Decio et al., 2014; Wang et al., 2014; Yang et al., 2014; Goussia et al., 2018). Activated LECs by VEGF-C suppress tumor specific CD8+ T cells (Lund et al., 2012).
PDGF, HIF1, ANGPT2,	Central angiogenic factors of tumor angiogenesis (Carmeliet and Jain, 2000; Potente et al., 2011).
FGF2	Inhibits the up-regulation of adhesion molecules (ICAM and VCAM) (Griffioen et al., 1999; Dirkx et al., 2003; Flati et al., 2006).
bFGF	Angiocrine factor promoting tumor cell metastasis (Maishi et al., 2019).
EGFL7	Down-regulates adhesion molecules expression and fosters tumor vessel development via NOTCH signaling (Delfortrie et al., 2011; Nichol and Stuhlmann, 2012).
TGFβ	Angiocrine factor contributing to tumor metastasis (Maishi et al., 2019).
Cytokines, interleukines, interferones, chemokines	
TNFα	EC activation relies on TNFα and endothelial anergy is characterized by unresponsiveness to TNFα (De Sanctis et al., 2018; Georganaki et al., 2018). Enhances PD-L1 up-regulation on ECs (Georganaki et al., 2018)
IFNγ	Endothelial anergy is characterized by unresponsiveness to IFNγ (De Sanctis et al., 2018). Enhances PD-L1 upregulation on ECs (Georganaki et al., 2018).
IL-6, IL-8	Secreted by activated ECs mediating immune cell trafficking (Aird, 2012; Klein, 2018).
CXCL12/13, CCL19/21, IL-7	Chemokines/interleukines that are associated with the formation of HEVs and TLS (Sautès-Fridman et al., 2019).
CCL2/18, CXCL10/11	Attractant chemokines down-regulated by TECs to prevent immune cell trafficking, therefore mediating immune cell anergy (Griffioen et al., 1996; Huang et al., 2015; Lambrechts et al., 2018).
CXCL4	Chemokine strongly involved in leukocyte activation and endothelial rolling (Ley et al., 2007).
Adhesion molecules	
VCAM1, ICAM1 PECAM1, E/P-selectin	Expressed on ECs mediating leukocyte recruitments (Ley et al., 2007). Important gatekeepers of transmigration to the tumor compartment (De Sanctis et al., 2018). Multiple regulation mechanisms by tumor cells/TECs.
Soluble adhesion molecules	sCD146 and Endoglin inhibit TIL recruitment by competitive mechanisms and show direct VEGF-synergistic effects (Rossi et al., 2013; Stalin et al., 2016).
Stemness factors, apoptosis-inducing/preventing molecules proteins	
FASL	Expressed by TECs induces apoptosis in $CD8^+$ T cells (Thommen and Schumacher, 2018).
Stemness molecules	TECs upregulate stem cell associated proteins (CD90, MDR1, ALP, Oct-4, and ALDH), which are associated with increased self renewal and high proliferative potential (Ohga et al., 2012; Ohmura-Kakutani et al., 2014; Hida et al., 2017)

(Continued)

TABLE 2 | Continued

Molecule	Function
Immune checkpoints	
PD-L1/2, TIM3	Expressed by TECs and promotes T cell arrest (Georganaki et al., 2018).
IDO1	Immune-regulatory molecule expressed by TECs in response to IFNγ restricting activation and inducing apoptosis of T cells (Georganaki et al., 2020).
Antigen-presenting molecules	
MHC I, II	Frequently expressed by ECs but lacking the co-stimulatory molecules (CD80 and CD86) (Shiao et al., 2007). MHC molecules can be down-regulated by TECs contributing to immune-evasion (Goveia et al., 2020).
Other	
NO	Affects leukocyte recruitment under malignant conditions (De Caterina et al., 1995). Directly suppresses effector T cells (De Sanctis et al., 2018). NO-antagonists restores T cell adhesion by up-regulation of adhesion molecules (Bouzin et al., 2007; Buckanovich et al., 2008).
ET1	Associated with ICAM1 expression and decreases TIL influx (Spinella et al., 2002; Buckanovich et al., 2008).
STING	Highly expressed on ECs of HEVs and impacts the expression of adhesion molecules (Demaria et al., 2015; Yang et al., 2019).
CLEVER1	Favors the selective influx of Treg and immuno-suppressive TAM (Nummer et al., 2007; Karikoski et al., 2014).
Biglycan	Preferentially expressed by high metastatic tumors and allows toll-like receptor expressing tumor cells to metastasize hematogenously (Maishi et al., 2016).
Galectins	Glycan-binding endogenous lectins which sustain tumor angiogenesis via autocrine and paracrine signaling (Elola et al., 2018). Directly interact with VEGFR2, bFGF, VEGFR3 and associated with resistance to anti-angiogenic therapies (Markowska et al., 2010; Markowska et al., 2011; Zhao et al., 2011; Laderach et al., 2013; Chen et al., 2016).
Jag1	Expressed by TECs to generate malignant vascular niche, which is associated with aggressive course and resistance to chemotherapy (Cao et al., 2016).
Slit2	Tumor-suppressive factor directly down-regulated by TECs via paracrine EphA2 signaling (Brantley-Sieders et al., 2011).

generating hypoxia and a shift to anaerobic glycolysis and acidosis (Cantelmo et al., 2016). This phenotype contributes to a pro-tumorigenic and immunosuppressive TME (Potente et al., 2011; Schaaf et al., 2018).

Endothelial Cells: Structure, Function, and Autophagy

Endothelial cells are a highly heterogenous cell population, showing structural differences (e.g., direction of cell alignment within the epithelium, type of intercellular junctions, fenestrated vs. non-fenestrated) as well as a great genetic variability (Aird, 2012). They play a critical role in numerous physiological processes, including vascular stabilization and tonus control, hemostasis, angiogenesis as well as regulation of immune cell trafficking between blood and tissues, thus they are key in maintaining vessel homeostasis (Aird, 2012). In a healthy state ECs are typically found in a non-proliferating form (quiescence). However, they become activated on environmental stressors, such as pro-angiogenic signals and hypoxia to initiate angiogenesis, or inflammatory triggers (e.g., TNF and interleukins) to enhance immune cell trafficking. Activated ECs are able to up-regulate the expression of cell surface antigens (e.g., HLA molecules), adhesion molecules (selectins and integrins) and cytokines (e.g., IL-6 and IL-8) as well as to exhibit pro-thrombotic functions, ultimately resulting in a pro-immunogenic phenotype (Aird, 2012; Klein, 2018). Importantly, activated ECs have the ability to return quiescent once the activated stressor is removed. This functional plasticity is a key physiologic characteristic of NEC and is, at least partially, regulated by EC-intrinsic autophagy.

Autophagy comprises the mechanism of lysosomal degradation of potentially toxic cytoplasmatic compounds and is a major physiologic cytoprotective and pro-homeostatic cellular pathway. The molecular mechanisms of how autophagy mediates EC reactiveness and quiescence are complex, however, redox-homeostasis is a key determinant (Schaaf et al., 2019). In healthy and pre-malignant cells, autophagy depicts various onco-suppressive functions (reviewed in Rybstein et al., 2018), however, in cancer cells autophagy seems to be carcinogenic and tumor growth promoting, especially in hypoxic und nutrient-deprived areas of the TME (Schaaf et al., 2019). The functional mechanisms of autophagy in the tumor stromal cells, especially in TECs have not been clarified so far.

Tumor Endothelial Cells

Tumor endothelial cells show distinct phenotypic differences on the molecular, structural, and functional level when compared to NECs (**Table 1**). Morphologically they present with irregular surfaces, excessively fenestrated cell walls and loose intercellular junctions to neighboring cells [e.g., pericytes (Morikawa et al., 2002)]. They may arrange in multiple layers (as opposed to single layer endothelial sheets in health vessels) which impedes proper function and promotes leakiness. On a molecular level, TECs are transcriptionally highly active, mirrored by an up to fourfold increase in RNA content when compared to NECs, potentially due to the high metabolic demands of nucleotide biosynthesis or glycolysis. Moreover, Myc-target synthesis is highly enriched in TECs, which is a well-recognized transcriptional factor associated with tumor-aggressiveness and known to impact angiogenesis (Lin et al., 2012; Lambrechts et al., 2018).

Tumor endothelial cells depict a wide inter- and intra-tumoral heterogeneity and already around 20 years ago TEC specific genes, called tumor endothelial markers (TEMs), were identified with, however, unclear function (St Croix et al., 2000). Only recent technological advances in RNA profiling by applying single cell transcriptome sequencing analysis (sc-RNA-seq) have allowed to characterize small cellular subpopulations of TECs and to provide functional information of the detected gene expression profiles. Lately, a single cell atlas of human NSCLC samples including NEC and TEC demonstrated the high phenotypic and functional heterogeneity of blood vascular ECs, distinguishing distinct subgroups of arterial, capillary and tip ECs (Goveia et al., 2020).

Moreover, Sievert et al. (2014) could not only show differences in the expression of surface markers when analyzing primary ECs originating from different organs, but also demonstrated a detailed analysis of phenotypic and functional differences

between TECs and NECs. Interestingly, TECs were of larger size, could spread faster in a more chaotic architecture and were less able to adapt to blood flow conditions. In addition, tubular structure formed by TECs showed significantly less branching and looping compared to NECs as well as they up-regulated the stem cell marker CD34 as well as the angiogenesis promoting markers CD61 (Integrin β3) and CD105 (Endoglin) (Sievert et al., 2014).

Neo-angiogenesis within the tumor stroma is also known to be orchestrated tip cells. They are known to exhibit discrete marker genes, e.g., CXCR4, PGF, or ANGPT2 and on a functional level these signatures have been associated with EC migration, matrix remodeling and VEGF signaling. Therefore, tip cells have long been in the spotlight of traditional antiangiogenic therapies that mainly target VEGF. Stalk ECs have been shown to express genes involved in vessel maturation and integrity as well as DII4-Notch signaling (Blanco and Gerhardt, 2013; Goveia et al., 2020).

TECs Are Highly Proliferative, Exhibit Abnormal Gene Expression Profiles Suggesting a Distinct Stem-Like Origin

Next to describing vessel structures and phenotypes, the characterization of possible TEC-specific genetic signatures is of particular interest. Growing evidence from both human and murine experiments show that ECs of malignant tumors prominently differ from NECs, exhibiting abnormal gene expression profiles and chromosomal instability (Akino et al., 2009; Schaaf et al., 2018). For instance, TECs display cytogenetic abnormalities such as larger nuclei and aneuploidic karyotypes as well as karyotypic abnormalities including deletions, translocations and abnormal centrosomes (Hida et al., 2004). Moreover, epigenetic alterations of TECs have been proposed. In prostate cancer Deeb et al. (2011) showed a different methylation of the gene promoter CYP24A1 in the endothelium of prostate cancer and the surrounding benign tissue, indicating an epigenetic influence on the gene expression profile of TECs. Also, Luo et al. (2013) found marked differences in the transcriptome and methylome of ECs in malignant and benign prostate tissue. Furthermore, TECs are highly proliferative in cell culture, suggesting a lack of normal mitosis inhibiting cell cycle proteins (Hida et al., 2004). All these aberrations may be rooted in the aggressive composition of the TME as it seems evident that hypoxia and reactive oxygen species (ROS) induce genetic and chromosomal instability *via* a higher mutational frequency (Kondoh et al., 2013; Hojo et al., 2017). On the other hand, pro-angiogenic factors such as VEGF can induce genetic reprogramming of TECs and their mode of interacting with immune cells (Albini et al., 2018; Maishi et al., 2019). In particular, up-regulation of angiogenic receptors as well as the close interaction with tumor cells and pro-inflammatory immune cells results in an inflamed and activated TEC state, inducing a highly proliferative phenotype with increased tendency for migration (Matsuda et al., 2010; Ohga et al., 2012).

Moreover, we want to discuss the diverse origins of TECs as a series of recent publications by Lambrechts et al. (2018); Goveia et al. (2020), and Rohlenova et al. (2020) using scRNAseq contributed to elucidate the heterogeneous origin of TECs and their corresponding untransformed EC types. These studies demonstrated that especially those NECs/TECs with capillary and venous genetic phenotype are relevant in active immune surveillance and distinguished the genetically differing subgroups of postcapillary venous ECs and activated postcapillary ECs. The postcapillary EC subgroup was mainly found within NECs and showed gene expression involved in leukocyte recruitment and tissue perfusion. Contrarily, the activated postcapillary vein EC phenotype was almost exclusively detected within the TEC population and exhibited up-regulation of immunomodulatory genes and ribosomal proteins, which have previously been described as characteristic of high endothelial venules (HEV) in inflammatory tissues. Intriguingly, VEGF blockade increased the presence of activated postcapillary venular ECs and was associated with molecular vessel normalization. Another study showed that VEGF could suppress leukocyte migration by inhibiting EC activation (*via* interference with NF-κB pathway components) or by modulating EC expression of several immunomodulatory genes, such as T cell attracting-chemokines (CXCL10 and CXCL11) (Huang et al., 2015). These data underline the immunomodulatory functions of VEGF and that targeting the VEGF-R/VEGF pathway not only impedes angiogenesis but also increases EC activation and function. Recently, by using a scRNAseq based dataset Ma et al. distinguished intra-tumoral transcriptomic heterogeneity as an unfavorable prognostic factor in primary liver cancer. In the context of tumor angiogenesis they found that VEGF is able to adversely reprogram the TME and that T cells from more heterogenous tumors had lower cytolytic activities (Ma et al., 2019).

Furthermore, it is controversially discussed if stem cell-like ECs, also called endothelial progenitor cells [EPCs, characterized by Asahara et al. (1997)] as physiological origin of ECs, contribute to tumor-vasculogenesis as well. On the other hand, there are hints that cancer cells themselves can also *trans*-differentiate into endothelial-like cells and thereby generate "their own" vessel structures. Strikingly, it was found in glioblastoma and lymphoma that TECs and cancer cells share common cytogenetic and genomic alterations (Streubel et al., 2004; Wang et al., 2010). It has further been reported that cancer cells can form pericytes (Cheng et al., 2013) and that other cell types of the TME, such as dendritic cells (DCs) and monocytes, possess the ability to differentiate into ECs (Fernandez Pujol et al., 2001; Chen et al., 2009). Moreover, TECs are able to up-regulate stem cell-associated proteins like CD90, Sca-1, MDR1 ALP, Oct-4 and high ALDH acitivity (Ohga et al., 2012; Ohmura-Kakutani et al., 2014; Hida et al., 2017) and to form stem cell-like clusters allowing increased self-renewal and high proliferative potential, which has already been described for normal ECs (Matsuda et al., 2010; Ohga et al., 2012). Importantly, TECs of highly metastatic tumors exhibit greater genetic heterogeneity (Ohga et al., 2012; Ma et al., 2019).

Hypoxia-induced signaling cascades do not only contribute to the specific TEC phenotype such as irregular cell surfaces, fenestrated cell walls and loose intercellular junctions promoting

leakiness (Lin et al., 2012; Morikawa et al., 2002), but also take influence on many cellular functions of TECs, thus playing an important role in tumor development and progression. Firstly, it has been shown that ROS directly influence cellular and chromosomal instability (Kondoh et al., 2013) and that a hypoxic microenvironment is a key driver of the morphological and molecular profile of TECs (Albini et al., 2018; Maishi et al., 2019). For instance, single cell transcriptomic analyses of murine TECs revealed profoundly altered gene expression profiles under the influence of anti-angiogenic treatment, which again underlines endothelial heterogeneity (Zhao et al., 2018). Moreover, a hypoxic TME markedly enhances inflammation in TECs. Interestingly, Tellier et al. (2015) showed both *in vitro* and *in vivo* that ECs exposed to hypoxia expressed tumor-promoting pro-inflammatory cytokines such as IL-6, IL-8, CXCL1, and increased ICAM1. Likewise, hypoxia deeply influences immune responses as a hypoxic microenvironment promotes immune tolerance and angiogenesis *via* promotion of regulatory T cells (Treg) (Facciabene et al., 2011) as well as by repressing T effector cell functions (Tian et al., 2017). Furthermore, the distinct stem-like origin of TECs also depends on hypoxia. For example, Rocha et al. (2018) demonstrated that pharmacological blockade of hypoxia-associated changes prevents the differentiation of glioblastoma stem-like cells into TECs. Importantly, a hypoxic TME also impacts the regulation of angiogenesis. In this regard it has been shown that angiogenetic feedback loops by soluble VEGFR are compromised in colon cancer-derived TECs, indicating a pro-angiogenetic disposition of TECs (Jayasinghe et al., 2009). Lastly, a hypoxic micro-milieu affects protein glycosylation and may therefore impact immune cell migration to the tumor and signaling cascades within the TME (Chandler et al., 2019).

These different origins and stem cell populations of TECs and the impact of hypoxia on these cell populations may at least in part explain their striking heterogeneity and are associated with drug resistance and worse clinical outcome (Naito et al., 2016; Hida et al., 2017). Thus, TECs are certainly not normal EC, but are yet themselves abnormal in terms of their cytogenetics, genetic expression profile, methylation patterns and their functional behavior. Thus, TECs play a major role in driving the TME to be highly tumor-permissive.

TECs AND THEIR IMMUNOREGULATORY PROPERTIES

Tumor endothelial cells are strategically positioned at the TME-vessel interface and represent the first-line encounter for circulating immune cells and the tumor stroma (Lambrechts et al., 2018). TECs are known to impact TME immunogenicity by actively guiding adhesion, rolling and extravasation of circulating immune cells into the tumor stroma, but also by fulfilling immune regulatory properties themselves, such as T cell priming and activation as well as exerting antigen presenting functions (Kambayashi and Laufer, 2014; Georganaki et al., 2018). The functional immunomodulatory characteristics will be discussed in the following (**Figure 1**).

Immune Cell Transmigration: Attraction, Adhesion, and Perfusion

In healthy as in cancerous sites, the process of immune cell extravasation and tissue infiltration involves a complex process of multi-staged adhesion and transcellular migration and is greatly mediated by activated venous ECs.

The leukocyte adhesion cascade includes the three main steps of selectin-mediated rolling, chemokine-mediated activation (e.g., CXCL4) and integrin-mediated arrest of leukocytes. ECs play a major role in leucokyte adhesion by expressing selectins (e.g., E-selectin and P-selectin), integrin ligands (e.g., VCAM1 and ICAM1) and adhesion molecules (e.g., glycosaminoglycans). Following adhesion, leukocyte transendothelial migration may be paracellular or transcellular. The more frequent paracellular form of migration involves the adhesion molecules PECAM1 (also known as CD31) and CD99. Transcellular migration mainly takes place in areas of thin endothelial linings (e.g., central nervous system) and involves vesiculo-vacuolar organelles (VVO) that form intracellular channels for leucokyte migration. As a last step, leukocytes pass the endothelial basement membrane and pericyte sheath, mostly in areas of low protein deposition and through pericyte gaps (Ley et al., 2007). Importantly, only activated ECs can initiate leukocyte migration and EC activation itself greatly relies upon stimulation by TNFα, mainly *via* activation of the NF-κB pathway (Huang et al., 2015). Quiescent ECs do generally not interfere with leukocytes by suppressing transcription of adhesion molecules (e.g., ICAM1, VCAM1, and E-selectin) (Pober and Sessa, 2007).

Tumor Endothelial Anergy

Inhibiting leukocyte migration into the TME at the endothelial level depicts one major way of cancer cells to escape anti-tumor host immune responses. Endothelial anergy describes the unresponsiveness of TECs to pro-inflammatory stimulation (TNFα, IFNγ, and IL-1[10]), which greatly impedes the adhesion and migration of immune cells. Accompanying the anergetic state, TECs can actively downregulate the expression of adhesion molecules (e.g., ICAM1, VCAM1, and E-selectin) or chemokines (e.g., CCL2, CCL18, CXCL10, and CXCL11) themselves (Griffioen et al., 1996; Lambrechts et al., 2018), thus reducing immune cell trafficking. Moreover, TECs select specific subsets of immune cells to infiltrate the tumor tissue, favoring Treg and macrophage precursors (MDSC), but inhibiting $CD4^+$ and $CD8^+$ effector T cells, DCs, natural killer (NK) cells and neutrophil granulocytes (De Sanctis et al., 2018). This consequently promotes an immunosuppressive tumor milieu, ultimately giving rise to tumor progression (Zhang et al., 2003; Bouma-ter Steege et al., 2004).

Tumors can upregulate several cytokines and chemokines in order to promote endothelial anergy. Most prominent, members of the VEGF family and fibroblast growth factor 2 (FGF2) inhibit the upregulation of cell adhesion molecules on TECs (e.g., ICAM1, VCAM1, and selectins) under inflammatory stimulation (e.g., TNFα) (Griffioen et al., 1999; Dirkx et al., 2003; Flati et al., 2006). Additionally, EGF-like domain-containing protein 7 (EGFL7) downregulates adhesion molecule expression and

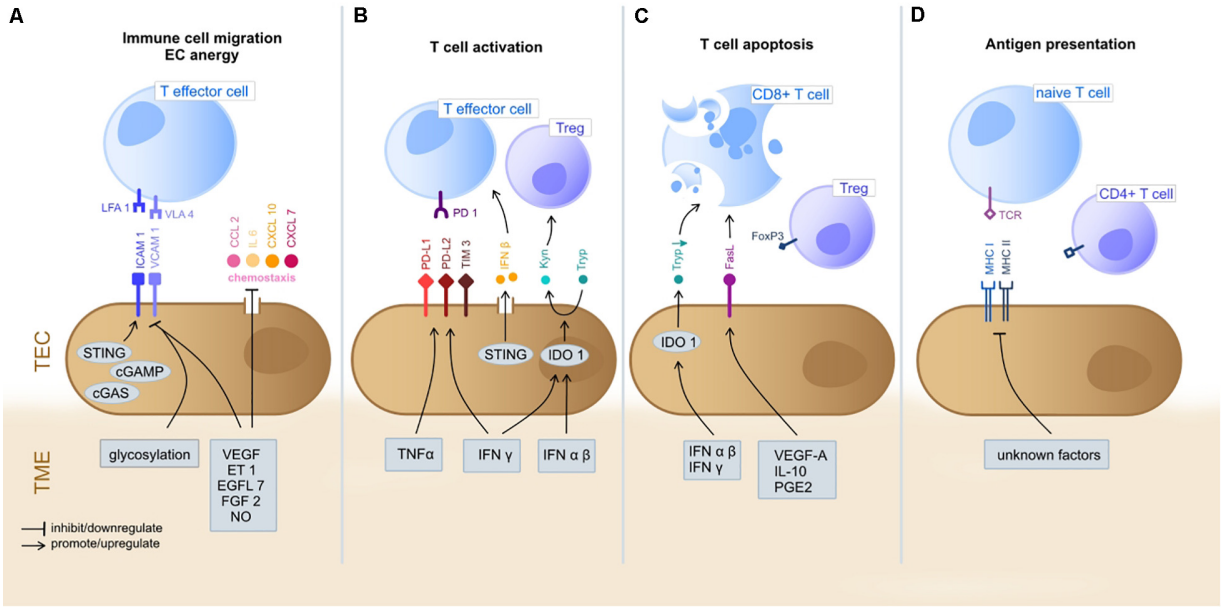


FIGURE 1 | Immunoregulatory functions of TEC in the TME. The gray boxes indicate TME-derived factors that inhibit/promote expression of mediators by TEC. **(A)** T cell extravasation into the TME starts with a multi-staged adhesion process and includes binding of integrins LFA1 and VLA4 on T cells to the respective ligands ICAM1 and VCAM1 on TEC (Ley et al., 2007; Georganaki et al., 2018). TEC can actively downregulate gene expression of adhesion molecules (e.g., ICAM1 and VCAM1) or chemostaxis themselves in order to control immune cell infiltration (Griffioen et al., 1996; Lambrechts et al., 2018). TME deriving cytokines (e.g., VEGF, ET1, EGFL7, and FGF2) inhibit TEC to upregulate expression of adhesion molecules and chemoattractants (e.g., CCL2, IL6, CXCL10, and CXCL7) (Flati et al., 2006; Buckanovich et al., 2008; Delfortrie et al., 2011). Also, NO has been shown to inhibit adhesion molecule and cytokine (IL6, IL8; not shown) expression (De Caterina et al., 1995). The intracellular cGAS-cGAMP-STING pathway is known to enhance adhesion molecule and promote T cell infiltration (Demaria et al., 2015). The glycosylation of surface molecules (referred to as glycocalyx) modulates the adhesive properties of TEC and can either enhance or reduce immune cell migration (Chandler et al., 2019). **(B)** T cell activation starts at the TEC/TME interface. Binding of inhibitory immune checkpoints (e.g., PD-1) on CD8+ cells with their ligands (e.g., PD-L1 and PD-L2) on TEC inhibits T cell activation and these ligands can be upregulated by TEC on proinflammatory TME derived factors (IFNγ and TNFα) (Georganaki et al., 2018). STING (see above) acts T cell activating via IFNβ secretion (Demaria et al., 2015). The immunosuppressive cytosolic protein IDO1 promotes the metabolism of Tryp to Kyn. Kyn downstream metabolites promote Treg activation, whilst Tryp depletion promotes T cell apoptosis and inhibits T cell proliferation. TME derived type I IFN (alpha/beta) upregulate IDO1 expression by TEC (Munn and Mellor, 2016; Georganaki et al., 2020). **(C)** T cell apoptosis can be triggered by IDO1 depended depletion of Tryp (Georganaki et al., 2020). Moreover, the molecule FasL expressed by TEC promotes CD8+ T cell apoptosis whilst sparing Treg (due to expression of FoxP3) and is FasL expression is upregulated in response to TME derived VEGF-A, IL-10 and PGE2 (Motz et al., 2014). TEC, tumor endothelial cells; TME, tumor microenvironment; LFA1, lymphocyte function-associated antigen 1; VLA4, very late antigen-4; ICAM1, Intercellular Adhesion Molecule 1; VCAM1, Vascular cell adhesion protein 1; VEGF, Vascular Endothelial Growth Factor; ET-1, Endothelin-1; EGF-like domain-containing protein 7; FGF2, Fibroblast Growth Factor 2; NO, nitric oxide; PD-1, programd death receptor 1; PD-L1/2, programed death receptor ligand 1/2; Tryp, tryptophan; Kyn, kynurenine; IFN, interferon; IDO1, indoleamine 2,3-dioxygenase 1; MHC, major histocompatibility complex; FasL, Fas ligand. **(D)** TEC can present processed antigens to T cells via MHC I and MHC II molecules, yet they are lacking the co-stimulatory markers CD80 and CD86 required for naïve T cell activation, distinguishing them from professional APC (Kambayashi and Laufer, 2014). Contrarily, CD4+ T cells are not dependent on co-stimulation at may directly be activated by TEC (Shiao et al., 2007). TEC can actively downregulate HLA genes as immune evading strategy (Goveia et al., 2020), yet external TME-derived factors have not been specified yet.

directly fosters tumor vessel development *via* NOTCH signaling (Delfortrie et al., 2011; Nichol and Stuhlmann, 2012). Recently it has been shown that nitric oxide (NO), a known physiological factor of endothelial relaxation, seems to essentially regulate blood flow within tumors (Fukumura et al., 2006). NO also affects leukocyte recruitment by preventing rolling and adhesion of immune cells under malignant conditions (De Caterina et al., 1995) and has been reported to directly suppress effector T cells (De Sanctis et al., 2018). The vasoconstrictive peptide Endothelin 1 (ET1), known for its direct effect on angiogenesis *via* VEGF and HIF1 (Spinella et al., 2002), is also associated with ICAM1 expression and the decreased presence of tumor infiltrating leukocytes (TIL) (Buckanovich et al., 2008). Another mechanism of endothelial anergy includes the secretion of soluble adhesion molecules by tumor cells (MCAM/sCD146 and Endoglin) that indirectly inhibit TIL recruitment by competing with EC bound-receptors (Rossi et al., 2013; Stalin et al., 2016) and by direct VEGF-synergistic interactions with tumor angiogenesis (Zheng et al., 2009). Furthermore, TECs can upregulate the expression of specific adhesion markers in order to direct immune cells. In this regard the common lymphatic endothelial and vascular endothelial receptor (CLEVER 1) has been identified as immunosuppressive molecule, favoring the influx of Treg as well as tumor associated macrophages (TAM) (Nummer et al., 2007; Karikoski et al., 2014).

Of note, endothelial anergy is reversible and may be used as a therapeutic strategy. Pre-clinical data suggests that blocking the before mentioned mechanisms in TECs favors the influx of immune cells (Dirkx et al., 2006; Karikoski et al., 2014; Facciabene et al., 2017). Reports from clinical data suggest that endothelial

anergy is associated with inferior outcome (Takahashi et al., 2001; Wu et al., 2009; Huang et al., 2010) and that anti-VEGF therapies may contribute to reprogramming of the TME (Griffioen et al., 1999; Huang et al., 2015). The immune modulatory effects of anti-angiogenic therapies and immunotherapies such as immune checkpoint inhibitors (ICI) thus applies a dual targeting concept, allowing a more effective reinvigoration of the anti-cancer immune response. Endothelial cell anergy complements the concept of immune cell anergy, which is defined as the failure of immune cells to create an effective response against cancer cells (Thommen and Schumacher, 2018). Targeting both mechanisms thus may be additive or even synergistic in case the most appropriate targets are well defined.

TEC Affects T Cell Priming and Migration

Next to altering leukocyte extravasation, TECs can actively impact T lymphocyte priming and migration (**Figures 1A,B**). This fact is especially of clinical interest, as many novel therapeutic strategies include immune checkpoint inhibitors (ICI) up-front and thereby target effector T cells in the TME.

The role of TECs in T cell activation is two-sided as both stimulatory and inhibitory mechanisms have been identified. As for the latter, TECs can up-regulate a variety of inhibitory molecules in order to promote T cell arrest. One important mechanism involves immune checkpoint molecules (IC) that get expressed by various cells of the TME (e.g., cancer cells, immune cells, and stromal cells). The most (clinically) important immunoinhibitory IC involves programmed death receptor (PD-1) which is found on $CD8^+$ T cell surfaces. Binding of PD-1 with its ligands (PD-L1 and PD-L2) down-regulates T cell activation. Next to cancer cells, TECs have been shown to express PD-L1 and PD-L2 themselves, as well as other known inhibitory ICs, such as T-cell immunoglobulin domain and mucin domain (TIM-3), thereby having the potential to directly inhibit T cell activation at the vessel site. It has been shown that pro-inflammatory cytokines including IFNγ and TNFα enhance PD-L1 up-regulation on ECs. PD-L1 expression by cancer cells is currently the most frequently used biomarker in immune-oncology guiding treatment decisions and patient stratification, However, the therapeutic impact of PD-L1 expression by TECs has not yet been specified (Georganaki et al., 2018).

Another T cell inhibitory mechanism of TECs involves Fas ligand (FasL), a homeostatic mediator of T cell apoptosis. An interesting preclinical study found that TECs expressing FasL were able to effectively diminish $CD8^+$ T cells in tumors while sparing Treg cells. FasL expression was induced by tumor-derived VEGF-A, IL-10 and prostaglandin E2 and pharmacological inhibition of these factors resulted in lower FasL expression and consequently higher $CD8^+$ T cell infiltration by preventing effector T cell apoptosis (Motz et al., 2014) (**Figure 1C**).

Furthermore, the enzyme IDO1 is known for its immunosuppressive functions by restricting T cell proliferation and inducing T cell apoptosis as well as promoting Treg activation *via* its key role in tryptophan metabolism. Next to other cells of the TME (e.g., cancer and immune cells), TECs can express and up-regulate IDO1 in response to type I IFN (αβ) and type II IFN (γ) stimulation in the TME, which has been associated with impeded T cell activation, thus promoting an immunosuppressive microenvironment. IDO inhibition has been shown to enhance efficacy of ICI and trials to establish the clinical use are ongoing (Georganaki et al., 2020).

As to T cell promoting mechanisms, the intracellular STING (the stimulator of IFN genes) signaling represents an important innate immune pathway in healthy and cancerous tissues also promoting $CD8^+$ T cell activation *via* expression of type I IFN (especially IFN-β). STING gets ubiquitously expressed by various cells of the TME, and especially high expression has been documented for ECs of high endothelial venules (HEV). STING activation leads to $CD8^+$ T cell tumor infiltration and has also been shown to enhance the upregulation of adhesion molecules (e.g., ICAM1 and VCAM1) on ECs, alterations that altogether promote anti-cancer immune responses. Moreover, there is evidence that STING activation is involved in tumor vessel maturation and inhibition of vessel sprouting. Furthermore, it could be shown that the cGAMP-induced antitumor activity was found to be mediated by the STING-driven induction of IFN-β in the TME. Interestingly, TECs were the main producer of IFN-β in response to cGAMP injection in both mouse and human (Demaria et al., 2015). Therefore, STING agonists are interesting modulators of the TME and not only affecting immune cells, as mostly appreciated, and therapeutic development is already ongoing in the clinical setting (Yang et al., 2019).

Next to TECs, LECs of extra-tumoral lymph nodes are known to chemo-attract and cross-prime naïve $CD8^+$ T cells by acting as semi-professional APCs (see below). Recently, LECs have been shown to generate antigen-experienced T cells with memory-like function that can rapidly evolve effector functions upon pro-inflammatory stimulation. These results give first insights into the functional consequence of LEC-associated T cell priming (Vokali et al., 2020).

TEC as Antigen Presenting Cells

It is well established that ECs from non-cancerous vasculature can express MHC class I and II molecules enabling them to present processed antigens to T cells (**Figure 1D**). Yet, there is currently no evidence that ECs can express the co-stimulatory markers CD80 and CD86 required for naïve T cell activation (Kambayashi and Laufer, 2014), distinguishing them from professional APCs. It seems they can only stimulate antigen experienced T cells. As conversely shown in a xenograft-model, $CD4^+$ memory cell response is not strictly depended on co-stimulation but can directly react on EC stimulation (Shiao et al., 2007). This suggests a contributing role of ECs in $CD4^+$ T cells tolerance. *Vice versa* $CD4^+$ T cells have been identified to mediate vessel normalization processes by directly altering associated gene expression of TECs (e.g., decrease of adhesion and ECM molecules, increase of VEGFA) (Tian et al., 2017).

Interestingly, liver sinusoidal ECs bear the capacity to cross-present antigens to naïve T cells, causing their rapid differentiation into memory T cells. Although these were not TECs, this is another line of evidence for ECs as a T cell tuning cell type (Knolle and Wohlleber, 2016).

It was recently shown in single cell analysis of TEC and NEC that especially those ECs with capillary phenotype harbor

genes associated with MHC II-mediated antigen presentation. Moreover, TECs can downregulate genes responsible for MHC expression (HLA-DQB1 and HLA-DQA1) impeding their antigen presenting functions, thus contributing to tumor immune-evasion (Goveia et al., 2020). The exact role of ECs, and even more of TECs as APCs remains to be specified in future studies, however, one potential mechanism could be the cross presentation of antigens.

HIGH ENDOTHELIAL VENULES (HEVs) AND VASCULAR GROWTH FACTORS ARE INVOLVED IN THE FORMATION OF TERTIARY LYMPHOID STRUCTURES (TLS)

Tertiary lymphoid structures (TLS) have very recently been identified as an important prognosticator in many different tumor types such as melanoma, colorectal, pancreatic and lung cancer (Martinet et al., 2012; Goc et al., 2014; Hiraoka et al., 2015; Posch et al., 2018; Cabrita et al., 2020; Helmink et al., 2020). Moreover, TLS harbor a response-predictive potential, having been associated with enhanced response to ICI (Cabrita et al., 2020; Helmink et al., 2020).

TLS are defined as a neoformation of lymphoid tissues in peripheral inflammatory environments such as organ transplants, infectious, autoimmune and inflammatory diseases as well as in tumors (Sautès-Fridman et al., 2019). TLS share many characteristics with physiological secondary lymphoid organs (SLO) such as lymph nodes, which represent the main interface between peripherally matured DCs and $CD4^+$ and $CD8^+$ T cells (Mellman et al., 2011). This interaction instructs effector T cells (T_{eff}) to migrate to the tumor site and convey anti-cancer immune response (Mellman et al., 2011; Sautès-Fridman et al., 2019). Under malignant conditions, TLS similarly represent a crucial site for the interaction of tumor antigen-presenting DCs and T cells as well as for the proliferation of B and T cell subpopulations (Germain et al., 2014; Goc et al., 2014; Kroeger et al., 2016). Thus, it can be expected that the presence of TLS eases immune cell recruitment and instructs tumor immunity (Sautès-Fridman et al., 2019). Recent publications using multi-omic approaches (Cabrita et al., 2020; Helmink et al., 2020) described the coincident presence of $CD20^+$ tumor infiltrating B cells and tumor associated $CD8^+$ T_{eff} cells, particularly during ICI, suggesting that these B cells may eventually contribute to T cell instruction by functioning as APCs.

Similar to SLO, TLS are closely associated with specialized vessels called high endothelial venules (HEV), which are located in the peripheral zone of the TLS (Martinet et al., 2011) and characteristically express PNAd, MECA79 and selectin ligands (Jones et al., 2018), all of which are required for lymphocyte homing (Streeter et al., 1988; Girard and Springer, 1995) (**Figure 2A**). Tumor-secreted cytokines (CXCL13 and IL-7) induce the formation of lymphoid tissue inducer (LTi) cells (Meier et al., 2007), which interact with local stromal cells via Lymphotoxin-α1β2 (Colbeck et al., 2017a). Consequently, these cells release the angiogenic factor VEGF-C, which is essential for HEV formation (Furtado et al., 2007). Like under other circumstances, EC under the stimulus of various chemokines (CXCL12, CXCL13, CCL19, and CCL21) mediate the recruitment of central cell populations of anti-tumor immunity by the expression of surface markers (Luther et al., 2002; Fleige et al., 2014; Sautès-Fridman et al., 2019). HEV have been reported to express ICAMs, VCAMs, and MADCAMs in a great density fostering the recruitment of lymphocyte population and organize them into zones (Girard and Springer, 1995). Furthermore, the ECs of lymphatic vessels associated with TLS express Podoplanin and secrete CCL21, which favors emigration of educated lymphocytes to the draining lymph nodes (Ruddle, 2016). This fact could consequently promote recognition of tumor antigens and contribute to establish a sustained protective immunity. Therefore, the EC in HEV possess an important gatekeeper function in TLS-associated immune response.

The selective influx of lymphocyte populations by regulatory EC seems to be of particular importance in the context of immunotherapy. As an example, Treg depletion (known to induce immune-mediated cancer regression) is linked to HEV development and consequently increased formation of TLS (Hindley et al., 2012; Colbeck et al., 2017b). From a clinical perspective, there is evidence that the presence of HEV acts as a favorable prognostic factor in melanoma (Martinet et al., 2012), pancreatic cancer, NSCLC and colorectal cancer (CRC) (Goc et al., 2014; Hiraoka et al., 2015; Posch et al., 2018). Moreover, TLS density depicts a predictive factor of response to anti-PD-1/PD-L1 antibodies. The putative mechanisms remain poorly understood, but could at least in part be rooted in the enhanced functionality of tumor infiltrating B lymphocytes (Cabrita et al., 2020; Helmink et al., 2020). Interestingly, the combination of anti-PD-L1 therapy and anti-angiogenic therapies further increases HEV and subsequent TLS formation (Allen et al., 2017). The mechanisms behind the synergistic effect of dual VEGF- and PD-1/PD-L1-targeting therapies remain incompletely understood, yet the concept of transforming cold immune-suppressed tumors into hot immune-competent tumors is appealing (Sautès-Fridman et al., 2019). The concept is currently mainly based on pre-clinical observations, highlighting the potential of combined targeting of VEGFR2, PDL1 and LTβ in enhancing HEV and TLS formation and, consequently, in tumor destruction (Allen et al., 2017; Colbeck et al., 2017b). Thus, it has been formulated very recently that the induction of intratumor HEV and TLS represents an important future target to improve anti-cancer immunity (Sautès-Fridman et al., 2019).

THE ROLE OF TUMOR LYMPHOID VESSELS HARBORING ENDOTHELIAL CELLS WITH VEGF-C/VEGFR3 SIGNALING ACTIVITY IN THE OCCURRENCE OF LYMPHOID METASTASES

Endothelial cells not only coat blood vessels, but also form the inner lining of lymphatic vessels, which are usually dependent on

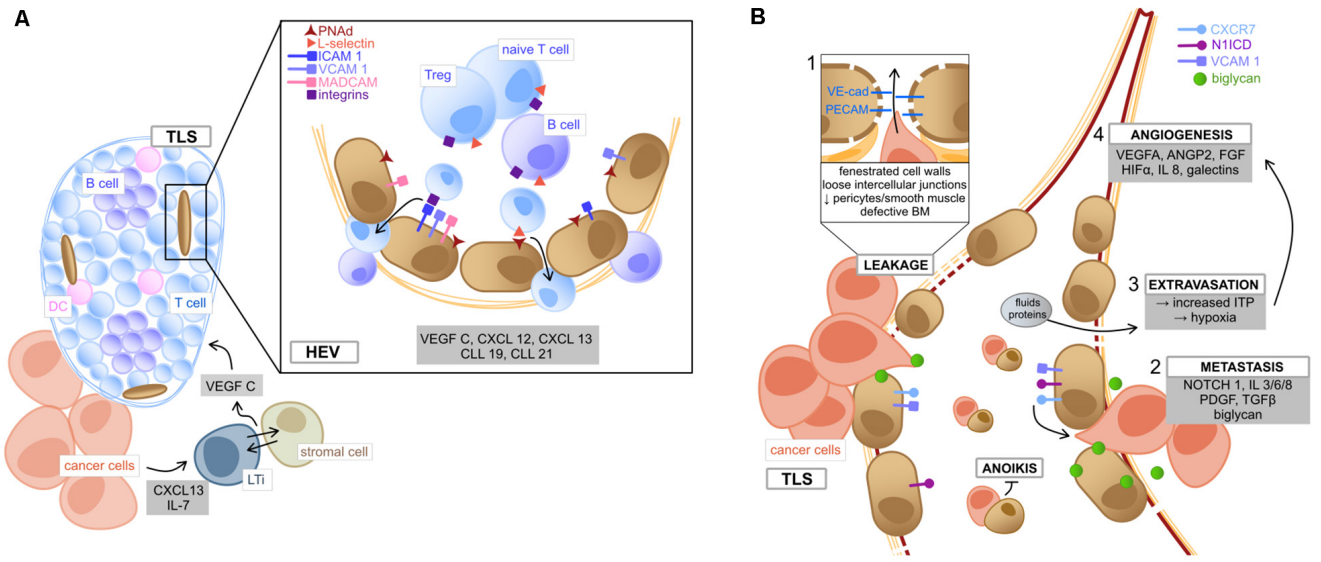


FIGURE 2 | Tumor endothelial cells **(A)** of high endothelial venules and **(B)** their role in metastasis. **(A)** Within the TME, tumor secreted cytokines (CXCL13 and IL7) induce the formation of LTi, which tightly interact with adjacent stromal cells to produce VEGF C, lastly inducing the formation of TLS (Furtado et al., 2007; Meier et al., 2007; Colbeck et al., 2017a). HEV are located in the TLS periphery and mediate immune cell transmigration. TEC lining HEV are characterized by the HEV specific marker PNAd. Binding of T cell expressed L-selectin to PNAd on TEC implies the first step of lymphocyte adhesion, followed by further interactions, such as binding of integrin ligands (ICAM1, VCAM1, and MADCAM1) with the respective receptors (LFA1, VLA4, and α4β7) (Jones et al., 2018). Lymphocytes can then transmigrate into the structured zones of the TLS, where they become activated upon interaction with local DC (Sautès-Fridman et al., 2019). TME deriving chemokines and angiocrine factors (gray box) mediate the migrating process as well as TLS organization (Sautès-Fridman et al., 2019). **(B)** The metastatic process in the TME is fired by the leaky and weak architecture of tumor vessels (box 1, involving loose connection of intercellular adhesion molecules VE-cad and PECAM), which facilitates cancer cells to disintegrate from the surrounding matrix and migrate into tumor vessels via binding to the chemokine receptor CXCR7 (Maishi et al., 2019). This metastatic process (box 2) is fostered by TME deriving pro-inflammatory cytokines (e.g., IL 3/6/8) and growth factors (e.g., PDGF and TGFβ) (Maishi et al., 2019). Moreover, NOTCH 1 signaling in TEC as well as expression of activated NOTCH 1 receptors (N1ICD) facilitate metastasis, e.g., via upregulation of integrin ligand VCAM1 (Wieland et al., 2017). Also, the TEC-expressed angiogenic molecule biglycan promotes hematogenous cancer cell metastasis (Maishi et al., 2016). Circulating TEC can bind to metastasizing cancer cells and exhibit protection from apoptosis (anoikis) (Cao et al., 2016). Besides, vessel leakiness leads to extravasation of fluids and proteins, which increases ITP, resulting in hypoxia and (box 3), the release of angiogenic factors by cellular TME components (box 4) and consequently promotes tumor neo-angiogenesis (Butler et al., 2010). TLS, tertiary lymphoid structures; TME, tumor microenvironment; LTi, lymphoid tissue inducer cells; HEV, high endothelial venules; PNAd, peripheral lymph node addressin; ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion protein 1; MADCAM1, mucosal addressin cell adhesion molecule-1; LFA1, lymphocyte function-associated antigen 1; VLA4, very late antigen-4; VE-cad, vascular endothelial cadherin; PECAM, platelet endothelial cell adhesion molecule; PDGF, platelet derived growth factor; TGFβ, transforming growth factor beta; N1ICD, NOTCH1 intracellular domain; ITP, intra-tumoral interstitial pressure.

VEGF-C and VEGFR3 signaling (Karkkainen et al., 2004). There is emerging insight that VEGFR3 positive ECs play an important (patho)physiological role not only in lymphangiogenesis of tumors as the structural determinant for lymph-node metastasis, but also in chronic inflammatory diseases (Hsu et al., 2019). The VEGF-C/VEGFR3 axis was found to be up-regulated in various tumor entities such as genitourinary tract, gastrointestinal and prostate cancer (Yu et al., 2013; Wang et al., 2014; Yang et al., 2014; Zhu et al., 2016). Pre-clinical and clinical evidence demonstrated that VEGF-C fosters the formation of lymph vessels by promoting lymphatic outgrowth, ultimately enabling lymphatic metastasis (Skobe et al., 2001; Mattila et al., 2002; Paiva et al., 2015; Zhu et al., 2016; Yeh et al., 2017). Consequently, the expression of VEGFR3 was correlated with lymph node and distant metastasis and poor prognosis in many different cancer types (Decio et al., 2014; Wang et al., 2014; Yang et al., 2014; Goussia et al., 2018). As shown in ovarian and breast cancer, tumor cell expression of VEGFR3 may generate a proliferative advantage by autocrine and paracrine signaling (Decio et al., 2014; Varney and Singh, 2015).

The VEGF-C/VEGFR3 axis may also be involved in anti-cancer immune responses. As shown in an interesting murine melanoma model, VEGF-C activated LECs exhibit immunomodulatory functions by suppressing tumor-specific $CD8^+$ T cells by lymphatic antigen presentation (Lund et al., 2012). Moreover, NK cells isolated from patients with acute myeloid leukemia (AML) were found to express increased levels of VEGFR3 and lower levels of IFN-γ (Lee et al., 2013). Importantly, resident macrophages in lung adenocarcinoma also expressed VEGFR3 and pharmacological inhibition of VEGFR3 signaling sensitized to chemotherapy (Li et al., 2017).

Several FDA-approved drugs target VEGF-C/VEGFR3, such as sorafenib, sunitinib pazopanib, axitinib (Hsu et al., 2019). For patients with renal cell carcinoma (RCC), hepatocellular

carcinoma (HCC), thyroid cancer, gastrointestinal stromal tumors (GIST) and soft tissue sarcoma treated with these TKIs improved outcomes were reported (Demetri et al., 2006; Escudier et al., 2007; Motzer et al., 2007, 2017; Llovet et al., 2008; Cheng et al., 2009; Sleijfer et al., 2009; Sternberg et al., 2010; Brose et al., 2014; Haas et al., 2016; Ravaud et al., 2016). Accordingly, the monoclonal antibodies bevaczizumab and ramucirumab targeting VEGF and VEGF signaling improved outcomes for patients with NSCLC, CRC and gastric cancer (Hurwitz et al., 2004; Sandler et al., 2006; Miller et al., 2007; Ohtsu et al., 2011; Garon et al., 2014; Tabernero et al., 2015). Conceivably, off-target effects on VEGF-C/VEGFR3 of these drugs may at least in part contribute to their anti-cancer efficacy, even though this has not formally been proven in the clinical setting.

TECs AND THEIR SIGNALING PROPERTIES (ANGIOCRINE FACTORS) MODULATE THE TME AND FOSTER TUMOR PROGRESSION AND METASTASIS AS WELL AS THERAPY RESISTANCE

Cancer progression and metastasis is a highly complex and multifactorial process depending on many factors and different cell types. Even though CAFs and suppressive immune cells very much contribute to cancer cell progression and metastatic spread (Quail and Joyce, 2013; Maishi et al., 2019), we will here focus on TEC (**Figure 2B**). Firstly, the abnormal and leaky architecture of tumor vessels facilitates the disengagement of tumor cells and the hematogenous metastatic spread of cancer cells (Maishi et al., 2019) (**Figure 2B**). Mechanistically, the adhesion molecule expression profile of TEC functions as scaffold guiding malignant cells to intravasation (Sökeland and Schumacher, 2019). Moreover, TEC were shown to use metalloproteases to break through the vessel basement membrane, thus directly impacting metastasis (Ohga et al., 2012).

Furthermore, angiocrine factors centrally contribute to cancer progression and metastasis (Butler et al., 2010), including numerous growth factors (bFGF, G/GM-CSF, IGF1, PDGF, and TGFβ), interleukins (IL-3, 6, 8) and other factors like NOTCH, calcineurin, biglycan, Jag1, and Slit2 (Maishi et al., 2019). Several mechanisms for the initiation of metastasis by angiocrine factors have been described. For instance, Notch1 signaling in TEC was observed to up-regulate chemokines and adhesion molecules (VCAM1) inducing leukocyte adhesion and metastatic outgrowth (Wieland et al., 2017). Moreover, the chemokine receptor CXCR7 initiates and mediates transendothelial migration of tumor cells under the control of TEC. In addition, angiocrine factors facilitate the blocking of tumor suppressive signals. For instance, the tumor suppressive factor Slit2 can directly be downregulated by TEC *via* their receptor EphA2 in a paracrine manner (Brantley-Sieders et al., 2011). However, TEC may also have an influence on therapy resistance *via* these angiocrine factors (Maishi et al., 2019). As an example, direct interaction of TEC and lymphoma cells *via* FGF4/FGFR1 has been shown. TEC express Jag1, which helps to generate a malignant vascular niche that correlates with aggressive course and resistance to chemotherapy (Cao et al., 2014).

Anoikis is defined as apoptosis of cells that have lost their contact to the extracellular matrix (ECM) (Frisch, 1994) and may play a crucial role in the formation of metastasis (Cao et al., 2016) (**Figure 2B**). EC use their surface molecules to connect with cell matrix contacts and it is thus speculated that circulating TEC attach to tumor cells and prevent anoikis-mediated apoptosis (Yadav et al., 2015).

Biglycan is a small leucin-rich repeat proteoglycan functioning as an angiocrine factor and is expressed by TEC of highly metastatic tumors (Maishi et al., 2016). It is suggested that biglycan allows toll-like receptor expressing tumor cells to metastasize hematogenously. Interestingly, biglycans seem to be also subject to epigenetic regulations (Maishi et al., 2016).

The glycosylation of surface proteins of cell populations in the TME seems to be another regulatory factor and mechanism of tumor progression and metastasis. EC exhibit a great extent of glycosylated proteins, which can be influenced by inflammation and hypoxia (Chandler et al., 2019). Glycosylation of endothelial adhesion molecules (e.g., ICAM1, VCAM1, PECAM, and lectins) affects the cellular adhesive properties of TEC. Thus, tumor infiltration by immune cells can either be favored or prevented (Chandler et al., 2019). Moreover, the signaling, adhesion and migration of EC can be dysregulated by altered glycosylation. Accordingly, VEGFR2 signaling can be negatively influenced and formation of abnormal vessels with an increased degree of leakage is favored (Chandler et al., 2019) finally impacting tumor progression and metastasis (Oliveira-Ferrer et al., 2017).

Galectins are a group of glycan-binding endogenous lectin proteins, which show interactions with fibroblasts, endothelial and immune cells. There is growing evidence that several isoforms of this protein family are involved in the function and regulation of the TME (Elola et al., 2018). Accordingly, it has been observed that dysregulated expression of galectins in human tumors is associated with greater extent of vascularization and unfavorable course such as rapid progression and metastatic disease (Liu and Rabinovich, 2005; Laderach et al., 2013). Here we want to focus on TEC derived from blood and lymphatic vessels. Firstly, members of the galectin family have been shown to facilitate tumor angiogenesis, as they are synthesized by activated ECs and are important mediators of EC migration and tube formation (Nangia-Makker et al., 2000; Thijssen et al., 2006; Hsieh et al., 2008; Delgado et al., 2011; Croci et al., 2014), in blood as in lymphatic vessels (Cueni and Detmar, 2009; Chen et al., 2016). Therefore, tumor angiogenesis is sustained by galectins *via* autocrine and paracrine mechanisms (Thijssen et al., 2010) as well as by direct interactions with VEGFR2 and HIF1α (Markowska et al., 2011; Zhao et al., 2011; Laderach et al., 2013; White et al., 2014). In addition, galectins seem to influence other prominent angiogenic stimuli, such as VEGF-A and bFGF (Markowska et al., 2010). Interestingly, Gal-1 activates VEGFR2 by phosphorylation resulting in unresponsiveness to anti-VEGF therapy, whereas elimination of Gal-1 restored sensitivity to

anti-angiogenic therapy (Croci et al., 2014). Moreover, depletion of Gal-1 contributed to vessel normalization by enhanced pericyte coverage and increased immune recruitment (Croci et al., 2014). This also holds true for lymphangiogenesis, as Gal-8 potentiates VEGFR3/VEGF-C signaling (Chen et al., 2016). Furthermore, galectins influence the immune compartment by inducing T cell apoptosis, impairing NK function as well as favoring the proliferation of Treg, tolerogenic DC and tumor-promoting macrophages and MDSC (Elola et al., 2018).

TECs INFLUENCE RESPONSE TO ANTI-ANGIOGENIC AND IMMUNE CHECKPOINT INHIBITOR THERAPIES THAT CONTRIBUTE TO RE-REGULATE THE TME

Throughout this review we already highlighted the immune regulatory properties of TEC and it seems obvious to discuss the additive effect and possible synergism of immunotherapy and anti-angiogenic therapies. As they mutually influence each other, reversing endothelial anergy to foster T cell infiltration and enhancing the efficacy of ICI seem promising (Uldry et al., 2017; De Sanctis et al., 2018).

The first aspect is that VEGFs are immunosuppressive, as they inhibit T cell infiltration and decrease maturation and function (antigen presentation) of DC, expand Treg and foster PD-1 expression (Ohm and Carbone, 2001; Voron et al., 2015). In addition, anti-angiogenic therapies were reported to up-regulate the expression of adhesion molecules and thereby promote the transmigration of immune cells (Griffioen et al., 1999; Dirkx et al., 2006). Thus, a better response to immunotherapy by decreasing TEC-driven immunosuppression *via* blockade of the VEGF axis is argued (De Sanctis et al., 2018). Accordingly, the formation of HEV and TLS as essential factors of anti-tumor immunity could be initiated by combining anti-VEGF and anti-PD1/PD-L1 therapies (Allen et al., 2017). Other mediators of endothelial anergy have been identified as promising therapeutic targets to restore anti-cancer immune responses (Uldry et al., 2017). For instance, NO antagonists were able to restore T cell adhesion to ECs *via* up-regulation of adhesion molecules (Bouzin et al., 2007; Buckanovich et al., 2008). Also, the stabilization of TNFα by vessel normalization lead to improved T cell function (Schmittnaegel et al., 2017). *Vice versa*, tumor angiogenesis is also driven by suppressive immune cells (MDSCs and TAM) and direct targeting of these cell populations increases sensitivity to anti-angiogenic therapy (Ugel et al., 2015a). Likewise, immunotherapy approaches were shown to induce the recognition and destruction of tumor vasculature (Ugel et al., 2015b), which was also demonstrated in a study applying CAR-T cells model targeting VEGFR2 (Chinnasamy et al., 2010; Kanagawa et al., 2013).

Vascular endothelial growth factor-targeted therapies rather reduce the tumor growth rate than inducing direct tumor shrinkage (Bagri et al., 2010) and contribute to blood flow normalization to the tumor. Consequently, the concept of vessel normalization is well established and is associated with increased sensitivity to chemo- and/or radiotherapy (Carmeliet and Jain, 2011; Uldry et al., 2017; De Sanctis et al., 2018). Retaining the TME from excessive hypoxia depicts an important factor for adequate anti-tumor immune responses (Uldry et al., 2017). For example, hypoxia induces the up-regulation of HIF1α, the increased expression of CXCL12/CXCR4 and subsequent recruitment of TAM, MDSC, and Treg (Ebos and Kerbel, 2011) as well as it favors an M2-phenotype (Facciabene et al., 2011; Colegio et al., 2014) and reduces cytotoxic T cell activity (Calcinotto et al., 2012; Barsoum et al., 2014). Anti-VEGF therapeutics therefore stabilize the tumor vasculature by vessel normalization which in turn increases anti-tumor immune responses by favoring an immune-competent TME (Huang et al., 2013; Uldry et al., 2017). To further optimize this approach, the combination of HIF1α blockers and anti-VEGF therapies further increase immune responses in pre-clinical mouse models (Chen et al., 2015) also decreasing tumor metastasis (Maes et al., 2014) via NOTCH1 mediated intravasation of cancer cells (Sonoshita et al., 2011). On the contrary, vessel normalization is also driven by the influence of immune cells, as TH1 cells are considered to crucially impact the regulation of tumor blood flow (Tian et al., 2017). Thus, vessel normalization substantially contributes to revise endothelial anergy and a mutual regulation of angiogenesis and immune response seems obvious. Combining immunotherapy strategies and blockade of the VEGF axis seems promising, changes tumor vasculature and immune responses in melanoma (Hodi et al., 2014) and has improved outcomes of NSCLC patients in clinical studies (Socinski et al., 2018).

CONCLUSION

TEC are central players orchestrating the TME. They phenotypically differ from NEC, are highly proliferative, exhibit genetic instability and a stemness gene signature. Functionally, TEC are deeply involved in immune-regulation of the TME as they can exert APC-like functions and may play a role in T cell priming or T cell anergization. They are immune-regulative, as they modulate trafficking of immune cells and by favoring recruitment of immunosuppressive rather than immune effector cells, contributing to cancer immune escape. Moreover, TEC are deeply involved in the formation of TLS, which correlate with improved prognosis and better response to ICI. In addition, EC of cancer associated lymphatic vessels and their VEGF-C/VEGFR3 signaling are critical for with lymphatic metastasis. Lastly, several angiocrine factors of TEC may directly foster cancer progression and the formation of distant metastasis.

Following these key immunomodulatory characteristics of TEC, it depicts a main future challenge to further functionally characterize TEC and TEC subtypes with respect to their cancer promoting properties. Technological advances like scRNAseq have already broadened the understanding of the cellular and molecular TME composition by deducing gene sequences and defining cell sub-clusters that strongly indicate their functional dysregulation in cancer. Nevertheless, it is of great importance

to sustain these findings *in vitro* and most notably *in vivo*, but the development of adequate pre-clinical models still remains challenging.

AUTHOR CONTRIBUTIONS

LN, LH, AP, and DW developed the concept of the review. LN and LH drafted the review. DW and AP corrected and reviewed the review. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by funds of the "In Memoriam Dr. Gabriel Salzner Privatstiftung, Innsbruck, Austria" (project number 56/LH/239051).

REFERENCES

Aird, W. C. (2012). Endothelial cell heterogeneity. *Cold Spring Harb. Perspect. Med.* 2:a006429.

Akino, T., Hida, K., Hida, Y., Tsuchiya, K., Freedman, D., Muraki, C., et al. (2009). Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors. *Am. J. Pathol.* 175, 2657–2667. doi: 10.2353/ajpath.2009.090202

Albini, A., Bruno, A., Noonan, D. M., and Mortara, L. (2018). Contribution to tumor angiogenesis from innate immune cells within the tumor microenvironment: implications for immunotherapy. *Front. Immunol.* 9:527. doi: 10.3389/fimmu.2018.00527

Allen, E., Jabouille, A., Rivera, L. B., Lodewijckx, I., Missiaen, R., Steri, V., et al. (2017). Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci. Transl. Med.* 9:eaak9679. doi: 10.1126/scitranslmed.aak9679

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., et al. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964–967.

Bagri, A., Berry, L., Gunter, B., Singh, M., Kasman, I., Damico, L. A., et al. (2010). Effects of anti-VEGF treatment duration on tumor growth, tumor regrowth, and treatment efficacy. *Clin .Cancer Res.* 16, 3887–3900. doi: 10.1158/1078-0432.ccr-09-3100

Barsoum, I. B., Smallwood, C. A., Siemens, D. R., and Graham, C. H. (2014). A mechanism of hypoxia-mediated escape from adaptive immunity in cancer cells. *Cancer Res.* 74, 665–674. doi: 10.1158/0008-5472.can-13-0992

Blanco, R., and Gerhardt, H. (2013). VEGF and Notch in tip and stalk cell selection. *Cold Spring Harb. Perspect. Med.* 3:a006569. doi: 10.1101/cshperspect.a006569

Bouma-ter Steege, J. C., Baeten, C. I., Thijssen, V. L., Satijn, S. A., Verhoeven, I. C., Hillen, H. F., et al. (2004). Angiogenic profile of breast carcinoma determines leukocyte infiltration. *Clin. Cancer Res.* 10, 7171–7178. doi: 10.1158/1078-0432.ccr-04-0742

Bouzin, C., Brouet, A., De Vriese, J., Dewever, J., and Feron, O. (2007). Effects of vascular endothelial growth factor on the lymphocyte-endothelium interactions: identification of caveolin-1 and nitric oxide as control points of endothelial cell anergy. *J. Immunol.* 178, 1505–1511. doi: 10.4049/jimmunol.178.3.1505

Brantley-Sieders, D. M., Dunaway, C. M., Rao, M., Short, S., Hwang, Y., Gao, Y., et al. (2011). Angiocrine factors modulate tumor proliferation and motility through EphA2 repression of Slit2 tumor suppressor function in endothelium. *Cancer Res.* 71, 976–987. doi: 10.1158/0008-5472.can-10-3396

Brose, M. S., Nutting, C. M., Jarzab, B., Elisei, R., Siena, S., Bastholt, L., et al. (2014). Sorafenib in radioactive iodine-refractory, locally advanced or metastatic differentiated thyroid cancer: a randomised, double-blind, phase 3 trial. *Lancet* 384, 319–328. doi: 10.1016/s0140-6736(14)60421-9

Buckanovich, R. J., Facciabene, A., Kim, S., Benencia, F., Sasaroli, D., Balint, K., et al. (2008). Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nat. Med.* 14, 28–36. doi: 10.1038/nm1699

Butler, J. M., Kobayashi, H., and Rafii, S. (2010). Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat. Rev. Cancer* 10, 138–146. doi: 10.1038/nrc2791

Cabrita, R., Lauss, M., Sanna, A., Donia, M., Skaarup Larsen, M., Mitra, S., et al. (2020). Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature* 577, 561–565. doi: 10.1038/s41586-019-1914-8

Calcinotto, A., Filipazzi, P., Grioni, M., Iero, M., De Milito, A., Ricupito, A., et al. (2012). Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes. *Cancer Res.* 72, 2746–2756. doi: 10.1158/0008-5472.can-11-1272

Cantelmo, A. R., Conradi, L. C., Brajic, A., Goveia, J., Kalucka, J., Pircher, A., et al. (2016). Inhibition of the glycolytic activator PFKFβ3 in endothelium induces tumor vessel normalization, impairs metastasis, and improves chemotherapy. *Cancer Cell* 30, 968–985. doi: 10.1016/j.ccell.2016.10.006

Cao, Z., Ding, B. S., Guo, P., Lee, S. B., Butler, J. M., Casey, S. C., et al. (2014). Angiocrine factors deployed by tumor vascular niche induce B cell lymphoma invasiveness and chemoresistance. *Cancer Cell* 25, 350–365. doi: 10.1016/j.ccr.2014.02.005

Cao, Z., Livas, T., and Kyprianou, N. (2016). Anoikis and EMT: lethal "Liaisons" during cancer progression. *Crit. Rev. Oncog.* 21, 155–168. doi: 10.1615/critrevoncog.2016016955

Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* 407, 249–257. doi: 10.1038/35025220

Carmeliet, P., and Jain, R. K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat. Rev. Drug Discov.* 10, 417–427. doi: 10.1038/nrd3455

Chandler, K. B., Costello, C. E., and Rahimi, N. (2019). Glycosylation in the tumor microenvironment: implications for tumor angiogenesis and metastasis. *Cells* 8:544. doi: 10.3390/cells8060544

Chen, H., Campbell, R. A., Chang, Y., Li, M., Wang, C. S., Li, J., et al. (2009). Pleiotrophin produced by multiple myeloma induces transdifferentiation of monocytes into vascular endothelial cells: a novel mechanism of tumor-induced vasculogenesis. *Blood* 113, 1992–2002. doi: 10.1182/blood-2008-02-133751

Chen, W. S., Cao, Z., Sugaya, S., Lopez, M. J., Sendra, V. G., Laver, N., et al. (2016). Pathological lymphangiogenesis is modulated by galectin-8-dependent crosstalk between podoplanin and integrin-associated VEGFR-3. *Nat. Commun.* 7:11302.

Chen, Y., Ramjiawan, R. R., Reiberger, T., Ng, M. R., Hato, T., Huang, Y., et al. (2015). CXCR4 inhibition in tumor microenvironment facilitates anti-programmed death receptor-1 immunotherapy in sorafenib-treated hepatocellular carcinoma in mice. *Hepatology* 61, 1591–1602. doi: 10.1002/hep.27665

Cheng, A. L., Kang, Y. K., Chen, Z., Tsao, C. J., Qin, S., Kim, J. S., et al. (2009). Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol.* 10, 25–34.

Cheng, L., Huang, Z., Zhou, W., Wu, Q., Donnola, S., Liu, J. K., et al. (2013). Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. *Cell* 153, 139–152. doi: 10.1016/j.cell.2013.02.021

Chinnasamy, D., Yu, Z., Theoret, M. R., Zhao, Y., Shrimali, R. K., Morgan, R. A., et al. (2010). Gene therapy using genetically modified lymphocytes targeting VEGFR-2 inhibits the growth of vascularized syngenic tumors in mice. *J. Clin. Invest.* 120, 3953–3968. doi: 10.1172/jci43490

Colbeck, E. J., Ager, A., Gallimore, A., and Jones, G. W. (2017a). Tertiary lymphoid structures in cancer: drivers of antitumor immunity, immunosuppression, or bystander sentinels in disease? *Front. Immunol.* 8:1830. doi: 10.3389/fimmu.2017.01830

Colbeck, E. J., Jones, E., Hindley, J. P., Smart, K., Schulz, R., Browne, M., et al. (2017b). Treg depletion licenses T Cell-Driven HEV neogenesis and promotes tumor destruction. *Cancer Immunol. Res.* 5, 1005–1015. doi: 10.1158/2326-6066.cir-17-0131

Colegio, O. R., Chu, N. Q., Szabo, A. L., Chu, T., Rhebergen, A. M., Jairam, V., et al. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 513, 559–563. doi: 10.1038/nature13490

Croci, D. O., Cerliani, J. P., Dalotto-Moreno, T., Méndez-Huergo, S. P., Mascanfroni, I. D., Dergan-Dylon, S., et al. (2014). Glycosylation-dependent lectin-receptor interactions preserve angiogenesis in anti-VEGF refractory tumors. *Cell* 156, 744–758. doi: 10.1016/j.cell.2014.01.043

Cueni, L. N., and Detmar, M. (2009). Galectin-8 interacts with podoplanin and modulates lymphatic endothelial cell functions. *Exp. Cell Res.* 315, 1715–1723. doi: 10.1016/j.yexcr.2009.02.021

De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., et al. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* 96, 60–68. doi: 10.1172/jci118074

De Palma, M., Biziato, D., and Petrova, T. V. (2017). Microenvironmental regulation of tumour angiogenesis. *Nat. Rev. Cancer* 17, 457–474. doi: 10.1038/nrc.2017.51

De Sanctis, F., Ugel, S., Facciponte, J., and Facciabene, A. (2018). The dark side of tumor-associated endothelial cells. *Semin. Immunol.* 35, 35–47. doi: 10.1016/j.smim.2018.02.002

Decio, A., Taraboletti, G., Patton, V., Alzani, R., Perego, P., Fruscio, R., et al. (2014). Vascular endothelial growth factor c promotes ovarian carcinoma progression through paracrine and autocrine mechanisms. *Am. J. Pathol.* 184, 1050–1061. doi: 10.1016/j.ajpath.2013.12.030

Deeb, K. K., Luo, W., Karpf, A. R., Omilian, A. R., Bshara, W., Tian, L., et al. (2011). Differential vitamin D 24-hydroxylase/CYP24A1 gene promoter methylation in endothelium from benign and malignant human prostate. *Epigenetics* 6, 994–1000. doi: 10.4161/epi.6.8.16536

Delfortrie, S., Pinte, S., Mattot, V., Samson, C., Villain, G., Caetano, B., et al. (2011). Egfl7 promotes tumor escape from immunity by repressing endothelial cell activation. *Cancer Res.* 71, 7176–7186. doi: 10.1158/0008-5472.can-11-1301

Delgado, V. M., Nugnes, L. G., Colombo, L. L., Troncoso, M. F., Fernández, M. M., Malchiodi, E. L., et al. (2011). Modulation of endothelial cell migration and angiogenesis: a novel function for the "tandem-repeat" lectin galectin-8. *FASEB J.* 25, 242–254. doi: 10.1096/fj.09-144907

Demaria, O., De Gassart, A., Coso, S., Gestermann, N., Di Domizio, J., Flatz, L., et al. (2015). STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15408–15413. doi: 10.1073/pnas.1512832112

Demetri, G. D., van Oosterom, A. T., Garrett, C. R., Blackstein, M. E., Shah, M. H., Verweij, J., et al. (2006). Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet* 368, 1329–1338. doi: 10.1016/s0140-6736(06)69446-4

Dirkx, A. E., oude Egbrink, M. G., Castermans, K., van der Schaft, D. W., Thijssen, V. L., and Dings, R. P. (2006). Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors. *FASEB J.* 20, 621–630. doi: 10.1096/fj.05-4493com

Dirkx, A. E., Oude Egbrink, M. G., Kuijpers, M. J., van der Niet, S. T., Heijnen, V. V., Bouma-ter Steege, J. C., et al. (2003). Tumor angiogenesis modulates leukocyte-vessel wall interactions in vivo by reducing endothelial adhesion molecule expression. *Cancer Res.* 63, 2322–2329.

Ebos, J. M., and Kerbel, R. S. (2011). Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nat. Rev. Clin. Oncol.* 8, 210–221. doi: 10.1038/nrclinonc.2011.21

Elola, M. T., Ferragut, F., Méndez-Huergo, S. P., Croci, D. O., Bracalente, C., and Rabinovich, G. A. (2018). Galectins: Multitask signaling molecules linking fibroblast, endothelial and immune cell programs in the tumor microenvironment. *Cell Immunol.* 333, 34–45. doi: 10.1016/j.cellimm.2018.03.008

Escudier, B., Eisen, T., Stadler, W. M., Szczylik, C., Oudard, S., Siebels, M., et al. (2007). Sorafenib in advanced clear-cell renal-cell carcinoma. *N. Engl. J. Med.* 356, 125–134.

Facciabene, A., De Sanctis, F., Pierini, S., Reis, E. S., Balint, K., Facciponte, J., et al. (2017). Local endothelial complement activation reverses endothelial quiescence, enabling t-cell homing, and tumor control during t-cell immunotherapy. *Oncoimmunology* 6:e1326442. doi: 10.1080/2162402x.2017.1326442

Facciabene, A., Peng, X., Hagemann, I. S., Balint, K., Barchetti, A., Wang, L. P., et al. (2011). Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature* 475, 226–230. doi: 10.1038/nature10169

Fernandez Pujol, B., Lucibello, F. C., Zuzarte, M., Lütjens, P., Müller, R., and Havemann, K. (2001). Dendritic cells derived from peripheral monocytes express endothelial markers and in the presence of angiogenic growth factors differentiate into endothelial-like cells. *Eur. J. Cell Biol.* 80, 99–110. doi: 10.1078/0171-9335-00136

Flati, V., Pastore, L. I., Griffioen, A. W., Satijn, S., Toniato, E., D'Alimonte, I., et al. (2006). Endothelial cell anergy is mediated by bFGF through the sustained activation of p38-MAPK and NF-kappaB inhibition. *Int. J. Immunopathol. Pharmacol.* 19, 761–773. doi: 10.1177/03946320060190 0406

Fleige, H., Ravens, S., Moschovakis, G. L., Bölter, J., Willenzon, S., Sutter, G., et al. (2014). IL-17-induced CXCL12 recruits B cells and induces follicle formation in BALT in the absence of differentiated FDCs. *J. Exp. Med.* 211, 643–651. doi: 10.1084/jem.20131737

Frisch, S. M. (1994). E1a induces the expression of epithelial characteristics. *J. Cell Biol.* 127, 1085–1096. doi: 10.1083/jcb.127.4.1085

Fukumura, D., Kashiwagi, S., and Jain, R. K. (2006). The role of nitric oxide in tumour progression. *Nat. Rev. Cancer.* 6, 521–534. doi: 10.1038/nrc1910

Furtado, G. C., Marinkovic, T., Martin, A. P., Garin, A., Hoch, B., Hubner, W., et al. (2007). Lymphotoxin beta receptor signaling is required for inflammatory lymphangiogenesis in the thyroid. *Proc. Natl. Acad .Sci.U.S.A.* 104, 5026–5031. doi: 10.1073/pnas.0606697104

Galon, J., and Bruni, D. (2020). Tumor immunology and tumor evolution: intertwined histories. *Immunity* 52, 55–81. doi: 10.1016/j.immuni.2019.12.018

Garon, E. B., Ciuleanu, T. E., Arrieta, O., Prabhash, K., Syrigos, K. N., Goksel, T., et al. (2014). Ramucirumab plus docetaxel versus placebo plus docetaxel for second-line treatment of stage IV non-small-cell lung cancer after disease progression on platinum-based therapy (REVEL): a multicentre, double-blind, randomised phase 3 trial. *Lancet* 384, 665–673. doi: 10.1016/s0140-6736(14)60845-x

Georganaki, M., Ramachandran, M., Tuit, S., Núñez, N. G., Karampatzakis, A., Fotaki, G., et al. (2020). Tumor endothelial cell up-regulation of IDO1 is an immunosuppressive feed-back mechanism that reduces the response to CD40-stimulating immunotherapy. *Oncoimmunology* 9:1730538. doi: 10.1080/2162402x.2020.1730538

Georganaki, M., van Hooren, L., and Dimberg, A. (2018). Vascular targeting to increase the efficiency of immune checkpoint blockade in Cancer. *Front. Immunol.* 9:3081. doi: 10.3389/fimmu.2018.03081

Germain, C., Gnjatic, S., Tamzalit, F., Knockaert, S., Remark, R., Goc, J., et al. (2014). Presence of B cells in tertiary lymphoid structures is associated with a protective immunity in patients with lung cancer. *Am. J. Respir. Crit. Care Med.* 189, 832–844. doi: 10.1164/rccm.201309-1611oc

Girard, J. P., and Springer, T. A. (1995). High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol. Today.* 16, 449–457. doi: 10.1016/0167-5699(95)80023-9

Goc, J., Germain, C., Vo-Bourgais, T. K., Lupo, A., Klein, C., Knockaert, S., et al. (2014). Dendritic cells in tumor-associated tertiary lymphoid structures signal a Th1 cytotoxic immune contexture and license the positive prognostic value of infiltrating CD8+ T cells. *Cancer Res.* 74, 705–715. doi: 10.1158/0008-5472.can-13-1342

Goussia, A., Simou, N., Zagouri, F., Manousou, K., Lazaridis, G., Gogas, H., et al. (2018). Associations of angiogenesis-related proteins with specific prognostic factors, breast cancer subtypes and survival outcome in early-stage breast cancer patients. A Hellenic Cooperative Oncology Group (HeCOG) trial. *PLoS One* 13:e0200302. doi: 10.1371/journal.pone.0200302

Goveia, J., Rohlenova, K., Taverna, F., Treps, L., Conradi, L. C., Pircher, A., et al. (2020). An integrated gene expression landscape profiling approach to identify lung tumor endothelial cell heterogeneity and angiogenic Candidates. *Cancer Cell* 37, 21.e13–36.e13.

Griffioen, A. W., Damen, C. A., Blijham, G. H., and Groenewegen, G. (1996). Tumor angiogenesis is accompanied by a decreased inflammatory response of tumor-associated endothelium. *Blood* 88, 667–673. doi: 10.1182/blood.v88.2.667.bloodjournal882667

Griffioen, A. W., Damen, C. A., Mayo, K. H., Barendsz-Janson, A. F., Martinotti, S., Blijham, G. H., et al. (1999). Angiogenesis inhibitors overcome tumor induced endothelial cell anergy. *Int. J. Cancer* 80, 315–319. doi: 10.1002/(sici)1097-0215(19990118)80:2<315::aid-ijc23>3.0.co;2-l

Haas, N. B., Manola, J., Uzzo, R. G., Flaherty, K. T., Wood, C. G., Kane, C., et al. (2016). Adjuvant sunitinib or sorafenib for high-risk, non-metastatic renal-cell carcinoma (ECOG-ACRIN E2805): a double-blind, placebo-controlled, randomised, phase 3 trial. *Lancet* 387, 2008–2016. doi: 10.1016/s0140-6736(16)00559-6

Helmink, B. A., Reddy, S. M., Gao, J., Zhang, S., Basar, R., Thakur, R., et al. (2020). B cells and tertiary lymphoid structures promote immunotherapy response. *Nature* 577, 549–555.

Hida, K., Hida, Y., Amin, D. N., Flint, A. F., Panigrahy, D., Morton, C. C., et al. (2004). Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* 64, 8249–8255. doi: 10.1158/0008-5472.can-04-1567

Hida, K., Maishi, N., Akiyama, K., Ohmura-Kakutani, H., Torii, C., Ohga, N., et al. (2017). Tumor endothelial cells with high aldehyde dehydrogenase activity show drug resistance. *Cancer Sci.* 108, 2195–2203. doi: 10.1111/cas.13388

Hindley, J. P., Jones, E., Smart, K., Bridgeman, H., Lauder, S. N., Ondondo, B., et al. (2012). T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. *Cancer Res.* 72, 5473–5482. doi: 10.1158/0008-5472.can-12-1912

Hiraoka, N., Ino, Y., Yamazaki-Itoh, R., Kanai, Y., Kosuge, T., and Shimada, K. (2015). Intratumoral tertiary lymphoid organ is a favourable prognosticator in patients with pancreatic cancer. *Br. J. Cancer* 112, 1782–1790. doi: 10.1038/bjc.2015.145

Hodi, F. S., Lawrence, D., Lezcano, C., Wu, X., Zhou, J., Sasada, T., et al. (2014). Bevacizumab plus ipilimumab in patients with metastatic melanoma. *Cancer Immunol. Res.* 2, 632–642.

Hojo, T., Maishi, N., Towfik, A. M., Akiyama, K., Ohga, N., Shindoh, M., et al. (2017). ROS enhance angiogenic properties via regulation of NRF2 in tumor endothelial cells. *Oncotarget* 8, 45484–45495. doi: 10.18632/oncotarget.17567

Hsieh, S. H., Ying, N. W., Wu, M. H., Chiang, W. F., Hsu, C. L., Wong, T. Y., et al. (2008). Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene* 27, 3746–3753. doi: 10.1038/sj.onc.1211029

Hsu, M. C., Pan, M. R., and Hung, W. C. (2019). Two birds, one stone: double hits on tumor growth and lymphangiogenesis by targeting vascular endothelial growth factor receptor 3. *Cells* 8:270. doi: 10.3390/cells8030270

Huang, C. H., Li, X. J., Zhou, Y. Z., Luo, Y., Li, C., and Yuan, X. R. (2010). Expression and clinical significance of EGFL7 in malignant glioma. *J. Cancer Res. Clin. Oncol.* 136, 1737–1743. doi: 10.1007/s00432-010-0832-9

Huang, H., Langenkamp, E., Georganaki, M., Loskog, A., Fuchs, P. F., Dieterich, L. C., et al. (2015). VEGF suppresses T-lymphocyte infiltration in the tumor microenvironment through inhibition of NF-κB-induced endothelial activation. *FASEB J.* 29, 227–238. doi: 10.1096/fj.14-250985

Huang, Y., Goel, S., Duda, D. G., Fukumura, D., and Jain, R. K. (2013). Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer Res.* 73, 2943–2948. doi: 10.1158/0008-5472.can-12-4354

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., et al. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* 350, 2335–2342.

Jayasinghe, C., Simiantonaki, N., Michel-Schmidt, R., and Kirkpatrick, C. J. (2009). Comparative study of human colonic tumor-derived endothelial cells (HCTEC) and normal colonic microvascular endothelial cells (HCMEC): hypoxia-induced sVEGFR-1 and sVEGFR-2 levels. *Oncol. Rep.* 21, 933–939.

Jones, E., Gallimore, A., and Ager, A. (2018). Defining high endothelial venules and tertiary lymphoid structures in Cancer. *Methods Mol. Biol.* 1845, 99–118. doi: 10.1007/978-1-4939-8709-2_7

Kambayashi, T., and Laufer, T. M. (2014). Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nat. Rev. Immunol.* 14, 719–730. doi: 10.1038/nri3754

Kanagawa, N., Yanagawa, T., Nakagawa, T., Okada, N., and Nakagawa, S. (2013). Tumor vessel-injuring ability improves antitumor effect of cytotoxic T lymphocytes in adoptive immunotherapy. *Cancer Gene Ther.* 20, 57–64. doi: 10.1038/cgt.2012.85

Karikoski, M., Marttila-Ichihara, F., Elima, K., Rantakari, P., Hollmén, M., Kelkka, T., et al. (2014). Clever-1/stabilin-1 controls cancer growth and metastasis. *Clin. Cancer Res.* 20, 6452–6464. doi: 10.1158/1078-0432.ccr-14-1236

Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., et al. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* 5, 74–80. doi: 10.1038/ni1013

Klein, D. (2018). The tumor vascular endothelium as decision maker in Cancer therapy. *Front. Oncol.* 8:367. doi: 10.3389/fonc.2018.00367

Knolle, P. A., and Wohlleber, D. (2016). Immunological functions of liver sinusoidal endothelial cells. *Cell Mol. Immunol.* 13, 347–353. doi: 10.1038/cmi.2016.5

Kondoh, M., Ohga, N., Akiyama, K., Hida, Y., Maishi, N., Towfik, A. M., et al. (2013). Hypoxia-induced reactive oxygen species cause chromosomal abnormalities in endothelial cells in the tumor microenvironment. *PLoS One* 8:e80349. doi: 10.1371/journal.pone.0080349

Kroeger, D. R., Milne, K., and Nelson, B. H. (2016). Tumor-infiltrating plasma cells are associated with tertiary lymphoid structures, cytolytic T-Cell responses, and superior prognosis in ovarian Cancer. *Clin. Cancer Res.* 22, 3005–3015. doi: 10.1158/1078-0432.ccr-15-2762

Laderach, D. J., Gentilini, L. D., Giribaldi, L., Delgado, V. C., Nugnes, L., Croci, D. O., et al. (2013). A unique galectin signature in human prostate cancer progression suggests galectin-1 as a key target for treatment of advanced disease. *Cancer Res.* 73, 86–96. doi: 10.1158/0008-5472.can-12-1260

Lambrechts, D., Wauters, E., Boeckx, B., Aibar, S., Nittner, D., Burton, O., et al. (2018). Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat. Med.* 24, 1277–1289. doi: 10.1038/s41591-018-0096-5

Lee, J. Y., Park, S., Kim, D. C., Yoon, J. H., Shin, S. H., Min, W. S., et al. (2013). A VEGFR-3 antagonist increases IFN-γ expression on low functioning NK cells in acute myeloid leukemia. *J. Clin. Immunol.* 33, 826–837. doi: 10.1007/s10875-013-9877-2

Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–689. doi: 10.1038/nri2156

Li, Y., Weng, Y., Zhong, L., Chong, H., Chen, S., Sun, Y., et al. (2017). VEGFR3 inhibition chemosensitizes lung adenocarcinoma A549 cells in the tumor-associated macrophage microenvironment through upregulation of p53 and PTEN. *Oncol. Rep.* 38, 2761–2773. doi: 10.3892/or.2017.5969

Lin, C. Y., Lovén, J., Rahl, P. B., Paranal, R. M., Burge, C. B., Bradner, J. E., et al. (2012). Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 151, 56–67. doi: 10.1016/j.cell.2012.08.026

Liu, F. T., and Rabinovich, G. A. (2005). Galectins as modulators of tumour progression. *Nat. Rev. Cancer* 5, 29–41. doi: 10.1038/nrc1527

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med.* 359, 378–390.

Lund, A. W., Duraes, F. V., Hirosue, S., Raghavan, V. R., Nembrini, C., Thomas, S. N., et al. (2012). VEGF-C promotes immune tolerance in B16 melanomas and cross-presentation of tumor antigen by lymph node lymphatics. *Cell Rep.* 1, 191–199. doi: 10.1016/j.celrep.2012.01.005

Luo, W., Hu, Q., Wang, D., Deeb, K. K., Ma, Y., Morrison, C. D., et al. (2013). Isolation and genome-wide expression and methylation characterization of CD31+ cells from normal and malignant human prostate tissue. *Oncotarget* 4, 1472–1483. doi: 10.18632/oncotarget.1269

Luther, S. A., Bidgol, A., Hargreaves, D. C., Schmidt, A., Xu, Y., Paniyadi, J., et al. (2002). Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J. Immunol.* 169, 424–433. doi: 10.4049/jimmunol.169.1.424

Ma, L., Hernandez, M. O., Zhao, Y., Mehta, M., Tran, B., Kelly, M., et al. (2019). Tumor cell biodiversity drives microenvironmental reprogramming in Liver Cancer. *Cancer Cell* 36, 418.e6–430.e6.

Maes, H., Kuchnio, A., Peric, A., Moens, S., Nys, K., De Bock, K., et al. (2014). Tumor vessel normalization by chloroquine independent of autophagy. *Cancer Cell* 26, 190–206. doi: 10.1016/j.ccr.2014.06.025

Maishi, N., Annan, D. A., Kikuchi, H., Hida, Y., and Hida, K. (2019). Tumor endothelial Heterogeneity in Cancer progression. *Cancers* 11:1511. doi: 10.3390/cancers11101511

Maishi, N., Ohba, Y., Akiyama, K., Ohga, N., Hamada, J., Nagao-Kitamoto, H., et al. (2016). Tumour endothelial cells in high metastatic tumours promote metastasis via epigenetic dysregulation of biglycan. *Sci. Rep.* 6:28039.

Markowska, A. I., Jefferies, K. C., and Panjwani, N. (2011). Galectin-3 protein modulates cell surface expression and activation of vascular endothelial growth factor receptor 2 in human endothelial cells. *J. Biol. Chem.* 286, 29913–29921. doi: 10.1074/jbc.m111.226423

Markowska, A. I., Liu, F. T., and Panjwani, N. (2010). Galectin-3 is an important mediator of VEGF- and bFGF-mediated angiogenic response. *J. Exp. Med.* 207, 1981–1993. doi: 10.1084/jem.20090121

Martinet, L., Garrido, I., Filleron, T., Le Guellec, S., Bellard, E., Fournie, J. J., et al. (2011). Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. *Cancer Res.* 71, 5678–5687. doi: 10.1158/0008-5472.can-11-0431

Martinet, L., Le Guellec, S., Filleron, T., Lamant, L., Meyer, N., Rochaix, P., et al. (2012). High endothelial venules (HEVs) in human melanoma lesions: major gateways for tumor-infiltrating lymphocytes. *Oncoimmunology* 1, 829–839. doi: 10.4161/onci.20492

Matsuda, K., Ohga, N., Hida, Y., Muraki, C., Tsuchiya, K., Kurosu, T., et al. (2010). Isolated tumor endothelial cells maintain specific character during long-term culture. *Biochem. Biophys. Res. Commun.* 394, 947–954. doi: 10.1016/j.bbrc.2010.03.089

Mattila, M. M., Ruohola, J. K., Karpanen, T., Jackson, D. G., Alitalo, K., and Härkönen, P. L. (2002). VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. *Int. J. Cancer* 98, 946–951. doi: 10.1002/ijc.10283

Meier, D., Bornmann, C., Chappaz, S., Schmutz, S., Otten, L. A., Ceredig, R., et al. (2007). Ectopic lymphoid-organ development occurs through interleukin 7-mediated enhanced survival of lymphoid-tissue-inducer cells. *Immunity* 26, 643–654. doi: 10.1016/j.immuni.2007.04.009

Mellman, I., Coukos, G., and Dranoff, G. (2011). Cancer immunotherapy comes of age. *Nature* 480, 480–489. doi: 10.1038/nature10673

Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E. A., et al. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N. Engl. J. Med.* 357, 2666–2676. doi: 10.1056/nejmoa072113

Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K., and McDonald, D. M. (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* 160, 985–1000. doi: 10.1016/s0002-9440(10)64920-6

Motz, G. T., Santoro, S. P., Wang, L. P., Garrabrant, T., Lastra, R. R., Hagemann, I. S., et al. (2014). Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. *Nat. Med.* 20, 607–615. doi: 10.1038/nm.3541

Motzer, R. J., Haas, N. B., Donskov, F., Gross-Goupil, M., Varlamov, S., Kopyltsov, E., et al. (2017). Randomized Phase III trial of adjuvant pazopanib versus placebo after nephrectomy in patients with localized or locally advanced renal cell carcinoma. *J. Clin. Oncol.* 35, 3916–3923. doi: 10.1200/jco.2017.73.5324

Motzer, R. J., Hutson, T. E., Tomczak, P., Michaelson, M. D., Bukowski, R. M., Rixe, O., et al. (2007). Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N. Engl. J. Med.* 356, 115–124.

Munn, D. H., and Mellor, A. L. (2016). IDO in the tumor microenvironment: inflammation, counter-regulation, and tolerance. *Trends Immunol.* 37, 193–207. doi: 10.1016/j.it.2016.01.002

Naito, H., Wakabayashi, T., Kidoya, H., Muramatsu, F., Takara, K., Eino, D., et al. (2016). Endothelial side population cells contribute to tumor angiogenesis and antiangiogenic drug resistance. *Cancer Res.* 76, 3200–3210. doi: 10.1158/0008-5472.can-15-2998

Nangia-Makker, P., Honjo, Y., Sarvis, R., Akahani, S., Hogan, V., Pienta, K. J., et al. (2000). Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am. J. Pathol.* 156, 899–909. doi: 10.1016/s0002-9440(10)64959-0

Nichol, D., and Stuhlmann, H. (2012). EGFL7: a unique angiogenic signaling factor in vascular development and disease. *Blood* 119, 1345–1352. doi: 10.1182/blood-2011-10-322446

Nummer, D., Suri-Payer, E., Schmitz-Winnenthal, H., Bonertz, A., Galindo, L., Antolovich, D., et al. (2007). Role of tumor endothelium in CD4+ CD25+ regulatory T cell infiltration of human pancreatic carcinoma. *J. Natl. Cancer Inst.* 99, 1188–1199. doi: 10.1093/jnci/djm064

Ohga, N., Ishikawa, S., Maishi, N., Akiyama, K., Hida, Y., Kawamoto, T., et al. (2012). Heterogeneity of tumor endothelial cells: comparison between tumor endothelial cells isolated from high- and low-metastatic tumors. *Am. J. Pathol.* 180, 1294–1307.

Ohm, J. E., and Carbone, D. P. (2001). VEGF as a mediator of tumor-associated immunodeficiency. *Immunol. Res.* 23, 263–272. doi: 10.1385/ir:23:2-3:263

Ohmura-Kakutani, H., Akiyama, K., Maishi, N., Ohga, N., Hida, Y., Kawamoto, T., et al. (2014). Identification of tumor endothelial cells with high aldehyde dehydrogenase activity and a highly angiogenic phenotype. *PLoS One* 9:e113910. doi: 10.1371/journal.pone.0113910

Ohtsu, A., Shah, M. A., Van Cutsem, E., Rha, S. Y., Sawaki, A., Park, S. R., et al. (2011). Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a randomized, double-blind, placebo-controlled phase III study. *J. Clin. Oncol.* 29, 3968–3976. doi: 10.1200/jco.2011.36.2236

Oliveira-Ferrer, L., Legler, K., and Milde-Langosch, K. (2017). Role of protein glycosylation in cancer metastasis. *Semin. Cancer Biol.* 44, 141–152. doi: 10.1016/j.semcancer.2017.03.002

Paiva, T. F., de Jesus, V. H., Marques, R. A., da Costa, A. A., de Macedo, M. P., Peresi, P. M., et al. (2015). Angiogenesis-related protein expression in bevacizumab-treated metastatic colorectal cancer: NOTCH1 detrimental to overall survival. *BMC Cancer* 15:643. doi: 10.1186/s12885-015-1648-4

Pober, J. S., and Sessa, W. C. (2007). Evolving functions of endothelial cells in inflammation. *Nat. Rev. Immunol.* 7, 803–815. doi: 10.1038/nri2171

Posch, F., Silina, K., Leibl, S., Mündlein, A., Moch, H., Siebenhüner, A., et al. (2018). Maturation of tertiary lymphoid structures and recurrence of stage II and III colorectal cancer. *Oncoimmunology* 7:e1378844. doi: 10.1080/2162402x.2017.1378844

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887. doi: 10.1016/j.cell.2011.08.039

Quail, D. F., and Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* 19, 1423–1437. doi: 10.1038/nm.3394

Ravaud, A., Motzer, R. J., Pandha, H. S., George, D. J., Pantuck, A. J., Patel, A., et al. (2016). Adjuvant sunitinib in high-risk renal-cell carcinoma after nephrectomy. *N. Engl. J. Med.* 375, 2246–2254.

Rocha, R., Torres, Á, Ojeda, K., Uribe, D., Rocha, D., Erices, J., et al. (2018). The Adenosine A3 receptor regulates differentiation of glioblastoma stem-like cells to endothelial cells under hypoxia. *Int. J. Mol. Sci.* 19:1228. doi: 10.3390/ijms19041228

Rohlenova, K., Goveia, J., García-Caballero, M., Subramanian, A., Kalucka, J., Treps, L., et al. (2020). Single-Cell RNA sequencing maps endothelial metabolic plasticity in pathological angiogenesis. *Cell Metab.* 31, 862.e14–877.e14.

Rossi, E., Sanz-Rodriguez, F., Eleno, N., Düwell, A., Blanco, F. J., Langa, C., et al. (2013). Endothelial endoglin is involved in inflammation: role in leukocyte adhesion and transmigration. *Blood* 121, 403–415. doi: 10.1182/blood-2012-06-435347

Ruddle, N. H. (2016). High endothelial venules and lymphatic vessels in tertiary lymphoid organs: characteristics. Functions, and regulation. *Front. Immunol.* 7:491. doi: 10.3389/fimmu.2016.00491

Rybstein, M. D., Bravo-San Pedro, J. M., Kroemer, G., and Galluzzi, L. (2018). The autophagic network and cancer. *Nat. Cell Biol.* 20, 243–251. doi: 10.1038/s41556-018-0042-2

Sandler, A., Gray, R., Perry, M. C., Brahmer, J., Schiller, J. H., Dowlati, A., et al. (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N. Engl. J. Med.* 355, 2542–2550.

Sautès-Fridman, C., Petitprez, F., Calderaro, J., and Fridman, W. H. (2019). Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat. Rev. Cancer* 19, 307–325. doi: 10.1038/s41568-019-0144-6

Schaaf, M. B., Garg, A. D., and Agostinis, P. (2018). Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis.* 9:115.

Schaaf, M. B., Houbaert, D., Meçe, O., and Agostinis, P. (2019). Autophagy in endothelial cells and tumor angiogenesis. *Cell Death Differ.* 26, 665–679. doi: 10.1038/s41418-019-0287-8

Schmittnaegel, M., Rigamonti, N., Kadioglu, E., Cassará, A., Wyser Rmili, C., Kiialainen, A., et al. (2017). Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci. Transl. Med.* 9:eaak9670. doi: 10.1126/scitranslmed.aak9670

Shiao, S. L., Kirkiles-Smith, N. C., Shepherd, B. R., McNiff, J. M., Carr, E. J., and Pober, J. S. (2007). Human effector memory CD4+ T cells directly recognize allogeneic endothelial cells in vitro and in vivo. *J. Immunol.* 179, 4397–4404. doi: 10.4049/jimmunol.179.7.4397

Sievert, W., Tapio, S., Breuninger, S., Gaipl, U., Andratschke, N., Trott, K. R., et al. (2014). Adhesion molecule expression and function of primary endothelial cells in benign and malignant tissues correlates with proliferation. *PLoS One* 9:e91808. doi: 10.1371/journal.pone.0091808

Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., et al. (2001). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.* 7, 192–198. doi: 10.1038/84643

Sleijfer, S., Ray-Coquard, I., Papai, Z., Le Cesne, A., Scurr, M., Schöffski, P., et al. (2009). Pazopanib, a multikinase angiogenesis inhibitor, in patients with relapsed or refractory advanced soft tissue sarcoma: a phase II study from the European organisation for research and treatment of cancer-soft tissue and bone sarcoma group (EORTC study 62043). *J. Clin. Oncol.* 27, 3126–3132. doi: 10.1200/jco.2008.21.3223

Socinski, M. A., Jotte, R. M., Cappuzzo, F., Orlandi, F., Stroyakovskiy, D., Nogami, N., et al. (2018). Atezolizumab for First-Line Treatment of Metastatic Nonsquamous NSCLC. *N. Engl. J. Med.* 378, 2288–2301.

Sökeland, G., and Schumacher, U. (2019). The functional role of integrins during intra- and extravasation within the metastatic cascade. *Mol. Cancer* 18:12.

Sonoshita, M., Aoki, M., Fuwa, H., Aoki, K., Hosogi, H., Sakai, Y., et al. (2011). Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. *Cancer Cell* 19, 125–137. doi: 10.1016/j.ccr.2010.11.008

Spinella, F., Rosanò, L., Di Castro, V., Natali, P. G., and Bagnato, A. (2002). Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. *J. Biol. Chem.* 277, 27850–27855. doi: 10.1074/jbc.m202421200

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., et al. (2000). Genes expressed in human tumor endothelium. *Science* 289, 1197–1202. doi: 10.1126/science.289.5482.1197

Stalin, J., Nollet, M., Garigue, P., Fernandez, S., Vivancos, L., Essaadi, A., et al. (2016). Targeting soluble CD146 with a neutralizing antibody inhibits vascularization, growth and survival of CD146-positive tumors. *Oncogene* 35, 5489–5500. doi: 10.1038/onc.2016.83

Sternberg, C. N., Davis, I. D., Mardiak, J., Szczylik, C., Lee, E., Wagstaff, J., et al. (2010). Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. *J. Clin. Oncol.* 28, 1061–1068. doi: 10.1200/jco.2009.23.9764

Streeter, P. R., Rouse, B. T., and Butcher, E. C. (1988). Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol.* 107, 1853–1862. doi: 10.1083/jcb.107.5.1853

Streubel, B., Chott, A., Huber, D., Exner, M., Jäger, U., Wagner, O., et al. (2004). Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. *N. Engl. J. Med.* 351, 250–259. doi: 10.1056/nejmoa033153

Tabernero, J., Yoshino, T., Cohn, A. L., Obermannova, R., Bodoky, G., Garcia-Carbonero, R., et al. (2015). Ramucirumab versus placebo in combination with second-line FOLFIRI in patients with metastatic colorectal carcinoma that progressed during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (RAISE): a randomised, double-blind, multicentre, phase 3 study. *Lancet Oncol.* 16, 499–508. doi: 10.1016/s1470-2045(15)70127-0

Takahashi, N., Kawanishi-Tabata, R., Haba, A., Tabata, M., Haruta, Y., Tsai, H., et al. (2001). Association of serum endoglin with metastasis in patients with colorectal, breast, and other solid tumors, and suppressive effect of chemotherapy on the serum endoglin. *Clin. Cancer Res.* 7, 524–532.

Tellier, C., Desmet, D., Petit, L., Finet, L., Graux, C., Raes, M., et al. (2015). Cycling hypoxia induces a specific amplified inflammatory phenotype in endothelial cells and enhances tumor-promoting inflammation in vivo. *Neoplasia* 17, 66–78. doi: 10.1016/j.neo.2014.11.003

Thijssen, V. L., Barkan, B., Shoji, H., Aries, I. M., Mathieu, V., Deltour, L., et al. (2010). Tumor cells secrete galectin-1 to enhance endothelial cell activity. *Cancer Res.* 70, 6216–6224. doi: 10.1158/0008-5472.can-09-4150

Thijssen, V. L., Postel, R., Brandwijk, R. J., Dings, R. P., Nesmelova, I., Satijn, S., et al. (2006). Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15975–15980. doi: 10.1073/pnas.0603883103

Thommen, D. S., and Schumacher, T. N. T. (2018). Cell Dysfunction in Cancer. *Cancer Cell* 33, 547–562. doi: 10.1016/j.ccell.2018.03.012

Tian, L., Goldstein, A., Wang, H., Ching, Lo H, Sun Kim, I., Welte, T., et al. (2017). . Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming. *Nature* 544, 250–254. doi: 10.1038/nature21724

Tilki, D., Kilic, N., Sevinc, S., Zywietz, F., Stief, C. G., and Ergun, S. (2007). Zone-specific remodeling of tumor blood vessels affects tumor growth. *Cancer* 110, 2347–2362. doi: 10.1002/cncr.23024

Ugel, S., De Sanctis, F., Mandruzzato, S., and Bronte, V. (2015a). Tumor-induced myeloid deviation: when myeloid-derived suppressor cells meet tumor-associated macrophages. *J. Clin. Invest.* 125, 3365–3376. doi: 10.1172/jci80006

Ugel, S., Facciponte, J. G., De Sanctis, F., and Facciabene, A. (2015b). Targeting tumor vasculature: expanding the potential of DNA cancer vaccines. *Cancer Immunol. Immunother.* 64, 1339–1348. doi: 10.1007/s00262-015-1747-8

Uldry, E., Faes, S., Demartines, N., and Dormond, O. (2017). Fine-Tuning Tumor Endothelial Cells to Selectively Kill Cancer. *Int. J. Mol. Sci.* 18:1401. doi: 10.3390/ijms18071401

Varney, M. L., and Singh, R. K. (2015). VEGF-C-VEGFR3/Flt4 axis regulates mammary tumor growth and metastasis in an autocrine manner. *Am. J. Cancer Res.* 5, 616–628.

Vokali, E., Yu, S. S., Hirosue, S., Rinçon-Restrepo, M., Duraes, V. F., Scherer, S., et al. (2020). Lymphatic endothelial cells prime naïve CD8+ T cells into memory cells under steady-state conditions. *Nat. Commun.* 11:538.

Voron, T., Colussi, O., Marcheteau, E., Pernot, S., Nizard, M., Pointet, A. L., et al. (2015). VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J. Exp. Med.* 212, 139–148. doi: 10.1084/jem.20140559

Wang, J., Taylor, A., Showeil, R., Trivedi, P., Horimoto, Y., Bagwan, I., et al. (2014). Expression profiling and significance of VEGF-A, VEGFR2, VEGFR3 and related proteins in endometrial carcinoma. *Cytokine* 68, 94–100. doi: 10.1016/j.cyto.2014.04.005

Wang, R., Chadalavada, K., Wilshire, J., Kowalik, U., Hovinga, K. E., Geber, A., et al. (2010). Glioblastoma stem-like cells give rise to tumour endothelium. *Nature* 468, 829–833. doi: 10.1038/nature09624

White, N. M., Masui, O., Newsted, D., Scorilas, A., Romaschin, A. D., Bjarnason, G. A., et al. (2014). Galectin-1 has potential prognostic significance and is implicated in clear cell renal cell carcinoma progression through the HIF/mTOR signaling axis. *Br. J. Cancer* 110, 1250–1259. doi: 10.1038/bjc.2013.828

Wieland, E., Rodriguez-Vita, J., Liebler, S. S., Mogler, C., Moll, I., Herberich, S. E., et al. (2017). Endothelial notch1 activity facilitates metastasis. *Cancer Cell* 31, 355–367. doi: 10.1016/j.ccell.2017.01.007

Wu, F., Yang, L. Y., Li, Y. F., Ou, D. P., Chen, D. P., and Fan, C. (2009). Novel role for epidermal growth factor-like domain 7 in metastasis of human hepatocellular carcinoma. *Hepatology* 50, 1839–1850. doi: 10.1002/hep.23197

Yadav, A., Kumar, B., Yu, J. G., Old, M., Teknos, T. N., and Kumar, P. (2015). Tumor-associated endothelial cells promote tumor metastasis by chaperoning circulating tumor cells and protecting them from Anoikis. *PLoS One* 10:e0141602. doi: 10.1371/journal.pone.0141602

Yang, H., Lee, W. S., Kong, S. J., Kim, C. G., Kim, J. H., Chang, S. K., et al. (2019). STING activation reprograms tumor vasculatures and synergizes with VEGFR2 blockade. *J. Clin. Invest.* 130, 4350–4364. doi: 10.1172/jci125413

Yang, Z. S., Xu, Y. F., Huang, F. F., and Ding, G. F. (2014). Associations of nm23H1, VEGF-C, and VEGF-3 receptor in human prostate cancer. *Molecules* 19, 6851–6862. doi: 10.3390/molecules19056851

Yeh, Y. W., Cheng, C. C., Yang, S. T., Tseng, C. F., Chang, T. Y., Tsai, S. Y., et al. (2017). Targeting the VEGF-C/VEGFR3 axis suppresses Slug-mediated cancer metastasis and stemness via inhibition of KRAS/YAP1 signaling. *Oncotarget* 8, 5603–5618. doi: 10.18632/oncotarget.13629

Yu, J. W., Wu, S. H., Lu, R. Q., Wu, J. G., Ni, X. C., Zhou, G. C., et al. (2013). Expression and significances of contactin-1 in human gastric cancer. *Gastroenterol. Res. Pract.* 2013:210205.

Zhang, L., Conejo-Garcia, J. R., Katsaros, D., Gimotty, P. A., Massobrio, M., Regnani, G., et al. (2003). Intratumoral T cells, recurrence, and survival

in epithelial ovarian cancer. *N. Engl. J. Med.* 348, 203–213. doi: 10.1056/nejmoa020177

Zhao, Q., Eichten, A., Parveen, A., Adler, C., Huang, Y., Wang, W., et al. (2018). Single-Cell transcriptome analyses reveal endothelial cell heterogeneity in tumors and changes following antiangiogenic treatment. *Cancer Res.* 78, 2370–2382. doi: 10.1158/0008-5472.can-17-2728

Zhao, X. Y., Zhao, K. W., Jiang, Y., Zhao, M., and Chen, G. Q. (2011). Synergistic induction of galectin-1 by CCAAT/enhancer binding protein alpha and hypoxia-inducible factor 1alpha and its role in differentiation of acute myeloid leukemic cells. *J. Biol. Chem.* 286, 36808–36819. doi: 10.1074/jbc.m111.247262

Zheng, C., Qiu, Y., Zeng, Q., Zhang, Y., Lu, D., Yang, D., et al. (2009). Endothelial CD146 is required for in vitro tumor-induced angiogenesis: the role of a disulfide bond in signaling and dimerization. *Int. J. Biochem. Cell Biol.* 41, 2163–2172. doi: 10.1016/j.biocel.2009.03.014

Zhu, G., Huang, Q., Zheng, W., Huang, Y., Hua, J., Yang, S., et al. (2016). LPS Upregulated VEGFR-3 expression promote migration and invasion in colorectal Cancer via a mechanism of increased NF-κB binding to the promoter of VEGFR-3. *Cell Physiol. Biochem.* 39, 1665–1678. doi: 10.1159/000447868

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 05 November 2020
doi: 10.3389/fcell.2020.594903

In vitro 3D Systems to Model Tumor Angiogenesis and Interactions With Stromal Cells

*Noémie Brassard-Jollive[1,2], Catherine Monnot[1], Laurent Muller[1] and Stéphane Germain[1]**

[1] Center for Interdisciplinary Research in Biology, College de France, CNRS UMR 7241, INSERM U1050, PSL Research University, Paris, France, [2] Sorbonne Université, Collège Doctoral, Paris, France

In vitro 3D culture systems provide promising tools for screening novel therapies and understanding drug resistance mechanisms in cancer because they are adapted for high throughput analysis. One of the main current challenges is to reproducibly culture patient samples containing cancer and stromal cells to faithfully recapitulate tumor microenvironment and move toward efficient personalized medicine. Tumors are composed of heterogeneous cell populations and characterized by chaotic vascularization in a remodeled microenvironment. Indeed, tumor angiogenesis occurs in a complex stroma containing immune cells and cancer-associated fibroblasts that secrete important amounts of cytokines, growth factors, extracellular vesicles, and extracellular matrix (ECM). This process leads to the formation of inflated, tortuous, and permeable capillaries that display deficient basement membrane (BM) and perivascular coverage. These abnormal capillaries affect responses to anti-cancer therapies such as anti-angiogenic, radio-, and immunotherapies. Current pre-clinical models are limited for investigating interactions between tumor cells and vascularization during tumor progression as well as mechanisms that lead to drug resistance. *In vitro* approaches developed for vascularization are either the result of engineered cell lining or based on physiological processes including vasculogenesis and sprouting angiogenesis. They allow investigation of paracrine and direct interactions between endothelial and tumor and/or stromal cells, as well as impact of biochemical and biophysical cues of the microenvironment, using either natural matrix components or functionalized synthetic hydrogels. In addition, microfluidic devices provide access to modeling the impact of shear stress and interstitial flow and growth factor gradients. In this review, we will describe the state of the art co-culture models of vascularized micro-tumors in order to study tumor progression and metastatic dissemination including intravasation and/or extravasation processes.

Keywords: 3D micro-tumors, microfluidic, angiogenesis, tumor microenvironment, tumor–stromal cell interactions, personalized medicine

OPEN ACCESS

Edited by:
Lucas Treps,
VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Enrique Boccardo,
University of São Paulo, Brazil
Ana Paula Lepique,
University of São Paulo, Brazil

***Correspondence:**
Stéphane Germain
stephane.germain@college-de-france.fr

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *14 August 2020*
Accepted: *05 October 2020*
Published: *05 November 2020*

Citation:
Brassard-Jollive N, Monnot C, Muller L and Germain S (2020) In vitro 3D Systems to Model Tumor Angiogenesis and Interactions With Stromal Cells. Front. Cell Dev. Biol. 8:594903. doi: 10.3389/fcell.2020.594903

INTRODUCTION

Angiogenesis in the Tumor Microenvironment, a Multidimensional Process

Understanding the mechanisms that govern tumor initiation, development, and response to therapy is one of the most obvious and challenging tasks in our quest to fight cancer. Every cancer originates from a single cell but tumors rapidly progress as heterogeneous populations of cells, since single cells acquire genetic and phenotypic differences from each other during expansion of the neoplastic cell population. In addition, oncogenes and tumor suppressor genes tend to show strong context specificity that depends on the tumor type, the cell-of-origin, and the environmental factors (Schneider et al., 2017). The desmoplastic reaction of the stroma, consisting of basement membrane (BM), extracellular matrix (ECM), fibroblasts, immune cells, and vasculature, can distinguish some tumor entities from other tumor types, such as sarcomas, even if they are driven by the same oncogene (Tomlinson et al., 1999). These distinct cell types establish complex dialogs that comprise both homotypic and heterotypic interactions. In addition, these cells secrete growth factors and cytokines, synthesize, and remodel a complex ECM that exerts physical constraints. These biochemical and mechanical signals are major components of a specific tumor microenvironment that profoundly affects tumor progression by modulating tumor cell proliferation but also by inducing angiogenesis aimed at providing oxygen and nutrients. Indeed, solid tumors depend on angiogenesis for growth and metastasis and numerous efforts have therefore been undertaken in the last decades to develop tools that target angiogenesis, some of which have been clinically developed. Nevertheless, these drugs often lack associated biomarkers of response and efficacy and might also be associated with acquisition of drug resistance and therapeutic failure in a long-term perspective. Therefore, reconstituting such a complex process as cancer and tumor angiogenesis *in vitro* is still rather technically challenging and represents an unmet medical need that must be addressed.

Current Angiogenesis Models and Their Limitations

Basement membrane extracts from Engelbreth-Holm-Swarm (EHS) mouse tumor cells, such as Matrigel^{TM}, have been extensively used for the so-called "tube formation assay" in order to investigate angiogenesis (Arnaoutova et al., 2009). This easy-to-perform assay is the most widely used *in vitro* angiogenesis assay. It should, however, be understood that the tube-like or capillary-like *in vitro* assays using endothelial cells (EC) plated on top of BM extract are not considered as actual angiogenesis models by the community since neither their structure nor the mechanisms of their formation are physiologically relevant (Simons et al., 2015; Nowak-Sliwinska et al., 2018). Furthermore, many mesenchymal cell types, including fibroblasts and smooth muscle cells, also organize in networks in response to the matrix alignment generated by tension forces of cellular traction (Vernon et al., 1992). These assays are commercially successful but nevertheless insufficient to address the complexity of tumor angiogenesis and only made it more important to develop relevant 3D models. Whereas more advanced *in vivo* and *in vitro* models aimed at mimicking tumor angiogenesis have led to the discovery of novel therapies, most of these have nevertheless failed in clinical trials, thus shedding light on the numerous limitations of current pre-clinical models. Angiogenesis actually undergoes multiple discrete steps that can be individually evaluated and quantified by a large number of bioassays that have been reviewed elsewhere (Nowak-Sliwinska et al., 2018). In this review, we will focus on integrated assays aimed at properly reproducing the morphogenetic events of the formation of new capillaries.

IN VITRO 3D SYSTEMS TO MODEL TUMOR AND STROMAL CELL INTERACTIONS WITH CAPILLARIES

Diversification of *in vitro* 3D culture methods including culture supports, cells, imaging, and quantification has led to a great diversity of models. Here, we will survey the existing 3D models and highlight those that are urgently needed in order to fill the gap between 2D models and animal models of human disease, and that could help the research community to address the high attrition rates in drug development and to fulfill the transition toward personalized medicine.

Relevant *in vitro* models of capillary formation recapitulate many of the steps of angiogenesis, including EC migration and proliferation, lumen formation, branching, and anastomosis (Nakatsu et al., 2003; Nakatsu and Hughes, 2008). Indeed, angiogenesis- and vasculogenesis-based methods allow the formation of functional capillaries displaying adherens and tight junctions containing VE-cadherin/β-catenin complexes and Zonula occludens-1 (ZO-1), respectively, as well as accurate apical-basal polarity characterized by the abluminal deposition of BM components including laminin and collagen IV (Nowak-Sliwinska et al., 2018; Marchand et al., 2019).

3D Assays of Capillary Formation

Endothelial Cells

The use of EC cultures for engineering capillaries remains an experimental challenge. The most commonly used cells are human umbilical EC (HUVEC) (Nowak-Sliwinska et al., 2018). Other primary sources of human EC are aortic or microvascular EC derived from various tissues, most frequently from skin, defined as human dermal microvascular EC (HDMEC). Endothelial progenitor cells (EPC) harvested as endothelial colony-forming cells (ECFC) from cord blood can also be used, but those from adult peripheral blood exhibit limited proliferation potential (Ferratge et al., 2017). Recently, Palikuqi et al. (2020) reported "reset" vascular EC that transiently express ETS variant transcription factor 2 (ETV2) and that self-assemble into vascular networks and arborized cancerous human colon organoids. Quite remarkably, only cells from human origin are used in relevant 3D models; while isolation and culture of mouse EC have been developed for years now, their ability to form

capillaries has proven to be more challenging (Nowak-Sliwinska et al., 2018). As example, mouse EC from the spleen stroma were used to explore the tumor-EC crosstalk in a 3D scaffold (Furlan et al., 2018). These cells were organized in elongated tubule-like structures but barely formed capillaries. Furthermore, neither human nor murine tumor EC have been reported to generate capillaries in 3D models, whether co-cultured with tumor cells or not.

Sprouting Angiogenesis- and Vasculogenesis-Based Assays

Two capillary morphogenesis models performed in 3D recapitulate sprouting angiogenesis and vasculogenesis processes.

The bead and spheroid assays are relevant 3D models for investigating the mechanisms of sprouting angiogenesis (Nakatsu et al., 2003; Heiss et al., 2015). In the first assay, EC are seeded on polysaccharide beads before embedding in hydrogel and co-culture with fibroblasts to promote sprouting and formation of lumen-containing capillaries within a few days (Nowak-Sliwinska et al., 2018). These capillaries are led by tip cells, while their stalk cells are polarized and deposit BM. In the second assay, EC spheroids are embedded in a hydrogel that supports the formation of lumenized capillary-like structures where tip cells precede the elongation of individual sprouts originating from single spheroids (Heiss et al., 2015). These 3D models have been used for investigating the involvement of enzymes that remodel the microenvironment (Beckouche et al., 2015; Umana-Diaz et al., 2020) and factors secreted by tumor or stromal cells at several steps of sprouting angiogenesis (Welti et al., 2011; Ramer et al., 2014; Hernandez-Fernaud et al., 2017) (**Figure 1**). The impact of anti-angiogenic drugs is also increasingly explored using these models (Welti et al., 2011; Ramer et al., 2014; Heiss et al., 2015).

Vasculogenesis occurs via EC properties of self-assembling into a 3D vascular network. Single EC are seeded in collagen I hydrogels and cultured in presence of VEGF, FGF-2, or fibroblast-conditioned medium in order to allow formation of lumen-containing capillaries within 4–5 days (Nowak-Sliwinska et al., 2018). The 3D vasculogenesis assay is appropriate for studying interactions between tumor and EC and engineering vascularized micro-tumors (Chen et al., 2013, 2016; Kim et al., 2013; Bray et al., 2015; Jeon et al., 2015; Sobrino et al., 2016; Taubenberger et al., 2016). In addition, co-culture with stromal pericytes in the hydrogel recapitulates a key step of vascular morphogenesis consisting in their perivascular recruitment (Baudino, 2013). This assay is adapted to the study of tumor angiogenesis when isolated cancer cells or spheroids are co-embedded with sparse EC in the hydrogel. Interestingly, number and shape of capillaries can be tuned via modulating the density of the microenvironment, either in collagen or fibrin (see below, Ghajar et al., 2008).

Biochemical and Biophysical Properties of Tumor Microenvironment Controlled in the 3D Gels

For engineering tumor microenvironment, a variety of natural and synthetic materials have been used as 3D scaffolds (Song et al., 2014). An important requirement for these scaffolds is their capacity to support simultaneously cancer cell proliferation/invasion and angiogenesis. Indeed, hydrogels have the ability to recapitulate many features of native ECM concerning their composition (either simple or composite), physical properties (stiffness, pore size, fiber length. . .), and biological functions (growth factors bioavailability, cell adhesion machinery. . .) (Li and Kumacheva, 2018). Modulation of these parameters does not only induce global changes of the 3D scaffold but also local modifications of the topology of cell microenvironment (Yamada and Sixt, 2019). Selection of the scaffold and associated culture medium is therefore a crucial step in engineering *in vitro* micro-tumors and their vasculature.

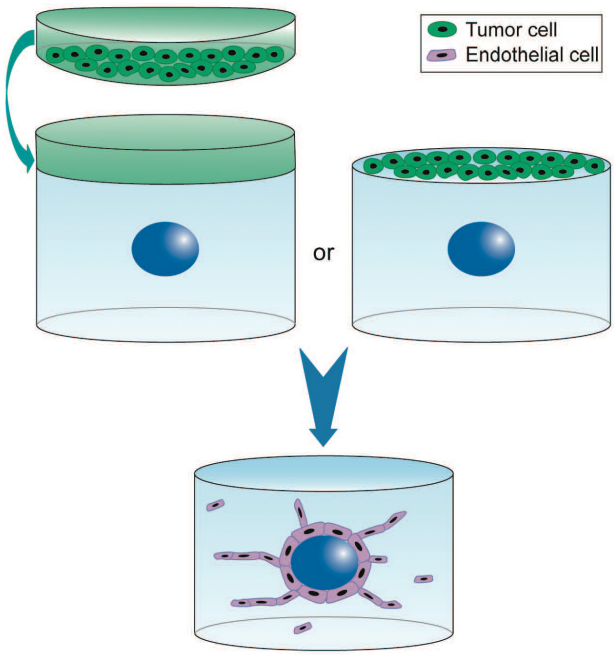


FIGURE 1 | 3D experimental settings for paracrine regulation of tumor cells on sprouting angiogenesis using the 3D angiogenesis assay. Endothelial cells are seeded on Cytodex beads (blue) and embedded in fibrin hydrogel. Culture is performed in presence of tumor cell conditioned medium (green) or tumor cells directly seeded on top of the hydrogel. EC proliferation and migration depend on the tumor paracrine regulation (Ramer et al., 2014). 3D cell culture is performed up to 6 days (blue arrow). This model is also used with endothelial spheroids. However, the absence of fibroblast contribution as much as the tumor paracrine effect is responsible for the chaotic structures often observed that barely form capillaries.

Natural Polymers for Hydrogels

Protein-based hydrogels consist in either purified single components of matrix proteins, including fibrin and collagen I or in crude extracts of natural matrices (Song et al., 2014; Li and Kumacheva, 2018).

Basement membrane extracts have been the most widely used biomaterial for many years, supporting growth for many tumor cell lines (Song et al., 2014). Assays combining 3D cultures of tumor spheroids overlayed with EC have thus also

been performed (Benton et al., 2015) but do not faithfully reproduce angiogenesis (see above). Furthermore, even when used for actual 3D culture of EC, Matrigel^{TM} was demonstrated to induce formation of multicellular tubes that display an inversed luminal/abluminal polarization with deposition of BM components like laminin α4 in the lumen (Kuèera et al., 2009). Quite remarkably, using mixtures of Matrigel^{TM} with natural polymers like fibrin turned out to noticeably improve capillary formation and resulted in efficient inosculation with the host vasculature and further blood perfusion after implantation in mice (Laib et al., 2009). Recently, Palikuqi et al. (2020) developed matrices made of mixture of laminin, entactin, and collagen IV that support vasculogenesis.

Polymerization of fibrin occurs from fibrinogen cleavage by thrombin and is controlled by calcium ions, temperature, and fibrinogen concentration. Fibrin hydrogels have been widely used for investigating angiogenesis in 3D settings since their nano/macro fibrous architecture mimics native ECM. They provide robust support for both sprouting angiogenesis (Nakatsu et al., 2003; Ghajar et al., 2006, 2008) and vasculogenesis (Rao et al., 2012). Ghajar et al. (2006) reported that fibrin hydrogel, at the concentration of 2.5 mg/ml, induces capillary network formation. Using a bead assay, the same authors further demonstrated that increasing fibrin density from 2.5 to 10 mg/ml leads to a decrease in capillary network formation. Soft fibrin gels of 90 Pa-stiffness corresponding to low density at 1 mg/ml were optimal for cancer cell proliferation and spheroid formation when compared to stiffer gels (Liu et al., 2012). These studies thus indicate that the fibrin hydrogel provides a relevant 3D scaffold for studying *in vitro* micro-tumors and their vasculature.

Collagen I, the major protein of the interstitial ECM (Blache and Ehrbar, 2017), is a biocompatible and biodegradable material that possesses the intrinsic capacity to self-assemble into hydrogels under physiological conditions (Sorushanova et al., 2019). Collagen gels can also be produced using multiple cross-linking methods resulting in fibrous architectures similar to those of native ECM. *In vitro* studies have demonstrated that collagen gel density and porosity are modulated by pH, temperature, and collagen concentration through modulation of fibrillogenesis, therefore offering ways to control structural properties (Doyle et al., 2015; Sapudom et al., 2015). Collagen hydrogels provide bioactive microenvironments supporting cellular processes, such as cell adhesion sites, proteolytic-degradable sites, and ECM crosslinking (Laib et al., 2009). Several *in vitro* studies have demonstrated that collagen hydrogels support capillary network formation using 3D vasculogenesis or EC spheroid assays (Heiss et al., 2015; Blache and Ehrbar, 2017). Collagen I also supports invasion and growth of numerous tumor cell lines (Jeong et al., 2016). MDA-MB 231 breast cancer cells cultured within collagen hydrogel formed spheroids displaying necrotic and hypoxic core (Szot et al., 2011; Song et al., 2014). Using collagen I of various origin (bovine dermal and rat tail), Wolf et al. (2013) demonstrated that matrix metalloproteinase (MMP)-independent migration of tumor cells into 3D hydrogel depends both on deformation of the nucleus and scaffold porosity in response to space constraints without any correlation with scaffold stiffness.

Natural materials have numerous limitations, though. They suffer from variability, either batch-to-batch or according to species or tissue differences as well as purification protocols. They provide limited control of ECM compound concentration and presence of degradation- or adhesion-sites. They thus fail to provide full control of both matrix density and stiffness (Song et al., 2014).

Synthetic Hydrogel Materials

Among the many synthetic biomaterials used for 3D cell culture (Blache and Ehrbar, 2017), polyethylene glycol-heparin (PEG) is one of the most commonly used for the development of micro-tumors (Bray et al., 2015; Chwalek et al., 2015; Roudsari et al., 2016; Taubenberger et al., 2016). PEG hydrogel aims to mimic the natural ECM and to offer control of its design and synthesis (Lutolf and Hubbell, 2005; Blache and Ehrbar, 2017). Scaffolds are generated using multi-armed PEG and heparin crosslinked via cytocompatible Michael-type addition that enables cell embedding in 3D (Chwalek et al., 2015). A major advantage of these synthetic scaffolds is the possibility to decouple mechanical and biochemical properties. Indeed, increasing PEG (or PEG-heparin) concentration allows to control mechanical properties over a broad range, while maintaining the heparin concentration constant. In addition, PEG can be functionalized for (i) reversible binding of multiple growth factors (VEGF, FGF-2, SDF-1), (ii) covalent conjugation of adhesion ligands (RGD motifs), or (iii) proteolytic sites (Kraehenbuehl et al., 2008; Bray et al., 2015; Chwalek et al., 2015; Blache et al., 2016; Taubenberger et al., 2016; Blache and Ehrbar, 2017). These PEG hydrogels support capillary network formation *in vitro*. Indeed, in several studies, co-culture of EC and mesenchymal cells leads to formation of capillaries displaying lumens, BM deposition (laminin and collagen IV), and perivascular localization of mesenchymal cells (Blache et al., 2016; Blache and Ehrbar, 2017). These vessels recapitulate many features of angiogenesis *in vitro* and are similar to those formed in natural hydrogels. Moreover, PEG hydrogels support 3D tumor spheroid formation and growth of a hepatocellular carcinoma (HCC) cell line (Liang et al., 2011) and of various others such as breast, prostate, and lung cancer cell lines (Bray et al., 2015; Roudsari et al., 2016; Taubenberger et al., 2016). This 3D scaffold is therefore readily available for the *in vitro* study of micro-tumors and their vasculature.

Static 3D Co-culture to Recapitulate Tumor–Stromal Cell Interactions With Capillaries

To date, multiple *in vitro* static 3D models have been engineered to recapitulate heterotypic interactions between tumor cells and capillaries. These are based on a large diversity of strategies using different scaffolds as well as tumor–stromal cell sources and methods for capillary formation. As described above, natural and synthetic scaffolds or tissue constructs are essential to support these cell interactions. Tumor cells are mainly human cell lines from breast, pancreatic, colorectal, skin, lung, and liver cancers cultured as single cells or in spheroids. Moreover, these models require stromal cells [e.g., fibroblasts, mesenchymal stem cells

(MSC)] or stromal conditioned medium since tumor cells alone do not support capillary formation. Basically, two types of approaches have been performed using vascularized spheroids in 3D hydrogels or vascularized tissue constructs.

Vascularized Spheroids in 3D Hydrogels

Cellular interactions

To recapitulate interactions between tumor cells and capillaries, Ehsan et al. developed vascularized tumor spheroids using various cancer cell lines from breast, lung, and colon. Multicellular spheroids containing tumor and EC were generated using the liquid overlay method and were co-embedded with isolated fibroblasts in fibrin hydrogels (Ehsan et al., 2014). The vascularized tumor spheroids exhibited sprouting angiogenesis, generating a capillary network that extends into the surrounding matrix within 7 days of culture (**Figure 2A**). In the case of colon cancer cell lines, capillaries localized within the spheroid have a distinct morphology and exhibit shorter, more branched, and irregular phenotype than capillaries that extend into the hydrogel. Interestingly, this model recapitulates specific pro-angiogenic capacities according to the type of tumor cell lines. Indeed, vascularized spheroids of breast tumor cell lines (MCF and MDA MB-231) exhibit more capillaries than those of lung or colon tumor cell lines. Quite surprisingly, co-seeding tumor cells, however, does not affect extent nor kinetics of sprouting and growth of the capillary network in the fibrin hydrogel. In addition, authors report that tumor cell-specific intravasation occurs in this model, as they detect SW620 cells localized and migrating in the capillary lumen, which is enhanced by low oxygen condition. It is, however, not determined whether presence of tumor cells in the lumen corresponds to actual trans-endothelial intravasation or engulfment of tumor cells upon initiation of capillary formation. This model nonetheless recapitulates tumor angiogenesis and interactions of tumor cells with capillaries in a fibroblast-containing hydrogel.

Bray et al. (2015) also investigated the interactions between tumor spheroids and capillaries by co-culture with MSC seeded in suspension in a synthetic hydrogel. First, spheroids were formed in hydrogel through proliferation of isolated tumor cells. After 7 days of culture, spheroids were harvested by collagenase treatment and seeded in a newly formed hydrogel containing both EC and MSC (**Figure 2B**). This protocol recapitulates the first avascular growing step of the tumor mass but requires a tough collagenase-based treatment for the co-culture model. Unlike the model described above (Ehsan et al., 2014), capillaries sprouted toward the tumor spheroids and did not invade the core of the spheroid but only established contacts at the spheroid surface (Bray et al., 2015).

Whereas both studies used spheroids devoid of invasive capacities, applying invasive spheroids in such models would be of major interest to monitor interactions between tumor protrusions and capillaries in the surrounding hydrogel in order to assess potential co-option processes.

ECM interactions

Models of vascularized spheroids in natural hydrogels effectively recapitulate cellular interactions within a 3D environment but

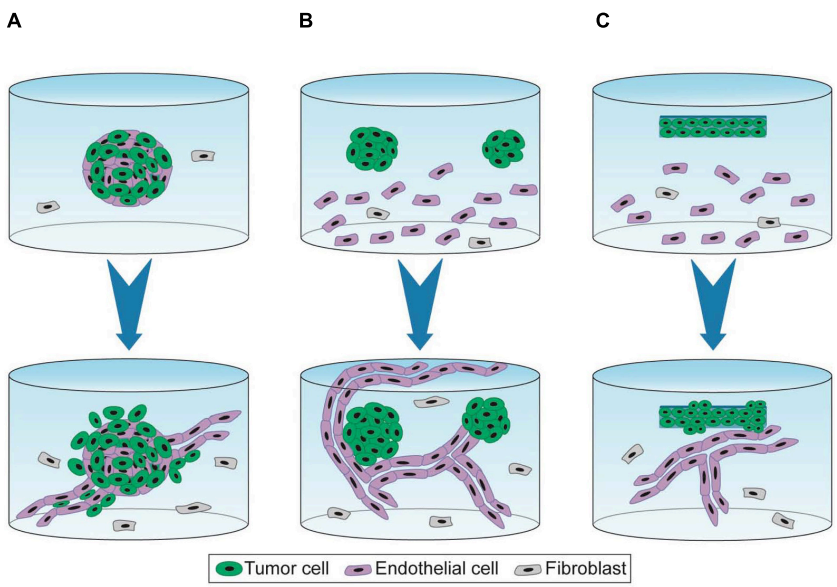


FIGURE 2 | 3D experimental settings of capillary formation and tumor growth. 3D cell co-culture is performed for 7 days **(A)**, 7–10 days **(B)**, and 10–21 days **(C)** (blue arrow). **(A)** Spheroids containing tumor and endothelial cells are embedded in fibrin hydrogel containing fibroblasts in suspensions. Capillaries are formed by a sprouting angiogenesis-mimicking process and grow away from the tumor mass (Ehsan et al., 2014). **(B)** Spheroid cells are embedded in functionalized PEG-derived hydrogel containing EC and fibroblasts in suspension. Capillaries formed by vasculogenesis, unfold the tumor mass but barely vascularize it (Bray et al., 2015; Taubenberger et al., 2016). **(C)** Dense artificial cancer mass (ACM) is obtained by plastic compression of collagen hydrogel containing tumor cells. ACM is then embedded in collagen hydrogel containing EC and fibroblasts in suspension. This setting is further compressed. Capillaries are formed by vasculogenesis (Magdeldin et al., 2017; Pape et al., 2019).

are limited for studying interactions between tumor cells and specific ECM proteins. Cell–ECM interactions play a major role in tumor growth, invasion, and angiogenesis. ECM proteins such as collagen I, fibronectin, and laminin are major components of the stromal microenvironment (Provenzano et al., 2006; Schedin and Keely, 2011). As mentioned above, synthetic hydrogels functionalized with specific cell adhesion domains of these matrix proteins are also useful tools to recapitulate a wide range of ECM interactions. Taubenberger et al. (2016) investigated the involvement of specific cell adhesion domains on interactions between tumor cells organized in spheroids and capillaries. For this purpose, an integrin binding domain present in fibronectin and vitronectin (RGD), a laminin-111 derived adhesion peptide (IKVAV), and an integrin binding domain present in collagen I (GFOGER) were conjugated to PEG hydrogel. Tumor spheroid formation and co-inclusion with EC and MSC in these functionalized hydrogels were performed as in Bray et al. (2015). While interactions between tumor cells and capillaries were observed in each condition, both tumor spheroid invasiveness and tumor-EC interactions inside spheroids increased only in IKVAV and GFOGER functionalized hydrogels. This 3D culture model therefore constitutes an interesting tool to decipher the effect of ECM interactions on tumor cell growth, invasion, and angiogenesis.

Composition but also density of the ECM are crucial features that impact cell proliferation/invasion and angiogenesis. To modulate the ECM density, a strategy named plastic compression consists in removing water from collagen hydrogel containing tumor cells (Magdeldin et al., 2017; Pape et al., 2019). This dense artificial cancer mass (ACM) is then seeded in collagen hydrogel supplemented with laminin and containing fibroblasts and EC, thereby mimicking the stromal compartment, before further compression. ECM density modulated by plastic compression as well as ECM composition of the stroma modulated by laminin concentrations both regulate cancer cell invasion (**Figure 2C**). A high density of collagen I is associated with increased tumor cell invasion. Moreover, presence of laminin stimulated both tumor cell invasion and vascular network formation. Authors reported that EC seeded in the stromal compartment formed "healthy" and "tumorigenic" vascular networks in absence or in presence of colorectal tumor cells, respectively (Magdeldin et al., 2017). Pape et al. (2019) compared two colorectal tumor cell lines (HT29 and HCT116) displaying distinct invasive properties. Authors reported that HCT116 was more invasive than HT29, displaying an increased distance and surface area of invasion into the stromal compartment. Spheroids containing highly invasive cells form less complex and less branched vascular networks.

Vascularized Tissue Constructs

3D *in vitro* models described above recapitulate the crosstalk between tumor cells, stromal microenvironment, and vessels but none of them present a tissue-specific environment including both blood and lymphatic capillaries. Indeed, the vast majority of articles published up to now, aiming to recapitulate interactions between tumor cells and capillaries, focus on blood vessels. However, it is commonly accepted that lymphatic vessels are essential for tumor spread and metastasis (Stacker et al., 2014). Furthermore, formation of functional lymphatic capillaries has been documented for some years now either within 3D hydrogel (Montaño et al., 2010; Marino et al., 2014) or in skin constructs (Gibot et al., 2016, 2017) using HDMEC or lymphatic EC (LEC) isolated from human foreskin. These capillaries are positive for Prox1 (lymphatic-specific transcription factor) and CD31 staining thereby confirming their lymphatic nature. To recapitulate specific interactions between tumor cells and lymphatic capillaries, stromal tissues as well as skin constructs were engineered by the layer-by-layer technique (Bourland et al., 2018; Nishiguchi et al., 2018). Using this approach, the authors investigated: (i) intravasation into lymphatic capillaries, (ii) MMP secretion profiles, (iii) response to therapies.

The stromal tissue developed by Nishiguchi et al. is composed of fibroblasts synthetizing their own ECM and of EC/LEC self-assembled in blood and lymph capillaries as originally described by Decher (1997). Single cancer cells are seeded onto these tissues to form primary micro-tumors. For studying intravasation in blood or lymphatic capillaries, pancreatic and colorectal cell lines displaying hematogenous (MiaPaCa-2 cells) or lymphogenous metastasis (BxPC3 cells) were used. The specificity of these cell lines for each type of vessel was indeed reproduced *in vitro* (Nishiguchi et al., 2018). Interestingly, tumor cells displayed distinct MMP secretion profiles according to their invasive properties and target vasculatures: hematogenous cells secreted MMP-1, -2, -3, and -10 while lymphogenous cells secreted MMP-1, -2, and -9. This model is thus relevant for recapitulating specific interactions of tumor cells displaying hematogenous and lymphogenous metastasis properties, tumor invasion, and intravasation/extravasation.

To mimic the melanoma microenvironment, Bourland et al. (2018) engineered a skin construct using the self-assembly method. This model mimics the human skin morphology characterized by a dermis enriched in ECM components, a differentiated epidermis composed by a proliferative basal layer and multiple suprabasal layers resulting in a *stratum corneum*. Microvascular EC self-organized to form two distinct capillary networks, one of lymphatic vessels positive for podoplanin and another of blood vessels positive for CD31 but negative for podoplanin. Melanoma spheroids integrated at the dermo-epidermal junction invaded the epidermal layer and localized in close contact with lymphatic capillaries. Chronic treatment with Vemurafenib (Zelboraf®), a selective inhibitor of BRAF frequently used to treat melanoma bearing the BRAF-V600E mutation, affected tumor cell proliferation and apoptosis, thereby recapitulating changes in tumor morphology, without leading to any toxicity in the surrounding tissue. Interestingly, some tumor cells localized in close contact with fibroblasts continue to proliferate even after 12 days of treatment suggesting an effect of the microenvironment on therapeutic resistance.

All in all, these approaches better recapitulate the global tissue organization than vascularized spheroids in 3D hydrogel.

Conclusion

Culture of vascularized tumor spheroids in hydrogel is suitable to mimic interactions between tumor cells and capillaries. Moreover, using functionalized synthetic scaffolds, 3D hydrogel

co-culture allows to study the impact of ECM proteins on tumor angiogenesis. Tissue constructs recapitulate the global tissue organization to create a more complex microenvironment than 3D culture in hydrogel. **Figure 1** illustrates some 3D experimental settings of capillary formation and tumor growth. **Table 1** is summing up the main features and applications of the static 3D models described above. Improvement of these models will consist in inclusion of other stromal cell types. Indeed, while fibroblasts are the major cell type used in these models, many other stromal cells such as immune cells, stem cells, or cancer associated fibroblasts (CAF) could be incorporated to recapitulate more complex features of the tumor microenvironment.

In addition, it is of major interest to include pericytes in these vascularized tumor spheroids to investigate the perivascular coverage defect observed in cancer. Other major factors involved in the vascularization process are shear forces and blood flow. These parameters require the use of microfluidic devices in order to be investigated *in vitro*.

Multiple Microfluidic Angiogenesis Models

Over the last decade, microfluidic organs-on-chips have been extensively developed to model cancer (Zervantonakis et al., 2012; Sontheimer-Phelps et al., 2019). These tumor-on-a-chip recapitulate the multicellular architecture, tumor–tissue interface, and physical microenvironment of cancers growing within human organs. Furthermore, a unique feature of microfluidic systems is perfusion of the vascular compartment, allowing the study of shear forces and blood flow in tumor vascularization (Haase and Kamm, 2017). Advantages of these systems are high spatial and temporal control over cell patterning, chemical gradients, and mechanical stimuli. In these tumor-on-chip technologies, engineering perfused micro-vessels has been a major challenge addressed by several *in vitro* approaches, such as EC lining-, vasculogenesis-, or angiogenesis-based methods (Haase and Kamm, 2017; Wang et al., 2018; Sontheimer-Phelps et al., 2019). Microfluidic tumor-on-a-chip models have been further developed to study tumor cell interactions with the vasculature as well as intravasation/extravasation processes.

Engineering Perfused Micro-Vessels

EC lining method

The EC lining method is based on endothelial monolayers established on the inner walls of microchannels (Sudo et al., 2009; Shin et al., 2012; Zhang et al., 2012; Tourovskaia et al., 2014; Pauty et al., 2018; Andrique et al., 2019). The most widely described microfluidic platform comprises hydrogel-incorporating chambers between surface-accessible microchannels made of polydimethylsiloxane (PDMS) and coated with ECM proteins (Shin et al., 2012). In other devices, microchannels are made of hydrogel which require the inclusion of a cylindrical rod into collagen I and its removal after polymerization to form a microchannel for infusion of EC (Tourovskaia et al., 2014; Wong and Searson, 2014). EC lining method is applied to explore processes of intravasation and extravasation by tumor cells under combination of multiple controllable biochemical and biophysical microenvironment parameters (Zervantonakis et al., 2012; Bersini et al., 2014; Wong and Searson, 2014). These models provide some advantages over common static assays used for studying migration in response to chemotactic gradients, such as Boyden chamber and wound assay as they allow integration of multiple microenvironmental factors (cells, secreted factors, 3D ECM, flow).

Angiogenesis-based method

In microfluidic systems, angiogenesis is induced from either EC monolayers or pre-existing vessels (Haase and Kamm, 2017). A typical microfluidic platform, initially developed by Song and Munn (2011), contains a central chamber incorporating a hydrogel that is in direct contact with two surface-accessible microchannels. The EC infused in these microchannels cover the surface of the central hydrogel. This microfluidic device incorporates features such as: (i) contact between the abluminal side of EC and the hydrogel to allow angiogenic sprouting; (ii) fluid flowing through two adjacent channels and controllable fluid convection through the hydrogel; (iii) co-culture with other cell types seeded either in the central hydrogel or in the opposite channel to EC; (iv) specific growth factors gradients. This microfluidic platform has given rise to many proximate designs that are now commonly used by many research groups (Yeon et al., 2012; Abe et al., 2019; van Duinen et al., 2019).

Vasculogenesis-based method

As previously described, vasculogenesis-based method produces microcirculation by formation of interconnected lumens via a process that relies on the capacity of EC to self-assemble into capillaries under proper culture conditions and stimulation (Haase and Kamm, 2017; Wang et al., 2018). The model contains one central hydrogel seeded with EC in suspension and bordered by micro-fabricated channels for medium perfusion (Shin et al., 2012; Kim et al., 2013; Moya et al., 2013; Wang et al., 2017). Stromal cells such as fibroblasts (Chen et al., 2013, 2016; Sobrino et al., 2016), MSC, osteoblasts-differentiated cells (Jeon et al., 2015), or perivascular cells (Sobrino et al., 2016) are seeded either with EC in the central hydrogel region or in the surrounding channels.

This method allows to engineer capillaries that are not only properly polarized and lumenized but also functional. Indeed, perfusion of the vascular network with FITC reveals its barrier integrity and therefore allows investigation of regulatory mechanisms of vascular permeability (Sobrino et al., 2016). In this respect, only a few microfluidic models, however, include pericytes wrapping the abluminal surface of the endothelium even though pericyte coverage is required for establishment of vascular permeability *in vivo* (Kim et al., 2013; Sobrino et al., 2016). Thus, this represents an important challenge for the development of physiologically relevant models.

Strategies to Recapitulate Tumor–Vasculature Interactions

Culture in separate compartments

Interstitial and blood flow. There is a distinction between the functional perfusion of the entire microvascular networks (Kim et al., 2013; van Duinen et al., 2019) and interstitial flow applied in the microfluidic device without perfused capillaries

TABLE 1 | *In vitro* 3D vascularized tumors in non-perfused models and applications.

	3D culture methods					
	Co-culture in 3D hydrogel				**Tissue constructs**	
Natural or synthetic scaffolds	Collagen I	PEG or PEG-heparin		Fibrin	Fibroblast-derived skin construct	
Methods						
Capillary formation	Vasculogenesis assay			Endothelial spheroid assay	Combined vasculogenesis (blood and lymphatic) sheet (tissue-construct) assays	
Tumor mass formation	Artificial cancer mass (ACM)	Spheroid growth		Multicellular spheroid growth	Spheroid growth	Individual tumor cell proliferation
Stromal cells	Fibroblasts	MSC		Fibroblasts	Fibroblasts Keratinocytes	Fibroblasts
Advantages	Physical properties (stiffness, density)	Controlled properties of 3D scaffolds: Functionalization (Growth factor binding sites and cell adhesion motifs)		Multicellular stromal microenvironment Relevant model for sprouting angiogenesis	Self-assembly method: Tumor growth in relevant complex cell-based microenvironment for skin	Specificity of hematogenous/ lymphogenous involvements in tumor invasion and intravasation
	Dissociation of ACM and Stromal Compartment	Cell BehaviorAssessment during Capillary and Tumor Growth				
Limits	Tumor growth and capillary formation affected by plastic compression	No capillary penetration into the tumor mass Weak assessment of the stromal (MSC) contribution	Weak assessment of the stromal (MSC) contribution	Low relevancy for capillary penetration into the tumor mass and intravasation mechanisms	Applicable for skin constructs	No relevancy of tumor mass and capillary penetration
Molecular and cell responses applications	Tumor–vessel interactions					
	Impact of specific ECM mechanical properties	Time-dependent drug testing	Impact of specific cell–ECM interactions	Sprouting angiogenesis	Drug screening	MMP secretion profile intravasation
References examples	Magdeldin et al., 2017 Pape et al., 2019	Bray et al., 2015	Taubenberger et al., 2016	Ehsan et al., 2014	Bourland et al., 2018	Nishiguchi et al., 2018

(Abe et al., 2019). Microfluidic devices allow investigation of specific mechanical factors consisting in forces generated by fluid flow through either the hydrogel or the microvascular network, respectively, mimicking the interstitial (Abe et al., 2019) and the blood flow (Jeon et al., 2015). For example, in the single gel channel device of Song and Munn (2011) described above, convective flow through media channels results in interstitial flow across the hydrogel region. These authors demonstrated that interstitial flow enhanced sprouting morphogenesis consisting in increased formation of filopodia extending against the flow (Song and Munn, 2011). However, it remains unclear whether interstitial flow magnitude can be optimized to control 3D vascular network formation in combination with various VEGF concentrations. Therefore, using a wide range of interstitial flow in a microfluidic device dedicated to angiogenesis, Abe et al. (2019) demonstrated a pro-angiogenic effect of the interstitial flow that is not substituted by increasing VEGF concentration. Indeed, density and length of the microvascular network as well as lumen diameter of capillaries and area of degraded gel increased specifically in response to flow magnitude. This study is in agreement with previous work performed on avian embryos where interstitial flow has been reported to affect VEGF distribution in the mesenchyme (Ghaffari et al., 2017). In tumor, interstitial fluid pressure is high due to plasma leakage from blood capillaries and non-functional lymphatic networks which limit drug delivery (Viallard and Larrivée, 2017). Microfluidic approaches are thus powerful tools to study the effect of interstitial pressure within the tumor microenvironment, but no study has been performed using lymphatic vessels so far, to the best of our knowledge.

Blood flow through capillaries results in shear stress, which is the major biomechanical factor depending on fluid flow (Haase and Kamm, 2017; Wang et al., 2018). Shear flow can be applied using EC lining, angiogenesis-, and vasculogenesis-based methods. For the EC-lining based method, it is easy to assess the shear stress applied on the capillary network because geometries of the device are known. However, for angiogenesis- and vasculogenesis-based methods, generating a network homogeneously perfused remains quite challenging, as much as modeling flow in a complex network produced by cell self-assembly. Kim et al. (2013) have demonstrated EC alignment in the direction of applied shear flow. Moreover, Jeon et al. (2015) revealed that capillaries under laminar flow displayed decreased vasculature permeability and tumor cell extravasation rates when compared to static conditions.

Paracrine factors secreted by tumor cells. Some *in vitro* microfluidic platforms are engineered to study the effect of factors secreted by tumor and stromal cells on angiogenesis using growth factor cocktails (van Duinen et al., 2019) or co-culture of cells in separate channels (Kim et al., 2013; Miller et al., 2018). In contrast to static culture, these models integrate both perfusion and generation of robust biomolecular gradients (van Duinen et al., 2019). For example, van Duinen et al. (2019) perfused a microfluidic device by passive leveling using a rocker platform thus applying the flow through all 40 units and resulting in reproducible gradient formation. They identified a combination of pro-angiogenic factors VEGF-A, and Sphingosine-1-phosphate, together with the PKC activator Phorbol 12-myristate 13-acetate, that were required to induce sprouting angiogenesis (van Duinen et al., 2019). These platforms could be used, in a high throughput manner, to perfuse stromal or tumor cell conditioned medium allowing the identification of secreted factors involved in tumor angiogenesis.

Another microfluidic device using EC co-cultured with human glioblastoma cells (U87MG) or fibroblasts (control condition) in independent channel reveals that an increased number of EC sprouts invade the matrix, displaying an aberrant morphology, in response to factors secreted by tumor cells (Kim et al., 2013). Indeed, when compared to fibroblasts-induced sprouts, glioblastoma cells fail to promote formation of a perfused vascular network, only inducing immature and rarely lumenized vessels.

Recapitulation of the tumor vascularized microenvironment

As described above for static models, co-culture of cells in 3D hydrogels recapitulates key features of the tumor microenvironment including crosstalk between tumor and stromal cells, ECM remodeling, and storage of paracrine factors. These culture conditions have therefore been transferred to microfluidics platforms for studying: (i) direct tumor–vasculature interactions; (ii) molecular interplay between tumor–stromal cells and the vasculature (Chung et al., 2017; Miller et al., 2018; Truong et al., 2019); (iii) response to standard therapies (Sobrino et al., 2016); (iv) tumor metabolism, which is an interesting marker for cellular state and response to drugs (Sobrino et al., 2016). For this purpose, a large variety of tumor cell lines and primary cultures from many origins, including breast, renal, and colorectal cancers have been incorporated in microfluidic devices. In addition, stromal cells such as fibroblasts, MSC, or osteoblasts are also included in these models either to support capillary formation or to generate tissue-specific microenvironments.

Sobrino et al. (2016) engineered vascularized micro-tumors to assess direct tumor–vasculature interactions as well as tumor metabolism, and response to chemotactic and anti-angiogenic therapies. Their platform, based on a vasculogenesis approach, consists in two channels connected by three tissue chambers where EC, tumor cells, and fibroblasts are injected. EC formed a capillary network covered with fibroblasts that are NG2 and PDGFR-B positive, consistent with a pericyte phenotype. Various colorectal and breast tumor cell lines proliferated to form cell aggregates in close contact with the capillary network. Perfusion of this tumor-associated vascular network was demonstrated using dextran. These vascularized micro-tumors respond to chemotherapeutics, displaying reduced growth or even regression. The model established the distinction between VEGF-specific drugs that were not effective in disrupting the vascular networks and drugs against several targets (VEGFR2, PDGFR, and Tie2) that induced effective regression of the vasculature. Interestingly, tumor cells, fibroblasts, and EC that composed the micro-tumors displayed a strong metabolic heterogeneity: tumor cells were more glycolytic than the

surrounding stroma, which was consistent with their known preferences for aerobic glycolysis.

One advantage of studying direct interactions within perfused vascularized micro-tumors is the possibility to easily include various stromal cells mimicking a specific tumor microenvironment. It is widely agreed that cells with stem-like properties form a fertile niche allowing tumor to grow and expand (Valkenburg et al., 2018). Indeed, in glioblastoma, a sub-population of cells with stem-like properties, glioma stem cell (GSC), has been reported as one of the major drivers of tumor recurrence, invasion of normal brain parenchyma, and resistance to therapies. Truong et al. (2019) developed a vascularized tumor-on-a-chip technology mimicking the GSC vascular niche to study EC impact on patient-derived GSC behavior. The signaling pathways governing invasion were thus identified. To this aim, EC were embedded in a hydrogel and infused within the microfluidic device to enclose the tumor region of the platform composed by patient-derived primary GSC encapsulated within Matrigel^{TM}. The established microvascular network enhanced GSC migration in the hydrogel, promoted invasive morphology, and maintained GSC proliferation rates and stemness (Nestin, SOX2, CD44). Using a specific CXCR4 antagonist, the role of the CXCL12-CXCR4 signaling axis on EC-mediated GSC invasion was demonstrated. This model could be adapted to many types of cancer.

Whereas perfused vascularized micro-tumors have been engineered with various tumor cell lines (Sobrino et al., 2016; Truong et al., 2019), the use of primary cells remains challenging. Miller et al. (2018) developed a vascularized flow-directed culture system for primary human clear cell renal cell carcinoma (ccRCC) cells isolated from six patients to study the angiogenic signaling axis between ccRCC cells and EC. Collagen I containing embedded tumor spheroids were infused into the matrix channel. A retaining rod was removed from the collagen gel after polymerization to form the lumen for infusion of EC. Under a medium flow through the EC-lined lumen, capillaries were higher in all devices containing tumor cells when compared to clusters formed from normal-adjacent renal cortex. Moreover, endothelial sprouting was not limited to migration oriented toward tumor clusters but was isotropically distributed in the outlet. Indeed, computational simulation demonstrated that the biochemical gradient of angiogenic growth factors favors sprouting in the outlet region of the flow-directed microphysiological system (Miller et al., 2018). Interestingly, depending on patients, factors secreted by tumor clusters induced formation of capillaries displaying different morphologies that could reflect the inter-patient heterogeneity. This device therefore recapitulates a primary tumor cell-derived biochemical gradient.

Intravasation and extravasation

During the metastasis cascade, tumor cells dissociate from the primary tumor, intravasate into capillaries and survive in circulation, extravasate from the microvasculature, and form metastases in the secondary parenchyma (Chen et al., 2013, 2016). Intravasation is the transmigration of cancer cells through BM and endothelium into capillaries close to the tumor. This process is regulated by tumor cell intrinsic factors and by stromal cells present in the tumor microenvironment such as macrophages and neutrophils. To study the interplay between tumor cells and the endothelium, Wong and Searson (2014) developed microvessels formed by an EC lining method and embedded in a hydrogel containing tumor cells. Using fluorescence live cell imaging, they deciphered various tumor–endothelium interactions such as invasion and intravasation. This platform could be used to investigate the role of tumor cell–endothelium interactions and biochemical factors involved in the intravasation process. Zervantonakis et al. developed a microfluidic model to mimic cancer cell intravasation across an impaired endothelial barrier by including soluble biochemical factors or macrophages. Using an EC lining method, they demonstrated that macrophage-secreted TNF-alpha stimulated intravasation of tumor cells due to impaired endothelial barrier function (Zervantonakis et al., 2012).

Extravasation is the reverse dynamic process that involves a cascade of events including tumor–EC adhesion, initiation and formation of tumor cell protrusions into the sub-endothelial ECM, interaction with the endothelial BM, and migration through the parenchyma. The study of this process requires real-time imaging at a single cell level in a model recapitulating the human microvasculature, which is possible only with microfluidic devices. Using a microfluidic model based on vasculogenesis, Chen et al. (2016) assessed the impact of β1 integrin depletion in cancer cells on the extravasation cascade. For this purpose, individual tumor cell lines invalidated or not for β1 integrin were directly perfused into the microvessels to monitor the dynamic extravasation events. The authors deciphered multiple steps of the extravasation process and their findings highlighted a critical role for beta1 integrin in adhesion to the sub-endothelial ECM, providing new insights into the mechanisms underlying metastasis.

Microfluidic systems recapitulate the trans-endothelial migration toward a non-organ-specific ECM. However, extravasation occurs in specific tumor microenvironment composed by several cell types which could impact this process. Bersini et al. (2014) and Jeon et al. (2015) explored breast cancer cell extravasation using two microfluidic approaches in a specific bone-mimicking microenvironment (BMi). These microfluidic devices are developed by an EC lining (Bersini et al., 2014) and a vasculogenesis-based method (Jeon et al., 2015). In the device developed by Bersini et al. (2014), osteo-differentiated-human bone marrow (OD-hBM)-derived MSC were embedded in hydrogel and cultured within an independent microfluidic channel for 2–3 weeks to allow proper deposition of bone ECM. EC were thus seeded into the adjacent channel inducing formation of a continuous monolayer at the 3D hydrogel–endothelial channel interface. Finally, breast cancer cells MDA-MB 231 were injected into the hollow channel mimicking the vascular lumen and the extravasation rate was monitored. Extravasation was significantly higher in the microenvironment conditioned by osteo-differentiated cells compared to collagen hydrogel. However, in this microfluidic device, the use of an endothelial monolayer does not recapitulate the parameters of a proper capillary. Aware of the limitations of this approach, they have developed another microfluidic system based on

a vasculogenesis approach. For this purpose, EC were co-embedded with hBM-MSC and OD-hBM-MSC in the central gel region of the microfluidic platform to generate a microvascular network enclosed in a BMi (Jeon et al., 2015). By perfusing individual breast tumor cell lines, the authors demonstrated that both extravasation rates and microvascular permeability were higher in the BMi compared to the muscle-mimicking microenvironment or to the unconditioned ECM. These results demonstrated a major role of the microenvironment in the extravasation process of tumor cells. This organ-specific vascularized 3D microfluidic model is an interesting tool to study cell extravasation in a stromal microenvironment and could be expanded to various cancer cells.

Conclusion

Microfluidic systems recapitulate many features of tumor microenvironment such as perfused and functional capillary networks, growth factor gradient, and shear stress. These models are powerful tools to study indirect and direct interactions between tumor and stromal cells with the vasculature. This approach is consistent with high resolution real-time imaging allowing to decipher each step of complex cellular processes such as intra- and extravasation (Zervantonakis et al., 2012; Chen et al., 2013, 2016). **Table 2** sums up the main features and applications of 3D microfluidic models of vascularized micro-tumors. As discussed below, these systems are adaptable to high-throughput analysis and drug screening (Sobrino et al., 2016; Miller et al., 2018; Ko et al., 2019; van Duinen et al., 2019). However, these perfused vascularized micro-tumors still fail to recapitulate the whole and complex composition of *in vivo* tumors, and they should in the next future include more specifically components of the immune compartment.

CHALLENGES AND FUTURE APPLICATIONS

High Throughput Screening of Drugs

For more than 50 years, 2D cultures of tumor cell lines have been widely used for drug screening. However, since the 2000s, many studies revealed more relevant responses to treatment of cells cultured in 3D. Tumor spheroids cultured in suspension or more recently in hydrogels have thus been proposed to bridge the gap between flat culture and *in vivo* experiments. Furthermore, vascularization as well as co-culture with stromal cells is of main interest to assess the drug delivery context. As described above, vascularized micro-tumors cultured within hydrogels (Bray et al., 2015), tissue constructs (Bourland et al., 2018; Nishiguchi et al., 2018), as well as microfluidic systems (Sobrino et al., 2016; Miller et al., 2018) are powerful platforms for cancer drug discovery and screening. However, in many cases, they are still at the proof-of-concept stage and are not designed for high throughput drug screening yet (Bray et al., 2015; Sobrino et al., 2016; Bourland et al., 2018; Miller et al., 2018; Nishiguchi et al., 2018).

Hughes's group has engineered a perfused vascularized micro-tumor model in a 96-well plate format compatible with standard robotic and fluorescent plate readers thus creating standardized and reproducible arrays (Sobrino et al., 2016; Phan et al., 2017). Adapted for high throughput screening, each unit can be filled by various drugs. The vascularized micro-tumors can be easily extracted for further gene expression analysis. As proof-of-concept, a full-blind test of 10 FDA-approved anti-tumor compounds and two negative control compounds has been performed on the vascularized micro-tumors. Anti-angiogenic, anti-tumor drugs and placebo were successfully discriminated according to their respective effects on tumor cells or on the vasculature. More recently, aware of the need to develop standardized assays, other groups have designed microfluidic platforms suitable for high throughput anti-tumor drug screening on vascularized micro-tumors (Ko et al., 2019; Meng et al., 2019). Interestingly, Meng et al. (2019) took advantage of 3D bioprinting to accurately position cluster of tumor cells, stromal cells, and EC in their device. These new platforms share some similar characteristics essential for high throughput analyses such as a simple and quick fabrication; a miniaturized format; a single vascularized micro-tumor per well; an automated imaging.

Toward Personalized Medicine

The development of perfused vascularized micro-tumors compatible with high throughput analysis is a major step toward personalized medicine in order to determine specific anti-tumor drugs for each individual patient.

However, up to now, personalized medicine remains challenging for many reasons: (1) the complex isolation of various cell types (tumor cells, EC, immune cells, CAF) from the same biopsy; (2) the maintenance of cell-specific functionality for a long time in culture; (3) the appropriate relative proportions of cell types; and (4) the optimal culture medium for the combined cell cultures (Sontheimer-Phelps et al., 2019). Indeed, specific isolation protocols and culture conditions are required for each cell type present in tumor biopsies. As described above, formation of *in vitro* capillaries with EC isolated from tumors is not achieved. The main improvement concerns the use of 3D-organoid technologies leading to the development of so-called "tumoroïds" that are composed by patient-derived 3D cultures of cells isolated from tumor biopsies. As previously reviewed by Drost and Clevers (2018), primary cells can be isolated from various cancer types such as colorectal, pancreatic, liver, breast, prostate, brain and bladder cancers to form these "tumoroïds." These cells derived from living tissue are more likely to reflect the properties of native cells *in vivo*. A major step to move toward personalized medicine will be to form vascularized micro-tumors, in a high throughput manner, by co-culturing "tumoroid" and EC.

Induced Pluripotent Stem Cells

Recent results suggest that cancer cells remain susceptible to transcription factor-mediated reprogramming (Utikal et al., 2009). As for angiogenesis, although human induced pluripotent stem cells (iPSC) can differentiate into functional EC, they still exhibit limited expansion potential compared with human embryonic stem cells-derived EC. However, individual lines of human embryonic stem cells and iPSC are distinct and can

TABLE 2 | *In vitro* 3D vascularized tumors in perfused models and applications.

3D scaffolds	**Fibrin**	**3D ECM (Collagen I)**			**Fibrin Fibrin/collagen I***			**Fibrin**	
Methods	EC lining				Vasculogenesis assay				
Capillary formation Tumor mass	Multicellular spheroid growth	Spheroid growth	Individual invading tumor cells		Perfused tumor cells			Individual tumor cells	
Stromal cells	Fibroblasts	–	Macrophages	–	Osteo-differentiated (OD) hBM-MSC		Fibroblasts	Fibroblasts Pericytes	–
Advantages	Sprouting angiogenesis	Paracrine and juxtacrine regulations mediated by 3D microenvironment			Cell-based complexity and ECM deposition Dynamic analysis	Bone mimicking environment	Temporal Resolution		Glioblastoma mimicking environment Stem cell involvement
	Simultaneous use of lymphatic and blood EC	Culture of patient-derived tumor cells	Live imaging				Extravasation events Association with endothelial barrier function	Peri-mural cell behavior	
			Assessment of intravasation process Endothelial barrier integrity						
Limits	No lymphatic or blood capillary penetration of tumor mass	Undefined cell type content in 3D patient-derived tumor Uncharacterized vessels	No relevancy for capillary penetration of tumor mass		No angiogenic process No tumor cell migration	Bone-specific metastasis	No capillary invasion		
				Limited cell-based complexity			No tumor cell migration		Fibrin–matrigel interface Uncharacterized vessels
Molecular and cell responses Applications	Angio- and lymph-angiogenesis Angiogenic regulation by tumor–fibroblasts interactions	Tumor cell-derived gradient of angiogenic factors	Paracrine factors involvement Intravasation		Extravasation		Extravasation Involvement of signaling pathways	Tumor–vessel interactions	
						Drug screening platform		Tumor metabolism Drug screening	Paracrine factors involvement
References examples	Chung et al., 2017	Miller et al., 2018	Zervantonakis et al., 2012	Wong and Searson, 2014	Bersini et al., 2014	Jeon et al., 2015	Chen et al., 2013, 2016	Sobrino et al., 2016; Phan et al., 2017	Truong et al., 2019

**Chen et al. (2016) is associated with fibrin/collagenI.*

often respond very differently to the same microenvironment cues. Thus, efficient and robust induction of EC from human pluripotent stem cells and multiple human iPSC lines or reset EC might also constitute a renewable and infinite cell source (Jahan and McCloskey, 2020; Palikuqi et al., 2020). These findings open new perspectives which should facilitate the study of epigenetic changes in human cancer and angiogenesis studies using pre-clinical complex *in vitro* 3D systems.

CRISPR/Cas9 Gene Editing

CRISPR-Cas9 genome editing has recently been used in tumor organoids to introduce mutations into genes commonly found mutated such as *Trp53*, *Brca1*, *Nf1*, and *Pten* in high-grade serous ovarian cancer (Lõhmussaar et al., 2020). This technology could be further used on organoids to investigate the nature and extent of intra-tumor diversification but also to study the role of mutational diversification associated with response variability to anticancer drugs between even closely related cells of the same tumor as reported by Roerink et al. (2018). In addition, although this is not the case yet, such a technology will undoubtedly be very useful to study angiogenesis in *in vitro* 3D systems.

CONCLUSION

Since unperfused models constituted powerful tool for deciphering tumor–vessel interactions, perfused models are more relevant for deciphering extra- and intravasation mechanisms. However, both types of these models present limits such as (i) low representation of relevant capillaries displaying a pericyte coverage, (ii) low level of concomitant occurrence of tumor migratory processes and capillary growth for analyzing the co-option processes or the regulation of migratory strategies, and (iii) rare investigation of the interaction types between tumor mass and capillaries as well as underlying mechanisms.

A further present limit of these models is the absence of a wide panel of stromal cells since most of the studies are based on 3D microenvironment constituted by ECM and cells such as fibroblasts, MSC, macrophages, and pericytes. These is an obvious increasing need of engineering *in vitro* 3D vascularized micro-tumors for investigating the role of inflammatory cells as well as cytokines recapitulating the complex immune compartment. Moreover, another feature to consider is the EC origins and heterogeneities. As example, it has been recently reported that EC display metabolic transcriptome heterogeneity and plasticity in tumor angiogenesis (Rohlenova et al., 2020).

AUTHOR CONTRIBUTIONS

NB-J, CM, LM, and SG wrote the review and the tables. All authors contributed to the article and approved the submitted version.

FUNDING

NB-J was supported by the Sorbonne Université.

ACKNOWLEDGMENTS

The authors wish to thank France Maloumian for designing the figures.

REFERENCES

Abe, Y., Watanabe, M., Chung, S., Kamm, R. D., Tanishita, K., and Sudo, R. (2019). Balance of interstitial flow magnitude and vascular endothelial growth factor concentration modulates three-dimensional microvascular network formation. *APL Bioengin.* 3:036102. doi: 10.1063/1.5094735

Andrique, L., Recher, G., Alessandri, K., Pujol, N., Feyeux, M., Bon, P., et al. (2019). A model of guided cell self-organization for rapid and spontaneous formation of functional vessels. *Sci. Adv.* 5:eaau6562. doi: 10.1126/sciadv.aau6562

Arnaoutova, I., George, J., Kleinman, H. K., and Benton, G. (2009). The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis* 12, 267–274. doi: 10.1007/s10456-009-9146-4

Baudino, T. A. (ed.) (2013). *Cell-Cell Interactions*. Totowa, NJ: Humana.

Beckouche, N., Bignon, M., Lelarge, V., Mathivet, T., Pichol-Thievend, C., Berndt, S., et al. (2015). The interaction of heparan sulfate proteoglycans with endothelial transglutaminase-2 limits VEGF $_{165}$ -induced angiogenesis. *Sci. Signal.* 8, ra70–ra70. doi: 10.1126/scisignal.aaa0963

Benton, G., DeGray, G., Kleinman, H. K., George, J., and Arnaoutova, I. (2015). In vitro microtumors provide a physiologically predictive tool for breast cancer therapeutic screening. *PLoS One* 10:e0123312. doi: 10.1371/journal.pone.0123312

Bersini, S., Jeon, J. S., Dubini, G., Arrigoni, C., Chung, S., Charest, J. L., et al. (2014). A microfluidic 3D in vitro model for specificity of breast cancer metastasis to bone. *Biomaterials* 35, 2454–2461. doi: 10.1016/j.biomaterials.2013.11.050

Blache, U., and Ehrbar, M. (2017). Inspired by nature: hydrogels as versatile tools for vascular engineering. *Adv. Wound Care* 7, 232–246. doi: 10.1089/wound.2017.0760

Blache, U., Metzger, S., Vallmajo-Martin, Q., Martin, I., Djonov, V., and Ehrbar, M. (2016). Dual Role of Mesenchymal Stem Cells Allows for Microvascularized Bone Tissue-Like Environments in PEG Hydrogels. *Adv. Healthcare Mater.* 5, 489–498. doi: 10.1002/adhm.201500795

Bourland, J., Fradette, J., and Auger, F. A. (2018). Tissue-engineered 3D melanoma model with blood and lymphatic capillaries for drug development. *Sci. Rep.* 8:13191. doi: 10.1038/s41598-018-31502-6

Bray, L. J., Binner, M., Holzheu, A., Friedrichs, J., Freudenberg, U., Hutmacher, D. W., et al. (2015). Multi-parametric hydrogels support 3D in vitro bioengineered microenvironment models of tumour angiogenesis. *Biomaterials* 53, 609–620. doi: 10.1016/j.biomaterials.2015.02.124

Chen, M. B., Lamar, J. M., Li, R., Hynes, R. O., and Kamm, R. D. (2016). Elucidation of the Roles of Tumor Integrin 1 in the Extravasation Stage of the Metastasis Cascade. *Cancer Res.* 76, 2513–2524. doi: 10.1158/0008-5472.CAN-15-1325

Chen, M. B., Whisler, J. A., Jeon, J. S., and Kamm, R. D. (2013). Mechanisms of tumor cell extravasation in an in vitro microvascular network platform. *Integr. Biol.* 5:1262. doi: 10.1039/c3ib40149a

Chung, M., Ahn, J., Son, K., Kim, S., and Jeon, N. L. (2017). Biomimetic Model of Tumor Microenvironment on Microfluidic Platform. *Adv. Healthcare Mater.* 6:1700196. doi: 10.1002/adhm.201700196

Chwalek, K., Tsurkan, M. V., Freudenberg, U., and Werner, C. (2015). Glycosaminoglycan-based hydrogels to modulate heterocellular communication in in vitro angiogenesis models. *Sci. Rep.* 4:4414. doi: 10.1038/srep04414

Decher, G. (1997). Fuzzy Nanoassemblies: Toward Layered Polymeric Multicomposites. *Science* 227, 1232–1237. doi: 10.1126/science.277.5330.1232

Doyle, A. D., Carvajal, N., Jin, A., Matsumoto, K., and Yamada, K. M. (2015). Local 3D matrix microenvironment regulates cell migration through spatiotemporal dynamics of contractility-dependent adhesions. *Nat. Commun.* 6:8720. doi: 10.1038/ncomms9720

Drost, J., and Clevers, H. (2018). Organoids in cancer research. *Nat. Rev. Cancer* 18, 407–418. doi: 10.1038/s41568-018-0007-6

Ehsan, S. M., Welch-Reardon, K. M., Waterman, M. L., Hughes, C. C. W., and George, S. C. (2014). A three-dimensional in vitro model of tumor cell intravasation. *Integr. Biol.* 6:603. doi: 10.1039/c3ib40170g

Ferratge, S., Ha, G., Carpentier, G., Arouche, N., Bascetin, R., Muller, L., et al. (2017). Initial clonogenic potential of human endothelial progenitor cells is predictive of their further properties and establishes a functional hierarchy related to immaturity. *Stem Cell Res.* 21, 148–159. doi: 10.1016/j.scr.2017.04.009

Furlan, A., Vercamer, C., Heliot, L., Wernert, N., Desbiens, X., and Pourtier, A. (2018). Ets-1 drives breast cancer cell angiogenic potential and interactions between breast cancer and endothelial cells. *Int. J. Oncol.* 54(1), 29–40.doi: 10.3892/ijo.2018.4605

Ghaffari, S., Leask, R. L., and Jones, E. A. V. (2017). Blood flow can signal during angiogenesis not only through mechanotransduction, but also by affecting growth factor distribution. *Angiogenesis* 20, 373–384. doi: 10.1007/s10456-017-9553-x

Ghajar, C. M., Blevins, K. S., Hughes, C. C. W., George, S. C., and Putnam, A. J. (2006). Mesenchymal stem cells enhance angiogenesis in mechanically viable prevascularized tissues via early matrix metalloproteinase upregulation. *Tissue Eng.* 12, 2875–2888. doi: 10.1089/ten.2006.12.2875

Ghajar, C. M., Chen, X., Harris, J. W., Suresh, V., Hughes, C. C. W., Jeon, N. L., et al. (2008). The effect of matrix density on the regulation of 3-D capillary morphogenesis. *Biophys. J.* 94, 1930–1941. doi: 10.1529/biophysj.107.120774

Gibot, L., Galbraith, T., Bourland, J., Rogic, A., Skobe, M., and Auger, F. A. (2017). Tissue-engineered 3D human lymphatic microvascular network for in vitro studies of lymphangiogenesis. *Nat. Protoc.* 12, 1077–1088. doi: 10.1038/nprot.2017.025

Gibot, L., Galbraith, T., Kloos, B., Das, S., Lacroix, D. A., Auger, F. A., et al. (2016). Cell-based approach for 3D reconstruction of lymphatic capillaries in vitro reveals distinct functions of HGF and VEGF-C in lymphangiogenesis. *Biomaterials* 78, 129–139. doi: 10.1016/j.biomaterials.2015.11.027

Haase, K., and Kamm, R. D. (2017). Advances in on-chip vascularization. *Regener. Med.* 12, 285–302. doi: 10.2217/rme-2016-0152

Heiss, M., Hellström, M., Kalén, M., May, T., Weber, H., Hecker, M., et al. (2015). Endothelial cell spheroids as a versatile tool to study angiogenesis *in vitro*. *FASEB J.* 29, 3076–3084. doi: 10.1096/fj.14-267633

Hernandez-Fernaud, J. R., Ruengeler, E., Casazza, A., Neilson, L. J., Pulleine, E., Santi, A., et al. (2017). Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity. *Nat. Commun.* 8:14206. doi: 10.1038/ncomms14206

Jahan, B., and McCloskey, K. E. (2020). Differentiation and expansion of endothelial cells requires pre-optimization of KDR+ expression kinetics. *Stem Cell Res.* 42:101685. doi: 10.1016/j.scr.2019.101685

Jeon, J. S., Bersini, S., Gilardi, M., Dubini, G., Charest, J. L., Moretti, M., et al. (2015). Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. *Proc. Natl. Acad. Sci. U S A.* 112, 214–219. doi: 10.1073/pnas.1417115112

Jeong, S.-Y., Lee, J.-H., Shin, Y., Chung, S., and Kuh, H.-J. (2016). Co-culture of tumor spheroids and fibroblasts in a collagen matrix-incorporated microfluidic chip mimics reciprocal activation in solid tumor microenvironment. *Plos One* 11:e0159013. doi: 10.1371/journal.pone.0159013

Kim, S., Lee, H., Chung, M., and Jeon, N. L. (2013). Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab. Chip.* 13:1489. doi: 10.1039/c3lc41320a

Ko, J., Ahn, J., Kim, S., Lee, Y., Lee, J., Park, D., et al. (2019). Tumor spheroid-on-a-chip: a standardized microfluidic culture platform for investigating tumor angiogenesis. *Lab. Chip.* 19, 2822–2833. doi: 10.1039/C9LC00140A

Kraehenbuehl, T. P., Zammaretti, P., Van der Vlies, A. J., Schoenmakers, R. G., Lutolf, M. P., Jaconi, M. E., et al. (2008). Three-dimensional extracellular matrix-directed cardioprogenitor differentiation: Systematic modulation of a synthetic cell-responsive PEG-hydrogel. *Biomaterials* 29, 2757–2766. doi: 10.1016/j.biomaterials.2008.03.016

Kuèera, T., Striliæ, B., Regener, K., Schubert, M., Laudet, V., and Lammert, E. (2009). Ancestral Vascular Lumen Formation via Basal Cell Surfaces. *PLoS One* 4:e4132. doi: 10.1371/journal.pone.0004132

Laib, A. M., Bartol, A., Alajati, A., Korff, T., Weber, H., and Augustin, H. G. (2009). Spheroid-based human endothelial cell microvessel formation in vivo. *Nat. Protoc.* 4, 1202–1215. doi: 10.1038/nprot.2009.96

Li, Y., and Kumacheva, E. (2018). Hydrogel microenvironments for cancer spheroid growth and drug screening. *Sci. Adv.* 4:eaas8998. doi: 10.1126/sciadv.aas8998

Liang, Y., Jeong, J., DeVolder, R. J., Cha, C., Wang, F., Tong, Y. W., et al. (2011). A cell-instructive hydrogel to regulate malignancy of 3D tumor spheroids with matrix rigidity. *Biomaterials* 32, 9308–9315. doi: 10.1016/j.biomaterials.2011.08.045

Liu, J., Tan, Y., Zhang, H., Zhang, Y., Xu, P., Chen, J., et al. (2012). Soft fibrin gels promote selection and growth of tumorigenic cells. *Nat. Mater* 11, 734–741. doi: 10.1038/nmat3361

Lõhmussaar, K., Kopper, O., Korving, J., Begthel, H., Vreuls, C. P. H., van Es, J. H., et al. (2020). Assessing the origin of high-grade serous ovarian cancer using CRISPR-modification of mouse organoids. *Nat. Commun.* 11:2660. doi: 10.1038/s41467-020-16432-0

Lutolf, M. P., and Hubbell, J. A. (2005). Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotech.* 23, 47–55. doi: 10.1038/nbt1055

Magdeldin, T., López-Dávila, V., Pape, J., Cameron, G. W. W., Emberton, M., Loizidou, M., et al. (2017). Engineering a vascularised 3D in vitro model of cancer progression. *Sci. Rep.* 7:44045. doi: 10.1038/srep44045

Marchand, M., Monnot, C., Muller, L., and Germain, S. (2019). Extracellular matrix scaffolding in angiogenesis and capillary homeostasis. *Semin. Cell Dev. Biol.* 89, 147–156. doi: 10.1016/j.semcdb.2018.08.007

Marino, D., Luginbuhl, J., Scola, S., Meuli, M., and Reichmann, E. (2014). Bioengineering Dermo-Epidermal Skin Grafts with Blood and Lymphatic Capillaries. *Sci. Transl. Med.* 6, ra14–ra221. doi: 10.1126/scitranslmed.3006894

Meng, F., Meyer, C. M., Joung, D., Vallera, D. A., McAlpine, M. C., and Panoskaltsis-Mortari, A. (2019). 3D Bioprinted In Vitro Metastatic Models via Reconstruction of Tumor Microenvironments. *Adv. Mater.* 31:1806899. doi: 10.1002/adma.201806899

Miller, C. P., Tsuchida, C., Zheng, Y., Himmelfarb, J., and Akilesh, S. (2018). A 3D Human Renal Cell Carcinoma-on-a-Chip for the Study of Tumor Angiogenesis. *Neoplasia* 20, 610–620. doi: 10.1016/j.neo.2018.02.011

Montaño, I., Schiestl, C., Schneider, J., Pontiggia, L., Luginbühl, J., Biedermann, T., et al. (2010). Formation of human capillaries *in vitro*: The Engineering of Prevascularized Matrices. *Tissue Engin. Part A.* 16, 269–282. doi: 10.1089/ten.tea.2008.0550

Moya, M. L., Hsu, Y.-H., Lee, A. P., Hughes, C. C. W., and George, S. C. (2013). *In vitro* perfused human capillary networks. *Tissue Engin. Part C Methods* 19, 730–737. doi: 10.1089/ten.tec.2012.0430

Nakatsu, M. N., and Hughes, C. C. W. (2008). An optimized three−dimensional in vitro model for the analysis of angiogenesis. *Methods Enzymol.* 443, 65–82. doi: 10.1016/S0076-6879(08)02004-1

Nakatsu, M. N., Sainson, R. C. A., Aoto, J. N., Taylor, K. L., Aitkenhead, M., Pérez-del-Pulgar, S., et al. (2003). Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1?. *Microvasc. Res.* 66, 102–112. doi: 10.1016/S0026-2862(03)00045-1

Nishiguchi, A., Matsusaki, M., Kano, M. R., Nishihara, H., Okano, D., Asano, Y., et al. (2018). In vitro 3D blood/lymph-vascularized human stromal tissues for preclinical assays of cancer metastasis. *Biomaterials* 179, 144–155. doi: 10.1016/j.biomaterials.2018.06.019

Nowak-Sliwinska, P., Alitalo, K., Allen, E., Anisimov, A., Aplin, A. C., Auerbach, R., et al. (2018). Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis* 21, 425–532. doi: 10.1007/s10456-018-9613-x

Palikuqi, B., Nguyen, D.-H. T., Li, G., Schreiner, R., Pellegata, A. F., Liu, Y., et al. (2020). Adaptable haemodynamic endothelial cells for organogenesis and tumorigenesis. *Nature* 585, 426–432. doi: 10.1038/s41586-020-2712-z

Pape, J., Magdeldin, T., Ali, M., Walsh, C., Lythgoe, M., Emberton, M., et al. (2019). Cancer invasion regulates vascular complexity in a three-dimensional biomimetic model. *Eur. J. Cancer* 119, 179–193. doi: 10.1016/j.ejca.2019.07.005

Pauty, J., Usuba, R., Cheng, I. G., Hespel, L., Takahashi, H., Kato, K., et al. (2018). A Vascular Endothelial Growth Factor-Dependent Sprouting Angiogenesis Assay Based on an In Vitro Human Blood Vessel Model for the Study of Anti-Angiogenic Drugs. *EBioMedicine* 27, 225–236. doi: 10.1016/j.ebiom.2017.12.014

Phan, D. T. T., Wang, X., Craver, B. M., Sobrino, A., Zhao, D., Chen, J. C., et al. (2017). A vascularized and perfused organ-on-a-chip platform for large-scale drug screening applications. *Lab. Chip.* 17, 511–520. doi: 10.1039/C6LC01422D

Provenzano, P. P., Eliceiri, K. W., Campbell, J. M., Inman, D. R., White, J. G., and Keely, P. J. (2006). Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med.* 4:38. doi: 10.1186/1741-7015-4-38

Ramer, R., Fischer, S., Haustein, M., Manda, K., and Hinz, B. (2014). Cannabinoids inhibit angiogenic capacities of endothelial cells via release of tissue inhibitor of matrix metalloproteinases-1 from lung cancer cells. *Biochem. Pharmacol.* 91, 202–216. doi: 10.1016/j.bcp.2014.06.017

Rao, R. R., Peterson, A. W., Ceccarelli, J., Putnam, A. J., and Stegemann, J. P. (2012). Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. *Angiogenesis* 15, 253–264. doi: 10.1007/s10456-012-9257-1

Roerink, S. F., Sasaki, N., Lee-Six, H., Young, M. D., Alexandrov, L. B., Behjati, S., et al. (2018). Intra-tumour diversification in colorectal cancer at the single-cell level. *Nature* 556, 457–462. doi: 10.1038/s41586-018-0024-3

Rohlenova, K., Goveia, J., García-Caballero, M., Subramanian, A., Kalucka, J., Treps, L., et al. (2020). Single-Cell RNA Sequencing Maps Endothelial Metabolic Plasticity in Pathological Angiogenesis. *Cell Metab.* 31, 862–877.e. doi: 10.1016/j.cmet.2020.03.009

Roudsari, L. C., Jeffs, S. E., Witt, A. S., Gill, B. J., and West, J. L. (2016). A 3D poly(ethylene glycol)-based tumor angiogenesis model to study the influence of vascular cells on lung tumor cell behavior. *Sci. Rep.* 6:32726. doi: 10.1038/srep32726

Sapudom, J., Rubner, S., Martin, S., Kurth, T., Riedel, S., Mierke, C. T., et al. (2015). The phenotype of cancer cell invasion controlled by fibril diameter and pore size of 3D collagen networks. *Biomaterials* 52, 367–375. doi: 10.1016/j.biomaterials.2015.02.022

Schedin, P., and Keely, P. J. (2011). Mammary Gland ECM Remodeling, Stiffness, and Mechanosignaling in Normal Development and Tumor Progression. *Cold Spring Harbor Perspect. Biol.* 3, a003228–a003228. doi: 10.1101/cshperspect.a003228

Schneider, G., Schmidt-Supprian, M., Rad, R., and Saur, D. (2017). Tissue-specific tumorigenesis: context matters. *Nat. Rev. Cancer* 17, 239–253. doi: 10.1038/nrc.2017.5

Shin, Y., Han, S., Jeon, J. S., Yamamoto, K., Zervantonakis, I. K., Sudo, R., et al. (2012). Microfluidic assay for simultaneous culture of multiple cell types on surfaces or within hydrogels. *Nat. Protoc.* 7, 1247–1259. doi: 10.1038/nprot.2012.051

Simons, M., Alitalo, K., Annex, B. H., Augustin, H. G., Beam, C., Berk, B. C., et al. (2015). State-of-the-Art Methods for Evaluation of Angiogenesis and Tissue Vascularization: A Scientific Statement From the American Heart Association. *Circ. Res.* 116, e99-132. doi: 10.1161/RES.0000000000000054

Sobrino, A., Phan, D. T. T., Datta, R., Wang, X., Hachey, S. J., Romero-López, M., et al. (2016). 3D microtumors in vitro supported by perfused vascular networks. *Sci. Rep.* 6:31589. doi: 10.1038/srep31589

Song, H.-H. G., Park, K. M., and Gerecht, S. (2014). Hydrogels to model 3D in vitro microenvironment of tumor vascularization. *Adv. Drug Deliv. Rev.* 7, 19–29. doi: 10.1016/j.addr.2014.06.002

Song, J. W., and Munn, L. L. (2011). Fluid forces control endothelial sprouting. *Proc. Natl Acad. Sci.* 108, 15342–15347. doi: 10.1073/pnas.1105316108

Sontheimer-Phelps, A., Hassell, B. A., and Ingber, D. E. (2019). Modelling cancer in microfluidic human organs-on-chips. *Nat. Rev. Cancer* 19, 65–81. doi: 10.1038/s41568-018-0104-6

Sorushanova, A., Delgado, L. M., Wu, Z., Shologu, N., Kshirsagar, A., Raghunath, R., et al. (2019). The collagen suprafamily: from biosynthesis to advanced biomaterial development. *Adv. Mater.* 31:1801651. doi: 10.1002/adma.201801651

Stacker, S. A., Williams, S. P., Karnezis, T., Shayan, R., Fox, S. B., and Achen, M. G. (2014). Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat. Rev. Cancer* 14, 159–172. doi: 10.1038/nrc3677

Sudo, R., Chung, S., Zervantonakis, I. K., Vickerman, V., Toshimitsu, Y., Griffith, L. G., et al. (2009). Transport−mediated angiogenesis in 3D epithelial coculture. *FASEB J.* 23, 2155–2164. doi: 10.1096/fj.08-122820

Szot, C. S., Buchanan, C. F., Freeman, J. W., and Rylander, M. N. (2011). 3D in vitro bioengineered tumors based on collagen I hydrogels. *Biomaterials* 32, 7905–7912. doi: 10.1016/j.biomaterials.2011.07.001

Taubenberger, A. V., Bray, L. J., Haller, B., Shaposhnykov, A., Binner, M., Freudenberg, U., et al. (2016). 3D extracellular matrix interactions modulate tumour cell growth, invasion and angiogenesis in engineered tumour microenvironments. *Acta Biomater* 36, 73–85. doi: 10.1016/j.actbio.2016.03.017

Tomlinson, J., Barsky, S. H., Nelson, S., Singer, S., Pezeshki, B., Lee, M. C., et al. (1999). Different Patterns of Angiogenesis in Sarcomas and Carcinomas. *Clin. Cancer Res.* 5, 3516–3522.

Tourovskaia, A., Fauver, M., Kramer, G., Simonson, S., and Neumann, T. (2014). Tissue-engineered microenvironment systems for modeling human vasculature. *Exp. Biol. Med.* 239, 1264–1271. doi: 10.1177/1535370214539228

Truong, D., Fiorelli, R., Barrientos, E. S., Melendez, E. L., Sanai, N., Mehta, S., et al. (2019). A three-dimensional (3D) organotypic microfluidic model for glioma stem cells – Vascular interactions. *Biomaterials* 198, 63–77. doi: 10.1016/j.biomaterials.2018.07.048

Umana-Diaz, C., Pichol-Thievend, C., Marchand, M. F., Atlas, Y., Salza, R., Malbouyres, M., et al. (2020). Scavenger Receptor Cysteine-Rich domains of Lysyl Oxidase-Like2 regulate endothelial ECM and angiogenesis through non-catalytic scaffolding mechanisms. *Matr. Biol.* 88, 33–52. doi: 10.1016/j.matbio.2019.11.003

Utikal, J., Maherali, N., Kulalert, W., and Hochedlinger, K. (2009). Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J. Cell Sci.* 122, 3502–3510. doi: 10.1242/jcs.054783

Valkenburg, K. C., de Groot, A. E., and Pienta, K. J. (2018). Targeting the tumour stroma to improve cancer therapy. *Nat. Rev. Clin. Oncol.* 15, 366–381. doi: 10.1038/s41571-018-0007-1

van Duinen, V., Zhu, D., Ramakers, C., van Zonneveld, A. J., Vulto, P., and Hankemeier, T. (2019). Perfused 3D angiogenic sprouting in a high-throughput in vitro platform. *Angiogenesis* 22, 157–165. doi: 10.1007/s10456-018-9647-0

Vernon, R., Angello, J., Iruela-Arispe, L., Lane, T., and Sage, H. (1992). Reorganization of Basement Membrane Matrices by Cellular Traction Promotes the Formation of Cellular Networks in vitro. *Lab. Invest.* 66(5), 536–47.

Viallard, C., and Larrivée, B. (2017). Tumor angiogenesis and vascular normalization: alternative therapeutic targets. *Angiogenesis* 20, 409–426. doi: 10.1007/s10456-017-9562-9

Wang, X., Phan, D. T. T., George, S. C., Hughes, C. C. W., and Lee, A. P. (2017). "3D Anastomosed Microvascular Network Model with Living Capillary Networks and Endothelial Cell-Lined Microfluidic Channels," in *3D Cell Culture Methods in Molecular Biology*, ed. Z. Koledova (New York, NY: Springer), 325–344. doi: 10.1007/978-1-4939-7021-6_24

Wang, X., Sun, Q., and Pei, J. (2018). Microfluidic-Based 3D Engineered Microvascular Networks and Their Applications in Vascularized Microtumor Models. *Micromachines* 9:493. doi: 10.3390/mi9100493

Welti, J. C., Gourlaouen, M., Powles, T., Kudahetti, S. C., Wilson, P., Berney, D. M., et al. (2011). Fibroblast growth factor 2 regulates endothelial cell sensitivity to sunitinib. *Oncogene* 30, 1183–1193. doi: 10.1038/onc.2010.503

Wolf, K., te Lindert, M., Krause, M., Alexander, S., te Riet, J., Willis, A. L., et al. (2013). Physical limits of cell migration: Control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J. Cell Biol.* 201, 1069–1084. doi: 10.1083/jcb.201210152

Wong, A. D., and Searson, P. C. (2014). Live-Cell Imaging of Invasion and Intravasation in an Artificial Microvessel Platform. *Cancer Res.* 74, 4937–4945. doi: 10.1158/0008-5472.CAN-14-1042

Yamada, K. M., and Sixt, M. (2019). Mechanisms of 3D cell migration. *Nat. Rev. Mol. Cell Biol.* 20, 738–752. doi: 10.1038/s41580-019-0172-9

Yeon, J. H., Ryu, H. R., Chung, M., Hu, Q. P., and Jeon, N. L. (2012). In vitro formation and characterization of a perfusable three-dimensional tubular capillary network in microfluidic devices. *Lab. Chip*. 12:2815. doi: 10.1039/c2lc40131b

Zervantonakis, I. K., Hughes-Alford, S. K., Charest, J. L., Condeelis, J. S., Gertler, F. B., and Kamm, R. D. (2012). Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function. *Proc. Natl. Acad. Sci.* 109, 13515–13520. doi: 10.1073/pnas.1210182109

Zhang, Q., Liu, T., and Qin, J. (2012). A microfluidic-based device for study of transendothelial invasion of tumor aggregates in realtime. *Lab. Chip*. 12:2837. doi: 10.1039/c2lc00030j

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 30 November 2020
doi: 10.3389/fonc.2020.564298

Forkhead Box Q1 Is Critical to Angiogenesis and Macrophage Recruitment of Colorectal Cancer

*Hui Tang[1,2], Ji Zheng[2,3], Xuan Bai[1,2], Ke-Lin Yue[1,2], Jian-Hua Liang[2], Dan-Yang Li[2], Lin-Ping Wang[1,2], Jin-Li Wang[1,2] and Qiang Guo[1,2]**

[1] Yunnan Digestive Endoscopy Clinical Medical Center, Department of Gastroenterology, The First People's Hospital of Yunnan Province, Kunming, China, [2] Medical Faculty, Kunming University of Science and Technology, Kunming, China, [3] Genetic Testing Center, Qingdao Women and Children's Hospital, Qingdao, China

Angiogenesis and the tumor microenvironment (TME) play important roles in tumorigenesis. Forkhead box Q1 (FOXQ1) is a well-established oncogene in multiple tumors, including colorectal cancer (CRC); however, whether FOXQ1 contributes to angiogenesis and TME modification in CRC remains largely uncharacterized. Here, we demonstrate an essential role of FOXQ1-induced angiogenesis and macrophage recruitment in CRC that is related to its ability to promote the migration of endothelial cells and macrophages through activation of the EGF/PDGF pathway and the Twist1/CCL2 axis. We also provide evidence showing that the clinical significance between FOXQ1, Twist1, CCL2, and macrophage infiltration is associated with reduced 8-year survival in CRC patients. Our findings suggest FOXQ1 plays critical roles in the malignancy and progression of CRC, Therefore, FOXQ1 may serve as a therapeutic target for inhibiting angiogenesis and reducing macrophage recruitment in CRC.

Keywords: FOXQ1, colorectal cancer, tumor angiogenesis, macrophages, tumor microenvironment

OPEN ACCESS

Edited by:
Lucas Treps, VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Anantha Koteswararao Kanugula, University of Massachusetts Medical School, United States
Ann Ager, Cardiff University, United Kingdom

***Correspondence:**
Qiang Guo
gqkj003@sina.com

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *21 May 2020*
Accepted: *23 October 2020*
Published: *30 November 2020*

Citation:
Tang H, Zheng J, Bai X, Yue K-L, Liang J-H, Li D-Y, Wang L-P, Wang J-L and Guo Q (2020) Forkhead Box Q1 Is Critical to Angiogenesis and Macrophage Recruitment of Colorectal Cancer. Front. Oncol. 10:564298. doi: 10.3389/fonc.2020.564298

INTRODUCTION

Tumor initiation and malignancy are closely associated with angiogenesis (1), and pathological neovascularization initiates tumor tissue ischaemia, growth, and metastasis (2). In angiogenesis, endothelial cell (EC) proliferation and migration cause new capillaries to develop from preexisting capillaries (3). In addition, macrophages within the tumor microenvironment (TME) facilitate angiogenesis and extracellular-matrix breakdown, thus remodelling and promoting tumor cell migration, invasion, and metastasis (4). Angiogenesis involves complex signalling pathways and is associated with the production of many key regulatory factors, such as epidermal growth factor (EGF)/platelet-derived growth factor (PDGF) (5), vascular endothelial growth factor (VEGF), epidermal growth factor-like growth factor (HB-EGF) (6), angiopoietins, tissue inhibitor of metalloproteinases 1 (TIMP-1), and basic fibroblast growth factor (bFGF) (7). Therefore, angiogenesis and the inflammatory microenvironment play important roles in tumorigenesis.

Forkhead box Q1 (FOXQ1) is a member of the forkhead transcription factor family (8) with demonstrated functional roles in hair follicle morphogenesis and gastric epithelial differentiation (9). Studies have indicated that FOXQ1 is also an oncogene in multiple tumors, including colorectal cancer (CRC) (10–12), non-small cell lung cancer (13), breast cancer (14, 15), ovarian cancer (16),

bladder carcinoma (17), stomach cancer (18), liver cancer (19), and neuroglioma (20). Recent studies suggest that the tumorogenic function of FOXQ1 may be related to its ability to promote cell cycle progression (10, 16), tumor angiogenesis (10), cell proliferation (11, 21), stem cell-like properties (14), resistance to chemotherapy-induced apoptosis (14), modification of the TME (19), epithelial-mesenchymal transition (EMT) (22, 23), senescence-associated inflammation (24), and Wnt signaling activation (25). We previously demonstrated that aberrant expression of FOXQ1 is correlated with metastasis in CRC (12). Furthermore, FOXQ1 has been shown to be a regulator of cancer invasion and metastasis in CRC and a modulator of Twist1 expression (11). The role of Twist1-induced CCL2 in angiogenesis has been demonstrated (26), which raises the possibility that FOXQ1 may induce angiogenesis in CRC by inducing Twist1; however, a role for FOXQ1 in inducing tumor angiogenesis and TME modification in CRC has not been evaluated.

In this study, we demonstrate that FOXQ1 promotes angiogenesis in CRC cells by activating the expression of angiogenic factors while reducing the expression of angiogenic inhibitors. In addition, FOXQ1 can promote recruitment of macrophages by activating the Twist1/CCL2 axis. Our results indicate that FOXQ1 overexpression correlates clinically with overall survival in CRC, suggesting that it might serve as a new target for anti-angiogenic and anti-inflammation therapy.

MATERIALS AND METHODS

Cell Culture

The human CRC cell lines LS174T, Colo320, SW480, HCT116, DLD1, HT29, and LoVo; HEK293 cells; and human macrophage U937 cells were purchased and authenticated from the Cell Bank of the Chinese Academy of Science in Shanghai, China. HUVECs were purchased from Yingrun Biotechnology Co. Ltd. in Changsha, China. All cells were cultured as recommended by the manufacturer.

Plasmid Construction and Transfection

Three siRNAs targeting the human FOXQ1 sequence (NM_033260.3) were designed using the siRNA Target Finder (InvivoGen, San Diego, CA, USA), and one scrambled siRNA was designed as a negative control (**Table S1**). The corresponding primers used for plasmid construction were synthesized by Sangon (Shanghai, China) (**Table S2**) and then ligated to the lentiviral PLKO.1 vector. A FOXQ1 cDNA plasmid purchased from GeneChem (Shanghai, China) was cloned into a pLVX-IRES-Puro lentiviral vector, and the recombinant plasmid was named lv-FOXQ1. Lentiviral vectors and packing vectors (pRSV-rev, pMDlg-pRRE and pCMV-VSV-G) were co-transfected into HEK293 cells. Lentivirus was collected to infect DLD1 and HCT116 cells. Stable cells were generated after selection with puromycin (12 mg/ml) (Solarbio, Beijing, China) for 20 days. The most effective knockdown/overexpression cells were designated DLD1-shFOXQ1/HCT116-FOXQ1, and the corresponding controls were named DLD1-shControl/HCT116-vec. For transient transfection, either siRNA targeting Twist1 or vector overexpressing Twist1 (pcDNA3.1-Twist1), or the respective controls (si-Scramble and pcDNA3.1), were purchased from GeneChem (Shanghai, China) and transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). qRT-PCR was performed using the LightCycler 480 (Roche, USA) with SYBR Premix Ex Taq II (Takara, China). Each sample was analysed in duplicate with GAPDH as a reference. Oligonucleotide sequences are provided in supplemental file 1 (**Table S3**). Quantitative results were calculated using the $2^{-\triangle\triangle CT}$ method.

Transcriptional Signature Analysis

Transcriptional signatures of DLD1-shFOXQ1 and DLD1-shControl cells were obtained and compared using the Human GE 4 × 44K Microarray platform (Agilent Technologies, Santa Clara, CA, USA). Microarray experiments were performed by KangChen Bio-tech, Shanghai, China. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies, Santa Clara, CA, USA). Differentially expressed genes (DEGs) with statistical significance were identified by volcano plot filtering. KEGG pathway analysis was performed using the NIH gene annotation software DAVID.

Western Blot Analysis

Cells were washed with PBS and lysed in ice-cold lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Lysates were separated by electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore; Bedford, MA, USA). The membranes were blocked and incubated with primary antibodies. Antibodies against FOXQ1 (ab51340), PDGF (ab178409), HB-EGF (ab92620), and Twist1 (ab175430) were purchased from Abcam (England). The antibody against EGFR (3265S) was purchased from Cell Signaling (Cold Spring Harbor, NY, USA), and antibodies against ANG (18302-1-AP), PDGFRB (13449-1-AP), ANGPT1 (23302-1-AP), PLAUR (10286-1-AP), tPA (10147-1-AP), VEGF (19003-1-AP), and β-actin (66009-1-Ig) were purchased from Proteintech (Wuhan, China). β-Actin was used as the loading control. The immunoreactive proteins were visualized with SuperSignal West Dura Chemiluminescent Substrate (ThermoFisher, Waltham, MA, USA).

Cell Proliferation Assay and Clone Formation Assay

The DLD1-shFOXQ1 and DLD1-shControl cells (2,000 cells/well in a 96-well plate) were incubated with medium containing

10% FBS at 37°C for 24 h, 48 h, 72 h, and 98 h, respectively. At the end of incubation, 10 µl Cell Counting Kit-8 (CCK-8) solutions (Beyotime Biotech, Shanghai, China) were added and incubation for another 4 h at 37°C, and then OD_{450nm} was measured by Microplate Reader (BioTek, Winooski, VT, USA). For clone formation assay, 500 cells were seeded into 6-well plates and incubated for 14 days (with medium replaced every three days). Then, the cells were fixed and stained with 1% crystal violet at room temperature for 20 min. Photos were taken and the number of clones was counted.

Cell Migration Assay and Wound Healing Assay

Analysis of cell migration was done by using Transwell insert with 8.0 µm membrane pores (BD, San Jose, CA, USA) according to the manufacturer's protocol. Migration was additionally evaluated with the wound healing assay. Briefly the DLD1-shFOXQ1 and DLD1-shControl cells were seeded in 24 well plates at a density that enabled a confluency of 80% to be attained 24 h after plating. A 200 µl filter tip was used to gently scratch the cell monolayer across the center of the well. The cells were then gently washed with PBS to remove the dislodged cells, and then replenished with fresh medium, after which the first images were acquired. The cells were incubated for a further 24 h after which a second set of images were acquired to determine the extent of wound closure.

Preparation of Conditioned Medium (CM) From CRC

HCT116-FOXQ1 and DLD1-shFOXQ1 cells were cultured in RPMI-1640/5% FBS medium. At 90% confluence, the culture medium was switched to either serum-free GT-T551 (Takara, Dalian, China) or fresh RPMI-1640/5% FBS medium and was incubated for 48 h before the CM was collected. Serum-free GT-T551 CM was collected for protein array analysis, and RPMI-1640/5% FBS CM was collected for EC migration assay and ELISA.

EC Migration Assay

The HUVEC migration assay was performed as described previously with minor modifications (27). HUVECs are regarded as a cell model in angiogenesis assays *in vitro*, for which they have been widely used to detect tube formation abilities on matrigel (28, 29). HUVECs at passage 5 or less were serum-starved for 5 h in serum-free EBM-2 (Lonza Cologne, Walkersville, MD, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, 10 ng/ml heparin, and 0.1% FBS. On the following day, a Transwell migration assay was performed using BD cell culture inserts (3.0 µm membrane pores) according to the manufacturer's protocol. Transwells were assembled in 12-well plates, and the lower chambers were filled with 1,500 µl of medium containing 50% fresh EBM-2/10% FBS and 50% CRC-CM. Then, 60,000 HUVECs resuspended in 500 µl serum-free EBM-2 were inoculated onto the upper chamber of each Transwell, and the plates were placed at 37°C in a 5% CO_2 incubator for 4 or 8 h. After removing the non-migrating cells with a cotton swab, the cells that had migrated to the lower surface of the filters were fixed with cold 4% paraformaldehyde and stained with 0.1% crystal violet/20% (v/v) methanol. Then, the migrated cells on the bottom of the Transwell inserts were counted. All assays were performed in triplicate. Three random fields were chosen for each insert, and the cells were counted and imaged under a light microscope.

ELISA

To measure CCL2 secretion, CM from CRC cells cultured for 48 h was collected, and CCL2 concentrations were measured using the CCL2 ELISA detection kit (eBioscience, Houston, Texas, USA) according to the manufacturer's protocol.

Microvessel Morphogenesis Assay

A microvessel formation assay was performed as described previously with minor modifications (27). HCT116-FOXQ1, DLD1-FOXQ1-shRNA cells, and HUVECs were cultured in reduced serum conditions in RPMI 1640 medium containing 1% FBS for 12 h, and then 2 x 10^5 HUVECs were mixed with either 2 x 10^5 HCT116-FOXQ1 or 2 x 10^5 DLD1-shFOXQ1 cells and grown in 24-well plates precoated with growth factor-reduced Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA). After 20 h incubation, images were taken, and microvessel formation abilities were quantified by measuring the cumulative tube length using ImageJ software (NIH, Bethesda, MD, USA). The number of intact or damaged microvessels was quantified. For the CM experiment, HCT116-FOXQ1 and DLD1-FOXQ1-shRNA cells were cultured in complete medium containing l0% FBS for 48 h, and then cell culture supernatants were collected as CM. At the same time, HUVECs were cultured in reduced serum conditions in RPMI 1640 medium containing 1% FBS for 12 h. Then, 2 × 10^5 HUVECs were resuspended in either the HCT116-FOXQ1 CM or DLD1-shFOXQ1 CM and grown in 24-well plates precoated with Matrigel. Microvessel formation was documented after 20 h by microscopy (Axiovert 200; Carl Zeiss, Gttingen, Germany).

Tumor Xenograft Model and *In Vivo* Angiogenesis Assay

Female BALB/c nude mice (6–7 weeks old) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Ten mice were randomly divided into two groups. A total of 200 µl of a 3:1 mixture of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) and 1 × 10^7 DLD1-shFOXQ1 or DLD1-shControl cells in DMEM were injected into the dorsal flank of each mouse. The date at which the first grossly visible tumor appeared was recorded, and the tumor size was measured every 3 days thereafter. Mice were sacrificed on day 16, and tumors were dissected and fixed for histological examination and microvessel density (MVD) analysis. The animal study was reviewed and approved by Institutional Animal Care and Use Committee, the First People's Hospital of Yunnan Province (Yunnan, China).

Immunohistochemistry and MVD Analysis

Antibodies against FOXQ1 (ab51340), CD31 (ab28364), CD34 (ab81289), and F4/80 (ab6640) were purchased from Abcam

(England), and paraffin-embedded mouse tumor serial sections (4 μm) were stained with anti-FOXQ1 antibody to confirm the efficiency of FOXQ1 gene knockdown. Haematoxylin and eosin (H&E) staining was performed to verify the morphological characteristics of xenograft tumor tissues. Anti-CD31 and anti-CD34 antibodies were used for EC staining. The macrophage content was measured by staining for the mature macrophage marker F4/80. For MVD analysis, $CD31^+$ or $CD34^+$ blood vessels in tumor sections were counted in 10 random fields (hpfs, 400×) in vascular hot spots, as previously described (30). For macrophage quantification, five random fields in $F4/80^+$ hot spots were scored on a scale of 0-6 for staining intensity and distribution within a field: 0, undetectable; 1, faint, discrete patches; 2 faint, all over; 3 medium, discrete patches; 4 medium, all over; 5 intense, discrete patches; 6 intense, all over. The images were documented by two pathologists blinded and processed using Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA, USA).

Protein Array Analysis

HUVECs at passage 5 or less were seeded at a density of 10,000 cells per well in a 12-well plate and then switched for 48 h to RPMI-1640/5% FBS medium. Then, the RPMI-1640/5% FBS medium was replaced with medium consisting of 50% RPMI-1640/5% FBS and 50% CRC-CM and cultured for another 48 h. The cells were lysed in NP-40 lysis buffer (Beyotime, Beijing, China) containing a protease inhibitor cocktail (Promega, Madison, WI, USA). CM of CRC cells and cell lysates of HUVECs were then assayed for angiogenesis factor levels by using protein arrays (QAH-ANG-2 and QAH-ANG-3, Quantibody Human Angiogenesis Array, RayBiotech, Norcross, GA, USA), which can quantitatively measure 60 well-established angiogenic proteins by comparing fluorescent signals to the standard curve. Analysis was performed according to the manufacturer's instructions, and the detected signals were quantified using a gel documentation system (UVItec, Cambridge, MA, USA).

In Vitro Macrophage Cell Migration Assay

U937, a human monocytic cell line, was differentiated into macrophages by using 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA, P8139) as described previously (31). Then, macrophage migration assays were performed as described (32). Briefly, macrophages were seeded (5×10^4 cells/insert) onto the upper well of Transwell inserts with 8.0 μm membrane pores (BD, San Jose, CA, USA) for 2 h to allow attachment to the membrane, and then Transwells were moved to 24-well plates containing 0.7 ml CM either from HCT116-FOXQ1 (co-transfected either with si-Twist1 or si-Scramble) or from DLD1-shFOXQ1 (co-transfected either with pcDNA3.1-Twist1 or pcDNA3.1) and further incubated for 4 h. Cells in the upper chamber were removed with a cotton swab after fixation in 4% paraformaldehyde and stained with 0.1% crystal violet, while the migrated macrophages in the lower chamber were quantified using 12–15 random fields. Three independent experiments were performed.

CRC Tissue Microarray

Tissue microarrays containing a total of 90 pairs of colorectal tumor tissues and matched adjacent normal tissues, together with pathological staging data in accordance with TNM classification of the American Joint Committee on Cancer (2010) and follow-up survival time after surgery, were obtained from Shanghai Biochip Co., Ltd., Shanghai, China (HCol-Ade180Sur-06). Antibodies against FOXQ1 (ab51340), CD31 (ab28364), Twist 1 (ab175430), CCL2 (ab73680), and CD68 (ab955) were purchased from Abcam (Cambridge, MA, USA), and tissue microarray analysis was performed using a standard immunohistochemistry protocol. The median value of the immunoreactivity score (IRS) was chosen as the cut-off for high and low protein expression levels based on a measure of heterogeneity according to the log-rank test with respect to disease-specific survival (DSS), as described previously (33). Cut-off values for the scoring system were assigned as follows: high expression of FOXQ1, Twist1, and CCL2 were defined as an IRS of ≥ 4 (4, 6, 8, 9, and 12), and low expression was defined as an IRS of < 4 (0, 1, 2, and 3). High expression of CD31 and CD68 was defined as an IRS of ≥ 150 and of ≥ 400, respectively, and low expression was defined as an IRS of <150 and of <400, respectively. Immunostained sections were scanned using a microscope (Axiovert 200; Carl Zeiss, Gttingen, Germany). Data for seven patients were excluded because the dots were off the chips during the experiment. Data for a total of 83 patients with CRC were therefore included in the final analysis.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6.01 and SPSS v.19. An unpaired two-tailed Student's *t*-test was performed for two-group comparisons, and one-way analysis of variance (ANOVA) was performed for multiple group comparisons. Survival curves were calculated using the Kaplan-Meier algorithm and log-rank test.

RESULTS

Transcriptional Signature Analysis of CRC Cells With FOXQ1 Overexpression or Knockdown

To elucidate functional roles for FOXQ1 in CRC, we generated CRC cell lines with stable FOXQ1 overexpression or shRNA. Among the CRC cell lines we tested, HCT116 had relatively low endogenous FOXQ1 expression, a feature already confirmed in several previous studies (10, 11, 14, 21, 25). Conversely, DLD1 had relatively high endogenous FOXQ1 expression. Both the HCT116 and DLD1 were CRC cell lines with malignant epithelial properties and originated from colorectal carcinoma. We therefore selected these two cell lines for over expression and knock down studies, respectively (**Figure 1A**). RT-PCR and Western blotting assays verified the successful preparation of HCT116-FOXQ1-2$^{\#}$ (designated "HCT116-FOXQ1"; **Figure 1B**) and DLD1-shFOXQ1-3$^{\#}$ (designated "DLD1-shFOXQ1";

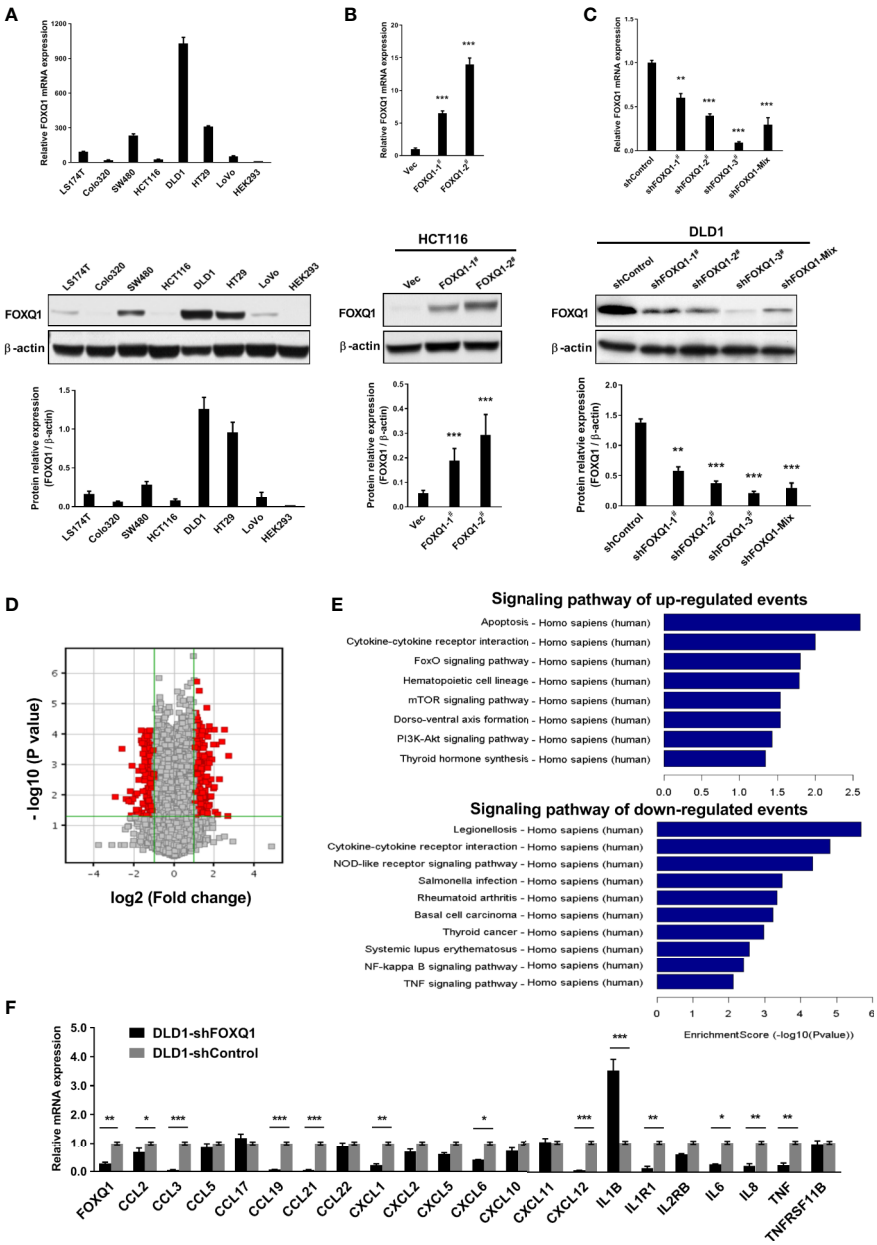


FIGURE 1 | FOXQ1 modulates the expression of genes that affect the tumor microenvironment (TME) during colorectal cancer (CRC) tumorigenesis. **(A)** Relative endogenous FOXQ1 expression in 7 CRC cell lines; HEK293 was used as internal control. **(B)** Increased expression of FOXQ1 after transfection of HCT116 with either FOXQ1-1$^{\#}$ or FOXQ1-2$^{\#}$; HCT116 transfected with Vec was used as control. **(C)** Reduction in FOXQ1 after transfection of DLD1 with shFOXQ1-1$^{\#}$, shFOXQ1-2$^{\#}$, shFOXQ1-3$^{\#}$, or shFOXQ1-Mix; DLD1 transfected with shControl was used as control. **(D)** Volcano Plot visualizing differentially expressed genes (DEGs) between DLD1-shFOXQ1 and DLD1-shControl. The red point in the plot represents the DEGs with statistical significance. **(E)** Significant pathway analysis of up- and down-regulated DEG events; pathways are sorted in descending order based on enrichment score. Left, Pathway Name; right, bar graph representing the enrichment score [-log10 (*P* value)]. **(F)** Validation of the expression profiles of a subset of DEGs involved in cytokine-cytokine receptor interaction and the TNF signaling pathway by using qRT-PCR. **P*<0.05, ***P*<0.01, ****P*<0.001 indicated a significant difference as compared to the control group (two-tailed, unpaired Student's *t*-test). Bars represent mean ± S.E. of three independent experiments.

Figure 1C). Transcriptional microarray analysis identified 431 DEGs (255 upregulated and 176 downregulated) between DLD1-shFOXQ1 and DLD1-shControl cells (**Figure 1D**, **Table S4**, GSE74223). Furthermore, pathway analysis revealed several pathways related to oncogenesis. Notably, "Cytokine-cytokine receptor interactions" was identified as both significantly up- and downregulated, and the "TNF signaling pathway" was identified as downregulated, which suggests that FOXQ1 modulates the TME (**Figure 1E**, **Table S5**). To verify these effects of shFOXQ1, the expression profiles of a subset of 21 genes involved in TME

modification were further validated by qRT-PCR. The results showed that mRNA expression of CCL2, CXCL12, IL6, IL8, and TNF, all of which are implicated in macrophage recruitment and inflammation, were dramatically downregulated in DLD1-shFOXQ1 compared with control cells (**Figure 1F**; CCL2 and IL6, $P<0.05$; IL8 and TNF, $P<0.01$; CXCL12, $P<0.001$). The results also showed that mRNA expression of cytokines/chemokines relevant to chemotaxis of T cells, such as IL1B, was also dramatically upregulated in DLD1-shFOXQ1 compared with control cells (**Figure 1F**; IL1B, $P<0.001$). These results suggest that FOXQ1 may play an important role in mediating TME modification during CRC tumorigenesis.

Inhibition of FOXQ1 in CRC Cells Induces Suppressed Proliferation and Migration of CRC Cells *In Vitro*

To dissect the impact that FOXQ1 had on tumor progression and malignancy, we appraised its influence on cell proliferation and migration. We observed significant inhibition in both cell proliferation (**Figure 2A**; $P<0.05$) and clone formation (**Figure 2B**; $P<0.01$), and a concurrent decrease in migration (**Figure 2C**; $P<0.01$) and wound healing ability (**Figure 2D**; $P<0.01$), when FOXQ1 was suppressed in DLD1 cells. The *in vitro* results showed that inhibition of FOXQ1 could suppress the proliferation and migration of DLD1 cells.

FOXQ1 Activates the Recruitment of HUVECs and Promotes Microvessel Morphogenesis *In Vitro*

Given the ability of FOXQ1 in CRC cells to modify the expression levels of genes related to the TME, we speculated that it might be involved in EC recruitment. Therefore, the effect of the FOXQ1 gene on the recruitment of HUVECs was detected using a Transwell system. HUVECs were cultured with CM collected from either HCT116-FOXQ1 or DLD1-shFOXQ1 cells. The results indicated that treatment of HUVECs with CM from HCT116-FOXQ1 for 8h displayed a higher ability to recruit HUVECs than those with CM from HCT116-Vec (**Figure 3A**; $P<0.01$). Conversely, blocking expression of FOXQ1 in DLD1-shFOXQ1 for 8h resulted in less recruitment of HUVECs than that in the control group (**Figure 3B**; $P<0.001$). Therefore, these results suggest that FOXQ1 mediates the recruitment of ECs, which comprises an initial step of angiogenesis.

Angiogenesis also entails the *de novo* formation of microvessels (3). To assess the function of FOXQ1 in regulating microvessel morphogenesis, *in vitro* microvessel formation assays were performed by co-culturing HUVECs with CRC cells or CM collected from CRC cells. The results show that HCT116-FOXQ1 cells elicited a strong angiogenic response and induced HUVECs to differentiate into microvessel structures; a similar angiogenic response was also observed with HCT116-FOXQ1-CM, though the effect was less obvious (**Figure 3C**). Consistently, ectopic expression of FOXQ1 in HCT116 cells increased the microvessel length of HUVECs (left panel of **Figure 3D**; both $P<0.01$) and the abundance of intact microvessels (right panel of **Figure 3D**; $P<0.01$ for HCT116 cells and $P<0.05$ for CM). Conversely, FOXQ1 knockdown in DLD1 resulted in a reduced angiogenic response and less HUVECs differentiating into microvessel structures either with DLD1-shFOXQ1 cells or DLD1-shFOXQ1-CM than in the control groups (**Figure 3E**). Blocking the expression in DLD1 cells resulted in microvessels of reduced length (left panel of

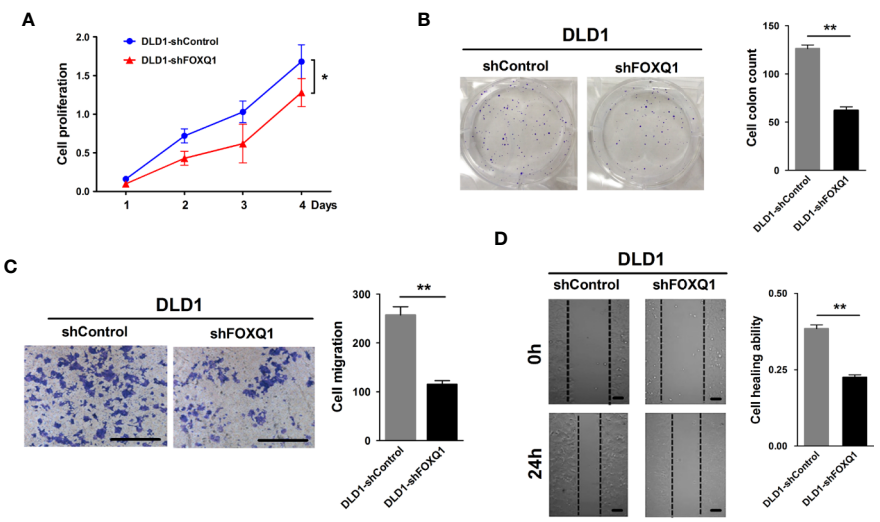


FIGURE 2 | Inhibition of FOXQ1 induces suppressed proliferation and migration of colorectal cancer (CRC) cells *in vitro*. **(A)** FOXQ1 inhibition significantly supressed cell proliferation of DLD1-shFOXQ1 compared with control. **(B)** Significant decrease of clone formation in DLD1-shFOXQ1 compared with control. **(C)** Representative images of inhibition in migration DLD1-shFOXQ1 compared with control. **(D)** Representative images of inhibition in wound healing ability in DLD1-shFOXQ1 compared with control. All scale bars represent 100 μm. *$P<0.05$, **$P<0.01$ signify a significant difference between the indicated groups (two-tailed, unpaired Student's *t*-test). *Bars* represent mean ± S.E. of three independent experiments.

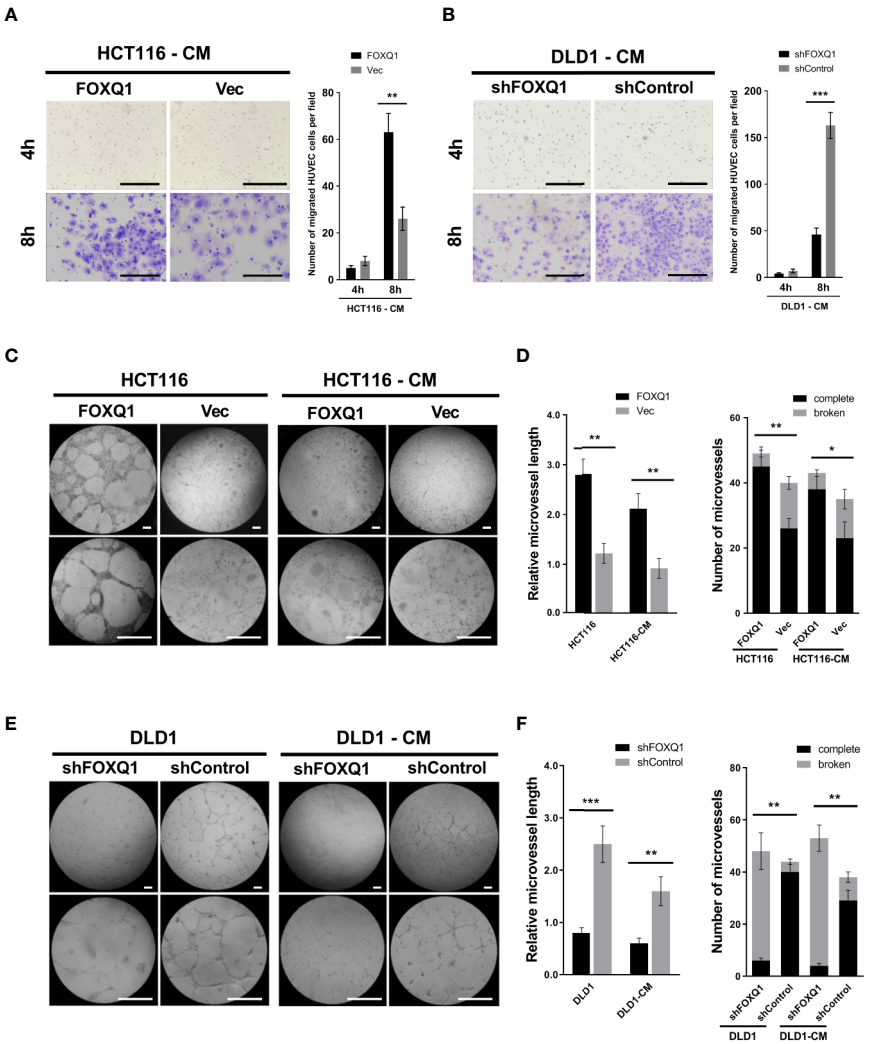


FIGURE 3 | FOXQ1 promotes colorectal cancer (CRC) angiogenesis by activating recruitment of HUVECs and promotes microvessel morphogenesis *in vitro*. HUVEC migration was imaged at 4 h and 8 h after cell seeding into the Transwells. The number of HUVECs that had migrated was counted and normalized to that of the control group. **(A)** Treatment of HUVECs with CM from HCT116-FOXQ1 displayed a higher ability to recruit HUVECs than those with CM from HCT116-Vec. **(B)** Conversely, blocking expression of FOXQ1 in DLD1-shFOXQ1 resulted in less recruitment of HUVECs than that in the control group at 8 h. **(C)** Representative images of microvessel formation for HUVECs either co-cultured with either HCT116-FOXQ1 or HCT116-Vec, or CM of either HCT116-FOXQ1 or HCT116-Vec. **(D)** The relative microvessel length and number of complete and broken microvessels in HCT116-FOXQ1 cultures was compared with that in HCT116-Vec cultures. FOXQ1 overexpression significantly increased both the relative microvessel length and number of complete microvessels in CRC. **(E)** Representative images of microvessel formation for HUVECs co-cultured with either DLD1-shFOXQ1 or DLD1-shControl, or CM of either DLD1-shFOXQ1 or DLD1-shControl. **(F)** The relative microvessel length and number of complete and broken microvessels in DLD1-shFOXQ1 was compared with that in DLD1-shControl. FOXQ1 knockdown significantly decreased both the relative microvessel length and the number of complete microvessels in CRC. All scale bars represent 100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001 signify a significant difference between the indicated groups (two-tailed, unpaired Student's *t*-test). CM, conditioned media.

Figure 3F; $P<0.001$ and $P<0.01$ for DLD1 cells and CM, respectively) and decreased abundance (right panel of **Figure 3F**; both $P<0.01$). These results suggest that FOXQ1 is essential for microvessel morphogenesis in CRC. The ability of the CM to confer a weaker angiogenic response than that of co-cultured cells implies that factors secreted by CRC cells can promote tumor angiogenesis, but that intercellular interactions between tumor cells and epithelial cells may also play important roles in promoting tumor angiogenesis.

FOXQ1 Inhibition in CRC Cells Results in Inhibited Tumor Angiogenesis and Intratumoral Macrophage Infiltration *In Vivo*

To determine whether FOXQ1 affects tumor angiogenesis and intratumoral macrophage infiltration *in vivo*, tumor xenografts were obtained by implanting DLD1-shFOXQ1 or DLD1-shControl cells subcutaneously in nude mice. DLD1-shFOXQ1 resulted in approximately 2.68-fold decrease in tumor size

relative to that of DLD1-shControl 16 days after implantation (**Figure 4A**; $P<0.01$). The sizes of dissected tumors reflected the differences in the tumor volumes (**Figure 4B**). Furthermore, the morphological features of CRC were verified by H&E staining of xenograft tumor tissues (**Figure 4C**), and immunostaining of FOXQ1 in dissected tumor tissues confirmed the reduction of FOXQ1 in DLD1-shFOXQ1 (**Figure 4D**; $P<0.01$). Thus, these results suggest that inhibition of FOXQ1 suppresses the tumor proliferation capacity *in vivo*.

To determine the *in vivo* effect of FOXQ1 on tumor angiogenesis, MVD was evaluated by immunohistochemical staining of tumor specimens for the blood vessel markers CD31 and CD34, and the number of $CD31^+$ (left panel of **Figure 4E**; $P<0.05$) and $CD34^+$ (right panel of **Figure 4E**; $P<0.05$) blood vessels was significantly decreased in DLD1-shFOXQ1 compared with DLD1-shControl tumors. To determine the effect of FOXQ1 downregulation on macrophage chemotaxis, the macrophage content in transplanted CRC tissues

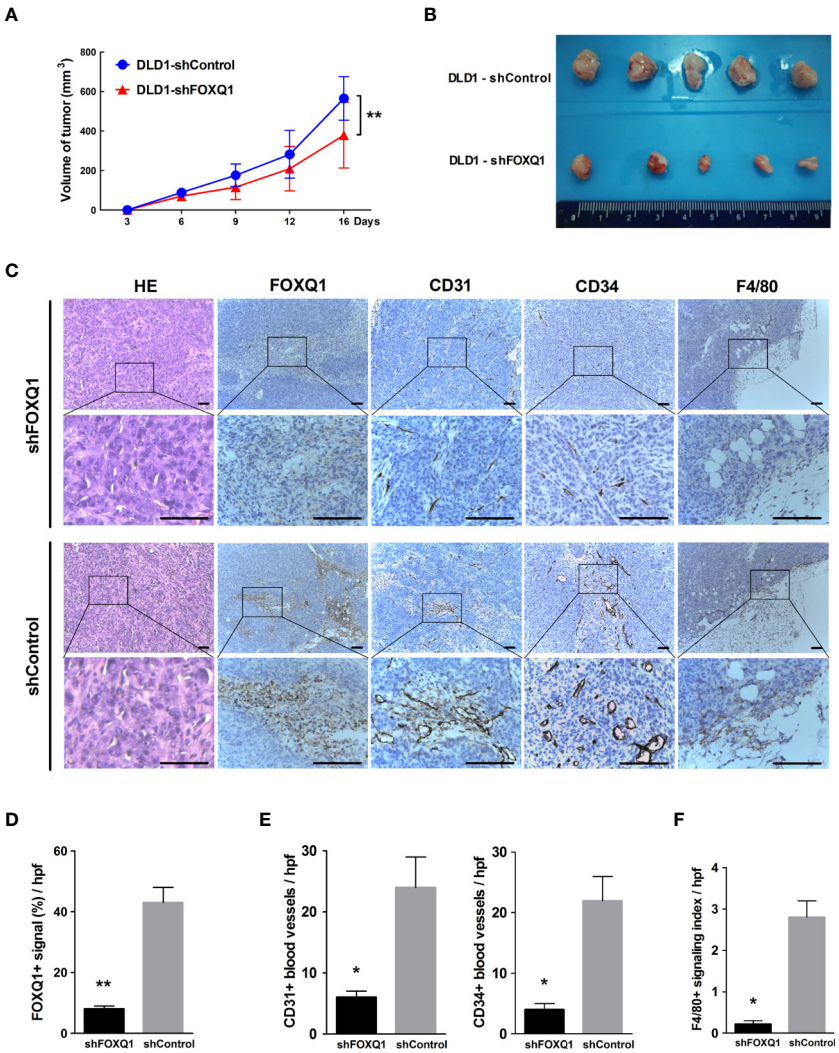


FIGURE 4 | Inhibition of FOXQ1 results in inhibited tumor angiogenesis and intratumoral macrophage infiltration *in vivo*. Athymic nu-/nu-mice were implanted with 1×10^7 DLD1-shFOXQ1 or DLD1-shControl cells subcutaneously (n = 5 per group); tumor volume was documented by caliper measurement. **(A)** FOXQ1 inhibition significantly suppressed the tumor proliferation capacity *in vivo*. **(B)** Resulting plugs were harvested and processed for IHC staining. **(C)** Representative HE staining images of tissue sections and IHC staining for FOXQ1; analysis of microvessel density (MVD) by IHC staining for CD31 or CD34; detection of the content of macrophages by IHC staining for F4/80. Scale bars represent 100 μm. **(D)** Reduction of FOXQ1 in dissected tumor tissue from the DLD1-shFOXQ1 group as determined by IHC staining quantification. **(E)** MVD was determined by counting the number of $CD31^+$ or $CD34^+$ vessels in tumor specimens; the number of $CD31^+$ or $CD34^+$ blood vessels was significantly decreased in DLD1-shFOXQ1 compared with DLD1-shControl. **(F)** Intratumoral macrophage quantification was determined by scoring for F4/80 staining intensity and distribution; DLD1 cells with downregulated FOXQ1 significantly decreased the number of infiltrating macrophages. 5 random hpf/section, 2-3 representative sections/plug, 5 plugs/group were scored for positive signal quantification. *$P<0.05$, **$P<0.01$ signifies a significant difference between the indicated groups (two-tailed, unpaired Student's *t*-test). HE, Hematoxylin and eosin; IHC, immunohistochemical; hpf, high-powered fields.

was measured by staining for the mature murine macrophage marker F4/80. The results indicate that the TME of DLD1-shFOXQ1 tumors had significantly decreased numbers of infiltrating macrophages (**Figure 4F**; $P<0.05$). Therefore, these results verify that FOXQ1 contributes to CRC angiogenesis and the CRC TME *in vivo*.

FOXQ1 Inhibition in CRC Cells Downregulates Angiogenic Factors and the Chemoattractant CCL2 and Upregulates Angiogenic Inhibitors

As the secretion of regulatory factors and cytokines by tumor cells are known to promote angiogenesis and macrophage recruitment, we speculated that FOXQ1 might increase EC migration, microvessel morphogenesis, and macrophage recruitment by affecting the secretion of an array of regulatory factors and cytokines in CRC cells. Therefore, we quantified the expression and secretion of 60 well-established angiogenic factors in CM from CRC cells by protein array. Thirty-seven of the 60 angiogenic proteins were below the detectable concentration range, and the remaining 23 proteins (including 17 angiogenic factors and 6 angiogenic inhibitors) that were within the detection range were further analysed (**Figures 5A, B**). Our results indicate that inhibition of FOXQ1 expression decreased the expression of important angiogenic factors in lysates from HUVECs that were cultured in CM from DLD1-shFOXQ1 (left panel of (**Figure 5C**), as well as in CM from CRC cells (left panel of **Figure 5D**), these decreased angiogenic factors include ANGPTL4, bFGF, Leptin, CCL2, CXCL16, Follistatin, and VEGF, most of which belong to EGF/PDGF pathway (34). In addition, CD31, a blood vessel marker, was decreased in HUVECs cultured with CM from DLD1-shFOXQ1 compared with those cultured with CM from DLD1-shControl (left panel of **Figure 5C**). On the other hand, the inhibition of FOXQ1 expression promoted the secretion of 3 out of 6 angiogenic inhibitors including ANG-2, TIMP-1, and IL-12 both in HUVECs cell lysates (right panel of **Figure 5C**) and CM from CRC cells (right panel of **Figure 5D**).

To further verify that FOXQ1 inhibition downregulates angiogenic factors while up-regulating angiogenic inhibitors, we performed Western blotting assays to evaluate the effect of FOXQ1 knockdown on the expression of selected proteins from the protein array (ANG, PDGF, PLAUR, ANGPT1, and VEGF), as well as additional proteins of the EGF/PDGF pathway that have been established to play important roles in tumor angiogenesis (PDGFRB, tPA, EGFR, and HB-EGF) (34). We also evaluated the expression of a downstream target gene previously shown to be regulated by FOXQ1 in CRC: Twist1 (11). Consistent with the findings from our protein array analysis and previous reports (10, 11), the protein levels of PDGF, PDGFRB, PLAUR, VEGF, EGFR, HB-EGF, and Twist1 were positively correlated with FOXQ1 expression, while the protein levels of ANG and tPA were not changed, and the protein level of ANGPT1 displayed the opposite trend (**Figure 5E**). In summary, these results suggest that FOXQ1 inhibition in CRC cells induces downregulation of angiogenic factors while upregulating angiogenic inhibitors.

To further verify that FOXQ1 inhibition downregulates CCL2, a well-known macrophage chemoattractant (35), we performed ELISA analysis in CRC cells. The results confirm that shFOXQ1 prevented the autocrine secretion of CCL2 by DLD1 cells (**Figure 5F**; $P<0.001$), whereas overexpression of FOXQ1 enhanced the secretion of CCL2 by HCT116 cells (**Figure 5F**; $P<0.001$). These results indicate that FOXQ1 expression positively correlates with the ability of CRC cells to secrete CCL2, which could explain the increased macrophage infiltration in tumor cells (**Figure 4F**).

Twist1 Is Essential for FOXQ1-Mediated Macrophage Recruitment in CRC

FOXQ1 is an established modulator of Twist1 expression and a regulator of cancer invasion and metastasis in CRC (11), and the role of Twist1-induced CCL2 in angiogenesis has been previously demonstrated (26). We therefore speculated that FOXQ1 might regulate macrophage infiltration by activating the Twist1/CCL2 axis. To further substantiate this hypothesis and to investigate the functional importance of the Twist1/CCL2 axis in macrophage infiltration, we measured CCL2 secretion in CM from HCT116-FOXQ1 and DLD1-shFOXQ1 cells that were co-transfected with si-Twist1 or pcDNA3.1-Twist1 (**Figure 6A**). The results indicate that Twist1 knockdown abolishes FOXQ1-mediated CCL2 upregulation in HCT116-FOXQ1 cells (**Figure 6B**; $P<0.01$). Conversely, upregulation of Twist1 increased CCL2 secretion in DLD1-shFOXQ1 cells (**Figure 6B**; $P<0.05$). We next sought to explore the effect of Twist1 on FOXQ1-dependent macrophage infiltration induced by CM from CRCs. The results reveal that Twist1 knockdown eliminates the FOXQ1-induced inhibition of macrophage recruitment in HCT116-FOXQ1cells (**Figure 6C**; $P<0.01$), whereas upregulation of Twist1 rescues the decreased macrophage infiltration ability induced by FOXQ1 (**Figure 6C**; $P<0.001$). Taken together, these studies suggest that FOXQ1-mediated CCL2 secretion is dependent on Twist1 and that the Twist1/CCL2 axis is essential for FOXQ1-mediated macrophage recruitment in CRC.

FOXQ1 Expression Is Positively Correlated With Twist1 and CCL2 Expression in Human CRC Tissues, and Their Positive Co-Expression Is Correlated With a Lower 8-Year Survival Rate

To verify the clinical relevance of our findings, we evaluated the expression of FOXQ1, CD31, Twist1, CCL2, and CD68 in human CRC tissue biopsies (cohort, n=83). IHC results showed that FOXQ1, Twist1, and CCL2 each were significantly upregulated in CRC tissues compared with adjacent nontumorous tissues, and that CD31 and CD68 were moderately upregulated (**Figure 7A**). Furthermore, the overexpression of FOXQ1 was significantly correlated with lymph node metastasis or higher TNM stage (**Table 1**), which is consistent with our previous study (12). Further analysis

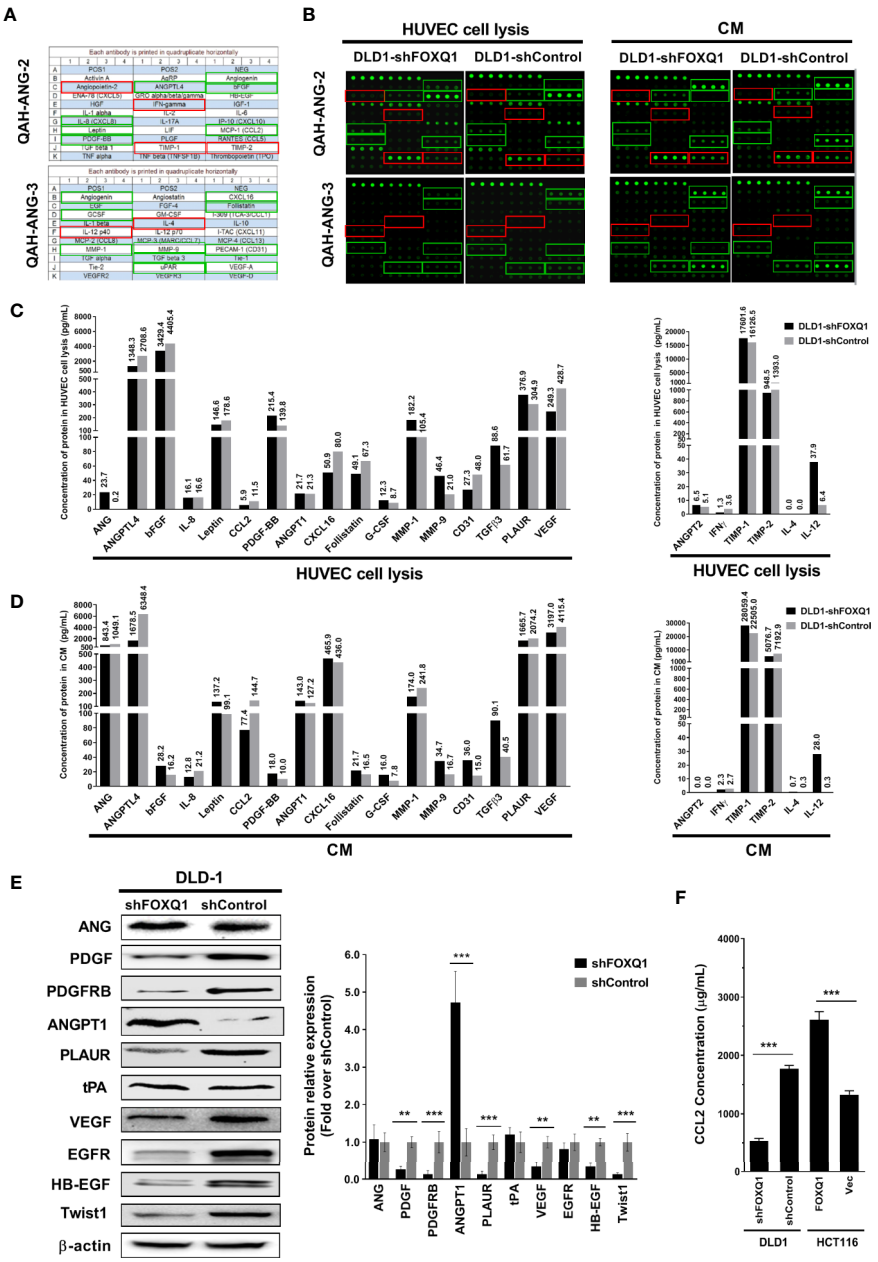


FIGURE 5 | Inhibition of FOXQ1 induces downregulation of angiogenic factors and upregulation of angiogenic inhibitors. **(A)** Matrix distribution of 60 well-established angiogenic proteins on a Quantibody Human Angiogenesis Array (QAH-ANG-2 and QAH-ANG-3); each protein is distributed in quadruplicate horizontally. **(B)** Fluorescence detection of protein arrays for either cell lysate of HUVECs or conditioned medium (CM) of colorectal cancer (CRC) cells. CM was collected from either DLD1-shFOXQ1 or DLD1-shControl and added to HUVECs. After 48 h, HUVECs were harvested and lysed. Both the cell lysates and CM were collected, and the secretion of 60 angiogenic proteins were measured by hybridization with QAH-ANG-2 and QAH-ANG-3. The 17 green and 6 red rectangles on **(A, B)** show 17 angiogenesis factors and 6 angiogenic inhibitors within the detection range of the protein array. **(C)** Quantitative concentration of the 17 angiogenic factors and 6 angiogenic factors in cell lysates of HUVECs (pg/ml). **(D)** Quantitative concentration of the 17 angiogenic factors and 6 angiogenic factors in CM collected from either DLD1-shFOXQ1 or DLD1-shControl (pg/ml). The values above each bar on **(C, D)** represent quantitative concentration of corresponding protein, is the average of quadruplicates (n = 1 per group). **(E)** Western blot analyses for ANG, PDGF, PDGFRB, ANGPT1, PLAUR, tPA, VEGF, EGFR, HB-EGF, and Twist1 were performed with DLD1-shFOXQ1 and DLD1-shControl cell lysates. β-actin was used as the loading control. **(F)** Inhibition of FOXQ1 prevented the autocrine secretion of CCL2, whereas overexpression of FOXQ1 enhanced the secretion of CCL2, as determined by ELISA analysis. $^{**}P<0.01$, $^{***}P<0.001$ signifies a significant difference between the indicated groups (two-tailed, unpaired Student's *t*-test). *Bars* represent mean ± S.E. of three independent experiments. HUVEC, human umbilical vein endothelial cell; CM, conditioned media.

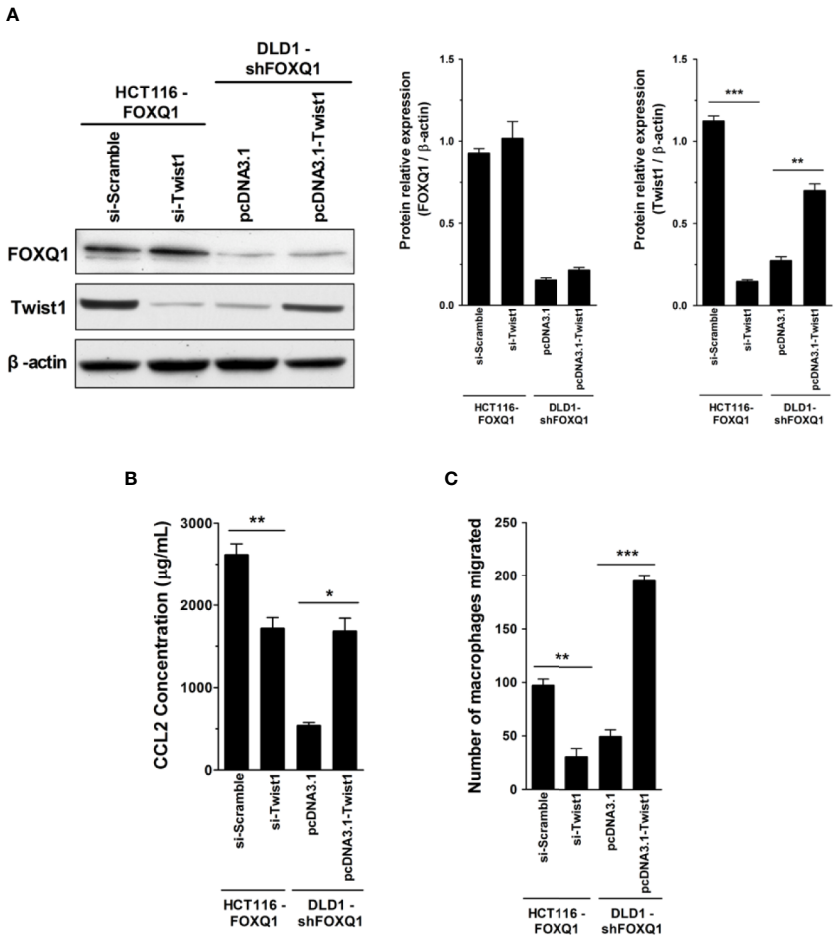


FIGURE 6 | Twist1 is essential for FOXQ1-mediated macrophage recruitment in CRC. **(A)** HCT116-FOXQ1 cells were co-transfected either with si-Twist1 or si-Scramble; DLD1-shFOXQ1 was co-transfected either with pcDNA3.1-Twist1 or pcDNA3.1. After 48 h culture, Western blot analysis was performed for detection of FOXQ1 and Twist1 proteins. Twist1 knockdown abolished FOXQ1-mediated CCL2 upregulation, whereas upregulation of Twist1 rescued the decreased secretion of CCL2 induced by FOXQ1 knockdown. **(B)** Twist1 knockdown abolished FOXQ1-mediated CCL2 upregulation, whereas upregulation of Twist1 rescued the decreased secretion of CCL2 induced by FOXQ1 knockdown. ELISA analysis of CCL2 secretion was detected in CM. **(C)** Twist1 knockdown eliminated the FOXQ1-induced inhibition of macrophage recruitment, whereas upregulation of Twist1 rescued the decreased macrophage infiltration ability induced by FOXQ1. Chemotactic properties of CCL2 secreted by CRC cells on macrophage infiltration were determined by Transwell assay. *P<0.05, **P<0.01, ***P<0.001 signifies a significant difference between the indicated groups (two-tailed, unpaired Student's t-test). *Bars* represent mean ± S.E. of three independent experiments.

verified the statistically significant correlation between FOXQ1 and Twist1 (P<0.01) **(Table 2)**. Twist1 expression was also positively correlated with CCL2 expression in cohort CRC tissues **(Table 3)**, but not CD68 expression **(Table 4)**. Finally, Kaplan-Meier survival analysis was performed to further validate the role of FOXQ1 in promoting tumor angiogenesis and TME modification. The results show that CRC patients with positive expression of either FOXQ1 (P = 0.012) or CCL2 (P = 0.002) had shorter overall survival than those with negative expression of FOXQ1 or CCL2 **(Figure 7B)**. Similarly, CRC patients with higher expression levels of CD31 had shorter overall survival than those with lower expression levels of CD31 (P = 0.002). Furthermore, CRC patients with positive co-expression of both FOXQ1/Twist1 (P<0.001), or FOXQ1/CCL2 (P<0.01), Twist1/CCL2 (P<0.001), or CCL2/CD68 (P<0.05) had the shortest overall survival times compared with the corresponding single negative or double negative groups **(Figure 7C)**. Thus, expression of FOXQ1 and its co-regulated proteins may have prognostic relevance in CRC.

DISCUSSION

Tumor angiogenesis is an important component of cancer development, involving a multistep process of EC migration and tubular formation (36). Macrophages are one of the main infiltrating cell groups in the cancer stroma, promoting the progression of tumors by releasing growth and angiogenic

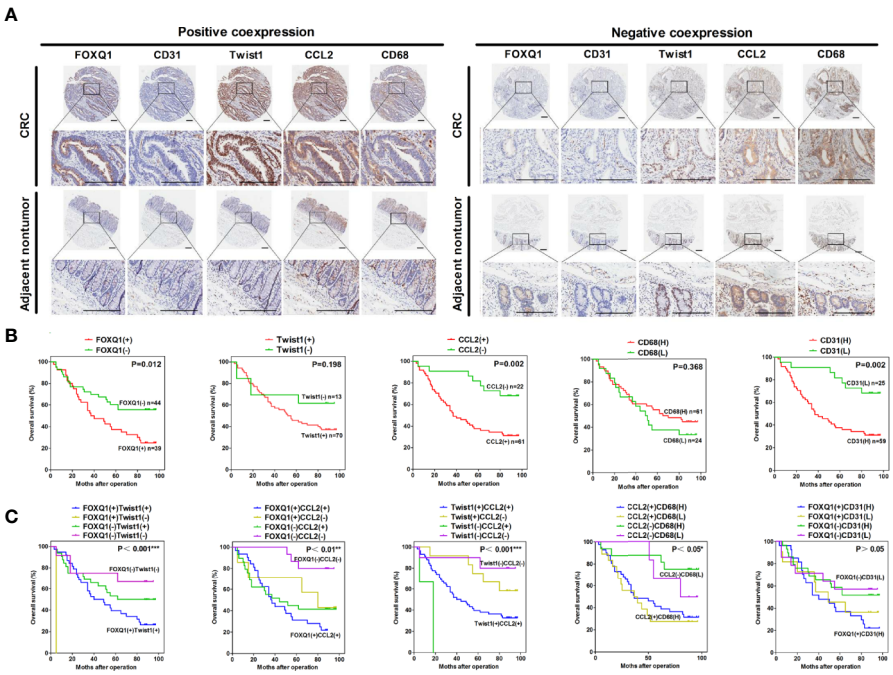


FIGURE 7 | FOXQ1 is associated with enhanced cancer angiogenesis and macrophage recruitment in human colorectal cancer (CRC). **(A)** Representative immunohistochemical images of FOXQ1, CD31, Twist1, CCL2, and CD68 positive co-expression and negative co-expression in human CRC tissues and adjacent nontumorous tissues. Scale bars represent 200 μm. **(B)** Kaplan-Meier survival analysis of CRC patients with positive expression of either FOXQ1 or CCL2 had shorter overall survival than those with negative expression of FOXQ1 or CCL2 in a cohort of 83 CRC patients. **(C)** Kaplan-Meier survival analysis of CRC patients with positive co-expression of both FOXQ1/Twist1, or FOXQ1/CCL2, Twist1/CCL2, or CCL2/CD68 had the shortest overall survival times compared with the corresponding single negative or double negative groups in the cohort of 83 CRC patients. *P<0.05, **P<0.01, ***P<0.001 signifies a significant difference between the indicated groups (log-rank test).

TABLE 1 | Correlation between FOXQ1 expression and clinicopathological characteristics of colorectal cancers (CRCs) in cohort of human CRC tissues.

Clinicopathological variables		Cohort tumor FOXQ1 expression		*P* Value
		Negative (n=44)	Positive (n=39)	
Age		69.28 (12.36)	70.54 (9.41)	
Gender	Female	19	21	> 0.05
	Male	25	18	
Maximal tumor size	≤10	10	9	>0.05
	>10	34	30	
Lymphatic metastasis	Absent	32	20	<0.05*
	Present	12	19	
Tumor differentiation	I–II	21	24	>0.05
	III–IV	23	15	
TNM stage	I–II	17	6	<0.05*
	III-IV	27	33	
AJCC clinical stage according to 7th issue	1 & 2A	22	24	>0.05
	3 & 3B	12	15	
Histological subtype	Adenocarcinoma (information about histological subtypes unavailable)	16	18	>0.05
	Tubular adenocarcinoma	23	19	
	Tubular adenocarcinoma with partial mucinous adenocarcinoma,	4	0	
	Mucinous adenocarcinoma	0	1	
	Papillary adenocarcinoma	0	1	
	Adenocarcinoma with squamous epithelial metaplasia	1	0	

*AJCC, American Journal of Critical Care. *P<0.05.*

TABLE 2 | Correlation analysis of FOXQ1 and Twist1, CCL2, or CD31 expression in cohort (n=83) colorectal cancer (CRC) tissues.

		FOXQ1		x^2	*P* value
		negative (n=44)	positive (n=39)		
Twist1	negative (n=13)	12	1	9.56	<0.01**
	positive (n=70)	32	38		
CCL2	negative (n=22)	15	7	2.77	>0.05
	positive (n=61)	29	32		
CD31	low (n=24)	16	8	2.53	>0.05
	high (n=59)	28	31		

***P<0.01.*

TABLE 3 | Correlation analysis of Twist1 and CCL2 expression in cohort (n=83) colorectal cancer (CRC) tissues.

		Twist1		x^2	*P* value
		negative (n=13)	positive (n=70)		
CCL2	negative (n=22)	10	12	17.16	<0.01**
	positive (n=61)	3	58		

***P<0.01.*

TABLE 4 | Correlation analysis of CCL2 and CD68 expression in cohort (n=83) colorectal cancer (CRC) tissues.

		CCL2		x^2	*P* value
		negative (n=22)	positive (n=61)		
CD68	low (n=22)	3	20	2.96	>0.05
	high (n=61)	19	41		

factors (37, 38). Angiogenesis and macrophage recruitment are closely related to cancer progression, and these two biological processes share common pathways (39).

In this study, we found that FOXQ1 inhibition in CRC cells results in suppressed proliferation and migration of CRC cells *in vitro* **(Figure 2)**, this result was consistent with ours (12) and other previous reports in CRC (11, 21). Furthermore, the impact of FoxQ1 on promoting tumor cell proliferation was also well established in other solid tumors including ovarian cancer (16), neuroblastoma (20), lung cancer (22), gastric cancer (23), and liver cancer (40). We also demonstrated that CRC cells with decreased FOXQ1 expression, as well as CM from these cells, can activate migration and microvessel morphogenesis of HUVECs *in vitro* **(Figure 3)**. The effect of FOXQ1 knockdown was further confirmed in *in vivo* experiments, demonstrating that FOXQ1 inhibition in CRC cells results in slower xenograft tumor growth and angiogenesis. Interestingly, FOXQ1 inhibition in CRC cells also evidently reduced recruitment of macrophages in our mouse model **(Figure 4)**. A limitation, however, should be taken into account when the findings of the present study are interpreted. *in vivo* study was only performed by using FOXQ1 knockdown DLD1 cells, an independent xenograft study by using FOXQ1 overexpressed CRC cell lines would make the results more convincing.

To evaluate which angiogenesis and macrophage recruitment factors are induced by FOXQ1 in CRC cells, we performed protein array analysis. The inhibition of FOXQ1 expression in CRC cells caused a pronounced decrease in the secretion of several angiogenic factors, whereas it caused a significant increase in the endogenous angiogenic inhibitor ANGPT1. Of particular interest, our results indicated VEGF was downregulated both in CM from DLD1-shFOXQ1 cells and in HUVECs cultured with this CM **(Figures 5C–E)**, this result was consistent with previous report, in which identified VEGFA as a candidate target gene of FOXQ1 (10). VEGF is the most crucial factor involved in angiogenesis, controlling the early steps that trigger the angiogenic cascade, which promotes EC migration and proliferation (41), VEGF play its roles *via* VEGF receptor 2 (VEGFR2), VEGF/VEGFR2 signalling is a key signalling event in angiogenesis and vascular permeability (42), further studies should be performed to demonstrate the effect of FOXQ1 in VEGF/VEGFR2 signalling.

Several additional critical angiogenic factors were also decreased with FOXQ1 inhibition in CRC cells, including PDGF and its receptor PDGFRB, as well as HB-EGF, one of the critical ligands of EGFR (6). Most of these angiogenic factors are molecular components of the EGF/PDGF pathway, which plays an important role in activating tumor angiogenesis (5). These results suggest that FOXQ1 overexpression in CRC cells can promote tumor angiogenesis either by promoting secretion of tumor cell-produced angiogenic factors mainly in the EGF/PDGF pathway, while reducing the expression of angiogenic inhibitors **(Figure 5)**. This notion was supported by our data showing that expression of the vascular marker CD31 were also decreased in EC cultured in CM from FOXQ1-inhibited CRC cells **(Figure 5C)**.

It has been well established that the TME plays an important role in tumorigenesis (4, 43). Macrophages, which are the most abundant immune-related stromal cells in the TME (37, 43), are key orchestrators of the TME, directly affecting neoplastic cell growth, angiogenesis, and extracellular matrix remodeling (44). The pro-tumor role of macrophages in CRC is controversial. Some studies have indicated that macrophages in CRC appear to have antitumor activity and are associated with improved disease-free survival (45). In contrast, other studies demonstrate that macrophages in CRC often display an alternatively activated phenotype, promoting tumor progression and disease aggressiveness (46, 47), and are associated with poor prognosis in CRC patients (48). In addition, cancer cells can actively modulate macrophages in the TME to enhance cancer development and metastasis (49). In the current study, we found that FOXQ1 inhibition in CRC cells results in inhibited intratumoral macrophage infiltration *in vivo*. This observation revealed that FOXQ1 accelerates tumor growth not only by strengthening tumor angiogenesis but also by promoting macrophage recruitment **(Figure 4)**.

Given the effect of FOXQ1 on macrophage recruitment, we sought to evaluate a potential role for CCL2, a well-established macrophage chemoattractant, in FOXQ1-dependent CRC secretion (26). CCL2 was decreased both in CM from DLD1

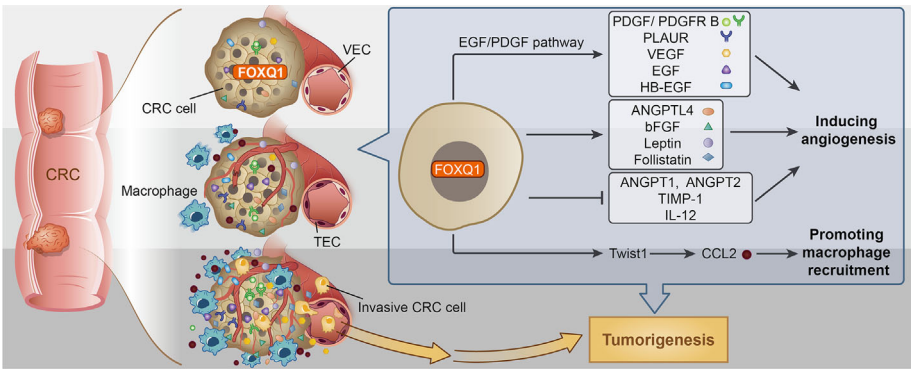


FIGURE 8 | Potential mechanism for Forkhead box Q1 (FOXQ1) induced angiogenesis and macrophage infiltration in colorectal cancer (CRC) tumorigenesis. We speculate that FOXQ1 can promote the migration of endothelial cell in tumor microenvironment (TME), improve microvessel morphogenesis, as well as strengthen intercellular interactions between tumor and epithelial cells mainly through activation of EGF/PDGF pathway. We also propose that FOXQ1 can promote macrophage infiltration in TME by activating the Twist1/CCL2 axis. Thus, the combined mechanisms of FOXQ1 described in this study support a dual role of FOXQ1 in promoting CRC tumorigenesis.

cells with FOXQ1 inhibition and HUVECs cultured with this CM (**Figures 5C, E**), and CCL2 secretion positively correlated with FOXQ1 expression in CRC cells (**Figure 5F**). We also evaluated the effects of FOXQ1 expression on Twist1, which has been established as a direct target of FOXQ1 in cancers including CRC (11, 50, 51). Evidence suggests that Twist1 can directly activate CCL2 and promote angiogenesis by increasing macrophage recruitment (26). Twist1 overexpression can also increase the synthesis of VEGF, promote vascular expansion and permeability, and accelerate tumor progression (52). Our macrophage migration results demonstrated that FOXQ1 overexpression in CRC cells can stimulate the production of CCL2, thus promoting macrophage infiltration within the TME, whereas Twist1 knockdown reversed the increased CCL2 expression and macrophage infiltration induced by FOXQ1 overexpression. In contrast, knockdown of FOXQ1 in CRC cells decreased CCL2 expression and macrophage infiltration, whereas upregulation of Twist1 rescued the decreased macrophage infiltration and CCL2 expression induced by FOXQ1 knockdown (**Figure 6**). Taken together, our data suggest that FOXQ1-mediated macrophage infiltration is dependent on the Twist1/CCL2 axis.

To further confirm that FOXQ1 promotes tumor angiogenesis and modifies the TME in CRC, tumor tissues from 83 patients diagnosed with CRC were used to evaluate the expression of FOXQ1, Twist1, CCL2, CD68, and CD31. IHC results showed that the expression of FOXQ1, Twist1, and CCL2 in CRC tissues was significantly higher than that in para-carcinoma tissues (**Figure 7A**). The expression of FOXQ1 was demonstrated to be positively correlated with lymph node metastasis and TNM stage (**Table 1**). Although we did not found any differential expression pattern of FOXQ1 in CRC subtypes statistically in present work. A cohort study based on larger sample needed to be conduct to characterize the potential relationship between expression pattern of FOXQ1 and CRC histologic subtypes (**Table 1**). Furthermore, there was a positive correlation between FOXQ1 and the expression of Twist1 (**Table 2**); and between Twist1 and the expression of CCL2 (**Table 3**). These studies suggest that overexpression of the FOXQ1-induced Twist1/CCL2 axis plays an important role in promoting CRC macrophage infiltration. The role of Twist1-induced CCL2 in angiogenesis has been elucidated previously (26). Furthermore, CCL2 was originally identified as a tumor-derived chemotactic factor for macrophages (53), and the levels of tumor-derived CCL2 significantly correlate with macrophage density and the depth of invasion in various cancers (54). Moreover, experimental studies using xenotransplanted tumors have revealed the involvement of the CCL2/CCR2 axis in cancer metastasis (55). In present study, Kaplan-Meier survival analysis further confirmed that FOXQ1-induced Twist1/CCL2 axis is closely associated with a lower 8-year survival in CRC patients (**Figures 7B, C**). However, we failed to find a positive correlation between FOXQ1 and the endothelial marker CD31 (**Table 2**), and their co-expression was not associated with poorer prognosis in CRC patients (**Figure 7C**). Our interpretation of these findings is that CD31 is a universal marker of EC but not a specific marker of tumor vessels. More specific markers for tumor vessels will be beneficial in confirming these findings.

In summary, there are two major steps during the pathological angiogenic process. First, tumor vascular formation involves the migration and differentiation of endothelial progenitors through vasculogenesis (56). Second, host inflammatory cells, including macrophages, infiltrate tumor tissues, alter the microenvironment, and promote tumor angiogenesis (57). Here, we confirmed that FOXQ1 directly contributes to both major steps. The essential role of FOXQ1-induced angiogenesis and macrophage recruitment in CRC is likely to be related to its ability to promote the migration of ECs and macrophages in the TME through activation of the EGF/PDGF pathway and the Twist1/CCL2 axis, respectively (**Figure 8**), thus supporting a dual role for FOXQ1 in promoting CRC progression. Based on our findings, FOXQ1 may serve as a

therapeutic target for CRC treatment by inhibiting tumor angiogenesis and reducing macrophage recruitment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study, these can be found in the NCBI Gene Expression Omnibus (GSE74223).

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, the First People's Hospital of Yunnan Province (Yunnan, China).

AUTHOR CONTRIBUTIONS

HT and QG designed the project, analyzed data, and wrote the manuscript. JZ performed xenograft model assay, IHC and MVD analysis. XB performed microvessel formation assay. K-LY generated stable FOXQ1 overexpress/knockdown cell lines. J-HL and D-YL performed *in vitro* macrophages migration experiments. L-PW and J-LW carried out HUVECs migration assay, Elisa, and Western blotting. HT analyzed colorectal tissue microarray data. All authors contributed to the article and approved the submitted version.

FUNDING

This study was founded by the National Natural Science Foundation of China [Grants 81502556, 81460463 and 81260323]; the Medical Academic Talents Cultivation Foundation for Health Commission of Yunnan Province [Grant D-201642]; the Foundation of Kunming Key Laboratory of Tumor Molecular and Immune Prevention [2018-1-A-17334]; and the Yunnan Digestive Endoscopy Clinical Medical Center Foundation for Health Commission of Yunnan Province [2X2019-01-02].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.564298/full#supplementary-material

REFERENCES

1. Folk man J. Angiogenesis: An organizing principle for drug discovery? *Nat Rev Drug Discovery* (2007) 6:273–86. doi: 10.1038/nrd2115
2. Bielenberg DR, Zetter BR. The contribution of angiogenesis to the process of metastasis. *Cancer J* (2015) 21:267–73. doi: 10.1097/PPO.0000000000000138
3. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* (2000) 407:249–57. doi: 10.1038/35025220
4. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* (2006) 124:263–6. doi: 10.1016/j.cell.2006.01.007
5. Bruce D, Tan PH. Vascular endothelial growth factor receptors and the therapeutic targeting of angiogenesis in cancer: where do we go from here? *Cell Commun Adhes* (2011) 18:85–103. doi: 10.3109/15419061.2011.619673
6. Rapisarda A, Melillo G. Role of the VEGF/VEGFR axis in cancer biology and therapy. *Adv Cancer Res* (2012) 114:237–67. doi: 10.1016/j.humpath.2008.08.019
7. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* (2011) 17:1359–70. doi: 10.1038/nm.2537
8. Myatt SS, Law EW. The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer* (2007) 7:847–59. doi: 10.1038/nrc2223
9. Feuerborn A, Srivastava PK, Kuffer S, Grandy WA, Sijmonsma TP, Gretz N, et al. The Forkhead factor FOXQ1 influences epithelial differentiation. *J Cell Physiol* (2011) 226:710–9. doi: 10.1002/jcp.22385
10. Kaneda H, Arao T, Tanaka K, Tamura D, Aomatsu K, Kudo K, et al. FOXQ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth. *Cancer Res* (2010) 70:2053–63. doi: 10.1158/0008-5472.CAN-09-2161
11. Abba M, Patil N, Rasheed K, Nelson LD, Mudduluru G, Leupold JH, et al. Unraveling the role of FOXQ1 in colorectal cancer metastasis. *Mol Cancer Res* (2013) 11:1017–28. doi: 10.1158/1541-7786.MCR-13-0024
12. Tang H, Guo Q, Zhang C, Zhu J, Yang H, Zou YL, et al. Identification of an intermediate signature that marks the initial phases of the colorectal adenoma-carcinoma transition. *Int J Mol Med* (2010) 26:631–41. doi: 10.3892/ijmm_00000508
13. Feng J, Zhang X, Zhu H, Wang X, Ni S, Huang J. FOXQ1 overexpression influences poor prognosis in non-small cell lung cancer, associates with the phenomenon of EMT. *PloS One* (2012) 7:e39937. doi: 10.1371/journal.pone.0039937
14. Qiao Y, Jiang X, Lee ST, Karuturi RK, Hooi SC, Yu Q. FOXQ1 regulates epithelial-mesenchymal transition in human cancers. *Cancer Res* (2011) 71:3076–86. doi: 10.1158/0008-5472.CAN-10-2787
15. Zhang H, Meng F, Liu G, Zhang B, Zhu J, Wu F, et al. Forkhead transcription factor FOXQ1 promotes epithelial-mesenchymal transition and breast cancer metastasis. *Cancer Res* (2011) 71:1292–301. doi: 10.1158/0008-5472.CAN-10-2825
16. Gao M, IeM S, Wang TL. The role of forkhead box q1 transcription factor in ovarian epithelial carcinomas. *Int J Mol Sci* (2012) 13:13881–93. doi: 10.3390/ijms131113881
17. Zhu Z, Zhu Z, Pang Z, Xing Y, Wan F, Lan D, et al. Short hairpin RNA targeting FOXQ1 inhibits invasion and metastasis via thereversal of epithelial-mesenchymal transition in bladder cancer. *Int J Oncol* (2013) 42:1271–8. doi: 10.3892/ijo.2013.1807
18. Li F, Liu B, Gao Y, Liu Y, Xu Y, Tong W, et al. Upregulation of microRNA-107 induces proliferation in human gastric cancer cells by targeting the transcription factor FOXO1. *FEBS Lett* (2014) 588:538–44. doi: 10.1016/j.febslet.2013.12.009
19. Xia L, Huang W, Tian D, Zhang L, Qi X, Chen Z, et al. Forkhead box Q1 promotes hepatocellular carcinoma metastasis by transactivating ZEB2 and VersicanV1 expression. *Hepatology* (2014) 59:958–73. doi: 10.1002/hep.26735
20. Sun HT, Cheng SX, Tu Y, Li XH, Zhang S. FoxQ1 promotes glioma cells proliferation and migration by regulating NRXN3 expression. *PloS One* (2013) 8:e55693. doi: 10.1371/journal.pone.0055693
21. Weng W, Okugawa Y, Toden S, Toiyama Y, Kusunoki M, Goel A. FOXM1 and FOXQ1 are promising prognostic biomarkers and novel targets of tumor suppressive miR-342 in human colorectal cancer. *Clin Cancer Res* (2016) 22:4947–57. doi: 10.1158/1078-0432.CCR-16-0360
22. Xiao B, Liu H, Gu Z, Ji C. Expression of microRNA-133 inhibits epithelial-mesenchymal transition in lung cancer cells by directly targeting FOXQ1. *Arch Bronconeumol* (2016) 52:505–11. doi: 10.1016/j.arbres.2015.10.016
23. Zhang J, Liu Y, Zhang J, Cui X, Li G, Wang J, et al. FOXQ1 promotes gastric cancer metastasis through upregulation of Snail. *Oncol Rep* (2016) 35:3607–13. doi: 10.3892/or.2016.4736

24. Wang P, Lv C, Zhang T, Liu J, Yang J, Guan F, et al. FOXQ1 regulates senescence-associated inflammation via activation of SIRT1 expression. *Cell Death Dis* (2017) 8:e2946. doi: 10.1038/cddis.2017.340
25. Christensen J, Bentz S, Sengstag T, Shastri VP, Anderle P. FOXQ1, a novel target of the Wnt pathway and a new marker for activation of Wnt signaling in solid tumors. *PloS One* (2013) 8:e60051. doi: 10.1371/journal.pone.0060051
26. Low-Marchelli JM, Ardi VC, Vizcarra EA, van Rooijen N, Quigley JP, Yang J. Twist1 induces CCL2 and recruits macrophages to promote angiogenesis. *Cancer Res* (2013) 73:662–71. doi: 10.1158/0008-5472.CAN-12-0653
27. DeCicco-Skinner KL, Henry GH, Cataisson C, Tabib T, Gwilliam JC, Watson NJ, et al. Endothelial cell tube formation assay for the in vitro study of angiogenesis. *J Vis Exp* (2014) 91:e51312. doi: 10.3791/51312
28. Heiss M, Hellström M, Kalén M, May T, Weber H, Hecker M, et al. Endothelial cell spheroids as a versatile tool to study angiogenesis in vitro. *FASEB J* (2015) 29:3076–84. doi: 10.1096/fj.14-267633
29. Tetzlaff F, Fischer A. Human endothelial cell spheroid-based sprouting angiogenesis assay in collagen. *Bio-protocol* (2018) 8:e2995. doi: 10.21769/BioProtoc.2995
30. Rogatsch H, Hittmair A, Reissigl A, Mikuz G, Feichtinger H. Microvessel density in core biopsies of prostatic adenocarcinoma: a stage predictor. *J Pathol* (1997) 182:205–10. doi: 10.1002/(SICI)1096-9896(199706)182:2<205::AID-PATH846>3.0.CO;2-5
31. Taniguchi K, Hikiji H, Okinaga T, Hashidate-Yoshida T, Shindou H, Ariyoshi W, et al. Essential role of lysophosphatidylcholine acyltransferase 3 in the induction of macrophage polarization in PMA-treated U937 cells. *J Cell Biochem* (2015) 116:2840–8. doi: 10.1002/jcb.25230
32. Ou YQ, Chen LH, Li XJ, Lin ZB, Li WD. Sinomenine influences capacity for invasion and migration in activated human monocytic THP-1 cells by inhibiting the expression of MMP-2, MMP-9, and CD147. *Acta Pharmacol Sin* (2009) 30:435–41. doi: 10.1038/aps.2009.21
33. Li W, Yu CP, Xia JT, Zhang L, Weng GX, Zheng HQ, et al. Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. *Clin Cancer Res* (2009) 15:1393–9. doi: 10.1158/1078-0432.CCR-08-1158
34. Zhao Y, Adjei AA. Targeting angiogenesis in cancer therapy: moving beyond vascular endothelial growth factor. *Oncologist* (2014) 20:660–73. doi: 10.1634/theoncologist.2014-0465
35. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* (2011) 475:222–5. doi: 10.1038/nature10138
36. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* (2003) 3:401–10. doi: 10.1038/nrc1093
37. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* (2010) 141:39–51. doi: 10.1016/j.cell.2010.03.014
38. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J Leukoc Biol* (2009) 86:1065–73. doi: 10.1189/jlb.0609385
39. Ono M. Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. *Cancer Sci* (2008) 99:1501–6. doi: 10.1111/j.1349-7006.2008.00853.x
40. Zhang J, Yang Y, Yang T, Yuan S, Wang R, Pan Z, et al. Double-negative feedback loop between microRNA-422a and forkhead box (FOX) G1/Q1/E1 regulates hepatocellular carcinoma tumor growth and metastasis. *Hepatology* (2015) 61:561–73. doi: 10.1002/hep.27491
41. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* (2008) 8:579–91. doi: 10.1038/nrc2403
42. Law AY, Wong CK. Stanniocalcin-1 and -2 promote angiogenic sprouting in HUVECs via VEGF/VEGFR2 and angiopoietin signaling pathways. *Mol Cell Endocrinol* (2013) 374:73–81. doi: 10.1016/j.mce.2013.04.024
43. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol* (2008) 66(1):1–9. doi: 10.1016/j.critrevonc.2007.07.004
44. Solinas G, Schiarea S, Liguori M, Fabbri M, Pesce S, Zammataro L, et al. Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. *J Immunol* (2010) 185:642–52. doi: 10.4049/jimmunol.1000413
45. Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A, Palmqvist R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin Cancer Res* (2007) 13:1472–9. doi: 10.1158/1078-0432.CCR-06-2073
46. Barbera-Guillem E, Nyhus JK, Wolford CC, Friece CR, Sampsel JW. Vascular endothelial growth factor secretion by tumor-infiltrating macrophages essentially supports tumor angiogenesis, and IgG immune complexes potentiate the process. *Cancer Res* (2002) 62:7042–9. doi: 10.1097/00002820-200212000-00014
47. Jedinak A, Dudhgaonkar S, Sliva D. Activated macrophages induce metastatic behavior of colon cancer cells. *Immunobiology* (2010) 215:242–9. doi: 10.1016/j.imbio.2009.03.004
48. Pancione M, Forte N, Sabatino L, Tomaselli E, Parente D, Febbraro A, et al. Reduced beta-catenin and peroxisome proliferator-activated receptor-gamma expression levels are associated with colorectal cancer metastatic progression: correlation with tumor-associated macrophages, cyclooxygenase 2, and patient outcome. *Hum Pathol* (2009) 40:714–25. doi: 10.1016/j.humpath.2008.08.019
49. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer* (2009) 9:239–52. doi: 10.1038/nrc2618
50. Huang W, Chen Z, Shang X, Tian D, Wang D, Wu K, et al. Sox12, a direct target of FoxQ1, promotes hepatocellular carcinoma metastasis through up-regulating Twist1 and FGFBP1. *Hepatology* (2015) 61:1920–33. doi: 10.1002/hep.27756
51. Ross JB, Huh D, Noble LB, Tavazoie SF. Identification of molecular determinants of primary and metastatic tumour re-initiation in breast cancer. *Nat Cell Biol* (2015) 17:651–64. doi: 10.1038/ncb3148
52. Mironchik Y, Winnard PTJr, Vesuna F, Kato Y, Wildes F, Pathak AP, et al. Twist1 overexprssion induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer. *Cancer Res* (2005) 65:10801–9. doi: 10.1158/0008-5472.CAN-05-0712
53. Graves DT, Jiang YL, Williamson MJ, Valente AJ. Identification of monocyte chemotactic activity produced by malignant cells. *Science* (1989) 245:1490–3. doi: 10.1126/science.2781291
54. Sica A, Allavena P, Mantovani A. Cancer related inflammation: the macrophage connection. *Cancer Lett* (2008) 267:204–15. doi: 10.1016/j.canlet.2008.03.028
55. Loberg RD, Ying C, Craig M, Day LL, Sargent E, Neeley C, et al. Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer regression in vivo. *Cancer Res* (2007) 67:9417–24. doi: 10.1158/0008-5472.CAN-07-1286
56. Kowanetz M, Ferrara N. Vascular endothelial growth factor signaling pathways: therapeutic perspective. *Clin Cancer Res* (2006) 12:5018–22. doi: 10.1158/1078-0432.CCR-06-1520
57. Yang L, Lin PC. Mechanisms that drive inflammatory tumor microenvironment, tumor heterogeneity, and metastatic progression. *Semin Cancer Biol* (2017) 47:185–95. doi: 10.1016/j.semcancer.2017.08.001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 09 December 2020
doi: 10.3389/fcell.2020.610661

Resistance Mechanisms of Anti-angiogenic Therapy and Exosomes-Mediated Revascularization in Cancer

Ye Zeng[1†] *and Bingmei M. Fu*[2*†]

[1] *Institute of Biomedical Engineering, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, Chengdu, China,* [2] *Department of Biomedical Engineering, The City College of the City University of New York, New York, NY, United States*

OPEN ACCESS

Edited by:
Lucas Treps, VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Anantha Koteswararao Kanugula, University of Massachusetts Medical School, United States
Bryan E. Strauss, University of São Paulo, Brazil

***Correspondence:**
Bingmei M. Fu
fu@ccny.cuny.edu

†ORCID:
Ye Zeng
orcid.org/0000-0001-5440-6194
Bingmei M. Fu
orcid.org/0000-0001-9343-5895

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *26 September 2020*
Accepted: *20 November 2020*
Published: *09 December 2020*

Citation:
Zeng Y and Fu BM (2020) Resistance Mechanisms of Anti-angiogenic Therapy and Exosomes-Mediated Revascularization in Cancer. Front. Cell Dev. Biol. 8:610661. doi: 10.3389/fcell.2020.610661

Anti-angiogenic therapies (AATs) have been widely used for cancer treatment. But the beneficial effects of AATs are short, because AAT-induced tumor revascularization facilitates the tumor relapse. In this mini-review, we described different forms of tumor neovascularization and revascularization including sprouting angiogenesis, vessel co-option, intussusceptive angiogenesis, and vasculogenic mimicry, all of which are closely mediated by vascular endothelial growth factor (VEGF), angiopoietins, matrix metalloproteinases, and exosomes. We also summarized the current findings for the resistance mechanisms of AATs including enhancement in pro-angiogenic cytokines, heterogeneity in tumor-associated endothelial cells (ECs), crosstalk between tumor cells and ECs, masking of extracellular vesicles, matrix stiffness and contributions from fibroblasts, macrophages and adipocytes in the tumor microenvironment. We highlighted the revascularization following AATs, particularly the role of exosome stimulating factors such as hypoxia and miRNA, and that of exosomal cargos such as cytokines, miRNAs, lncRNAs, and circRNAs from the tumor ECs in angiogenesis and revascularization. Finally, we proposed that renormalization of tumor ECs would be a more efficient cancer therapy than the current AATs.

Keywords: anti-angiogenesis, tumor endothelial cell, resistance, revascularization, therapy failure

INTRODUCTION

Current anticancer therapies are hindered by two critical processes. One process is the local invasion and metastasis of cancer cells from either primary tumors or distant lesions. Another is imposed by the resistance to therapies including surgery, radiation and chemotherapy (Weinstein et al., 1991; Meads et al., 2009; Alexander and Friedl, 2012). For example, anti-angiogenic therapies (AATs) which aim at shrinking solid tumors by disrupting pre-existing blood vessels around tumors have not achieved expected therapeutic effects (Park et al., 2016; Cully, 2017).

The blood vascular system is essential for nutrients and oxygen supply, waste removal and immune surveillance (Carmeliet and Jain, 2011; Li et al., 2019b). The tumor vasculature is also essential for nutrients and oxygen supply for tumor tissue. Angiogenesis is a crucial process for the

growth and metastasis of tumors. A solid tumor cannot exceed a few (2–3) mm^3 in the absence of angiogenesis. Therefore, AATs including vessel pruning, disruption and normalization have been developed to suppress the tumor growth and prevent it from metastatic dissemination (Cully, 2017; Cloughesy et al., 2020).

Certain isoforms of vascular endothelial growth factor (VEGF) are promoting angiogenesis and VEGF receptors (VEGFR) of endothelial cells play an important role in this process. Thus, AATs employing the inhibitor of VEGF such as bevacizumab, and many VEGFR tyrosine kinase inhibitors (VEGFR-TKIs) have been widely used in clinic. Bevacizumab induces vessel normalization and reduces vascular leakage. VB-111 destroys the tumor vasculature and promotes the recruitment of immune cells (Cloughesy et al., 2020). Immune checkpoint blockade could increase vessel normalization (Tian et al., 2017). However, AATs did not yield satisfactory efficacy as promised. The beneficial effects of AATs do not last because tumor revascularization that arises from vascular co-option, intussusceptive angiogenesis and vasculogenic mimicry (VM) facilitates the tumor relapse (Holash et al., 1999; Hendrix et al., 2003; Wagenblast et al., 2015; Kuczynski et al., 2016; Angara et al., 2017). Excessive AATs can aggravate hypoxia, which instead promote tumor metastasis and revascularization (De Bock et al., 2011).

Anti-angiogenic therapies have also been developed to battle against tumor metastasis (Hosein et al., 2020). However, AAT disrupts the vascular barrier to facilitate the invasion and metastasis of tumor cells, which drives acquired AAT resistance in cancers such as hepatocellular carcinoma (HCC) (Kuczynski et al., 2016; Angara et al., 2017).

The AAT resistance is closely associated with the tumor microenvironment. The tumor microenvironment is a local pathological environment formed by tumor cells, immune cells, fibroblasts, adipocytes, smooth muscle cells and endothelial cells (ECs), as well as extracellular matrix (ECM), which not only modulate the biochemical but also the mechanical environment. Angiogenesis is regulated by hypoxia, low pH, high pressure as well as a large number of growth factors and proteolytic enzymes in the tumor microenvironment. In addition, microRNA (miR)-9 is enriched in tumor ECs, and it promotes angiogenesis (Zeng et al., 2019; Yao et al., 2020). Although AATs suppress the angiogenesis of tumor ECs at the primary site, they trigger VEGF-enriched exosomes released from tumor ECs to facilitate the tumor vasculogenesis for relapse (Zeng et al., 2019). Most recently, it was reported that tissue stiffness increased by metastasis-associated fibroblasts could enhance both angiogenesis and AAT resistance to bevacizumab in metastatic colorectal cancer (Shen et al., 2020).

Thus, the aim of this mini-review is to summarize the key characteristics and roles of angiogenesis in cancer and highlight the acquired AAT resistance due to revascularization.

ANGIOGENESIS IN TUMOR

The tumor vasculatures are characterized by disorganized and tortuous architecture with abnormal and leaky ECs. There are at least four different forms of tumor neovascularization, sprouting angiogenesis, intussusceptive angiogenesis, vessel co-option, and vasculogenic mimicry (VM) (Potente et al., 2011; Eelen et al., 2020; **Figure 1**). The angiogenesis arises from a complex array of genetic, functional and microenvironmental factors such as interstitial fluid pressure, hypoxia and acidosis (Jain, 2005). During the angiogenesis, cell proliferation, differentiation and migration, cell-ECM adhesions, intercellular signaling cascades, and tumor cell-stromal cell interactions all participate and are coordinated with each other. The epigenetic regulation of angiogenesis, and proangiogenic factors-associated angiogenic switch of ECs were extensively reviewed in Ciesielski et al. (2020) and Tan et al. (2020). In this mini review, we only summarized the specific angiogenesis related to the resistance of AATs.

It was believed that VEGF triggers the activation of quiescent ECs and tip ECs to guide the sprouting angiogenesis toward hypoxic and nutrient-deprived microenvironment. VEGF also involves in tumor cell growth and immunosuppression (Sheng et al., 2019; Wilky et al., 2019), suggesting the benefit of a combination therapy of AATs with immune checkpoint inhibitors. A recent study found that tumor growth in some patients with glioma is independent of the VEGF pathway (García-Romero et al., 2020), while other instances (e.g., high dose of bevacizumab in glioma) indicated that AATs suppress tumor independently from vascular regression (von Baumgarten et al., 2011), showing complex roles of VEGF in tumor progression. The release of VEGF by cancer cells and angiogenesis were promoted by activation of various transient receptor potential (TRP) cation channels including TRPC1, TRPC5 and TRPC6 (Ge et al., 2009; Prevarskaya et al., 2018), and the activation of VEGF receptor-2 (VEGFR2) was proposed to be mediated by two-pore channel 2 (TPC2) and downregulation of mechanosensitive TRPV4 (Favia et al., 2014; Kanugula et al., 2019).

In vessel co-option, tumor incorporates the surrounding vessels from normal tissue such as brain, liver, skin, lymph node, and lungs (Latacz et al., 2020) to obtain the nutrients and oxygen. Electron microscopy demonstrated that cancer cells interacted closely with the co-opted blood vessel walls in glioblastoma (Baker et al., 2014). The co-opted vessels lack angiogenic markers as in normal vessels, e.g., αvβ3 integrin is poorly expressed in non-angiogenic non-small cell lung carcinomas (Passalidou et al., 2002). The adhesion molecules such as L1 cell adhesion molecule (L1CAM) mediated attachment of cancer cells to the vascular surface is critical for vessel co-option (Valiente et al., 2014), while the cancer cell motility is enhanced via activation of metabolic signaling pathways (Fack et al., 2015) and cytoskeleton elements such as Arp2/3 complex (Frentzas et al., 2016). It was believed that vessel co-option is driven by angiopoietin and VEGF (Voutouri et al., 2019). Vascular leakage could be mediated by VEGF and angiopoietin-associated inflammatory signaling (Stewart et al., 2016). Therapeutic approach targeting the angiopoietin pathway has been developed to overcome the toxicity and resistance of VEGF-dependent AATs (Monk et al., 2016).

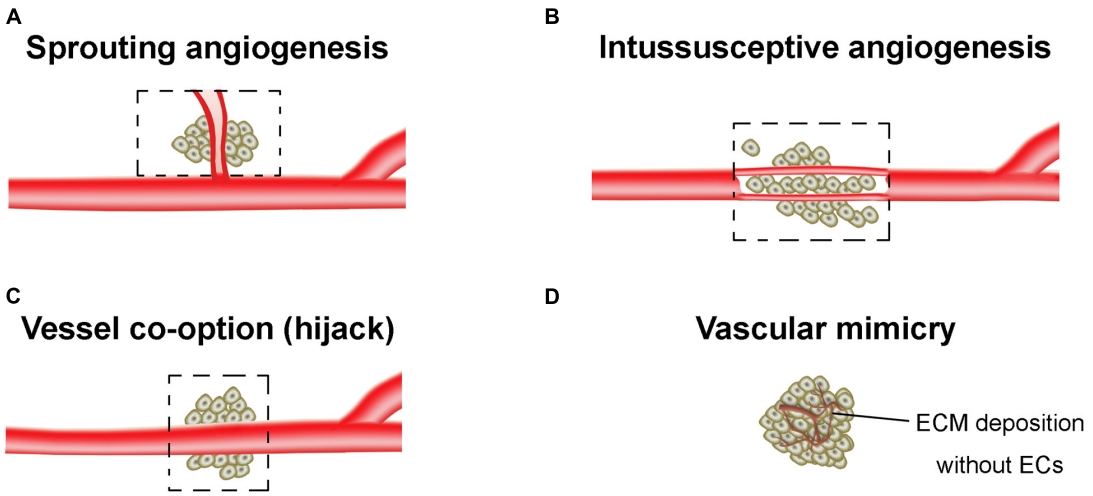


FIGURE 1 | Tumor vasculogenesis. **(A)** Sprouting angiogenesis. Angiogenic sprouting from the pre-existing vessels. **(B)** Intussusceptive angiogenesis. Internal splitting of the pre-existing capillary plexus without sprouting. **(C)** Vessel co-option. Hijacking of pre-existing vessels by tumors. **(D)** Vascular mimicry. The vessel-like network forms by tumor cells, which is closely associated with the extracellular matrix (ECM) deposition. Dashed boxes indicate the new blood vessels (angiogenesis) hijack or form from pre-existing vessels of the surrounding normal tissue and incorporate into the tumor for blood supply.

The formation of capillary plexus by intussusception vessels is faster than sprouting angiogenesis, less dependent on EC actions and requires less energy (De Spiegelaere et al., 2012). A recent study demonstrated that membrane type 1 (MT1)-matrix metalloproteinases (MMPs) activity in ECs regulated intussusceptive angiogenesis (Esteban et al., 2020). However, the detailed signaling mechanism in intussusceptive angiogenesis remains unclear.

It was suggested that an EC-like phenotype supports the malignant cancer cell to form a VM network that is rich in ECM proteins. But this remains an unconfirmed phenomenon (Weis and Cheresh, 2011). Malignant phenotypes switching by epithelial to mesenchymal transition contributed to a mosaic endothelial/non-endothelial VM vasculature. In human HCC HepG2 cells, Twist1 enhanced the cancer stem-like cell behaviors to adopt an EC-like phenotype, prompting cell motility, invasiveness, and VM formation (Sun et al., 2010). VE-cadherin was transported by extracellular vehicles (EVs) from human umbilical vein endothelial cells (HUVECs) to breast cancer cells, which promotes the VM in a chicken chorioallantois membrane tumor model (Rezaei et al., 2020).

Degradation and regulation of the ECM also play an essential role in EC tissue invasion and generation of new blood vessels (Chung et al., 2010). One earlier finding implicated that EVs containing membrane-associated MMPs and other ECM-degrading proteases can be secreted by both cancer cells and ECs (Ginestra et al., 1997; Dolo et al., 1998; Taraboletti et al., 2002). Later, it has been found that cancer cells can derive EVs with other types of membrane proteins, which stimulate tumor angiogenesis by directly triggering signaling pathways in ECs that promote EC survival, migration and/or tube formation. An example is epidermal growth factor receptor (EGFR). Lung, colorectal and skin cancer cells release EGFR-enriched EVs that can transfer oncogenic EGFR to ECs to activate EGFR-dependent MAPK and AKT, triggering ECs to express VEGF. This in turn induces autocrine activation of VEGFR2. VEGFR2 regulates most of the angiogenic effects of VEGF (Al-Nedawi et al., 2009).

AATs AND AAT RESISTANCE MECHANISMS

Tumor angiogenesis is regulated by a variety of cytokines including VEGF, placenta growth factor (PLGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and angiopoietin (Galvano et al., 2019). Given the abundance of VEGF in tumors and the critical role of VEGF and its receptors (VEGFRs) in promoting angiogenesis, the ideas of targeting VEGF and VEGFRs were widely used in AATs. Although the inhibitor of VEGF such as bevacizumab, and many VEGFR-TKIs including vandetanib, carbozantinib, sunitinib, sorafenib, etc., have been widely used in the clinic (Haibe et al., 2020), however, current AATs are not overly successful in providing systematic and durable control for tumors (Eelen et al., 2020). Resistance often occurs in patients with long-term AATs, which lead to tumor recurrence. Resistance is likely to be associated with a particular tumor EC subtype whose proliferation could not be inhibited by AATs (Zhao et al., 2018; Goveia et al., 2020).

Heterogeneity in Tumor ECs

It was initially thought that AATs were less likely to produce drug resistance, since researchers believed that ECs have a very stable genome, and endogenous angiogenesis inhibitors target ECs rather than tumor cells (Jayson et al., 2016). The enthusiasm was tempered by the fact that tumor ECs contain abnormal

genomics that are differentially expressed or do not exist in a healthy vascular system. miR-9 is rich in HCC tissues and tumor-cocultured ECs, while the miR-9 was lowly expressed in normal ECs (Zhuang et al., 2012; Zeng et al., 2019). miR-9-2 was significantly higher in ECs from Wnt- and Shh-medulloblastoma and glioblastoma xenograft than in normal ECs (Yao et al., 2020). The G protein-coupled sphingosine-1-phosphate (S1P) receptor one $S1P_1$ was significantly lower in ECs from Wnt-medulloblastoma, secondary colorectal cancer, and HCC than in normal ECs, and negatively correlated to miR-9-2 in Shh-medulloblastoma ECs (Yao et al., 2020). $S1P_1$ has been found to control the sorting of cargo into exosomes derived from ECs and tumor cells (Kajimoto et al., 2013).

Exosomes

With the development of single cell endothelial transcriptomes, the changes of miR-9 and $S1P_1$ in other types of tumors will be identified, as well as other abnormal genetic and epigenetic changes. It is very important to clarify where the abnormal genetic and epigenetic changes originate from and how they activate. Recent studies found that exosomes can transfer the miR-9 from tumor cells to co-cultured ECs (Zhuang et al., 2012), and transfer the angiogenic proteins such as VEGF from ECs to tumors (Zeng et al., 2019). Increasing evidence has shown exosomes as mediators of tumor cell-to-ECs crosstalk in angiogenesis, tumor progression and metastasis. Exosomal cargos include proteins, mRNAs, long non-coding RNAs (lncRNAs), miRs, etc. miR-135b delivered by exosomes from gastric tumor to ECs promotes angiogenesis in gastric cancer (Bai et al., 2019). miR-205 delivered by exosomes from ovarian cancer to ECs also promotes angiogenesis and tumor growth (He et al., 2019). Exosomal-miR-629-5p derived from lung adenocarcinoma cells increases endothelial monolayers permeability (Li et al., 2020a). Exosome derived from Hela cells could significantly increase the endoplasmic reticulum stress in HUVECs (Lin et al., 2020). Exosomal miR-155 derived from gastric cancer cells promotes VEGF expression and tube formation of ECs (Deng et al., 2020). Studies from liver cancer models also showed that anti-VEGR treatment triggered the release of VEGF-enriched exosomes that stimulate angiogenesis (Zeng et al., 2019). EVs can also activate VEGF-independent pathways such as those mediated by hepatocyte growth factor (HGF) to enable TKI-induced cell death (Qu et al., 2016). Exosomal miR-19b derived from cancer stem cells could promote angiogenesis (Wang et al., 2020). Exosomal circular RNAs (circRNAs) such as circ-CCAC1 (Xu et al., 2020) and circSHKBP1 (Xie et al., 2020) also involved in angiogenesis.

Hypoxia

The resistance of tumors to AATs is also due to the tumor hypoxia coming from too much blood vessel disruption by AATs (Pàez-Ribes et al., 2009). Hypoxia and acidosis induce tumor angiogenesis via overexpression of IL-8 (Shi et al., 1999). Hypoxia can stimulate cells to secrete higher numbers of exosomes (King et al., 2012) and to shed microvesicles from the plasma membrane (Wang et al., 2014). Due to hypoxia-induced transcriptional reprogramming, hypoxic glioblastoma cells secreted pro-angiogenic cytokines-enriched EVs (enriched in MMPs, IL-8, etc.) that are more effective in stimulating tumor growth and vessel formation than the EVs secreted by normoxic glioblastoma cells (Kucharzewska et al., 2013). Hypoxic colorectal cancer cells derived exosomes were found to stimulate EC proliferation and migration by inducing β-catenin signaling (Huang and Feng, 2017). Exosomes secreted by hypoxic pancreatic cancer promotes EC migration and tube formation through upregulation of angiomotin-like protein 2 (AMOTL2) (Guo et al., 2020). Effects of exosome stimulating factors and exosomal cargos on angiogenesis and AAT resistance are summarized in **Table 1**.

Masking of EVs

Besides AAT-induced hypoxia and changes in EV release and EV compositions, masking the therapeutic agent by EVs could decrease efficacy of AATs. One study showed that glioblastoma cells engulfed bevacizumab which were trafficked into endosomes but later displayed on the EVs released by these cancer cells. However, the bevacizumab associated with the EVs was unable to bind VEGF (Simon et al., 2018). Bevacizumab was believed to neutralize all isoforms of VEGF, but the binding to VEGF was mostly characterized in soluble VEGF. Recently, it was found that EV-associated VEGF is able in signaling but cannot be neutralized by bevacizumab (Ko et al., 2019).

Tumor Microenvironment

The mechanical properties of a tissue are determined by ECM (Zanotelli and Reinhart-King, 2018). ECM stiffness and density were increased in many solid tumors, and abnormal ECM dynamics promotes the progression of cancer (Zanotelli and Reinhart-King, 2018). ECM maintains the morphology of capillary, promotes angiogenesis via increasing MMP activity (Bordeleau et al., 2017). Matrix stiffness and cyclic compression could increase angiogenesis via inducing secretion of VEGF from human mesenchymal stem cells via Yes-associated protein (YAP) pathway (Bandaru et al., 2020). Dynamic hydrogels could induce clustering of integrin β1 and MMP expression to promote angiogenesis (Wei et al., 2020). Metastasis-associated fibroblasts could increase ECM stiffness (Shen et al., 2020). Inhibiting fibroblast contraction and ECM deposition could reduce liver cancer hardening and improve response to bevacizumab in metastatic colorectal cancer (Shen et al., 2020). Besides fibroblasts, other types of cells including tumor-associated macrophages (Li et al., 2019a; Pathria et al., 2019) and tumor-associated adipocytes (Wagner et al., 2012) in tumor environment contribute to angiogenesis.

Within human solid tumors, the interstitial fluid pressure is ranged from 1 to 60 mmHg, but it is only −7 to 14 mmHg in normal tissues (Boucher and Jain, 1992; Zanotelli and Reinhart-King, 2018). High interstitial fluid pressure may induce VEGF expression and facilitate the VEGF distribution in tumor (Rofstad et al., 2010; Vilanova et al., 2018). AATs could reduce tumor interstitial fluid pressure (Willett et al., 2009) and lessen the AAT resistance.

TABLE 1 | Effects of exosome stimulating factors and exosomal cargos on angiogenesis and anti-angiogenesis resistance.

Factors/Exosomal cargos	Function
miR-9	Improves AATs resistance by releasing VEGF-enriched exosomes in HCC (Zeng et al., 2019). Promotes angiogenesis via targeting $S1P_1$ (Yao et al., 2020).
EVs (e.g., MMP, IL-8, PDGFs, caveolin 1 and lysyl oxidase) from hypoxic glioblastoma multiforme	Promotes tumor growth and angiogenesis (Kucharzewska et al., 2013).
Exosomes from hypoxic colorectal cancer cells	Promotes angiogenesis via Wnt4-induced β-catenin signaling (Huang and Feng, 2017).
Exosomal lncRNA MALAT1 (YAP1 depletion in EC)	Increases the HCC cell invasion and metastasis via activation of ERK1/2 signaling (Li et al., 2020b).
Exosomal miR-135b	Promotes angiogenesis in gastric cancer via downregulation of forkhead box O1 (FOXO1) (Bai et al., 2019).
Exosomal miR-205	Promotes angiogenesis in ovarian cancer via the PTEN-AKT pathway (He et al., 2019).
Exosomal miR-629-5p	Promotes EC tumor cell invasion by targeting PPWD1 and increases EC permeability via suppressing CELSR1 in lung adenocarcinoma (Li et al., 2020a).
Exosome from Hela cells	Increases endoplasmic reticulum stress in EC and breaks down the endothelial integrity via downregulation of ZO-1 and Claudin-5 by a miRNA-independent manner (Lin et al., 2020).
Exosomal miR-155	Promotes angiogenesis via downregulating C-MYB and increasing of VEGF in gastric carcinoma (Deng et al., 2020).
Hypoxic exosomal lncRNA UCA1	Promotes angiogenesis and tumor growth through sponging miR-96-5p to upregulate angiomotin-like protein 2 (AMOTL2) in pancreatic cancer (Guo et al., 2020).
Exosomal miR-19b	Associates with angiogenesis and tumorigenesis of cancer stem cells (Wang et al., 2020).
Exosomal circSHKBP1	Promotes tumor growth and angiogenesis via sponging miR-582-3p to increase HUR expression in gastric cancer (Xie et al., 2020).
Exosomal circ-CCAC1	Increases cell progression by sponging miR-514a-5p to upregulate YY1, and induces angiogenesis in cholangiocarcinoma (Xu et al., 2020).

Based on the above findings, the resistance mechanisms of AATs are summarized as: (1) Induction of hypoxia by pruning tumor blood vessels and subsequently increasing proangiogenic cytokines including PLGF, FGF and VEGF (Eelen et al., 2020; Ko and Naora, 2020); (2) Heterogeneity in tumor ECs, different phenotypic characteristics exist between tumor ECs and normal ECs; (3) Close crosstalk between the tumor cells and ECs, which is mediated by exosomes derived by these cells; (4) Masking of EVs by rendering VEGF unrecognizable to the therapeutic agent (Ko and Naora, 2020); (5) Contributions from other types of cells including tumor associated fibroblasts, macrophages and adipocytes in tumor environment to angiogenesis; and (6) Stiffness of tumors. The tumor microenvironment plays a critical role in AAT resistance, which requires further understanding and clarification to develop tailored and efficient anti-proangiogenic strategies (Vasudev and Reynolds, 2014).

It was postulated that the dual-inhibition of the ECs and tumor cells/tumor stem cells, or targeting multiple growth factors could significantly inhibit tumorigenesis and angiogenesis. A recent study found that dual-inhibition of the ECs and tumor stem cells significantly inhibits the tumorigenesis and angiogenesis in renal cancer patient-derived xenograft mice (Wang et al., 2020). Besides the resistance of AATs, the benefit of AATs is transient, and efficacy is limited by a fast relapse. In this respect, we summarized below the side effects or failure of AATs due to revascularization after AATs.

REVASCULARIZATION FOLLOWING AATs

Preclinical and clinical studies have reported revascularization following AATs stoppage (Cacheux et al., 2008). The tumor growth rate in the patients with metastatic colorectal cancer was faster after bevacizumab (anti-VEGF) treatment and also possibly accelerated by surgery due to increased angiogenic cytokines such as plasmatic VEGF (Cacheux et al., 2008). Discontinuous schedule of bevacizumab accelerated tumor growth and revascularization in a patient-derived colorectal cancer subcutaneous xenograft in mice (Becherirat et al., 2018). Sunitinib or bevacizumab pretreatment significantly reduced microvessel density in primary renal cell carcinoma, but the proliferating ECs were dramatically increased in the sunitinib-pretreated tissues (Griffioen et al., 2012). Fibronectin networks provide a scaffold for revascularization and contribute to determining the proper direction of angiogenesis (Morita et al., 2020). It is still unclear why AATs promote EC proliferation and how. Also, adverse effects/toxicity of AATs on ECs are largely understudied. Recent studies revealed that vandetanib (anti-VEGFR2) inhibited the tumor EC (which overexpresses miR-9) migration, invasion and angiogenesis, and promoted cell autophagy (Zeng et al., 2019). Autophagy activation promotes bevacizumab resistance (Zhao et al., 2018; Chandra et al., 2019). Bevacizumab (100 μg/mL) enhanced migration, invasion and permeation ability of HUVECs via upregulation of TGF-β1 (Jia et al., 2019). Bevacizumab was also found to promote migration and tube formation of HUVECs via activation of the TGF-β1 pathway (Zhang et al., 2020). Using rat cornea model of revascularization, it was observed that revascularization occurred in partially regressed vessels, while fully regressed vessels retained non-functional empty basement membrane sleeves (Mukwaya et al., 2019). It remains intriguing whether and how the AATs kill all the tumor ECs to achieve full regression of vessels. Even when all the original tumor ECs are killed, AATs still can trigger a dramatic release of VEGF-enriched exosomes from these ECs to promote EC vasculogenesis and VM in other locations (Zeng et al., 2019; **Figure 2**). Yes-associated protein 1 (YAP1)/Notch pathway was reported to be involved in tumor vasculogenesis (Sivaraj et al., 2020).

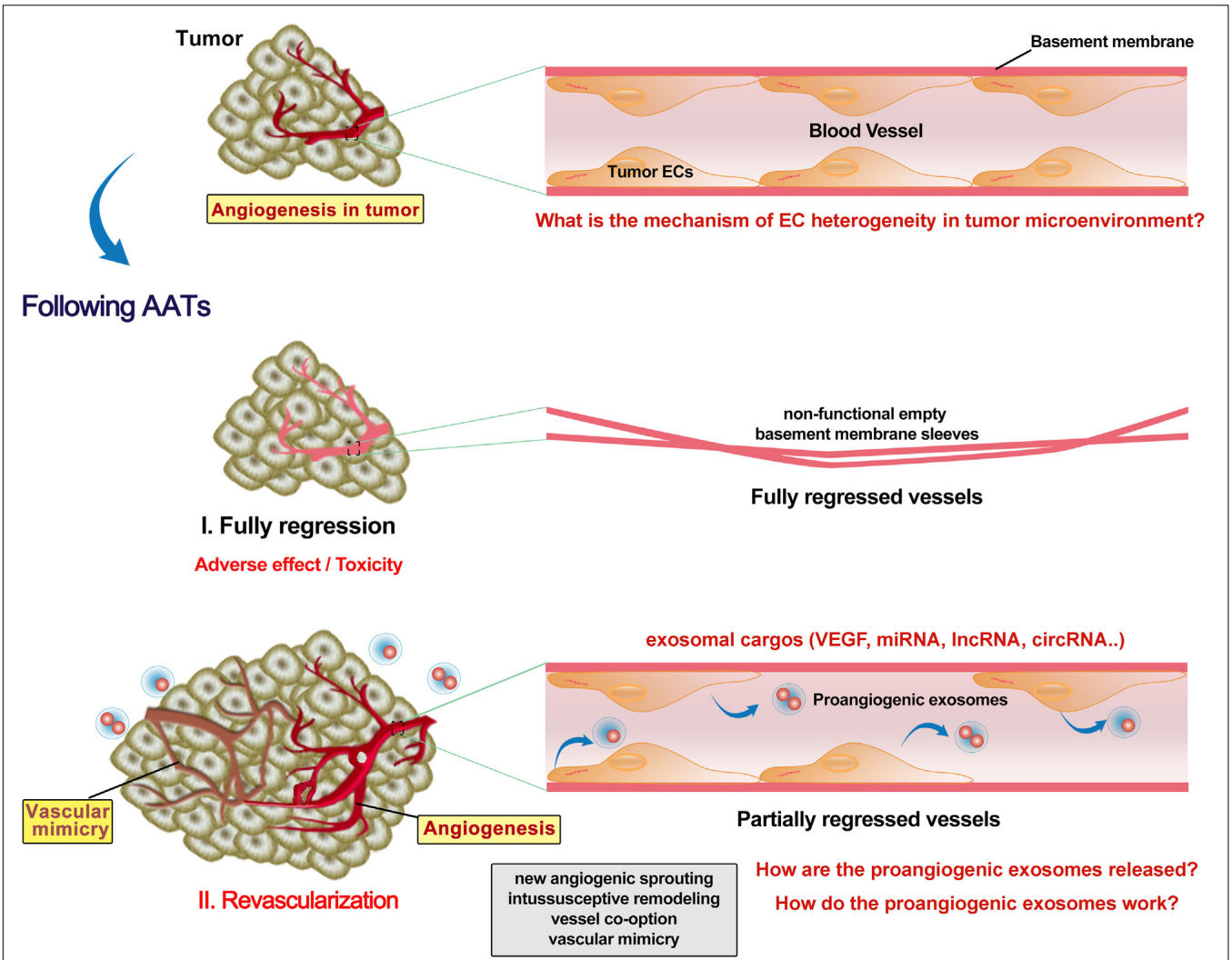


FIGURE 2 | Revascularization following AATs. Tumor endothelial cells (ECs) have different phenotypic characteristics from normal ECs. Anti-angiogenesis therapies (AATs) fully or partially regressed the vessel in the tumor. Non-functional empty basement membrane sleeves were found in fully regressed vessels, while EC degeneracy was shown in partially regressed persistent vessels. Although fully regressing the vessel in the tumor might inhibit the reuse of ECs, severe adverse effect and toxicity on the cardiovascular system are not negligible. Moreover, AATs trigger the ECs to release various exosomes with enriched proangiogenic molecules such as VEGF. Increases in proangiogenic exosomes and EC proliferation following AATs stoppage support the subsequent revascularization including new angiogenic sprouting, intussusceptive remodeling, vessel co-option and mimics, and thus promoting the tumor progression and metastasis. Future studies need to identify more key exosomal cargos as well as elucidate the mechanisms by which proangiogenic exosomes are released and how they contribute to the revascularization.

Depleting YAP1 from vascular ECs promotes the release of exosomes containing lncRNA MALAT1, and increases the HCC cell invasion and metastasis (Li et al., 2020b). Collectively, AATs trigger the release of exosomes with proangiogenic factors from tumor ECs to promote later vascularization, however, the underneath molecular mechanisms have not yet completely elucidated.

It was also suggested that vessel co-option is associated with AAT resistance (Kuczynski and Reynolds, 2020). MT1-MMP induces nitric oxide production to promote the vasodilation in arterioles and initiate the process of intussusceptive remodeling (D'Amico et al., 2020), which might be associated with changes in blood flow dynamics. Anti-intussusceptive angiogenesis may be a potential strategy for vascular diseases and AAT resistance.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Tumor vasculogenesis is critical in tumor growth, progression and metastatic dissemination. AATs targeting primary tumor vasculature have good clinical applications, but faster tumor relapse arises from tumor revascularization after AATs. To improve AAT efficacy, the most important and earliest step is to identify biomarkers for sensitive and resistant population

screening. The resistance of AATs is closely associated with an increase in pro-angiogenic cytokines, ECs heterogeneity, and tumor cell/tumor stem cell-EC crosstalk in the tumor environment. Detecting the VEGF-enriched exosomes in the blood following anti-VEGR2 therapy may allow us to predict the potential metastasis. Dual-inhibition of the ECs and tumor cells/tumor stem cells, targeting multiple growth factors, and new players in angiogenesis such as YAP1 or autophagy can be utilized to overcome the AAT resistance. Therefore, better understanding in the underlining mechanism of sprouting angiogenesis, vessel co-option, intussusceptive angiogenesis, and VM is critical for the development of novel AATs without the resistance and failure. In addition, modulation of tumor mechanical environment as a therapeutic approach is a direction toward personalized medicine. Moreover, tumor EC normalization by trimming the genetically modified ECs rather than tumor vessel normalization is a promising AAT strategy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was partially supported by the United States National Institutes of Health (1UH3TR002151-01) and the National Natural Science Foundation of China (No. 11932014).

REFERENCES

Alexander, S., and Friedl, P. (2012). Cancer invasion and resistance: interconnected processes of disease progression and therapy failure. *Trends Mol. Med.* 18, 13–26. doi: 10.1016/j.molmed.2011.11.003

Al-Nedawi, K., Meehan, B., Kerbel, R. S., Allison, A. C., and Rak, J. (2009). Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3794–3799. doi: 10.1073/pnas.0804543106

Angara, K., Borin, T. F., and Arbab, A. S. (2017). Vascular mimicry: a novel neovascularization mechanism driving anti-angiogenic therapy (AAT) resistance in glioblastoma. *Transl. Oncol.* 10, 650–660. doi: 10.1016/j.tranon.2017.04.007

Bai, M., Li, J., Yang, H., Zhang, H., Zhou, Z., Deng, T., et al. (2019). miR-135b delivered by gastric tumor exosomes inhibits FOXO1 expression in endothelial cells and promotes angiogenesis. *Mol. Ther.* 27, 1772–1783. doi: 10.1016/j.ymthe.2019.06.018

Baker, G. J., Yadav, V. N., Motsch, S., Koschmann, C., Calinescu, A. A., Mineharu, Y., et al. (2014). Mechanisms of glioma formation: iterative perivascular glioma growth and invasion leads to tumor progression, VEGF-independent vascularization, and resistance to antiangiogenic therapy. *Neoplasia* 16, 543–561. doi: 10.1016/j.neo.2014.06.003

Bandaru, P., Cefaloni, G., Vajhadin, F., Lee, K., Kim, H. J., Cho, H. J., et al. (2020). Mechanical cues regulating proangiogenic potential of human mesenchymal stem cells through YAP-Mediated mechanosensing. *Small* 16:e2001837. doi: 10.1002/smll.202001837

Becherirat, S., Valamanesh, F., Karimi, M., Faussat, A. M., Launay, J. M., Pimpie, C., et al. (2018). Discontinuous schedule of bevacizumab in colorectal cancer induces accelerated tumor growth and phenotypic changes. *Transl. Oncol.* 11, 406–415. doi: 10.1016/j.tranon.2018.01.017

Bordeleau, F., Mason, B. N., Lollis, E. M., Mazzola, M., Zanotelli, M. R., Somasegar, S., et al. (2017). Matrix stiffening promotes a tumor vasculature phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 114, 492–497. doi: 10.1073/pnas.1613855114

Boucher, Y., and Jain, R. K. (1992). Microvascular pressure is the principal driving force for interstitial hypertension in solid tumors: implications for vascular collapse. *Cancer Res.* 52, 5110–5114.

Cacheux, W., Boisserie, T., Staudacher, L., Vignaux, O., Dousset, B., Soubrane, O., et al. (2008). Reversible tumor growth acceleration following bevacizumab interruption in metastatic colorectal cancer patients scheduled for surgery. *Ann. Oncol.* 19, 1659–1661. doi: 10.1093/annonc/mdn540

Carmeliet, P., and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307. doi: 10.1038/nature10144

Chandra, A., Rick, J., Yagnik, G., and Aghi, M. K. (2019). Autophagy as a mechanism for anti-angiogenic therapy resistance. *Semin. Cancer Biol.* 66, 75–88. doi: 10.1016/j.semcancer.2019.08.031

Chung, A. S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat. Rev. Cancer* 10, 505–514. doi: 10.1038/nrc2868

Ciesielski, O., Biesiekierska, M., Panthu, B., Vialichka, V., Pirola, L., and Balcerczyk, A. (2020). The epigenetic profile of tumor endothelial cells. effects of combined therapy with antiangiogenic and epigenetic drugs on cancer progression. *Int. J. Mol. Sci.* 21:2606. doi: 10.3390/ijms21072606

Cloughesy, T. F., Brenner, A., de Groot, J. F., Butowski, N. A., Zach, L., Campian, J. L., et al. (2020). A randomized controlled phase III study of VB-111 combined with bevacizumab vs bevacizumab monotherapy in patients with recurrent glioblastoma (GLOBE). *Neuro Oncol.* 22, 705–717. doi: 10.1093/neuonc/noz232

Cully, M. (2017). Cancer: tumour vessel normalization takes centre stage. *Nat. Rev. Drug Discov.* 16:87. doi: 10.1038/nrd.2017.4

D'Amico, G., Muñoz-Félix, J. M., Pedrosa, A. R., and Hodivala-Dilke, K. M. (2020). "Splitting the matrix": intussusceptive angiogenesis meets MT1-MMP. *EMBO Mol. Med.* 12:e11663. doi: 10.15252/emmm.201911663

De Bock, K., Mazzone, M., and Carmeliet, P. (2011). Antiangiogenic therapy, hypoxia, and metastasis: risky liaisons, or not? *Nat. Rev. Clin. Oncol.* 8, 393–404. doi: 10.1038/nrclinonc.2011.83

De Spiegelaere, W., Casteleyn, C., Van den Broeck, W., Plendl, J., Bahramsoltani, M., Simoens, P., et al. (2012). Intussusceptive angiogenesis: a biologically relevant form of angiogenesis. *J. Vasc. Res.* 49, 390–404. doi: 10.1159/000338278

Deng, T., Zhang, H., Yang, H., Wang, H., Bai, M., Sun, W., et al. (2020). Exosome miR-155 derived from gastric carcinoma promotes angiogenesis by targeting the c-MYB/VEGF Axis of endothelial cells. *Mol. Ther. Nucleic Acids* 19, 1449–1459. doi: 10.1016/j.omtn.2020.01.024

Dolo, V., Ginestra, A., Cassarà, D., Violini, S., Lucania, G., Torrisi, M. R., et al. (1998). Selective localization of matrix metalloproteinase 9, beta1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells. *Cancer Res.* 58, 4468–4474.

Eelen, G., Treps, L., Li, X., and Carmeliet, P. (2020). Basic and therapeutic aspects of angiogenesis updated. *Circ. Res.* 127, 310–329. doi: 10.1161/circresaha.120.316851

Esteban, S., Clemente, C., Koziol, A., Gonzalo, P., Rius, C., Martínez, F., et al. (2020). Endothelial MT1-MMP targeting limits intussusceptive angiogenesis and colitis via TSP1/nitric oxide axis. *EMBO Mol. Med.* 12:e10862. doi: 10.15252/emmm.201910862

Fack, F., Espedal, H., Keunen, O., Golebiewska, A., Obad, N., Harter, P. N., et al. (2015). Bevacizumab treatment induces metabolic adaptation toward anaerobic metabolism in glioblastomas. *Acta Neuropathol.* 129, 115–131. doi: 10.1007/s00401-014-1352-5

Favia, A., Desideri, M., Gambara, G., D'Alessio, A., Ruas, M., Esposito, B., et al. (2014). VEGF-induced neoangiogenesis is mediated by NAADP and two-pore channel-2-dependent Ca2+ signaling. *Proc. Natl. Acad. Sci. U.S.A.* 111, E4706–E4715. doi: 10.1073/pnas.1406029111

Frentzas, S., Simoneau, E., Bridgeman, V. L., Vermeulen, P. B., Foo, S., Kostaras, E., et al. (2016). Vessel co-option mediates resistance to anti-angiogenic therapy in liver metastases. *Nat. Med.* 22, 1294–1302. doi: 10.1038/nm.4197

Galvano, A., Guarini, A., Iacono, F., Castiglia, M., Rizzo, S., Tarantini, L., et al. (2019). An update on the conquests and perspectives of cardio-oncology in the field of tumor angiogenesis-targeting TKI-based therapy. *Expert Opin. Drug Saf.* 18, 485–496. doi: 10.1080/14740338.2019.1613371

García-Romero, N., Palacín-Aliana, I., Madurga, R., Carrión-Navarro, J., Esteban-Rubio, S., Jiménez, B., et al. (2020). Bevacizumab dose adjustment to improve clinical outcomes of glioblastoma. *BMC Med.* 18:142. doi: 10.1186/s12916-020-01610-0

Ge, R., Tai, Y., Sun, Y., Zhou, K., Yang, S., Cheng, T., et al. (2009). Critical role of TRPC6 channels in VEGF-mediated angiogenesis. *Cancer Lett.* 283, 43–51. doi: 10.1016/j.canlet.2009.03.023

Ginestra, A., Monea, S., Seghezzi, G., Dolo, V., Nagase, H., Mignatti, P., et al. (1997). Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT1080 fibrosarcoma cells. *J. Biol. Chem.* 272, 17216–17222. doi: 10.1074/jbc.272.27.17216

Goveia, J., Rohlenova, K., Taverna, F., Treps, L., Conradi, L. C., Pircher, A., et al. (2020). An integrated gene expression landscape profiling approach to identify lung tumor endothelial cell heterogeneity and angiogenic candidates. *Cancer Cell* 37, 21.e13–36.e13. doi: 10.1016/j.ccell.2019.12.001

Griffioen, A. W., Mans, L. A., de Graaf, A. M. A., Nowak-Sliwinska, P., de Hoog, C., de Jong, T. A. M., et al. (2012). Rapid angiogenesis onset after discontinuation of sunitinib treatment of renal cell carcinoma patients. *Clin. Cancer Res.* 18, 3961–3971. doi: 10.1158/1078-0432.Ccr-12-0002

Guo, Z., Wang, X., Yang, Y., Chen, W., Zhang, K., Teng, B., et al. (2020). Hypoxic tumor-derived exosomal long noncoding RNA UCA1 promotes angiogenesis via miR-96-5p/AMOTL2 in Pancreatic Cancer. *Mol. Ther. Nucleic Acids* 22, 179–195. doi: 10.1016/j.omtn.2020.08.021

Haibe, Y., Kreidieh, M., El Hajj, H., Khalifeh, I., Mukherji, D., Temraz, S., et al. (2020). Resistance mechanisms to anti-angiogenic therapies in Cancer. *Front. Oncol.* 10:221. doi: 10.3389/fonc.2020.00221

He, L., Zhu, W., Chen, Q., Yuan, Y., Wang, Y., Wang, J., et al. (2019). Ovarian cancer cell-secreted exosomal miR-205 promotes metastasis by inducing angiogenesis. *Theranostics* 9, 8206–8220. doi: 10.7150/thno.37455

Hendrix, M. J., Seftor, E. A., Hess, A. R., and Seftor, R. E. (2003). Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat. Rev. Cancer* 3, 411–421. doi: 10.1038/nrc1092

Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., et al. (1999). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* 284, 1994–1998. doi: 10.1126/science.284.5422.1994

Hosein, A. N., Brekken, R. A., and Maitra, A. (2020). Pancreatic cancer stroma: an update on therapeutic targeting strategies. *Nat. Rev. Gastroenterol. Hepatol.* 17, 487–505. doi: 10.1038/s41575-020-0300-1

Huang, Z., and Feng, Y. (2017). Exosomes derived from hypoxic colorectal cancer cells promote angiogenesis through Wnt4-Induced β-Catenin signaling in endothelial cells. *Oncol. Res.* 25, 651–661. doi: 10.3727/096504016x14752792816791

Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307, 58–62. doi: 10.1126/science.1104819

Jayson, G. C., Kerbel, R., Ellis, L. M., and Harris, A. L. (2016). Antiangiogenic therapy in oncology: current status and future directions. *Lancet* 388, 518–529. doi: 10.1016/s0140-6736(15)01088-0

Jia, Y., Qin, T., Zhang, X., Liu, S., Liu, Z., Zhang, C., et al. (2019). Effect of bevacizumab on the tight junction proteins of vascular endothelial cells. *Am. J. Transl. Res.* 11, 5546–5559.

Kajimoto, T., Okada, T., Miya, S., Zhang, L., and Nakamura, S. (2013). Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes. *Nat. Commun.* 4:2712. doi: 10.1038/ncomms3712

Kanugula, A. K., Adapala, R. K., Midha, P., Cappelli, H. C., Meszaros, J. G., Paruchuri, S., et al. (2019). Novel noncanonical regulation of soluble VEGF/VEGFR2 signaling by mechanosensitive ion channel TRPV4. *FASEB J.* 33, 195–203. doi: 10.1096/fj.201800509R

King, H. W., Michael, M. Z., and Gleadle, J. M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* 12:421. doi: 10.1186/1471-2407-12-421

Ko, S. Y., Lee, W., Kenny, H. A., Dang, L. H., Ellis, L. M., Jonasch, E., et al. (2019). Cancer-derived small extracellular vesicles promote angiogenesis by heparin-bound, bevacizumab-insensitive VEGF, independent of vesicle uptake. *Commun. Biol.* 2:386. doi: 10.1038/s42003-019-0609-x

Ko, S. Y., and Naora, H. (2020). Extracellular vesicle membrane-associated proteins: emerging roles in tumor angiogenesis and anti-angiogenesis therapy resistance. *Int. J. Mol. Sci.* 21:5418. doi: 10.3390/ijms21155418

Kucharzewska, P., Christianson, H. C., Welch, J. E., Svensson, K. J., Fredlund, E., Ringnér, M., et al. (2013). Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7312–7317. doi: 10.1073/pnas.1220998110

Kuczynski, E. A., and Reynolds, A. R. (2020). Vessel co-option and resistance to anti-angiogenic therapy. *Angiogenesis* 23, 55–74. doi: 10.1007/s10456-019-09698-6

Kuczynski, E. A., Yin, M., Bar-Zion, A., Lee, C. R., Butz, H., Man, S., et al. (2016). Co-option of liver vessels and not sprouting angiogenesis drives acquired sorafenib resistance in hepatocellular carcinoma. *J. Natl. Cancer Inst.* 108:djw030. doi: 10.1093/jnci/djw030

Latacz, E., Caspani, E., Barnhill, R., Lugassy, C., Verhoef, C., Grünhagen, D., et al. (2020). Pathological features of vessel co-option versus sprouting angiogenesis. *Angiogenesis* 23, 43–54. doi: 10.1007/s10456-019-09690-0

Li, X., Liu, R., Su, X., Pan, Y., Han, X., Shao, C., et al. (2019a). Harnessing tumor-associated macrophages as aids for cancer immunotherapy. *Mol. Cancer* 18:177. doi: 10.1186/s12943-019-1102-3

Li, X., Sun, X., and Carmeliet, P. (2019b). Hallmarks of endothelial cell metabolism in health and disease. *Cell Metab.* 30, 414–433. doi: 10.1016/j.cmet.2019.08.011

Li, Y., Zhang, H., Fan, L., Mou, J., Yin, Y., Peng, C., et al. (2020a). MiR-629-5p promotes the invasion of lung adenocarcinoma via increasing both tumor cell invasion and endothelial cell permeability. *Oncogene* 39, 3473–3488. doi: 10.1038/s41388-020-1228-1

Li, Y., Zhang, X., Zheng, Q., Zhang, Y., Ma, Y., Zhu, C., et al. (2020b). YAP1 inhibition in HUVECs is associated with released exosomes and increased hepatocarcinoma invasion and metastasis. *Mol. Ther. Nucleic Acids* 21, 86–97. doi: 10.1016/j.omtn.2020.05.021

Lin, Y., Zhang, C., Xiang, P., Shen, J., Sun, W., and Yu, H. (2020). Exosomes derived from HeLa cells break down vascular integrity by triggering endoplasmic reticulum stress in endothelial cells. *J. Extracell Vesicles* 9:1722385. doi: 10.1080/20013078.2020.1722385

Meads, M. B., Gatenby, R. A., and Dalton, W. S. (2009). Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nat. Rev. Cancer* 9, 665–674. doi: 10.1038/nrc2714

Monk, B. J., Minion, L. E., and Coleman, R. L. (2016). Anti-angiogenic agents in ovarian cancer: past, present, and future. *Ann. Oncol.* 27(Suppl. 1), i33–i39. doi: 10.1093/annonc/mdw093

Morita, A., Goko, T., Matsumura, M., Asaso, D., Arima, S., Mori, A., et al. (2020). The process of revascularization in the neonatal mouse retina following short-term blockade of vascular endothelial growth factor receptors. *Cell Tissue Res.* 382, 529–549. doi: 10.1007/s00441-020-03276-9

Mukwaya, A., Mirabelli, P., Lennikov, A., Thangavelu, M., Ntzouni, M., Jensen, L., et al. (2019). Revascularization after angiogenesis inhibition favors new sprouting over abandoned vessel reuse. *Angiogenesis* 22, 553–567. doi: 10.1007/s10456-019-09679-9

Pàez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Viñals, F., et al. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15, 220–231. doi: 10.1016/j.ccr.2009.01.027

Park, J. S., Kim, I. K., Han, S., Park, I., Kim, C., Bae, J., et al. (2016). Normalization of tumor vessels by Tie2 activation and Ang2 inhibition enhances drug delivery and produces a favorable tumor microenvironment. *Cancer Cell* 30, 953–967. doi: 10.1016/j.ccell.2016.10.018

Passalidou, E., Trivella, M., Singh, N., Ferguson, M., Hu, J., Cesario, A., et al. (2002). Vascular phenotype in angiogenic and non-angiogenic lung non-small cell carcinomas. *Br. J. Cancer* 86, 244–249. doi: 10.1038/sj.bjc.6600015

Pathria, P., Louis, T. L., and Varner, J. A. (2019). Targeting tumor-associated macrophages in cancer. *Trends Immunol.* 40, 310–327. doi: 10.1016/j.it.2019.02.003

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887. doi: 10.1016/j.cell.2011.08.039

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion channels in cancer: are cancer hallmarks oncochannelopathies? *Physiol. Rev.* 98, 559–621. doi: 10.1152/physrev.00044.2016

Qu, Z., Wu, J., Wu, J., Luo, D., Jiang, C., and Ding, Y. (2016). Exosomes derived from HCC cells induce sorafenib resistance in hepatocellular carcinoma both in vivo and in vitro. *J. Exp. Clin. Cancer Res.* 35:159. doi: 10.1186/s13046-016-0430-z

Rezaei, M., Martins Cavaco, A. C., Stehling, M., Nottebaum, A., Brockhaus, K., Caliandro, M. F., et al. (2020). Extracellular vesicle transfer from endothelial cells drives VE-Cadherin expression in breast cancer cells, thereby causing heterotypic cell contacts. *Cancers* 12:2138. doi: 10.3390/cancers12082138

Rofstad, E. K., Ruud, E. B., Mathiesen, B., and Galappathi, K. (2010). Associations between radiocurability and interstitial fluid pressure in human tumor xenografts without hypoxic tissue. *Clin. Cancer Res.* 16, 936–945. doi: 10.1158/1078-0432.Ccr-09-2718

Shen, Y., Wang, X., Lu, J., Salfenmoser, M., Wirsik, N. M., Schleussner, N., et al. (2020). Reduction of liver metastasis stiffness improves response to bevacizumab in metastatic Colorectal Cancer. *Cancer Cell* 37, 800.e7–817.e7. doi: 10.1016/j.ccell.2020.05.005

Sheng, X., Yan, X., Chi, Z., Si, L., Cui, C., Tang, B., et al. (2019). Axitinib in combination with Toripalimab, a humanized immunoglobulin G4 monoclonal antibody against programmed cell death-1, in patients with metastatic mucosal melanoma: an open-label phase ib trial. *J. Clin. Oncol.* 37, 2987–2999. doi: 10.1200/jco.19.00210

Shi, Q., Abbruzzese, J. L., Huang, S., Fidler, I. J., Xiong, Q., and Xie, K. (1999). Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. *Clin. Cancer Res.* 5, 3711–3721.

Simon, T., Pinioti, S., Schellenberger, P., Rajeeve, V., Wendler, F., Cutillas, P. R., et al. (2018). Shedding of bevacizumab in tumour cells-derived extracellular vesicles as a new therapeutic escape mechanism in glioblastoma. *Mol. Cancer* 17:132. doi: 10.1186/s12943-018-0878-x

Sivaraj, K. K., Dharmalingam, B., Mohanakrishnan, V., Jeong, H. W., Kato, K., Schröder, S., et al. (2020). YAP1 and TAZ negatively control bone angiogenesis by limiting hypoxia-inducible factor signaling in endothelial cells. *eLife* 9:e50770. doi: 10.7554/eLife.50770

Stewart, E. A., Saker, S., and Amoaku, W. M. (2016). Dexamethasone reverses the effects of high glucose on human retinal endothelial cell permeability and proliferation in vitro. *Exp. Eye Res.* 151, 75–81. doi: 10.1016/j.exer.2016.08.005

Sun, T., Zhao, N., Zhao, X. L., Gu, Q., Zhang, S. W., Che, N., et al. (2010). Expression and functional significance of Twist1 in hepatocellular carcinoma: its role in vasculogenic mimicry. *Hepatology* 51, 545–556. doi: 10.1002/hep.23311

Tan, H. W., Xu, Y. M., Qin, S. H., Chen, G. F., and Lau, A. T. Y. (2020). Epigenetic regulation of angiogenesis in lung cancer. *J. Cell Physiol.* doi: 10.1002/jcp.30104 [Epub ahead of print].

Taraboletti, G., D'Ascenzo, S., Borsotti, P., Giavazzi, R., Pavan, A., and Dolo, V. (2002). Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am. J. Pathol.* 160, 673–680. doi: 10.1016/s0002-9440(10)64887-0

Tian, L., Goldstein, A., Wang, H., Lo, H. C., Kim, I. S., Welte, T., et al. (2017). Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming. *Nature* 544, 250–254. doi: 10.1038/nature21724

Valiente, M., Obenauf, A. C., Jin, X., Chen, Q., Zhang, X. H. F., Lee, D. J., et al. (2014). Serpins promote cancer cell survival and vascular co-option in brain metastasis. *Cell* 156, 1002–1016. doi: 10.1016/j.cell.2014.01.040

Vasudev, N. S., and Reynolds, A. R. (2014). Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. *Angiogenesis* 17, 471–494. doi: 10.1007/s10456-014-9420-y

Vilanova, G., Burés, M., Colominas, I., and Gomez, H. (2018). Computational modelling suggests complex interactions between interstitial flow and tumour angiogenesis. *J. R. Soc. Interface* 15:20180415. doi: 10.1098/rsif.2018.0415

von Baumgarten, L., Brucker, D., Tirniceru, A., Kienast, Y., Grau, S., Burgold, S., et al. (2011). Bevacizumab has differential and dose-dependent effects on glioma blood vessels and tumor cells. *Clin. Cancer Res.* 17, 6192–6205. doi: 10.1158/1078-0432.Ccr-10-1868

Voutouri, C., Kirkpatrick, N. D., Chung, E., Mpekris, F., Baish, J. W., Munn, L. L., et al. (2019). Experimental and computational analyses reveal dynamics of tumor vessel cooption and optimal treatment strategies. *Proc. Natl. Acad. Sci. U.S.A.* 116, 2662–2671. doi: 10.1073/pnas.1818322116

Wagenblast, E., Soto, M., Gutierrez-Angel, S., Hartl, C. A., Gable, A. L., Maceli, A. R., et al. (2015). A model of breast cancer heterogeneity reveals vascular mimicry as a driver of metastasis. *Nature* 520, 358–362. doi: 10.1038/nature14403

Wagner, M., Bjerkvig, R., Wiig, H., Melero-Martin, J. M., Lin, R. Z., Klagsbrun, M., et al. (2012). Inflamed tumor-associated adipose tissue is a depot for macrophages that stimulate tumor growth and angiogenesis. *Angiogenesis* 15, 481–495. doi: 10.1007/s10456-012-9276-y

Wang, L., Lv, Y., Li, C., Yang, G., Fu, B., Peng, Q., et al. (2020). Transformable dual-inhibition system effectively suppresses renal cancer metastasis through blocking endothelial cells and cancer stem cells. *Small* 16:e2004548. doi: 10.1002/smll.202004548

Wang, T., Gilkes, D. M., Takano, N., Xiang, L., Luo, W., Bishop, C. J., et al. (2014). Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3234–E3242. doi: 10.1073/pnas.1410041111

Wei, Z., Schnellmann, R., Pruitt, H. C., and Gerecht, S. (2020). Hydrogel network dynamics regulate vascular morphogenesis. *Cell Stem Cell* 27, 798.e6–812.e6. doi: 10.1016/j.stem.2020.08.005

Weinstein, R. S., Jakate, S. M., Dominguez, J. M., Lebovitz, M. D., Koukoulis, G. K., Kuszak, J. R., et al. (1991). Relationship of the expression of the multidrug resistance gene product (P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis. *Cancer Res.* 51, 2720–2726.

Wilky, B. A., Trucco, M. M., Subhawong, T. K., Florou, V., Park, W., Kwon, D., et al. (2019). Axitinib plus pembrolizumab in patients with advanced sarcomas including alveolar soft-part sarcoma: a single-centre, single-arm, phase 2 trial. *Lancet Oncol.* 20, 837–848. doi: 10.1016/s1470-2045(19)30153-6

Weis, S. M., and Cheresh, D. A. (2011). Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat. Med.* 17, 1359–1370. doi: 10.1038/nm.2537

Willett, C. G., Duda, D. G., di Tomaso, E., Boucher, Y., Ancukiewicz, M., Sahani, D. V., et al. (2009). Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II study. *J. Clin. Oncol.* 27, 3020–3026. doi: 10.1200/jco.2008.21.1771

Xie, M., Yu, T., Jing, X., Ma, L., Fan, Y., Yang, F., et al. (2020). Exosomal circSHKBP1 promotes gastric cancer progression via regulating the miR-582-3p/HUR/VEGF axis and suppressing HSP90 degradation. *Mol. Cancer* 19:112. doi: 10.1186/s12943-020-01208-3

Xu, Y., Leng, K., Yao, Y., Kang, P., Liao, G., Han, Y., et al. (2020). A novel circular RNA, circ-CCAC1, contributes to CCA progression, induces angiogenesis, and disrupts vascular endothelial barriers. *Hepatology* doi: 10.1002/hep.31493 [Epub ahead of print].

Yao, X., Xie, L., and Zeng, Y. (2020). MiR-9 promotes angiogenesis via targeting on Sphingosine-1- phosphate Receptor 1. *Front. Cell Dev. Biol.* 8:755. doi: 10.3389/fcell.2020.00755

Zanotelli, M. R., and Reinhart-King, C. A. (2018). Mechanical forces in tumor angiogenesis. *Adv. Exp. Med. Biol.* 1092, 91–112. doi: 10.1007/978-3-319-95294-9_6

Zeng, Y., Yao, X., Liu, X., He, X., Li, L., Liu, X., et al. (2019). Anti-angiogenesis triggers exosomes release from endothelial cells to promote tumor vasculogenesis. *J. Extracell Vesicles* 8:1629865. doi: 10.1080/20013078.2019.1629865

Zhang, X., Zhang, Y., Jia, Y., Qin, T., Zhang, C., Li, Y., et al. (2020). Bevacizumab promotes active biological behaviors of human umbilical vein endothelial cells by activating TGFβ1 pathways via off-VEGF signaling. *Cancer Biol. Med.* 17, 418–432. doi: 10.20892/j.issn.2095-3941.2019.0215

Zhao, Q., Eichten, A., Parveen, A., Adler, C., Huang, Y., Wang, W., et al. (2018). Single-cell transcriptome analyses reveal endothelial cell heterogeneity in tumors and changes following antiangiogenic

treatment. *Cancer Res.* 78, 2370–2382. doi: 10.1158/0008-5472.Can-17-2728

Zhao, Z., Xia, G., Li, N., Su, R., Chen, X., and Zhong, L. (2018). autophagy inhibition promotes bevacizumab-induced apoptosis and proliferation inhibition in colorectal cancer cells. *J. Cancer* 9, 3407–3416. doi: 10.7150/jca.24201

Zhuang, G., Wu, X., Jiang, Z., Kasman, I., Yao, J., Guan, Y., et al. (2012). Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *EMBO J.* 31, 3513–3523. doi: 10.1038/emboj.2012.183

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 09 December 2020
doi: 10.3389/fcell.2020.611039

Extracellular Vesicles Are Key Regulators of Tumor Neovasculature

*Naoya Kuriyama[1,2], Yusuke Yoshioka[1], Shinsuke Kikuchi[2], Nobuyoshi Azuma[2] and Takahiro Ochiya[1]**

[1] Division of Molecular and Cellular Medicine, Institute of Medical Science, Tokyo Medical University, Tokyo, Japan,
[2] Department of Vascular Surgery, Asahikawa Medical University, Asahikawa, Japan

Tumor progression involves a series of biologically important steps in which the crosstalk between cancer cells and the surrounding environment is an important issue. Angiogenesis is a key tumorigenic phenomenon for cancer progression. Tumor-related extracellular vesicles (EVs) modulate the tumor microenvironment (TME) through cell-to-cell communication. Tumor cells in a hypoxic TME release more EVs than cells in a normoxic environment due to uncontrollable tumor proliferation. Tumor-derived EVs in the TME influence endothelial cells (ECs), which then play multiple roles, contributing to tumor angiogenesis, loss of the endothelial vascular barrier by binding to ECs, and subsequent endothelial-to-mesenchymal transition. In contrast, they also indirectly induce tumor angiogenesis through the phenotype switching of various cells into cancer-associated fibroblasts, the activation of tumor-associated ECs and platelets, and remodeling of the extracellular matrix. Here, we review current knowledge regarding the involvement of EVs in tumor vascular-related cancer progression.

Keywords: extracellular vesicles, cancer, tumor microenvironment, tumor metastasis, tumor angiogenesis

OPEN ACCESS

Edited by:
Kyoko Hida,
Hokkaido University, Japan

Reviewed by:
Mitsuhiko Osaki,
Tottori University, Japan
Benedetta Bussolati,
University of Turin, Italy

***Correspondence:**
Takahiro Ochiya
tochiya@tokyo-med.ac.jp

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 September 2020*
Accepted: *16 November 2020*
Published: *09 December 2020*

Citation:
Kuriyama N, Yoshioka Y, Kikuchi S, Azuma N and Ochiya T (2020) Extracellular Vesicles Are Key Regulators of Tumor Neovasculature. Front. Cell Dev. Biol. 8:611039. doi: 10.3389/fcell.2020.611039

INTRODUCTION

Extracellular vesicles (EVs) were first discovered in 1967 and initially called "platelet dust," which was defined as subcellular coagulant materials (Wolf, 1967; Cufaro et al., 2019). Thereafter, platelet dust has been called microparticles (MPs) or microvesicles (MVs) in general. However, accumulating evidence for mechanisms associated with EVs indicate that EVs are involved in various cell-to-cell signaling pathways and act as important molecular messengers in various pathophysiological processes (Cufaro et al., 2019). EVs are released from almost all cells and phospholipid bilayer membranous vesicles (Thery et al., 2018). According to the International Society for Extracellular Vesicles (ISEV), EVs are categorized into several subtypes according to their size or biogenesis: exosomes, MVs (microvesicles, ectosomes, or microparticles), and apoptotic bodies (Witwer et al., 2013; Cufaro et al., 2019). Exosomes are believed to originate from intraluminal vesicles by merging intracellular multivesicular bodies (MVBs) with the plasma membrane (Witwer et al., 2013; Cufaro et al., 2019). MVBs contain vesicles and combine with lysosomes for degradation of their contents or combine with the plasma membrane, resulting in the effusion of intraluminal vesicles, defined as exosomes, into the extracellular space (Harding et al., 1983; Pan et al., 1985). Exosomes

are approximately 100 nm in diameter and smaller than MVs (Witwer et al., 2013). The membranous proteins of exosomes are enriched with heat shock proteins (HSP70, HSP90), integrins, Alix, tetraspanins (CD9, CD63, CD81, CD82, and CD151), MHC class II proteins, epithelial cell adhesion molecules, and members of the human epidermal receptor family (Zhang and Grizzle, 2014; Kalluri, 2016). In contrast, MVs are a few nm to a few μm in diameter and originate from outward budding from the plasma membrane (Witwer et al., 2013; Kalluri, 2016; Tkach and Théry, 2016). Apoptotic bodies are released by apoptotic cells; they are 1–5 μm in diameter and the largest EVs (van der Pol et al., 2012; Cufaro et al., 2019). However, there are currently no indubitable markers to specifically identify these vesicles. Thus, the ISEV consensus recommends using the generic term EV in the nomenclature (Gould and Raposo, 2013). EVs contain various types of molecules, such as proteins, lipids, carbohydrates, DNAs, and coding and non-coding RNAs (Nawaz et al., 2018). EVs have various types of functions and can mediate cell-to-cell communication. They are taken up by various cells via autocrine, paracrine, and endocrine processes (Zhang and Grizzle, 2014). EVs are involved in various physiological processes, such as atherosclerosis (Hafiane and Daskalopoulou, 2018; Deng et al., 2019), neurodegenerative diseases (Grad et al., 2014), autoimmune diseases (Beyer and Pisetsky, 2010; Willis et al., 2019; Wu et al., 2019), and cancer progression (Cufaro et al., 2019).

It is clear that the majority of cancer-related mortalities are the result of cancer metastasis (Eccles and Welch, 2007). Cancer metastasis is a complex process that includes detachment from the primary tumor and invasion into adjacent tissues, evasion from the immune system, transport into the circulation, and extravasation and growth at a secondary organ (Mehlen and Puisieux, 2006; Li et al., 2019). The tumor microenvironment (TME) is the environment around the tumor and plays important roles in cancer progression and metastasis (Guo and Deng, 2018). The TME consists is highly organized and includes blood vessels, lymph vessels, endothelial cells (ECs), pericytes, fibroblasts, macrophages, and lymphocytes (Balkwill et al., 2012; Baghban et al., 2020). The TME also includes non-cellular components. For example, the extracellular matrix (ECM) is one of the important components of the TME and includes collagen, fibronectin, hyaluronic acid, laminin and other molecules (Baghban et al., 2020). In addition to these molecules, studies on the TME have also focused on EVs in. Numerous studies have suggested that tumor cells and stromal cells in the TME release a large number of EVs that mediate cell-to-cell communication and that the surrounding microenvironment plays essential roles in cancer metastasis (Tominaga et al., 2015a; Kalluri, 2016; Li et al., 2019).

The TME is more hypoxic than the normal internal environment due to the uncontrollable growth of cancer cells (Jing et al., 2019; Tan et al., 2020). Solid tumors, which require nutrients and oxygen under hypoxic conditions, develop vessels that supply the tumor (Vaupel and Harrison, 2004; Jing et al., 2019). Cancer-related neovessels, such as those observed during angiogenesis and lymphangiogenesis, are needed for invasive tumor growth and metastasis. Angiogenesis refers to the growth of new blood vessels and is one of the most important processes for invasive tumor growth and cancer metastasis. Tumor angiogenesis supplies oxygen and nutrients to not only cancer cells but also the premetastatic niche, and the primary tumor prepares the microenvironment for distant organs to promote cancer metastasis, angiogenesis and vascular permeability (Liu and Cao, 2016). Tumor angiogenesis is also associated with various types of cells, such as tumor cells, ECs, pericytes, lymphocytes, monocytes/macrophages, fibroblasts, and platelets (De Palma et al., 2017). According to recent studies, EVs secreted by these cells are substantially involved in cancer neovasculature and metastasis. In this review article, we summarize recent findings on the relationship between cancer-related EVs and the TME, tumor-related vasculature, and metastasis.

FUNCTIONAL EVs RELEASED BY TUMORS UNDER HYPOXIA

Solid tumor cells near functional blood vessels, in which the normoxic area has an oxygen level of 38 mmHg (5%) (McKeown, 2014), are viable and proliferative. However, as a result of growing solid tumors at a distance of approximately 200 μm from normal functional vessels, tumor cells are exposed to a hypoxic environment induced by an inadequate oxygen supply due to an increase in diffusion distance (Wilson and Hay, 2011). Hypoxia due to this uncontrollable proliferation of tumor cells results in an oxygen level below 10 mmHg (1.3%) (McKeown, 2014). This hypoxic environment may become anoxic and give rise to cell necrosis. Furthermore, a tumor mass exceeding 1–2 mm in diameter without a vascular supply can prevent cancer cell proliferation (Mao et al., 2019). Genomic changes in tumor cells induced by a hypoxic environment lead to the adaptation of tumor cells to poor nutrition and a poor oxygen supply (Al Tameemi et al., 2019). Tumor cells lead to the formation of new blood vessels that bring nutrients and oxygen to survive and overcome proliferation limitations in a hypoxic microenvironment. Hypoxia is one of the first steps of cancer metastasis, one of the most intensively studied characteristics of the TME (Meng et al., 2019), and is strongly associated with immune evasion, the evasion of apoptosis, tumor growth, angiogenesis, and metastasis (Walsh et al., 2014; Al Tameemi et al., 2019).

Tumor cells in a hypoxic environment produce more EVs than cells in a normoxic environment, and this environment substantially alters the protein and nucleic acid profiles of EVs (King et al., 2012; Wang T. et al., 2014; **Figure 1**). EVs induced by hypoxia may have a strong effect on cancer metastasis and angiogenesis. Tumor-derived EVs and stromal cell-derived EVs under hypoxic conditions can mediate crosstalk between each other (Meng et al., 2019). For example, hypoxia-related EVs increase the expression of miR-210, which promotes tumor progression through EC tubulogenesis (Fasanaro et al., 2008; Jung et al., 2016). In ovarian cancer, hypoxic conditions induce STAT3, which downregulates Rab7 and upregulates Rab27,

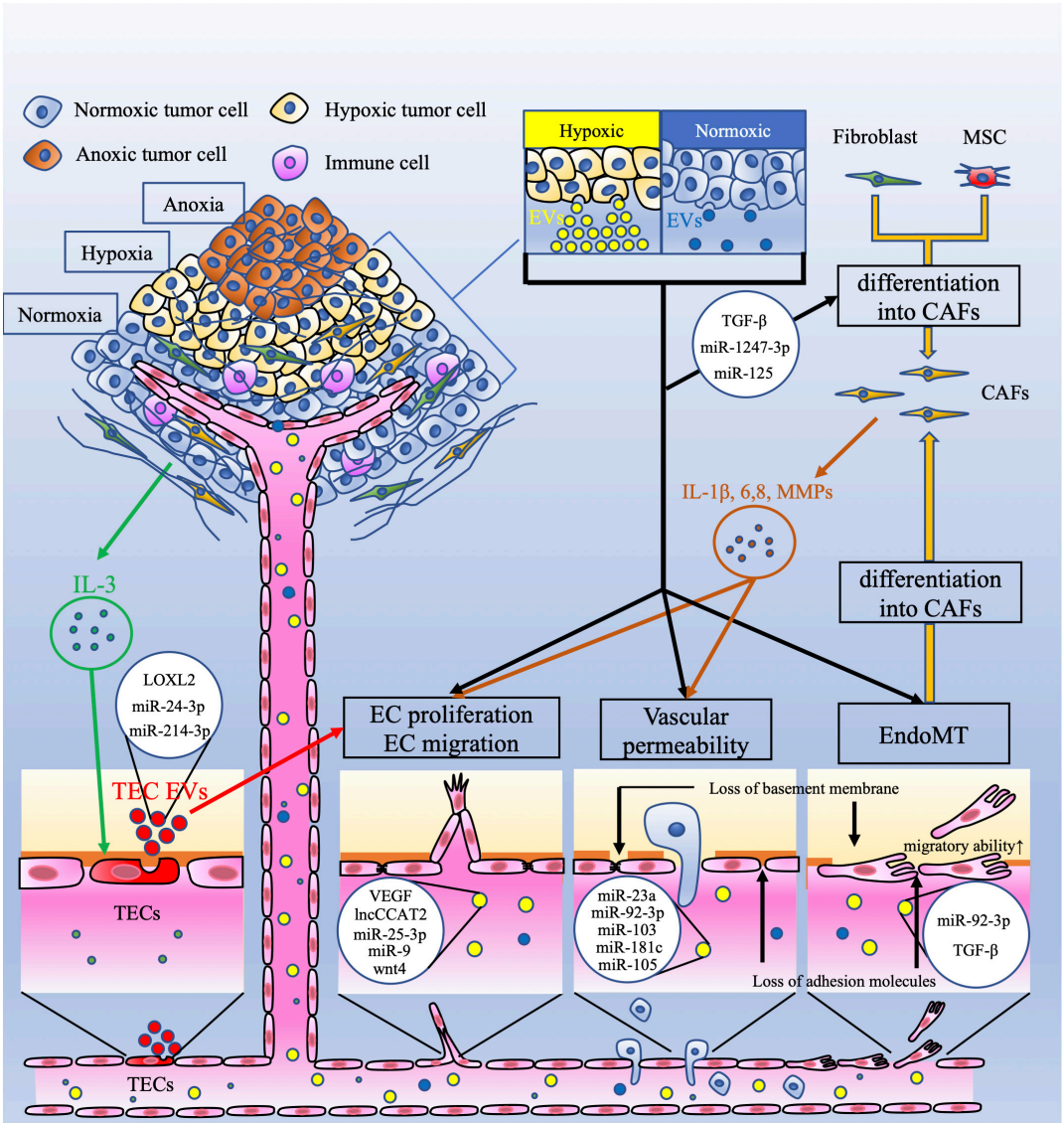


FIGURE 1 | Crosstalk between tumor cells and endothelial cells via extracellular vesicles. This figure shows the vascular-related tumor microenvironment (TME) and extracellular vesicles (EVs). Tumor cells under hypoxia produce more extracellular vesicles (EVs) than those under normoxia. These EVs have several effects on endothelial cells (ECs). EC proliferation and migration are induced by EV-related vascular endothelial growth factor (VEGF), the long non-coding (lnc) RNA CCAT2, miR-25-3p, miR-9, and wnt4. Vascular permeability is increased by EV-related miR-23a, miR-92-3p, miR-103, miR-181c, and miR-105. Interleukin 3 (IL-3), secreted by tumor cells, activates ECs (TECs), which secrete EVs and promote neovessel formation. These EVs contain lysyl oxidase like-2 (LOXL2), miR-24-3p, and miR-214-3p. EVs derived from tumor cells, which contain miR-92-3p and/or TGF-β, downregulate endothelial characteristics and acquire mesenchymal characteristics in ECs; this is referred to as endothelial-mesenchymal transition (EndoMT). EndoMT is also induced by tumor-derived EVs. EndoMT can be the source of cancer-associated fibroblasts (CAFs). Fibroblasts and mesenchymal stem cells (MSCs) can also differentiate into CAFs by tumor-derived EVs. CAFs secrete interleukin-1β (IL-1β), IL-6, IL-8, and matrix metalloproteinases (MMPs). These factors induce EC proliferation and migration and increase vascular permeability. These mechanisms mediated by EVs contribute to tumor angiogenesis and metastasis.

resulting in an increase in EV release (Dorayappan et al., 2018). The EV-associated miR-135b derived from hypoxia-resistant multiple myeloma cells suppresses the expression of factor inhibiting hypoxia-inducible factor 1 (FIH-1), a negative regulator of the transcriptional activity of HIF-1α that promotes endothelial tube formation (Umezu et al., 2014). These studies suggest that tumor cell-derived EVs, which increase under hypoxia, can directly enhance tumor angiogenesis.

Several studies have shown that tumor cells release more EVs under hypoxic conditions than under normoxic conditions in a hypoxia inducible factor (HIF)-dependent manner. HIFs have various effects on cellular functions and control various types of oxygen responsive target genes. HIF-1α induces various angiogenetic growth factors, including vascular endothelial growth factor (VEGF), stromal derived factor 1 (SDF1), angiopoietin 2, placental growth factor, platelet derived growth

factor (PDGF), and stem cell factor (Rey and Semenza, 2010). These factors stimulate ECs, pericytes, and vascular smooth muscle cells and promote the formation of new capillary vessels (Rey and Semenza, 2010).

The release of MVs or EVs is promoted by the HIF pathway. Dimethyloxalylglycine (DMOG), which inhibits HIF hydroxylase, mediates the activation of hypoxic signaling and results in a significant increase in EV release (King et al., 2012). Another study revealed that hypoxia induced breast cancer cells to release more MVs than normoxia. This mechanism is mediated by HIF-dependent RAB22A gene expression, resulting in an increase in MV generation (Wang T. et al., 2014). In nasopharyngeal carcinoma, latent membrane protein 1 (LMP1) is the primary oncogene, as in Epstein-Barr virus (EBV) (Aga et al., 2014). LMP1 promotes the transformation and migration of epithelial cells. Aga et al. (2014) showed that LMP1 also increased the levels of HIF-1 in EVs. HIF-1 recovered from EVs is taken up by recipient cells and mediates the migration and invasion of nasopharyngeal carcinoma cells (Aga et al., 2014).

Hypoxia induced by tumor growth further limits cell proliferation. However, tumor cells in hypoxic areas produce EVs, which have several effects on the stroma. Additionally, EVs derived from hypoxic tumor cells influence important steps in cancer metastasis, such as angiogenesis. The HIF pathway is one of the most important pathways for tumor cells to survive in a hypoxic environment and mediates tumor angiogenesis in the TME. EVs containing HIF-1α can also induce tumor angiogenesis and metastasis.

THE INVOLVEMENT OF EVs DERIVED FROM TUMOR CELLS IN ANGIOGENESIS AND LYMPHANGIOGENESIS IN ENDOTHELIAL CELLS

Endothelial cells play essential roles in microvessel sprouting and angiogenesis associated with the TME and cancer metastasis. Angiogenesis is defined as the formation of new blood vessels from the existing vasculature to supply nutrients and oxygen to hypoxic organs (Maishi and Hida, 2017). VEGF is one of the key regulators of angiogenesis and promotes EC proliferation and migration and decreases EC apoptosis (Apte et al., 2019). The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PGF (Ferrara and Adamis, 2016). VEGF-A plays an important role in regulating angiogenesis during homeostasis and under disease conditions (Ferrara and Adamis, 2016). In a hypoxic TME, tumor cells and stromal cells secrete VEGF, leading to the survival of ECs and the formation of new blood vessels that may be structurally abnormal and leaky (Apte et al., 2019). It has been reported that tumor- and/or stroma-derived EVs promote angiogenesis related to VEGF. For example, VEGF on the surface of EVs derived from cancer cells promotes tumor angiogenesis (Ko et al., 2019). In glioma cells, EVs derived from U83-MG upregulate VEGF-A in HUVECs (Lang et al., 2017). These EVs contain the long non-coding (lnc) RNA-CCAT2, which is overexpressed in glioma tissues (Guo et al., 2016). The authors showed that EV-delivered lncRNA CCAT2 promoted angiogenesis by increasing the expression of VEGF-A in HUVECs (Lang et al., 2017). Colorectal cancer cell-derived EVs containing miR-25-3p target Krüppel-like factor 2 and regulate the expression of VEGF receptor 2, a VEGF receptor and the main signaling receptor for VEGF (Ferrara and Adamis, 2016); this results in vascular angiogenesis (Zeng et al., 2018). Thus, it has been well established that cancer cell-derived EVs directly transport VEGF or upregulate the VEGF pathway in ECs, resulting in tumor angiogenesis. In contrast, several reports have shown that various cancer cell-derived EVs regulate angiogenesis, EC proliferation and migration without stimulating VEGF expression. For example, EVs derived from breast cancer cells containing miR-9 effectively decreased suppressor of cytokine signaling 5 (SOCS5) expression in HUVECs. This decrease in SOCS5 led to EC migration by activating the JAK-STAT pathway (Zhuang et al., 2012). EV-delivered Wnt4 derived from colorectal cancer promotes angiogenesis through the Wnt/β-catenin signaling pathway in endothelial cells (Huang and Feng, 2017). Various types of tumor cells release EVs involved in the regulation of EC proliferation and migration through the VEGF- or non-VEGF-mediated pathway. The effects of tumor-derived EVs on ECs are summarized in **Table 1**.

Tumor cells promote the loosening of intercellular adhesions between ECs by impairing adhesion molecules and gap junctions. Promotion of vascular permeability is related to enhanced cancer growth and metastasis by supplying enough nutrients and oxygen to growing tumors and promoting intravasation and extravasation. Accumulating evidence has revealed that tumor-associated EVs can modify vascular permeability (**Figure 1**). miR-23a in EVs is secreted by hypoxic lung cancer cells and suppresses prolyl hydroxylase (PHD) 1 and 2. PHD-1 and PHD-2 are regulators of HIF-1α (Berra et al., 2003). The suppression of PHD-1 and PHD-2 leads to the accumulation of HIF-1α in HUVECs (Hsu et al., 2017). miR-23a also downregulates ZO-1 expression in HUVECs and is related to tight junctions in the endothelium (Hsu et al., 2017). In addition, EV-delivered miR-92a-3p derived from colorectal cancer cells downregulates Claudin-11 (Yamada et al., 2019). Claudin-11 belongs to the claudin gene family and is one of the cell-to-cell adhesion molecules located at the tight junctions between ECs (Tsukita et al., 2019). VE-cadherin and p120-catenin are also adhesion molecules that contribute to endothelial junctions. Hepatoma cell-secreted EVs containing miRNA-103 impair endothelial junctions by directly inhibiting the expression of VE-cadherin, p120-catenin and ZO-1 (Choi and Moon, 2018). Thus, tumor-derived EVs influence vascular permeability and promote metastasis from the primary tumor.

An increase in vascular permeability in distant organs also promotes the extravasation of cancer cells and formation of the premetastatic niche. EV-delivered miR-181c from breast cancer cells downregulates 3-phosphoinositide-dependent protein kinase-1 (PDPK1) and disrupts the blood-brain barrier and consists of tight junctions between ECs and surrounding cells that contribute to low permeability and promote cancer

metastasis in distant organs (Tominaga et al., 2015b). Another study showed that EV-delivered miR-105, which is a key regulator of ZO-1 and secreted by metastatic breast cancer cells, downregulated and destroyed tight junctions in endothelial monolayers. The same study also showed that treatment with anti-miR-105 suppressed brain metastasis *in vivo* (Zhou et al., 2014). ECs essentially have the ability of a physical barrier to separate blood from tissue and prevent tumor cell intravasation and extravasation. Tumor-derived EVs can lead to a disruption of adhesion molecules in ECs and promote not only cancer growth at the primary tumor but also the extravasation of tumor cells and formation of the premetastatic niche at distant organs. Tumor cells modify the structure of ECs to lose their physical barrier, resulting in tumor metastasis.

Tumor-derived EVs can also enhance tumor lymphangiogenesis and lymph node (LN) metastasis. It has been reported that different types of human cancer promote lymphangiogenesis, which is correlated with LN metastasis, and the presence of LN metastasis often reflects a poor prognosis (Karaman and Detmar, 2014). VEGF-C is a member of the VEGF family of angiogenic factors and is characterized as a lymphangiogenic growth factor (Su et al., 2007). Several studies have shown that VEGF-C promotes tumor-associated lymphangiogenesis and LN metastasis (Mandriota et al., 2001; Skobe et al., 2001). VEGFR-2 and VEGFR-3 are receptors for VEGF-C and VEGF-D. VEGFR-2 and VEGFR-3 also play important roles in the survival, proliferation and migration of lymphatic endothelial cells (LECs) (Joukov et al., 1996). There is no evidence that tumor-related EVs regulate lymphangiogenesis through the VEGF-C pathway. However, Fu et al. (2015) reported that miR-221-5p promoted the expression of VEGF-C in bladder cancer cells. Yang et al. (2017) reported that tumor-derived EVs contained miR-221, which contributed to tumor progression in glioma. miR-221-5p in tumor-derived EVs may induce lymphangiogenesis via the VEGF-C pathway.

Bladder cancer is one of the most commonly diagnosed urological malignancies (Bray et al., 2018). LN metastasis in patients with bladder cancer is related to a poor prognosis (Hautmann et al., 2012). Although VEGF-C plays an important role in lymphangiogenesis (Tacconi et al., 2015), LN metastasis in 20% of bladder cancers in a VEGF-C-independent manner (Chen et al., 2020). Chen et al. (2020) suggested that EV-related lymph node metastasis associated transcript 2 (LNMAT2) induced human LEC tube formation and migration *in vitro* and enhanced tumor lymphangiogenesis and LN metastasis *in vivo* through the upregulation of prospero homeobox 1 (PROX1). Another study showed that cervical squamous cell carcinoma-derived EVs promoted LEC migration and tube formation *in vitro* and facilitated lymphangiogenesis and LN metastasis *in vivo* (Zhou et al., 2019). These EVs contained miR-221-3p, which downregulated vasohibin-1 expression in LECs. This miR-221-3p-vasohibin-1 axis activated the AKT/ERK signaling pathway, resulting in lymphangiogenesis without VEGF-C (Zhou et al., 2019). These mechanisms may explain why tumor-derived EVs induce tumor lymphagenesis and LN metastasis in a VEGF-C-independent manner. There is little evidence of tumor- or stromal cell-derived EVs associated with lymphangiogenesis and LN metastasis.

TABLE 1 | The effect of tumor-derived EVs on ECs.

Function	Cargo mediators	Origination	Mechanism	References
ECs migration and/or proliferation	VEGF	Several cancer cells	Upregulate VEGF pathway	Ko et al., 2019
	lncCCAT2	Glioma	Upregulate VEGF-A	Lang et al., 2017
	miR-25-3p	Colorectal cancer	Downregulate KLF2 leading to VEGFR2 upregulation	Zeng et al., 2018
	miR-9	Breast cancer	Downregulation SOCS5 leading to activated JAK-STAT pathway	Zhuang et al., 2012
	wnt4	Colorectal cancer	Increase β-catenin nuclear translocation leading to activated wnt/β-catenin pathway	Huang and Feng, 2017
Vascular permeability	miR-23a	Lung cancer	Suppress PHD-1,2 leading to accumulation of HIF-1α and downregulate ZO-1	Hsu et al., 2017
	miR-92-3p	Colorectal cancer	Downregulate Claudin 11	Yamada et al., 2019
	miR-103	HCC	Downregulate VE-cadherin, p120-catenin, and ZO-1	Choi and Moon, 2018
	miR-181c	Breast cancer	Downregulate PDPK1 leading to phosphorylated cofilin	Tominaga et al., 2015b
	miR-105	Breast cancer	Downregulate ZO-1	Zhou et al., 2014
lymphangiogenesis	LNMAT2	Bladder cancer	Upregulate PROX1	Chen et al., 2020
	miR-221-3p	Cervical cancer	Downregulate vasohibin-1 leading to activated ERK/AKT pathway	Zhou et al., 2019
EndoMT	miR-92-3p	Colorectal cancer	Upregulate snail and vimentin and downregulate Claudin 11	Yamada et al., 2019
	TGF-β	Melanoma	Induce TGF-β signaling	Yeon et al., 2018
ECM remodeling	mRNA MMP-2, 9	Renal cell carcinoma	Upregulate MMP-2, 9	Grange et al., 2011

EC, endothelial cells; VEGF, vascular endothelial growth factor; lnc, long non-coding; KLF2, Krüppel-like factor; VEGFR2, vascular endothelial growth factor receptor 2; SOCS5; suppressor of cytokine signaling 5; PHD, prolyl hydroxylase; HIF, hypoxia-inducible factor; PDPK, 3-phosphoinositide-dependent protein kinase-1; LNMAT2, lymph node metastasis-associated transcript 2; PROX1, prospero homeobox 1; EndoMT, endothelial mesenchymal transition; TGF-β, transforming growth factor-β; ECM, extracellular matrix; MMP, matrix metalloproteinase.

Tumor cells may regulate lymphangiogenesis through the EV-mediated VEGF-C or non-VEGF-C pathway. If tumor lymphangiogenesis-mediated tumor-related EVs are elucidated, we can predict LN metastasis in various tumor cell types by sampling various biofluids and prevent LN metastasis by inhibiting lymphangiogenesis-mediated EV secretion.

THE CONTRIBUTIONS OF EVs DERIVED FROM TUMOR-ASSOCIATED ENDOTHELIAL CELLS TO ANGIOGENESIS

Numerous studies have shown that tumor-derived EVs influence ECs to promote vascular permeability, angiogenesis and metastasis. On the other hand, ECs and tumor-associated ECs (TECs) also secrete EVs, and several studies have suggested that these EVs contribute to angiogenesis and metastasis. ECs in a hypoxic environment secrete EVs, which include lysyl oxidase-like 2 (LOXL2) (de Jong et al., 2016). LOXL2 is a member of the LOX family and regulates extracellular matrix (ECM) remodeling, angiogenesis, and premetastatic niche formation. EV-associated LOXL2 derived from TECs in a hypoxic environment induces angiogenesis. Another study showed that breast and ovarian cancer tissue released IL-3, which influenced ECs in the TME. IL-3-treated ECs secrete EVs, serving as a paracrine mechanism for neighboring ECs (Lombardo et al., 2018). When TECs were treated with an anti-IL-3Rα blocking antibody, EV-delivered miR-214-3p was upregulated, and miR-24-3p was downregulated. These miRNAs regulate neovessel formation through the Wnt/β-catenin pathway (**Table 2**). Anti-IL-3R antibody-treated EVs derived from TECs regressed tumor-treated EC-induced neovessels *in vivo* (Lombardo et al., 2018). In addition, TECs also contribute to immune suppression via EVs. Lopatina et al. (2020) showed that EVs collected from ECs associated to head and neck squamous cell carcinoma were able to increase IL-6 and TGF-β in peripheral blood mononuclear cells (PBMC) and increase the T regulatory cell population. TEC-EVs also increased the expression of MALAT1 in PBMC resulting in M2-type differentiation of macrophages. These studies indicate that EVs derived from ECs in the TME carry several factors, resulting in tumor angiogenesis and immune suppression (**Figure 1**).

Data from several clinical studies have suggested that EV-derived ECs are involved in cancer prognosis. In patients with lung cancer, circulating levels of endothelial-derived EVs, especially MVs, are independent predictors of mortality (Wang C.C et al., 2014). In breast cancer patients treated by chemotherapy, the decrease in endothelial-derived EV (defined as CD144-positive MV) levels after chemotherapy is associated with prolonged overall and disease-free survival (García Garre et al., 2018). Although tumor cells can educate ECs (TECs) and promote angiogenesis in neighboring ECs, the mechanism by which TEC-associated EVs contribute to tumor angiogenesis and metastasis remains unclear. However, these previous studies indicate that crosstalk between tumor cells and ECs via EVs mediates tumor progression and angiogenesis.

TUMOR-DERIVED EVs PROMOTE ENDOTHELIAL TO MESENCHYMAL TRANSITION (EndoMT)

Epithelial cells in tumors can change their phenotype, downregulate epithelial characteristics and acquire mesenchymal characteristics (Lamouille et al., 2014). This cellular program is called epithelial-mesenchymal transition (EMT). EMT is an important step in the malignant progression of almost all types of carcinoma (Dongre and Weinberg, 2019). When EMT occurs, cell-to-cell tight junctions and adhesive molecules in epithelial cells are lost, and cancer cells acquire migration ability (Quintero-Fabián et al., 2019).

Endothelial cells in the TME can also modify their endothelial phenotype switch to the mesenchymal profile in a manner similar to that of epithelial cells. This phenotype switch in ECs is referred to as endothelial-to-mesenchymal transition (EndoMT) (Platel et al., 2019). Recently, EndoMT has been described as an important process in the development and dissemination of cancer. In a way similar to that during EMT, ECs induced to switch to the mesenchymal phenotype lose capillaries and endothelial markers such as CD31, Tie-2 and VE-cadherin, acquire mesenchymal markers such as N-cadherin, fibroblast-specific protein-1, vimentin, α-SMA, and type I/III collagen and become more invasive, with increased migratory abilities (Nie et al., 2014; Platel et al., 2019). Although various molecules, such as interleukin-1β (IL-1β) and TNF-α, can promote EndoMT, transforming growth factor-β (TGF-β) plays a major role in EC conversion during EndoMT (Medici et al., 2011; Kovacic et al., 2012; Sanchez-Duffhues et al., 2018). TGF-β stimulates the Smad signaling pathway, which regulates the differentiation transcription factors Snail, Twist, Slug, and ZEB (Miyazono, 2000; Dongre and Weinberg, 2019). These transcriptional factors inhibit the expression of genes related to the epithelial state and promote the expression of genes related to the mesenchymal state (Dongre and Weinberg, 2019). In addition, Snail and ZEB2 activate the expression of matrix metalloproteinase (MMP), which promotes degradation of the basement membrane, causing vascular invasion in the primary tumor (Miyoshi et al., 2004, 2005). EndoMT functions to promote the extravasation of tumor cells by increasing endothelial vascular permeability and causing tumor metastasis. A recent study showed that EVs containing miR-92a-3p derived from colon cancer cells upregulated mesenchymal markers such as snail and vimentin and downregulated the tight junction marker ZO-1 in HUVECs. This biological process is referred to as "partial-EndoMT" (Yamada et al., 2019). This process promotes angiogenesis by increasing proliferation, motility, and tube formation in HUVECs. In addition, TGF-β on the surface of melanoma-derived EVs trigger the phenotypic

switch from an endothelial phenotype to a mesenchymal phenotype concerning gene expression (Yeon et al., 2018). These phenomena transdifferentiate ECs into cancer-associated fibroblasts (CAFs) (Togo et al., 2013). CAFs are the most abundant cells in the TME, secrete several proangiogenic signaling factors and play an important role in metastasis and angiogenesis (Wang et al., 2019).

The phenotypic switch in ECs via tumor-related EVs is thought to contribute to the intravasation and extravasation of tumor cells via an impairment in vascular permeability and becomes a resource for CAFs (**Figure 1**). EndoMT also promotes the proliferation and migration of ECs (i.e., mesenchymal abilities), resulting in tumor angiogenesis. These findings suggest that ECs that undergo the phenotypic switching migrate to distant organs via tumor-derived EVs and induce tumor angiogenesis in the premetastatic niche as well as in the primary tumor.

THE FUNCTION OF EVs ASSOCIATED WITH CANCER-ASSOCIATED FIBROBLASTS AND TUMOR CELLS IN TUMOR ANGIOGENESIS

Cancer-associated fibroblasts are the main component of stromal cells in the TME. They originate from various types of cells, such as epithelial cells, resident fibroblasts, bone marrow derived from mesenchymal stem cells, hematopoietic stem cells, and ECs (Quante et al., 2011; Mitra et al., 2012). Myofibroblasts are one of the main subtypes of CAFs that express α-smooth muscle actin (α-SMA) (Sappino et al., 1988; Kraman et al., 2010). Because there are various subtypes of CAFs, it is not clear what markers are specific for CAFs. However, α-SMA is currently the most commonly used specific marker for CAFs. CAFs have several important functions in tumor angiogenesis. They secrete various proinflammatory cytokines that promote angiogenesis and vasculogenesis, such as IL-1β, IL-6, and IL-8, and recruit inflammatory cells, such as macrophages (Erez et al., 2010). MMPs secreted by CAFs degrade the extracellular matrix and basal membrane and lead to tumor angiogenesis (Erez et al., 2010). Several proangiogenic signaling factors derived from CAFs, such as VEGF, TGF-β, hepatocyte growth factor (HGF), and SDF-1, also promote angiogenesis and vasculogenesis (Orimo et al., 2005; Chowdhury et al., 2015). PDGF-β and basic fibroblast growth factor (bFGF) can be secreted by cancer cells to activate CAFs indirectly to promote tumor angiogenesis (Shao et al., 2000; Strutz et al., 2000).

Evidence of the mechanism by which stromal cells are activated by tumor cells via EVs is gradually accumulating (**Table 2**). According to a recent study, a highly metastatic type of diffuse-type gastric cancer cell-derived EVs contain

TABLE 2 | The function of tumor cells and stromal cells-derived EVs.

Function	Cargo mediators	Origination	Target	Mechanism	References
ECs migration and/or proliferation	miR-24-3p miR-214-3p	TECs	ECs	Induce wnt/β-catenin pathway	Lombardo et al., 2018
Differentiation into CAFs	several miRNAs	Gastric cancer	Fibroblasts	Induce CAFs to secrete CXCL1, 8	Naito et al., 2019
	TGF-β	Prostate cancer	MSC	Upregulate α-SMA in MSC	Chowdhury et al., 2015
	TGF-β	Bladder cancer	Fibroblasts	Induce Smad pathway in fbroblasts	Ringuette Goulet et al., 2018
	miR-125	Breast cancer	Fibroblasts	Upregulate α-SMA in fibroblasts	Vu et al., 2019
	miR-1247-3p	HCC	Fibroblasts	Upregulate B4GALT3 leading to activated β1-integrin-NF-κB pathway in fibroblasts	Fang et al., 2018
ECM remodeling	LOXL2	EC	none	Directly ECM remodeling	de Jong et al., 2016
	mRNA MMP-1	Ovarian cancer	mesothelial cells	Upregulate MMP-1 in mesothelial cells	Yokoi et al., 2017
	mRNA MMP-9	Platelets	ECs	Upregulate MMP-9 in ECs	Janowska-Wieczorek et al., 2005
	ADAM 10	CAFs	several cancer cells	Induce RhoA-Notch signaling	Shimoda et al., 2014
	miR-139	CAFs	Gastric cancer	Suppress MMP-11 in gastric cancer	Xu et al., 2019
	Unknown	CAFs	Gastric cancer	Upregulate MMP-2 in gastric cancer	Miki et al., 2018
Coagulability	TF	Ovarian cancer		Hypercoagulability	Koizume et al., 2016
	TF	Platelets		Hypercoagulability	Freyssinet and Toti, 2010
VM formation	VEGF	TECs	HCC	Upregulate VEGF	Zeng et al., 2019

EC, endothelial cell; TEC, tumor associated endothelial cell; CAF, cancer associated fibroblast; CXCL, chemokine (C-X-C motif) ligand; TGF-β, transforming growth factor-β; SMA, smooth muscle actin; MSC, mesenchymal stem cell; HCC, hepatocellular carcinoma; B4GALT3, β-1,4-galactosyltransferases III; ECM, extracellular matrix; LOXL2, lysyl oxidase-like 2; MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase; TF, tissue factor; VM, vasculogenic mimicry; VEGF, vascular endothelial growth factor.

several miRNAs (Naito et al., 2019). CAFs take up these EVs, which effect CAFs, to secrete chemokine (C-X-C motif) ligand (CXCL) 1 and CXCL8 via miR-155, miR-193b, and miR-210. Another study suggested that cancer cell-derived EVs promote the phenotypic switch of cancer stromal cells into CAFs. For example, EVs containing TGF-β secreted by prostate cancer cells induced mesenchymal stem cell differentiation into CAFs that exhibited α-SMA-positive myofibroblast cells (Chowdhury et al., 2015). In addition, bladder cancer cell-derived EVs contain TGF-β. These EVs induced normal fibroblasts into CAFs through the Smad pathway (Ringuette Goulet et al., 2018). Moreover, EVs derived from breast cancer cells are taken up by cancer stromal cells at a high rate. These EVs contain miR-125, which is transferred to normal fibroblasts and upregulates α-SMA (Vu et al., 2019). EndoMT can also be the source of CAFs via cancer cell-derived EVs, as mentioned above. In primary tumors, tumor-derived EVs promote the conversion of several types of cells into CAFs, and CAFs secrete various types of proangiogenic factors, resulting in tumor angiogenesis.

Cancer cell-derived EVs contribute to the conversion of fibroblasts into CAFs in the premetastatic niche. In HCC, miR-1247-3p is carried to the premetastatic niche in the lung through EVs and converts fibroblasts to CAFs by decreasing the expression of its target, β-1,4-galactosyltransferase III (B4GALT3), to activate the β1-integrin-NF-κB pathway (Fang et al., 2018). Integrins promote the nuclear translocation of NF-κB, and the NF-κB signaling pathway promotes the growth and migration of epithelial cells (Nikolopoulos et al., 2005). B4GALT3 also inhibits β1-integrin activation and stability (Liao et al., 2015). Serum EVs containing miR-1247-3p are elevated in HCC patients with lung metastasis compared with healthy individuals or HCC patients without lung metastasis (Fang et al., 2018). The NF-κB pathway mediates various proangiogenic factors, such as IL-1, IL-8, tumor necrosis factor, IL-6, VEGF, MMP-2, and MMP-9 (Maeda and Omata, 2008). This study indicates that cancer cell-derived EVs induce angiogenesis in the premetastatic niche through the NF-κ B pathway.

Not only cancer cells but also CAFs secrete EVs, which may stimulate cancer metastasis and angiogenesis. Miki et al. (2018) revealed that CD9-positive EVs from CAFs were taken into scirrhous-type gastric cancer cells. MMP-2 expression in scirrhous-type gastric cancer cells was significantly decreased by CD9-siRNA. CD9 positivity was significantly related to LN metastasis and venous invasion. MMP-2 is well known as a modulator of dynamic remodeling of the ECM (Quintero-Fabián et al., 2019). MMP-2 correlates with neocapillary network growth, HUVEC migration and angiogenesis. CAF-derived EVs may induce angiogenesis and lymphangiogenesis through MMP-2. Crosstalk between cancer cells and CAFs plays an important role in angiogenesis. Recent studies have revealed that cancer cell-derived EVs might be associated with converting stromal cells into CAFs, which upregulate angiogenesis in primary tumors and distant organs. Evidence of the mechanisms of interaction between tumor cells and CAFs via EVs has accumulated. CAFs constitute the majority of cells in the TME and are thought to secrete various angiogenic factors, including EVs.

THE ROLES OF MATRIX REMODELING AND ANGIOGENESIS ASSOCIATED WITH EVs

The ECM consists of collagen, proteoglycans, elastin, fibronectin and integrins that provide structural and biochemical support for cancer cell growth (De Palma et al., 2017; Nawaz et al., 2018). The ECM is versatile and continues to be remodeled. Tumor cells, CAFs, ECs and other stromal cells are associated with matrix remodeling in the primary tumor microenvironment (Shuman Moss et al., 2012; Eble and Niland, 2019). Tumor cells invade the ECM as a barrier and result in cancer cell migration. Moreover, they remodel the ECM in distant organs to facilitate formation of the premetastatic niche. Angiogenesis also requires a disruption in endothelial-lined vessels through the sprouting of ECs (Quintero-Fabián et al., 2019). Sprouting angiogenesis is related to the degradation of intercellular relationships, cell-matrix adhesion molecules, the ECM, and basement membrane components by MMPs, plasmin and other proteases (De Palma et al., 2017; Quintero-Fabián et al., 2019). Thus, it is important to understand ECM remodeling in the TME to elucidate cancer metastasis and angiogenesis.

Recently, relevant studies in which tumor-derived EVs containing MMPs were associated with angiogenesis have gradually accumulated (**Table 2**). The involvement of MMPs in tumor angiogenesis is highly important and well known. During the angiogenesis process, ECs secrete MMPs to remodel the basement membrane (De Palma et al., 2017). MMPs are a family of 23 zinc-dependent endopeptidases that degrade ECM components and the basement membrane and are related to tumor cell migration into adjacent tissue. Not only ECs but also several types of cells, such as cancer cells, CAFs, and platelets, secrete EVs containing MMPs and result in angiogenesis. For example, EVs derived from CD105-positive cells in renal cell carcinoma trigger angiogenesis and promote formation of the premetastatic niche (Grange et al., 2011). These EVs contain several mRNAs, such as VEGF, FGF, MMP-2, and MMP-9. In addition, EVs derived from highly metastatic ovarian cancer cells contain MMP-1 mRNA, resulting in destruction of the peritoneal mesothelium barrier (Yokoi et al., 2017). Platelets activated by tumor cells also secrete MVs that stimulate the mRNA expression of MMP-9, VEGF, and IL-8 in HUVECs (Janowska-Wieczorek et al., 2005). CAFs secrete ADAM10-rich EVs, which promote the RhoA and Notch signaling pathways in cancer cells, and play an essential role in enhancing cell motility (Shimoda et al., 2014). Based on the findings described above, cancer cells and cancer stromal cells can create favorable conditions for growth through ECM remodeling and promote tumor angiogenesis (**Figure 2A**).

However, cancer stromal cells may suppress cancer progression. Xu et al. (2019) suggested that EVs containing miR-139 derived from CAFs in gastric cancer suppressed MMP-11 secretion via EVs derived from CAFs. They also showed that miR-139 in EVs inhibited tumor growth and metastasis *in vitro*

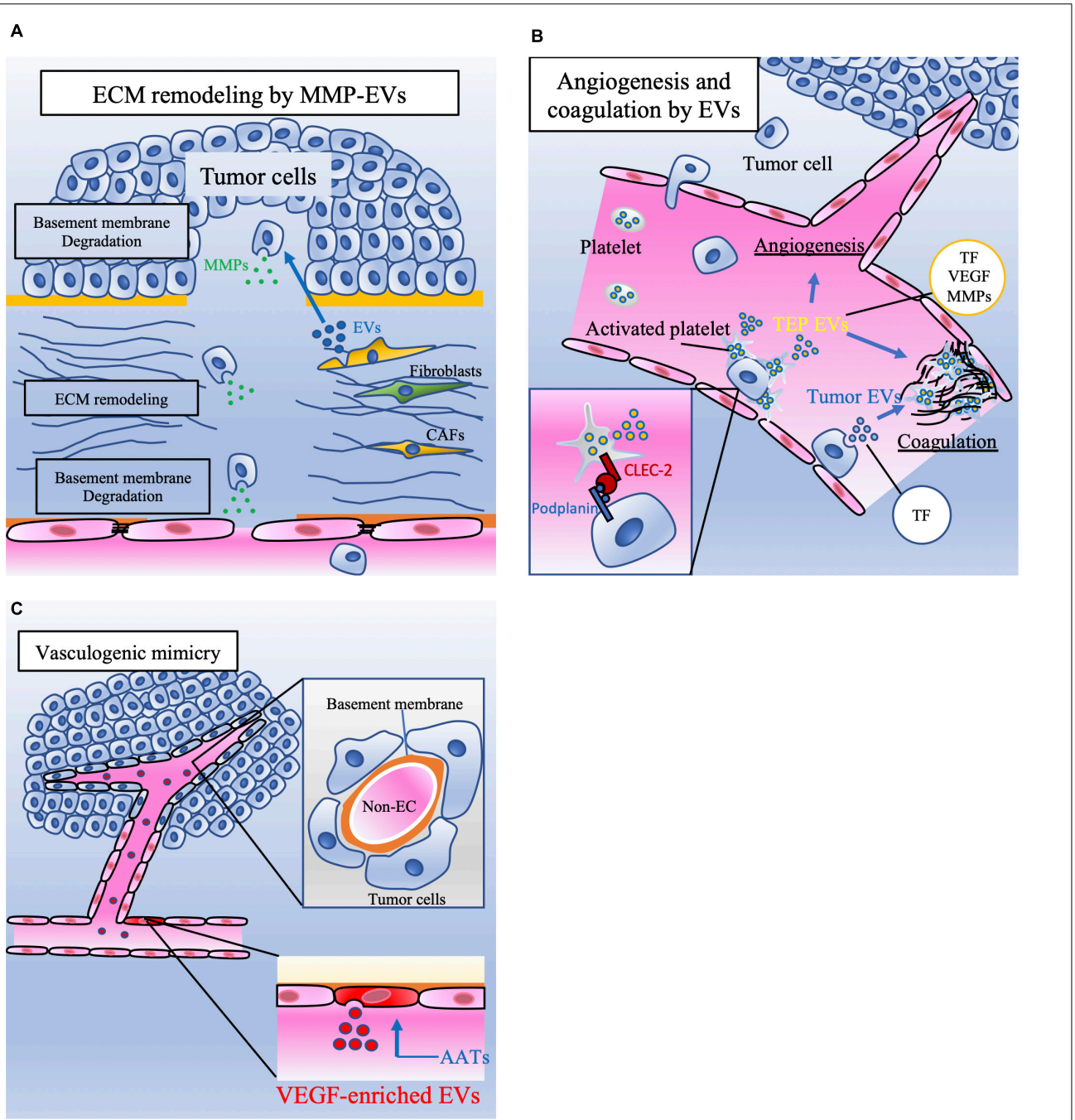


FIGURE 2 | The involvement of EVs released by tumor cells and stromal cells in tumor metastasis. **(A)** Extracellular matrix (ECM) remodeling is mediated by tumor cells and cancer-associated fibroblasts (CAFs). Extracellular vesicles (EVs) contain matrix metalloproteinases (MMPs), which degrade the basement membrane. ECM remodeling induces tumor cell migration and vascular sprouting, resulting in tumor progression. **(B)** Platelet microparticles (PMPs) are the most abundant EVs in the blood and are secreted by activated platelets. Platelets can be activated by tumor cells. Podoplanin is expressed on the surface of tumor cells, and C-type lectin-like receptor 2 (CLEC-2) is expressed on the surface of platelets. The interaction between CLEC-2 and podoplanin activates platelets referred to as tumor-educated platelets (TEPs). TEPs play important roles in angiogenesis and coagulation through the secretion of these EVs or PMPs containing tissue factor (TF), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs). Tumor cells also directly secrete procoagulant EVs that contain TF. TF activates plasmatic coagulation cascades and induces platelet activation. **(C)** Although anti-angiogenic targeted therapy (AAT) is a key therapy for malignant tumors, the effect of AATs is transient. One of the mechanisms of AAT resistance is vasculogenic mimicry (VM). VM consists of tumor cells that have capillary-like structures and basement membranes but not endothelial cells. ECs treated with AATs secrete vascular endothelial growth factor (VEGF)-enriched EVs. These EVs enhance VM, which supplies nutrients and oxygen to tumor cells and contributes to tumor progression.

and *in vivo* (Xu et al., 2019). CAFs may contribute to cancer progression and/or suppression through EVs, but the exact mechanism is unclear.

THE LINK BETWEEN CANCER CELLS AND PLATELETS IN EVs

Platelets contribute to pathological conditions such as inflammation, coagulation and tumor progression. The expression of podoplanin on cancer cells is one of the representative mechanisms of platelet activation by cancer cells (Haemmerle et al., 2018). C-type lectin-like receptor 2 (CLEC-2) on the surface of platelets can bind to podoplanin. After an interaction between CLEC-2 and podoplanin activates platelets, activated platelets promote hypercoagulation, inflammation, tumor growth, and metastasis (Takagi et al., 2013). Activated platelets, which are referred to as tumor-educated platelets (TEPs), were discovered in the 19th century (In 't Veld and Wurdinger, 2019). Platelet microparticles (PMPs) are a predominant population of not only platelet-derived EVs but also all EVs in the blood, accounting for approximately 70–90% (Zmigrodzka et al., 2016). PMPs secreted by activated platelets enhance angiogenesis, vascular permeability, and coagulation (Wojtukiewicz et al., 2017; Haemmerle et al., 2018). In the 1860s, Armand Trousseau observed that an increase in venous thrombosis and/or blood hypercoagulability was associated with certain cancers; this is now referred to as "Trousseau's syndrome" (Trousseau, 1865). Trousseau's syndrome causes venous thromboembolism (VTE), which includes deep venous thrombosis and pulmonary embolism (Campello et al., 2011). PMPs are involved in coagulation as a source of tissue factor (TF) and phosphatidylserine (Freyssinet and Toti, 2010). High PMP levels are associated with aggressive tumors and thrombotic events. It has been reported that EVs containing TF from aggressive breast cancer cell lines exhibit more platelet aggregation activity than those from less invasive breast cancer cell lines and are associated with an increased risk of thrombosis (Gomes et al., 2017), and the procoagulant activity is of PMPs in cancer patients with VTE is high at baseline, suggesting that this activity can be used to predict VTE (van Doormaal et al., 2012). Not only platelets but also tumor cells secrete EVs and have procoagulant ability. Koizume et al. (2016) showed that ovarian clear-cell carcinoma (CCC), a subtype of ovarian cancer, secreted higher levels of TF within EVs than breast cancer. These EVs contributed to the increased plasma coagulability of CCC tumor-bearing mice. A hypoxic environment enhances CCC to secrete EVs enriched with TF. Although TEPs play an important role in tumor hypercoagulability, tumor cells also directly secrete procoagulant EVs and promote VTE. TF activates plasmatic coagulation cascades and induces platelet activation (Han et al., 2014). Platelet activation by tumor cells induces tumor invasion, immune evasion, vascular arrest, and extravasation (Schlesinger, 2018). Thus, hypercoagulability induced by TEPs and tumor cells contributes to tumor progression.

Several studies have suggested that PMPs are associated with not only VTE but also cancer angiogenesis and patient prognosis (**Figure 2B**). PMPs in lung cancer cells can induce the mRNA expression of MMP-9, VEGF, IL-8, and HGF in HUVECs (Janowska-Wieczorek et al., 2005), mediate angiogenic functions by VEGF, PDGF, TGF-β, and bFGF (Haemmerle et al., 2018), and stimulate the proliferation, chemotaxis and tube formation of HUVECs (Kim et al., 2004). In addition, these contents of PMPs are predictors of cancer metastasis in gastric cancer (Kim et al., 2003). In patients with prostate cancer, a significant relationship between high levels of PMPs and poor survival rates was found (Helley et al., 2009). TEPs are thought to promote angiogenesis and be associated with the prognosis of patients with cancer. Therefore, tumor cells utilize platelets to create a beneficial microenvironment for themselves. TEPs contribute to several important steps in tumor metastasis. Although antiplatelet therapy can be used as anticancer therapy, there is no reliable clinical evidence for antiplatelet therapy for tumor metastasis (Xu et al., 2018). Clinical evidence, such as that from a large randomized control trial, is needed before antiplatelet therapy can be translated into anticancer therapy.

THE MECHANISM OF OVERCOMING ANTI-ANGIOGENIC TARGETED THERAPY: VASCULOGENIC MIMICRY

Anti-angiogenic targeted therapy (AAT) is a key therapy for many tumor types and a well-established research area (Reck et al., 2018). AATs help starve tumors by suppressing tumor angiogenesis and destroying preexisting tumor-associated blood vessels (Zeng et al., 2019). As mentioned above, VEGF and its receptors play an important role in EC survival and tumor angiogenesis. Bevacizumab is one of the first commercially used AATs and a humanized monoclonal antibody against VEGF-165 (Pinto et al., 2016). Currently, several AAT drugs can be used and have been well established. However, the effect of AATs is transient. Tumor cells can escape angiogenesis inhibition through different forms of neovasculature and acquire resistance to AATs (Pinto et al., 2016). These processes may result in limited outcomes in patients with aggressive tumors and lead to cancer-related death.

One of the mechanisms by which AATs function is vascular mimicry (VM) (Maniotis et al., 1999; Folberg et al., 2000). In 1999, it was described that the formation of tubular structures was consistent not with that of ECs but of cancer cells within melanomas (Valdivia et al., 2019). Thereafter, the concept of VM has been reported in various types of solid tumors until now and was significantly associated with cancer differentiation, LN metastasis (Sun B. et al., 2017; Hujanen et al., 2020), distant organ metastasis, and poor prognosis (Yang et al., 2016). VM is described as tumor cells acquiring the endothelial-like capillary form and providing oxygen and nutrients to hypoxic tumor cells (Maniotis et al., 1999; Folberg et al., 2000). The basal membrane consists not only of blood and lymph vessels but also a variety of glycoproteins that are stained by periodic acid-Schiff (PAS) (Folberg and Maniotis, 2004). However, one characteristic of VM is the absence of CD31 and CD34, which are defined as endothelial markers (Seftor et al., 2001). VM is defined as PAS

positive and CD31 or CD34 negative (Angara et al., 2017; Valdivia et al., 2019; Hujanen et al., 2020).

Vascular mimicry can be induced by a hypoxic environment. Several studies have suggested that HIF-1α is involved in VM (Serova et al., 2016). The expression of HIF-1α, MMP-2, VE-cadherin, and Twist-1 in triple-negative breast cancer cells is higher than that in non-triple-negative breast cancer cells (Sun H. et al., 2017). HIF-1α activation is correlated with EBV-associated epithelial cancers, such as nasopharyngeal carcinoma and gastric cancer (Xiang et al., 2018). On the other hand, VM associated with VEGF is controversial. Some reports suggested that anti-VEGF agents limited VM (Xiang et al., 2018). Another report suggested that the suppression of Flk-1 (VEGFR-2) activity and gene expression inhibited VM *in vivo* (Scully et al., 2012). Further investigation is needed to understand the mechanism underlying the relationship between VM and signaling pathways.

On the other hand, Zeng et al. (2019) showed that EVs derived from TECs might underlie the mechanism of resistance to anti-VEGF therapy (**Table 2**). miR-9 induces angiogenesis through the VEGF signaling pathway and promotes autophagy (Fu et al., 2015). The authors transfected HUVECs with miR-9 to mimic TECs in HCC. When vandetanib, an angiogenesis inhibitor, was administered to TECs, miR-9-induced angiogenesis and autophagy were inhibited. Instead, TECs secreted a high level of VEGF-enriched EVs. These EVs induced vascular network formation *in vitro* and enhanced endothelial vasculogenesis and VM *in vivo* (Zeng et al., 2019; **Figure 2C**). This mechanism may be one of the accountable phenomena by which tumor cells acquire resistance to AATs. Although there are few studies on the involvement of EVs in VM, tumor-associated EVs are thought to be related to VM, leading to resistance to AATs and tumor progression. A clinical study suggested that VM was related to tumor progression. Hujanen et al. (2020) showed in a meta-analysis that vascular mimicry positivity was strongly associated with poor overall survival in patients with squamous cell carcinoma of the head and neck or esophagus.

These studies indicate that EVs in the TME are implicated in VM after AATs, resulting in AAT resistance. If VM can be prevented, AATs will be beneficial to patients with cancer. The prevention of EV release in TECs induced by AATs can be one of the target therapies for VM and AAT resistance. Another study showed that miR-124 inhibit VM formation of cervical cancer through inhibition of MMP-2, MMP-9, and VEGF (Wan et al., 2014). miR-9 also inhibits VM formation by regulating stathmin expression in glioma cells (Song et al., 2013). These studies are not associated with EVs. However, these miRNAs are delivered by EVs in several cancer cells (Sueta et al., 2017; Lu et al., 2018). EVs loading these miRNAs can be also therapeutic candidates for VM formation and AAT resistance.

CONCLUDING REMARKS

A hypoxic environment due to the rapid and uncontrollable growth of tumor cells influences tumor cells and stromal cells to secrete not only several chemical mediators but also EVs, which contribute to neovasculature and metastasis. Neovasculature is one of the most important steps in metastasis and components of the TME. Tumor and stromal cells communicate with each other via EVs in the TME. Tumor-derived EVs induce angiogenesis in ECs in a VEGF-dependent or VEGF-independent manner. EVs secreted by tumor cells impair EC adhesion molecules and gap junctions to promote vascular permeability. The loss of endothelial markers and the acquisition of mesenchymal markers is referred to as EndoMT, which is activated by tumor-related EVs, contributes to the intravasation and extravasation of tumor cells and becomes a resource for CAFs. CAFs are derived from various types of cells and secrete EVs related to angiogenesis. Several types of cells, such as ECs, CAFs, platelets, and tumor cells, secrete EVs containing MMPs and result in ECM remodeling and angiogenesis. Platelets educated by tumor cells secrete PMPs that induce inflammation and coagulation. PMPs are the most abundant EVs in the blood and are associated with tumor angiogenesis, VTE, and patient prognosis. AAT is an important therapy for tumor progression. However, tumor cells induced by AATs establish capillary forms without vascular-related cells; this is referred to as VM.

As mentioned in this review article, we provide evidence that tumor and stromal cells are involved in tumor neovasculature through crosstalk with each other via EVs. A number of studies have suggested that tumor cells rearrange surrounding cells and the ECM using EVs, and these studies have helped understand the mechanism of tumor angiogenesis. In addition, recent studies on the involvement of non-malignant cells in cancer progression and regression have gradually accumulated.

The function of EVs derived from tumor cells and stromal cells may shed light on the mechanisms of cancer angiogenesis and progression. Tumor-derived EVs may also contribute to tumor cells becoming resistant to anti-tumor therapy, such as VM induced by AATs. Therefore, if we discover a method to prevent the release of EVs associated with tumor angiogenesis, lymphangiogenesis, and VM, it will be a candidate as effective treatment methods to improve not only cancer angiogenesis and metastasis but also cancer prognosis. In fact, accumulating evidence for therapeutic candidates associated with EVs indicate that several biomolecules in EVs have significant advantages for cancer diagnosis and prognosis (Wang et al., 2018). In addition, EVs can be loaded with several molecules such as chemotherapy agents (Agrawal et al., 2017) and nucleic acids including mRNA, miRNA, and interfering RNA (Jiang et al., 2017). Although further investigation is needed to utilize these techniques for tumor therapy, the approaches to EVs will provide a step forward in the overcoming of cancer.

AUTHOR CONTRIBUTIONS

NK, YY, SK, NA, and TO developed the concept of the review. NK, YY, and TO drafted the review. SK and NA corrected and reviewed the review. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Project for Cancer Research and Therapeutic Evolution (P-CREATE) grant number: JP20cm0106402 (to TO) from the Japan Agency for Medical Research and Development (AMED), Practical Research Project for Life-Style related Diseases including Cardiovascular Diseases and Diabetes Mellitus grant number: JP20ek0210145 (to TO) from AMED, and the Center of Open Innovation Network for Smart Health (COINS) (to TO) from the Japan Science and Technology Agency (JST).

REFERENCES

Aga, M., Bentz, G. L., Raffa, S., Torrisi, M. R., Kondo, S., Wakisaka, N., et al. (2014). Exosomal HIF1alpha supports invasive potential of nasopharyngeal carcinoma-associated LMP1-positive exosomes. *Oncogene* 33, 4613–4622. doi: 10.1038/onc.2014.66

Agrawal, A. K., Aqil, F., Jeyabalan, J., Spencer, W. A., Beck, J., Gachuki, B. W., et al. (2017). Milk-derived exosomes for oral delivery of paclitaxel. *Nanomedicine* 13, 1627–1636. doi: 10.1016/j.nano.2017.03.001

Al Tameemi, W., Dale, T. P., Al-Jumaily, R. M. K., and Forsyth, N. R. (2019). Hypoxia-modified cancer cell metabolism. *Front. Cell Dev. Biol.* 7:4. doi: 10.3389/fcell.2019.00004

Angara, K., Borin, T. F., and Arbab, A. S. (2017). Vascular mimicry: a novel neovascularization mechanism driving Anti-Angiogenic Therapy (AAT) resistance in glioblastoma. *Transl. Oncol.* 10, 650–660. doi: 10.1016/j.tranon.2017.04.007

Apte, R. S., Chen, D. S., and Ferrara, N. (2019). VEGF in signaling and disease: beyond discovery and development. *Cell* 176, 1248–1264. doi: 10.1016/j.cell.2019.01.021

Baghban, R., Roshangar, L., Jahanban-Esfahlan, R., Seidi, K., Ebrahimi-Kalan, A., Jaymand, M., et al. (2020). Tumor microenvironment complexity and therapeutic implications at a glance. *Cell Commun. Signal.* 18:59. doi: 10.1186/s12964-020-0530-4

Balkwill, F. R., Capasso, M., and Hagemann, T. (2012). The tumor microenvironment at a glance. *J. Cell Sci.* 125(Pt 23), 5591–5596. doi: 10.1242/jcs.116392

Berra, E., Benizri, E., Ginouvès, A., Volmat, V., Roux, D., and Pouysségur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. *EMBO J.* 22, 4082–4090. doi: 10.1093/emboj/cdg392

Beyer, C., and Pisetsky, D. S. (2010). The role of microparticles in the pathogenesis of rheumatic diseases. *Nat. Rev. Rheumatol.* 6, 21–29. doi: 10.1038/nrrheum.2009.229

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Campello, E., Spiezia, L., Radu, C. M., Bulato, C., Castelli, M., Gavasso, S., et al. (2011). Endothelial, platelet, and tissue factor-bearing microparticles in cancer patients with and without venous thromboembolism. *Thromb. Res.* 127, 473–477. doi: 10.1016/j.thromres.2011.01.002

Chen, C., Luo, Y., He, W., Zhao, Y., Kong, Y., Liu, H., et al. (2020). Exosomal long noncoding RNA LNMAT2 promotes lymphatic metastasis in bladder cancer. *J. Clin. Invest.* 130, 404–421. doi: 10.1172/JCI130892

Choi, H., and Moon, A. (2018). Crosstalk between cancer cells and endothelial cells: implications for tumor progression and intervention. *Arch. Pharm. Res.* 41, 711–724. doi: 10.1007/s12272-018-1051-1

Chowdhury, R., Webber, J. P., Gurney, M., Mason, M. D., Tabi, Z., and Clayton, A. (2015). Cancer exosomes trigger mesenchymal stem cell differentiation into pro-angiogenic and pro-invasive myofibroblasts. *Oncotarget* 6, 715–731. doi: 10.18632/oncotarget.2711

Cufaro, M. C., Pieragostino, D., Lanuti, P., Rossi, C., Cicalini, I., Federici, L., et al. (2019). Extracellular vesicles and their potential use in monitoring cancer progression and therapy: the contribution of proteomics. *J. Oncol.* 2019:1639854. doi: 10.1155/2019/1639854

de Jong, O. G., van Balkom, B. W., Gremmels, H., and Verhaar, M. C. (2016). Exosomes from hypoxic endothelial cells have increased collagen crosslinking activity through up-regulation of lysyl oxidase-like 2. *J. Cell Mol. Med.* 20, 342–350. doi: 10.1111/jcmm.12730

De Palma, M., Biziato, D., and Petrova, T. V. (2017). Microenvironmental regulation of tumour angiogenesis. *Nat. Rev. Cancer* 17, 457–474. doi: 10.1038/nrc.2017.51

Deng, W., Tang, T., Hou, Y., Zeng, Q., Wang, Y., Fan, W., et al. (2019). Extracellular vesicles in atherosclerosis. *Clin. Chim. Acta* 495, 109–117. doi: 10.1016/j.cca.2019.04.051

Dongre, A., and Weinberg, R. A. (2019). New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat. Rev. Mol. Cell Biol.* 20, 69–84. doi: 10.1038/s41580-018-0080-84

Dorayappan, K. D. P., Wanner, R., Wallbillich, J. J., Saini, U., Zingarelli, R., Suarez, A. A., et al. (2018). Hypoxia-induced exosomes contribute to a more aggressive and chemoresistant ovarian cancer phenotype: a novel mechanism linking STAT3/Rab proteins. *Oncogene* 37, 3806–3821. doi: 10.1038/s41388-018-0189-0

Eble, J. A., and Niland, S. (2019). The extracellular matrix in tumor progression and metastasis. *Clin. Exp. Metast.* 36, 171–198. doi: 10.1007/s10585-019-09966-1

Eccles, S. A., and Welch, D. R. (2007). Metastasis: recent discoveries and novel treatment strategies. *Lancet* 369, 1742–1757. doi: 10.1016/s0140-6736(07)60781-8

Erez, N., Truitt, M., Olson, P., Arron, S. T., and Hanahan, D. (2010). Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell* 17, 135–147. doi: 10.1016/j.ccr.2009.12.041

Fang, T., Lv, H., Lv, G., Li, T., Wang, C., Han, Q., et al. (2018). Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nat. Commun.* 9:191. doi: 10.1038/s41467-017-02583-0

Fasanaro, P., D'Alessandra, Y., Di Stefano, V., Melchionna, R., Romani, S., Pompilio, G., et al. (2008). MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J. Biol. Chem.* 283, 15878–15883. doi: 10.1074/jbc.M800731200

Ferrara, N., and Adamis, A. P. (2016). Ten years of anti-vascular endothelial growth factor therapy. *Nat. Rev. Drug Discov.* 15, 385–403. doi: 10.1038/nrd.2015.17

Folberg, R., Hendrix, M. J. C., and Maniotis, A. J. (2000). Vasculogenic Mimicry and Tumor Angiogenesis. *Am. J. Pathol.* 156, 361–381. doi: 10.1016/S0002-9440(10)64739-6

Folberg, R., and Maniotis, A. J. (2004). Vasculogenic mimicry. *Apmis* 112, 508–525. doi: 10.1111/j.1600-0463.2004.apm11207-0810.x

Freyssinet, J.-M., and Toti, F. (2010). Formation of procoagulant microparticles and properties. *Thromb. Res.* 125, S46–S48. doi: 10.1016/j.thromres.2010.01.036

Fu, B., Wang, Y., Zhang, X., Lang, B., Zhou, X., Xu, X., et al. (2015). MiR-221-induced PUMA silencing mediates immune evasion of bladder cancer cells. *Int. J. Oncol.* 46, 1169–1180. doi: 10.3892/ijo.2015.2837

García Garre, E., Luengo Gil, G., Montoro García, S., Gonzalez Billalabeitia, E., Zafra Poves, M., García Martinez, E., et al. (2018). Circulating small-sized endothelial microparticles as predictors of clinical outcome after chemotherapy for breast cancer: an exploratory analysis. *Breast Cancer Res. Treat.* 169, 83–92. doi: 10.1007/s10549-017-4656-z

Gomes, F. G., Sandim, V., Almeida, V. H., Rondon, A. M. R., Succar, B. B., Hottz, E. D., et al. (2017). Breast-cancer extracellular vesicles induce platelet activation and aggregation by tissue factor-independent and -dependent mechanisms. *Thromb. Res.* 159, 24–32. doi: 10.1016/j.thromres.2017.09.019

Gould, S. J., and Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. *J. Extracell. Ves.* 2:20389. doi: 10.3402/jev.v2i0.20389

Grad, L. I., Yerbury, J. J., Turner, B. J., Guest, W. C., Pokrishevsky, E., O'Neill, M. A., et al. (2014). Intercellular propagated misfolding of wild-type Cu/Zn superoxide dismutase occurs via exosome-dependent and -independent

mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* 111, 3620–3625. doi: 10.1073/pnas.1312245111

Grange, C., Tapparo, M., Collino, F., Vitillo, L., Damasco, C., Deregibus, M. C., et al. (2011). Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. *Cancer Res.* 71, 5346–5356. doi: 10.1158/0008-5472.Can-11-0241

Guo, H., Hu, G., Yang, Q., Zhang, P., Kuang, W., Zhu, X., et al. (2016). Knockdown of long non-coding RNA CCAT2 suppressed proliferation and migration of glioma cells. *Oncotarget* 7, 81806–81814. doi: 10.18632/oncotarget.13242

Guo, S., and Deng, C. X. (2018). Effect of stromal cells in tumor microenvironment on metastasis initiation. *Int. J. Biol. Sci.* 14, 2083–2093. doi: 10.7150/ijbs.25720

Haemmerle, M., Stone, R. L., Menter, D. G., Afshar-Kharghan, V., and Sood, A. K. (2018). The platelet lifeline to cancer: challenges and opportunities. *Cancer Cell* 33, 965–983. doi: 10.1016/j.ccell.2018.03.002

Hafiane, A., and Daskalopoulou, S. S. (2018). Extracellular vesicles characteristics and emerging roles in atherosclerotic cardiovascular disease. *Metabolism* 85, 213–222. doi: 10.1016/j.metabol.2018.04.008

Han, X., Guo, B., Li, Y., and Zhu, B. (2014). Tissue factor in tumor microenvironment: a systematic review. *J. Hematol. Oncol.* 7:54. doi: 10.1186/s13045-014-0054-8

Harding, C., Heuser, J., and Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97, 329–339. doi: 10.1083/jcb.97.2.329

Hautmann, R. E., de Petriconi, R. C., Pfeiffer, C., and Volkmer, B. G. (2012). Radical cystectomy for urothelial carcinoma of the bladder without neoadjuvant or adjuvant therapy: long-term results in 1100 patients. *Eur. Urol.* 61, 1039–1047. doi: 10.1016/j.eururo.2012.02.028

Helley, D., Banu, E., Bouziane, A., Banu, A., Scotte, F., Fischer, A. M., et al. (2009). Platelet microparticles: a potential predictive factor of survival in hormone-refractory prostate cancer patients treated with docetaxel-based chemotherapy. *Eur. Urol.* 56, 479–484. doi: 10.1016/j.eururo.2008.06.038

Hsu, Y. L., Hung, J. Y., Chang, W. A., Lin, Y. S., Pan, Y. C., Tsai, P. H., et al. (2017). Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1. *Oncogene* 36, 4929–4942. doi: 10.1038/onc.2017.105

Huang, Z., and Feng, Y. (2017). Exosomes derived from hypoxic colorectal cancer cells promote angiogenesis through wnt4-induced beta-catenin signaling in endothelial cells. *Oncol. Res.* 25, 651–661. doi: 10.3727/096504016X14752792816791

Hujanen, R., Almahmoudi, R., Karinen, S., Nwaru, B. I., Salo, T., and Salem, A. (2020). Vasculogenic mimicry: a promising prognosticator in head and neck squamous cell carcinoma and esophageal cancer? a systematic review and meta-analysis. *Cells* 9:507. doi: 10.3390/cells9020507

In 't Veld, S., and Wurdinger, T. (2019). Tumor-educated platelets. *Blood* 133, 2359–2364. doi: 10.1182/blood-2018-12-852830

Janowska-Wieczorek, A., Wysoczynski, M., Kijowski, J., Marquez-Curtis, L., Machalinski, B., Ratajczak, J., et al. (2005). Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Intern. J. Cancer* 113, 752–760. doi: 10.1002/ijc.20657

Jiang, L., Vader, P., and Schiffelers, R. M. (2017). Extracellular vesicles for nucleic acid delivery: progress and prospects for safe RNA-based gene therapy. *Gene Ther.* 24, 157–166. doi: 10.1038/gt.2017.8

Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H., et al. (2019). Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol. Cancer* 18:157. doi: 10.1186/s12943-019-1089-9

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., et al. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* 15, 290–298. doi: 10.1002/j.1460-2075.1996.tb00359.x

Jung, K. O., Youn, H., Lee, C.-H., Kang, K. W., and Chung, J.-K. (2016). Visualization of exosome-mediated miR-210 transfer from hypoxic tumor cells. *Oncotarget* 8, 9899–9910. doi: 10.18632/oncotarget.14247

Kalluri, R. (2016). The biology and function of exosomes in cancer. *J. Clin. Invest.* 126, 1208–1215. doi: 10.1172/jci81135

Karaman, S., and Detmar, M. (2014). Mechanisms of lymphatic metastasis. *J. Clin. Invest.* 124, 922–928. doi: 10.1172/JCI71606

Kim, H. K., Song, K. S., Chung, J.-H., Lee, K. R., and Lee, S.-N. (2004). Platelet microparticles induce angiogenesis in vitro. *Br. J. Haematol.* 124, 376–384. doi: 10.1046/j.1365-2141.2003.04773.x

Kim, H. K., Song, K. S., Park, Y. S., Kang, Y. H., Lee, Y. J., Lee, K. R., et al. (2003). Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *Eur. J. Cancer* 39, 184–191. doi: 10.1016/S0959-8049(02)00596-8

King, H. W., Michael, M. Z., and Gleadle, J. M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* 12:421. doi: 10.1186/1471-2407-12-421

Ko, S. Y., Lee, W., Kenny, H. A., Dang, L. H., Ellis, L. M., Jonasch, E., et al. (2019). Cancer-derived small extracellular vesicles promote angiogenesis by heparin-bound, bevacizumab-insensitive VEGF, independent of vesicle uptake. *Commun. Biol.* 2, 386–386. doi: 10.1038/s42003-019-0609-x

Koizume, S., Ito, S., Yoshioka, Y., Kanayama, T., Nakamura, Y., Yoshihara, M., et al. (2016). High-level secretion of tissue factor-rich extracellular vesicles from ovarian cancer cells mediated by filamin-A and protease-activated receptors. *Thromb. Haemost.* 115, 299–310. doi: 10.1160/th15-03-0213

Kovacic, J. C., Mercader, N., Torres, M., Boehm, M., and Fuster, V. (2012). Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: from cardiovascular development to disease. *Circulation* 125, 1795–1808. doi: 10.1161/CIRCULATIONAHA.111.040352

Kraman, M., Bambrough, P. J., Arnold, J. N., Roberts, E. W., Magiera, L., Jones, J. O., et al. (2010). Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science* 330, 827–830. doi: 10.1126/science.1195300

Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* 15, 178–196. doi: 10.1038/nrm3758

Lang, H. L., Hu, G. W., Zhang, B., Kuang, W., Chen, Y., Wu, L., et al. (2017). Glioma cells enhance angiogenesis and inhibit endothelial cell apoptosis through the release of exosomes that contain long non-coding RNA CCAT2. *Oncol. Rep.* 38, 785–798. doi: 10.3892/or.2017.5742

Li, K., Chen, Y., Li, A., Tan, C., and Liu, X. (2019). Exosomes play roles in sequential processes of tumor metastasis. *Int. J. Cancer* 144, 1486–1495. doi: 10.1002/ijc.31774

Liao, W. C., Liu, C. H., Chen, C. H., Hsu, W. M., Liao, Y. Y., Chang, H. M., et al. (2015). β-1,4-Galactosyltransferase III suppresses extravillous trophoblast invasion through modifying β1-integrin glycosylation. *Placenta* 36, 357–364. doi: 10.1016/j.placenta.2015.01.008

Liu, Y., and Cao, X. (2016). Characteristics and significance of the pre-metastatic niche. *Cancer Cell* 30, 668–681. doi: 10.1016/j.ccell.2016.09.011

Lombardo, G., Gili, M., Grange, C., Cavallari, C., Dentelli, P., Togliatto, G., et al. (2018). IL-3R-alpha blockade inhibits tumor endothelial cell-derived extracellular vesicle (EV)-mediated vessel formation by targeting the beta-catenin pathway. *Oncogene* 37, 1175–1191. doi: 10.1038/s41388-017-0034-x

Lopatina, T., Favaro, E., Danilova, L., Fertig, E. J., Favorov, A. V., Kagohara, L. T., et al. (2020). Extracellular vesicles released by tumor endothelial cells spread immunosuppressive and transforming signals through various recipient cells. *Front. Cell Dev. Biol.* 8:698. doi: 10.3389/fcell.2020.00698

Lu, J., Liu, Q. H., Wang, F., Tan, J. J., Deng, Y. Q., Peng, X. H., et al. (2018). Exosomal miR-9 inhibits angiogenesis by targeting MDK and regulating PDK/AKT pathway in nasopharyngeal carcinoma. *J. Exp. Clin. Cancer Res.* 37:147.

Maeda, S., and Omata, M. (2008). Inflammation and cancer: role of nuclear factor-kappaB activation. *Cancer Sci.* 99, 836–842. doi: 10.1111/j.1349-7006.2008.00763.x

Maishi, N., and Hida, K. (2017). Tumor endothelial cells accelerate tumor metastasis. *Cancer Sci.* 108, 1921–1926. doi: 10.1111/cas.13336

Mandriota, S. J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., et al. (2001). Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J.* 20, 672–682. doi: 10.1093/emboj/20.4.672

Maniotis, A. J., Folberg, R., Hess, A., Seftor, E. A., Gardner, L. M. G., Pe'er, J., et al. (1999). Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am. J. Pathol.* 155, 739–752. doi: 10.1016/S0002-9440(10)65173-5

Mao, Y., Wang, Y., Dong, L., Zhang, Y., Zhang, Y., Wang, C., et al. (2019). Hypoxic exosomes facilitate angiogenesis and metastasis in esophageal squamous cell carcinoma through altering the phenotype and transcriptome of endothelial cells. *J. Exper. Clin. Cancer Res.* 38:389. doi: 10.1186/s13046-019-1384-8

McKeown, S. R. (2014). Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *Br. J. Radiol.* 87:20130676. doi: 10.1259/bjr.20130676

Medici, D., Potenta, S., and Kalluri, R. (2011). Transforming growth factor-β2 promotes Snail-mediated endothelial-mesenchymal transition through convergence of Smad-dependent and Smad-independent signalling. *Biochem. J.* 437, 515–520. doi: 10.1042/bj20101500

Mehlen, P., and Puisieux, A. (2006). Metastasis: a question of life or death. *Nat. Rev. Cancer* 6, 449–458. doi: 10.1038/nrc1886

Meng, W., Hao, Y., He, C., Li, L., and Zhu, G. (2019). Exosome-orchestrated hypoxic tumor microenvironment. *Mol. Cancer* 18:57. doi: 10.1186/s12943-019-0982-6

Miki, Y., Yashiro, M., Okuno, T., Kitayama, K., Masuda, G., Hirakawa, K., et al. (2018). CD9-positive exosomes from cancer-associated fibroblasts stimulate the migration ability of scirrhous-type gastric cancer cells. *Br. J. Cancer* 118, 867–877. doi: 10.1038/bjc.2017.487

Mitra, A. K., Zillhardt, M., Hua, Y., Tiwari, P., Murmann, A. E., Peter, M. E., et al. (2012). MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. *Cancer Discov.* 2, 1100–1108. doi: 10.1158/2159-8290.Cd-12-0206

Miyazono, K. (2000). TGF-beta signaling by Smad proteins. *Cytokine Growth Fact. Rev.* 11, 15–22. doi: 10.1016/s1359-6101(99)00025-8

Miyoshi, A., Kitajima, Y., Kido, S., Shimonishi, T., Matsuyama, S., Kitahara, K., et al. (2005). Snail accelerates cancer invasion by upregulating MMP expression and is associated with poor prognosis of hepatocellular carcinoma. *Br. J. Cancer* 92, 252–258. doi: 10.1038/sj.bjc.6602266

Miyoshi, A., Kitajima, Y., Sumi, K., Sato, K., Hagiwara, A., Koga, Y., et al. (2004). Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *Br. J. Cancer* 90, 1265–1273. doi: 10.1038/sj.bjc.6601685

Naito, Y., Yamamoto, Y., Sakamoto, N., Shimomura, I., Kogure, A., Kumazaki, M., et al. (2019). Cancer extracellular vesicles contribute to stromal heterogeneity by inducing chemokines in cancer-associated fibroblasts. *Oncogene* 38, 5566–5579. doi: 10.1038/s41388-019-0832-4

Nawaz, M., Shah, N., Zanetti, B. R., Maugeri, M., Silvestre, R. N., Fatima, F., et al. (2018). Extracellular vesicles and matrix remodeling enzymes: the emerging roles in extracellular matrix remodeling, progression of diseases and tissue repair. *Cells* 7:167. doi: 10.3390/cells7100167

Nie, L., Lyros, O., Medda, R., Jovanovic, N., Schmidt, J. L., Otterson, M. F., et al. (2014). Endothelial-mesenchymal transition in normal human esophageal endothelial cells cocultured with esophageal adenocarcinoma cells: role of IL-1β and TGF-β2. *Am. J. Physiol.-Cell Physiol.* 307, C859–C877. doi: 10.1152/ajpcell.00081.2014

Nikolopoulos, S. N., Blaikie, P., Yoshioka, T., Guo, W., Puri, C., Tacchetti, C., et al. (2005). Targeted deletion of the integrin beta4 signaling domain suppresses laminin-5-dependent nuclear entry of mitogen-activated protein kinases and NF-kappaB, causing defects in epidermal growth and migration. *Mol. Cell Biol.* 25, 6090–6102. doi: 10.1128/mcb.25.14.6090-6102.2005

Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., et al. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121, 335–348. doi: 10.1016/j.cell.2005.02.034

Pan, B. T., Teng, K., Wu, C., Adam, M., and Johnstone, R. M. (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* 101, 942–948. doi: 10.1083/jcb.101.3.942

Pinto, M. P., Sotomayor, P., Carrasco-Avino, G., Corvalan, A. H., and Owen, G. I. (2016). Escaping antiangiogenic therapy: strategies employed by cancer cells. *Int. J. Mol. Sci.* 17:1489. doi: 10.3390/ijms17091489

Platel, V., Faure, S., Corre, I., and Clere, N. (2019). Endothelial-to-mesenchymal transition (EndoMT): roles in Tumorigenesis, metastatic extravasation and therapy resistance. *J. Oncol.* 2019:8361945. doi: 10.1155/2019/8361945

Quante, M., Tu, S. P., Tomita, H., Gonda, T., Wang, S. S., Takashi, S., et al. (2011). Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell* 19, 257–272. doi: 10.1016/j.ccr.2011.01.020

Quintero-Fabián, S., Arreola, R., Becerril-Villanueva, E., Torres-Romero, J. C., Arana-Argáez, V., Lara-Riegos, J., et al. (2019). Role of matrix metalloproteinases in angiogenesis and cancer. *Front. Oncol.* 9:1370. doi: 10.3389/fonc.2019.01370

Reck, M., Garassino, M. C., Imbimbo, M., Shepherd, F. A., Socinski, M. A., Shih, J. Y., et al. (2018). Antiangiogenic therapy for patients with aggressive or refractory advanced non-small cell lung cancer in the second-line setting. *Lung Cancer* 120, 62–69. doi: 10.1016/j.lungcan.2018.03.025

Rey, S., and Semenza, G. L. (2010). Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling. *Cardiovasc. Res.* 86, 236–242. doi: 10.1093/cvr/cvq045

Ringuette Goulet, C., Bernard, G., Tremblay, S., Chabaud, S., Bolduc, S., and Pouliot, F. (2018). Exosomes induce fibroblast differentiation into cancer-associated fibroblasts through TGFbeta signaling. *Mol. Cancer Res.* 16, 1196–1204. doi: 10.1158/1541-7786.MCR-17-0784

Sanchez-Duffhues, G., Garcia de Vinuesa, A., and Ten Dijke, P. (2018). Endothelial-to-mesenchymal transition in cardiovascular diseases: developmental signaling pathways gone awry. *Dev. Dyn.* 247, 492–508. doi: 10.1002/dvdy.24589

Sappino, A. P., Skalli, O., Jackson, B., Schürch, W., and Gabbiani, G. (1988). Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. *Int. J. Cancer* 41, 707–712. doi: 10.1002/ijc.2910410512

Schlesinger, M. (2018). Role of platelets and platelet receptors in cancer metastasis. *J. Hematol. Oncol.* 11:125. doi: 10.1186/s13045-018-0669-2

Scully, S., Francescone, R., Faibish, M., Bentley, B., Taylor, S. L., Oh, D., et al. (2012). Transdifferentiation of glioblastoma stem-like cells into mural cells drives vasculogenic mimicry in glioblastomas. *J. Neurosci.* 32, 12950–12960. doi: 10.1523/jneurosci.2017-12.2012

Seftor, R. E., Seftor, E. A., Koshikawa, N., Meltzer, P. S., Gardner, L. M., Bilban, M., et al. (2001). Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res.* 61, 6322–6327.

Serova, M., Tijeras-Raballand, A., Dos Santos, C., Martinet, M., Neuzillet, C., Lopez, A., et al. (2016). Everolimus affects vasculogenic mimicry in renal carcinoma resistant to sunitinib. *Oncotarget* 7, 38467–38486. doi: 10.18632/oncotarget.9542

Shao, Z.-M., Nguyen, M., and Barsky, S. H. (2000). Human breast carcinoma desmoplasia is PDGF initiated. *Oncogene* 19, 4337–4345. doi: 10.1038/sj.onc.1203785

Shimoda, M., Principe, S., Jackson, H. W., Luga, V., Fang, H., Molyneux, S. D., et al. (2014). Loss of the Timp gene family is sufficient for the acquisition of the CAF-like cell state. *Nat. Cell Biol.* 16, 889–901. doi: 10.1038/ncb3021

Shuman Moss, L. A., Jensen-Taubman, S., and Stetler-Stevenson, W. G. (2012). Matrix metalloproteinases: changing roles in tumor progression and metastasis. *Am. J. Pathol.* 181, 1895–1899. doi: 10.1016/j.ajpath.2012.08.044

Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., et al. (2001). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.* 7, 192–198. doi: 10.1038/84643

Song, Y., Mu, L., Han, X., Li, Q., Dong, B., Li, H., et al. (2013). MicroRNA-9 inhibits vasculogenic mimicry of glioma cell lines by suppressing Stathmin expression. *J. Neurooncol.* 115, 381–390. doi: 10.1007/s11060-013-1245-9

Strutz, F., Zeisberg, M., Hemmerlein, B., Sattler, B., Hummel, K., Becker, V., et al. (2000). Basic fibroblast growth factor expression is increased in human renal fibrogenesis and may mediate autocrine fibroblast proliferation. *Kidney Int.* 57, 1521–1538. doi: 10.1046/j.1523-1755.2000.00997.x

Su, J. L., Yen, C. J., Chen, P. S., Chuang, S. E., Hong, C. C., Kuo, I. H., et al. (2007). The role of the VEGF-C/VEGFR-3 axis in cancer progression. *Br. J. Cancer* 96, 541–545. doi: 10.1038/sj.bjc.6603487

Sueta, A., Yamamoto, Y., Tomiguchi, M., Takeshita, T., Yamamoto-Ibusuki, M., and Iwase, H. (2017). Differential expression of exosomal miRNAs between breast cancer patients with and without recurrence. *Oncotarget* 8, 69934–69944. doi: 10.18632/oncotarget.19482

Sun, B., Zhang, D., Zhao, N., and Zhao, X. (2017). Epithelial-to-endothelial transition and cancer stem cells: two cornerstones of vasculogenic mimicry in malignant tumors. *Oncotarget* 8, 30502–30510. doi: 10.18632/oncotarget.8461

Sun, H., Zhang, D., Yao, Z., Lin, X., Liu, J., Gu, Q., et al. (2017). Anti-angiogenic treatment promotes triple-negative breast cancer invasion via vasculogenic mimicry. *Cancer Biol. Ther.* 18, 205–213. doi: 10.1080/15384047.2017.1294288

Tacconi, C., Correale, C., Gandelli, A., Spinelli, A., Dejana, E., D'Alessio, S., et al. (2015). Vascular endothelial growth factor C disrupts the endothelial lymphatic barrier to promote colorectal cancer invasion. *Gastroenterology* 148, 1438–1451.e1438. doi: 10.1053/j.gastro.2015.03.005

Takagi, S., Sato, S., Oh-hara, T., Takami, M., Koike, S., Mishima, Y., et al. (2013). Platelets promote tumor growth and metastasis via direct interaction between Aggrus/Podoplanin and CLEC-2. *PLoS One* 8:e73609. doi: 10.1371/journal.pone.0073609

Tan, S., Xia, L., Yi, P., Han, Y., Tang, L., Pan, Q., et al. (2020). Exosomal miRNAs in tumor microenvironment. *J. Exp. Clin. Cancer Res.* 39:67. doi: 10.1186/s13046-020-01570-6

Thery, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *J. Extracell Ves.* 7:1535750. doi: 10.1080/20013078.2018.1535750

Tkach, M., and Théry, C. (2016). Communication by extracellular vesicles: where we are and where we need to go. *Cell* 164, 1226–1232. doi: 10.1016/j.cell.2016.01.043

Togo, S., Polanska, U. M., Horimoto, Y., and Orimo, A. (2013). Carcinoma-associated fibroblasts are a promising therapeutic target. *Cancers* 5, 149–169. doi: 10.3390/cancers5010149

Tominaga, N., Katsuda, T., and Ochiya, T. (2015a). Micromanaging of tumor metastasis by extracellular vesicles. *Semin. Cell Dev. Biol.* 40, 52–59. doi: 10.1016/j.semcdb.2015.02.016

Tominaga, N., Kosaka, N., Ono, M., Katsuda, T., Yoshioka, Y., Tamura, K., et al. (2015b). Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood-brain barrier. *Nat. Commun.* 6:6716. doi: 10.1038/ncomms7716

Trousseau, A. (1865). Plegmasia alba dolens. *Lectur. Clin. Med.* 5, 281–332.

Tsukita, S., Tanaka, H., and Tamura, A. (2019). The claudins: from tight junctions to biological systems. *Trends Biochem. Sci.* 44, 141–152. doi: 10.1016/j.tibs.2018.09.008

Umezu, T., Tadokoro, H., Azuma, K., Yoshizawa, S., Ohyashiki, K., and Ohyashiki, J. H. (2014). Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. *Blood* 124, 3748–3757. doi: 10.1182/blood-2014-05-576116

Valdivia, A., Mingo, G., Aldana, V., Pinto, M. P., Ramirez, M., Retamal, C., et al. (2019). Fact or fiction, it is time for a verdict on vasculogenic mimicry? *Front. Oncol.* 9:680. doi: 10.3389/fonc.2019.00680

van der Pol, E., Böing, A., Harrison, P., Sturk, A., and Nieuwland, R. (2012). Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol. Rev.* 64, 676–705. doi: 10.1124/pr.112.005983

van Doormaal, F., Kleinjan, A., Berckmans, R. J., Mackman, N., Manly, D., Kamphuisen, P. W., et al. (2012). Coagulation activation and microparticle-associated coagulant activity in cancer patients. An exploratory prospective study. *Thromb. Haemost.* 108, 160–165. doi: 10.1160/th12-02-0099

Vaupel, P., and Harrison, L. (2004). Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *Oncologist* 9(Suppl. 5), 4–9. doi: 10.1634/theoncologist.9-90005-4

Vu, L. T., Peng, B., Zhang, D. X., Ma, V., Mathey-Andrews, C. A., Lam, C. K., et al. (2019). Tumor-secreted extracellular vesicles promote the activation of cancer-associated fibroblasts via the transfer of microRNA-125b. *J. Extracell Ves.* 8:1599680. doi: 10.1080/20013078.2019.1599680

Walsh, J. C., Lebedev, A., Aten, E., Madsen, K., Marciano, L., and Kolb, H. C. (2014). The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities. *Antioxid. Redox Signal.* 21, 1516–1554. doi: 10.1089/ars.2013.5378

Wan, H. Y., Li, Q. Q., Zhang, Y., Tian, W., Li, Y. N., Liu, M., et al. (2014). MiR-124 represses vasculogenic mimicry and cell motility by targeting amotL1 in cervical cancer cells. *Cancer Lett.* 355, 148–158. doi: 10.1016/j.canlet.2014.09.005

Wang, C.C., Tseng, C.-C., Hsiao, C.-C., Chang, H.-C., Chang, L.-T., Fang, W.-F., et al. (2014). Circulating endothelial-derived activated microparticle: a useful biomarker for predicting one-year mortality in patients with advanced non-small cell lung cancer. *Biomed. Res. Intern.* 2014:173401. doi: 10.1155/2014/173401

Wang, T., Gilkes, D. M., Takano, N., Xiang, L., Luo, W., Bishop, C. J., et al. (2014). Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3234–E3242. doi: 10.1073/pnas.1410041111

Wang, F. T., Sun, W., Zhang, J. T., and Fan, Y. Z. (2019). Cancer-associated fibroblast regulation of tumor neo-angiogenesis as a therapeutic target in cancer. *Oncol. Lett.* 17, 3055–3065. doi: 10.3892/ol.2019.9973

Wang, M., Ji, S., Shao, G., Zhang, J., Zhao, K., Wang, Z., et al. (2018). Effect of exosome biomarkers for diagnosis and prognosis of breast cancer patients. *Clin. Transl. Oncol.* 20, 906–911. doi: 10.1007/s12094-017-1805-0

Willis, C. M., Nicaise, A. M., Menoret, A., Ryu, J. K., Mendiola, A. S., Jellison, E. R., et al. (2019). Extracellular vesicle fibrinogen induces encephalitogenic CD8++ T cells in a mouse model of multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 116, 10488–10493. doi: 10.1073/pnas.1816911116

Wilson, W. R., and Hay, M. P. (2011). Targeting hypoxia in cancer therapy. *Nat. Rev. Cancer* 11, 393–410. doi: 10.1038/nrc3064

Witwer, K. W., Buzas, E. I., Bemis, L. T., Bora, A., Lasser, C., Lotvall, J., et al. (2013). Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J. Extracell Ves.* 2:20360. doi: 10.3402/jev.v2i0.20360

Wojtukiewicz, M. Z., Sierko, E., Hempel, D., Tucker, S. C., and Honn, K. V. (2017). Platelets and cancer angiogenesis nexus. *Cancer Metast. Rev.* 36, 249–262. doi: 10.1007/s10555-017-9673-1

Wolf, P. (1967). The nature and significance of platelet products in human plasma. *Br. J. Haematol.* 13, 269–288. doi: 10.1111/j.1365-2141.1967.tb08741.x

Wu, X., Liu, Y., Wei, W., and Liu, M. L. (2019). Extracellular vesicles in autoimmune vasculitis - Little dirts light the fire in blood vessels. *Autoimmun. Rev.* 18, 593–606. doi: 10.1016/j.autrev.2018.12.007

Xiang, T., Lin, Y. X., Ma, W., Zhang, H. J., Chen, K. M., He, G. P., et al. (2018). Vasculogenic mimicry formation in EBV-associated epithelial malignancies. *Nat. Commun.* 9:5009. doi: 10.1038/s41467-018-07308-5

Xu, G., Zhang, B., Ye, J., Cao, S., Shi, J., Zhao, Y., et al. (2019). Exosomal miRNA-139 in cancer-associated fibroblasts inhibits gastric cancer progression by repressing MMP11 expression. *Int. J. Biol. Sci.* 15, 2320–2329. doi: 10.7150/ijbs.33750

Xu, X. R., Yousef, G. M., and Ni, H. (2018). Cancer and platelet crosstalk: opportunities and challenges for aspirin and other antiplatelet agents. *Blood* 131, 1777–1789. doi: 10.1182/blood-2017-05-743187

Yamada, N. O., Heishima, K., Akao, Y., and Senda, T. (2019). Extracellular vesicles containing MicroRNA-92a-3p facilitate partial endothelial-mesenchymal transition and angiogenesis in endothelial cells. *Int. J. Mol. Sci.* 20:4406. doi: 10.3390/ijms20184406

Yang, J.-K., Yang, J.-P., Tong, J., Jing, S.-Y., Fan, B., Wang, F., et al. (2017). Exosomal miR-221 targets DNM3 to induce tumor progression and temozolomide resistance in glioma. *J. Neurooncol.* 131, 255–265. doi: 10.1007/s11060-016-2308-5

Yang, J. P., Liao, Y. D., Mai, D. M., Xie, P., Qiang, Y. Y., Zheng, L. S., et al. (2016). Tumor vasculogenic mimicry predicts poor prognosis in cancer patients: a meta-analysis. *Angiogenesis* 19, 191–200. doi: 10.1007/s10456-016-9500-2

Yeon, J. H., Jeong, H. E., Seo, H., Cho, S., Kim, K., Na, D., et al. (2018). Cancer-derived exosomes trigger endothelial to mesenchymal transition followed by the induction of cancer-associated fibroblasts. *Acta Biomater.* 76, 146–153. doi: 10.1016/j.actbio.2018.07.001

Yokoi, A., Yoshioka, Y., Yamamoto, Y., Ishikawa, M., Ikeda, S. I., Kato, T., et al. (2017). Malignant extracellular vesicles carrying MMP1 mRNA facilitate peritoneal dissemination in ovarian cancer. *Nat. Commun.* 8:14470. doi: 10.1038/ncomms14470

Zeng, Y., Yao, X., Liu, X., He, X., Li, L., Liu, X., et al. (2019). Anti-angiogenesis triggers exosomes release from endothelial cells to promote tumor vasculogenesis. *J. Extracell Ves.* 8:1629865. doi: 10.1080/20013078.2019.1629865

Zeng, Z., Li, Y., Pan, Y., Lan, X., Song, F., Sun, J., et al. (2018). Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing

vascular permeability and angiogenesis. *Nat. Commun.* 9:5395. doi: 10.1038/s41467-018-07810-w

Zhang, H. G., and Grizzle, W. E. (2014). Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *Am. J. Pathol.* 184, 28–41. doi: 10.1016/j.ajpath.2013.09.027

Zhou, C. F., Ma, J., Huang, L., Yi, H. Y., Zhang, Y. M., Wu, X. G., et al. (2019). Cervical squamous cell carcinoma-secreted exosomal miR-221-3p promotes lymphangiogenesis and lymphatic metastasis by targeting VASH1. *Oncogene* 38, 1256–1268. doi: 10.1038/s41388-018-0511-x

Zhou, W., Fong, M. Y., Min, Y., Somlo, G., Liu, L., Palomares, M. R., et al. (2014). Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* 25, 501–515. doi: 10.1016/j.ccr.2014.03.007

Zhuang, G., Wu, X., Jiang, Z., Kasman, I., Yao, J., Guan, Y., et al. (2012). Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *EMBO J.* 31, 3513–3523. doi: 10.1038/emboj.2012.183

Zmigrodzka, M., Guzera, M., Miskiewicz, A., Jagielski, D., and Winnicka, A. (2016). The biology of extracellular vesicles with focus on platelet microparticles and their role in cancer development and progression. *Tumour Biol.* 37, 14391–14401. doi: 10.1007/s13277-016-5358-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 05 January 2021
doi: 10.3389/fcell.2020.610903

The Role of Anti-angiogenesis in the Treatment Landscape of Non-small Cell Lung Cancer – New Combinational Approaches and Strategies of Neovessel Inhibition

*Sophia Daum[1], Hannes Hagen[1], Erin Naismith[1], Dominik Wolf[1,2] and Andreas Pircher[1]**

[1] Internal Medicine V, Department of Hematology and Oncology, Medical University Innsbruck, Innsbruck, Austria, [2] Medical Clinic 3, Department of Oncology, Hematology, Immunoncology and Rheumatology, University Hospital Bonn (UKB), Bonn, Germany

OPEN ACCESS

Edited by:
Lucas Treps,
VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Anca Maria Cimpean,
Victor Babes University of Medicine and Pharmacy, Romania
Girolamo Ranieri,
Istituto Nazionale dei Tumori (IRCCS), Italy
Patrycja Monika Nowak-Sliwinska,
Université de Genève, Switzerland

***Correspondence:**
Andreas Pircher
andreas.pircher@i-med.ac.at

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 27 September 2020
Accepted: 07 December 2020
Published: 05 January 2021

Citation:
Daum S, Hagen H, Naismith E, Wolf D and Pircher A (2021) The Role of Anti-angiogenesis in the Treatment Landscape of Non-small Cell Lung Cancer – New Combinational Approaches and Strategies of Neovessel Inhibition. Front. Cell Dev. Biol. 8:610903. doi: 10.3389/fcell.2020.610903

Tumor progression depends primarily on vascular supply, which is facilitated by angiogenic activity within the malignant tissue. Non-small cell lung cancer (NSCLC) is a highly vascularized tumor, and inhibition of angiogenesis was projected to be a promising therapeutic approach. Over a decade ago, the first anti-angiogenic agents were approved for advanced stage NSCLC patients, however, they only produced a marginal clinical benefit. Explanations why anti-angiogenic therapies only show modest effects include the highly adaptive tumor microenvironment (TME) as well as the less understood characteristics of the tumor vasculature. Today, advanced methods of in-depth characterization of the NSCLC TME by single cell RNA sequencing (scRNA-Seq) and preclinical observations enable a detailed characterization of individual cancer landscapes, allowing new aspects for a more individualized inhibition of angiogenesis to be identified. Furthermore, the tumor vasculature itself is composed of several cellular subtypes, which closely interact with other cellular components of the TME, and show distinct biological functions such as immune regulation, proliferation, and organization of the extracellular matrix. With these new insights, combinational approaches including chemotherapy, anti- angiogenic and immunotherapy can be developed to yield a more target-oriented anti-tumor treatment in NSCLC. Recently, anti-angiogenic agents were also shown to induce the formation of high endothelial venules (HEVs), which are essential for the formation of tertiary lymphoid structures, and key components in triggering anti-tumor immunity. In this review, we will summarize the current knowledge of tumor-angiogenesis and corresponding anti-angiogenic therapies, as well as new aspects concerning characterization of tumor-associated vessels and the resulting new strategies for anti-angiogenic therapies and vessel inhibition in NSCLC. We will further discuss why anti-angiogenic therapies form an interesting backbone strategy for combinational therapies and how anti-angiogenic approaches could be further developed in a more personalized tumor-oriented fashion with focus on NSCLC.

Keywords: non-small cell lung cancer, angiogenesis, vascular endothelial growth factor, tumor microenvironment, tumor endothelial cells, immunotherapy, combinational therapy

INTRODUCTION

Angiogenesis is regulated by the balance of pro-angiogenic and anti-angiogenic factors present in a tissue, if vascular remodeling is required this balance shifts to an activating state, called the "angiogenic switch" (Bergers and Benjamin, 2003). In progressing tumors, a similar activated angiogenic phenotype occurs which promotes endothelial cell (EC) proliferation, migration, elongation and dissemination of metastases to distant organs (Teleanu et al., 2019). These findings proposed inhibiting angiogenesis as a highly potent anti-cancer therapeutical approach and have intensified the research for agents to hamper vessel formation in diverse tumors over the past decades (Augustine et al., 2019). Conclusively, inhibiting pro-angiogenic molecules including vascular endothelial growth factors (VEGFs) or their cognate receptors (VEGFRs), served as anti-angiogenic therapy approaches in advanced stage NSCLC patients, as well as other cancer entities, at the beginning of this century (Sandler et al., 2006) and reviewed in Jayson et al. (2016). High expectations for these anti-cancer drugs were shattered rapidly as they exhibited only marginal benefits in early clinical trials due to the acquisition of evasive or primary resistance mechanisms consequently leading to a transient therapy benefit. Therapy failure could be attributed to the interplay of adaptive mechanisms of the TME (e.g., eliciting compensatory angiogenic pathways) and its interacting cellular compartments including TECs. In the previous years, a more detailed characterization identified the (tumor) endothelium as a heterogeneous cell population with distinct functional and organ-specific phenotypes indicating multiple pathological features of the tumor vasculature (Rafii et al., 2016). In addition to tumor endothelial heterogeneity, other vessel formation processes alongside vascular sprouting, such as vessel co-option or vasculogenic mimicry (VM) were less acknowledged in NSCLC but may play an important role in anti-angiogenic therapy resistance. Furthermore, the ability of tumors to compensate for absent signaling molecules by activating alternative pathways represents another resistance mechanism. The inhibition of VEGF/VEGFR, for example, was shown to prompt tumors to sustain angiogenesis via the secretion of substitute factors such as PDGF (Crawford et al., 2009), bFGF (Babina and Turner, 2017) and angiopoietin-2 (Rigamonti et al., 2014), or by the recruitment of pro-angiogenic cells such as tryptase secreting mast cells (Wroblewski et al., 2017) thus, resisting single-target therapies. Previous studies using dual or multi-target antibodies which simultaneously inhibit several angiogenic signals exhibited an incremental anti-angiogenic efficacy in different tumor types (Li et al., 2016; Peterson et al., 2016; Liu et al., 2018; Hosaka et al., 2020). However, many processes and factors contributing to inefficacy and resistance to angiogenesis inhibitors, in particular those involving the tumor endothelium, remain ambiguous.

The transient combinational efficacy of anti-angiogenic agents and chemotherapy (in first-line, as well as in second-line therapy) could possibly be attributed to a "vascular normalization" phenotype. Nevertheless, the time window of vessel re-organization and normalization is not well understood in the clinical setting but could play a major role in the transmission of chemical agents directly to the tumor, thereby enhancing anti-cancer efficacy (Johnson et al., 2004; Sandler et al., 2006; Garon et al., 2014; Reck et al., 2014). Additionally, cancer immunotherapies which inhibit immune checkpoints (ICs) such as programmed cell death protein 1/programmed cell death 1 ligand 1 (PD1/PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) have become landmarks in cancer treatment. The interaction of tumor vasculature with immune cells has a severe impact on the responsiveness and immunodeficiency of the tumor. Vascular normalization due to VEGF-inhibiting therapy exhibited increased lymphocyte infiltration and T-cell activation which, combined with immune checkpoint inhibitors (ICI), elicited an improved anti-tumor immunity in preclinical trials (Allen et al., 2017; Schmittnaegel et al., 2017). Additionally, combinational therapy of anti-angiogenic agents and ICI resulted in the formation of HEVs, which enhances activation of circulating B- and T-cells by mediating migration into secondary lymphoid organs (Ager and May, 2015). When surrounded by dense B- and T-cell rich areas, HEV can further adapt to tertiary lymphoid structures (TLS) thereby triggering potent anti-tumor immunity, which can significantly improve patient outcomes (Martinet and Girard, 2013). We are confronted with a network of considerable aspects when it comes to anti-angiogenic therapy, many of which still require thorough investigation. Further characterization of the TME and the associated endothelium can help improve anti-angiogenic therapies and optimize the proposed powerful synergic efficacy of combinational therapeutical approaches in NSCLC.

THE ROLE OF TUMOR ANGIOGENESIS IN NSCLC

Physiological angiogenesis has already been characterized in detail and previously reviewed elsewhere (Góth et al., 2003; Senger and Davis, 2011). The process of tumor angiogenesis, which occurs early during tumor progression, is similar to physiological vessel formation, but with differences in regulation and grade of activity (Hanahan and Folkman, 1996; Raica et al., 2009; Hanahan and Weinberg, 2011). Firstly, tumor associated ECs acquire a chronic activated state, the "angiogenic switch," a result of the upregulation of angiogenic receptors and activation of the PI3K-AKT signaling axis (Phung et al., 2006). This activation results in increased proliferation, survival and migration, leading to distortion of the basement membrane as well as pericyte coverage in the tumor vasculature (Hida et al., 2016). Consequently, TECs exhibit dysregulated behavior and polarization resulting in leaky, hemorrhagic, and dysfunctional vessels. Thus, oxygen levels, nutrient availability and waste disposal is diminished, which has severe effects on the TME (Colegio et al., 2014; Sanctis et al., 2018). Furthermore, dysfunctional TECs severely impact lymphocyte adhesion, trafficking and migration to the local tissue, resulting in a highly immunosuppressive TME (Fridman et al., 2012).

Additionally, the tumor stroma, which consists of a mix of resident fibroblasts and pericytes as well as bone-marrow derived tumor infiltrating leukocytes (e.g., macrophages and mast

cells), regulates angiogenesis. M2 polarized tumor associated macrophages can either directly activate angiogenesis by releasing VEGF, bFGF and PlGF or indirectly via the release of matrix-metalloproteinases (MMPs), which in turn remodel the extracellular matrix for an enhanced endothelial migration (Kessenbrock et al., 2010; Olson and Joyce, 2015). Fibroblasts, as well as myeloid derived suppressor cells (MDSCs) promote angiogenesis through expression of growth factors such as VEGF and bFGF (Shi et al., 2017). CSF-1, a cytokine crucial for the survival and differentiation of monocytes and macrophages, mediates the recruitment of MDSCs into the tumor niche, which in turn increases angiogenesis due to growth factor release (Shojaei et al., 2007). By blocking the CSF-1 signaling in combination with anti-VEGFR2 therapy, tumor growth could be markedly decreased in murine lung carcinoma models (Priceman et al., 2010). Mast cells comprise a major compartment of inflammatory cells present in the TME and exhibit important regulatory features regarding angiogenesis (Ribatti and Crivellato, 2012). Their granules contain various proteases, cytokines and growth factors including pro-angiogenic molecules such as VEGF, bFGF, PDGF and the potent angiogenic factor tryptase, which is released upon activation of IgE or c-kit receptors (Ribatti and Ranieri, 2015). Tryptase induces vascularization and vessel tube formation by stimulating proliferation of ECs and activation of MMPs (Ribatti and Crivellato, 2012). In NSCLC the number of tryptase positive MCs linearly correlates with microvascular density, confirming the important role of this enzyme in regulating tumor angiogenesis (Ibaraki et al., 2005; Carlini et al., 2010). Inhibition of c-kit and its ligand SCF could hamper mast cell infiltration into the TME, preventing degranulation and thereby producing a synergizing anti-angiogenic effect (Huang et al., 2008; Overed-Sayer et al., 2020).

Current vessel-inhibiting therapies for treating advanced NSCLC mainly focus on repressing the process of vessel sprouting predominantly triggered by VEGF signaling. In the past years, however, non-angiogenic processes in the TME have gained attention as they are suggested to significantly contribute to tumor progression while being resistant to traditional angiogenesis inhibitors. In highly vascularized organs such as the lung, it was observed that cancer cells start to grow along existing vessels to preserve access to essential nutrients and gases without the need to form new vasculature. This process is referred to as vessel co-option (Pezzella et al., 1997; Coelho et al., 2017). In contrast to the chaotic growth of angiogenic tumor vessels, co-opted vasculature remains well organized as deduced from normal tissues (Adighibe et al., 2006). So far, vessel co-option is suggested to result, at least in part, of differential mitochondrial metabolism, but it may also involve reduced inflammation (Donnem et al., 2013). The ECs of co-opted vessels experience severe molecular changes during this process, for e.g., starting to express angiopoietin-2, which results in strong regression of vessels in the tumor core (Coelho et al., 2017). Thereupon, the tumor core becomes hypoxic, which consequently activates the angiogenic switch in tumor vessels (Holash et al., 1999). *In vitro* studies of glioma cells suggest that tumor cells that facilitate vessel co-option are dependent on the endoplasmic reticulum based stress sensing protein IRE1 (Auf et al., 2010). Furthermore the MMP-activating protein B2R was shown to serve as a chemoattractant during the migration of glioma cells towards blood vessels (Montana and Sontheimer, 2011). Finally, CDC42, a protein involved in actin-dependent formation of cytoplasmatic extensions, together with CD44, a protein crucial for establishing cell-cell contact, enable the connection between tumor cells and vessel covering pericytes for vessel co-option (Caspani et al., 2014).

Another non-angiogenic mechanism termed "VM" describes the process where cancer cells gain endothelial abilities to form their own circulatory network consisting of microvascular tubes to preserve blood supply (Pinto et al., 2016). So far, the molecular mechanism behind VM is not yet understood, however, it appears that VE-cadherin, the most prominent receptor on ECs, may play an important role. VE-cadherin on tumor cells can activate PI3K through the ERK1/ERK2 pathway which subsequently activates the metalloproteases MMP14 and pro-MMP2, resulting in remodeling of the ECM to enable cancer cells to be reorganized into vessel-like tubes (Paulis et al., 2010; Delgado-Bellido et al., 2017). VM networks resemble embryonic vasculogenesis, referring to a highly aggressive tumor cell phenotype that converted to an embryonic-like, undifferentiated state to facilitate tube formation (Maniotis et al., 1999). Gene expression analysis of VM networks in aggressive melanoma identified genes correlated with various cellular phenotypes such as fibroblasts, ECs and epithelial cells (Bittner et al., 2000; Seftor et al., 2002a,b). Tumors positive for VM show an increased expression of the ECM component laminin5γ2 and several MMPs, underlining the importance of ECM remodeling for initiating and promoting this non-angiogenic process (Seftor et al., 2001). Furthermore, VM is associated with poor prognosis as it is mainly observed in aggressive forms of melanoma and lung metastases (Williamson et al., 2016). Taking the potent impact of these non-angiogenic processes in cancer progression into consideration, may help us explain the occurring resistance of lung tumors to VEGF-inhibitors (Döme et al., 2007; Bergers and Hanahan, 2008).

In summary, the pathological features of tumor-associated ECs and non-ECs which result in a complex cancer promoting TME are diverse, and consequently contribute to therapy failure of angiogenesis inhibitors as well as other therapy approaches in a remarkable fashion. To better understand the biological mechanisms behind drug resistance or lack of clinical benefit, further investigation into the detailed characterization of the endothelial compartment in the TME are essential.

TRADITIONAL METHODS FOR VESSEL INHIBITION IN NSCLC

Currently used anti-angiogenic agents have been developed and approved for clinical application after intense study of their molecular, cellular, and physiological mode of action using various experimental approaches. In the following part we summarize currently available methods for investigating tumor

angiogenesis as well as anti-angiogenic agents that have already been accepted for treating NSCLC.

Methods to Study (Tumor) Angiogenesis

Experimental models remain the cornerstone for investigating tumor angiogenesis and the development of new anti-angiogenic therapies. As vessel sprouting is a multistep process there is a wide array of assays which enable individual evaluation of different stages, and each possesses specific advantages and disadvantages (Shahid et al., 2017; Stryker et al., 2019). To unravel these complex processes, it is crucial to understand the analytical potential of each model. *In vitro* methods represent the fundamental evaluation of tumor angiogenesis including basic functional analysis such as proliferation, migration, and tube formation. The big advantages of *in vitro* assays are their simplicity, high reproducibility, and cost effectiveness, while the disadvantages include the incomplete representation of the cellular heterogeneity and prevailing conditions present in human organs. Although findings from *in vitro* assays may never be conclusive alone, they serve as a preliminary projection of angiogenic processes upon treatment of choice and provide first insights into a testing hypothesis.

Ex vivo assays such as the thoracic aorta ring and retina angiogenesis methods represent the link between *in vitro* and *in vivo* analysis. Here, functional vasculature fragments of aorta/retina derived from mice or rats are immersed in a three-dimensional culturing system for evaluating vessel sprouting outgrowth under specific conditions. The advantage of this method over *in vitro* assays is the preservation of original EC properties within the tissue that are normally modified due to isolation processes and repeated passaging. The absence of blood flow and circulating EC progenitors or other factors constitute the main disadvantages of these methods.

For more accurate information regarding angiogenic processes upon treatment in a biological system or to perform long-term studies, *in vivo* methods are necessary. The most common systems to investigate angiogenesis in a living organism are the chicken chorioallantoic membrane (CAM) assay, matrigel plugs, and tumor xenograft models. CAM assays, which have already been in use for decades, utilize chorioallantoic membranes of fertilized chicken eggs to evaluate angiogenic processes. While this method is cost effective, highly reproducible and the outcomes are easily visualized, it must be taken into consideration that vessel growth is evaluated during developmental stages, which can affect studies investigating mechanisms in mature vasculature. Matrigel plug assays enable the use of an *in vitro* tool in an *in vivo* setting. Here, vascular growth is evaluated by injection of matrigel, a synthesized substrate resembling basement membrane matrix, into an animal model which allows easy stimulation, subsequent excision, and investigation of the plug with, for example, immunohistological stainings. Compared with CAM assays, the matrigel plug can be used in more analytical methods and provides a fast and reliable representation of angiogenic processes in a biological system. Nevertheless, this method may require more replicates due to higher variability of results and is therefore more expensive. Lastly, transplantation xenografts represent the most advanced method to investigate tumor angiogenesis in a living organism. Tumor cells, mostly of human origin, are injected into immunodeficient mice to induce formation of a cancer mass that can be further treated and monitored for changes regarding tumor angiogenesis. This method most suitably reflects the pathological mechanism of vessel growth *in vivo* in the presence of blood circulation, as well as diverse environmental factors. Furthermore, it enables the long-term study of diverse processes associated with angiogenesis that are observed in a biological system such as tissue invasion, distant metastasis formation as well as non-angiogenic processes like vessel co-option and VM, which are known to promote resistance mechanisms in various cancers. Aside from the ethical aspect, a considerable disadvantage of this method is the incomplete or lacking representation of the immune system due to immunosuppression of the study organism.

Examining which experimental assay is most suitable for investigating a chosen angiogenic process under certain conditions, necessitates extensive deliberation with the desired endpoint, required technical equipment, level of experimental throughput, cost, and ethics kept in mind. Additionally, the complexity of angiogenesis cannot be unraveled using a single analytical method but the thought-out application of multiple overlapping analyses, ranging from cellular to physiological levels, are necessary to obtain robust findings worth testing in the clinical setting.

Anti-angiogenic Therapies Approved for Treating NSCLC

In 2004, the first VEGFA-inhibiting antibody, bevacizumab, was approved for use in advanced colorectal cancer in combination with chemotherapy and was followed in 2006 in NSCLC (Sandler et al., 2006). Since then, diverse anti-angiogenic antibodies or tyrosine kinase inhibitors (TKIs) have been developed, which block either VEGF-A binding to the receptor or directly inhibit VEGFR-2 to hamper vascularization in tumors. VEGF-pathway inhibition has a broad anti-angiogenic effect in tumors: (1) it primarily inhibits vessel growth which induces regional cancer cell death and delays progression of the tumor rather than diminishing its size (Escudier et al., 2007); (2) it induces EC apoptosis as VEGF acts as a survival factor on the endothelium by activating BCL2, Akt signaling, or apoptosis inhibitors (Gerber et al., 1998; Fujio and Walsh, 1999); (3) it blocks the recruitment of hematopoietic or endothelial progenitor cells for new vessel formation which provides an essential function in neovascularization in growing tumors (Rafii et al., 2002; Bertolini et al., 2006). Angiogenesis inhibitors in combination with either chemotherapeutics, targeted therapies or ICI, in first or second-line therapies in NSCLC, have exhibited improved efficacy and feasible safety, which significantly improved response rates and prolonged progression free survival (PFS) in a large number of patients. Currently three anti-angiogenic agents, namely bevacizumab, ramucirumab and nintedanib are FDA/EMA approved for use in advanced stage NSCLC and are summarized in **Table 1** (Hall et al., 2015; Alshangiti et al., 2018; Janning and Loges, 2018) while more are in clinical testing. Despite the

TABLE 1 | Clinical studies of anti-angiogenesis based therapies in NSCLC that led to FDA/EMA approval.

Line	Population	Therapy-combination	Experimental arms	Phase	References
First	Stage IIIB-IV NSCLC	AAT + ChT	Bevacizumab + Carboplatin + Paclitaxel (*n* = 434)	III	Sandler et al., 2006
			Carboplatin + Paclitaxel (*n* = 444)		
	Advanced non-squamous NSCLC	AAT + ChT	Bevacizumab (low) + Cisplatin + Gemcitabine (*n* = 345)	III	Reck et al., 2009; Reck et al., 2010
			Bevacizumab (high) + Cisplatin + Gemcitabine (*n* = 351)		
			Cisplatin + Gemcitabine (*n* = 347)		
	Stage IIIB-IV non-squamous NSCLC	AAT + ChT	Bevacizumab + standard chemotherapy (*n* = 2212)	IV	Crinò et al., 2010
	Locally advanced, recurrent or metastatic NSCLC	AAT + ChT	Bevacizumab + Platin-based chemotherapy (*n* = 1313)	II/III	Soria et al., 2013
			Platin-based chemotherapy (*n* = 881)		
	Stage IIIB-IV EGFR-mutated non-squamous NSCLC	AAT + TT	Bevacizumab + Erlotinib (*n* = 75)	II	Seto et al., 2014; Yamamoto et al., 2018
			Erlotinib (*n* = 77)		
	Stage IIIB-IV EGFR-mutated non-squamous NSCLC	AAT + TT	Bevacizumab + Erlotinib (*n* = 114)	III	Saito et al., 2019
			Erlotinib (*n* = 114)		
	Stage IV EGFR-mutated NSCLC	AAT + TT	Ramucirumab + Erlotinib (*n* = 224)	II	Nakagawa et al., 2019
			Erlotinib (*n* = 225)		
	Metastatic non-squamous NSCLC	AAT + ChT + ICI	Bevacizumab + Atezolizumab + Carboplatin + Paclitaxel (*n* = 356)	III	Socinski et al., 2018
			Bevacizumab + Carboplatin + Paclitaxel (*n* = 336)		
Second	Stage IV squamous and non-squamous NSCLC	AAT + ChT	Ramucirumab + Docetaxel (*n* = 1253)	III	Garon et al., 2014; Reck et al., 2017
			Docetaxel (*n* = 625)		
	Stage IIIB-IV NSCLC	AAT + ChT	Nintedanib + Docetaxel (*n* = 655)	III	Reck et al., 2014; Novello et al., 2015
			Docetaxel (*n* = 654)		

remarkable clinical benefits of these combinational approaches on response rate and PFS, the overall survival (OS) benefits were modest due to acquired drug resistance. It is important to mention that in most lung cancer studies anti-angiogenic therapy is administered until the onset of severe drug related adverse effects or disease progression. So far, there is only preclinical evidence that discontinued angiogenesis inhibition results in TME reorganization and perhaps causes a rebound effect of tumor angiogenesis. In tumor and healthy mouse models, it could be shown that anti-VEGF therapy withdrawal resulted in rapid tissue revascularization and long lasting structural changes including vessel hyper-permeability and increased metastasis in the diseased cohort (Yang et al., 2016). The treatment-triggered hypoxia which induces angiogenesis especially during therapy-withdrawal is one possible explanation to this tumor promoting off-drug effect. The benefit of continuous anti-angiogenic therapy beyond disease progression in the clinical setting was first analyzed in a phase 3b trail in 2018 which included 485 advanced NSCLC patients (Gridelli et al., 2018). Here, bevacizumab was administered in addition to standard of care therapy beyond disease progression. While, the treatment continuation of bevacizumab yielded no substantial therapy benefit, improvements in efficacy, and no new safety signals were observed. Based on these findings, the approach of continuous angiogenesis inhibition should be further investigated but may be recommended at a certain degree in the future. Nevertheless, treatment decisions should be based on individual therapeutic efficacy, which needs to be tracked throughout the entire therapy. However, the absence of reliable biomarkers with predictive features for anti-angiogenic therapies hamper further therapy improvement, thus molecular screening for markers associated with tumor angiogenesis is currently of great value.

NEW MOLECULAR CANDIDATES TO PREDICT AND TRACK ANTI-ANGIOGENIC EFFICACY IN NSCLC

As previously mentioned, there is a great need for biomarkers to predict and track anti-angiogenic therapy efficacy, to help

overcome innate and acquired resistance as it is still the main obstacle that restrains clinical success (Bergers and Hanahan, 2008). So far, predictive angiogenesis-associated biomarkers in NSCLC are lacking, highlighting the need for further investigation to improve this anti-tumor approach.

In a recent study, it was demonstrated that immunohistochemically confirmed TTF-1 expression in advanced non-squamous NSCLC samples, which is a known prognostic biomarker of lung adenocarcinomas, could be linked to therapy success of bevacizumab in combination with pemetrexed plus platinum derivatives (Takeuchi et al., 2018). TTF-1 positive tumors exhibited enhanced clinical benefits when bevacizumab was combined with the basic therapy whereas TTF-1 negative tumors did not benefit from this addition.

Furthermore, despite the previous results of the IMpower150 study, where significant clinical benefits of bevacizumab in combination with ICI and chemotherapy were shown, regardless of PDL-L1 expression, a phase 1b study by Herbst et al. observed contrary results. They reported a beneficial efficacy of ramucirumab in combination with the PD-L1 inhibitor pembrolizumab especially in patients with a PD-L1 expression above 50% (Herbst et al., 2019). According to this, PD-L1 expression remains a predictive marker of ICI therapy or ICI therapy in combination with anti-angiogenesis agents in NSCLC. Qiu et al. recently examined the benefit of anti-angiogenic therapies (bevacizumab, anlotinib or others) with anti-PD-L1 agents (nivolumab or pembrolizumab) in a real-world study including 69 NSCLC patients. Subgroup analyses in the cohort revealed that the response and PFS of this combinational therapy was significantly higher when it was administered as first-line therapy compared to other lines of treatment, and when the therapy was initiated within the first 6 months of diagnosis compared to later time points (Qiu et al., 2020). Additionally, patients with EGFR wildtype tumors exhibited significantly prolonged PFS after the combinational therapy compared to patients with EGFR mutated tumors. Interestingly, no correlation between PDL-1 expression levels and the efficacy of this combinational therapy has been observed so far, however, follow up will be continued. In short, these study results can help to optimize the use of anti-angiogenic agents in combination with PD-L1 inhibitors, however, more factors need to be investigated to yield an optimal benefit.

Another potent multi-targeted anti-angiogenic TKI, anlotinib, has already shown profound benefits as third-line combinational therapy in advanced NSCLC (Han et al., 2018a,b). A transcriptomics study of an anlotinib-resistant lung cancer cell line, indicated that CXCL2, a cytokine involved in wound healing and angiogenesis, was also involved in anlotinib-resistance (Lu et al., 2019a). *In vitro* assays demonstrated that exogenous CXCL2 could recover anti-angiogenic-induced inhibition of migration and invasion and prevent apoptosis of anlotinib-resistant cells. Furthermore, in a retrospective analysis, anlotinib-induced decrease of the inflammatory cytokine CCL2 in serum correlated with prolonged PFS and OS (Lu et al., 2019b). Nevertheless, resistance and poor response to anlotinib hinder drug efficacy. While the underlying mechanisms are still unknown, elevated serum-levels of two angiogenesis-related markers KLK5 and L1CAM were recently correlated with poor response to anlotinib (Lu et al., 2019b).

Easily available predictive biomarkers, e.g., liquid biopsy, which allow the continuous track of response to angiogenesis inhibition are highly desired to optimize efficacy, as most of the current methods involve invasive procedures (biopsy or surgery) which limit analytical accessibility.

Several studies suggested a potential prognostic value of VEGF in NSCLC but so far investigations into circulating VEGF levels have not yielded consistent results (Rodríguez Garzotto et al., 2016). In the E4599 study, high VEGF levels in pretreatment plasma of 878 patients with advanced stage NSCLC, who received combinational treatment of bevacizumab plus chemotherapy, correlated with increased overall response but had no predictive outcome on survival (Dowlati et al., 2008). Another study observed contrary results when baseline plasma biomarkers of 303 non-squamous NSCLC patients undergoing similar therapy were evaluated (Mok et al., 2014). Here, baseline VEGFA levels in the plasma correlated with prolonged PFS and OS but showed no association with response rates to the therapy. The predictive value of VEGF or other proangiogenic factors on anti-angiogenic drug response is a highly discussed matter revealing vastly variable results. This is partly due to analytical variability, including sample collection and handling, as well as the disagreements regarding the most suitable sample choice for evaluating circulating factors (Rodríguez Garzotto et al., 2016). For example, serum or platelet rich plasma may not adequately represent the physiological VEGF level as it has been shown that the clotting processes initiates VEGF release in platelets (Webb et al., 1998). Moreover, the pathological situation can impact VEGF levels, as patients with more advanced tumors or several metastatic tumor sites exhibit a higher baseline level of plasma VEGFA, suggesting that VEGFA is linked to the tumor burden (Mok et al., 2014). Previously proposed correlations of circulating angiogenic factor levels with anti-angiogenic therapy efficacy in lung cancer seem to reflect tumor biology thus, have an important prognostic role rather than to be predictive (Crohns et al., 2010). The observed trend of increasing circulating factors in response to angiogenesis inhibition on one hand was shown to depend considerably on the TME and may represent therapy-induced hypoxia (Zaman et al., 2006; Kut et al., 2007). On the other hand, high VEGFA levels could also be attributed to TP53 mutated lung tumors which correlated with improved efficacy of bevacizumab (Schwaederlé et al., 2015). A currently identified alternative biomarker for bevacizumab-based chemotherapy combinations in patients with advanced NSCLC is CXCL16. In the analyzed sera of 40 advanced staged NSCLC patients therapy-induced decrease of CXCL16 levels correlated with prolonged OS compared with patients exhibiting only moderate decrement (Shibata et al., 2020).

However, confirming if any of these molecular markers indeed exhibit adequate predictive features necessitates further investigation. New aspects of processes which promote tumor angiogenesis, and a better understanding of the endothelium as driving force can help identify reliable biomarkers and overcome therapy failure in NSCLC.

MECHANISMS OF TUMOR VASCULARIZATION IN NSCLC

There are several mechanisms on both the cellular and environmental levels which can promote vessel formation in human tumors, many of which are not yet been completely elucidated. Although angiogenesis may represent the most important part of tumor vascularization, other processes that result in perfusion of the tumor tissue should be investigated in more detail and considered when designing new anti-angiogenic approaches in NSCLC.

In the following part we summarize various levels of tumor vascularization that may represent new targets for vessel inhibition in NSCLC. All mentioned mechanisms are summarized in **Figure 1**.

TEC Characteristics That Promote Tumor Vascularization in NSCLC

The endothelium is postulated to be a large contributor to the therapeutic efficacy of anti-angiogenic therapies, and therefore represents a possible source of therapy response or failure. It is well known that the process of angiogenesis is comprised of different EC phenotypes which execute distinct functions. During the elongation of the sprouting vessel VEGF-sensitive tip ECs migrate into avascular tissue regions, thus leading the proliferating trailing stalk ECs, which built up the growing vessel. Newly formed vasculature finally adapts a mature and quiescent phenotype referred to as phalanx ECs (Carmeliet and Jain, 2011; Betz et al., 2016). The EC phenotypes involved are highly dynamic and can reprogram the gene expression to meet their current physiological requirements. However, the tumor

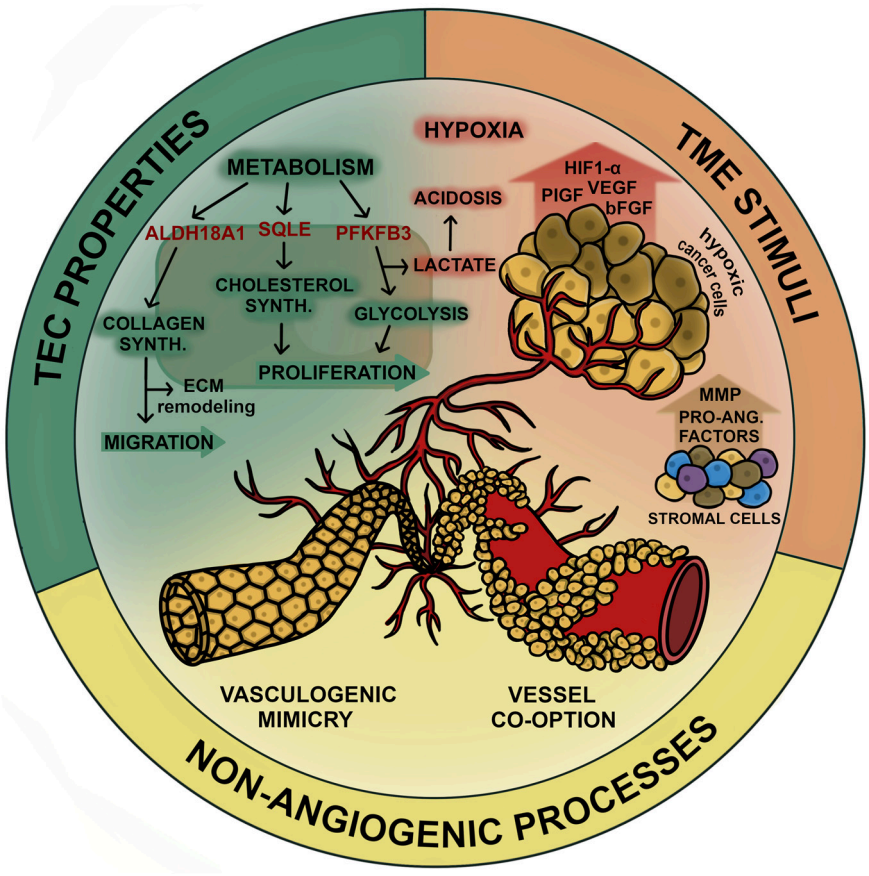


FIGURE 1 | Mechanisms of tumor vascularization in NSCLC. Tumor vascularization in lung cancer can be promoted by various processes which overlap during cancer progression. In general tumor vascularization/angiogenesis can be stimulated on the cellular level (TEC properties), the environmental level (TME stimuli) or facilitated in absence of angiogenic signaling (non-angiogenic processes). TECs exhibit upregulated metabolism to enable high angiogenic activity which includes processes involved in proliferation (cholesterol synthesis and glycolysis) and processes that enable migration via ECM remodeling (collagen synthesis). Potential targets involved in these pathways (SQLE, PFKFB3, and ALDH18A1, respectively) are considered to increase the angiogenic potential of TECs in NSCLC. Hypoxia and acidosis induced by high levels of lactate due to upregulated glycolysis constitute to a highly pro-angiogenic tumor environment. Angiogenesis stimulating factors (VEGF, bFGF, PDGF, HIF-1α, tryptase, and MMPs) are released by both, cancer cells and stromal cells, including fibroblasts, pericytes, tumor associated macrophages and ECs. Non-angiogenic processes constitute to tumor vascularization and are inaccessible for anti-angiogenic agents, thus contributing to therapy resistance. VM comprises the formation of tubular structures arising from cancer cells that gain endothelial like properties to maintain vascular supply during cancer progression. Another mechanism of cancer cells to persist in circulation is to grow along existing vasculature, which is referred to as vessel co-option. In this figure we summarized the various mechanism of tumor vascularization that should be considered when targeting the inhibition of tumor vessels in NSCLC.

endothelium was not studied in depth and a recent single-cell RNA sequencing (scRNA-Seq) study identified even more EC phenotypes from both healthy and tumor tissue from lung cancer samples as already known, indicating a much more complex phenotypic heterogeneity of the (tumor) vasculature than initially presumed (Goveia et al., 2020). Interestingly, although phenotype proportions differed strongly between analyzed NSCLC patients, they collectively observed a low abundance of tip and proliferating TECs, which represent the main targets of traditional anti-angiogenic therapy. Furthermore, they identified a so-far-unknown tumor exclusive phenotype of activated postcapillary vein EC that upregulated features known from HEVs in inflamed tissues such as immunomodulatory factors and ribosomal proteins. The unexpected finding that activated and proliferating TECs only represent a minority of the pathological EC phenotypes found in NSCLC, allows us to reconsider currently used anti-angiogenic therapy as less of a vessel-inhibiting strategy, and more of a strategy to modulate the higher proportion of mature TECs into potent participants of tumor surveillance.

In order to develop new angiogenesis-inhibiting therapies, the molecular differences between physiological and pathological ECs will need to be elaborated. Genetically TEC and NEC phenotypes significantly differ in gene expression affecting diverse cellular mechanisms such as proliferation, migration, inflammation, and angiogenesis (**Figure 2**). Previous studies have shown that one key feature of TECs is a highly active metabolism, which permits pathological processes as increased

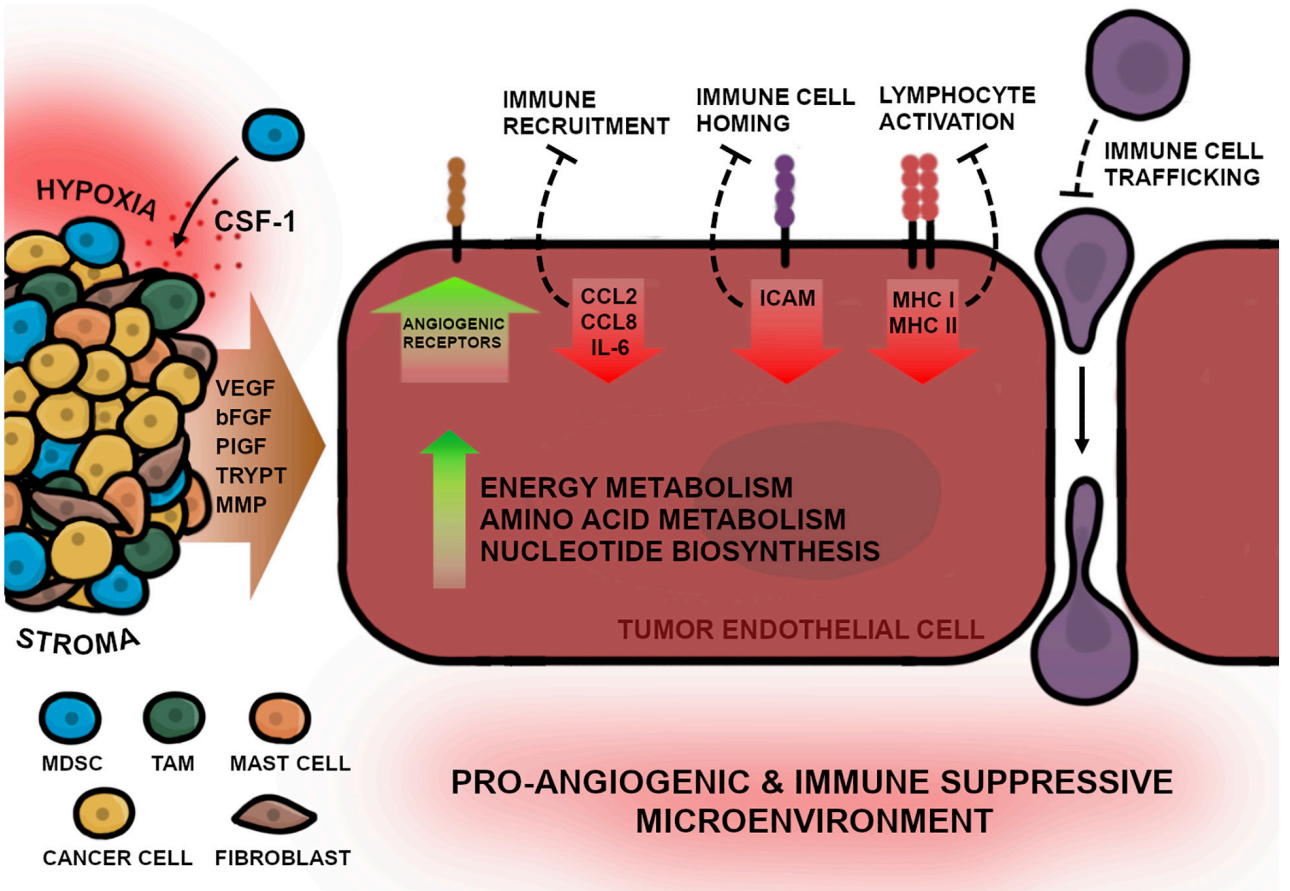


FIGURE 2 | The multifaced picture of TECs in NSCLC. TECs possess features that enable continuous angiogenic activity for progressing vascularization of the tumor. These features are ensured by genetical changes in the tumor endothelium that are triggered by diverse stimuli of the TME e.g., hypoxia and growth factor release. The stroma, consisting of various cells, promote angiogenesis by directly releasing signaling molecules into the adjacent tissue, thereby stimulating TECs. Fibroblasts and myeloid derived suppressor cells (MDSCs) activate angiogenesis by releasing VEGF and bFGF into the TME. Additionally, CSF-1 molecules, expressed by cancer cells, further recruit MDSCs into the tumor niche. Tumor associated macrophages (TAMs) can directly induce angiogenesis by releasing VEGF, bFGF, and PIGF, or indirectly by releasing matrix metalloproteinases (MMPs) which promote endothelial migration. Mast cells secrete tryptase (TRYPT) into the TME which stimulates EC proliferation and enables ECM remodeling. Furthermore, to facilitate enhanced angiogenesis, TECs upregulate the surface expression of angiogenic receptors as well as increase metabolic activity including energy and amino acid metabolism and the biosynthesis of nucleotides. In addition to the high angiogenic activity, TECs can directly suppress inflammatory responses by downregulation of inflammatory cytokines for immune cell recruitment (CCL2, CCL8, and IL-6), receptors required for immune cell homing (ICAM) or lymphocyte activation (MHC I and MHC II) which results in impaired immune cell trafficking and migration into the TME. In summary the complex interaction of tumor-protecting environmental conditions and the pathological features of TECs lead to a pro-angiogenic and immune suppressive TME in NSCLC.

proliferation and angiogenesis (Cantelmo et al., 2016). TECs exhibit upregulated glycolysis due to elevated expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), which regulates proliferation and migration during tumor angiogenesis (van der Veldt et al., 2012; de Bock et al., 2013). Hyperglycolytic TECs subsequently release high amounts of lactate into the environment, which in turn, further stimulates EC proliferation and angiogenesis (Annan et al., 2019). It could be demonstrated that inhibition of PFKFB3 resulted in improved drug efficacy and decreased metastatic events in tumor mouse models (Cantelmo et al., 2016). Another study in xenograft NSCLC mouse models exhibited that PFKFB3 mRNA silencing in combination with docetaxel results in a chemoenhancing effect and increases anti-cancer efficacy compared with monotherapies alone (Chowdhury et al., 2017). Furthermore, to sustain upregulated proliferative capacity, TECs exhibit elevated nucleotide biosynthesis including upstream pathways that are involved in serine and lipid synthesis (Cantelmo et al., 2016; Bruning et al., 2018; Li et al., 2019). In addition, Lambrechts et al. (2018) showed that MYC-targets, which are involved in transcription processes, were most upregulated in TECs of human NSCLC samples. Interestingly, c-MYC expression induces angiogenesis in combination with HIF-1α and VEGF (Lee and Wu, 2015) and recruits tryptase positive mast cells into the tumor niche (Soucek et al., 2007), therefore, MYC inhibition may have a potential anti-cancer effect.

Focusing on endothelial metabolism in cancer, a recent study could identify at least two metabolic signatures which are highly upregulated in angiogenic endothelium and TECs. One for proliferation, which includes gene sets associated with biomass production e.g., glycolysis, TCA cycle, fatty acid oxidation, cholesterol biosynthesis and amino acid metabolism, and one for remodeling of the extracellular matrix including gene sets for collagen biosynthesis in particular proline synthesis (Rohlenova et al., 2020). These results educed two new possible metabolic targets to hamper tumor angiogenesis; aldehyde dehydrogenase 18 family member A1 (ALDH18A1), an enzyme essential for *de novo* biosynthesis of proline; and squalene epoxidase (SQLE), the rate-limiting enzyme in cholesterol biosynthesis. Silencing of ALDH18A as well as SQLE impaired EC proliferation, migration and vessel sprouting in *in vitro* assays. Summarized, targeting endothelial metabolism in cancer is an interesting therapeutic option that could possibly assist an anti-angiogenic approach for treating NSCLC.

Another key feature of TECs in lung cancer is the downregulation of inflammatory responses thus contributing to tumor-associated immune escape. Single-cell analysis of NSCLC samples identified the most downregulated genes of the tumor endothelium in connection to inflammation, which included CCL2, CCL18, and IL6, essential for immune cell recruitment; MHC I and II, essential for immune cell activation; and ICAM, required for immune cell homing (Lambrechts et al., 2018). As the endothelium represents the primary connection between the immune system and tumor cells, these results indicate the important role of TECs in immunomodulatory processes that hamper anti-tumor immunity. It has been demonstrated that angiogenesis inhibition can restore proinflammatory surface proteins on TECs during a therapy-induced process termed "vessel normalization" (Goveia et al., 2020). Vessel normalization not only improves immune cell activation and infiltration, but is also suggested to enhance drug delivery to the tumor sites, thus improving its efficacy (Allen et al., 2017; Schmittnaegel et al., 2017). Additionally, combinational therapy of angiogenesis inhibitors and immunotherapy (anti-PD-L1) in previous studies could elicit the formation of unique blood vessels in treated tumors that resemble HEVs typically found in lymphoid tissues, which implicated increased treatment efficacy (Allen et al., 2017; Schmittnaegel et al., 2017). HEVs can mediate immune cell adhesion and migration into the tumor, which may be important for bypassing TEC-induced immune escape (Ager and May, 2015). In the already discussed scRNA-Seq study by Goveia et al., they demonstrated that VEGFR inhibition could induce vessel normalization by shifting invasive, low immunogenic TEC phenotypes to a more quiescent, immune-modulatory phenotype resembling HEVs (Goveia et al., 2020). These remarkable observations indicate that TECs comprise the ability to transform into HEVs to promote immune cell infiltration into the tumor and induce a potent anti-tumor response. This extends the previous observations of favorable synergistic effects of immune therapy in combination with angiogenesis inhibitors in NSCLC, especially when it results in HEV formation. Furthermore, direct induction of HEV formation could be a promising new strategy in anti-angiogenic approaches that may attain great clinical importance. However, currently there are no reliable biomarkers to track the process of vessel normalization or HEV formation in NSCLC which could help to predict and optimize this new treatment strategy.

Non-angiogenic Mechanisms in Association With Neovessel Inhibition in NSCL

As mentioned above, in some cases tumor vascularization can be facilitated by non-ECs which adapt certain properties to sustain access to the circulation, which may support anti-angiogenic drug resistance. During tumor progression, processes that lead to vascularization of the malignant tissue can vary locally as well as temporarily and involve angiogenic as well as non-angiogenic mechanisms even in the same lesion (Bridgeman et al., 2017). In lung tumors, where non-angiogenic tumor growth occurs most commonly, previous studies primarily located non-angiogenic processes in the tumor periphery, whereas angiogenesis is typically localized in the hypoxic tumor core (Pezzella et al., 1997; Donnem et al., 2018). Here, we briefly discuss the impact of non-angiogenic processes in NSCLC on anti-angiogenic drug efficacy based on previous studies.

VEGF-A inhibition using bevacizumab failed to inhibit VM in breast cancer cells *in vitro*, furthermore, sunitinib, a multi targeting anti-VEGFR inhibitor, even promoted VM in breast cancer mouse models (Dey et al., 2015; Sun et al., 2017). Additionally it could be demonstrated that VM in NSCLC depends on expression of Sema4D and its receptor plexinB1 which activate RhoA and downstream ROCK, comprising an already known angiogenesis-promoting process in tumors (Basile

et al., 2006). Inhibition of Sema4D or downregulation of plexinB1 resulted in RhoA/ROCK pathway inhibition and could reduce VM formation in human NSCLC cell lines (Xia et al., 2019). Although the role of VM in NSCLC is not fully understood, previous observations suggest that it may contribute to anti-angiogenic therapy failure and may serve as an option to treat aggressive lung tumors.

Vessel co-option on the other hand is a common phenomenon especially observed in lung metastases when tumor cells start to invade perivascular tissues (Jensen, 2016). Anti-angiogenic therapy with sunitinib could induce a switch from angiogenic vessel formation to vessel co-option in a lung metastatic mouse model, which ultimately resulted in sunitinib resistance (Bridgeman et al., 2017). Unfortunately, regulative mechanisms of vessel co-option in human tumors remain unknown in large part, however, predicting the occurrence of either VM or vessel co-option could be a useful tactic to prevent anti-angiogenic drug resistance in some patients. According to these and other results, it could be confirmed that non-angiogenic tumors contribute to anti-angiogenic therapy resistance which reveals the undoubted importance of targeting both angiogenic, but also non-angiogenic vessel growth to treat NSCLC (Donnem et al., 2018).

NEW ASPECTS FOR VESSEL INHIBITION IN NSCLC

Increasing knowledge of the physiological processes of tumor vascularization in addition to traditional angiogenesis has enlightened a variety of adaptive mechanisms which can promote anti-angiogenic therapy resistances. This awareness fortifies the necessity for alternative anti-angiogenic agents besides traditional anti-VEGF therapy.

New Targets for Vessel Inhibition in NSCLC

As previously examined, tumor angiogenesis depends on upregulated metabolic activity e.g., elevated cholesterol levels in TECs. Cholesterol not only represents a fundamental structural component of cell membranes and serves as precursor for several steroid hormones, it is also crucial for membrane function and angiogenic signaling, making it a favorable target for tumor vessel inhibition (Lyu et al., 2017). Inhibition of intracellular cholesterol trafficking with anti-inflammatory drug chepharantine was shown to hamper angiogenesis and tumor growth in lung cancer xenograft mice while improving anti-tumor activity of standard chemotherapeutics (Lyu et al., 2017). Another study has shown that pharmacological lowering of intracellular cholesterol levels with pitavastatin could reduce growth and migration and induced apoptosis in human lung tumor-associated ECs *in vitro* (Hu et al., 2020). *In vivo* experiments using lung cancer xenograft mice exhibited that pitavastatin-treatment could completely arrest tumor growth in these animals when combined with cisplatin and delayed tumor growth and impaired angiogenesis in cisplatin-resistant mouse models.

Another potential angiogenic target for cancer treatment is tie1. While the second tie receptor, tie2, is well characterized as a regulator during late stages of angiogenesis (e.g. vascular maturation or in quiescent ECs) via the angiopoetin/tie signaling pathway, an associated ligand for the less studied tie1, has not yet been identified (Augustin et al., 2009). In contrast to tie2, tie1 is upregulated in angiogenic vessels and downregulated in quiescent ECs and is somehow involved in the regulation of the angiopoietin/tie signaling cascade (Sato et al., 1995; Kim et al., 2016; Korhonen et al., 2016; La Porta et al., 2018). As tie1 is also upregulated in intratumoral vasculature, its deletion on ECs successfully produced a potent anti-angiogenic effect in different cancers (Kaipainen et al., 1994; Aguayo et al., 2001). In fact, EC-specific deletion of tie1 in lung carcinoma and melanoma mouse models resulted in delayed cancer growth, predominantly in late-stage tumors (La Porta et al., 2018). Furthermore, it inhibited neovessel sprouting and a reduced intratumoral vessel density, while the remaining mature vasculature became strongly normalized, which limited further metastatic formation. These findings, and the fact that tie1 expression is increased in angiogenic endothelium compared with resting vasculature, presents tie1 as a highly potent angiogenic target, especially in the treatment of advanced staged NSCLC.

Another considerable strategy of anti-angiogenic therapy could include targeting micro RNAs (miRNAs) as they represent a new paradigm in molecular cancer therapy. The impact of miRNAs in post-transcriptional regulation has already been associated with pathways involved in cancer and vascular disease as summarized in Sun et al., 2018. The following studies evaluated the potential role of specific angiogenesis-related miRNAs as targets in lung cancer. Hsu et al. observed that miR-23a, a micro RNA known to be hypoxia-associated, was overexpressed in exosomes of oxygen depleted CL1-5 lung cancer cells (Hsu et al., 2017). Furthermore, these cancer-cell derived exosomes could induce angiogenesis via HIF-1α signaling *in vitro* when internalized by HUVECs. Additionally, miR-23a transfection increased permeability and transendothelial migration of cancer cells *in vitro* by downregulation of the tight junction protein ZO-1 and stimulated neovascularization and tumor growth *in vivo* in CL1-5 xenograft mice, proposing it to be an appealing target for anti-angiogenic therapy. Upregulation of miR-195 in squamous lung cancer cells *in vitro* on the other hand could be associated with impaired VEGF expression and hampered migration and invasion, thereby facilitating a tumor-suppressive function. Additionally, overexpression of miR-195 in HUVECs was observed to inhibit tube formation and reduced the expression of VEGF, which hampered their angiogenesis activity *in vitro* (Liu et al., 2019).

As it is an essential process during vessel growth, targeting ECM remodeling may also be an interesting approach to inhibit tumor angiogenesis in NSCLC. The most prominent enzymes involved in this process are matrix-metalloporoteinases (MMPs) which are inhibited under physiological conditions by tissue inhibitors of metalloproteinases (TIMPs). miR-130b could be identified as a promotor of MMP-2 activity and invasion of NSCLC cancer cells *in vitro* by downregulation of TIMP-2. Additionally, it could be observed that miR-130b was significantly upregulated in tumor tissue of NSCLC patients with vascular cancer cell invasion (Hirono et al., 2019). According to these

findings, targeting miR-130b could be a strategy to impede angiogenesis and cancer cell invasion in lung cancer.

Uribesalgo et al. suggested targeting the apelin signaling pathway to inhibit tumor vessel formation in lung cancer (Uribesalgo et al., 2019). Apelin is a conserved peptide involved in developmental angiogenesis and is also upregulated in ECs within the TME. Previous studies could associate high apelin levels with a poor clinical outcome in patients with NSCLC (Györffy et al., 2010). In murine lung cancer models, apelin knockout reduced tumor burden and prolonged survival by inhibiting VEGF, TGF-β1, and TNF-α and simultaneously decreased MDSC infiltration in the TME (Uribesalgo et al., 2019). The combination of pharmacological inhibition of apelin with the anti-angiogenic drug sunitinib in lung cancer and mammary cancer mouse models, significantly delayed tumor growth and could almost double the survival, even in the KRAS driven or p53 mutated tumors, when compared with sunitinib treatment alone. Finally, apelin loss also reduced vessel density and prevented sunitinib-induced hypoxia and poor vessel structure in the TME. Conclusively, apelin inhibition may provide a potent synergistic anti-tumor effect when combined with anti-angiogenic agents, while, and most importantly, avoiding therapy-induced hypoxia of the TME, thus decreasing the chance of metastases, and bypassing potential therapy resistances.

New Therapy Approaches for Vessel Inhibition in NSCLC

Single-target anti-angiogenic agents have already shown their limitations in clinical settings (Jayson et al., 2016). Even in combination with other therapy approaches like standard chemotherapy or immune therapy, treatment success remains largely marginal. Targeting several pro-angiogenic molecules with recombinant fusion proteins could therefore increase the anti-angiogenic effect of such therapies. Zhang et al. (2020) could successfully establish a multi-epitope peptibody containing bFGF and VEGF sequences, which could provoke a potent anti-bFGF/VEGF response by inhibiting proliferation and migration of lung cancer cells as well as HUVECs *in vitro*. When injected into lung cancer mouse models, autologous generated anti-peptibody antibodies inhibited tumor progression and angiogenesis and decreased expression of bFGF, VEGFA and PDGF in the tumor tissue. Targeting angiogenesis with fusion proteins exhibited potent anti-tumor efficacy in murine models and may represent a new approach for vessel inhibition in NSCLC, especially in combination with other therapy agents aimed at important angiogenic factors, previously discussed potential TEC specific markers or cellular mechanisms (**Table 2**).

The instability of tumor vessels due to morphological abnormalities (e.g., incomplete pericyte coverage) impedes drug delivery to the local lesion. Although anti-angiogenic therapy can temporarily restore tissue perfusion and drug delivery by vascular normalization, treatment withdrawal often results in vessel hyper-permeability and can even induce a rebound effect of tumor angiogenesis (Yang et al., 2016). As continuous inhibition of angiogenesis remains difficult to implement for health or economic reasons, an alternative or more independent delivery system of anti-angiogenic agents could help to overcome these issues. Nanomaterials have become an emerging field in cancer therapy in recent years, as their unique molecular properties make them suitable targeted drug delivery-systems. Physiochemically, these nanoparticles match the size of inter-endothelial junctions of blood vessels in the TME and therefore increase permeation and retention (EPR) resulting in a passive drug delivery (Chauhan and Jain, 2013). Nanomaterials such as liposomes or nanotube carbon structures are used to deliver anti-angiogenic agents and improve drug specificity while reducing cytotoxic side effects, drug clearance and resistance mechanisms in the treatment of NSCLC (Seshadri and Ramamurthi, 2018). In the past, studies using biodegradable polymers as nanocarriers

TABLE 2 | Pro-angiogenic factors involved in angiogenesis.

Factor	Abbreviation	Angiogenic function
Vascular endothelial growth factor	VEGF	Inducing angiogenesis by stimulating proliferation, survival, and migration of ECs
Basic fibroblast growth factor	bFGF	Inducing angiogenesis by stimulating proliferation and migration of ECs and extracellular matrix degradation
Hypoxia-inducible factor 1-alpha	HIF-1α	Regulating proangiogenic factor expression under oxygen depletion
Platelet-derived growth factor	PDGF	Inducing angiogenesis by stimulating proliferation, migration and tube formation of ECs and regulating VEGF signaling
Tryptase	TRYPT	Inducing angiogenesis by stimulating proliferation of ECs and vascular tube formation
Colony stimulating factor 1	CSF-1	Inducing release of proangiogenic factors by MDSC
Placental growth factor	PlGF	Inducing angiogenesis by stimulating proliferation, survival and migration of ECs and recruiting proangiogenic macrophages
Angiopoietin-2	Ang2	Regulating neovascular remodeling, vessel maturation and sensitizing ECs to cytokines
Matrix metalloproteases	MMPs	Remodeling of ECM for endothelial migration
6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase	PFKFB3	Metabolic regulation of proliferation and migration in ECs
MYC		Inducing angiogenesis in combination with HIF-1α and VEGF
Aldehyde dehydrogenase 18 family member A1	ALDH18A1	Regulating collagen biosynthesis for remodeling of ECM for endothelial migration
Squalene epoxidase	SQLE	Regulating cholesterol biosynthesis for EC proliferation
Apelin	APLN	Involved in developmental angiogenesis and expressed in angiogenic ECs
miR-23a		Inducing angiogenesis via HIF-1α signaling
Fibronectin/EIIIB		Remodeling of ECM for endothelial migration

to deliver chemotherapeutics and targeted drugs exhibited significant anti-tumor efficacy *in vitro* and *in vivo*. For example, paclitaxel encapsulated aldehyde polyethylene glycol-polylactide (PEG-PLGA) conjugated to a VEGFR2-inhibiting peptide showed increased internalization in HUVECs *in vitro* as well as potent activity against breast cancer models *in vivo* (Yu et al., 2010). Although there are several peptide motifs that are suggested to target tumor endothelium such as RGD or NGR which can bind integrin heterodimers CD51 and CD61, or aminopeptidase N, respectively, their targeting with nanomaterial is not yet applied for treating NSCLC (Sakurai et al., 2019). Furthermore, non-angiogenic mechanisms such as VM or vessel co-option could also represent possible targets for nanomaterial-based therapy as the EPR effect of such molecules could help to overcome delivery and infiltration issues of traditional cancer therapeutics. However, nanotherapeutics may provide a new potential anti-angiogenic therapeutical approach, but as already discussed, there is still a need for more specific biomarkers to exclusively target tumor vasculature in an organ specific manner. Taking this into consideration, chimeric antigen receptor (CAR) T-cell therapy, which serves as personalized immune therapy using autologous T-lymphocytes, engineered to target specific antigens present in a tumor, could be used to exclusively eliminate TECs without damaging healthy vasculature. CAR T-cells already have shown remarkable clinical success in liquid malignancies like B-cell acute leukemia with response rates above 80%, however, this outstanding efficacy has not yet been translated into the solid tumor setting. The therapy failure can, at least in part, be attributed to the impaired accessibility of the tumor mass due to dysfunctional vasculature and immunosuppressive conditions in the TME. Targeting tumor vessels directly with CAR T-cells could therefore be a good strategy to overcome these issues, which at best, can normalize the defective vasculature and improve drug efficacy in combinational therapy settings. In a recent study Xie et al. (2019) generated VHH-based CAR T-cells targeting EIIIB, an alternatively spliced domain of fibronectin, which is strongly expressed during angiogenesis. Injected EIIIB-targeting CAR T-cells could delay tumor growth and improve survival in immunocompetent mouse models harboring aggressive melanoma, whereas colorectal cancer mouse models did not respond to the treatment. Here, the expression levels of EIIIB in the different tissues had impact on the therapy outcome which again highlights the importance of organ specific vascular markers as well as the impact of organ specific angiogenic activity when targeting tumor vessel formation. Other studies investigated the anti-angiogenic efficacy of TEM8-specific CAR T cells in solid cancer mouse models. TEM8 is one of the first discovered TEC markers and represents a promising target in anti-angiogenic therapy strategies (St Croix et al., 2000). In 2018, a study reported that TEM8-specific CAR T-cells could improve survival and significantly decreased vascularization in triple negative breast cancer mouse models and induced tumor regression in mice with lung metastases (Byrd et al., 2018). A more recent study, however, observed contrasting results where TEM8-sepcific CAR T-cells triggered high toxicity and induced inflammation in lung and spleen when injected into healthy mice (Petrovic et al., 2019). It is suggested that the engineered T-cells cross-reacted with other antigens or targeted TEM8 in healthy tissues, although it is normally expressed at a much lower quantity compared with pathological levels. However, both processes resulted in severe toxicity *in vivo* and again emphasize the need for more adequate, highly specific tumor-vessel exclusive markers that can be targeted with either CAR T-cells or other previously discussed inhibiting molecules.

So far, the main obstacles of anti-angiogenic therapy in NSCLC are evading- or intrinsic resistance mechanisms which still remain elusive. We have discussed a wide array of possible therapies and therapy systems that could improve anti-angiogenic efficacy when combined with standard treatment. The principal goal would be to expand the therapeutical effect of angiogenesis-inhibiting drugs on vessel normalization and render the tumor more vulnerable to additional agents such as chemotherapy or immunotherapy. In a recent study, Hosaka et al. could show that dual angiogenesis inhibition could sensitize resistant off-target tumors to therapy. Therefore they created mouse models of breast cancer or fibrosarcoma, both resistant to anti-VEGF and anti-PDGF treatment due to increased tumor associated expression of bFGF, a molecule which modulates the vasculature via pericyte recruitment in a PDGF-dependent process (Hosaka et al., 2020). Neither anti-VEGF nor anti-PDGF monotherapy had a significant anti-tumor effect on bFGF-positive tumors, but the combination of both agents produced a superior benefit, inhibiting cancer growth by suppressing proliferation and triggering apoptosis of tumor cells. Interestingly, even the pan-blocking of FGF-receptors did not yield a comparable benefit. To explain this unexpected effect, angiogenesis has to be considered as an interacting network of various signaling pathways which cannot be disrupted by blocking a single molecule. In this study the off-target anti-VEGF/PDGF therapy generated a synergistical effect in which PDGF inhibition ablated bFGF-dependent perivascular coverage which further sensitized tumor vessels to anti-VEGF inhibition. These findings demonstrate that the disruption of interacting angiogenic pathways by simultaneously targeting multiple angiogenic factors can provoke a highly potent anti-tumor effect which is able to circumvent mechanisms of therapy resistance, and thus should be considered as new approach to improve neovessel inhibition in cancer. While physiological, as well as pathological vascularization is comprised of diverse molecular pathways, several of which may serve as new targets, some in particular, such as VEGF/VEGFR signaling, represent key players of angiogenesis and should remain an irreplaceable anchor of anti-angiogenic therapy approaches in NSCLC.

DISCUSSION

Angiogenesis is a main therapeutic concept in oncology, especially in NSCLC, where three approved agents are available in combination with chemotherapy or immunotherapy. Nevertheless, the therapeutic efficacy of the current anti-angiogenic therapies is not satisfying and needs a more personalized/individualized approach. Increasing knowledge

in angiogenic processes and non-angiogenic processes that contribute to tumor vascularization, provide precise targets for novel therapy strategies and pave the way for developing new anti-angiogenic treatment concepts that target e.g., TEC metabolism, TEC specific factors, tumor vessel normalization and combinational approaches with CAR T-cells. These therapeutic concepts need to be evaluated for synergistic effects as, in our view, modern anti-angiogenesis represents the concept of shaping the TME rather than being a direct anti-tumor therapy itself. However, these therapeutic strategies are very promising in preclinical setting and the translation into a clinical setting is not only warranted but highly desired. Furthermore, a new horizon of targeted and functional TEC characterization was opened by scRNA-Seq studies, which proved that the tumor vasculature is highly heterogenous and differs from the normal adjacent vasculature more than primarily assumed in terms of metabolic activity, immune suppression and heterogeneity for example. In addition, new synergistic effects of TECs in their role of immunomodulation were identified and induction of HEV formation for immune priming is suggested to be a new therapeutic strategy. Next the organ specific context of the vasculature plays an important role and has to be further studied for better therapy allocation.

In conclusion the concept and goal of anti-angiogenesis in NSCLC in the future can be reshaped by abolishing the traditional vessel priming concept and moving toward a side specific molding of the TME, using the tumor vasculature as a tool, like a trojan horse.

AUTHOR CONTRIBUTIONS

SD, HH, EN, AP, and DW developed the concept of the review. SD, HH, and EN drafted the review. DW and AP corrected and reviewed the review. All authors contributed to the article and approved the submitted version.

REFERENCES

Adighibe, O., Micklem, K., Campo, L., Ferguson, M., Harris, A., Pozos, R., et al. (2006). Is nonangiogenesis a novel pathway for cancer progression? A study using 3-dimensional tumour reconstructions. *Br. J. Cancer* 94, 1176–1179. doi: 10.1038/sj.bjc.6603039

Ager, A., and May, M. J. (2015). Understanding high endothelial venules: lessons for cancer immunology. *Oncoimmunology* 4:e1008791. doi: 10.1080/2162402X.2015.1008791

Aguayo, A., Manshouri, T., O'Brien, S., Keating, M., Beran, M., Koller, C., et al. (2001). Clinical relevance of Flt1 and Tie1 angiogenesis receptors expression in B-cell chronic lymphocytic leukemia (CLL). *Leukemia Res.* 25, 279–285. doi: 10.1016/S0145-2126(00)00139-9

Allen, E., Jabouille, A., Rivera, L. B., Lodewijckx, I., Missiaen, R., Steri, V., et al. (2017). Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci. Transl. Med.* 9:eaak9679. doi: 10.1126/scitranslmed.aak9679

Alshangiti, A., Chandhoke, G., and Ellis, P. M. (2018). Antiangiogenic therapies in non-small-cell lung cancer. *Curr. Oncol.* 25, S45–S58. doi: 10.3747/co.25.3747

Annan, D. A., Maishi, N., Soga, T., Dawood, R., Li, C., Kikuchi, H., et al. (2019). Carbonic anhydrase 2 (CAII) supports tumor blood endothelial cell survival under lactic acidosis in the tumor microenvironment. *Cell Commun. Signal.* 17:169. doi: 10.1186/s12964-019-0478-4

Auf, G., Jabouille, A., Guérit, S., Pineau, R., Delugin, M., Bouchecareilh, M., et al. (2010). Inositol-requiring enzyme 1alpha is a key regulator of angiogenesis and invasion in malignant glioma. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15553–15558. doi: 10.1073/pnas.0914072107

Augustin, H. G., Koh, G. Y., Thurston, G., and Alitalo, K. (2009). Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat. Rev.. Mol. Cell Biol.* 10, 165–177. doi: 10.1038/nrm2639

Augustine, R., Prasad, P., and Khalaf, I. M. N. (2019). Therapeutic angiogenesis: from conventional approaches to recent nanotechnology-based interventions. *Mater. Sci. Eng. C Mater. biol. Appl.* 97, 994–1008. doi: 10.1016/j.msec.2019.01.006

Babina, I. S., and Turner, N. C. (2017). Advances and challenges in targeting FGFR signalling in cancer. *Nat. Rev. Cancer* 17, 318–332. doi: 10.1038/nrc.2017.8

Basile, J. R., Castilho, R. M., Williams, V. P., and Gutkind, J. S. (2006). Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9017–9022. doi: 10.1073/pnas.0508825103

Bergers, G., and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* 3, 401–410. doi: 10.1038/nrc1093

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Bertolini, F., Shaked, Y., Mancuso, P., and Kerbel, R. S. (2006). The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat. Rev. Cancer* 6, 835–845. doi: 10.1038/nrc1971

Betz, C., Lenard, A., Belting, H.-G., and Affolter, M. (2016). Cell behaviors and dynamics during angiogenesis. *Development* 143, 2249–2260. doi: 10.1242/dev.135616

Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., et al. (2000). Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 406, 536–540. doi: 10.1038/35020115

de Bock, K., Georgiadou, M., Schoors, S., Kuchnio, A., Wong, B. W., Cantelmo, A. R., et al. (2013). Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 154, 651–663. doi: 10.1016/j.cell.2013.06.037

Bridgeman, V. L., Vermeulen, P. B., Foo, S., Bilecz, A., Daley, F., Kostaras, E., et al. (2017). Vessel co-option is common in human lung metastases and mediates resistance to anti-angiogenic therapy in preclinical lung metastasis models. *J. Pathol.* 241, 362–374. doi: 10.1002/path.4845

Bruning, U., Morales-Rodriguez, F., Kalucka, J., Goveia, J., Taverna, F., Queiroz, K. C. S., et al. (2018). Impairment of Angiogenesis by Fatty Acid Synthase Inhibition Involves mTOR Malonylation. *Cell Metab.* 28, 866.e–880.e. doi: 10.1016/j.cmet.2018.07.019

Byrd, T. T., Fousek, K., Pignata, A., Szot, C., Samaha, H., Seaman, S., et al. (2018). TEM8/ANTXR1-Specific CAR T cells as a targeted therapy for triple-negative breast cancer. *Cancer Res.* 78, 489–500. doi: 10.1158/0008-5472.CAN-16-1911

Cantelmo, A. R., Conradi, L.-C., Brajic, A., Goveia, J., Kalucka, J., Pircher, A., et al. (2016). Inhibition of the Glycolytic Activator PFKFB3 in Endothelium Induces Tumor Vessel Normalization. Impairs Metastasis, and Improves Chemotherapy. *Cancer Cell* 30, 968–985. doi: 10.1016/j.ccell.2016.10.006

Carlini, M. J., Dalurzo, M. C. L., Lastiri, J. M., Smith, D. E., Vasallo, B. C., Puricelli, L. I., et al. (2010). Mast cell phenotypes and microvessels in non-small cell lung cancer and its prognostic significance. *Hum. Pathol.* 41, 697–705. doi: 10.1016/j.humpath.2009.04.029

Carmeliet, P., and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307. doi: 10.1038/nature10144

Caspani, E. M., Crossley, P. H., Redondo-Garcia, C., and Martinez, S. (2014). Glioblastoma: a pathogenic crosstalk between tumor cells and pericytes. *PLoS One* 9:e101402. doi: 10.1371/journal.pone.0101402

Chauhan, V. P., and Jain, R. K. (2013). Strategies for advancing cancer nanomedicine. *Nat. Mater.* 12, 958–962. doi: 10.1038/nmat3792

Chowdhury, N., Vhora, I., Patel, K., Doddapaneni, R., Mondal, A., and Singh, M. (2017). Liposomes co-Loaded with 6-Phosphofructo-2-Kinase/Fructose-2, 6-Biphosphatase 3 (PFKFB3) shRNA Plasmid and Docetaxel for the Treatment of non-small Cell Lung Cancer. *Pharm. Res.* 34, 2371–2384. doi: 10.1007/s11095-017-2244-x

Coelho, A. L., Gomes, M. P., Catarino, R. J., Rolfo, C., Lopes, A. M., Medeiros, R. M., et al. (2017). Angiogenesis in NSCLC: is vessel co-option the trunk that sustains the branches? *Oncotarget* 8, 39795–39804. doi: 10.18632/oncotarget.7794

Colegio, O. R., Chu, N.-Q., Szabo, A. L., Chu, T., Rhebergen, A. M., Jairam, V., et al. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 513, 559–563. doi: 10.1038/nature13490

Crawford, Y., Kasman, I., Yu, L., Zhong, C., Wu, X., Modrusan, Z., et al. (2009). PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* 15, 21–34. doi: 10.1016/j.ccr.2008.12.004

Crinò, L., Dansin, E., Garrido, P., Griesinger, F., Laskin, J., Pavlakis, N., et al. (2010). Safety and efficacy of first-line bevacizumab-based therapy in advanced non-squamous non-small-cell lung cancer (SAiL, MO19390): a phase 4 study. *Lancet Oncol.* 11, 733–740. doi: 10.1016/S1470-2045(10)70151-0

Crohns, M., Saarelainen, S., Laine, S., Poussa, T., Alho, H., and Kellokumpu-Lehtinen, P. (2010). Cytokines in bronchoalveolar lavage fluid and serum of lung cancer patients during radiotherapy - Association of interleukin-8 and VEGF with survival. *Cytokine* 50, 30–36. doi: 10.1016/j.cyto.2009.11.017

Delgado-Bellido, D., Serrano-Saenz, S., Fernández-Cortés, M., and Oliver, F. J. (2017). Vasculogenic mimicry signaling revisited: focus on non-vascular VE-cadherin. *Mol. Cancer* 16:65. doi: 10.1186/s12943-017-0631-x

Dey, N., De, P., and Brian, L.-J. (2015). Evading anti-angiogenic therapy: resistance to anti-angiogenic therapy in solid tumors. *Am. J. Transl. Res.* 7, 1675–1698.

Döme, B., Hendrix, M. J. C., Paku, S., Tóvári, J., and Tímár, J. (2007). Alternative vascularization mechanisms in cancer: pathology and therapeutic implications. *Am. J. Pathol.* 170, 1–15. doi: 10.2353/ajpath.2007.060302

Donnem, T., Hu, J., Ferguson, M., Adighibe, O., Snell, C., Harris, A. L., et al. (2013). Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? *Cancer Med.* 2, 427–436. doi: 10.1002/cam4.105

Donnem, T., Reynolds, A. R., Kuczynski, E. A., Gatter, K., Vermeulen, P. B., Kerbel, R. S., et al. (2018). Non-angiogenic tumours and their influence on cancer biology. *Nat. Rev.. Cancer* 18, 323–336. doi: 10.1038/nrc.2018.14

Dowlati, A., Gray, R., Sandler, A. B., Schiller, J. H., and Johnson, D. H. (2008). Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small cell lung cancer treated with chemotherapy with or without bevacizumab–an Eastern Cooperative Oncology Group Study. *Clin. Cancer Res.* 14, 1407–1412. doi: 10.1158/1078-0432.CCR-07-1154

Escudier, B., Eisen, T., Stadler, W. M., Szczylik, C., Oudard, S., Siebels, M., et al. (2007). Sorafenib in advanced clear-cell renal-cell carcinoma. *New Eng. J. Med.* 356, 125–134. doi: 10.1056/NEJMoa060655

Fridman, W. H., Pagès, F., Sautès-Fridman, C., and Galon, J. (2012). The immune contexture in human tumours: impact on clinical outcome. *Nat. Rev.. Cancer* 12, 298–306. doi: 10.1038/nrc3245

Fujio, Y., and Walsh, K. (1999). Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J. Biol. Chem.* 274, 16349–16354. doi: 10.1074/jbc.274.23.16349

Garon, E. B., Ciuleanu, T.-E., Arrieta, O., Prabhash, K., Syrigos, K. N., Goksel, T., et al. (2014). Ramucirumab plus docetaxel versus placebo plus docetaxel for second-line treatment of stage IV non-small-cell lung cancer after disease progression on platinum-based therapy (REVEL): a multicentre, double-blind, randomised phase 3 trial. *Lancet* 384, 665–673. doi: 10.1016/S0140-6736(14)60845-X

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V., et al. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* 273, 30336–30343. doi: 10.1074/jbc.273.46.30336

Góth, M. I., Hubina, E., Raptis, S., Nagy, G. M., and Tóth, B. E. (2003). Physiological and pathological angiogenesis in the endocrine system. *Microscopy Res. Tech.* 60, 98–106. doi: 10.1002/jemt.10248

Goveia, J., Rohlenova, K., Taverna, F., Treps, L., Conradi, L.-C., Pircher, A., et al. (2020). An integrated gene expression landscape profiling approach to identify lung tumor endothelial cell heterogeneity and angiogenic candidates. *Cancer Cell* 37, 21.e–36.e. doi: 10.1016/j.ccell.2019.12.001

Gridelli, C., Castro Carpeno, J., de, Dingemans, A.-M. C., Griesinger, F., Grossi, F., et al. (2018). Safety and efficacy of bevacizumab plus standard-of-care treatment beyond disease progression in patients with advanced non-small cell lung cancer: the AvaALL randomized clinical trial. *JAMA Oncol.* 4:e183486. doi: 10.1001/jamaoncol.2018.3486

Győrffy, B., Lanczky, A., Eklund, A. C., Denkert, C., Budczies, J., Li, Q., et al. (2010). An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treatment* 123, 725–731. doi: 10.1007/s10549-009-0674-9

Hall, R. D., Le, T. M., Haggstrom, D. E., and Gentzler, R. D. (2015). Angiogenesis inhibition as a therapeutic strategy in non-small cell lung cancer (NSCLC). *Transl. Lung Cancer Res.* 4, 515–523. doi: 10.3978/j.issn.2218-6751.2015.06.09

Han, B., Li, K., Wang, Q., Zhang, L., Shi, J., Wang, Z., et al. (2018a). Effect of anlotinib as a third-line or further treatment on overall survival of patients with advanced non-small cell lung cancer: the alter 0303 phase 3 randomized clinical trial. *JAMA Oncol.* 4, 1569–1575. doi: 10.1001/jamaoncol.2018.3039

Han, B., Li, K., Zhao, Y., Li, B., Cheng, Y., Zhou, J., et al. (2018b). Anlotinib as a third-line therapy in patients with refractory advanced non-small-cell lung cancer: a multicentre, randomised phase II trial (ALTER0302). *Br. J. Cancer* 118, 654–661. doi: 10.1038/bjc.2017.478

Hanahan, D., and Folkman, J. (1996). Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis. *Cell* 86, 353–364. doi: 10.1016/s0092-8674(00)80108-7

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Herbst, R. S., Arkenau, H.-T., Santana-Davila, R., Calvo, E., Paz-Ares, L., Cassier, P. A., et al. (2019). Ramucirumab plus pembrolizumab in patients with previously treated advanced non-small-cell lung cancer, gastro-oesophageal cancer, or urothelial carcinomas (JVDF): a multicohort, non-randomised, open-label, phase 1a/b trial. *Lancet Oncol.* 20, 1109–1123. doi: 10.1016/S1470-2045(19)30458-9

Hida, K., Maishi, N., Torii, C., and Hida, Y. (2016). Tumor angiogenesis–characteristics of tumor endothelial cells. *Int. J. Clin. Oncol.* 21, 206–212. doi: 10.1007/s10147-016-0957-1

Hirono, T., Jingushi, K., Nagata, T., Sato, M., Minami, K., Aoki, M., et al. (2019). MicroRNA-130b functions as an oncomiRNA in non-small cell lung cancer by targeting tissue inhibitor of metalloproteinase-2. *Sci. Rep.* 9:6956. doi: 10.1038/s41598-019-43355-8

Holash, J., Wiegand, S. J., and Yancopoulos, G. D. (1999). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 18, 5356–5362. doi: 10.1038/sj.onc.1203035

Hosaka, K., Yang, Y., Seki, T., Du, Q., Jing, X., He, X., et al. (2020). Therapeutic paradigm of dual targeting VEGF and PDGF for effectively treating FGF-2 off-target tumors. *Nat. Commun.* 11:3704. doi: 10.1038/s41467-020-17525-6

Hsu, Y.-L., Hung, J.-Y., Chang, W.-A., Lin, Y.-S., Pan, Y.-C., Tsai, P.-H., et al. (2017). Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1. *Oncogene* 36, 4929–4942. doi: 10.1038/onc.2017.105

Hu, T., Shen, H., Huang, H., Yang, Z., Zhou, Y., and Zhao, G. (2020). Cholesterol-lowering drug pitavastatin targets lung cancer and angiogenesis via suppressing prenylation-dependent Ras/Raf/MEK and PI3K/Akt/mTOR signaling. *Anti Cancer Drugs* 31, 377–384. doi: 10.1097/CAD.0000000000000885

Huang, B., Lei, Z., Zhang, G.-M., Li, D., Song, C., Li, B., et al. (2008). SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment. *Blood* 112, 1269–1279. doi: 10.1182/blood-2008-03-147033

Ibaraki, T., Muramatsu, M., Takai, S., Jin, D., Maruyama, H., Orino, T., et al. (2005). The relationship of tryptase- and chymase-positive mast cells to angiogenesis in stage I non-small cell lung cancer. *Eur. J. Cardio-Thorac. Surg.* 28, 617–621. doi: 10.1016/j.ejcts.2005.06.020

Janning, M., and Loges, S. (2018). Anti-Angiogenics: their Value in Lung Cancer Therapy. *Oncol. Res. Treat.* 41, 172–180. doi: 10.1159/000488119

Jayson, G. C., Kerbel, R., Ellis, L. M., and Harris, A. L. (2016). Antiangiogenic therapy in oncology: current status and future directions. *Lancet* 388, 518–529. doi: 10.1016/S0140-6736(15)01088-0

Jensen, L. D. (2016). When tumors are (co-)opting to resist anti-angiogenic treatment. *Transl. Cancer Res.* 5, S1433–S1436. doi: 10.21037/tcr.2016.12.35

Johnson, D. H., Fehrenbacher, L., Novotny, W. F., Herbst, R. S., Nemunaitis, J. J., Jablons, D. M., et al. (2004). Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J. Clin Oncol* 22, 2184–2191. doi: 10.1200/JCO.2004.11.022

Kaipainen, A., Vlaykova, T., Hatva, E., Böhling, T., Jekunen, A., Pyrhönen, S., et al. (1994). Enhanced expression of the tie receptor tyrosine kinase mesenger RNA in the vascular endothelium of metastatic melanomas. *Cancer Res.* 54, 6571–6577.

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 141, 52–67. doi: 10.1016/j.cell.2010.03.015

Kim, M., Allen, B., Korhonen, E. A., Nitschké, M., Yang, H. W., Baluk, P., et al. (2016). Opposing actions of angiopoietin-2 on Tie2 signaling and FOXO1 activation. *J. Clin. Invest.* 126, 3511–3525. doi: 10.1172/JCI84871

Korhonen, E. A., Lampinen, A., Giri, H., Anisimov, A., Kim, M., Allen, B., et al. (2016). Tie1 controls angiopoietin function in vascular remodeling and inflammation. *J. Clin. Invest.* 126, 3495–3510. doi: 10.1172/JCI84923

Kut, C., Mac Gabhann, F., and Popel, A. S. (2007). Where is VEGF in the body? A meta-analysis of VEGF distribution in cancer. *Br. J. Cancer* 97, 978–985. doi: 10.1038/sj.bjc.6603923

La Porta, S., Roth, L., Singhal, M., Mogler, C., Spegg, C., Schieb, B., et al. (2018). Endothelial Tie1-mediated angiogenesis and vascular abnormalization promote tumor progression and metastasis. *J. Clin. Invest.* 128, 834–845. doi: 10.1172/JCI94674

Lambrechts, D., Wauters, E., Boeckx, B., Aibar, S., Nittner, D., Burton, O., et al. (2018). Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat. Med.* 24, 1277–1289. doi: 10.1038/s41591-018-0096-5

Lee, J. G., and Wu, R. (2015). Erlotinib-cisplatin combination inhibits growth and angiogenesis through c-MYC and HIF-1α in EGFR-mutated lung cancer in vitro and in vivo. *Neoplasia* 17, 190–200. doi: 10.1016/j.neo.2014.12.008

Li, D., Xie, K., Zhang, L., Yao, X., Li, H., Xu, Q., et al. (2016). Dual blockade of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) exhibits potent anti-angiogenic effects. *Cancer Lett.* 377, 164–173. doi: 10.1016/j.canlet.2016.04.036

Li, X., Sun, X., and Carmeliet, P. (2019). Hallmarks of endothelial cell metabolism in health and disease. *Cell Metab.* 30, 414–433. doi: 10.1016/j.cmet.2019.08.011

Liu, H., Chen, Y., Li, Y., Li, C., Qin, T., Bai, M., et al. (2019). miR-195 suppresses metastasis and angiogenesis of squamous cell lung cancer by inhibiting the expression of VEGF. *Mol. Med. Rep.* 20, 2625–2632. doi: 10.3892/mmr.2019.10496

Liu, T., Ma, W., Xu, H., Huang, M., Zhang, D., He, Z., et al. (2018). PDGF-mediated mesenchymal transformation renders endothelial resistance to anti-VEGF treatment in glioblastoma. *Nat. Commun.* 9:3439. doi: 10.1038/s41467-018-05982-z

Lu, J., Xu, W., Qian, J., Wang, S., Zhang, B., Zhang, L., et al. (2019a). Transcriptome profiling analysis reveals that CXCL2 is involved in anlotinib resistance in human lung cancer cells. *BMC Med. Genomics* 12:38. doi: 10.1186/s12920-019-0482-y

Lu, J., Zhong, H., Chu, T., Zhang, X., Li, R., Sun, J., et al. (2019b). Role of anlotinib-induced CCL2 decrease in anti-angiogenesis and response prediction for nonsmall cell lung cancer therapy. *Eur. Respirat. J.* 53:1801562. doi: 10.1183/13993003.01562-2018

Lyu, J., Yang, E. J., Head, S. A., Ai, N., Zhang, B., Wu, C., et al. (2017). Pharmacological blockade of cholesterol trafficking by cepharanthine in endothelial cells suppresses angiogenesis and tumor growth. *Cancer Lett.* 409, 91–103. doi: 10.1016/j.canlet.2017.09.009

Maniotis, A. J., Folberg, R., Hess, A., Seftor, E. A., Gardner, L. M. G., Pe'er, J., et al. (1999). Vascular channel formation by human melanoma cells in Vivo and in Vitro: vasculogenic Mimicry. *Am. J. Pathol.* 155, 739–752. doi: 10.1016/S0002-9440(10)65173-5

Martinet, L., and Girard, J.-P. (2013). Regulation of tumor-associated high-endothelial venules by dendritic cells: a new opportunity to promote lymphocyte infiltration into breast cancer? *Oncoimmunology* 2:e26470. doi: 10.4161/onci.26470

Mok, T., Gorbunova, V., Juhasz, E., Szima, B., Burdaeva, O., Orlov, S., et al. (2014). A correlative biomarker analysis of the combination of bevacizumab and carboplatin-based chemotherapy for advanced nonsquamous non-small-cell lung cancer: results of the phase II randomized ABIGAIL study (BO21015). *J. Thoracic Oncol.* 9, 848–855. doi: 10.1097/JTO.0000000000000160

Montana, V., and Sontheimer, H. (2011). Bradykinin promotes the chemotactic invasion of primary brain tumors. *J. Neurosci.* 31, 4858–4867. doi: 10.1523/JNEUROSCI.3825-10.2011

Nakagawa, K., Garon, E. B., Seto, T., Nishio, M., Ponce Aix, S., Paz-Ares, L., et al. (2019). Ramucirumab plus erlotinib in patients with untreated, EGFR-mutated, advanced non-small-cell lung cancer (RELAY): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* 20, 1655–1669. doi: 10.1016/S1470-2045(19)30634-5

Novello, S., Kaiser, R., Mellemgaard, A., Douillard, J.-Y., Orlov, S., Krzakowski, M., et al. (2015). Analysis of patient-reported outcomes from the LUME-Lung 1 trial: a randomised, double-blind, placebo-controlled, Phase III study of second-line nintedanib in patients with advanced non-small cell lung cancer. *Eur. J. Cancer* 51, 317–326. doi: 10.1016/j.ejca.2014.11.015

Olson, O. C., and Joyce, J. A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nat. Rev. Cancer* 15, 712–729. doi: 10.1038/nrc4027

Overed-Sayer, C., Miranda, E., Dunmore, R., Liarte Marin, E., Beloki, L., Rassl, D., et al. (2020). Inhibition of mast cells: a novel mechanism by which nintedanib may elicit anti-fibrotic effects. *Thorax* 75, 754–763. doi: 10.1136/thoraxjnl-2019-214000

Paulis, Y. W. J., Soetekouw, P. M. M. B., Verheul, H. M. W., Tjan-Heijnen, V. C. G., and Griffioen, A. W. (2010). Signalling pathways in vasculogenic mimicry. *Biochim. Biophys. Acta* 1806, 18–28. doi: 10.1016/j.bbcan.2010.01.001

Peterson, T. E., Kirkpatrick, N. D., Huang, Y., Farrar, C. T., Marijt, K. A., Kloepper, J., et al. (2016). Dual inhibition of Ang-2 and VEGF receptors normalizes tumor vasculature and prolongs survival in glioblastoma by altering macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4470–4475. doi: 10.1073/pnas.1525349113

Petrovic, K., Robinson, J., Whitworth, K., Jinks, E., Shaaban, A., and Lee, S. P. (2019). TEM8/ANTXR1-specific CAR T cells mediate toxicity in vivo. *PLoS One* 14:e0224015. doi: 10.1371/journal.pone.0224015

Pezzella, F., Pastorino, U., Tagliabue, E., Andreola, S., Sozzi, G., Gasparini, G., et al. (1997). Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. *Am. J. Pathol.* 151, 1417–1423.

Phung, T. L., Ziv, K., Dabydeen, D., Eyiah-Mensah, G., Riveros, M., Perruzzi, C., et al. (2006). Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell* 10, 159–170. doi: 10.1016/j.ccr.2006.07.003

Pinto, M. P., Sotomayor, P., Carrasco-Avino, G., Corvalan, A. H., and Owen, G. I. (2016). Escaping antiangiogenic therapy: strategies employed by cancer cells. *Int. J. Mol. Sci.* 17:1489. doi: 10.3390/ijms17091489

Priceman, S. J., Sung, J. L., Shaposhnik, Z., Burton, J. B., Torres-Collado, A. X., Moughon, D. L., et al. (2010). Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. *Blood* 115, 1461–1471. doi: 10.1182/blood-2009-08-237412

Qiu, L., Zhao, X., Shi, W., Sun, S., Zhang, G., Sun, Q., et al. (2020). Real-world treatment efficacy of anti-programmed death-1 combined with anti-angiogenesis therapy in non-small cell lung cancer patients. *Medicine* 99:e20545. doi: 10.1097/MD.0000000000020545

Rafii, S., Butler, J. M., and Ding, B.-S. (2016). Angiocrine functions of organ-specific endothelial cells. *Nature* 529, 316–325. doi: 10.1038/nature17040

Rafii, S., Lyden, D., Benezra, R., Hattori, K., and Heissig, B. (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat. Rev. Cancer* 2, 826–835. doi: 10.1038/nrc925

Raica, M., Cimpean, A. M., and Ribatti, D. (2009). Angiogenesis in pre-malignant conditions. *Eur. J. Cancer* 45, 1924–1934. doi: 10.1016/j.ejca.2009.04.007

Reck, M., Kaiser, R., Mellemgaard, A., Douillard, J.-Y., Orlov, S., Krzakowski, M., et al. (2014). Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): a phase 3, double-blind, randomised controlled trial. *Lancet Oncol.* 15, 143–155. doi: 10.1016/S1470-2045(13)70586-2

Reck, M., Pawel, J., von, Zatloukal, P., Ramlau, R., Gorbounova, V., et al. (2009). Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab

as first-line therapy for nonsquamous non-small-cell lung cancer: AVAil. *J. Clin. Oncol.* 27, 1227–1234. doi: 10.1200/JCO.2007.14.5466

Reck, M., Pawel, J., von, Zatloukal, P., Ramlau, R., Gorbounova, V., et al. (2010). Overall survival with cisplatin-gemcitabine and bevacizumab or placebo as first-line therapy for nonsquamous non-small-cell lung cancer: results from a randomised phase III trial (AVAiL). *Ann. Oncol.* 21, 1804–1809. doi: 10.1093/annonc/mdq020

Reck, M., Paz-Ares, L., Bidoli, P., Cappuzzo, F., Dakhil, S., Moro-Sibilot, D., et al. (2017). Outcomes in patients with aggressive or refractory disease from REVEL: a randomized phase III study of docetaxel with ramucirumab or placebo for second-line treatment of stage IV non-small-cell lung cancer. *Lung Cancer* 112, 181–187. doi: 10.1016/j.lungcan.2017.07.038

Ribatti, D., and Crivellato, E. (2012). Mast cells, angiogenesis, and tumour growth. *Biochim. Biophys. Acta* 1822, 2–8. doi: 10.1016/j.bbadis.2010.11.010

Ribatti, D., and Ranieri, G. (2015). Tryptase, a novel angiogenic factor stored in mast cell granules. *Exp. Cell Res.* 332, 157–162. doi: 10.1016/j.yexcr.2014.11.014

Rigamonti, N., Kadioglu, E., Keklikoglou, I., Wyser Rmili, C., Leow, C. C., and de Palma, M. (2014). Role of angiopoietin-2 in adaptive tumor resistance to VEGF signaling blockade. *Cell Rep.* 8, 696–706. doi: 10.1016/j.celrep.2014.06.059

Rodríguez Garzotto, A., Díaz-García, C. V., Agudo-López, A., Prieto García, E., Ponce, S., López-Martín, J. A., et al. (2016). Blood-based biomarkers for monitoring antiangiogenic therapy in non-small cell lung cancer. *Med. Oncol.* 33:105. doi: 10.1007/s12032-016-0824-y

Rohlenova, K., Goveia, J., García-Caballero, M., Subramanian, A., Kalucka, J., Treps, L., et al. (2020). Single-Cell RNA Sequencing Maps Endothelial Metabolic Plasticity in Pathological Angiogenesis. *Cell Metab.* 31, 862.e14–877.e14. doi: 10.1016/j.cmet.2020.03.009

Saito, H., Fukuhara, T., Furuya, N., Watanabe, K., Sugawara, S., Iwasawa, S., et al. (2019). Erlotinib plus bevacizumab versus erlotinib alone in patients with EGFR-positive advanced non-squamous non-small-cell lung cancer (NEJ026): interim analysis of an open-label, randomised, multicentre, phase 3 trial. *Lancet Oncol.* 20, 625–635. doi: 10.1016/S1470-2045(19)30035-X

Sakurai, Y., Akita, H., and Harashima, H. (2019). Targeting Tumor Endothelial Cells with Nanoparticles. *Int. J. Mol. Sci.* 20:5819. doi: 10.3390/ijms20235819

Sanctis, F., de, Ugel, S., Facciponte, J., and Facciabene, A. (2018). The dark side of tumor-associated endothelial cells. *Semin. Immunol.* 35, 35–47. doi: 10.1016/j.smim.2018.02.002

Sandler, A., Gray, R., Perry, M. C., Brahmer, J., Schiller, J. H., Dowlati, A., et al. (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *New Engl. J. Med.* 355, 2542–2550. doi: 10.1056/NEJMoa061884

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., et al. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376, 70–74. doi: 10.1038/376070a0

Schmittnaegel, M., Rigamonti, N., Kadioglu, E., Cassará, A., Wyser Rmili, C., Kiialainen, A., et al. (2017). Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci. Transl. Med.* 9:eaak9670. doi: 10.1126/scitranslmed.aak9670

Schwaederlé, M., Lazar, V., Validire, P., Hansson, J., Lacroix, L., Soria, J.-C., et al. (2015). VEGF-A expression correlates with TP53 mutations in non-small cell lung cancer: implications for antiangiogenesis therapy. *Cancer Res.* 75, 1187–1190. doi: 10.1158/0008-5472.CAN-14-2305

Seftor, E. A., Meltzer, P. S., Kirschmann, D. A., Pe'er, J., Maniotis, A. J., Trent, J. M., et al. (2002a). Molecular determinants of human uveal melanoma invasion and metastasis. *Clin. Exp. Metastasis* 19, 233–246. doi: 10.1023/a:1015591624171

Seftor, E. A., Meltzer, P. S., Schatteman, G. C., Gruman, L. M., Hess, A. R., Kirschmann, D. A., et al. (2002b). Expression of multiple molecular phenotypes by aggressive melanoma tumor cells: role in vasculogenic mimicry. *Crit. Rev. Oncol.* 44, 17–27. doi: 10.1016/S1040-8428(01)00199-8

Seftor, R. E., Seftor, E. A., Koshikawa, N., Meltzer, P. S., Gardner, L. M., Bilban, M., et al. (2001). Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res.* 61, 6322–6327.

Senger, D. R., and Davis, G. E. (2011). Angiogenesis. *Cold Spring Harbor Perspect. Biol.* 3:a005090. doi: 10.1101/cshperspect.a005090

Seshadri, D. R., and Ramamurthi, A. (2018). Nanotherapeutics to modulate the compromised micro-environment for lung cancers and chronic obstructive pulmonary disease. *Front. Pharmacol.* 9:759. doi: 10.3389/fphar.2018.00759

Seto, T., Kato, T., Nishio, M., Goto, K., Atagi, S., Hosomi, Y., et al. (2014). Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced non-squamous non-small-cell lung cancer harbouring EGFR mutations (JO25567): an open-label, randomised, multicentre, phase 2 study. *Lancet Oncol.* 15, 1236–1244. doi: 10.1016/S1470-2045(14)70381-X

Shahid, I., AlMalki, W. H., AlRabia, M. W., Ahmed, M., Imam, M. T., Saifullah, M. K., et al. (2017). "Recent Advances in Angiogenesis Assessment Methods and their Clinical Applications," in *Physiologic and Pathologic Angiogenesis. Signaling Mechanisms and Targeted Therapy*, eds D. Simionescu and A. Simionescu (Rijeka: InTech).

Shi, Y., Du, L., Lin, L., and Wang, Y. (2017). Tumour-associated mesenchymal stem/stromal cells: emerging therapeutic targets. *Nature reviews. Drug Discov.* 16, 35–52. doi: 10.1038/nrd.2016.193

Shibata, Y., Kobayashi, N., Sato, T., Nakashima, K., and Kaneko, T. (2020). The clinical significance of CXCL16 in the treatment of advanced non-small cell lung cancer. *Thorac. Cancer* 11, 1258–1264. doi: 10.1111/1759-7714.13387

Shojaei, F., Wu, X., Malik, A. K., Zhong, C., Baldwin, M. E., Schanz, S., et al. (2007). Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat. Biotechnol.* 25, 911–920. doi: 10.1038/nbt1323

Socinski, M. A., Jotte, R. M., Cappuzzo, F., Orlandi, F., Stroyakovskiy, D., Nogami, N., et al. (2018). Atezolizumab for First-Line Treatment of Metastatic Nonsquamous NSCLC. *New Engl. J. Med.* 378, 2288–2301. doi: 10.1056/NEJMoa1716948

Soria, J.-C., Mauguen, A., Reck, M., Sandler, A. B., Saijo, N., Johnson, D. H., et al. (2013). Systematic review and meta-analysis of randomised, phase II/III trials adding bevacizumab to platinum-based chemotherapy as first-line treatment in patients with advanced non-small-cell lung cancer. *Ann. Oncol.* 24, 20–30. doi: 10.1093/annonc/mds590

Soucek, L., Lawlor, E. R., Soto, D., Shchors, K., Swigart, L. B., and Evan, G. I. (2007). Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat. Med.* 13, 1211–1218. doi: 10.1038/nm1649

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., et al. (2000). Genes expressed in human tumor endothelium. *Science* 289, 1197–1202. doi: 10.1126/science.289.5482.1197

Stryker, Z. I., Rajabi, M., Davis, P. J., and Mousa, S. A. (2019). Evaluation of angiogenesis assays. *Biomedicines* 7:37. doi: 10.3390/biomedicines7020037

Sun, H., Zhang, D., Yao, Z., Lin, X., Liu, J., Gu, Q., et al. (2017). Anti-angiogenic treatment promotes triple-negative breast cancer invasion via vasculogenic mimicry. *Cancer Biol. Ther.* 18, 205–213. doi: 10.1080/15384047.2017.1294288

Sun, L.-L., Li, W.-D., Lei, F.-R., and Li, X.-Q. (2018). The regulatory role of microRNAs in angiogenesis-related diseases. *J. Cell. Mol. Med.* 22, 4568–4587. doi: 10.1111/jcmm.13700

Takeuchi, A., Oguri, T., Yamashita, Y., Sone, K., Fukuda, S., Takakuwa, O., et al. (2018). TTF-1 expression predicts the merit of additional antiangiogenic treatment in non-squamous non-small cell lung cancer. *Anticancer Res.* 38, 5489–5495. doi: 10.21873/anticanres.12882

Teleanu, R. I., Chircov, C., Grumezescu, A. M., and Teleanu, D. M. (2019). Tumor angiogenesis and anti-angiogenic strategies for cancer treatment. *J. Clin. Med.* 9:84. doi: 10.3390/jcm9010084

Uribesalgo, I., Hoffmann, D., Zhang, Y., Kavirayani, A., Lazovic, J., Berta, J., et al. (2019). Apelin inhibition prevents resistance and metastasis associated with anti-angiogenic therapy. *EMBO Mol. Med.* 11:e9266. doi: 10.15252/emmm.201809266

van der Veldt, A. A. M., Lubberink, M., Bahce, I., Walraven, M., Boer, M. P., de, et al. (2012). Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. *Cancer Cell* 21, 82–91. doi: 10.1016/j.ccr.2011.11.023

Webb, N. J., Bottomley, M. J., Watson, C. J., and Brenchley, P. E. (1998). Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. *Clin. Sci.* 94, 395–404. doi: 10.1042/cs0940395

Williamson, S. C., Metcalf, R. L., Trapani, F., Mohan, S., Antonello, J., Abbott, B., et al. (2016). Vasculogenic mimicry in small cell lung cancer. *Nat. Commun.* 7:13322. doi: 10.1038/ncomms13322

Wroblewski, M., Bauer, R., Cubas Córdova, M., Udonta, F., Ben-Batalla, I., Legler, K., et al. (2017). Mast cells decrease efficacy of anti-angiogenic therapy by secreting matrix-degrading granzyme B. *Nat. Commun.* 8:269. doi: 10.1038/s41467-017-00327-8

Xia, Y., Cai, X.-Y., Fan, J.-Q., Zhang, L.-L., Ren, J.-H., Li, Z.-Y., et al. (2019). The role of sema4D in vasculogenic mimicry formation in non-small cell lung cancer and the underlying mechanisms. *Int. J. Cancer* 144, 2227–2238. doi: 10.1002/ijc.31958

Xie, Y. J., Dougan, M., Jailkhani, N., Ingram, J., Fang, T., Kummer, L., et al. (2019). Nanobody-based CAR T cells that target the tumor microenvironment inhibit the growth of solid tumors in immunocompetent mice. *Proc. Natl. Acad. Sci. U.S.A.* 116, 7624–7631. doi: 10.1073/pnas.1817147116

Yamamoto, N., Seto, T., Nishio, M., Goto, K., Okamoto, I., Yamanaka, T., et al. (2018). Erlotinib plus bevacizumab (EB) versus erlotinib alone (E) as first-line treatment for advanced EGFR mutation–positive non-squamous non–small-cell lung cancer (NSCLC): Survival follow-up results of JO25567. *J. Clin. Oncol.* 36:9007. doi: 10.1200/JCO.2018.36.15_suppl.9007

Yang, Y., Zhang, Y., Iwamoto, H., Hosaka, K., Seki, T., Andersson, P., et al. (2016). Discontinuation of anti-VEGF cancer therapy promotes metastasis through a liver revascularization mechanism. *Nat. Commun.* 7:12680. doi: 10.1038/ncomms12680

Yu, D.-H., Lu, Q., Xie, J., Fang, C., and Chen, H.-Z. (2010). Peptide-conjugated biodegradable nanoparticles as a carrier to target paclitaxel to tumor neovasculature. *Biomaterials* 31, 2278–2292. doi: 10.1016/j.biomaterials.2009.11.047

Zaman, K., Driscoll, R., Hahn, D., Werffeli, P., Goodman, S. L., Bauer, J., et al. (2006). Monitoring multiple angiogenesis-related molecules in the blood of cancer patients shows a correlation between VEGF-A and MMP-9 levels before treatment and divergent changes after surgical vs. *conservative therapy*. *Int. J. Cancer* 118, 755–764. doi: 10.1002/ijc.21408

Zhang, L., Deng, Y., Zhang, Y., Liu, C., Zhang, S., Zhu, W., et al. (2020). The Design, Characterizations, and Tumor Angiogenesis Inhibition of a Multi-Epitope Peptibody With bFGF/VEGFA. *Front. Oncol.* 10:1190. doi: 10.3389/fonc.2020.01190

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor LT declared a past co-authorship with one of the authors AP.

frontiers in Oncology

REVIEW
published: 04 February 2021
doi: 10.3389/fonc.2020.612802

Endothelial YAP/TAZ Signaling in Angiogenesis and Tumor Vasculature

Aukie Hooglugt[1,2†], *Miesje M. van der Stoel*[1†], *Reinier A. Boon*[2,3,4] *and Stephan Huveneers*[1*]

[1] Department of Medical Biochemistry, Amsterdam Cardiovascular Sciences, Amsterdam UMC, University of Amsterdam, Amsterdam, Netherlands, [2] Department of Physiology, Amsterdam Cardiovascular Sciences, Amsterdam UMC, VU University Medical Center, Amsterdam, Netherlands, [3] German Center for Cardiovascular Research (DZHK), Partner Site Rhein-Main, Berlin, Germany, [4] Institute of Cardiovascular Regeneration, Goethe University, Frankfurt am Main, Germany

OPEN ACCESS

Edited by:
Lucas Treps,
VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Carmen Ruiz De Almodovar,
Heidelberg University, Germany
Jinlong He,
Tianjin Medical University, China

***Correspondence:**
Stephan Huveneers
s.huveneers@amsterdamumc.nl

†These authors share first authorship

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 30 September 2020
Accepted: 07 December 2020
Published: 04 February 2021

Citation:
Hooglugt A, van der Stoel MM, Boon RA and Huveneers S (2021) Endothelial YAP/TAZ Signaling in Angiogenesis and Tumor Vasculature. Front. Oncol. 10:612802. doi: 10.3389/fonc.2020.612802

Solid tumors are dependent on vascularization for their growth. The hypoxic, stiff, and pro-angiogenic tumor microenvironment induces angiogenesis, giving rise to an immature, proliferative, and permeable vasculature. The tumor vessels promote tumor metastasis and complicate delivery of anti-cancer therapies. In many types of tumors, YAP/TAZ activation is correlated with increased levels of angiogenesis. In addition, endothelial YAP/TAZ activation is important for the formation of new blood and lymphatic vessels during development. Oncogenic activation of YAP/TAZ in tumor cell growth and invasion has been studied in great detail, however the role of YAP/TAZ within the tumor endothelium remains insufficiently understood, which complicates therapeutic strategies aimed at targeting YAP/TAZ in cancer. Here, we overview the upstream signals from the tumor microenvironment that control endothelial YAP/TAZ activation and explore the role of their downstream targets in driving tumor angiogenesis. We further discuss the potential for anti-cancer treatments and vascular normalization strategies to improve tumor therapies.

Keywords: yes-associated protein (YAP), TAZ, tumor vasculature, endothelium, mechanotransduction, cancer, Angiogenic therapy, tumor angiogenesis

Abbreviations: AMOT, Angiomotin; Ang1, Angiopoietin-1; Ang2, Angiopoietin-2; ANKRD1, Ankyrin repeat domain 1; CAFs, Cancer-associated fibroblast; CTGF, Connective tissue growth factor; CYR61, Cysteine-rich angiogenic inducer 61; ECM, Extracellular matrix; EndoMT, Endothelial to mesenchymal transition; ECs, Endothelial cells; FA, Focal adhesion; FAK, Focal Adhesion Kinase; FGFR, FGF receptor; FGF2, Fibroblast growth factor 2; GPCR, G-protein-coupled receptor; HIF1α, Hypoxia inducible transcription factor 1α; IFP, Interstitial fluid pressure; LATS, Large tumor suppressor kinase; MMPs, Matrix metalloproteases; RTK, Receptor tyrosine kinase; SAV1, Salvador family WW-domain-containing-protein-1; STAT3, Signal Transducer and Activator of Transcription-3; STK, Serine/threonine kinases; TAZ, Transcriptional Co-Activator With PDZ-binding Motif; TEAD, TEA domain family member; TME, Tumor microenvironment; VEGF, Vascular endothelial growth factor; VEGFR2, VEGF receptor 2; YAP, Yes-associated protein.

INTRODUCTION

It is estimated that solid tumors can grow to a size of approximately 2 mm^3 without being vascularized (1). For further growth, tumors require blood vessels that deliver oxygen and nutrients. Tumors use several mechanisms for neovascularization, including angiogenesis, vessel co-option, vascular mimicry, trans-differentiation of cancer cells into endothelial cells (ECs), and through the recruitment of endothelial progenitor cells (2, 3). Angiogenesis, the formation of new vessels from pre-existing ones, is essential for tumor progression and growth and is promoted by pro-angiogenic signals secreted by the tumor cells and the tumor microenvironment (TME) (4, 5). The TME consists of cancer-associated fibroblast (CAFs), mesenchymal stromal cells, immune cells, ECs, as well as extracellular matrix (ECM) components, growth factors and cytokines (6–8). The tumor cells together with the TME, generate a hypoxic, acidic and inflammatory environment that further drives tumor angiogenesis, tumor growth and contributes to drug resistance (8, 9).

The tumor vasculature is morphologically different compared to the normal blood vessels (5, 9–11). Tumor angiogenesis gives rise to a dense and disorganized vessel network, in which the vessels are immature, dilated, hyperpermeable, and lack the support of pericytes or normal basement membrane. Tumor vessels are further characterized by irregular blood flow, which fails to supply sufficient oxygen and nutrients to the tumor tissue (12, 13). The tumor vessels contribute to malignancy by maintaining the hypoxic, acidic, and inflammatory environment, thereby fueling a vicious cycle that prevents normalization of the tumor vasculature and maintains a pro-metastatic environment (14, 15).

Targeting of tumor angiogenesis as treatment for cancer has been extensively investigated in pre-clinical and clinical settings. The first commercially available anti-angiogenic drug, Bevacizumab improves survival of patients with metastatic colorectal cancer by targeting of vascular endothelial growth factor (VEGF) (16). Currently, several VEGF-targeting FDA-approved drugs are used as anti-angiogenic cancer treatments, including for gastrointestinal cancer, glioblastoma, non-small lung carcinoma, breast cancer, and renal cancer (17, 18). Patient studies have shown increased survival after combining anti-VEGF therapy with chemotherapy (17, 19, 20). Unfortunately, long term administration of anti-VEGF treatments raises therapy resistance (21, 22) and major reductions in tumor blood vessels are not achieved, likely due to the activation of alternative neovascularization events in tumors (23, 24). Furthermore, pre-clinical *in vivo* studies have shown that after termination of anti-VEGF treatments, the tumor vessels rapidly return to an angiogenic and disorganized state (25).

Anti-angiogenic agents are often prescribed in high doses, which effectively leads to tumor vessel pruning and consequently decreases drug delivery to the TME (26). Moreover, local hypoxia in the TME, such as induced by vascular regression, enhances tumor invasiveness, chemo- and immunotherapy resistance, and metastasis (27). Hypoxia also induces expression of alternative angiogenic cytokines and compensatory mechanisms of neovascularization, further limiting the anti-angiogenic potential of anti-VEGF treatments (2, 28). Alternative angiogenic pathways are suspected to enhance tumor invasion and metastasis in response to anti-VEGF treatment (29).

Upon anti-angiogenic treatments, there is typically a short "window" during which vascular normalization is achieved, restoring normal blood vessel function and reducing hypoxia in the TME (21, 30–32). During this time frame, radiation- and immunotherapies were found to be most effective (33, 34). Because high doses and long-term treatment of anti-angiogenic drugs promote hypoxia in the tumor tissue, it is thought that lowering of drug dosage may reduce the levels of angiogenic factors and normalize the tumor vasculature accordingly (30). Experimental tumorigenesis studies using low doses or short-term treatments of anti-angiogenic drugs, indeed observed increased functional blood vessels, improved immunotherapy efficacy, and reduced metastatic activity of tumor cells (26, 35, 36). The discovery of additional therapeutic strategies are pursued to try to overcome anti-angiogenic resistance and to better control normalization of tumor vessels (37, 38).

The oncogene Yes-associated protein (YAP) and its paralogue Transcriptional Co-Activator With PDZ-binding Motif (TAZ or WWTR1) have been considered as attractive pharmacological targets, as they are highly activated in many forms of cancers and contribute to tumor growth and invasion (39). YAP/TAZ are also well known for their regulatory role during physiological and developmental angiogenesis and have recently gained attention in the context of endothelial-driven tumor angiogenesis (40–42). In this review we aim to understand how YAP/TAZ signaling affects the (tumor) endothelium. We will further discuss the potential mechanisms of YAP/TAZ activation by the TME and the downstream transcriptional program of YAP/TAZ that controls angiogenesis.

MOLECULAR REGULATION OF YAP/TAZ ACTIVITY

In general, in normal quiescent adherent cells YAP/TAZ are inhibited by the Hippo pathway and located in the cytoplasm. Upon various activating signals YAP/TAZ translocate toward the nucleus and act as transcriptional co-factors for the regulation of tissue homeostasis and organ growth (43, 44). In addition, YAP/TAZ respond to mechanical stimuli derived from cell spreading, contact inhibition, cytoskeletal contractility, ECM stiffness and fluid shear forces (45, 46).

The Hippo signaling pathway consists of a phosphorylation cascade with several effectors. Serine/threonine kinases 3 and 4 (STK3 and STK4, also called MST1/2) interact with the scaffolding protein Salvador family WW-domain-containing-protein-1 (SAV1). If Hippo signaling is turned "on", the MST-SAV1 complex phosphorylates MOB kinase activator 1A and 1B (MOB1A and MOB1B). This leads to an interaction of MOB1A and MOB1B with large tumor suppressor kinase 1 and 2 (LATS1/2) (47, 48). Once in complex with MOB1A/1B, LATS1/2 become autophosphorylated and phosphorylated by MST1/2 (47, 49). In turn, active LATS1/2 kinases phosphorylate YAP on 5 serine residues (S61, S109, S127, S164, and S381) and TAZ on 4 serine residues (S66, S89, S117, S311) (50). Serine phosphorylated YAP/TAZ bind to 14-3-3 proteins, which sequesters the proteins in the cytoplasm or targets YAP/TAZ for ubiquitin-mediated proteasomal degradation (51, 52).

Alternatively, YAP/TAZ can be sequestered in the cytoplasm by Angiomotin (AMOT) proteins that interact with YAP/TAZ or Hippo pathway effectors. If Hippo signaling is turned "off", YAP/TAZ act as transcriptional co-activators in the nucleus, where they primarily interact with TEA domain family member (TEAD) transcriptional factors to regulate genes involved in proliferation, migration and survival (53). The Hippo pathway crosstalks with major signaling routes that control tissue remodeling and growth, including the Wnt/β-catenin, TGFβ, and Notch pathways (54–56).

YAP/TAZ are also activated in various force-dependent manners (43, 45, 51). Upon cytoskeletal-driven cellular adaptations, such as during ECM stiffening, shear stress sensing or upon G-protein-coupled receptor (GPCR) signaling, the AMOT proteins enhance their interaction with F-actin, allowing YAP/TAZ to translocate toward the nucleus (57–60). ECM stiffening also remodels the integrin-based focal adhesions (FA). Cell adhesion promotes activation of Focal Adhesion Kinase (FAK) and SRC tyrosine kinases, that impinge on the Hippo pathway through direct activation of YAP/TAZ and inhibition of LATS1/2 and MOB1 through FAK/Rac and SRC/PI3K signaling (51, 61). The stiffness-sensing integrin receptors transduce forces to the cytoskeleton-anchored nucleus, opening the nuclear pores and driving YAP/TAZ nuclear translocation (46). Mechanical forces at cell-cell junctions also control YAP/TAZ, as strain on epithelial monolayers induce β-catenin and YAP1 nuclear localization (62, 63). Moreover, high tension inferred at the junctional cadherin-catenin complex, triggers the interaction of α-catenin with TRIP6 and LIMD1, which recruit LATS1/2 to the junctions and inhibit their kinase activity, leading to nuclear translocation of YAP/TAZ (64–66). The various Hippo and mechanotransduction pathways that regulate YAP/TAZ have been elegantly discussed previously (45, 63, 67, 68). These molecular pathways have been investigated in great detail in normal epithelia or tumor cells and are expected to be responsible for YAP/TAZ regulation in the endothelium as well (45).

THE ROLE OF YAP/TAZ IN DEVELOPMENTAL ANGIOGENESIS

Angiogenesis is driven by endothelial proliferation, collective cell migration, and cellular rearrangements (69–71). Angiogenic stimuli, such as VEGF-A and FGF2, activate the ECs to promote the formation of endothelial tip cells that migrate toward the angiogenic cue. Tip cells are followed by proliferative endothelial stalk cells, which shape the developing sprouts and the vascular lumen (2, 72, 73). The tip and stalk cell rearrangements are regulated through feedback loops between VEGF and Dll4/Notch signaling (74).

YAP/TAZ are activated in the sprouting ECs of the developing vasculature in the mouse retina (75). YAP/TAZ are expressed in the entire retinal vasculature, but they reside in the endothelial cytoplasm in the central vascular region, while YAP/TAZ are mainly nuclear in sprouting ECs and the remodeling vascular plexus (76–78). Especially, TAZ nuclear localization is prominent in spouting ECs of the developing retinal vasculature (76, 77). Importantly, the activation of YAP/TAZ in ECs is crucial for angiogenesis (75, 77). The nuclear translocation of endothelial YAP/TAZ is regulated by VEGF signaling, VE-cadherin-based adherens junctions, and cytoskeletal remodeling (45, 75, 79, 80). The VE-cadherin complex sequesters YAP/TAZ and (force-dependent) remodeling of the cell-cell junctions leads to YAP/TAZ activation (81, 82). VEGF-A signaling stimulates YAP/TAZ through cytoskeletal remodeling and inactivation of the Hippo effectors LATS1/2 (79). In turn, active endothelial YAP/TAZ induce a downstream transcriptional program which regulates proliferation, actin cytoskeleton contractility, cell adhesion, and collective cell migration (76, 77, 79, 80, 83).

The importance of YAP/TAZ function for vascular development has been studied by several groups using (inducible) endothelial-specific YAP/TAZ double knock out mouse models (76, 77, 79, 83). Endothelial-specific depletion of YAP/TAZ reduces the number of tip cells and angiogenic sprouts, and leads to excessive vessel crossing in the developing vasculature of the mouse retina (76, 77, 79, 83). Moreover, once the vasculature in the YAP/TAZ endothelial-specific knockout mice makes it to the stage of larger vessels, the vessels turn out to be leaky due to perturbation of endothelial cell-cell junctions (76, 77). Interestingly, knocking out only the YAP or TAZ genes from the endothelium resulted in mild vascular defects, indicating that YAP and TAZ have redundant functions and can compensate for each other in the endothelium (77).

Endothelial-specific overexpression of YAP or TAZ induced retinal vessel growth through increased angiogenic sprouting (77, 84). Interestingly, endothelial-specific YAP overexpression did not affect the vasculature of quiescent tissue in adult mice (84). Constitutive activation of YAP/TAZ, induced by knockout of LATS kinases or overexpression of an active mutant of YAP or TAZ, resulted in endothelial hypersprouting *in vivo* (77, 83). *In vitro* it was found that overexpression of an active form of YAP promotes hypersprouting *via* the angiogenic growth factor angiopoietin-2 (Ang2) signaling (75).

In agreement with the knockout mouse models that demonstrate an important role for YAP/TAZ in vascular development, depleting YAP/TAZ from zebrafish resulted in embryonic lethality due to severe developmental and vascular malformations (85, 86). YAP1 null mutant zebrafish showed a drastic reduction in transcriptional activity of TEAD2, while in TAZ null mutant zebrafish TEAD2 transcriptional activity was unaffected (86), suggesting that YAP is the major transcriptional regulator for vascular development in zebrafish. YAP1 null mutant zebrafish showed increased vessel regression and lumen stenosis, suggesting an important role for YAP1 in lumen maintenance in response to blood flow (86). Moreover, truncation of the cranial and ocular vasculature is observed (85). By contrast, TAZ null mutants did not display clear vascular defects (85). Transgenic mutant zebrafish in which the binding of the YAP/TAZ-TEAD complex to the DNA has been prevented, display altered vascular remodeling (87). Interestingly, expression of constitutively active YAP (YAP-5SA), TAZ (TAZ-4SA), or TEAD mutants initially promote vessel sprouting, but the sprouts fail to anastomose or stabilize at later stages (85). In summary, the regulation of endothelial YAP/TAZ activity is critical during

vascular development and both the down- and upregulation of YAP/TAZ activity leads to aberrant sprouting angiogenesis and blood vessel formation.

After new blood vessels are formed during development, angiogenic growth factor levels drop and vessels mature through the stabilization of cell-cell junctions and the recruitment of mural pericytes. In mature vessels, ECs are quiescent (88–90) and endothelial YAP/TAZ are inactivated (79). During wound healing in physiological and pathological conditions, YAP/TAZ are activated on demand to induce angiogenesis (53). The TME is somewhat comparable to the tissue of an inflamed wound (91), but in tumors endothelial YAP/TAZ remain activated and its vasculature does not evolve into a mature state (79) (**Figure 1**).

YAP/TAZ ACTIVATION IN TUMOR ANGIOGENESIS

Many types of cancer are accompanied by increased levels and activity of YAP/TAZ, including breast, pancreatic, liver and colorectal cancer (39, 92–94). Increased expression or activation of YAP/TAZ in cancer is associated with poor prognosis and reduced survival (93, 95). Increased YAP/TAZ levels are often observed both in tumor and stromal cells, including the CAFs, ECs, immune cells, and pericytes (96–99). YAP/TAZ activation in the TME promotes tumor growth, metastasis and angiogenesis (95, 97–99). For instance, in glioblastoma, high expression of TAZ in tumor endothelium is correlated with increased blood vessel density and tumor malignancy (100). There is an intricate connection between YAP/TAZ activation in the TME and the tumor vasculature.

Oncogenic activation of YAP/TAZ in tumor cells drives the ECs toward a pro-angiogenic state (**Figure 1**). Conditioned medium from (YAP positive) breast cancer cells induced endothelial YAP activation, which in turn promoted tumor angiogenesis (101). Also, ECs treated with conditioned medium from cholangiocarcinoma cells containing a constitutively active YAP mutation (YAP S127A), showed increased tube formation capacity *in vitro* (102), suggesting enhanced endothelial activity. Moreover, YAP activation in mesenchymal stromal cells has been

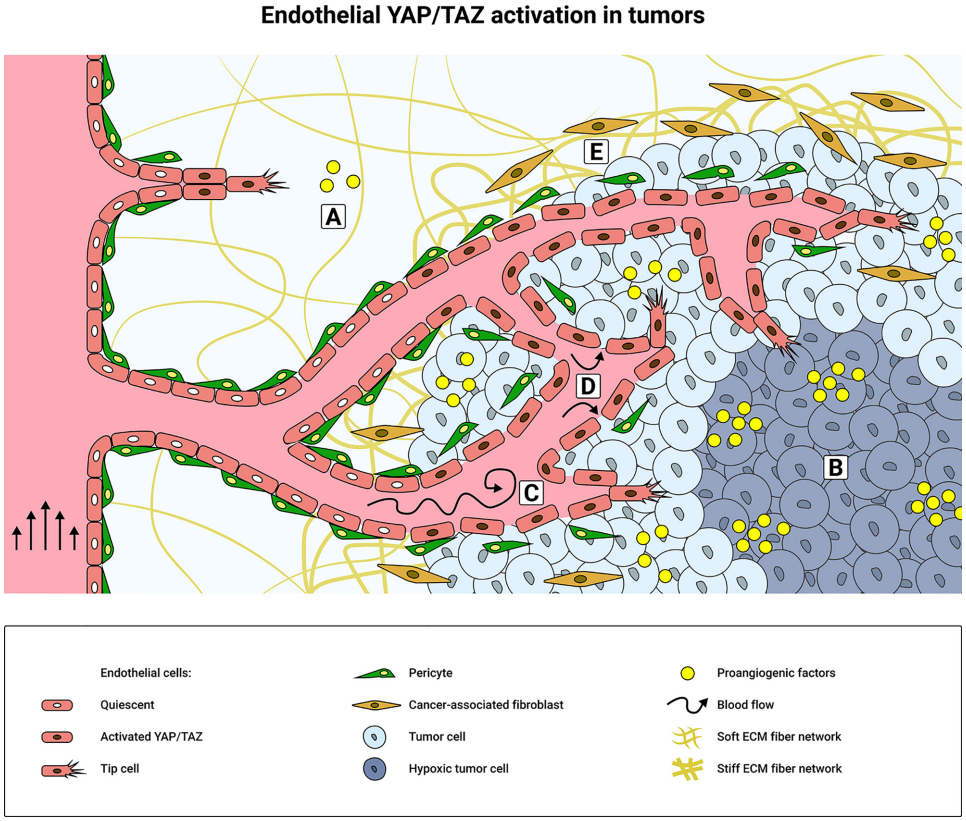


FIGURE 1 | Schematic overview of tumor microenvironment factors that promote endothelial YAP/TAZ activity. **(A)** During physiological angiogenesis, pro-angiogenic cytokines and growth factors activate YAP/TAZ signaling in endothelial cells (ECs) through GPCRs and RTKs, such as VEGFR2 or Tie2. **(B)** Hypoxia induces secretion of pro-angiogenic factors to promote tumor angiogenesis and consequently YAP/TAZ activity in the tumor microenvironment (TME). Elevated levels of pro-angiogenic factors promote the formation of a dense and disorganized tumor vasculature, resulting in disturbed blood flow patterns **(C)** and increased interstitial fluid pressure **(D)** that mechanically activate YAP/TAZ. The integrity of the tumor endothelium is weakened due to decreased cell-cell interactions between the ECs and/or pericytes. YAP/TAZ are further mechanically activated in the TME by ECM stiffening **(E)**, which is promoted by the cancer-associated fibroblasts (CAFs).

shown to enhance the crosstalk between gastric cancer cells and the tumor endothelium (103). Importantly, transplantations of Lewis Lung carcinoma allografts in transgenic mice with endothelial-specific YAP overexpression, resulted in an increase in tumor size and tumor vasculature (84). Vice versa, YAP knockdown in renal cell carcinoma, inhibited the angiogenic capacity of ECs *via* paracrine VEGF signaling (104). Overall, these findings emphasize the critical involvement of endothelial YAP/TAZ signaling during tumor angiogenesis following the interplay between tumor tissue and the ECs. Of note, YAP/TAZ activation does not always increase tumor angiogenesis and differences between tumor types should be considered. For instance, in angiosarcoma, a rare type of cancer derived from the vasculature, inhibition of PECAM-1 raised YAP levels, but decreased the tubulogenic potential of the angiosarcoma cells (105).

TUMOR MICROENVIRONMENTAL FACTORS THAT ACTIVATE YAP/TAZ

In solid tumors many microenvironmental properties have changed compared to in healthy tissue, for example increased interstitial fluid pressure (IFP), inflammation and ECM stiffness. These TME properties are key drivers of YAP/TAZ and promote the immature characteristics of the tumor vasculature (43, 106) (**Figure 1**).

Hypoxia

One of the prominent angiogenic features of solid tumors is their hypoxic condition. Hypoxia stabilizes hypoxia inducible transcription factor 1α (HIF1α) in tumor cells, initiating the transcription and secretion of pro-angiogenic factors, such as VEGF and Ang2 (107). Also in ECs, HIF1α promotes the transcription of autocrine pro-angiogenic molecules and matrix metalloproteases (MMPs) (40). Importantly, the endothelial-specific depletion of HIF1α resulted in reduced tumor growth and angiogenesis in experimental Lewis lung carcinoma (108). The onset of hypoxia in the retinal vasculature is known to inhibit Hippo pathway effectors and activates endothelial YAP (109). In turn, YAP is able to interact with HIF1α to sustain HIF1α signaling (110). In hypoxic colorectal cancer cells, HIF1α induces the transcription of GPCR5A, which in turn activates YAP to promote cell survival (111). Of note, in hepatocellular carcinoma cells, hypoxia was shown to activate YAP through a HIF1α independent manner (112). The intricate crosstalk between YAP/TAZ and HIF1α signaling in cancer has recently been overviewed (113).

Hypoxia also induces the activation of Signal Transducer and Activator of Transcription-3 (STAT3), which forms a complex with YAP in ECs to drive expression of angiogenic factors VEGF and Ang2 (84, 109) (**Figure 2**). Furthermore, the transcription factor SNAIL is a direct target of HIF1α, and hypoxia-induced expression of SNAIL promotes endothelial to mesenchymal transition (EndoMT) (114). YAP has been shown to induce EndoMT during cardiac development by upregulating expression of SNAIL (115). YAP activation potentially promotes hypoxia-induced EndoMT in the TME by facilitating SNAIL expression. Taken together, the hypoxic conditions within tumors activate YAP/TAZ-dependent programs that promote tumor vascularization. Notably, the described crosstalk between YAP/TAZ and HIF1α may differ between tissues, because in the hypoxic environment of the bone marrow, endothelial YAP/TAZ function to inhibit angiogenesis (116).

Angiogenic Growth Factors and Cytokines

A second important TME property that promotes endothelial YAP/TAZ activity and angiogenesis is the increased presence of angiogenic and inflammatory cytokines (i.e., VEGF, TGFβ and TNFα). Key is the (hypoxia-driven) VEGF production by tumor cells, ECs, and CAFs (117, 118). VEGF interaction with VEGF receptor 2 (VEGFR2) induces downstream signaling toward SRC kinases, PI3K/Akt, and MEK/ERK. These signaling pathways inactivate the Hippo pathway effectors MST1/2 and LATS1/2, leading to activation of endothelial YAP/TAZ (79, 119). The stimulation of ECs by VEGF also remodels the actin cytoskeleton and endothelial cell-cell junctions (120, 121), which further modulates YAP/TAZ activity (79). Actomyosin contractility is a well-established regulator of YAP/TAZ activity in a variety of cell types, acting directly on YAP/TAZ or *via* Hippo pathway effectors (45, 46, 122). In summary, VEGF-VEGFR2 signaling can induce endothelial YAP activation through various pathways, directly by interrupting the Hippo signaling cascade, or indirectly by its effect on cell-cell junctions and the actin cytoskeleton. Similar mechanisms may take place in the tumor endothelium.

Another important growth factor that affects YAP/TAZ signaling in tumors is TGFβ. Hypoxia together with TGFβ-mediated SMAD transcriptional activation induces complex formation between Zyxin, LATS2 and SIAH2 (123). The latter being an E3 ligase that mediates LATS2 ubiquitination and degradation (123). Furthermore, SMAD2 can interact with YAP *via* the RASS1FA scaffold protein, which helps to retain the SMAD2/YAP complex in the cytoplasm in quiescent cells (124). TGFβ induces the degradation of RASS1FA, which enables the SMAD2/YAP complex to translocate to the nucleus, leading to transcription of their target genes (124). YAP plays a crucial role in the nuclear translocation of SMADs (125). Most of these molecular mechanisms have been studied in epithelial or tumor cells. Yet, the tumor ECs express high levels of Endoglin (126), a BMP9 receptor that is part of the TGFβ receptor complex and an important receptor for endothelial migration and angiogenesis (127–129). BMP9-endoglin signaling induces YAP/SMAD nuclear translocation driving the expression of inflammatory genes in ECs (130), implying that endothelial YAP-SMAD may regulate tumor angiogenesis. Taken together, there are multiple angiogenic growth factors and cytokines within the TME that control YAP/TAZ and contribute to the immature and proliferative tumor vasculature.

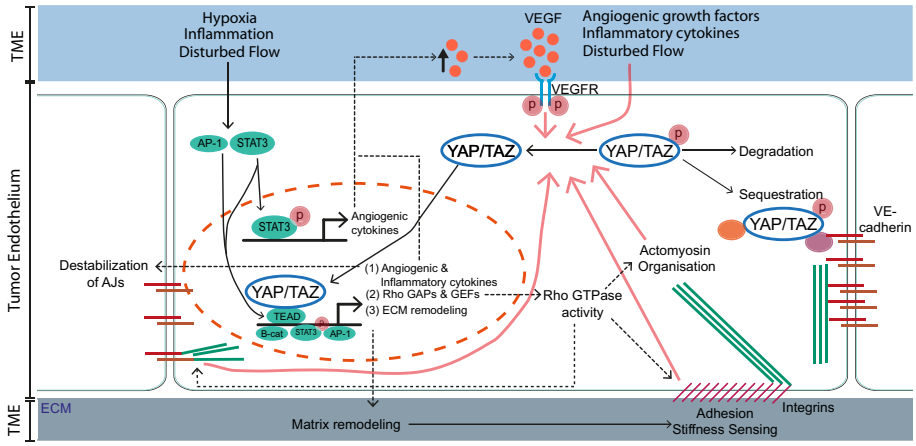


FIGURE 2 | The tumor microenvironment (TME) and downstream transcriptional targets of YAP/TAZ engage in a positive feedback program that sustains YAP/TAZ activity in the endothelium. In quiescent endothelial cells YAP/TAZ are kept inactive and sequestered in the cytoplasm *via* interaction with 14-3-3 proteins, Angiomotin (AMOT) proteins or the VE-cadherin complex. Alternatively, inactive YAP/TAZ can be targeted for ubiquitin-mediated degradation. External cues from the tumor microenvironment (TME), including pro-angiogenic growth factors, inflammatory cytokines, stiff extracellular matrix (ECM), hypoxia, and disturbed blood flow activate YAP/TAZ leading to their translocation to the nucleus (**Figure 1**). In parallel, multiple TME factors activate the transcription factors STAT3 and AP-1. Within the nucleus, YAP/TAZ interacts with several transcription factors, most notably the TEA domain family members (TEADs), but also with STAT3 and β-catenin to induce transcription of downstream target genes. Activated YAP/TAZ induce the expression of angiogenic and inflammatory cytokines, Rho GAPs and GEFs and extracellular matrix (ECM) remodeling proteins that engage in a positive feedback system that sustains YAP/TAZ activity. (1) angiogenic and inflammatory cytokines maintain the pro-angiogenic and inflammatory TME. Moreover, the angiogenic effectors of YAP/TAZ (such as Ang2 and VEGF) destabilize VE-cadherin-based adherens junctions (AJs), further promoting the activity of YAP/TAZ. (2) Rho GAPs and GEFs control the level of Rho-GTPase activities, crucial switches that organize the actomyosin cytoskeleton, AJs and integrin-based focal adhesions (FA). In turn, the FAs and actomyosin cytoskeleton transduce forces from stiff ECM and maintain the nuclear translocation of YAP/TAZ. (3) ECM remodeling proteins are secreted and remodel the TME in favor of YAP/TAZ activity.

Tumor Microenvironment Stiffness

Overall, solid tumors are stiffer in comparison to normal tissue (106). The stiffening is caused by increased collagen deposition and cross-linking (131). In tumors, ECM stiffening promotes sprouting angiogenesis and gives rise to a dense and hyperpermeable vasculature (106). Integrin adhesion receptors sense the increased ECM stiffness and induce the assembly of integrin-based FAs (132, 133). In turn, FAK/SRC signaling downstream of integrins inhibits LATS1/2 and activates YAP/TAZ (51, 134–136). The activation of endothelial FAK through phosphorylation of its autophosphorylated Y397 residue is crucial for tumor angiogenesis and tumor progression (137). Interestingly, targeting of endothelial FAK activity improved the efficacy of DNA-damaging chemotherapeutics, providing proof-of-principle for normalization of tumor vasculature as an adjuvant approach in cancer therapies (138). Importantly, integrin-mediated stiffness-sensing also activates YAP/TAZ independently of the Hippo pathway through activation of Rho-GTPases and actomyosin contractility (45, 46). YAP/TAZ activation, in turn, regulates FA turnover (139), a feedback mechanism that is crucial for endothelial collective migration and angiogenic sprouting (80, 140). Taken together, it is likely that direct stiffness-sensing through the tumor endothelium promotes endothelial YAP/TAZ activation and tumor angiogenesis. To prove this concept more experimental work is needed.

In addition, TME stiffening and tumor cell contractility promote MMP activity (141). MMP activity and subsequent matrix degradation controls endothelial sprouting through the stiff ECM (131). Vice versa, inhibition of lysyl oxidase, a matrix cross-linking enzyme involved in tumor stiffening, was shown to reduce the tumor stiffness and tumor vasculature in a mouse mammary tumor model (131). It was recently reported that metastasis-associated fibroblasts promote TME stiffening and angiogenesis in particular in liver metastases from colorectal cancer (142). Intriguingly, inhibition of renin-angiotensin signaling in combination with the anti-VEGF drug Bevacizumab, inactivated tumor endothelial YAP and reduced the fibroblast-induced metastatic TME stiffness and tumor vasculature (142). Moreover, the activation of YAP in CAFs is known to increase ECM stiffening, tumor cell invasion and tumor angiogenesis (97). Thus CAFs participate in a self-sustaining YAP-dependent feed forward loop that aggravates tumorigenesis, through a mechanism in which tumor stiffening and angiogenesis take central roles (**Figure 1**).

Interstitial Fluid Pressure and Blood Flow

Tumor vessels are disorganized and hyperpermeable, which increases interstitial fluid pressure (IFP) and leads to disturbed blood flow (143), altogether hindering drug delivery to tumors (144). The high level of IFP also stretches the blood vessels and promotes metastasis (145–148). Mechanical stretching of tumor stromal cells, such as fibroblasts and ECs, is a well-known driver of YAP/TAZ nuclear translocation (46, 77, 149, 150). Consequently, IFP likely activates endothelial YAP/TAZ

signaling in the TME. The tumor vasculature is also poorly perfused and the blood flow is often turbulent (13, 151). Disturbed flow patterns activate endothelial YAP/TAZ and trigger proliferative and inflammatory responses *in vitro* (152–154). In the mouse aortic arch, a vascular area exposed to disturbed blood flow, YAP phosphorylation was lower and its nuclear localization higher, as in comparison to the thoracic aorta, an area exposed to laminar blood flow (153). Consequently, disturbed haemodynamics activate endothelial YAP/TAZ and we speculate that they sustain YAP/TAZ signaling in the tumor vasculature.

In summary, the pro-angiogenic, inflammatory, hypoxic, and stiff TME activates endothelial YAP/TAZ and promotes tumor angiogenesis. A major challenge is to tackle the essential events that sustain this oncogenic vascular environment. Investing in pre-clinical engineered tumor vascular models in which the TME cues can be manipulated in a defined manner are expected to greatly propel research aimed at developing anti-angiogenic therapies for cancer (155).

DOWNSTREAM EFFECTORS OF YAP/TAZ IN TUMOR ANGIOGENESIS

To date, the mechanisms through which YAP/TAZ control (tumor) angiogenesis remains unclear. Active YAP/TAZ act as co-factors and bind to transcription factors to modulate gene expression (156). YAP/TAZ both contain a TEAD-binding domain and one WW (TAZ) or two WW domains (YAP) that mediate the interaction with transcription factors (157). The interaction of YAP/TAZ with the transcriptional enhancer factor domain (TEAD) family has been considered as the primary axis through which YAP/TAZ regulate transcription. TEAD-dependent transcription include most of the classical YAP/TAZ target genes, including CTGF, CYR61, and ANKRD1 (158–160). The YAP-TEAD complex is important for tumorigenesis: mutating the TEAD binding domain in YAP suppresses its oncogenic capacity in cancer cells (159, 161). The ability of tumor tissue to promote tumor angiogenesis through expression of angiogenic factors occurs in a TEAD-dependent manner (102, 162). Furthermore, pharmacological inhibition of the YAP-TEAD complex with verteporfin suppresses tumor growth in pancreatic cancer, by inhibiting the proliferation of pancreatic ductal adenocarcinoma cells and through inhibition of the angiogenic activity of associated tumor ECs (162).

The WW domains of YAP/TAZ bind to proline-rich sequences such as the PPXY motif, found in a variety of transcription factors, and they mediate the interaction with SMADs, AMOTs, ErbB4, β-catenin, RUNXs, and p73 (163). Mutations in the WW domain perturb YAP-controlled transcriptional programs and reduce its oncogenic capacity (161). While not yet investigated directly, the WW domains of YAP/TAZ are likely to modulate tumor angiogenesis, as YAP/TAZ WW-binding proteins, such as SMADs and AMOTs, have readily been linked to angiogenesis (164, 165).

Promoting gene expression through TEAD-binding or the WW domains are not the only mechanisms through which YAP/TAZ may regulate tumor angiogenesis. For instance TBX5, a transcription factor that lacks a PPXY-motif, can interact with YAP/TAZ *via* its carboxyl-terminus (166). Moreover, SMAD2/3 can bind the coiled-coil region of YAP/TAZ (167). Multiple YAP/TAZ interactors can synergize to promote gene expression. For instance, ErbB4 binds to YAP through the WW domain (168), which promotes the binding of TEAD to YAP. In this way, ErbB4 regulates the expression of the canonical YAP/TAZ-TEAD targets, including CTGF, CYR61, and ANKRD1 (169). AP-1 is a transcription factor present in most of YAP/TAZ-TEAD genomic binding sites and its presence greatly enhances oncogenic growth induced by active YAP/TAZ. Conversely, AP-1 inactivation inhibits YAP/TAZ-driven proliferation and tumorigenesis (170, 171). AP-1 does not directly bind to YAP/TAZ, but it controls TEAD-dependent gene expression in a cis-regulatory fashion (170–172) (**Figure 2**). Interestingly, TRPS1 also controls YAP/TAZ through regulatory elements, but decreases YAP transcriptional activity by recruiting co-repressor complexes (173). This shows that YAP/TAZ is capable of inducing gene expression, not only through direct interactions with transcription factors, but also through regulators which are in close proximity of nuclear YAP/TAZ. Which of these transcriptional regulators are activated in tumor ECs is currently still unclear.

Canonical YAP/TAZ Effectors in Tumor Angiogenesis

Tumor ECs express a different transcriptional program compared to normal ECs (174). Many genes which are upregulated during physiological angiogenesis, are also upregulated in ECs in the TME (175, 176); for example increased VEGF-VEGFR2 signaling (177, 178). In addition, the expression of various genes involved in the interaction of ECs with immune cells are downregulated, suggesting that tumor-associated ECs play an important role in the immunotherapeutic resistance of the TME (179, 180). To understand how YAP/TAZ influences tumor angiogenesis, we need to consider the (potential) function of their downstream transcriptional targets.

Activation of YAP/TAZ induce the transcription of a canonical set of genes involved in proliferation, migration and cytoskeletal rearrangement and suppresses genes related to apoptosis in a variety of cell types (67, 94). In **Table 1** we summarize the currently known YAP/TAZ target genes and their possible involvement in (tumor) angiogenesis (**Table 1**). These genes were included as part of the endothelial YAP/TAZ target gene signature if detected in a minimum of three independent YAP/TAZ transcriptome studies, and have been confirmed at least once in an endothelial context (77, 79, 153, 158, 244, 245). For an overview of all YAP/TAZ target genes from these studies, see **Supplemental Table 1**.

The well-established YAP/TAZ transcriptional targets connective tissue growth factor (CTGF), Cysteine-rich angiogenic inducer 61 (CYR61) and Ankyrin Repeat Domain 1 (ANKRD1) were upregulated in all of the above-mentioned transcriptome studies. CTGF and CYR61 are part of the CCN

TABLE 1 | Selection of YAP/TAZ-regulated genes involved in (tumor) angiogenesis.

Gene	Function	Role in (tumor) angiogenesis	References
CYR61	Matricellular protein of the CCN family, regulates inflammation, wound healing, and fibrosis.	Promotes angiogenic processes through integrin αvβ3, which in turn activates VEGFR2 and downstream MAPK/PI3K signaling pathways.	(154, 181)
ANKRD1	Transcriptional effector, highly expressed in cardiac, and skeletal muscle and able to interact with transcription factors of different pathways.	ANKRD1-/- mouse have angiogenic impairments. ANKRD1 has been proposed to mediate angiogenesis through control of MMP-mediated ECM remodeling.	(182–184)
CTGF	Matricellular protein of the CCN family. A known mediator of fibrosis in multiple diseases. Cofactor required for TGFβ activity.	Activates VEGF signaling by aiding MMP-mediated cleavage of VEGF and prolongs VEGF signaling. Moreover, CTGF binding to integrin αvβ3 activates RhoA and promotes cell migration.	(185–187)
AMOTL2	Scaffold protein, acts as a link between VE-cadherin and contractile F-actin. AMOTL2 plays a regulatory role in the Hippo pathway.	AMOTL2 is a negative regulator of YAP/TAZ by preventing their nuclear translocation. The role of AMOTL2 in tumor angiogenesis is still debated, but it has been shown to promote proliferation and migration during angiogenesis and to promote tumorigenesis in specific tissues.	(57, 188, 189)
FGF2	Growth factor, interacts with FGF receptors and integrins. Plays an important role in the regulation of cell survival, division, differentiation, and migration.	Angiogenic growth factor implicated in tumor resistance to anti-VEGF tumor therapies. ECs are activated through MAPK and PI3K/Akt pathways, inducing MMP production, migration, and proliferation.	(190–192)
SLIT2	Secreted protein that acts as a guidance cue in cellular migration. Functions through the ROBO family as an inhibitor in processes neuronal migration and leukocyte chemotaxis.	SLIT2 has been implicated in tumor angiogenesis and shown to regulate endothelial migration. SLIT2 was found to suppress endothelial migration *in vitro*. Loss of SLIT2 resulted in decreased tumor vessel density in a tumor growth mouse model. Loss of endothelial SLIT2 in mouse models of breast and lung cancer suppressed tumor cell migration and metastatic events.	(193–196)
DLC1	Rho-GTPase-activating protein, controls RhoA inactivation, and regulates the actin cytoskeleton, cell shape, adhesion, migration, and proliferation.	DLC1 has a regulatory role in cell-contact inhibition of proliferation, EC migration and (tumor) angiogenesis. Interference of the DLC1-RhoA axis in knockout models disrupted cell migration and caused angiogenic defects.	(140, 197–199)
SERPINE1	Serine protease inhibitor regulating fibrinolysis through inhibition of tissue- and urokinase-type plasminogen activator. Major downstream effector of TGFβ signaling and upregulated in inflammatory, fibrotic, and thrombotic events.	SERPINE1 expression is positively correlated to tumorigenesis. Independent of its protease inhibitor activity, SERPINE1 regulates angiogenic related processes, such as matrix degradation, migration, proliferation, and cytoskeleton changes. Furthermore, inhibition of urokinase plasminogen activator was shown to inhibit angiogenesis.	(200–203)
CRIM1	(Putative) transmembrane protein containing IGF-binding domain. Plays a role in development and in different tissues by binding secreted growth factors.	CRIM1 plays a role in vascular development, capillary formation and angiogenesis by augmenting VEGF-A signaling through VEFGR2. In cancer, CRIM1 was shown to regulate cell adhesion and migration.	(204–207)
AXL	AXL is a receptor tyrosine kinase (RTK), that upon binding its ligand (growth factor GAS6) activates the PI3K/Akt pathway.	AXL plays a role in neovascularization by regulating EC proliferation, survival and migration. *In vivo* models demonstrated its importance in angiogenesis and tumor formation. AXL regulates angiogenesis by modulating Ang2 and DKK3 levels.	(208–212)
BIRC5	Mitotic spindle checkpoint gene, component of the mitotic apparatus, involved in chromosome alignment and segregation during mitosis and cytokinesis. Cell cycle dependent expression, promoting cell proliferation and inhibition of apoptosis.	Several studies found a correlation between tumor angiogenesis and BIRC5 expression. BIRC5 promotes endothelial proliferation and migration and inhibits apoptosis. BIRC5-dependent VEGF and FGF expression and modulation of the PI3K/Akt pathway are the proposed mechanisms for the angiogenic effect of BIRC5.	(213–217)
SHCBP1	Adaptor protein associated with cell surface receptors, involved in various signaling pathways, such as FGF, NF-κB, TGFβ-1/Smad, and β-catenin signaling.	SHCBP1 is upregulated in several cancers and promotes proliferation and migration. SHCBP1 overexpression increased VEGF expression and promoted angiogenesis through TGFβ/SMAD signaling.	(218–221)
SGK1	Serine/threonine-protein kinase, downstream effector of PI3K/mTORC2 signaling. Anti-apoptotic gene regulating cell growth, proliferation, survival, and migration.	Role in angiogenesis through phosphorylation of SGK1 target NDRG1 (also a transcriptional target of YAP/TAZ), which modulates NF-κB signaling and expression of VEGF. Activated NDRG1 induces expression of angiogenic CXC cytokines, such as IL-8. High microvessel density in tumors correlates with NDRG1 nuclear activity. Knockdown of SGK1/NDRG1 reduced tumor angiogenesis.	(222–225)
DDAH1	Role in the regulation of nitric oxide generation. Inhibits degradation of nitric oxide synthase.	Upregulated in tumor tissues, increases VEGF expression as a result of elevated nitric oxide levels and correlates with tumor growth and angiogenesis *in vivo*. Targeting of DDAH1 with a therapeutic compound resulted in regression of tumor size and tumor vasculature density.	(226–232)
TK1	Thymidine kinase, involved in cell division. Activity of the cytosolic enzyme is highest during the S-phase. Marker for proliferating cells.	TK1 is upregulated in tumor-associated ECs. Exact role of TK1 in tumor angiogenesis is still unknown, but it was found to act as both an angiogenic and angiostatic factor.	(233–235)

(Continued)

TABLE 1 | Continued

Gene	Function	Role in (tumor) angiogenesis	References
TGFB-2	Ligand activating the TGFβ receptor/SMAD pathway; activation of this pathway results in an increase in the deposition of ECM, angiogenesis, immunosuppression and alterations in cell adhesion.	Multifunctional protein, involved in regulation of angiogenesis. Implicated in tumor angiogenesis through activation of YAP/TAZ and upregulation of angiogenic factors. Also able to indirectly promote angiogenesis by reorganizing the ECM and promoting the immune system.	(164, 236)
ECT2	Rho GEF activating Rho-GTPases, like RhoA, RhoC, Rac1, and CDC42, plays a role in cell division.	During angiogenesis, ECT2 controls VEGF-induced activation of Rho-GTPases. Knockdown of ECT2 inhibits sprouting angiogenesis. During tumorigenesis, deregulated ECT2 drives tumor cell proliferation.	(237–240)
COL4A3	Subunit of type IV collagen, structural component of the basement membrane.	Expressed in angiogenic endothelial cultures and considered as an angiogenic factor. Upregulated in tumor tissue and COL4A3 expression levels are correlated to tumor progression. In contrast, Tumstatin (MMP product of COL4A3) suppresses angiogenesis.	(241–243)

Table 1 *highlights the most common YAP/TAZ target genes and provides an overview of the different mechanisms through which they regulate (tumor) angiogenesis. These genes were considered as common YAP/TAZ targets when found in a minimum of three independent YAP/TAZ transcriptome studies, and have been confirmed at least once in an endothelial cell context (77, 79, 153, 158, 244, 245). The full list of genes can be found in* ***Supplemental data file*** *1. The variety of effectors through which YAP/TAZ affect tumor angiogenesis currently makes it difficult to discern the significance of each protein to the process.* ***Table 1*** *demonstrates the diversity of ways through which YAP/TAZ transcriptional targets are able to regulate tumor angiogenesis, e.g., as an extracellular matrix (ECM) component, a secreted factor, a modulator of a signaling pathway, a transcription factor in the nucleus or as a combination of such mechanisms. Furthermore, these YAP/TAZ targets are also expressed by other cells in the tumor microenvironment (TME), such as tumor cells and fibroblasts, and often work through a common mode of action to promote tumor angiogenesis.*

protein family (246). CTGF is a known mediator of fibrosis in a wide range of diseases (247). During tumor progression, CTGF plays a role in ECM deposition and promotes proliferation and epithelial to mesenchymal transition (EMT) (159, 248). Stromal expression of CTGF increases micro-vessel density in prostate cancer xenografts (249). CTGF was found to promote angiogenesis by inducing VEGF-A secretion of TGFβ-stimulated fibroblasts (250). CTGF also promotes tumor angiogenesis through regulation of Ang2 (251).

The YAP/TAZ target CYR61 was demonstrated to promote angiogenesis and improve tissue perfusion in ischemic models (252). CYR61 null mice are embryonically lethal caused by vascular defects (253). CYR61 is expressed in angiogenic ECs at sites of neovascularization (254). Increased expression of CYR61 is found in many forms of cancer and is linked to an increase of size and vascularization of tumors (255–257). CYR61 promotes tumor angiogenesis through its interaction with integrin αvβ3, which in turn regulates endothelial adhesions, migration, proliferation, and activates VEGFR2 (154).

ANKRD1 is a transcriptional effector of YAP/TAZ. ANKRD1 has been shown to have an anti-inflammatory role through inhibition of NF-κB (182). ANKRD1 may act as a co-activator of the tumor suppressor p53, and the presence of p53 maintains the expression levels of ANKRD1 through a positive feedback loop (183, 258). In cancer ANKRD1 can be epigenetically inactivated (258), which may explain how such a well-established YAP/TAZ target does not attenuate YAP/TAZ-mediated tumorigenesis. ANKRD1 has been proposed to mediate angiogenesis through MMP-mediated ECM remodeling (184). ANKRD1 promotes angiogenesis after acute wounding of mouse skin (259, 260), but currently little is known about the potential role of ANKRD1 in tumor angiogenesis.

Endothelial-Specific YAP/TAZ Angiogenic Effectors

YAP/TAZ also induce the expression of endothelial-specific genes to modulate the tumor vasculature. While not always being referred to as YAP/TAZ effectors in the literature, these pro-angiogenic proteins are readily investigated for their role in tumor angiogenesis and their potential as therapeutic targets.

Ephrin-Eph System

The Ephrin receptor genes EphA2-4 and EphB4 and Ephrin genes EFNB2-3 were found to be downstream targets of endothelial YAP/TAZ in developmental angiogenesis (79). Ephrins and Eph receptors are upregulated in almost all tumors and are considered as promising targets for cancer therapy (261). Interestingly, in YAP/TAZ transcriptome studies focusing on expression in cancer cell lines, the Ephrin family does not seem to be a prominent transcriptional target of YAP/TAZ (158, 244, 245), which indicates that Ephrins may be specifically derived from YAP/TAZ activation in the tumor endothelium.

The Eph receptors and Ephrin ligands are crucial for vascular specification during development *via* their signaling toward Rho-GTPases, cytoskeletal remodeling, and cell migration (262). Expression of the EphA2 receptor has been reported to promote tumor size and vascular density (263, 264). Genetic silencing or blocking activation of the EphA receptors attenuated tumor angiogenesis and decreased tumor vessel density (265, 266). Signaling through EphrinB2 and EphB4 has been directly associated with tumor angiogenesis and with tumor resistance to anti-angiogenic therapy (267, 268). Inhibition of EphB4 signaling through the use of soluble ligands, reduced the growth and vascularization of tumors (269). Another study showed that EphB4 suppresses sprouting angiogenesis and induces circumferential growth of blood vessels in tumor xenografts (270). Furthermore, the Ephrins and their receptors differ in expression levels between tumor types. For instance, EphA2 and EphB2 both promote tumor angiogenesis, but EphA2 is upregulated in prostate cancer, while EphB2 is downregulated (261). Before considering Ephrin signaling as therapeutic target to normalize the tumor vasculature, more understanding is needed of the downstream mechanisms of Eph receptors, as

they induce vascularization in some cancers, but restrict tumor growth in others.

Angiopoietin-Tie System

Ang2 is a downstream transcriptional target of YAP/TAZ in ECs (75, 79). The ligands Angiopoietin-1 (Ang1) and Ang2, bind to Tie receptors and control the angiogenic activation of ECs to finely tune vascular development and homeostasis (271, 272). The Ang2 antagonist Ang1 is an important regulator of vessel maturation and Ang1-Tie2 signaling induces endothelial quiescence (272). Conversely, interaction of Ang2 with Tie2, results in disruption of EC monolayer integrity, making ECs more responsive to inflammatory and pro-angiogenic cytokines (273–275). Ang2 is produced by ECs and signals in an autocrine manner (274). Ang2 expression is induced in response to inflammatory cytokines, hypoxia, and haemodynamic forces (275, 276). Ang2 is highly expressed in ECs of remodeling vessels, indicating an important role for Ang2 in angiogenesis (275, 277). Interestingly, Ang2 may exert both pro- and anti-angiogenic functions. In the presence of VEGF, Ang2 has a pro-angiogenic effect on ECs, while in the absence of VEGF, apoptosis, and vessel regression is induced by Ang2 (278–281). Ang1 binding to Tie2 induces Tie2 autophosphorylation and downstream PI3K/Akt and ERK signaling pathways and inhibits NF-κB activation (271, 273). Moreover, Ang1-Tie2 signaling inhibits Ang2 expression, maintaining endothelial quiescence (273). Upregulation of Ang2 competes with Ang1-Tie2 signaling, resulting in destabilization of the vascular endothelium (273).

Ang2 is described to be a mediator of YAP-induced angiogenesis in mouse retinal vasculature (75, 84). Ang2 levels correlate with YAP activation in sprouting vessels in the retina (75). Supplementation of Ang2 rescues angiogenic defects in the retinal vasculature of YAP/TAZ knockdown mouse and marks Ang2 as a prominent downstream effector of YAP/TAZ-regulated angiogenesis (75). Blocking Ang2 was able to inhibit endothelial YAP-induced angiogenic sprouting (84). Ang2 is upregulated in several types of cancer and is a mediator of tumor angiogenesis (282). Interestingly, YAP and Ang2 association is also observed in the tumor vessels of melanoma (75). In astrocytomas, Ang2 upregulation was correlated with increased vascular growth and an abnormal tumor vasculature (283). Furthermore, angiogenic tumor vessels of human squamous cell carcinoma and skin carcinogenesis xenografts showed an upregulation of Ang2 (284). Interestingly, Ang1 overexpression inhibits tumor growth in these cancer models. The amount of vascularization was unchanged, however more pericyte coverage of the tumor vessels was observed, pointing toward vessel maturation (284, 285). Alternatively, in tumor ECs, Ang2 has been described to induce pro-angiogenic effects by triggering integrin adhesion signaling (286). Ang2 inhibition and Tie2 activation in experimental glioma models resulted in normalization of the tumor vessels, reduced hypoxia and acidosis in the TME, and reduced tumor growth (287). In experimental glioblastoma models, a combination of VEGF- and Ang2-inhibition, was also found to induce tumor vessel normalization (288). Overall, these findings indicate that the YAP/TAZ effector Ang2 might be a promising therapeutic target in cancer.

Fibroblast Growth Factor 2

Fibroblast growth factor 2 (FGF2) is a well-defined YAP/TAZ target expressed in both tumor cells and ECs (77, 79, 158, 244, 245). FGF2 signals through the FGF receptor (FGFR) tyrosine kinase family and induces a broad range of cellular functions, including proliferation, migration, and angiogenesis. Tumor-secreted FGF2, in conjunction with VEGF, promotes tumor angiogenesis (190, 191). The FGF2 pathway is being considered as an important angiogenic pathway involved in bypassing tumor resistance to anti-angiogenic therapies that target VEGF signaling (289, 290). Inhibiting FGF2-FGFR signaling in mice indeed improved tumor sensitivity to anti-VEGF therapy, suggesting that therapeutic strategies that target both growth factors could form a stronger anti-angiogenic intervention in cancer (192).

Deleted-in-Liver-Cancer 1

We recently discovered that Deleted-in-liver-cancer-1 (DLC1), a Rho GAP protein, functions as a direct target of YAP/TAZ in ECs (140). DLC1 is a potential tumor suppressor in various cancer types (291, 292). DLC1 is recruited to integrin-based adhesions through binding to the FA proteins talin, tensin and/or FAK (293, 294). DLC1 knock out mice are embryonically lethal, and the depletion of DLC1 leads to vascular defects (197, 295). DLC1 expression is upregulated by ECM stiffening and angiogenic VEGF signaling (140, 296), and it functions as a prominent target of YAP/TAZ by driving endothelial FA turnover, collective cell migration and sprouting angiogenesis (140). Perturbation of YAP/TAZ signaling and DLC1 levels affect endothelial contact inhibition and promote the development of angiosarcoma (198, 297). Intriguingly, depletion of DLC1 from normal epithelial cells, resulted in increased production of VEGF and upregulation of active HIF1α, suggesting that the absence of DLC1 in oncogenic cells can drive angiogenesis in a paracrine fashion (298). Whether DLC1 is an important target of YAP/TAZ in the tumor endothelium remains to be addressed.

CXCL Chemokines

Various chemokine CXC family members (e.g., CXCL1, CXCL6, CXCL12) are regulated by YAP/TAZ in the endothelium (79, 153). CXCL1, CXCL2, CXCL3, and CXCL8 are part of an angiogenic subset of the CXC family that regulate chemotaxis and angiogenesis through the GPCR CXCR2 (299). In physiological context, CXCL1 primarily targets ECs and neutrophils (299). However, CXCL1 and CXCR2 are also upregulated in different tumor tissues and induce tumor angiogenesis (300–303). It is thought that the inflammatory cytokines contribute to the TME by recruiting inflammatory cells and inducing stromal cell senescence (304–307).

Are There Tumor Angiogenesis-Specific YAP/TAZ Effectors?

The YAP/TAZ-regulated transcriptome is tissue specific. In the endothelium, YAP/TAZ modulate angiogenesis by regulating

angiogenic genes. Interestingly, Wang et al. compared RNA-sequencing data from endothelial-specific YAP/TAZ KO mice with that from VEGF-treated HUVECs. Intriguingly, the VEGF-regulated genes were enriched in the gene set that was downregulated upon YAP/TAZ depletion (79). This indicates that YAP/TAZ activation and VEGF signaling synergize to promote angiogenesis.

The transcriptome of tumor-associated ECs in human lung tumors were recently investigated at single-cell resolution (179, 180, 308). Tumor ECs upregulate genes involved in transcription, oxidative phosphorylation and glycolysis, whereas they suppress inflammatory genes. Interestingly, TEAD1 was found as one of the two transcription factors responsible for the tumor-associated endothelial phenotype (179). A large number of the upregulated genes in tumor-associated ECs as reported by Lambrechts et al. are also controlled by YAP/TAZ (**Supplemental table 1**). Furthermore, tumor-associated ECs strongly activate VEGF and Notch signaling (180), which is likely mediated by YAP/TAZ through crosstalk between these pathways. Treatment with anti-VEGF therapies converts the transcriptomic profile of tumor-associated ECs into a quiescent EC type (308), emphasizing that the activity of tumor ECs is sensitive to therapeutic interventions.

To address if tumor angiogenesis is promoted through specific genes, other than those employed during physiological angiogenesis, the gene expression profiles of ECs in resting liver, regenerating liver, and tumor-bearing liver were compared (233). This study confirmed the upregulation of established angiogenic genes involved in proliferation, such as Top2a, TK1, and Ki67. Incidentally, these genes have also been reported as downstream targets of YAP/TAZ (77, 79, 158, 244, 245). The study further identified two genes that were markedly upregulated during tumor angiogenesis and have been reported as YAP/TAZ targets, namely, SH2D5 (158) and Apelin (79).

SH2 domain containing protein 5, or SH2D5, is a transcriptional target of YAP/TAZ (158) and promotes tumor growth through interaction with transketolase, a regulator of the STAT3 signaling pathway (309). The STAT3 signaling pathway is essential during physiological and tumor angiogenesis (310). In tumors, sustained STAT3 signaling promotes VEGF expression and angiogenesis (311). Furthermore, STAT3 interacts with YAP to promote angiogenesis in a synergistic manner (109). By contrast, a recent paper described YAP to downregulate STAT3 activity and inhibit VEGF expression (312). By regulating STAT3 and VEGF signaling, SH2D5 might be involved in regulating YAP/TAZ activity during tumor angiogenesis.

Apelin is a secreted protein and its expression is regulated by endothelial YAP/TAZ (79). Apelin is required during vascular development (313) and controls initiation of angiogenesis (314). In tumors, increased levels of Apelin promote tumor angiogenesis (314–317). In addition, Apelin drives angiogenesis of lymphatic vessels (318). Interestingly, the GPCR of Apelin, is upregulated in ECs taking part in pathological angiogenesis (308). Because of the anti-angiogenic and anti-lymphangiogenic abilities of Apelin, Apelin has been proposed as a potential therapeutic target for tumor therapies (319).

YAP/TAZ SUSTAIN TUMOR ANGIOGENESIS THROUGH FEEDBACK MECHANISMS

Physiological angiogenesis and tumor angiogenesis are largely driven by common mechanisms (174, 233). The tumor vasculature is however very different in its organization and function compared to healthy vasculature (10). YAP/TAZ promote the formation of a disorganized and dense tumor vasculature network and simultaneously prevent vessel maturation and specification by sustaining angiogenic signaling (40). In this section we provide an overview of the potential mechanisms through which endothelial YAP/TAZ sustain angiogenic signaling in the TME.

Feedback Signals that Fine Tune YAP/TAZ

YAP/TAZ are regulated by genes that feedback on the upstream elements of the signaling pathway. For instance, the YAP/TAZ target NUAK2 sustains YAP/TAZ activity in breast cancer cells through inhibition of LATS1/2 (320). Pharmacological inhibition of NUAK2 reduces tumor growth in mice, indicating its activity is important to enforce tumorigenic YAP/TAZ signaling (320). By contrast, in collectively migrating ECs, NUAK2 provides negative feedback to YAP/TAZ by reducing actomyosin contractility (80), indicating that this YAP/TAZ target's feedback function is tissue-dependent. Members of the AMOT family are known regulators of YAP/TAZ (321), and likely also play a role in regulating endothelial YAP/TAZ activity through feedback; since AMOT and AMOTL2 were identified as YAP/TAZ transcriptional targets in endothelial RNA-sequencing studies (77, 79). The p130 isoform of AMOT increases YAP transcriptional activity by binding to YAP and preventing LATS1-mediated YAP phosphorylation (322). In contrast, AMOTL2 inhibits YAP/TAZ activity by binding directly to YAP or to LATS1/2 kinases (57, 188). In most cancers the AMOT family promotes tumorigenesis, while in others its effect is inhibitory (321), indicating AMOT function to be tissue-specific. Altogether the involvement of the AMOT family in the regulation of YAP/TAZ activity and tumor angiogenesis is controversial and should be studied more closely in endothelial context. Interestingly, LATS1/2 kinases were found to phosphorylate AMOT and to inhibit angiogenesis in ECs, independent of YAP/TAZ transcriptional activity (165). These debated findings highlight the currently limited understanding behind the regulatory switches in the YAP/TAZ pathway.

YAP/TAZ activity is further defined by other transcriptional regulators. β-catenin binds to YAP/TAZ in the nucleus and the complex drives tumorigenesis in β-catenin-driven tumors (323). BIRC5 is one of the transcriptional targets of the β-catenin-YAP/TAZ complex that promotes tumorigenesis (323) and is also a target of endothelial YAP/TAZ (**Supplemental table 1**) (77). BIRC5 was found to upregulate VEGF expression in esophageal cancer cells (213), and may therefore potentially sustain tumor

angiogenesis through VEGF signaling. The interaction of YAP/TAZ with β-catenin is regulated by the Wnt pathway: in the absence of Wnt activity, the APC/axin destruction complex degrades the β-catenin-YAP/TAZ complex (324, 325). Moreover, Wnt5a/b and Wnt3a promote YAP/TAZ activity through Rho-GTPase-mediated inactivation of LATS (326). YAP/TAZ, in turn, promote expression of DKK1, BMP4, and IGFBP4, inhibitors of the canonical Wnt/β-catenin signaling (326). Thus, the crosstalk events that take place between YAP/TAZ and Wnt signaling could be an interesting element in the sustained YAP/TAZ signaling in tumor growth and angiogenesis.

YAP/TAZ-Triggered Positive Feedback Loops

Angiogenic Growth Factor Signaling

In angiogenic tissue, YAP/TAZ generate positive feedback by driving a transcriptional response that sustains VEGF-VEGFR2 signaling (79). VEGF signaling itself activates endothelial YAP/TAZ and promotes YAP-dependent STAT3 activation (79, 84, 109). In turn, activated STAT3 elevates VEGF expression (109, 311). YAP/TAZ further enhance VEGF signaling through the YAP/TAZ target CRIM1, a transmembrane receptor that is highly expressed in angiogenic ECs (204). CRIM1 interacts with VEGF, and promotes VEGFR2 phosphorylation (204, 205).

Several YAP/TAZ effectors amplify VEGF signaling downstream of the VEGFR. For instance, the YAP/TAZ effector Rho GEF ECT2 is essential for VEGF-mediated activation of RhoA and endothelial migration (237, 327). In addition, increased VEGF signaling induces MMP expression, which is important for matrix degradation and basement membrane remodeling during angiogenesis (328). MMPs are also upregulated by the YAP/TAZ transcriptional program (329, 330), and may in turn modulate VEGF signaling by controlling VEGFR2 expression (331, 332).

Active endothelial YAP/TAZ drive the expression of other angiogenic cytokines as well, such as FGF2, CXCL1 and TGFβ-2 (see **Supplemental table 1**). TGFβ-2 activates the TGFβ receptor/SMAD signaling axis to promote angiogenesis, whereas TGFβ-1 promotes angiogenesis through VEGF expression (164). Thus, endothelial YAP/TAZ activation sustains tumor angiogenesis by enhancing VEGF signaling and related angiogenic factors.

TME Stiffening and Cellular Contractility

YAP/TAZ promote a transcriptional program that increases TME stiffening and intracellular contractility, which in turn reinforces YAP/TAZ signaling (67, 97). Activation of YAP/TAZ in fibroblasts is well known to promote deposition of ECM proteins, secretion of MMPs and cross-linking enzymes (78, 97). Within tumors, the CAFs synthesize fibronectin and collagens, which are key constituents of the stiff tumor tissue and modulate tumor angiogenesis (78, 97). ECM stiffening induces endothelial YAP/TAZ signaling to promote angiogenic sprouting (131, 140). In addition, ECM stiffening and YAP/TAZ signaling jointly induce EndoMT, during which ECs undergo mesenchymal transformation and start to actively participate in TME stiffening and vascular remodeling (333). Along that line, YAP/TAZ signaling in cholangiocarcinoma cells promotes expression and deposition of MFAP5, which is an component of the elastin fibrils in the ECM and promotes tumor vasculature formation (102). The YAP/TAZ target SERPINE1 is another secreted factor that correlates with tumor progression and modulates angiogenesis by competing with ECM proteins for binding to integrins (200, 201).

Finally, YAP/TAZ also regulate expression of genes that increase cytoskeletal contractility to mechanoactivate and further enforce their signaling. Endothelial YAP/TAZ induce expression of well-known modulators of intracellular tension, including the Ephrin-Eph system and the Rho-GTPase family and their regulators, such as the Rho GAP DLC1 and Rho GEF ECT2 (77, 79, 140).

Inflammatory Factors

Inflammatory cytokines in the TME activate YAP/TAZ and sustain angiogenesis (334–338). Inflammatory cytokines activate AP-1 (339), which promotes oncogenic growth in conjunction with YAP/TAZ (171). Expression of the YAP/TAZ effector CYR61 is upregulated by inflammatory cytokines such as IL-1 and TNF-α (340). CYR61 enhances VEGFR2 activity and consequently endothelial YAP/TAZ activity through an integrin αvβ3-VEGFR2-MAPK/PI3K-YAP/TAZ axis and enhanced STAT3 activation (154). However, the details of the reciprocal regulatory mechanism between CYR61 and YAP/TAZ need further investigations, as another study described CYR61 to negatively regulate YAP/TAZ activity (341).

Inflammatory stimuli also trigger the release of the YAP/TAZ target Ang2. Interestingly, blocking the function of Ang2, impaired the interaction between ECs and immune cells and reduced tumor neovascularization (285), suggesting that Ang2 is one of the targets of the YAP/TAZ pathway that might be amenable to normalize the tumor vasculature. Ang2-Tie2 signaling weakens the junctional integrity between ECs (273, 274). The destabilization of cell-cell contacts is known to activate YAP/TAZ *via* the inhibition of LATS1/2 (62, 63). Moreover, the destabilization of VE-cadherin-based cell-cell junctions activates YAP and induces Ang2 expression (342), pointing toward a positive feedback loop between endothelial YAP and Ang2 signaling.

In conclusion, various factors of the TME activate YAP/TAZ in the endothelium. Activated YAP/TAZ promote angiogenesis through a subset of downstream effectors, while other targets further aggravate the TME conditions. **Figure 2** gives an overview of the positive feedback loops that likely sustain YAP/TAZ activation in the TME.

OUTLOOK

The oncogenes YAP/TAZ are interesting targets for cancer therapy as they play an essential role during tumor vascularization. YAP/TAZ have readily been investigated as

therapeutic targets for the tumor stroma. Therapeutic interventions have focused on inhibiting YAP/TAZ by targeting upstream Hippo effectors or the YAP/TAZ-TEAD interaction; such strategies have been reviewed in great detail in recent years (343–345). One of the major challenges of targeting strategies is that YAP/TAZ modulate multiple signaling pathways and that completely blocking YAP/TAZ signaling likely has large side effects on tissue homeostasis (95). In this review, we highlight how YAP/TAZ is able to maintain a hyperactive endothelial state within the TME and how this leads to aberrant tumor angiogenesis. Targeting the transcriptional downstream targets that reinforce YAP/TAZ activity in the endothelium may provide an interesting approach to normalize tumor vasculature and improve the efficacy of cancer therapies.

When determining the role of transcriptional targets downstream of YAP/TAZ in a biological process, such as tumor angiogenesis, there are a few things to consider. First, besides promoting gene expression, YAP/TAZ are capable of silencing genes through recruitment of inhibitory co-factors. Therefore YAP/TAZ effectively downregulate a significant number of target genes (173, 346). Secondly, YAP and TAZ each control unique (as well as overlapping) transcriptomes (158) and could therefore affect tumor angiogenesis differently. While YAP and TAZ are considered closely related paralogs, structural differences between YAP and TAZ proteins likely affect their specific transcriptional activities, which may be relevant for cancer subtypes. Finally, endothelial YAP was shown to have a cytoplasmic function, from where it regulates EC migration through interaction with CDC42 in the mouse retinal neovasculature (83). Hence, future research aimed to understand the cytoplasmic role of YAP/TAZ during tumor angiogenesis could provide important new insights.

Finally, different microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) have been reported to regulate YAP/TAZ activity (347–350). Surprisingly, little is known regarding noncoding RNAs downstream of YAP/TAZ. In renal cell carcinoma, expression of lncRNA lncARSR is increased in a YAP/TEAD-dependent manner. In turn, the binding of lncARSR to YAP prevented LATS-mediated phosphorylation and activated YAP (351). TAZ upregulates the miRNAs miR-224 and miR-135, which promote tumorigenesis through inhibition of tumor suppressor SMAD4 (352) and suppression of LATS kinases (353), respectively. Furthermore, YAP was found to downregulate the lncRNA MT1DP, a tumor suppressor that inhibits YAP expression (354). A recent review of Tu et al. nicely summarizes the crosstalk between lncRNAs and YAP/TAZ during tumorigenesis (355). The role of noncoding RNAs as effectors of YAP/TAZ in tumor angiogenesis and their promise as therapeutic application remains a topic to address in the nearby future.

Here, we have described upstream mechanisms of YAP/TAZ activation in (tumor) ECs and provided an overview of the downstream transcriptional effectors of YAP/TAZ that participate in the development of tumor vasculature. Many YAP/TAZ downstream targets drive a stiff, pro-inflammatory, hypoxic TME, creating a self-sustained positive loop of YAP/TAZ activity and tumor angiogenesis. Interfering with the crucial events in such YAP/TAZ signal amplification steps are expected to put a brake on pathological angiogenesis in tumors and help to inhibit tumor growth and progression.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This study was financially supported by the Netherlands Organization of Scientific Research (NWO-VIDI grant 016.156.327) and the Amsterdam Cardiovascular Sciences institute.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.612802/full#supplementary-material

REFERENCES

1. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* (1992) 3:65–71. doi: 10.1053/sonc.2002.37263
2. Lugano R, Ramachandran M, Dimberg A. Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cell Mol Life Sci* (2019) 77:1745–70. doi: 10.1007/s00018-019-03351-7
3. Zuazo-Gaztelu I, Casanovas O. Unraveling the Role of Angiogenesis in Cancer Ecosystems. *Front Oncol* (2018) 8:248. doi: 10.3389/fonc.2018.00248
4. Hanahan D, Folkman J. Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis. *Cell* (1996) 86:353–64. doi: 10.1016/S0092-8674(00)80108-7
5. Carmeliet P, Jain RK. Angiogenesis in cancer and other disease. *Nature* (2000) 407:249–57. doi: 10.1038/35025220
6. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumor microenvironment in tumorigenesis. *J Cancer* (2017) 8:761–73. doi: 10.7150/jca.17648
7. Franco PIR, Rodrigues AP, de Menezes LB, Pacheco Miguel M. Tumor microenvironment components: Allies of cancer progression. *Pathol - Res Pract* (2020) 216:152729. doi: 10.1016/j.prp.2019.152729
8. Baghban R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor microenvironment complexity and therapeutic implications at a glance. *Cell Commun Signal* (2020) 18:59. doi: 10.1186/s12964-020-0530-4
9. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* (2011) 17:1359–70. doi: 10.1038/nm.2537
10. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* (2005) 15:102–11. doi: 10.1016/j.gde.2004.12.005

11. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, et al. Openings between Defective Endothelial Cells Explain Tumor Vessel Leakiness. *Am J Pathol* (2000) 156:1363–80. doi: 10.1016/S0002-9440(10)65006-7
12. Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* (2005) 437:497–504. doi: 10.1038/nature03987
13. McDonald DM, Baluk P. Significance of Blood Vessel Leakiness in Cancer. *Cancer Res* (2002) 62:5381–5.
14. Goel S, Wong AH-K, Jain RK. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harb Perspect Med* (2012) 2:a006486. doi: 10.1101/cshperspect.a006486
15. Martin JD, Seano G, Jain RK. Normalizing Function of Tumor Vessels: Progress, Opportunities, and Challenges. *Annu Rev Physiol* (2019) 81:505–34. doi: 10.1146/annurev-physiol-020518-114700
16. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* (2005) 23:1011–27. doi: 10.1200/JCO.2005.06.081
17. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus Irinotecan, Fluorouracil, and Leucovorin for Metastatic Colorectal Cancer. *N Engl J Med* (2004) 350:2335–42. doi: 10.1056/NEJMoa032691
18. Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, et al. Paclitaxel plus Bevacizumab versus Paclitaxel Alone for Metastatic Breast Cancer. *N Engl J Med* (2007) 357:2666–76. doi: 10.1056/NEJMoa072113
19. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al. Paclitaxel–Carboplatin Alone or with Bevacizumab for Non–Small-Cell Lung Cancer. *N Engl J Med* (2006) 355:2542–50. doi: 10.1056/NEJMoa061884
20. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* (2011) 473:298–307. doi: 10.1038/nature10144
21. Ribatti D, Annese T, Ruggieri S, Tamma R, Crivellato E. Limitations of Anti-Angiogenic Treatment of Tumors. *Transl Oncol* (2019) 12:981–6. doi: 10.1016/j.tranon.2019.04.022
22. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the Vasculature for Treatment of Cancer and Other Diseases. *Physiol Rev* (2011) 91:1071–121. doi: 10.1152/physrev.00038.2010
23. van Beijnum JR, Nowak-Sliwinska P, Huijbers EJM, Thijssen VL, Griffioen AW. The great escape; the hallmarks of resistance to antiangiogenic therapy. *Pharmacol Rev* (2015) 67:441–61. doi: 10.1124/pr.114.010215
24. Hillen F, Griffioen AW. Tumour vascularization: sprouting angiogenesis and beyond. *Cancer Metastasis Rev* (2007) 26:489–502. doi: 10.1007/s10555-007-9094-7
25. Mancuso MR, Davis R, Norberg SM, O'Brien S, Sennino B, Nakahara T, et al. Rapid vascular regrowth in tumors after reversal of VEGF inhibition. *J Clin Invest* (2006) 116:2610–21. doi: 10.1172/JCI24612
26. Huang Y, Yuan J, Righi E, Kamoun WS, Ancukiewicz M, Nezivar J, et al. Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. *Proc Natl Acad Sci USA* (2012) 109:17561–6. doi: 10.1073/pnas.1215397109
27. Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer* (2011) 11:393–410. doi: 10.1038/nrc3064
28. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* (2008) 8:592–603. doi: 10.1038/nrc2442
29. Pàez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, et al. Antiangiogenic Therapy Elicits Malignant Progression of Tumors to Increased Local Invasion and Distant Metastasis. *Cancer Cell* (2009) 15:220–31. doi: 10.1016/j.ccr.2009.01.027
30. Jain RK. Antiangiogenesis Strategies Revisited: From Starving Tumors to Alleviating Hypoxia. *Cancer Cell* (2014) 26:605–22. doi: 10.1016/j.ccell.2014.10.006
31. Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: A new paradigm for combination therapy. *Nat Med* (2001) 7:987–9. doi: 10.1038/nm0901-987
32. Wong P-P, Bodrug N, Hodivala-Dilke KM. Exploring Novel Methods for Modulating Tumor Blood Vessels in Cancer Treatment. *Curr Biol* (2016) 26:R1161. doi: 10.1016/j.cub.2016.09.043
33. Winkler F, Kozin SV, Tong RT, Chae S-S, Booth MF, Garkavtsev I, et al. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: Role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* (2004) 6:553–63. doi: 10.1016/j.ccr.2004.10.011
34. Fukumura D, Kloepper J, Amoozgar Z, Duda DG, Jain RK. Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges. *Nat Rev Clin Oncol* (2018) 15:325–40. doi: 10.1038/nrclinonc.2018.29
35. Mpekris F, Voutouri C, Baish JW, Duda DG, Munn LL, Stylianopoulos T, et al. Combining microenvironment normalization strategies to improve cancer immunotherapy. *Proc Natl Acad Sci* (2020) 117:3728–37. doi: 10.1073/pnas.1919764117
36. Steins A, Klaassen R, Jacobs I, Schabel MC, van Lier MGJTB, Ebbing EA, et al. Rapid stromal remodeling by short-term VEGFR2 inhibition increases chemotherapy delivery in esophagogastric adenocarcinoma. *Mol Oncol* (2020) 14:704–20. doi: 10.1002/1878-0261.12599
37. Eelen G, Treps L, Li X, Carmeliet P. Basic and Therapeutic Aspects of Angiogenesis Updated. *Circ Res* (2020) 127:310–29. doi: 10.1161/CIRCRESAHA.120.316851
38. Wong P-P, Demircioglu F, Ghazaly E, Alrawashdeh W, Stratford MRL, Scudamore CL, et al. Dual-Action Combination Therapy Enhances Angiogenesis while Reducing Tumor Growth and Spread. *Cancer Cell* (2015) 27:123–37. doi: 10.1016/j.ccell.2014.10.015
39. Zanconato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer. *Cancer Cell* (2016) 29:783–803. doi: 10.1016/j.ccell.2016.05.005
40. Azad T, Ghahremani M, Yang X. The Role of YAP and TAZ in Angiogenesis and Vascular Mimicry. *Cells* (2019) 8:407. doi: 10.3390/cells8050407
41. Park JA, Kwon Y-G. Hippo-YAP/TAZ signaling in angiogenesis. *BMB Rep* (2018) 51:157–62. doi: 10.5483/bmbrep.2018.51.3.016
42. Elaimy AL, Mercurio AM. Convergence of VEGF and YAP/TAZ signaling: Implications for angiogenesis and cancer biology. *Sci Signal* (2018) 11:eaau1165. doi: 10.1126/scisignal.aau1165
43. Panciera T, Azzolin L, Cordenonsi M, Piccolo S. Mechanobiology of YAP and TAZ in physiology and disease. *Nat Rev Mol Cell Biol* (2017) 18:758–70. doi: 10.1038/nrm.2017.87
44. Zhao B, Li L, Lei Q, Guan K-L. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes Dev* (2010) 24:862–74. doi: 10.1101/gad.1909210
45. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. *Nature* (2011) 474:179. doi: 10.1016/j.yexcr.2015.10.034
46. Elosegui-Artola A, Andreu I, Beedle AEM, Lezamiz A, Uroz M, Kosmalska AJ, et al. Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores. *Cell* (2017) 171:1397–410. doi: 10.1016/j.cell.2017.10.008
47. Praskova M, Xia F, Avruch J. MOBKL1A/MOBKL1B Phosphorylation by MST1 and MST2 Inhibits Cell Proliferation. *Curr Biol* (2008) 18:311–21. doi: 10.1016/j.cub.2008.02.006
48. Meng Z, Moroishi T, Guan K-L. Mechanisms of Hippo pathway regulation. *Genes Dev* (2016) 30:1–17. doi: 10.1101/gad.274027.115
49. Chan EHY, Nousiainen M, Chalamalasetty RB, Schäfer A, Nigg EA, Silljé HHW. The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* (2005) 24:2076–86. doi: 10.1038/sj.onc.1208445
50. He M, Zhou Z, Shah AA, Hong Y, Chen Q, Wan Y. New insights into posttranslational modifications of Hippo pathway in carcinogenesis and therapeutics. *Cell Div* (2016) 11:4. doi: 10.1186/s13008-016-0013-6
51. Dobrokhotov O, Samsonov M, Sokabe M, Hirata H. Mechanoregulation and pathology of YAP/TAZ via Hippo and non-Hippo mechanisms. *Clin Transl Med* (2018) 7:23. doi: 10.1186/s40169-018-0202-9
52. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* (2007) 21:2747–61. doi: 10.1101/gad.1602907
53. Boopathy GTK, Hong W. Role of Hippo Pathway-YAP/TAZ Signaling in Angiogenesis. *Front Cell Dev Biol* (2019) 7:49. doi: 10.3389/fcell.2019.00049
54. Totaro A, Castellan M, Di Biagio D, Piccolo S. Crosstalk between YAP/TAZ and Notch Signaling. *Trends Cell Biol* (2018) 28:560–73. doi: 10.1016/j.tcb.2018.03.001
55. Konsavage WM, Yochum GS. Intersection of Hippo / YAP and Wnt / b-catenin signaling pathways Hippo / YAP Signaling Pathway. *Acta Biochim*

Biophys Sin Adv Access (2012) 45:71–9. doi: 10.1093/abbs/gms084.Intersection
56. Attisano L, Wrana JL. Signal integration in TGF-β, WNT, and Hippo pathways. *F1000Prime Rep* (2013) 5:1–8. doi: 10.12703/P5-17
57. Wang W, Huang J, Chen J. Angiomotin-like Proteins Associate with and Negatively Regulate YAP1. *J Biol Chem* (2011) 286:4364–70. doi: 10.1074/jbc.C110.205401
58. Zhao B, Li L, Lu Q, Wang LH, Liu CY, Lei Q, et al. Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes Dev* (2011) 25:51–63. doi: 10.1101/gad.2000111
59. Chan SW, Lim CJ, Chong YF, Pobbati AV, Huang C, Hong W. Hippo Pathway-independent Restriction of TAZ and YAP by Angiomotin. *J Biol Chem* (2011) 286:7018–26. doi: 10.1074/jbc.C110.212621
60. Sun S, Irvine KD. Cellular Organization and Cytoskeletal Regulation of the Hippo Signaling Network. *Trends Cell Biol* (2016) 26:694–704. doi: 10.1016/j.tcb.2016.05.003
61. Kim N-G, Gumbiner BM. Adhesion to fibronectin regulates Hippo signaling via the FAK-Src-PI3K pathway. *J Cell Biol* (2015) 210:503–15. doi: 10.1083/jcb.201501025
62. Benham-Pyle BW, Pruitt BL, Nelson WJ. Mechanical strain induces E-cadherin-dependent Yap1 and β-catenin activation to drive cell cycle entry. *Sci (80-)* (2015) 348:1024–7. doi: 10.1126/science.aaa4559
63. Dasgupta I, McCollum D. Control of cellular responses to mechanical cues through YAP/TAZ regulation. *J Biol Chem* (2019) 294:17693–706. doi: 10.1074/jbc.REV119.007963
64. Venkatramanan S, Ibar C, Irvine KD. TRIP6 is required for tension at adherens junctions. *bioRxiv* (2020) 2020.04.19.049569. doi: 10.1101/2020.04.19.049569. 2020.04.19.049569.
65. Dutta S, Mana-Capelli S, Paramasivam M, Dasgupta I, Cirka H, Billiar K, et al. TRIP6 inhibits Hippo signaling in response to tension at adherens junctions. *EMBO Rep* (2018) 19:337–50. doi: 10.15252/embr.201744777
66. Ibar C, Kirichenko E, Keepers B, Enners E, Fleisch K, Irvine KD. Tension-dependent regulation of mammalian Hippo signaling through LIMD1. *J Cell Sci* (2018) 131:jcs214700. doi: 10.1242/jcs.214700
67. Totaro A, Panciera T, Piccolo S. YAP/TAZ upstream signals and downstream responses. *Nat Cell Biol* (2018) 20:888–99. doi: 10.1038/s41556-018-0142-z
68. Aragona M, Panciera T, Manfrin A, Giulitti S, Michielin F, Elvassore N, et al. A Mechanical Checkpoint Controls Multicellular Growth through YAP/TAZ Regulation by Actin-Processing Factors. *Cell* (2013) 154:1047–59. doi: 10.1016/j.cell.2013.07.042
69. Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci* (2008) 121:2115–22. doi: 10.1242/jcs.017897
70. Potente M, Gerhardt H, Carmeliet P. Basic and Therapeutic Aspects of Angiogenesis. *Cell* (2011) 146:873–87. doi: 10.1016/J.CELL.2011.08.039
71. Szymborska A, Gerhardt H. Hold Me, but Not Too Tight—Endothelial Cell–Cell Junctions in Angiogenesis. *Cold Spring Harb Perspect Biol* (2018) 10: a029223. doi: 10.1101/cshperspect.a029223
72. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* (2003) 161:1163–77. doi: 10.1083/jcb.200302047
73. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer* (2010) 10:505–14. doi: 10.1038/nrc2868
74. Jakobsson L, Bentley K, Gerhardt H. VEGFRs and Notch: a dynamic collaboration in vascular patterning. *Biochem Soc Trans* (2009) 37:1233–6. doi: 10.1042/BST0371233
75. Choi H-J, Zhang H, Park H, Choi KS, Lee HW, Agrawal V, et al. Yes-associated protein regulates endothelial cell contact-mediated expression of angiopoietin-2. *Nat Commun* (2015) 6:1–14. doi: 10.1038/ncomms7943
76. Kim J, Kim YH, Kim J, Park DY, Bae H, Lee D-H, et al. YAP/TAZ regulates sprouting angiogenesis and vascular barrier maturation. *J Clin Invest* (2017) 127:3441–61. doi: 10.1172/JCI93825
77. Neto F, Klaus-Bergmann A, Ong YT, Alt S, Vion A-C, Szymborska A, et al. YAP and TAZ regulate adherens junction dynamics and endothelial cell distribution during vascular development. *Elife* (2018) 7:e31037. doi: 10.7554/eLife.31037
78. Liu F, Lagares D, Choi KM, Stopfer L, Marinković A, Vrbanac V, et al. Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. *Am J Physiol - Lung Cell Mol Physiol* (2015) 308:L344–57. doi: 10.1152/ajplung.00300.2014
79. Wang X, Freire Valls A, Schermann G, Shen Y, Moya IM, Castro L, et al. YAP/TAZ Orchestrate VEGF Signaling during Developmental Angiogenesis. *Dev Cell* (2017) 42:462–78. doi: 10.1016/j.devcel.2017.08.002
80. Mason DE, Collins JM, Dawahare JH, Nguyen TD, Lin Y, Voytik-Harbin SL, et al. YAP and TAZ limit cytoskeletal and focal adhesion maturation to enable persistent cell motility. *J Cell Biol* (2019) 218:1369–89. doi: 10.1083/jcb.201806065
81. Giampietro C, Disanza A, Bravi L, Barrios-Rodiles M, Corada M, Frittoli E, et al. The actin-binding protein EPS8 binds VE-cadherin and modulates YAP localization and signaling. *J Cell Biol* (2015) 211:1177–92. doi: 10.1083/jcb.201501089
82. Bornhorst D, Xia P, Nakajima H, Dingare C, Herzog W, Lecaudey V, et al. Biomechanical signaling within the developing zebrafish heart attunes endocardial growth to myocardial chamber dimensions. *Nat Commun* (2019) 10:4113. doi: 10.1038/s41467-019-12068-x
83. Sakabe M, Fan J, Odaka Y, Liu N, Hassan A, Duan X, et al. YAP/TAZ-CDC42 signaling regulates vascular tip cell migration. *Proc Natl Acad Sci USA* (2017) 114:10918–23. doi: 10.1073/pnas.1704030114
84. He J, Bao Q, Zhang Y, Liu M, Lv H, Liu Y, et al. Yes-associated protein promotes angiogenesis via signal transducer and activator of transcription 3 in endothelial cells. *Circ Res* (2018) 122:591–605. doi: 10.1161/CIRCRESAHA.117.311950
85. Astone M, Lai JKH, Dupont S, Stainier DYR, Argenton F, Vettori A. Zebrafish mutants and TEAD reporters reveal essential functions for Yap and Taz in posterior cardinal vein development. *Sci Rep* (2018) 8:10189. doi: 10.1038/s41598-018-27657-x
86. Nakajima H, Yamamoto K, Agarwala S, Terai K, Fukui H, Fukuhara S, et al. Flow-Dependent Endothelial YAP Regulation Contributes to Vessel Maintenance. *Dev Cell* (2017) 40:523–36.e6. doi: 10.1016/J.DEVCEL.2017.02.019
87. Nagasawa-Masuda A, Terai K. Yap/Taz transcriptional activity is essential for vascular regression via Ctgf expression and actin polymerization. *PloS One* (2017) 12:e0174633. doi: 10.1371/journal.pone.0174633
88. Raza A, Franklin MJ, Dudek AZ. Pericytes and vessel maturation during tumor angiogenesis and metastasis. *Am J Hematol* (2010) 85:593–8. doi: 10.1002/ajh.21745
89. Jain RK. Molecular regulation of vessel maturation. *Nat Med* (2003) 9:685–93. doi: 10.1038/nm0603-685
90. Abraham S, Yeo M, Montero-Balaguer M, Paterson H, Dejana E, Marshall CJ, et al. VE-Cadherin-Mediated Cell-Cell Interaction Suppresses Sprouting via Signaling to MLC2 Phosphorylation. *Curr Biol* (2009) 19:668–74. doi: 10.1016/j.cub.2009.02.057
91. Dvorak HF. "Tumors: Wounds that do not heal." *New Engl J Med* (1986) 314:144–9. doi: 10.1056/NEJM198107023050102
92. Moroishi T, Hansen CG, Guan K-L. The emerging roles of YAP and TAZ in cancer. *Nat Rev Cancer* (2015) 15:73–9. doi: 10.1038/nrc3876
93. Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. *Nat Rev Cancer* (2013) 13:246–57. doi: 10.1038/nrc3458
94. Thompson BJ. YAP/TAZ: Drivers of Tumor Growth, Metastasis, and Resistance to Therapy. *BioEssays* (2020) 42:1900162. doi: 10.1002/bies.201900162
95. Warren JSA, Xiao Y, Lamar JM. YAP/TAZ Activation as a Target for Treating Metastatic Cancer. *Cancers (Basel)* (2018) 10:115. doi: 10.3390/cancers10040115
96. Zanconato F, Cordenonsi M, Piccolo S. YAP and TAZ: a signalling hub of the tumour microenvironment. *Nat Rev Cancer* (2019) 19:454–64. doi: 10.1038/s41568-019-0168-y
97. Calvo F, Ege N, Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, et al. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nat Cell Biol* (2013) 15:637. doi: 10.1038/ncb2756
98. Pan Z, Tian Y, Cao C, Niu G. The emerging role of YAP/TAZ in tumor immunity. *Mol Cancer Res* (2019) 17:1777–86. doi: 10.1158/1541-7786.MCR-19-0375

99. Yang W, Yang S, Zhang F, Cheng F, Wang X, Rao J. Influence of the Hippo-YAP signalling pathway on tumor associated macrophages (TAMs) and its implications on cancer immunosuppressive microenvironment. *Ann Transl Med* (2020) 8:399. doi: 10.21037/atm.2020.02.11
100. Xu C, Mao L, Xiong J, Wen J, Wang Y, Geng D, et al. TAZ Expression on Endothelial Cells Is Closely Related to Blood Vascular Density and VEGFR2 Expression in Astrocytomas. *J Neuropathol Exp Neurol* (2019) 78:172–80. doi: 10.1093/jnen/nly122
101. Yan Y, Song Q, Yao L, Zhao L, Cai H. YAP Overexpression in Breast Cancer Cells Promotes Angiogenesis Through Activating Yap Signaling in Vascular Endothelial Cells. *Researchsquare* PREPRINT (Version 1) (2020). doi: 10.21203/rs.3.rs-27591/v1
102. Marti P, Stein C, Blumer T, Abraham Y, Dill MT, Pikiolek M, et al. YAP promotes proliferation, chemoresistance, and angiogenesis in human cholangiocarcinoma through TEAD transcription factors. *Hepatology* (2015) 62:1497–510. doi: 10.1002/hep.27992
103. Pan Z, Tian Y, Zhang B, Zhang X, Shi H, Liang Z, et al. YAP signaling in gastric cancer-derived mesenchymal stem cells is critical for its promoting role in cancer progression. *Int J Oncol* (2017) 51:1055–66. doi: 10.3892/ijo.2017.4101
104. Xu S, Zhang H, Chong Y, Guan B, Guo P. YAP Promotes VEGFA Expression and Tumor Angiogenesis Though Gli2 in Human Renal Cell Carcinoma. *Arch Med Res* (2019) 50:225–33. doi: 10.1016/j.arcmed.2019.08.010
105. Venkataramani V, Küffer S, Cheung KCP, Jiang X, Trümper L, Wulf GG, et al. CD31 Expression Determines Redox Status and Chemoresistance in Human Angiosarcomas. *Clin Cancer Res* (2018) 24:460–73. doi: 10.1158/1078-0432.CCR-17-1778
106. Zanotelli MR, Reinhart-King CA. Mechanical Forces in Tumor Angiogenesis. *Adv Exp Med Biol* (2018) 1092:91–112. doi: 10.1007/978-3-319-95294-9_6
107. Ajith TA. Current insights and future perspectives of hypoxia-inducible factor-targeted therapy in cancer. *J Basic Clin Physiol Pharmacol* (2018) 30:11–8. doi: 10.1515/jbcpp-2017-0167
108. Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber H-P, et al. Loss of HIF-1α in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* (2004) 6:485–95. doi: 10.1016/j.ccr.2004.09.026
109. Zhu M, Liu X, Wang Y, Chen L, Wang L, Qin X, et al. YAP via interacting with STAT3 regulates VEGF-induced angiogenesis in human retinal microvascular endothelial cells. *Exp Cell Res* (2018) 373:155–63. doi: 10.1016/j.yexcr.2018.10.007
110. Zhang X, Li Y, Ma Y, Yang L, Wang T, Meng X, et al. Yes-associated protein (YAP) binds to HIF-1α and sustains HIF-1α protein stability to promote hepatocellular carcinoma cell glycolysis under hypoxic stress. *J Exp Clin Cancer Res* (2018) 37:216. doi: 10.1186/s13046-018-0892-2
111. Greenhough A, Bagley C, Heesom KJ, Gurevich DB, Gay D, Bond M, et al. Cancer cell adaptation to hypoxia involves a HIF-GPRC5A-YAP axis. *EMBO Mol Med* (2018) 10:e8699. doi: 10.15252/emmm.201708699
112. Dai X-Y, Zhuang L-H, Wang D-D, Zhou T-Y, Chang L-L, Gai R-H, et al. Nuclear translocation and activation of YAP by hypoxia contributes to the chemoresistance of SN38 in hepatocellular carcinoma cells. *Oncotarget* (2016) 7:6933–47. doi: 10.18632/oncotarget.6903
113. Zhao C, Zeng C, Ye S, Dai X, He Q, Yang B, et al. Yes-associated protein (YAP) and transcriptional coactivator with a PDZ-binding motif (TAZ): a nexus between hypoxia and cancer. *Acta Pharm Sin B* (2019) 10:947–60. doi: 10.1016/j.apsb.2019.12.010
114. Xu X, Tan X, Tampe B, Sanchez E, Zeisberg M, Zeisberg EM. Snail Is a direct target of hypoxia-inducible factor 1α (HIF1α) in hypoxia-induced endothelial to mesenchymal transition of human coronary endothelial cells. *J Biol Chem* (2015) 290:16553–664. doi: 10.1074/jbc.M115.636944
115. Zhang H, Von Gise A, Liu Q, Hu T, Tian X, He L, et al. Yap1 Is required for endothelial to mesenchymal transition of the atrioventricular cushion. *J Biol Chem* (2014) 289:18681–92. doi: 10.1074/jbc.M114.554584
116. Sivaraj KK, Dharmalingam B, Mohanakrishnan V, Jeong H-W, Kato K, Schröder S, et al. YAP1 and TAZ negatively control bone angiogenesis by limiting hypoxia-inducible factor signaling in endothelial cells. *Elife* (2020) 9:e50770. doi: 10.7554/eLife.50770
117. Kugeratski FG, Atkinson SJ, Neilson LJ, Lilla S, Knight JRP, Serneels J, et al. Hypoxic cancer-associated fibroblasts increase NCBP2-AS2/HIAR to promote endothelial sprouting through enhanced VEGF signaling. *Sci Signal* (2019) 12:eaan8247. doi: 10.1126/scisignal.aan8247
118. Tamura R, Tanaka T, Akasaki Y, Murayama Y, Yoshida K, Sasaki H. The role of vascular endothelial growth factor in the hypoxic and immunosuppressive tumor microenvironment: perspectives for therapeutic implications. *Med Oncol* (2019) 37:2. doi: 10.1007/s12032-019-1329-2
119. Azad T, Janse van Rensburg HJ, Lightbody ED, Neveu B, Champagne A, Ghaffari A, et al. A LATS biosensor screen identifies VEGFR as a regulator of the Hippo pathway in angiogenesis. *Nat Commun* (2018) 9:1061. doi: 10.1038/s41467-018-03278-w
120. Weis SM, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J Cell Biol* (2004) 167:223–9. doi: 10.1083/jcb.200408130
121. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the β-arrestin-dependent endocytosis of VE-cadherin. *Nat Cell Biol* (2006) 8:1223–34. doi: 10.1038/ncb1486
122. Yu F-X, Zhao B, Panupinthu N, Jewell JL, Lian I, Wang LH, et al. Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell* (2012) 150:780–91. doi: 10.1016/j.cell.2012.06.037
123. Ma B, Cheng H, Gao R, Mu C, Chen L, Wu S, et al. Zyxin-Siah2–Lats2 axis mediates cooperation between Hippo and TGF-β signalling pathways. *Nat Commun* (2016) 7:11123. doi: 10.1038/ncomms11123
124. Pefani D-E, Pankova D, Abraham AG, Grawenda AM, Vlahov N, Scrace S, et al. TGF-β Targets the Hippo Pathway Scaffold RASSF1A to Facilitate YAP/SMAD2 Nuclear Translocation. *Mol Cell* (2016) 63:156–66. doi: 10.1016/j.molcel.2016.05.012
125. Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J, et al. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nat Cell Biol* (2008) 10:837–48. doi: 10.1038/ncb1748
126. ten Dijke P, Goumans M-J, Pardali E. Endoglin in angiogenesis and vascular diseases. *Angiogenesis* (2008) 11:79–89. doi: 10.1007/s10456-008-9101-9
127. Tual-Chalot S, Mahmoud M, Allinson KR, Redgrave RE, Zhai Z, Oh SP, et al. Endothelial Depletion of Acvrl1 in Mice Leads to Arteriovenous Malformations Associated with Reduced Endoglin Expression. *PloS One* (2014) 9:e98646. doi: 10.1371/journal.pone.0098646
128. Lebrin F, Goumans M-J, Jonker L, Carvalho RLC, Valdimarsdottir G, Thorikay M, et al. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* (2004) 23:4018–28. doi: 10.1038/sj.emboj.7600386
129. Park S, Dimaio TA, Liu W, Wang S, Sorenson CM, Sheibani N. Endoglin regulates the activation and quiescence of endothelium by participating in canonical and non-canonical TGF-β signaling pathways. *J Cell Sci* (2013) 126:1392–405. doi: 10.1242/jcs.117275
130. Young K, Tweedie E, Conley B, Ames J, FitzSimons M, Brooks P, et al. BMP9 Crosstalk with the Hippo Pathway Regulates Endothelial Cell Matricellular and Chemokine Responses. *PloS One* (2015) 10:e0122892–e0122892. doi: 10.1371/journal.pone.0122892
131. Bordeleau F, Mason BN, Lollis EM, Mazzola M, Zanotelli MR, Somasegar S, et al. Matrix stiffening promotes a tumor vasculature phenotype. *Proc Natl Acad Sci USA* (2017) 114:492–7. doi: 10.1073/pnas.1613855114
132. Schwartz MA, Ginsberg MH. Networks and crosstalk: integrin signalling spreads. *Nat Cell Biol* (2002) 4:E65–8. doi: 10.1038/ncb0402-e65
133. Kechagia JZ, Ivaska J, Roca-Cusachs P. Integrins as biomechanical sensors of the microenvironment. *Nat Rev Mol Cell Biol* (2019) 20:457–73. doi: 10.1038/s41580-019-0134-2
134. Wada KI, Itoga K, Okano T, Yonemura S, Sasaki H. Hippo pathway regulation by cell morphology and stress fibers. *Development* (2011) 138:3907–14. doi: 10.1242/dev.070987
135. Feng X, Arang N, Rigiracciolo DC, Lee JS, Yeerna H, Wang Z, et al. A Platform of Synthetic Lethal Gene Interaction Networks Reveals that the GNAQ Uveal Melanoma Oncogene Controls the Hippo Pathway through FAK. *Cancer Cell* (2019) 35:457–72. doi: 10.1016/j.ccell.2019.01.009
136. Lamar JM, Xiao Y, Norton E, Jiang Z-G, Gerhard GM, Kooner S, et al. SRC tyrosine kinase activates the YAP/TAZ axis and thereby drives tumor growth

and metastasis. *J Biol Chem* (2019) 294:2302–17. doi: 10.1074/jbc.RA118.004364
137. Pedrosa A-R, Bodrug N, Gomez-Escudero J, Carter EP, Reynolds LE, Georgiou PN, et al. Tumor Angiogenesis Is Differentially Regulated by Phosphorylation of Endothelial Cell Focal Adhesion Kinase Tyrosines-397 and -861. *Cancer Res* (2019) 79:4371–86. doi: 10.1158/0008-5472.CAN-18-3934
138. Tavora B, Reynolds LE, Batista S, Demircioglu F, Fernandez I, Lechertier T, et al. Endothelial-cell FAK targeting sensitizes tumours to DNA-damaging therapy. *Nature* (2014) 514:112–6. doi: 10.1038/nature13541
139. Nardone G, Oliver-De La Cruz J, Vrbsky J, Martini C, Pribyl J, Skládal P, et al. YAP regulates cell mechanics by controlling focal adhesion assembly. *Nat Commun* (2017) 8:15321. doi: 10.1038/ncomms15321
140. van der Stoel M, Schimmel L, Nawaz K, van Stalborch A-M, de Haan A, Klaus-Bergmann A, et al. DLC1 is a direct target of activated YAP/TAZ that drives collective migration and sprouting angiogenesis. *J Cell Sci* (2020) 133:jcs239947. doi: 10.1242/jcs.239947
141. Haage A, Schneider IC. Cellular contractility and extracellular matrix stiffness regulate matrix metalloproteinase activity in pancreatic cancer cells. *FASEB J* (2014) 28:3589–99. doi: 10.1096/fj.13-245613
142. Shen Y, Wang X, Lu J, Salfenmoser M, Wirsik NM, Schleussner N, et al. Reduction of Liver Metastasis Stiffness Improves Response to Bevacizumab in Metastatic Colorectal Cancer. *Cancer Cell* (2020) 37:800–17. doi: 10.1016/j.ccell.2020.05.005
143. Boucher Y, Jain RK. Microvascular Pressure Is the Principal Driving Force for Interstitial Hypertension in Solid Tumors: Implications for Vascular Collapse. *Cancer Res* (1992) 52:5110–4.
144. Schaaf MB, Garg AD, Agostinis P. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis* (2018) 9:115. doi: 10.1038/s41419-017-0061-0
145. Hofmann M, Guschel M, Bernd A, Bereiter-Hahn J, Kaufmann R, Tandi C, et al. Lowering of tumor interstitial fluid pressure reduces tumor cell proliferation in a xenograft tumor model. *Neoplasia* (2006) 8:89–95. doi: 10.1593/neo.05469
146. Haessler U, Teo JCM, Foretay D, Renaud P, Swartz MA. Migration dynamics of breast cancer cells in a tunable 3D interstitial flow chamber. *Integr Biol* (2011) 4:401–9. doi: 10.1039/c1ib00128k
147. Piotrowski-Daspit AS, Tien J, Nelson CM. Interstitial fluid pressure regulates collective invasion in engineered human breast tumors via Snail, vimentin, and E-cadherin. *Integr Biol (Camb)* (2016) 8:319–31. doi: 10.1039/c5ib00282f
148. Huang YL, Tung C, Zheng A, Kim BJ, Wu M. Interstitial flows promote amoeboid over mesenchymal motility of breast cancer cells revealed by a three dimensional microfluidic model. *Integr Biol* (2015) 7:1402–11. doi: 10.1039/c5ib00115c
149. Hoffman LM, Smith MA, Jensen CC, Yoshigi M, Blankman E, Ullman KS, et al. Mechanical stress triggers nuclear remodeling and the formation of transmembrane actin nuclear lines with associated nuclear pore complexes. *Mol Biol Cell* (2020) 31:1774–87. doi: 10.1091/mbc.E19-01-0027
150. Yamashiro Y, Thang BQ, Ramirez K, Shin SJ, Kohata T, Ohata S, et al. Matrix mechanotransduction mediated by thrombospondin-1/integrin/YAP signaling pathway in the remodeling of blood vessels. *Proc Natl Acad Sci* (2020) 117:9896–905. doi: 10.1073/pnas.1919702117
151. Gillies RJ, Schornack PA, Secomb TW, Raghunand N. Causes and effects of heterogeneous perfusion in tumors. *Neoplasia* (1999) 1:197–207. doi: 10.1038/sj.neo.7900037
152. Wang KC, Yeh YT, Nguyen P, Limqueco E, Lopez J, Thorossian S, et al. Flow-dependent YAP/TAZ activities regulate endothelial phenotypes and atherosclerosis. *Proc Natl Acad Sci USA* (2016) 113:11525–30. doi: 10.1073/pnas.1613121113
153. Wang L, Luo J-Y, Li B, Tian XY, Chen L-J, Huang Y, et al. Integrin-YAP/TAZ-JNK cascade mediates atheroprotective effect of unidirectional shear flow. *Nature* (2016) 540:579. doi: 10.1038/nature20602
154. Park M-H, Kim AK, Manandhar S, Oh S-Y, Jang G-H, Kang L, et al. CCN1 interlinks integrin and hippo pathway to autoregulate tip cell activity. *Elife* (2019) 8:e46012. doi: 10.7554/eLife.46012
155. Cui X, Morales R-TT, Qian W, Wang H, Gagner J-P, Dolgalev I, et al. Hacking macrophage-associated immunosuppression for regulating glioblastoma angiogenesis. *Biomaterials* (2018) 161:164–78. doi: 10.1016/j.biomaterials.2018.01.053
156. Kim M, Jang J, Bae S. DNA binding partners of YAP / TAZ. *BMB Rep* (2018) 51:126–33. doi: 10.5483/bmbrep.2018.51.3.015
157. Piccolo S, Dupont S, Cordenonsi M. The Biology of YAP/TAZ: Hippo Signaling and Beyond. *Physiol Rev* (2014) 94:1287–312. doi: 10.1152/physrev.00005.2014
158. Zhang H, Liu CY, Zha ZY, Zhao B, Yao J, Zhao S, et al. TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. *J Biol Chem* (2009) 284:13355–62. doi: 10.1074/jbc.M900843200
159. Zhao B, Ye X, Yu J, Li L, Li W, Li S, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* (2008) 22:1962–71. doi: 10.1101/gad.1664408
160. Stein C, Bardet AF, Roma G, Bergling S, Clay I, Ruchti A, et al. YAP1 Exerts Its Transcriptional Control via TEAD-Mediated Activation of Enhancers. *PloS Genet* (2015) 11:e1005465. doi: 10.1371/journal.pgen.1005465
161. Zhao B, Kim J, Ye X, Lai ZC, Guan KL. Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein. *Cancer Res* (2009) 69:1089–98. doi: 10.1158/0008-5472.CAN-08-2997
162. Wei H, Wang F, Wang Y, Li T, Xiu P, Zhong J, et al. Verteporfin suppresses cell survival, angiogenesis and vasculogenic mimicry of pancreatic ductal adenocarcinoma via disrupting the YAP-TEAD complex. *Cancer Sci* (2017) 108:478–87. doi: 10.1111/cas.13138
163. Callus BA, Finch-Edmondson ML, Fletcher S, Wilton SD. YAPping about and not forgetting TAZ. *FEBS Lett* (2019) 593:253–76. doi: 10.1002/1873-3468.13318
164. Bertolino P, Deckers M, Lebrin F, ten Dijke P. Transforming Growth Factor-beta Signal Transduction in Angiogenesis and Vascular Disorders. *Chest* (2006) 128:585S–90S. doi: 10.1378/chest.128.6
165. Dai X, She P, Chi F, Feng Y, Liu H, Jin D, et al. Phosphorylation of angiomotin by Lats1/2 kinases inhibits F-actin binding, cell migration, and angiogenesis. *J Biol Chem* (2013) 288:34041–51. doi: 10.1074/jbc.M113.518019
166. Murakami M, Nakagawa M, Olson EN, Nakagawa O. A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. *Proc Natl Acad Sci USA* (2005) 102:18034–9. doi: 10.1073/pnas.0509109102
167. Varelas X, Samavarchi-Tehrani P, Narimatsu M, Weiss A, Cockburn K, Larsen BG, et al. The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-β-SMAD pathway. *Dev Cell* (2010) 19:831–44. doi: 10.1016/j.devcel.2010.11.012
168. Schuchardt BJ, Bhat V, Mikles DC, McDonald CB, Sudol M, Farooq A. Molecular Basis of the Binding of YAP Transcriptional Regulator to the ErbB4 Receptor Tyrosine Kinase. *Biochimie* (2014) 101:192–202. doi: 10.1016/j.biochi.2014.01.011
169. Haskins JW, Nguyen DX, Stern DF. Neuregulin 1-activated ERBB4 interacts with YAP to induce Hippo pathway target genes and promote cell migration. *Sci Signal* (2014) 7:ra116. doi: 10.1126/scisignal.2005770
170. Koo JH, Plouffe SW, Meng Z, Lee D-H, Yang D, Lim D-S, et al. Induction of AP-1 by YAP/TAZ contributes to cell proliferation and organ growth. *Genes Dev* (2020) 34:72–86. doi: 10.1101/gad.331546.119
171. Zanconato F, Forcato M, Battilana G, Azzolin L, Quaranta E, Bodega B, et al. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nat Cell Biol* (2015) 17:1218–27. doi: 10.1038/ncb3216
172. Liu X, Li H, Rajurkar M, Li Q, Cotton JL, Ou J, et al. Tead and AP1 Coordinate Transcription and Motility. *Cell Rep* (2016) 14:1169–80. doi: 10.1016/j.celrep.2015.12.104
173. Elster D, Tollot M, Schlegelmilch K, Ori A, Rosenwald A, Sahai E. TRPS1 shapes YAP/TEAD-dependent transcription in breast cancer cells. *Nat Commun* (2018) 9:3115. doi: 10.1038/s41467-018-05370-7
174. Rohlenova K, Goveia J, García-Caballero M, Subramanian A, Kalucka J, Treps L, et al. Single-Cell RNA Sequencing Maps Endothelial Metabolic Plasticity in Pathological Angiogenesis. *Cell Metab* (2020) 31:862–77. doi: 10.1016/j.cmet.2020.03.009

175. Hida K, Maishi N, Annan DA, Hida Y. Contribution of Tumor Endothelial Cells in Cancer Progression. *Int J Mol Sci* (2018) 19:1272. doi: 10.3390/ijms19051272
176. Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, et al. Genes Expressed in Human Tumor Endothelium. *Sci (80-)* (2000) 289:1197–202. doi: 10.1126/science.289.5482.1197
177. Matsuda K, Ohga N, Hida Y, Muraki C, Tsuchiya K, Kurosu T, et al. Isolated tumor endothelial cells maintain specific character during long-term culture. *Biochem Biophys Res Commun* (2010) 394:947–54. doi: 10.1016/j.bbrc.2010.03.089
178. Bussolati B, Assenzio B, Deregibus MC, Camussi G. The proangiogenic phenotype of human tumor-derived endothelial cells depends on thrombospondin-1 downregulation via phosphatidylinositol 3-kinase/Akt pathway. *J Mol Med* (2006) 84:852–63. doi: 10.1007/s00109-006-0075-z
179. Lambrechts D, Wauters E, Boeckx B, Aibar S, Nittner D, Burton O, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat Med* (2018) 24:1277–89. doi: 10.1038/s41591-018-0096-5
180. Kim N, Kim HK, Lee K, Hong Y, Cho JH, Choi JW, et al. Single-cell RNA sequencing demonstrates the molecular and cellular reprogramming of metastatic lung adenocarcinoma. *Nat Commun* (2020) 11:2285. doi: 10.1038/s41467-020-16164-1
181. Lau LF. CCN1 / CYR61 : the very model of a modern matricellular protein. *Cell Mol Life Sci* (2011) 1:3149–63. doi: 10.1007/s00018-011-0778-3
182. Liu X, Bauman WA, Cardozo C. ANKRD1 modulates inflammatory responses IN C2C12 myoblasts through feedback inhibition OF NF-κB signaling activity. *Biochem Biophys Res Commun* (2015) 464:208–13. doi: 10.1016/j.bbrc.2015.06.118
183. Kojic S, Nestorovic A, Rakicevic L, Belgrano A, Stankovic M, Divac A, et al. A novel role for cardiac ankyrin repeat protein Ankrd1 / CARP as a co-activator of the p53 tumor suppressor protein. *Arch Biochem Biophys* (2010) 502:60–7. doi: 10.1016/j.abb.2010.06.029
184. Almodovar-Garcia K, Kwon M, Samaras SE, Davidson JM. ANKRD1 Acts as a Transcriptional Repressor of MMP13 via the AP-1 Site. *Mol Cell Biol* (2014) 34:1500–11. doi: 10.1128/MCB.01357-13
185. Lee MS, Ghim J, Kim SJ, Yun YS, Yoo SA, Suh PG, et al. Functional interaction between CTGF and FPRL1 regulates VEGF-A-induced angiogenesis. *Cell Signal* (2015) 27:1439–48. doi: 10.1016/j.cellsig.2015.04.001
186. Mochizuki S, Tanaka R, Shimoda M, Onuma J, Fujii Y, Jinno H, et al. Connective tissue growth factor is a substrate of ADAM28. *Biochem Biophys Res Commun* (2010) 402:651–7. doi: 10.1016/j.bbrc.2010.10.077
187. Aguiar DP, Correa de Farias G, Branco de Sousa E, De Mattos Coelho-Aguiar J, Lobo JC, Casado PL, et al. New strategy to control cell migration and metastasis regulated by CCN2 CTGF. *Cancer Cell Int* (2014) 14:61. doi: 10.1186/1475-2867-14-61
188. Kim M, Kim M, Park S, Lee C, Lim D. Role of Angiomotin-like 2 mono-ubiquitination on YAP inhibition. *EMBO Rep* (2016) 17:64–78. doi: 10.15252/embr.201540809
189. Wang Y, Li Z, Xu P, Huang L, Tong J, Huang H, et al. Angiomotin-like2 Gene (amotl2) Is Required for Migration and Proliferation of Endothelial Cells during Angiogenesis *. *J Biol Chem* (2011) 286:41095–104. doi: 10.1074/jbc.M111.296806
190. Compagni A, Wilgenbus P, Impagnatiello M, Cotten M, Christofori G. Fibroblast Growth Factors Are Required for Efficient Tumor Angiogenesis. *Cancer Res* (2000) 1:7163–9.
191. Presta M, Era PD, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor / fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev* (2005) 16:159–78. doi: 10.1016/j.cytogfr.2005.01.004
192. Incio J, Ligibel JA, Mcmanus DT, Suboj P, Jung K, Kawaguchi K, et al. Obesity promotes resistance to anti-VEGF therapy in breast cancer by up-regulating IL-6 and potentially FGF-2. *Sci Transl Med* (2018) 10:eaag0945. doi: 10.1126/scitranslmed.aag0945
193. Seth P, Lin Y, Hanai JI, Shivalingappa V, Duyao MP, Sukhatme VP. Magic roundabout, a tumor endothelial marker: Expression and signaling. *Biochem Biophys Res Commun* (2005) 332:533–41. doi: 10.1016/j.bbrc.2005.03.250
194. Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Bin CC, et al. Robo4 is a vascular-specific receptor that inhibits endothelial migration. *Dev Biol* (2003) 261:251–67. doi: 10.1016/S0012-1606(03)00258-6
195. Wang B, Xiao Y, Ding BB, Zhang N, Bin YX, Gui L, et al. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell* (2003) 4:19–29. doi: 10.1016/S1535-6108(03)00164-8
196. Tavora B, Mederer T, Wessel KJ, Ruffing S, Sadjadi M, Missmahl M, et al. Tumoural activation of TLR3–SLIT2 axis in endothelium drives metastasis. *Nature* (2020) 586:299–304. doi: 10.1038/s41586-020-2774-y
197. Shih Y-P, Yuan SY, Lo SH. Down-regulation of DLC1 in endothelial cells compromises the angiogenesis process. *Cancer Lett* (2017) 398:46–51. doi: 10.1016/j.canlet.2017.04.004
198. Sánchez-Martín D, Otsuka A, Kabashima K, Ha T, Wang D, Qian X, et al. Effects of DLC1 Deficiency on Endothelial Cell Contact Growth Inhibition and Angiosarcoma Progression. *JNCI J Natl Cancer Inst* (2018) 110:390–9. doi: 10.1093/jnci/djx219
199. Shih Y-P, Sun P, Wang A, Lo SH. Tensin1 positively regulates RhoA activity through its interaction with DLC1. *Biochim Biophys Acta* (2015) 1853:3258–65. doi: 10.1016/j.bbamcr.2015.09.028
200. Kjøller L, Kanse SM, Kirkegaard T, Rodenburg KW, Rønne E, Goodman SL, et al. Plasminogen activator inhibitor-1 represses integrin-and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. *Exp Cell Res* (1997) 232:420–9. doi: 10.1006/excr.1997.3540
201. Degryse B, Orlando S, Resnati M, Rabbani SA, Blasi F. Urokinase/urokinase receptor and vitronectin/αvβ3 integrin induce chemotaxis and cytoskeleton reorganization through different signaling pathways. *Oncogene* (2001) 20:2032–43. doi: 10.1038/sj.onc.1204261
202. Subramanian R, Gondi CS, Lakka SS, Jutla A, Rao JS. siRNA-mediated simultaneous downregulation of uPA and its receptor inhibits angiogenesis and invasiveness triggering apoptosis in breast cancer cells. *Int J Oncol* (2006) 28:831–9. doi: 10.3892/ijo.28.4.831
203. Degryse B, Neels JG, Czekay RP, Aertgeerts K, Kamikubo YI, Loskutoff DJ. The low density lipoprotein receptor-related protein is a motogenic receptor for plasminogen activator inhibitor-1. *J Biol Chem* (2004) 279:22595–604. doi: 10.1074/jbc.M313004200
204. Fan J, Ponferrada VG, Sato T, Vemaraju S, Fruttiger M, Gerhardt H, et al. Crim1 maintains retinal vascular stability during development by regulating endothelial cell Vegfa autocrine signaling. *Dev* (2014) 141:448–59. doi: 10.1242/dev.097949
205. Wilkinson L, Gilbert T, Kinna G, Ruta LA, Pennisi D, Kett M, et al. Crim1KST264/KST264 mice implicate Crim1 in the regulation of vascular endothelial growth factor-A activity during glomerular vascular development. *J Am Soc Nephrol* (2007) 18:1697–708. doi: 10.1681/ASN.2006091012
206. Zeng H, Zhang Y, Yi Q, Wu Y, Wan R, Tang L. CRIM1, a newfound cancer-related player, regulates the adhesion and migration of lung cancer cells. *Growth Factors* (2015) 33:384–92. doi: 10.3109/08977194.2015.1119132
207. Glienke J, Sturz A, Menrad A, Thierauch K-H. CRIM1 is involved in endothelial cell capillary formation in vitro and is expressed in blood vessels in vivo. *Mech Dev* (2002) 119:165–75. doi: 10.1016/s0925-4773(02)00355-6
208. Li Y, Ye X, Tan C, Hongo JA, Zha J, Liu J, et al. Axl as a potential therapeutic target in cancer: Role of Axl in tumor growth, metastasis and angiogenesis. *Oncogene* (2009) 28:3442–55. doi: 10.1038/onc.2009.212
209. Xu MZ, Chan SW, Liu AM, Wong KF, Fan ST, Chen J, et al. AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. *Oncogene* (2011) 30:1229–40. doi: 10.1038/onc.2010.504
210. Linger RMA, Keating AK, Earp HS, Graham DK. Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. *Expert Opin Ther Targets* (2010) 14:1073–90. doi: 10.1517/14728222.2010.515980
211. Holland SJ, Powell MJ, Franci C, Chan EW, Friera AM, Atchison RE, et al. Multiple roles for the receptor tyrosine kinase Axl in tumor formation. *Cancer Res* (2005) 65:9294–303. doi: 10.1158/0008-5472.CAN-05-0993
212. Gallicchio M, Mitola S, Valdembri D, Fantozzi R, Varnum B, Avanzi GC, et al. Inhibition of vascular endothelial growth factor receptor 2-mediated endothelial cell activation by Axl tyrosine kinase receptor. *Blood* (2005) 105:1970–6. doi: 10.1182/blood-2004-04-1469
213. Shang X, Liu G, Zhang Y, Tang P, Zhang H, Jiang H, et al. Downregulation of BIRC5 inhibits the migration and invasion of esophageal cancer cells by

interacting with the PI3K/Akt signaling pathway. *Oncol Lett* (2018) 16:3373–9. doi: 10.3892/ol.2018.8986
214. Tu SP, Jiang XH, Lin MCM, Cui JT, Yang Y, Lum CT, et al. Suppression of Survivin Expression Inhibits in Vivo Tumorigenicity and Angiogenesis in Gastric Cancer. *Cancer Res* (2003) 63:7724–32.
215. Kawasaki H, Toyoda M, Shinohara H, Okuda J, Watanabe I, Yamamoto T, et al. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. *Cancer* (2001) 91:2026–32. doi: 10.1002/1097-0142(20010601)91:11<2026::AID-CNCR1228>3.0.CO;2-E
216. O'Connor DS, Schechner JS, Adida C, Mesri M, Rothermel AL, Li F, et al. Control of apoptosis during angiogenesis by survivin expression in endothelial cells. *Am J Pathol* (2000) 156:393–8. doi: 10.1016/S0002-9440(10)64742-6
217. Wang P, Zhen H, Zhang J, Zhang W, Zhang R, Cheng X, et al. Survivin promotes glioma angiogenesis through vascular endothelial growth factor and basic fibroblast growth factor in vitro and in vivo. *Mol Carcinog* (2012) 51:586–95. doi: 10.1002/mc.20829
218. Zhou Y, Tan Z, Chen K, Wu W, Zhu J, Wu G, et al. Overexpression of SHCBP1 promotes migration and invasion in gliomas by activating the NF-κB signaling pathway. *Mol Carcinog* (2018) 57:1181–90. doi: 10.1002/mc.22834
219. Feng W, Li H, Xu K, Chen Y, Pan L, Mei Y, et al. SHCBP1 is over-expressed in breast cancer and is important in the proliferation and apoptosis of the human malignant breast cancer cell line. *Mol Carcinog* (2018) 57:1181–90. doi: 10.1016/j.gene.2016.04.046
220. Peng C, Zhao H, Song Y, Chen W, Wang X, Liu X, et al. SHCBP1 promotes synovial sarcoma cell metastasis via targeting TGF- β 1 / Smad signaling pathway and is associated with poor prognosis. *Mol Carcinog* (2018) 57:1181–90. doi: 10.1186/s13046-017-0616-z
221. Tao H-C, Wang H, Dai M, Gu C, Wang Q, Han Z, et al. Targeting SHCBP1 Inhibits Cell Proliferation in Human Hepatocellular Carcinoma Cells. *Mol Carcinog* (2018) 57:1181–90. doi: 10.7314/apjcp.2013.14.10.5645
222. Catela C, Kratsios P, Hede M, Lang F, Rosenthal N. Serum and glucocorticoid-inducible kinase 1 (SGK1) is necessary for vascular remodeling during angiogenesis. *Dev Dyn* (2010) 239:2149–60. doi: 10.1002/dvdy.22345
223. Zarrinpashneh E, Poggioli T, Sarathchandra P, Lexow J, Terracciano C, Lang F, et al. Ablation of SGK1 Impairs Endothelial Cell Migration and Tube Formation Leading to Decreased Neo-Angiogenesis Following Myocardial Infarction. *PloS One* (2013) 8:1–13. doi: 10.1371/journal.pone.0080268
224. Amato R, Antona LD, Porciatti G, Agosti V, Menniti M, Rinaldo C, et al. Sgk1 activates MDM2-dependent p53 degradation and affects cell proliferation, survival, and differentiation. *J Mol Med* (2009) 87:1221–39. doi: 10.1007/s00109-009-0525-5
225. Zhang L, Cui R, Cheng X, Du J. Antiapoptotic Effect of Serum and Glucocorticoid-Inducible Protein Kinase Is Mediated by Novel Mechanism Activating IκB Kinase. *Cancer Res* (2005) 65:457–64.
226. Buijs N, Oosterink JE, Jessup M, Schierbeek H, Stolz DB, Houdijk AP, et al. A new key player in VEGF-dependent angiogenesis in human hepatocellular carcinoma: dimethylarginine dimethylaminohydrolase 1. *Angiogenesis* (2017) 20:557–65. doi: 10.1007/s10456-017-9567-4
227. Zhang P, Xu X, Hu X, Wang H, Fassett J, Huo Y, et al. DDAH1 deficiency attenuates endothelial cell cycle progression and angiogenesis. *PloS One* (2013) 8:1–9. doi: 10.1371/journal.pone.0079444
228. Kami Reddy KR, Dasari C, Vandavasi S, Natani S, Supriya B, Jadav SS, et al. Novel Cellularly Active Inhibitor Regresses DDAH1 Induced Prostate Tumor Growth by Restraining Tumor Angiogenesis through Targeting DDAH1/ADMA/NOS Pathway. *ACS Comb Sci* (2019) 21:241–56. doi: 10.1021/acscombsci.8b00133
229. Trittmann JK, Almazroue H, Jin Y, Nelin LD. DDAH1 regulates apoptosis and angiogenesis in human fetal pulmonary microvascular endothelial cells. *Physiol Rep* (2019) 7:1–14. doi: 10.14814/phy2.14150
230. Reddy K, Reddy K, Dasari C, Duscharla D, Supriya B, Ram NS. Dimethylarginine dimethylaminohydrolase−1 (DDAH1) is frequently upregulated in prostate cancer, and its overexpression conveys tumor growth and angiogenesis by metabolizing asymmetric dimethylarginine (ADMA). *Angiogenesis* (2018) 21:79–94. doi: 10.1007/s10456-017-9587-0
231. Lange C, Mowat F, Sayed H, Mehad M, Duluc L, Piper S, et al. Dimethylarginine dimethylaminohydrolase-2 deficiency promotes vascular regeneration and attenuates pathological angiogenesis. *Exp Eye Res* (2016) 147:148–55. doi: 10.1016/j.exer.2016.05.007
232. Kostourou V, Robinson SP, Cartwright JE, Whitley G. Dimethylarginine dimethylaminohydrolase I enhances tumour growth and angiogenesis. *Br J Cancer* (2002) 87:673–80. doi: 10.1038/sj.bjc.6600518
233. Seaman S, Stevens J, Yang MY, Logsdon D, Graff-cherry C, Croix BS. Genes that Distinguish Physiological and Pathological Angiogenesis. *Cancer Cell* (2007) 11:539–54. doi: 10.1016/j.ccr.2007.04.017
234. Brockenbrough JS, Morihara JK, Hawes SE, Stern JE, Rasey JS, Wiens LW, et al. Thymidine kinase 1 and thymidine phosphorylase expression in non-small-cell lung carcinoma in relation to angiogenesis and proliferation. *J Histochem Cytochem* (2009) 57:1087–97. doi: 10.1369/jhc.2009.952804
235. Hisaka Y, Ieda M, Nakamura T, Kosai KI, Ogawa S, Fukuda K. Powerful and controllable angiogenesis by using gene-modified cells expressing human hepatocyte growth factor and thymidine kinase. *J Am Coll Cardiol* (2004) 43:1915–22. doi: 10.1016/j.jacc.2004.01.034
236. Nakagawa T, Li JH, Garcia G, Mu W, Piek E, Böttinger EP, et al. TGF-β induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways. *Kidney Int* (2004) 66:605–13. doi: 10.1111/j.1523-1755.2004.00780.x
237. Nacak TG, Alajati A, Leptien K, Fulda C, Weber H, Miki T, et al. The BTB-kelch protein KLEIP controls endothelial migration and sprouting angiogenesis. *Circ Res* (2007) 100:1155–63. doi: 10.1161/01.RES.0000265844.56493.ac
238. Justilien V, Fields AP. Ect2 links the PKC-Par6α complex to Rac1 activation and cellular transformation. *Oncogene* (2009) 28:3597–607. doi: 10.1038/onc.2009.217
239. Saito S, Liu XF, Kamijo K, Raziuddin R, Tatsumoto T, Okamoto I, et al. Deregulation and Mislocalization of the Cytokinesis Regulator ECT2 Activate the Rho Signaling Pathways Leading to Malignant Transformation. *J Biol Chem* (2004) 279:7169–79. doi: 10.1074/jbc.M306725200
240. Tatsumoto T, Xie X, Blumenthal R, Okamoto I, Miki T. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J Cell Biol* (1999) 147:921–7. doi: 10.1083/jcb.147.5.921
241. Georgiou GK, Igglezou M, Sainis I, Vareli K, Batsis H, Briasoulis E, et al. Impact of breast cancer surgery on angiogenesis circulating biomarkers: A prospective longitudinal study. *World J Surg Oncol* (2013) 11:1–8. doi: 10.1186/1477-7819-11-213
242. Bahramsoltani M, Slosarek I, De Spiegelaere W, Plendl J. Angiogenesis and Collagen Type IV Expression in Different Endothelial Cell Culture Systems. *J Vet Med Ser C Anat Histol Embryol* (2014) 43:103–15. doi: 10.1111/ahe.12052
243. Nie XC, Wang JP, Zhu W, Xu XY, Xing YN, Yu M, et al. COL4A3 expression correlates with pathogenesis, pathologic behaviors, and prognosis of gastric carcinomas. *Hum Pathol* (2013) 44:77–86. doi: 10.1016/j.humpath.2011.10.028
244. Cordenonsi M, Zanconato F, Azzolin L, Forcato M, Rosato A, Frasson C, et al. The Hippo Transducer TAZ Confers Cancer Stem Cell-Related Traits on Breast Cancer Cells. *Cell* (2011) 147:759–72. doi: 10.1016/j.cell.2011.09.048
245. Hiemer SE, Zhang L, Kartha VK, Packer TS, Almershed M, Noonan V, et al. A YAP/TAZ-Regulated Molecular Signature is Associated with Oral Squamous Cell Carcinoma. *Mol Cancer Res* (2016) 13:617–38. doi: 10.1158/1541-7786.MCR-14-0580.A
246. Li J, Ye L, Owen S, Weeks Ping H, Zhang Z, Jiang GW. Emerging role of CCN family proteins in tumorigenesis and cancer metastasis (Review). *Int J Mol Med* (2015) 36:1451–63. doi: 10.3892/ijmm.2015.2390
247. Lipson KE, Wong C, Teng Y, Spong S. CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis Tissue Repair* (2012) 5:S24–4. doi: 10.1186/1755-1536-5-S1-S24
248. Zhu X, Zhong J, Zhao Z, Sheng J, Wang J, Liu J, et al. Epithelial derived CTGF promotes breast tumor progression via inducing EMT and collagen I fibers deposition. *Oncotarget* (2015) 6:25320–38. doi: 10.18632/oncotarget.4659

249. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* (2005) 65:8887–95. doi: 10.1158/0008-5472.CAN-05-1702
250. Sakai N, Nakamura M, Lipson KE, Miyake T, Kamikawa Y, Sagara A, et al. Inhibition of CTGF ameliorates peritoneal fibrosis through suppression of fibroblast and myofibroblast accumulation and angiogenesis. *Sci Rep* (2017) 7:1–13. doi: 10.1038/s41598-017-05624-2
251. Wang L-H, Tsai H-C, Cheng Y-C, Lin C-Y, Huang Y-L, Tsai C-H, et al. CTGF promotes osteosarcoma angiogenesis by regulating miR-543/angiopoietin 2 signaling. *Cancer Lett* (2017) 391:28–37. doi: 10.1016/j.canlet.2017.01.013
252. Fataccioli V, Abergel V, Wingertsmann L, Neuville P, Spitz E, Adnot S, et al. Stimulation of Angiogenesis by Cyr61 gene: A New Therapeutic Candidate. *Hum Gene Ther* (2002) 1470:1461–70. doi: 10.1089/10430340260185094
253. Mo F-E, Muntean AG, Chen C-C, Stolz DB, Watkins SC, Lau LF. CYR61 (CCN1) Is Essential for Placental Development and Vascular Integrity. *Mol Cell Biol* (2002) 22:8709–20. doi: 10.1128/MCB.22.24.8709-8720.2002
254. Chintala H, Krupska I, Yan L, Lau L, Grant M, Chaqour B. The matricellular protein CCN1 controls retinal angiogenesis by targeting VEGF, Src homology 2 domain phosphatase-1 and Notch signaling. *Development* (2015) 142:2364–74. doi: 10.1242/dev.121913
255. Tsai M-S, Bogart DF, Castañeda JM, Li P, Lupu R. Cyr61 promotes breast tumorigenesis and cancer progression. *Oncogene* (2002) 21:8178–85. doi: 10.1038/sj.onc.1205682
256. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc Natl Acad Sci* (1998) 95:6355–60. doi: 10.1073/pnas.95.11.6355
257. Xie D, Miller CW, O'Kelly J, Nakachi K, Sakashita A, Said JW, et al. Breast Cancer: Cyr61 is Overexpressed, Estrogen- Inducible, and associated with more Advanced Disease. *J Biol Chem* (2001) 276:14187–94. doi: 10.1074/jbc.M009755200
258. Jiménez AP, Traum A, Boettger T, Hackstein H, Richter AM, Dammann RH. The tumor suppressor RASSF1A induces the YAP1 target gene ANKRD1 that is epigenetically inactivated in human cancers and inhibits tumor growth. *Oncotarget* (2017) 8:88437–52. doi: 10.18632/oncotarget.18177
259. Samaras SE, Almodóvar-García K, Wu N, Yu F, Davidson JM. Global deletion of ankrd1 results in a wound-healing phenotype associated with dermal fibroblast dysfunction. *Am J Pathol* (2015) 185:96–109. doi: 10.1016/j.ajpath.2014.09.018
260. Shi Y, Reitmaier B, Regenbogen J, Slowey RM, Opalenik SR, Wolf E, et al. CARP, a cardiac ankyrin repeat protein, is up-regulated during wound healing and induces angiogenesis in experimental granulation tissue. *Am J Pathol* (2005) 166:303–12. doi: 10.1016/S0002-9440(10)62254-7
261. Héroult M, Schaffner F, Augustin HG. Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. *Exp Cell Res* (2006) 312:642–50. doi: 10.1016/j.yexcr.2005.10.028
262. Lisabeth EM, Falivelli G, Pasquale EB. Eph receptor signaling and ephrins. *Cold Spring Harb Perspect Biol* (2013) 5:a009159. doi: 10.1101/cshperspect.a009159
263. Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* (2000) 19:6043–52. doi: 10.1038/sj.onc.1204004
264. Shao Z, Zhang WF, Chen XM, Shang ZJ. Expression of EphA2 and VEGF in squamous cell carcinoma of the tongue: Correlation with the angiogenesis and clinical outcome. *Oral Oncol* (2008) 44:1110–7. doi: 10.1016/j.oraloncology.2008.01.018
265. Dobrzanski P, Hunter K, Jones-bolin S, Chang H, Robinson C, Pritchard S, et al. Antiangiogenic and Antitumor Efficacy of EphA2 Receptor Antagonist. *Cancer Res* (2004) 64:910–9. doi: 10.1158/0008-5472.can-3430-2
266. Brantley-Sieders DM, Caughron J, Hicks D, Pozzi A, Ruiz JC, Chen J. EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation. *J Cell Sci* (2004) 117:2037–49. doi: 10.1242/jcs.01061
267. Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB. Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. *Proc Natl Acad Sci* (2004) 101:5583–8. doi: 10.1073/pnas.0401381101
268. Uhl C, Markel M, Broggini T, Nieminen M, Kremenetskaia I, Vajkoczy P, et al. EphB4 mediates resistance to antiangiogenic therapy in experimental glioma. *Angiogenesis* (2018) 21:873–81. doi: 10.1007/s10456-018-9633-6
269. Kertesz N, Krasnoperov V, Reddy R, Leshanski L, Kumar SR, Zozulya S, et al. The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth. *Blood* (2006) 107:2330–8. doi: 10.1182/blood-2005-04-1655
270. Erber R, Eichelsbacher U, Powajbo V, Korn T, Djonov V, Lin J, et al. EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. *EMBO J* (2006) 25:628–41. doi: 10.1038/sj.emboj.7600949
271. Thurston G. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. *Cell Tissue Res* (2003) 314:61–8. doi: 10.1007/s00441-003-0749-6
272. Thomas M, Augustin HG. The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis* (2009) 12:125. doi: 10.1007/s10456-009-9147-3
273. Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-α and has a crucial role in the induction of inflammation. *Nat Med* (2006) 12:235–9. doi: 10.1038/nm1351
274. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligand Angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. *J Cell Sci* (2005) 118:771–80. doi: 10.1242/jcs.01653
275. Augustin HG, Young Koh G, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin–Tie system. *Nat Rev Mol Cell Biol* (2009) 10:165–77. doi: 10.1038/nrm2639
276. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. Hypoxia and Vascular Endothelial Growth Factor Selectively Up-regulate Angiopoietin-2 in Bovine Microvascular Endothelial Cells. *J Biol Chem* (1999) 274:15732–9. doi: 10.1074/jbc.274.22.15732
277. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a Natural Antagonist for Tie2 That Disrupts in vivo Angiogenesis. *Sci (80-)* (1997) 277:55–60. doi: 10.1126/science.277.5322.55
278. Lobov IB, Brooks PC, Lang RA. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. *Proc Natl Acad Sci* (2002) 99:11205–10. doi: 10.1073/pnas.172161899
279. Oshima Y, Oshima S, Nambu H, Kachi S, Takahashi K, Umeda N, et al. Different effects of angiopoietin-2 in different vascular beds in the eye: new vessels are most sensitive. *FASEB J* (2005) 19:963–5. doi: 10.1096/fj.04-2209fje
280. Oshima Y, Deering T, Oshima S, Nambu H, Reddy PS, Kaleko M, et al. Angiopoietin-2 enhances retinal vessel sensitivity to vascular endothelial growth factor. *J Cell Physiol* (2004) 199:412–7. doi: 10.1002/jcp.10442
281. Daly C, Pasnikowski E, Burova E, Wong V, Aldrich TH, Griffiths J, et al. Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. *Proc Natl Acad Sci* (2006) 103:15491–6. doi: 10.1073/pnas.0607538103
282. Shim WSN, Ho IAW, Wong PEH. Angiopoietin : A TIE (d) Balance in Tumor Angiogenesis. *Mol Cancer Res* (2007) 5:655–66. doi: 10.1158/1541-7786.MCR-07-0072
283. Zadeh G, Koushan K, Baoping Q, Shannon P, Guha A. Role of angiopoietin-2 in regulating growth and vascularity of astrocytomas. *J Oncol* (2010) 2010:659231. doi: 10.1155/2010/659231
284. Hawighorst T, Skobe M, Streit M, Hong Y-K, Velasco P, Brown LF, et al. Activation of the Tie2 Receptor by Angiopoietin-1 Enhances Tumor Vessel Maturation and Impairs Squamous Cell Carcinoma Growth. *Am J Pathol* (2002) 160:1381–92. doi: 10.1016/S0002-9440(10)62565-5
285. Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell* (2011) 19:512–26. doi: 10.1016/j.ccr.2011.02.005
286. Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. *J Clin Invest* (2012) 122:1991–2005. doi: 10.1172/JCI58832
287. Park J-S, Kim I-K, Han S, Park I, Kim C, Bae J, et al. Normalization of Tumor Vessels by Tie2 Activation and Ang2 Inhibition Enhances Drug Delivery and

Produces a Favorable Tumor Microenvironment. *Cancer Cell* (2016) 30:953–67. doi: 10.1016/j.ccell.2016.10.018
288. Peterson TE, Kirkpatrick ND, Huang Y, Farrar CT, Marijt KA, Kloepper J, et al. Dual inhibition of Ang-2 and VEGF receptors normalizes tumor vasculature and prolongs survival in glioblastoma by altering macrophages. *Proc Natl Acad Sci* (2016) 113:4470–5. doi: 10.1073/pnas.1525349113
289. Jászai J, Schmidt M. Trends and Challenges in Tumor Anti-Angiogenic Therapies. *Cells* (2019) 8:1102. doi: 10.3390/cells8091102
290. Al-Abd AM, Alamoudi AJ, Abdel-Naim AB, Neamatallah TA, Ashour OM. Anti-angiogenic agents for the treatment of solid tumors: Potential pathways, therapy and current strategies – A review. *J Adv Res* (2017) 8:591–605. doi: 10.1016/j.jare.2017.06.006
291. Durkin ME, Yuan B-Z, Zhou X, Zimonjic DB, Lowy DR, Thorgeirsson SS, et al. DLC-1:a Rho GTPase-activating protein and tumour suppressor. *J Cell Mol Med* (2007) 11:1185–207. doi: 10.1111/j.1582-4934.2007.00098.x
292. Liao Y-C, Lo SH. Deleted in liver cancer-1 (DLC-1): a tumor suppressor not just for liver. *Int J Biochem Cell Biol* (2008) 40:843–7. doi: 10.1016/j.biocel.2007.04.008
293. Li G, Du X, Vass WC, Papageorge AG, Lowy DR, Qian X. Full activity of the deleted in liver cancer 1 (DLC1) tumor suppressor depends on an LD-like motif that binds talin and focal adhesion kinase (FAK). *Proc Natl Acad Sci* (2011) 108:17129–34. doi: 10.1073/pnas.1112122108
294. Haining AWM, Rahikainen R, Cortes E, Lachowski D, Rice A, von Essen M, et al. Mechanotransduction in talin through the interaction of the R8 domain with DLC1. *PloS Biol* (2018) 16:1–20. doi: 10.1371/journal.pbio.2005599
295. Durkin ME, Avner MR, Huh C-G, Yuan B-Z, Thorgeirsson SS, Popescu NC. DLC-1, a Rho GTPase-activating protein with tumor suppressor function, is essential for embryonic development. *FEBS Lett* (2005) 579:1191–6. doi: 10.1016/j.febslet.2004.12.090
296. Schimmel L, van der Stoel M, Rianna C, van Stalborch AM, de Ligt A, Hoogenboezem M, et al. Stiffness-Induced Endothelial DLC-1 Expression Forces Leukocyte Spreading through Stabilization of the ICAM-1 Adhesome. *Cell Rep* (2018) 24:3115–24. doi: 10.1016/j.celrep.2018.08.045
297. Ritchey L, Ha T, Otsuka A, Kabashima K, Wang D, Wang Y, et al. DLC1 deficiency and YAP signaling drive endothelial cell contact inhibition of growth and tumorigenesis. *Oncogene* (2019) 38:7046–59. doi: 10.1038/s41388-019-0944-x
298. Shih Y-P, Liao Y-C, Lin Y, Lo SH. DLC1 negatively regulates angiogenesis in a paracrine fashion. *Cancer Res* (2010) 70:8270–5. doi: 10.1158/0008-5472.CAN-10-1174
299. Le Y, Zhou Y, Iribarren P, Wang JM. Chemokines and Chemokine Receptors : Their Manifold Roles in Homeostasis and Disease. *Cell Mol Immunol* (2004) 1:95–104.
300. Bandapalli OR, Ehrmann F, Ehemann V, Gaida M, Macher-goeppinger S, Wente M, et al. Cytokine Down-regulation of CXCL1 inhibits tumor growth in colorectal liver metastasis. *Cytokine* (2012) 57:46–53. doi: 10.1016/j.cyto.2011.10.019
301. Dhawan P, Richmond A. Role of CXCL1 in tumorigenesis of melanoma. *J Leukoc Biol* (2002) 72:9–18.
302. Wang D, Wang H, Brown J, Daikoku T, Ning W, Shi Q, et al. CXCL1 induced by prostaglandin E 2 promotes angiogenesis in colorectal cancer. *J Exp Med* (2006) 203:941–51. doi: 10.1084/jem.20052124
303. Wang Y, Liu J, Jiang Q, Deng J, Xu F, Chen X, et al. Human Adipose-Derived Mesenchymal Stem Cell- Secreted CXCL1 and CXCL8 Facilitate Breast Tumor Growth by Promoting Angiogenesis. *Stem Cells* (2017) 35:2060–70. doi: 10.1002/stem.2643
304. Miyake M, Goodison S, Urquidi V, Giacoia EG, Rosser CJ. Expression of CXCL1 in human endothelial cells induces angiogenesis through the CXCR2 receptor and the ERK1 / 2 and EGF pathways. *Lab Invest* (2013) 93:768–78. doi: 10.1038/labinvest.2013.71
305. Wei Z, Xia G, Wu Y, Chen W, Xiang Z, Schwarz RE, et al. CXCL1 promotes tumor growth through VEGF pathway activation and is associated with inferior survival in gastric cancer. *Cancer Lett* (2015) 359:335–43. doi: 10.1016/j.canlet.2015.01.033
306. Scapini P, Morini M, Tecchio C, Di Carlo E, Tanghetti E, Albini A, et al. CXCL1/Macrophage Inflammatory Protein-2-Induced Angiogenesis In Vivo Is Mediated by Neutrophil-Derived Vascular Endothelial Growth Factor-A. *J Immunol* (2004) 172:5034–40. doi: 10.4049/jimmunol.172.8.5034
307. Cai L, Xu S, Piao C, Qiu S, Li H, Du J. Adiponectin Induces CXCL1 Secretion From Cancer Cells and Promotes Tumor Angiogenesis by Inducing Stromal Fibroblast Senescence. *Mol Carcinog* (2016) 55:1796–806. doi: 10.1002/mc.22428
308. Goveia J, Rohlenova K, Taverna F, Treps L, Conradi L-C, Pircher A, et al. An Integrated Gene Expression Landscape Profiling Approach to Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates. *Cancer Cell* (2020) 37:21–36. doi: 10.1016/j.ccell.2019.12.001
309. Zheng Y, Ming P, Zhu C, Si Y, Xu S, Chen A, et al. Hepatitis B virus X protein–induced SH2 domain– containing 5 (SH2D5) expression promotes hepatoma cell growth via an SH2D5–transketolase interaction. *J Biol Chem* (2019) 294:4815–27. doi: 10.1074/jbc.RA118.005739
310. Chen Z, Zhong CH. STAT3: A critical transcription activator in angiogenesis. *Med Res Rev* (2008) 28:185–200. doi: 10.1002/med.20101
311. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, et al. Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* (2002) 21:2000–8. doi: 10.1038/sj.onc.1205260
312. Fan X, Shan X, Jiang S, Wang S, Zhang F, Tian Q, et al. YAP promotes endothelial barrier repair by repressing STAT3/VEGF signaling. *Life Sci* (2020) 256:117884. doi: 10.1016/j.lfs.2020.117884
313. Kidoya H, Takakura N. Biology of the apelin-APJ axis in vascular formation. *J Biochem* (2012) 152:125–31. doi: 10.1093/jb/mvs071
314. Kälin RE, Kretz MP, Meyer AM, Kispert A, Heppner FL, Brändli AW. Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis. *Dev Biol* (2007) 305:599–614. doi: 10.1016/j.ydbio.2007.03.004
315. Muto J, Shirabe K, Yoshizumi T, Ikegami T, Aishima S, Ishigami K, et al. The Apelin–APJ System Induces Tumor Arteriogenesis in Hepatocellular Carcinoma. *Anticancer Res* (2014) 34:5313–20. doi: 10.1111/liv.12459
316. Sorli SC, Le Gonidec S, Knibiehler B, Audigier Y. Apelin is a potent activator of tumour neoangiogenesis. *Oncogene* (2007) 26:7692–9. doi: 10.1038/sj.onc.1210573
317. Berta J, Kenessey I, Dobos J, Tovari J, Klepetko W, Jan Ankersmit H, et al. Apelin expression in human non-small cell lung cancer: Role in angiogenesis and prognosis. *J Thorac Oncol* (2010) 5:1120–9. doi: 10.1097/JTO.0b013e3181e2c1ff
318. Berta J, Hoda MA, Laszlo V, Rozsas A, Garay T, Torok S, et al. Apelin promotes lymphangiogenesis and lymph node metastasis. *Oncotarget* (2014) 5:4426–37. doi: 10.18632/oncotarget.2032
319. Rayalam S A, Della-Fera M, Kasser T, Warren W, Baile C A. Emerging Role of Apelin as a Therapeutic Target in Cancer: A Patent Review. *Recent Pat Anticancer Drug Discovery* (2011) 6:367–72. doi: 10.2174/157489211796957856
320. Gill MK, Christova T, Zhang YY, Gregorieff A, Zhang L, Narimatsu M, et al. A feed forward loop enforces YAP/TAZ signaling during tumorigenesis. *Nat Commun* (2018) 9:3510. doi: 10.1038/s41467-018-05939-2
321. Lv M, Shen Y, Yang J, Li S, Wang B, Chen Z, et al. Angiomotin Family Members: Oncogenes or Tumor Suppressors? *Int J Biol Sci* (2017) 13:772–81. doi: 10.7150/ijbs.19603
322. Yi C, Shen Z, Stemmer-Rachamimov A, Dawany N, Troutman S, Showe LC, et al. The p130 isoform of angiomotin is required for yap-mediated hepatic epithelial cell proliferation and tumorigenesis. *Sci Signal* (2013) 6:ra77. doi: 10.1126/scisignal.2004060
323. Rosenbluh J, Nijhawan D, Cox AG, Li X, Neal JT, Schafer EJ, et al. β-Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell* (2012) 151:1457–73. doi: 10.1016/j.cell.2012.11.026
324. Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, et al. YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. *Cell* (2014) 158:157–70. doi: 10.1016/j.cell.2014.06.013
325. Azzolin L, Zanconato F, Bresolin S, Forcato M, Basso G, Bicciato S, et al. Role of TAZ as mediator of wnt signaling. *Cell* (2012) 151:1443–56. doi: 10.1016/j.cell.2012.11.027
326. Park HW, Kim YC, Yu B, Moroishi T, Mo JS, Plouffe SW, et al. Alternative Wnt Signaling Activates YAP/TAZ. *Cell* (2015) 162:780–94. doi: 10.1016/j.cell.2015.07.013
327. Miyamura N, Nishina H. YAP regulates liver size and function. *Cell Cycle* (2018) 17:267–8. doi: 10.1080/15384101.2017.1407390
328. Heo S-H, Choi Y-J, Ryoo H-M, Cho J-Y. Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: Role of MMP-10 in

VEGF-induced angiogenesis. *J Cell Physiol* (2010) 224:734–42. doi: 10.1002/jcp.22175
329. Nukuda A, Sasaki C, Ishihara S, Mizutani T, Nakamura K, Ayabe T, et al. Stiff substrates increase YAP-signaling-mediated matrix metalloproteinase-7 expression. *Oncogenesis* (2015) 4:1–11. doi: 10.1038/oncsis.2015.24
330. Tang Y, Rowe RG, Botvinick EL, Kurup A, Putnam AJ, Seiki M, et al. MT1-MMP-Dependent Control of Skeletal Stem Cell Commitment via a β1-Integrin/YAP/TAZ Signaling Axis. *Dev Cell* (2013) 25:402–16. doi: 10.1016/j.devcel.2013.04.011
331. Mazor R, Alsaigh T, Shaked H, Altshuler AE, Pocock ES, Kistler EB, et al. Matrix metalloproteinase-1-mediated up-regulation of vascular endothelial growth factor-2 in endothelial cells. *J Biol Chem* (2013) 288:598–607. doi: 10.1074/jbc.M112.417451
332. Quintero-Fabián S, Arreola R, Becerril-Villanueva E, Torres-Romero JC, Arana-Argáez V, Lara-Riegos J, et al. Role of Matrix Metalloproteinases in Angiogenesis and Cancer. *Front Oncol* (2019) 9:1370. doi: 10.3389/fonc.2019.01370
333. Souilhol C, Harmsen MC, Evans PC, Krenning G. Endothelial–mesenchymal transition in atherosclerosis. *Cardiovasc Res* (2018) 114:565–77. doi: 10.1093/cvr/cvx253
334. Numasaki M, Fukushi JI, Ono M, Narula SK, Zavodny PJ, Kudo T, et al. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* (2003) 101:2620–7. doi: 10.1182/blood-2002-05-1461
335. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol* (2005) 7:122–33. doi: 10.1215/s1152851704001061
336. Gopinathan G, Milagre C, Pearce OMT, Reynolds LE, Hodivala-Dilke K, Leinster DA, et al. Interleukin-6 Stimulates Defective Angiogenesis. *Cancer Res* (2015) 75:3098–107. doi: 10.1158/0008-5472.CAN-15-1227
337. Romagnani P, Lasagni L, Annunziato F, Serio M, Romagnani S. CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends Immunol* (2004) 25:201–9. doi: 10.1016/j.it.2004.02.006
338. Wang S, Zhou L, Ling L, Meng X, Chu F, Zhang S, et al. The Crosstalk Between Hippo-YAP Pathway and Innate Immunity. *Front Immunol* (2020) 11:323. doi: 10.3389/fimmu.2020.00323
339. Kyriakis JM. Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Expr J Liver Res* (1999) 7:217–31.
340. Gashaw I, Stiller S, Böing C, Kimmig R, Winterhager E. Premenstrual regulation of the pro-angiogenic factor CYR61 in human endometrium. *Endocrinology* (2008) 149:2261–9. doi: 10.1210/en.2007-1568
341. Lee S, Ahad A, Luu M, Moon S, Caesar J, Cardoso WV, et al. CCN1–Yes-Associated Protein Feedback Loop Regulates Physiological and Pathological Angiogenesis. *Mol Cell Biol* (2019) 39:e00107–19. doi: 10.1128/mcb.00107-19
342. Choi H-J, Kwon Y-G. Roles of YAP in mediating endothelial cell junctional stability and vascular remodeling. *BMB Rep* (2015) 48:429–30. doi: 10.5483/bmbrep.2015.48.8.146
343. Nguyen CDK, Yi C. YAP/TAZ Signaling and Resistance to Cancer Therapy. *Trends Cancer* (2019) 5:283–96. doi: 10.1016/j.trecan.2019.02.010
344. Pobbati AV, Hong W. A combat with the YAP/TAZ-TEAD oncoproteins for cancer therapy. *Theranostics* (2020) 10:3622–35. doi: 10.7150/thno.40889
345. Santucci M, Vignudelli T, Ferrari S, Mor M, Scalvini L, Bolognesi ML, et al. The Hippo Pathway and YAP/TAZ–TEAD Protein–Protein Interaction as Targets for Regenerative Medicine and Cancer Treatment. *J Med Chem* (2015) 58:4857–73. doi: 10.1021/jm501615v
346. Kim M, Kim T, Randy JL, Lim D-S. Transcriptional Co-repressor Function of the Hippo Pathway Transducers YAP and TAZ. *CellReports* (2015) 11:270–82. doi: 10.1016/j.celrep.2015.03.015
347. Guan Z-B, Cao Y-S, Li Y, Tong W-N, Zhuo A-S. Knockdown of lncRNA GHET1 suppresses cell proliferation, invasion and LATS1/YAP pathway in non small cell lung cancer. *Cancer Biomarkers* (2018) 21:557–63. doi: 10.3233/CBM-170431
348. Wang Y, Cui M, Sun B, Liu FB, Zhang XD, Ye LH. MiR-506 suppresses proliferation of hepatoma cells through targeting YAP mRNA 3′UTR. *Acta Pharmacol Sin* (2014) 35:1207–14. doi: 10.1038/aps.2014.59
349. Liu P, Zhang H, Liang X, Ma H, Luan F, Wang B, et al. HBV preS2 promotes the expression of TAZ via miRNA-338-3p to enhance the tumorigenesis of hepatocellular carcinoma. *Oncotarget* (2015) 6:29048–59. doi: 10.18632/oncotarget.4804
350. Zhang M, Zhao Y, Zhang Y, Wang D, Gu S, Feng W, et al. LncRNA UCA1 promotes migration and invasion in pancreatic cancer cells via the Hippo pathway. *Biochim Biophys Acta - Mol Basis Dis* (2018) 1864:1770–82. doi: 10.1016/j.bbadis.2018.03.005
351. Qu L, Wu Z, Li Y, Xu Z, Liu B, Liu F, et al. A feed-forward loop between lncARSR and YAP activity promotes expansion of renal tumour-initiating cells. *Nat Commun* (2016) 7:1–14. doi: 10.1038/ncomms12692
352. Ma J, Huang K, Ma Y, Zhou M, Fan S. The TAZ-miR-224-SMAD4 axis promotes tumorigenesis in osteosarcoma. *Cell Death Dis* (2017) 8:1–16. doi: 10.1038/cddis.2016.468
353. Shen S, Huang K, Wu Y, Ma Y, Wang J, Qin F, et al. A miR-135b-TAZ positive feedback loop promotes epithelial–mesenchymal transition (EMT) and tumorigenesis in osteosarcoma. *Cancer Lett* (2017) 407:32–44. doi: 10.1016/j.canlet.2017.08.005
354. Yu W, Qiao Y, Tang X, Ma L, Wang Y, Zhang X, et al. Tumor suppressor long non-coding RNA, MT1DP is negatively regulated by YAP and Runx2 to inhibit FoxA1 in liver cancer cells. *Cell Signal* (2014) 26:2961–8. doi: 10.1016/j.cellsig.2014.09.011
355. Tu C, Yang K, Wan L, He J, Qi L, Wang W, et al. The crosstalk between lncRNAs and the Hippo signalling pathway in cancer progression. *Cell Prolif* (2020) 53:e12887. doi: 10.1111/cpr.12887

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 05 February 2021
doi: 10.3389/fcell.2021.634853

Potential Roles of Muscle-Derived Extracellular Vesicles in Remodeling Cellular Microenvironment: Proposed Implications of the Exercise-Induced Myokine, Irisin

Samuel Darkwah[1], Eun Jeong Park[1], Phyoe Kyawe Myint[1], Atsushi Ito[1,2], Michael G. Appiah[1], Gideon Obeng[1], Eiji Kawamoto[1,3] and Motomu Shimaoka[1]**

[1] Department of Molecular Pathobiology and Cell Adhesion Biology, Mie University Graduate School of Medicine, Tsu, Japan, [2] Department of Cardiothoracic and Vascular Surgery, Mie University Graduate School of Medicine, Tsu, Japan, [3] Department of Emergency and Disaster Medicine, Mie University Graduate School of Medicine, Tsu, Japan

OPEN ACCESS

Edited by:
Lucas Treps,
VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Valentina Di Felice,
University of Palermo, Italy
Michele Guescini,
University of Urbino Carlo Bo, Italy
Alexis Forterre,
Stanford University, United States

***Correspondence:**
Samuel Darkwah
kwekuadarkwah@gmail.com
Motomu Shimaoka
motomushimaoka@gmail.com

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 29 November 2020
Accepted: 14 January 2021
Published: 05 February 2021

Citation:
Darkwah S, Park EJ, Myint PK, Ito A, Appiah MG, Obeng G, Kawamoto E and Shimaoka M (2021) Potential Roles of Muscle-Derived Extracellular Vesicles in Remodeling Cellular Microenvironment: Proposed Implications of the Exercise-Induced Myokine, Irisin. Front. Cell Dev. Biol. 9:634853. doi: 10.3389/fcell.2021.634853

Extracellular vesicles (EVs) have emerged as key players of intercellular communication and mediate crosstalk between tissues. Metastatic tumors release tumorigenic EVs, capable of pre-conditioning distal sites for organotropic metastasis. Growing evidence identifies muscle cell-derived EVs and myokines as potent mediators of cellular differentiation, proliferation, and metabolism. Muscle-derived EVs cargo myokines and other biological modulators like microRNAs, cytokines, chemokines, and prostaglandins hence, are likely to modulate the remodeling of niches in vital sites, such as liver and adipose tissues. Despite the scarcity of evidence to support a direct relationship between muscle-EVs and cancer metastasis, their indirect attribution to the regulation of niche remodeling and the establishment of pre-metastatic homing niches can be put forward. This hypothesis is supported by the role of muscle-derived EVs in findings gathered from other pathologies like inflammation and metabolic disorders. In this review, we present and discuss studies that evidently support the potential roles of muscle-derived EVs in the events of niche pre-conditioning and remodeling of metastatic tumor microenvironment. We highlight the potential contributions of the integrin-mediated interactions with an emerging myokine, irisin, to the regulation of EV-driven microenvironment remodeling in tumor metastasis. Further research into muscle-derived EVs and myokines in cancer progression is imperative and may hold promising contributions to advance our knowledge in the pathophysiology, progression and therapeutic management of metastatic cancers.

Keywords: extracellular vesicles, muscle, integrins, myokines, tumor metastasis, tissue microenvironment, homing niche, irisin

INTRODUCTION

Extracellular vesicles (EVs) are secreted by all cell types of the body, including tumor cells and can be isolated from various biological fluids (Neven et al., 2017). Initially thought to be cellular "trash bins" containing unwanted excretes, EVs in recent times have insightfully been recognized as significant players in proximal and distal intercellular communication. A peculiar

characteristic of EVs is their ability to cargo several biologic materials, capable of influencing physiological and pathological processes (Frühbeis et al., 2013; Regev-Rudzki et al., 2013; Abels and Breakefield, 2016) (**Figures 1**, **2**). In recent times, rapid progress is being made with regards to the implementation of EVs in therapeutic interventions in the areas of inflammation, metabolic disorders, vaccination and drug delivery (Viaud et al., 2010; Lee et al., 2012; Hagiwara et al., 2014). Muscle-derived EVs have gained attention owing to their beneficial function in modulating metabolism, cell differentiation and regeneration (Choi et al., 2016; Takafuji et al., 2020a). It remains yet to be confirmed, the direct role of muscle-derived EVs in regulating cancer progression and metastasis, however, the evidence of regulatory properties of muscle EVs and myokines on other cell types hold promising cues for their potential role in tumor spread (Gannon et al., 2015; Zhang et al., 2018). In this review, we summarize the biogenesis, characteristics and functions, as well as recent findings on how muscle-derived EVs mitigate or aggravate disease conditions. We also attempt to spotlight the potential role of muscle EVs in remodeling metastatic pre-conditioning events via irisin-triggered integrin signaling.

BIOGENESIS, CHARACTERISTICS, AND FUNCTION OF EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) describe the heterogenous collection of a highly conserved system for intercellular communication, within which cells are able to exchange information in the form of biologically functional components: nucleic acids, proteins, and lipids (Stahl and Raposo, 2018; Wiklander et al., 2019). EVs are released by all cell types as membrane-bound spherical structures that originate from the endosome or plasma membrane, and are present in many body fluids, such as blood, urine, semen, saliva, and breast milk (Neven et al., 2017). The term, "extracellular vesicles" is generic and hence specific properties, such as the origin/source, physical characteristics and biochemical properties are required to adequately define the various subtypes (Théry et al., 2018).

Classically, EVs are broadly categorized into three vesicular types depending on their biogenesis; outward budding or fusion of multivesicular bodies (MVB) with plasma membrane. This classification gives rise to three distinct vesicle types, namely, Exosomes (EXOs), microvesicles (MVs), and apoptotic bodies (Akers et al., 2013) (**Figure 1**). EVs are isolated from biological samples via a myriad of isolation methods, such as precipitation, filtration, centrifugation, and a combination of different method. However, specificity and recovery of EVs may greatly vary per the isolation methods used to obtain them. Additionally, the presence of EVs in biological samples are confirmed by specific protein and lipid markers (present or absent) on the various subtypes. Well-documented guidelines outlining the recommended protocols for EVs and their documentation (provided by the International Society of Extracellular Vesicles-ISEV) exist. These guidelines encompass protocols and recommendations designed for the efficient characterization, isolation, and functional studies of EVs (Théry et al., 2018).

Exosomes

Exosomes, which are nano-sized vesicles ranging between small diameters of about 30–150 nm, are derived from the endosomal system. The formation and secretion of exosomes begin with the invagination of the plasma membrane (endocytosis) to form a cup-like structure harboring cell-surface proteins and extracellular deposits (Kalluri and LeBleu, 2020). This invagination forms an early endosome which then mature to form late endosomes. Subsequently, intraluminal vesicles (ILVs) are formed by the progressive, inward budding of the late endosomal membrane. The accumulation of ILVs in the late endosome generates multivesicular bodies (MVBs) (Kalluri and LeBleu, 2020; Yue et al., 2020). At this stage, MVBs can fuse with autophagosomes or lysosomes for the degradation of their endosomal contents. Degraded components can then be recycled by the cell. Alternatively, MVBs (containing mature or late endosomes) that do not traffic to lysosomes move toward the cytoplasmic side of the plasma membrane with the aid of the cellular cytoskeletal and microtubule network (Kalluri and LeBleu, 2020). Here, MVBs are able to dock via MVB-docking proteins and fuse with the plasma membrane as exocytic MVBs. Exocytic MVBs release their lipid bi-layered ILV contents to the extracellular space as exosomes (Kalra et al., 2016) (**Figure 1**).

It still remains to be completely understood, the processes that govern the formation of ILVs in MVBs and their further release as exosomes. However, a proposed mechanism implicated in this process involves the Endosomal Sorting Complex Required for Transport (ESCRT). ESCRTs comprise of several proteins that assemble into four ESCRT complexes: namely ESCRT-0, -I, -II, and -III, in association with others like ALIX and vacuolar protein sorting-associated proteins (VPS4, VTA 1) (Colombo et al., 2013). Initiating this process is the organization of endosomal membranes into specialized units that are highly enriched for the tetraspanin class of membrane proteins. Tetraspanins (e.g., CD63, CD9, and CD81) function to cluster the required proteins for ILV formation and are considered common markers for the identification of exosomal vesicles (Pols and Klumperman, 2009). ESCRTs are then recruited to the site of ILV formation where ESCRT-0 recognizes and binds to ubiquitinated proteins present on the outside of the endosomal membrane. ESCRTs-I and -II are then assembled to the cytosolic area to initiate and drive intraluminal membrane budding. The assembling of ESCRT-I/II is reportedly stimulated by some factors, such as the abundance of phosphatidylinositol 3-phosphate (PIP3) in the membrane early endosomes, ESCRT-0 proteins (e.g., hepatocyte growth factor-regulated tyrosine kinase substrate-HRS) and the ubiquitination of the cytosolic tail of endocytosed proteins and/or their curved membrane topology (Henne et al., 2013; Abels and Breakefield, 2016). Upon activation of ESCRT-II, ESCRT-III is recruited through an associated protein, ALIX. ALIX binds to the tumor susceptibility gene 101 (TSG101) component of ESCRT-I complex and also to the charged MVB protein 4A (CHMP4A) components of ESCRT-III, thus, serving as an intermediate between ESCRT-III

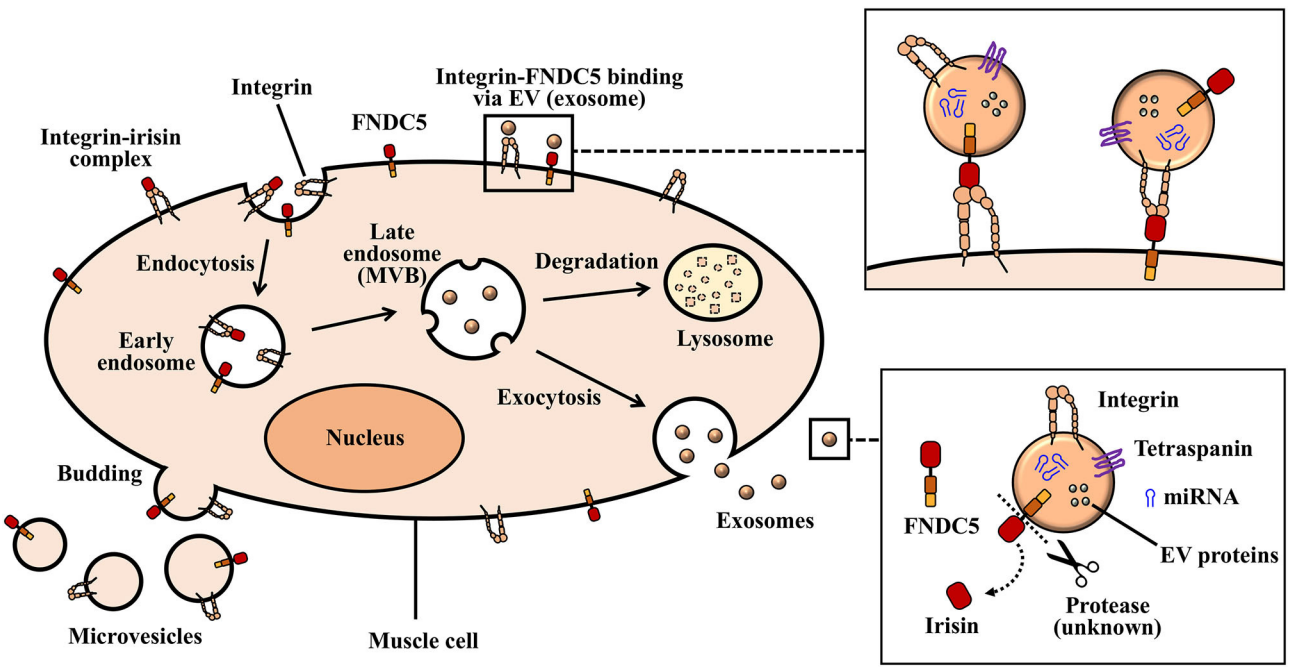


FIGURE 1 | Biogenesis of exosomes and microvesicles. Inward protrusions from the plasma membrane into the cytoplasm allows for the internalization of extracellular and transmembrane proteins in the form of early endosomes. Multivesicular bodies (MVBs), as well as intraluminal vesicles originate from invaginated early endosomes. Large MVBs fuse with the plasma membrane and release exosomes into extracellular space. Vesicles that arise from the outward budding and fusion of plasma membrane make up ectosomes (microvesicles).

and ESCRT-I association (McCullough et al., 2008). ESCRT-III in conjunction with deubiquitinating enzymes then finalize the processes that involve vesicle closure and the detachment of ILVs from the membrane. ESCRT-III forms structures that constrict budding neck followed by scissoring of the membrane (driven by accessory proteins, such as VPS4) and finally, the dissociation and recycling of the ESCRT machinery (Kalra et al., 2016; Schöneberg et al., 2017).

Recent studies identify an alternate ESCRT-independent pathway for ILV formation which involves clustering of sphingomyelin in lipid rafts where it is converted to ceramide by sphingomyelinases. The accumulation of ceramide subsequently induces the merging of microdomain, thus, triggering ILV formation (Stuffers et al., 2009; Kalra et al., 2016). As such, exosomes presumed to be formed through this pathway may lack the expression of common markers, such as ALIX and TSG 101 involved in the endosomal pathway. Small RAB GTPases facilitate the docking, fusion and secretion of exosomes. For example, RAB27A promotes docking of MVBs and fusion to the plasma membrane, whiles RAB27B is involved in vesicle transfer from the Golgi to MVBs, and mobilization of MVBs to the actin-rich cortex under the plasma membrane (Ostrowski et al., 2010; Kalra et al., 2016).

Microvesicles

Vesicles that arise through direct outward budding and fission of the plasma membrane are known as microvesicles (MVs) or ectosomes (**Figure 1**). These range between relatively larger diameters of about 50–2,000 nm (Akers et al., 2013). Ectosome biogenesis involves plasma membrane phospholipid re-arrangements/redistribution, as well as, contractions of cytoskeletal proteins.

Enzymes (collectively described as aminophospholipid translocases) involved in the exchange of lipids between the inner and outer leaflet of the cell membrane for asymmetric maintenance are activated to induce changes within the bilayer to allow for budding and membrane abscission. Flippases are translocases required for the transfer of phospholipids from the outer leaflet to the inner leaflet and floppases, on the other hand, transfer phospholipids from within out. Following this, actin and myosin interactions within the cytosol induce the contraction of cytoskeletal structures; a mechanism that underlies bud formation and subsequent release of ectosome portions. Microvesicles, like exosomes, can be highly enriched in sets of proteins and are particularly identified with markers, such as vesicle-associated membrane protein 3 (VAMP3) and ADP-ribosylation factor 6 (ARF6) (Hugel et al., 2005; Muralidharan-Chari et al., 2009).

Apoptotic Bodies

During programmed cell death, apoptotic cells undergo several stages. These stages begin with condensation of the nuclear chromatin and then membrane blebbing. Cellular components that undergo disintegration are then processed into distinct membrane-enclosed vesicles. These vesicles comprise of a type of EV known as apoptotic bodies that range in sizes between

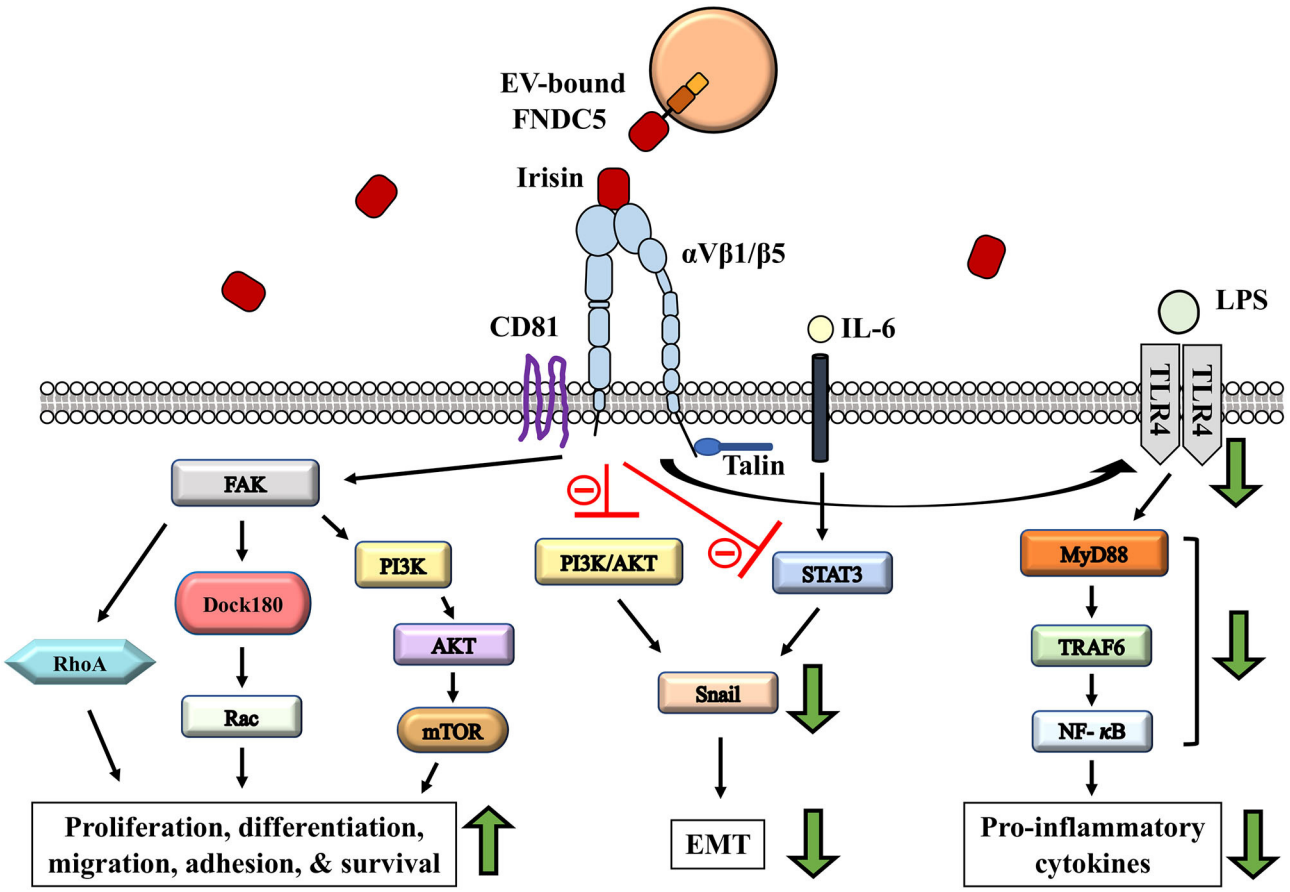


FIGURE 2 | Potential role of irisin-involved integrin signaling cascade in modulating tumor microenvironment remodeling. Irisin exerts anti-inflammatory and cancer suppressing effects through the reduction in proinflammatory cytokines; via regulation of TLR4/MyD88 downstream signaling (Mazur-Bialy et al., 2017a,b). IL-6 induced EMT is regulated by irisin through STAT3/Snail signaling pathways in osteosarcoma (Kong et al., 2017). EMT inhibition is mediated by irisin signaling via the inhibition of PI3K/AKT pathway in lung cancer cells (Shao et al., 2017). Activation of CD81/integrin-mediated FAK signaling in response to irisin regulate cell survival, proliferation and migration (Oguri et al., 2020).

500 and 4,000 nm. Apoptotic bodies pack organelles or cytosolic components and are capable of transferring genetic information from cell to cell via their uptake (Bergsmedh et al., 2001; Cocucci et al., 2009; Kalra et al., 2016).

CELLULAR MICROENVIRONMENTS (NICHES) AND EXTRACELLULAR VESICLES

Accumulating evidence has revealed that EVs are involved in the inter-cellular communication of a wide range of physiology and pathophysiology (Park et al., 2019a; Wortzel et al., 2019; Kalluri and LeBleu, 2020). In the following sections, we focus on an emerging topic (Myint et al., 2020): the roles of EVs in the regulations of microenvironments supporting the trafficking of normal (lymphocytes) and transformed (e.g., cancer) cells in sections 'Cellular Microenvironments (Niches) and Extracellular Vesicles' and 'EV-Mediated Remodeling of Cancer Microenvironment', respectively.

Efficient migration and retention of specific cell types in various compartments of the body are supported by a special microenvironment (homing niche). Such microenvironments have the ability to maintain specific cell populations away from apoptotic and differentiation signals, as well as provide an ambience (cellular structures, extracellular proteins and soluble factors) for proliferation (Moore and Lemischka, 2006).

EVs in Lymphocyte Homing

The regulated migration of lymphocytes is necessary for their effective function. This regulation phenomenon termed, "lymphocyte homing" enables for the trafficking of lymphocytes to specific sites of the body. Lymphocyte homing has been particularly demonstrated in gut immunology. Antigen-primed naïve T lymphocytes in the gut lymph nodes are imprinted to allow them home back into gut tissues as effector or memory T lymphocytes: a necessity for efficient adaptive immunity in the gut mucosa (Myint et al., 2020). High endothelial venules (HEV) of the gut associated lymphoid tissues (GALT) highly express mucosal addressin cell adhesion molecule-1 (MAdCAM-1) that

engages the receptor integrin α4β7 expressed on activated lymphocytes. This interaction enables for the homing of antigen-specific B and T lymphocytes into gut tissues (Streeter et al., 1988; Berlin et al., 1995). The inability of β7 integrin deficient mice to mount antigen-mediated humoral responses in the gut affirms the critical role of integrin α4β7 in gut mucosa B-cell immune responses (Schippers et al., 2012). Additionally, HEVs in GALT chemo-attract chemokine receptor 7 (CCR7)-bearing naïve and central memory T lymphocytes via CCL21 (Okada et al., 2002). Gut-tropic lymphocytes expressing chemokine receptor 9 (CCR9) are signaled by CCL25 and integrin α4β7/MADCAM-1 expressing epithelial cells of HEV to facilitate their migration and homing to the gut (Myint et al., 2020). Lymphocyte homing niche in the gut is physiologically regulated. The expression of MAdCAM-1 in the gut is stabilized in limited levels to maintain homeostasis (Pabst et al., 2000). Elevated signal from inflammatory mediators as seen in gut inflammatory diseases upregulate MAdCAM-1 expression and promote the accumulation of activated T lymphocytes that abundantly express integrin α4β7 (Myint et al., 2020).

Remodeling of homing niches to regulate the tissue-specific migration and recruitment of activated lymphocytes has been demonstrated in recent studies. One mechanism by which the homing niche is remodeled appears to be through gut-tropic derived exosomal secretions, demonstrated by Park et al. (2019b). Activated gut-tropic T lymphocytes secrete exosomes that highly express functional integrin α4β7 with the ability to bind MAdCAM-1 and preferentially home to the gut mucosa. These α4β7 expressing exosomes function to suppress MAdCAM-1 through the cargo and delivery of microRNA milieu that target the transcriptional factor, Nirenberg-Kim (NK) 2 homeobox 3 (NKX2.3) necessary for MAdCAM-1 regulation. Consequently, other homing niche factors, such as the chemokines CCL25 and CCL28 were suppressed by gut-tropic lymphocyte-derived exosomes (Park et al., 2019b). It is therefore not far-fetched to consider exosomes of lymphocytes and other host cells as key remodeling regulators of homing niches to maintain homeostasis.

Other Homing Niches

EV-mediated regulation of homing niches (by modifying the expression of adhesion molecules and chemokines) may operate in stem cell trafficking, and warrants further research. Here we explain the overview of how integrins and chemokines govern stem cell trafficking. Physiologically, stem cells home to specific tissue areas via chemoattraction. For example, hematopoietic stem cell precursors home to the bone marrow after selectin-mediated braking (E-selectins, P-selectins, and L-selectins) that facilitates migration on adhesion ligands expressed by vascular endothelium (Liesveld et al., 2020). Following braking, intracellular signaling cascades are activated through chemokine-chemokine receptor interactions (e.g., CXCL12/CXCR4) to trigger the activation states of integrins like $\alpha_L\beta_2$ (LFA-1) and $\alpha_4\beta_1$ (VLA-4). This facilitates the arrest of cells and their subsequent migration via integrin/cell adhesion molecule (CAM) interaction (Schweitzer et al., 1996; Huttenlocher and Horwitz, 2011; Sahin and Buitenhuis, 2012). Increased CXCL12/CXCR4 signaling is implicated in the retention of leukocytes, such as mature neutrophils at inflammatory sites (demonstrated in tissue-damaging inflammatory diseases) (Yamada et al., 2011; Isles et al., 2019), or in the bone marrow (demonstrated in Warts, Hypogammaglobulinemia, Infections, and Myelokathexis (WHIM) syndrome-associated neutropenia) (Kawai and Malech, 2009). Additionally, FoxP3$^+$regulatory T cells trafficking and homing to hematopoietic stem cell niche in the bone marrow involves the signaling of CXCR4 (Zou et al., 2004; Hirata et al., 2018). It has been demonstrated that, loss of adhesion molecules like integrins (e.g., β7-integrins: α4β7, αEβ7) and selectins (e.g., P-selectin) influences stem cell and lymphocyte trafficking (Frenette et al., 1996; Schön et al., 1999; Lucas, 2019).

The subventricular zone (SVZ) of the brain is another example of a host site that serves as a niche for neural stem cells (NSC), with inflammation playing a key role in the homing and recruitment of NSC for CNS regeneration. Increased expression of stromal derived factor 1α (SDF-1α or CXCL12) by astrocytes and endothelial cells of ischemic areas in the brain, as well as their constitutive expression and activation of CXCR4 (receptor of CXCL12), facilitate NSC migration toward ischemic brain explants (Imitola et al., 2004). LeX-positive NSCs (denoting a subpopulation of SVZ adult stem cells that express the extracellular matrix (ECM)-associated carbohydrate, Lewis X) potentially differentiate into cells with neuronal phenotypes following transplantation. These cells acquire enhanced migratory and proliferative characteristics upon exposure to SDF-1α (Corti et al., 2005). Other soluble factors involved in the migration and recruitment of NSCs include vascular endothelial growth factor (VEGF), interleukin-6, hepatocyte growth factor (HGF) and platelet derived growth factor (PDGF) (Garzón-Muvdi and Quiñones-Hinojosa, 2009). Just as with the effect of soluble growth factors on NSC homing, the expression of integrins, such as α6β1 in SVZ neurogenic niche play a vital role in NSC recruitment, demonstrated in a rodent model of cerebral ischemia (Prestoz et al., 2001).

EV-MEDIATED REMODELING OF CANCER MICROENVIRONMENT

Cancer cells secrete exosomes that remodel homing niches to promote migration, retention and proliferation of tumor cell and/or other cell types, thus, tumor exosomes form a part of the tumor-derived factors necessary for pro-tumor microenvironment formation and metastatic niche pre-conditioning (Hoshino et al., 2015). Exosomes released by breast cancer cells are delivered to distal cells of target organs, such as the lung and liver, where they induce the expression of proinflammatory genes through the activation of Src-kinase signaling. Thus, the initiation of exosome-mediated chronic inflammation contributes to the remodeling of distal tissues to favor tumor metastasis. Concomitantly, breast cancer exosomes highly expressing integrins αvβ5 and α6β4 distribute to the fibronectin-enriched ECM of the liver and laminin-enriched ECM of the lung, respectively. As a result, tissues within which these integrin-directed exosomes are specifically distributed

become prone to inflammatory-mediated remodeling to support the cultivation of pre-metastatic niche and consequently, predisposed to metastatic cancers (Hoshino et al., 2015; Myint et al., 2020).

Alluring to the evidence that non-tumor cells and their exosomes function to tightly regulate trafficking and homing to specific niches for regeneration, repair and homeostasis (Prestoz et al., 2001; Park et al., 2019b), cancer cells and cancer-derived exosomes on the other hand play a contributory role in remodeling niches to support cancer metastasis (via chronic inflammation, angiogenesis, increased vascular permeability, etc.) (Hoshino et al., 2015; Myint et al., 2020).

MUSCLE–DERIVED EVS AND MYOKINES IN MICROENVIRONMENT REMODELING

Growing evidence suggests that major risk factors of cancer development and progression include metabolic disorders that facilitate chronic inflammation, thus, metabolic syndrome is consistently associated with increased risk of common cancers (Esposito et al., 2012). Although mechanisms underlying the link between metabolic syndrome and cancer risk are not fully understood, metabolic syndrome represents a proxy marker for several risk factors, such as lack of physical activity, high dietary fat intake and oxidative stress (Alberti et al., 2009). Adipocytes and infiltrating immune cells induce a systemic low-grade chronic state of inflammation by producing inflammatory mediators/immune cell chemo-attractants (interleukin-1β [IL-lβ], interleukin-6 [IL-6], tumor necrosis factor-α [TNF-α], and monocyte chemoattractant protein-1[MCP-1]) and increasing the circulation of free fatty acids to promote the establishment of tumorigenic microenvironment. This is evident in excess adiposity, insulin resistance, aberrant glucose metabolism and particularly, central obesity (visceral adiposity) (Harvey et al., 2011). Hyperglycemia, a hallmark of metabolic syndrome, is implicated in the dysregulation of growth factors and metabolic hormones like insulin and Insulin-like growth factor (IGF-1). Hyperglycemia-induced suppression of IGF-binding protein synthesis and the promotion of IGF-1 synthesis increase the amounts of bioavailable IGF-1 in circulation; an established critical risk factor for several malignancies (Pollak, 2008). Downstream signaling pathways of activated receptor tyrosine kinases, such as IGF-1 receptor activate the Akt cascades which are commonly altered in epithelial cancers. Typical in tumors and tissues of diabetic rats, is the increased activation of downstream mediators, such as mammalian target of rapamycin (mTOR) (Wong et al., 2010; De Angel et al., 2013).

The association between obesity and physical inactivity has been shown in several studies (Gustat et al., 2002; Golbidi et al., 2012). Evidently, exercise and muscle training prove to be beneficial in the prevention and management of obesity or other related metabolic distress. Decreased daily physical activity in healthy individuals is linked to undesired consequences in metabolism, such as reduced insulin sensitivity and elevated adiposity (Olsen et al., 2008). Exercise induces anti-inflammatory effects, boosts antioxidant capacity, and regulates glucose/fat metabolism. Skeletal muscle activity has been found to be beneficial in regulating metabolism and inflammation within several tissues, including adipose tissues. Contracting muscles release myokines, such as interleukin 6 (IL-6) that positively impact inflammation. IL-6 acts as a pro-inflammatory and an anti-inflammatory mediator, however, studies have shown that exercise-induced muscle-derived IL-6 exerts anti-inflammatory effects. The anti-inflammatory property of IL-6 is shown by way of an inhibitory effect on TNF-α, IL-10, and IL-1β, to protect against TNF-induced insulin resistance (Festa et al., 2000; Febbraio and Pedersen, 2005). Myokines secreted by muscle tissues into the extracellular space are capable of modulating homeostasis in the bone, pancreas and adipose tissues. Such myokines like IL-6, irisin, myostatin, and interleukin-15 (IL-15) play a key role in the crosstalk between muscle and adipose tissues (Leal et al., 2018). Recent articles, such as that of Mika et al., review the potential benefits of exercise-induced release of myokines via the alterations in adipose tissue fatty acid metabolism (e.g., increased lipolysis and reduced fatty acid uptake). Chronic exercise impacts the profile of adipokines released from adipose tissues, as well as promote the "beiging" of adipocytes (Mika et al., 2019).

Intriguingly, the provision of beneficial myokines is not confined to muscle cells. Muscle-derived extracellular vesicles (EVs) cargo and distribute myokines, muscle specific microRNAs (myomiRs) and other soluble factors to proximal and/or distal tissues, thus, crosstalk between muscle and other tissues is at least in part, mediated by EVs (Whitham et al., 2018), from muscles. Exercise and physical activity have been demonstrated to augment the release of muscle EVs into circulation.

Skeletal Muscle EVs

Both myoblasts and non-proliferating myotube forms of skeletal tissue are capable of secreting EVs, *in vitro* (Forterre et al., 2014a; Choi et al., 2016). Skeletal muscle-derived EVs have been demonstrated to be key players in muscle physiology and systemic homeostasis via paracrine actions (Rome et al., 2019). The release of small EVs into circulation is particularly enhanced by physical exercise, inflammation/stress, and several muscle-related conditions (Frühbeis et al., 2015; Barone et al., 2016). Skeletal muscle is a major contributor of exercise-induced secreted molecules. Some of these secreted molecules were identified in EVs harvested from conditioned medium (CM) of myotube cultures (Forterre et al., 2014b; Deshmukh et al., 2015). Skeletal muscle-derived EVs play vital roles in the differentiation and regeneration of muscle tissue by triggering cues for myogenic processes and myofiber regeneration. Exosomes derived from human skeletal myoblasts were found to contain various myogenic factors, such as insulin-like growth factors (IGFs), fibroblast growth factor-2 (FGF2), and hepatocyte growth factor (HGF) that enhanced terminal myogenic differentiation of stem cells, *in vitro* (Choi et al., 2016). Conversely, skeletal muscle-derived exosomes may be implicated in suppressing myogenic events. During inflammatory conditions or tissue damage, inflammatory cells and the milieu of pro-inflammatory mediators, such as chemokine ligand 2 (CCL2) induce the production of muscle EVs which package more myostatin

(negative regulator of myoblast proliferation and differentiation) and less decorin (myostatin antagonist) (Tidball, 2017; Kim S. et al., 2018).

Crosstalk between the skeletal system and the peripheral nervous system have been demonstrated to be at least in part, mediated by skeletal muscle-derived exosomes. Motor neuron regeneration and survival has been found to be positively impacted by EVs released from muscle cells, *in vitro* (Madison et al., 2014). Additionally, skeletal muscle denervation induced the release of muscle EVs that preferentially support motor neuron regeneration accuracy. Muscle-derived EVs (containing muscle specific markers, such as α-sarcoglycan) were taken up by denervated, but not naïve nerve tissue around the neuro-muscular junction and significantly induced the exclusive projection of motor neurons to muscle branch, thus, ensuring anatomically accurate motor neuron regeneration in rats (Madison and Robinson, 2019). However, mechanisms underlying exactly how muscle-derived EVs are taken up and localized within the denervated nerve remain a knowledge gap incompletely bridged.

MicroRNAs, carried by muscle EVs have been implicated in the crosstalk between muscle and other cell types (Jalabert et al., 2016; Nie et al., 2019), particularly between muscle and bone in the events of bone remodeling. Skeletal muscle-derived exosomes enriched in miR-27a-3p positively impacted osteogenesis by delivering and increasing miR-27a-3p to target adenomatous polyposis coli (APC), thereby, activating the Wnt/β-catenin pathway to promote osteogenic differentiation of MC3T3-E1 pre-osteoblasts (Xu Q. et al., 2018). Another area of evidence for muscle-bone crosstalk mediated by muscle EVs is osteoclast biogenesis in osteoporosis. Mouse muscle cell line (C2C12 myoblasts)-derived EVs suppressed osteoclast formation and mitochondrial energy metabolism in bone marrow cells (Takafuji et al., 2020a). This group demonstrated that Myo-EVs were successfully taken up by bone cells in culture and suppressed the expression of RANKL-induced osteogenic factors, such as cathepsin K (CTSK), nuclear factor of activated T-cells 1 (NFATc1) and dendrocyte expressed seven transmembrane protein (DCSTAMP). Evidently, Myo-EVs suppressed the oxygen consumption rate and expressions of peroxisome proliferator-activated receptor co-activator 1 beta (PGC1β), NADH-ubiquinone oxidoreductase chain 4 (ND4) and cytochrome c in mouse bone cells (Takafuji et al., 2020a), possibly by an miRNA-mediated inhibition of CREB/PGC1β pathway in osteoclast precursors (Takafuji et al., 2020b). Oxidative stress has been shown to modify miRNA contents of Myo-EVs; miR-34a is elevated in C2C12 derived EVs following oxidative stress. Muscle EVs enriched in miR-34a home to the bone and induce senescence of bone marrow-derived stem cells by decreasing Sirtuin1 expression on primary bone marrow cells (Fulzele et al., 2019).

In metabolic abnormalities like diabetes and insulin resistance, paracrine and/or endocrine effects of Myo-EVs have been reported. A study revealed that the pancreas takes up muscle EVs *in vivo*. Such EVs transferred muscle specific miRNAs to pancreatic beta cells and islets, thereby, modulating gene expression and proliferation (demonstrated *in vitro* with MIN6B1 pancreatic cell line). In this study, muscle EVs derived from insulin resistant skeletal muscle were particularly enriched in miR-16 cargo that could be transferred to pancreatic cells and regulate PTCH1, potentially contributing to the pathogenesis of type-2 diabetes (Jalabert et al., 2016).

As positive regulators of metabolic function, exercise-induced skeletal muscle exosomes potentially promote adipocyte lipolysis. IL-15 is known to be highly expressed in skeletal muscle, and acts to decrease fat depots in adipose tissue (Quinn et al., 2005). Additionally, myonectin, a recently established myokine involved in lipid metabolism has been identified as a link between skeletal muscle and systemic lipid metabolism (Seldin and Wong, 2012). Considering the abundance of IL-15 mRNA in the contracting skeletal muscle (Nielsen et al., 2007) and the exercise-induced release of muscle IL-6 in vesicular structures (Lauritzen et al., 2013), it is not far-fetched to speculate the packaging and cargo of this lipolysis-influencing myokine in skeletal muscle derived exosomes. Alterations in skeletal muscle (e.g., muscle activity and injury) have been shown to influence the release of key microRNAs (e.g., miR-21, miR-148b, and miR-486 in tissues, plasma and exosomes) that regulate insulin responsiveness (D'Souza et al., 2018).

Cardiomyocyte Derived EVs

Although not classically described as typical secretory cells, cardiac muscle cells can be induced to release EVs, *in vitro* (Gupta and Knowlton, 2007; Chistiakov et al., 2016). Cardiomyocytes, which constitute the prime contractile cell type in cardiac tissue, display augmented secretions of EVs under several cellular stress conditions like inflammation, injury, hypoxia, alcohol exposure and glucose starvation (Garcia et al., 2015; Yu and Wang, 2019). Similar to skeletal muscles, cardiac muscle EVs cargo nucleic acids, including miRNAs that can regulate gene expression in recipient cells (Waldenström et al., 2012).

Oxygen and nutrient supply are imperative for the efficient function of the myocardium. As such, blood vessels feeding the myocardium play a key role in cardiac function. The crosstalk between cardiac myocytes and intra-cardiac endothelial cells has been established. This crosstalk is vital to meet the metabolic needs for efficient heart function (Garcia et al., 2016) and has been found to be mediated by paracrine signal molecules, direct cell-cell communication, and EVs (Colliva et al., 2020). In the light of cardiomyocyte-endothelial cell communication, cardiomyocyte-derived EVs effect both anti-angiogenic and pro-angiogenic regulatory functions (Ribeiro et al., 2013; Wang et al., 2014; Chistiakov et al., 2016). Heat shock proteins (Hsps) well-known to protect cardiomyocytes from stresses (Willis and Patterson, 2010; Edwards et al., 2011; Yu and Wang, 2019; Yu et al., 2019) are highly expressed in muscle cells and secreted from cardiomyocytes via exosomes. Particularly, cardiomyocyte Hsp20 released through exosomes by the non-classical endoplasmic pathway promote angiogenesis; enhanced proliferation, migration, and tube formation of endothelial cells by Hsp20 via the activation of vascular endothelial growth factor receptor (VEGFR) signaling cascade (Zhang et al., 2012). The crucial role of cardiac microvascular endothelial (CME) cells in protecting against myocardial injury

by way of activating endothelial nitric oxide synthase (eNOS) has been evidently linked to signals from cardiomyocytes. A study reported that, cardiomyocytes regulated CME cells via EV transfer of long intergenic non-protein coding RNA, Regulator of Reprogramming (Linc-ROR) to target miR-145-5p, ultimately resulting in the activation of eNOS pathway (Chen et al., 2020). Conversely, anti-angiogenic pathways are mediated by cardiomyocyte-derived EVs and contribute to endothelial dysfunction, insufficient myocardial angiogenesis and cardiovascular complications (Wang et al., 2014). Myocyte-derived exosomes from type-2 diabetic rats but not wild type inhibited proliferation, migration, and tube formation of cardiac endothelial cells in culture through miRNA regulation. It was observed that diabetes induced the release and transfer of exosomes harboring high levels of miR-320 (while being less enriched in miR-126) that target angiogenic factors (Hsp20 and insulin-like growth factor-1) in endothelial cells (Wang et al., 2009, 2014), potentially contributing to diabetes-related vascular dysfunction.

Cardiac remodeling is crucial in the pathophysiology of cardiac injury and hence, should be appropriately regulated to prevent irreversible changes to the structure and function of the myocardium (Tracy et al., 2020). Cardiac muscle exosomes play a key role in regulating myocardial remodeling by the transfer of encapsulated miRNAs to target fibrotic factors (Chaturvedi et al., 2015; Chistiakov et al., 2016). A study revealed that miR-133a from cardiomyocyte exosomes targeted type IA1 collagen (Col1A1) and connective tissue growth factor, resulting in decreased myocardial fibrosis in hypertensive rats (Castoldi et al., 2012). Other cardiomyocyte-derived exosome miRNAs that have been identified to promote fibrosis and fibroblast differentiation in animal models include miR-208a (Yang et al., 2018), miR-92a (Wang et al., 2020) and miR-195 (Morelli et al., 2019). While a number of exosome-derived miRNAs contribute to myocardial fibrosis, others, such as miR-378 tend to exhibit a protective effect by targeting mitogen-activated protein kinase kinase 6 (MKK6) to suppress p38 mitogen-activated protein kinase phosphorylation in cardiac fibroblasts (Yuan et al., 2018). Other miRNAs implicated in regulating the fibrotic cascades in this regard include miR-29b (Chaturvedi et al., 2015) and miR-373 (Xuan et al., 2019).

Recently, the roles of cardiomyocyte-derived exosomes have expanded to encompass a role in mechanisms that underlie the therapeutic efficacy of stem cells in myocardial infarction (Hu et al., 2018). Cardiomyocyte exosomes obtained from culture conditioned medium under stress microenvironment (peroxide-induced oxidative stress) hastened stress-induced injury of bone marrow-derived stem cells (Hu et al., 2018).

Smooth Muscle–Derived EVs

Vascular smooth muscles in a synthetic or proliferative, non-contractile state exhibit an increased production of extracellular vesicles (Kapustin and Shanahan, 2016; Schurgers et al., 2018), demonstrated by the inverse relationship between the expression of contractile vascular smooth muscle cell markers and exosome secretion (Kapustin et al., 2015). Inflammatory cytokines and growth factors like tumor necrosis factor-α (TNF-α) and platelet-derived growth factor (PDGF), respectively stimulate the phenotypic transition and exosome secretion by vascular smooth muscle cells (Kapustin and Shanahan, 2016). The activation of vascular smooth muscle cells (SMC) form one of the key events in atherogenesis and the onset of vascular complications, such as stroke and myocardial infarction (Bennett et al., 2016). Overexpression of Krüppel-like factor 5 (KLF5), a transcription factor requisite for mediating SMC proliferation and migration in vascular remodeling events, mediates the secretion of exosomes enriched on miR-155 from human smooth muscle cells. The transfer and uptake of SMC-derived exosomal miR-155 by endothelial cells have been shown to impair endothelial integrity by suppressing their proliferation, migration, tube formation (angiogenesis), and expression of tight junction proteins (Zheng et al., 2017).

Recently, smooth muscles of the pulmonary vasculature have been shown to release EVs that package a myriad of RNA transcripts transferrable to pulmonary arterial endothelial cells (de la Cuesta et al., 2019). Migration and apoptosis of pulmonary arterial smooth muscle cells account for the modulation of miR-143. As a result, exosomes derived from these cells selectively pack abundant miR-143-3p that exhibit pro-angiogenic and pro-migratory effects on recipient pulmonary arterial epithelial cells (Deng et al., 2015). In another recent finding, pulmonary arterial epithelial cells cultured with exosomes derived from platelet-derived growth factor-stimulated smooth muscle cells showed an enhanced migratory but not proliferative ability via the effect of exosomal miRNAs. It was observed that exosomes from PDGF-stimulated smooth muscle cells were deficient in miR-182, miR-1246, and miR-486 and this alteration proved critical for the enhanced migratory phenotype of endothelial cells in pathologic states (Heo et al., 2020).

The microenvironment or matrix of the vasculature is to a large extent regulated by microvesicles secreted from muscle cells, endothelial cells, and infiltrating immune cells of blood vessels (Reynolds et al., 2004; New et al., 2013). Small EVs secreted by vascular smooth muscle cells normally cargo various factors like the high affinity calcium ion-binding matrix Gla protein (MGP) and fetuin-A that function to inhibit calcification, thus protecting against vascular mineralization and the formation of atherosclerotic plagues (Reynolds et al., 2005). However, inflammation and mineral imbalance influence this protective role by inducing the release of EVs enriched in calcification promoters and depleted of inhibitors, thus, conditioning an environment conducive for vascular mineralization and stiffening (Reynolds et al., 2005; Kapustin et al., 2011).

EMERGENCE OF IRISIN SIGNALING AND POTENTIAL IMPLICATIONS FOR MICROENVIRONMENT REMODELING

Irisin, a recent discovery to the family of adipomyokines (exerting its effect on both adipose and muscle tissues) is a thermogenic protein thought to be vital in energy metabolism (Boström et al., 2012; Rodríguez et al., 2017). Irisin has been proposed

to bridge the communication between the muscle and other tissues of the body, and therefore has gained much attention in research relating to metabolism and tissue crosstalk (Pukajło et al., 2015). Irisin is a fragment of the fibronectin type III domain-containing protein 5 (FNDC5) on the cell membrane of myocytes, adipocytes, and other cell types (liver, brain, stomach, etc.) (Aydin et al., 2014). The FNDC5 protein has a cytoplasmic C-terminal portion and an N-terminal extracellular portion that is proteolytically cleaved and released into circulation as irisin (Panati et al., 2018). Physical activity, especially high intensity exercise and resistance training have been shown to increase the levels of irisin in circulation (Tsuchiya et al., 2014, 2015). Although the specific receptor for irisin was yet to be identified until recently, the pivotal study by Spiegelman and colleagues has demonstrated that irisin binds to integrins (Kim H. et al., 2018). Integrin-ligand interactions activate several downstream signaling pathways that regulate cellular processes (**Figures 2**, **3**). We have previously reviewed and discussed the emergence of irisin and integrin-ligand interactions in the context of cancer, metabolic disorders, and inflammation (Park et al., 2020). Irisin binds to several integrins, such as αVβ5 and α5β1 on bone and fat cells, however, binding affinity seems to be higher with the α_V family of integrins (Kim H. et al., 2018). Irisin increases the expression of thermogenin (UPC-1, a mitochondrial protein in adipose tissue lipid droplets) in matured fat cells and facilitates browning of white adipose tissue (WAT), leading to the formation of beige-adipose tissue. Although not directly inducing browning, irisin inhibits the formation of new adipose cells, and thus, negatively regulates adipogenesis via irisin-induced phosphorylation of mitogen-activated protein kinases (Zhang et al., 2014; Korta et al., 2019). *In vitro* studies suggest that irisin plays a protective anti-inflammatory role by suppressing the production of pro-inflammatory cytokines in fat cells and immune cells, and therefore alleviates obesity-induced inflammation. Adipocytes are sensitive to irisin and tend to release relatively low levels of IL-6, IL-1β, monocyte chemotactic protein 1 (MCP 1), and TNF-α under the effect of irisin *in vitro*, potentially via the regulation of downstream signaling of Toll-like receptor 4/myeloid differentiation primary response 88 (TLR4/MyD88) (Mazur-Bialy et al., 2017a,b) (**Figure 2**).

Aside adipocytes, bone cells (osteocytes) have been identified to be sensitive to irisin, and thus, irisin partly plays a role in bone remodeling. Upon irisin treatment, osteocytes under oxidative stress conditions show a reduced ratio of apoptotic cells in culture (Kim H. et al., 2018). Irisin directly regulates osteoclast function and differentiation by stimulating the release of osteoclastogenic promoters like RANKL and sclerostin, even at normal circulatory concentrations (Estell et al., 2020). Earlier studies had also identified osteoblasts as direct targets of irisin; an enhancer of osteoblast differentiation (Colaianni et al., 2014). Although irisin dually targets osteocytes and osteoblasts, the effect of irisin on different bone cell types greatly depends on the circulatory concentrations of the protein, since varying concentrations of irisin treatment have demonstrated contrasting effects in *in vitro* studies (Colaianni et al., 2014; Estell et al., 2020). Further studies in this area are needed to clearly understand the mechanisms by which irisin mediates bone metabolism.

Recently, the involvement of irisin in inflammatory disease has been demonstrated, particularly with intestinal inflammation. Binding of irisin to αVβ5 integrin units on intestinal epithelial cells have been shown to alleviate epithelial barrier dysfunction during gut injury. Irisin restored epithelial barrier function via the activation of αVβ5/AMPK-UCP 2 signaling pathway, while reducing oxidative stress and apoptosis in enterocytes (Bi et al., 2020). Taken together, irisin is a physiologically beneficial adipomyokine that possess properties contributing to oxidative stress alleviation (Bi et al., 2020), anti-inflammation (Mazur-Bialy et al., 2017a,b; Xiong et al., 2018), and anti-metastatic effects (Rabiee et al., 2020). It is worth mentioning that, there are a few conditions in which irisin has been demonstrated to promote inflammation. This is particularly shown in hepatocellular carcinoma (HCC) whereby, the increased hepatic mRNA levels of FNDC5/irisin in HCC patients correlated with increased expression of proinflammatory markers, such as IL-6 and TNF-α. However, mechanisms underlying the irisin-induced inflammation in hepatic carcinoma remains to be completely understood (Gaggini et al., 2017).

EXOSOMAL INTEGRIN-IRISIN SIGNALING (POTENTIAL IMPLICATION FOR CANCER METASTASIS)

The discovery of integrins belonging to the αV family as receptors for irisin signaling (Kim H. et al., 2018) has been a great leap toward understanding how irisin could regulate metabolism of both host and tumor cells, considering that, integrins are ubiquitously expressed. Although irisin has not yet been identified as a cargo content of exosomes released by muscle and adipose cells, circulating irisin may engage integrin receptors on exosomes to mediate various niche pre-conditioning (**Figure 3**). Taking into consideration that exosomes express and transport integrins (identified receptor for irisin) on their surfaces, the potential "cargoing" of irisin in muscle-derived exosomes can be proposed in a few ways. One of such possibilities lies in the endocytosis of surface proteins on muscle cells during EV biogenesis, leading to the formation of MVBs that may package membrane-bound integrin-irisin complexes (**Figure 1**). This mechanism has been proposed for the exosomal packaging of ECM proteins, such as fibronectin (Sung et al., 2015).

The expression of αV integrins is highly upregulated in several cancers and tends to augment the metastatic phenotype of tumor cells, thus promoting their migration (Teti et al., 2002; McCabe et al., 2007; Haeger et al., 2020). Expression of integrins is not confined to cancer cells only; exosomes obtained from tumors contain a myriad of functional tumor-derived integrins that precondition microenvironments for organ-specific metastasis (Hoshino et al., 2015). Tumor-derived exosomes are capable of transferring exosomal integrins to tumorigenic and non-tumorigenic cells. For example, Fedele et al. demonstrated that αVβ6 which is highly expressed on prostate cancer cells was transferable among different subsets of prostate cancer cell lines via their exosomes. Prostate cancer cell-derived exosomes which

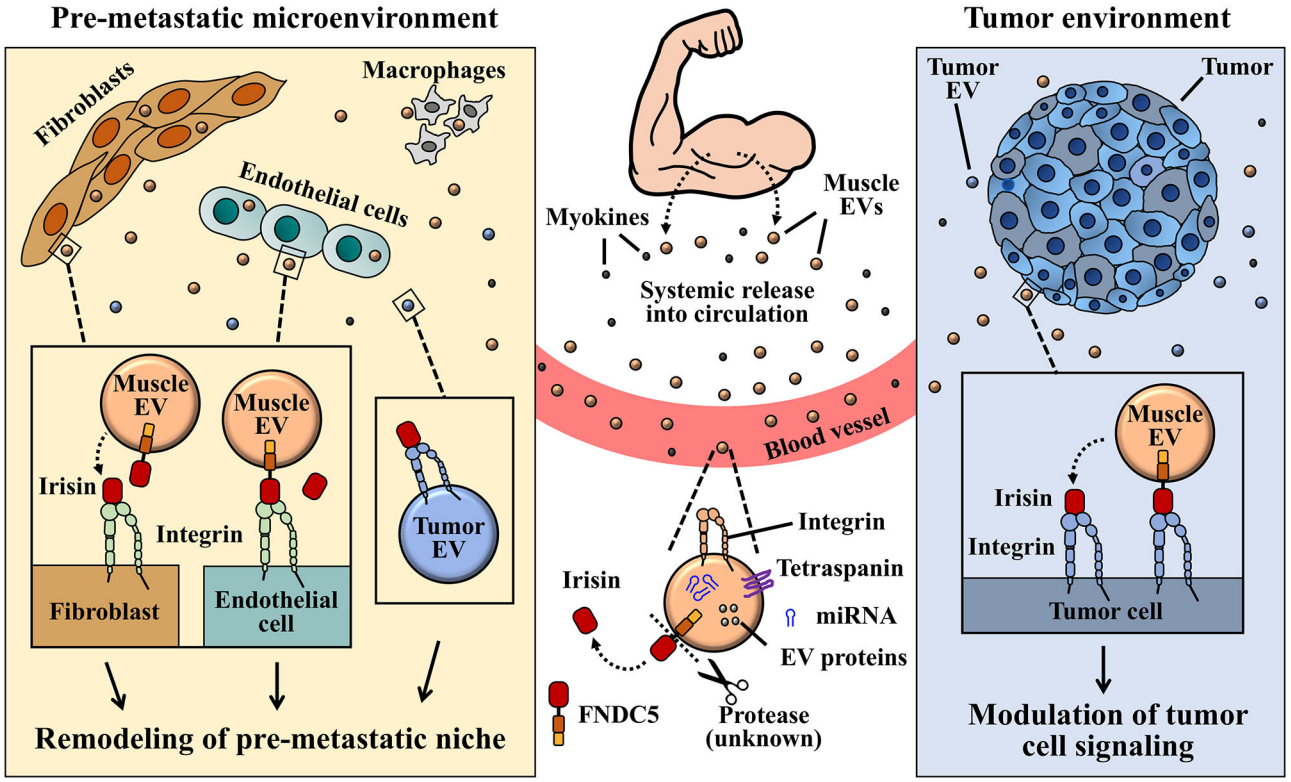


FIGURE 3 | A graphical illustration proposed for the interaction between muscle EV irisin and integrin, and its role in pre-metastatic tumor environment remodeling. Circulatory or EV-bound irisin derived from muscle cells can potentially bind to integrin receptors expressed on several tumor cells or tumor-derived EVs. EVs and irisin released by muscles into circulation, upon reaching pre-metastatic microenvironment, may engage tumor-derived EVs via EV-bound irisin/EV integrin and/or free-irisin/EV integrin interactions. Signaling from integrin-irisin interaction in tumor microenvironment could mediate functional regulation of tumor-derived exosomes in the remodeling of pre-metastatic microenvironment.

expressed functional integrin αVβ6 were taken up by recipient prostate cancer cells that subsequently re-expressed the integrin on their surfaces (Fedele et al., 2015). This group also showed that another member of the αV integrin family, αVβ3, was transferred from tumorigenic to non-tumorigenic prostate epithelial cells via exosomes and induced functional changes like enhanced migration and adhesion in recipient cells (Singh et al., 2016). Other studies confirm the *in vitro* transfer of exosomal αVβ3 integrin to β3-negative cells resulting in the acquisition of ligand binding activity (Krishn et al., 2019). Indeed, integrins expressed on the surface of exosomes are able to bind and adhere to cell surface, or ECM proteins, such as collagen and fibronectin, thus, support integrin binding-mediated cell adhesion (Park et al., 2019b; Altei et al., 2020).

Although the particular role of irisin in cancer progression still remains to be elucidated, serum levels of irisin is markedly altered in several cancers (Zhang et al., 2018). However, there are contrasting reports on the effects of irisin in association with cells. For example, irisin was found to suppress the viability, proliferation and migration of some malignant breast cancer cells (MCF-7 and MDA-MB-231, by way of induced apoptosis) but not others like MCF-10A breast epithelial cells (Gannon et al., 2015). Additionally, irisin signaling has been found to inhibit the proliferation, migration, and invasion of lung cancer (Shao et al., 2017). This effect results from the irisin-induced inhibition of Snail transcription factors via the PI3K/AKT pathway, resulting in the reduced expression of epithelial-mesenchymal transition (EMT) markers (Shao et al., 2017) (**Figure 2**). Nonetheless, these findings point to the potentially protective and organ specific role played by irisin in cancer progression and metastasis (Zhang et al., 2018).

Taken together, the evidence supporting exosomal carriage of functional and transferable integrin proteins, such as those belonging to the αV family (Fedele et al., 2015; Singh et al., 2016; Krishn et al., 2019), and the identification of integrins as signaling receptors (Kim H. et al., 2018) propose irisin-exosome interactions as potential players in niche regulation especially in cancer metastasis (**Figures 1–3**). Irisin may negatively impact cancer progression and metastasis indirectly via their interaction with tumor-derived exosomes. Extracellular vesicles designed to precondition distant microenvironment for organotropic metastasis could potentially be altered by the activation of integrin-irisin signaling cascades (**Figures 2**, **3**). Further studies in the area of irisin-exosomal integrin interaction is warranted to ascertain molecular mechanisms that underlie the remodeling of microenvironment and tumor metastasis. Muscle-derived

myokines and EVs may hold promising contributions toward therapeutic breakthroughs in cancer management.

PERSPECTIVE

With the growing evidence of their involvement in the autocrine/paracrine/endocrine regulation of metabolic pathways (Seldin and Wong, 2012) and inflammation (Febbraio and Pedersen, 2005), muscle cells and muscle cell-derived EVs deserve attention in our quest to demystify pathophysiological processes underlying various health condition, including cancers. Muscle-derived EVs that cargo myokines and other biologic modulators (nucleic acids, growth factors, etc.) could potentially modulate the remodeling of niches in vital sites, such as liver and adipose tissues. The anti-inflammatory effect of skeletal muscle activity mediated by myokines (e.g., IL-6) could negatively contribute to the establishment of favorable inflammatory niche conditions, necessary for cancer metastasis. Although more studies to support exosomal packaging and release of important myokines like irisin, IL-6, and myonectin are desired, the growing evidence of muscle EV secretion and remarkable encapsulation of muscle-specific biologic contents (transferable to various cell types and also implicated in cancer progression) (Singh et al., 2016; Wang et al., 2016; Li et al., 2018; Xu X. et al., 2018; Krishn et al., 2019) provide substantial cues for further investigations. Metabolic syndrome (including obesity, hyperlipidemia and diabetes) burden organs with pro-tumorigenic and metastatic cascades associated with chronic inflammation, increased oxidative stress, and deregulated cellular signals (Harvey et al., 2011), and therefore, potentially render tissues niche-ready for cancer metastasis. On one end, because muscle and muscle-derived EVs pack mediators that restitute the effects of metabolic dysregulation, their indirect attribution to the regulation of microenvironment remodeling and the establishment of pre-metastatic homing niches can be hypothesized. Little evidence exists to explain the relationship between muscle-derived EVs and tumor metastasis, however, findings from other pathologies, such as inflammation and metabolic disorders discussed in this review point out a plausible connection that may potentially benefit homeostatic regulation of homing niches against tumor progression metastasis. Further studies are imperative to better understand the role of muscle-derived exosomes in regulating the events of pre-metastatic niches and cancer progression. This may provide novel insights to the development of therapeutic interventions for the management of cancers.

AUTHOR CONTRIBUTIONS

SD and MS contributed to the conceptualization, scope, and outline of this review. SD, EJP, PKM, AI, MGA, GO, EK, and MS analyzed the referenced manuscripts in this manuscript and participated in preparing the manuscript. All authors read and approved the final version.

FUNDING

This work was supported by the JSPS KAKENHI Grants (EJP, 19K09392; AI, 19K18210; EK, 18K08917 and 19KK0224; and MS, 18H02622 and 19KK0196).

REFERENCES

Abels, E. R., and Breakefield, X. O. (2016). Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. *Cell Mol. Neurobiol*. 36, 301–312. doi: 10.1007/s10571-016-0366-z

Akers, J. C., Gonda, D., Kim, R., Carter, B. S., and Chen, C. C. (2013). Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J. Neurooncol*. 113, 1–11. doi: 10.1007/s11060-013-1084-8

Alberti, K. G., Eckel, R. H., Grundy, S. M., Zimmet, P. Z., Cleeman, J. I., Donato, K. A., et al. (2009). Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 120, 1640–1645. doi: 10.1161/CIRCULATIONAHA.109.192644

Altei, W. F., Pachane, B. C., Dos Santos, P. K., Ribeiro, L. N. M., Sung, B. H., Weaver, A. M., et al. (2020). Inhibition of αvβ3 integrin impairs adhesion and uptake of tumor-derived small extracellular vesicles. *Cell Commun Signal*. 18:158. doi: 10.1186/s12964-020-00630-w

Aydin, S., Kuloglu, T., Kalayci, M., Yilmaz, M., Cakmak, T., Albayrak, S., et al. (2014). A comprehensive immunohistochemical examination of the distribution of the fat-burning protein irisin in biological tissues. *Peptides* 61, 130–136. doi: 10.1016/j.peptides.2014.09.014

Barone, R., Macaluso, F., Sangiorgi, C., Campanella, C., Marino Gammazza, A., Moresi, V., et al. (2016). Skeletal muscle Heat shock protein 60 increases after endurance training and induces peroxisome proliferator-activated receptor gamma coactivator 1 α1 expression. *Sci Rep*. 6:19781. doi: 10.1038/srep19781

Bennett, M. R., Sinha, S., and Owens, G. K. (2016). Vascular smooth muscle cells in atherosclerosis. *Circ Res*. 118, 692–702. doi: 10.1161/CIRCRESAHA.115.306361

Bergsmedh, A., Szeles, A., Henriksson, M., Bratt, A., Folkman, M. J., Spetz, A. L., et al. (2001). Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl. Acad. Sci. U.S.A*. 98, 6407–6411. doi: 10.1073/pnas.101129998

Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hasslen, S. R., et al. (1995). alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 80, 413–422. doi: 10.1016/0092-8674(95)90491-3

Bi, J., Zhang, J., Ren, Y., Du, Z., Li, T., Wang, T., et al. (2020). Irisin reverses intestinal epithelial barrier dysfunction during intestinal injury via binding to the integrin αVβ5 receptor. *J. Cell. Mol. Med*. 24, 996–1009. doi: 10.1111/jcmm.14811

Boström, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., et al. (2012). A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* 481, 463–468. doi: 10.1038/nature10777

Castoldi, G., Di Gioia, C. R., Bombardi, C., Catalucci, D., Corradi, B., Gualazzi, M. G., et al. (2012). MiR-133a regulates collagen 1A1: potential role of miR-133a in myocardial fibrosis in angiotensin II-dependent hypertension. *J. Cell Physiol*. 227, 850–856. doi: 10.1002/jcp.22939

Chaturvedi, P., Kalani, A., Medina, I., Familtseva, A., and Tyagi, S. C. (2015). Cardiosome mediated regulation of MMP9 in diabetic heart: role of mir29b and mir455 in exercise. *J. Cell. Mol. Med*. 19, 2153–2161. doi: 10.1111/jcmm.12589

Chen, G., Xu, C., Gillette, T. G., Huang, T., Huang, P., Li, Q., et al. (2020). Cardiomyocyte-derived small extracellular vesicles can signal eNOS activation in cardiac microvascular endothelial cells to protect

against ischemia/reperfusion injury. *Theranostics* 10, 11754–11774. doi: 10.7150/thno.43163

Chistiakov, D. A., Orekhov, A. N., and Bobryshev, Y. V. (2016). Cardiac extracellular vesicles in normal and infarcted heart. *Int. J. Mol. Sci*. 17:63. doi: 10.3390/ijms17010063

Choi, J. S., Yoon, H. I., Lee, K. S., Choi, Y. C., Yang, S. H., Kim, I. S., et al. (2016). Exosomes from differentiating human skeletal muscle cells trigger myogenesis of stem cells and provide biochemical cues for skeletal muscle regeneration. *J. Control Release* 222, 107–115. doi: 10.1016/j.jconrel.2015.12.018

Cocucci, E., Racchetti, G., and Meldolesi, J. (2009). Shedding microvesicles: artefacts no more. *Trends Cell Biol*. 19, 43–51. doi: 10.1016/j.tcb.2008.11.003

Colaianni, G., Cuscito, C., Mongelli, T., Oranger, A., Mori, G., Brunetti, G., et al. (2014). Irisin enhances osteoblast differentiation *in vitro*. *Int. J. Endocrinol*. 2014:902186. doi: 10.1155/2014/902186

Colliva, A., Braga, L., Giacca, M., and Zacchigna, S. (2020). Endothelial cell-cardiomyocyte crosstalk in heart development and disease. *J. Physiol*. 598, 2923–2939. doi: 10.1113/JP276758

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., et al. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J. Cell Sci*. 126, 5553–5565. doi: 10.1242/jcs.128868

Corti, S., Locatelli, F., Papadimitriou, D., Donadoni, C., Del Bo, R., Fortunato, F., et al. (2005). Multipotentiality, homing properties, and pyramidal neurogenesis of CNS-derived LeX(ssea-1)+/CXCR4+ stem cells. *FASEB J*. 19, 1860–1862. doi: 10.1096/fj.05-4170fje

De Angel, R. E., Conti, C. J., Wheatley, K. E., Brenner, A. J., Otto, G., Degraffenried, L. A., et al. (2013). The enhancing effects of obesity on mammary tumor growth and Akt/mTOR pathway activation persist after weight loss and are reversed by RAD001. *Mol. Carcinog*. 52, 446–458. doi: 10.1002/mc.21878

de la Cuesta, F., Passalacqua, I., Rodor, J., Bhushan, R., Denby, L., and Baker, A. H. (2019). Extracellular vesicle cross-talk between pulmonary artery smooth muscle cells and endothelium during excessive TGF-β signalling: implications for PAH vascular remodelling. *Cell Commun. Signal*. 17:143. doi: 10.1186/s12964-019-0449-9

Deng, L., Blanco, F. J., Stevens, H., Lu, R., Caudrillier, A., McBride, M., et al. (2015). MicroRNA-143 activation regulates smooth muscle and endothelial cell crosstalk in pulmonary arterial hypertension. *Circ. Res*. 117, 870–883. doi: 10.1161/CIRCRESAHA.115.306806

Deshmukh, A. S., Murgia, M., Nagaraj, N., Treebak, J. T., Cox, J., and Mann, M. (2015). Deep proteomics of mouse skeletal muscle enables quantitation of protein isoforms, metabolic pathways, and transcription factors. *Mol. Cell Proteomics* 14, 841–853. doi: 10.1074/mcp.M114.044222

D'Souza, R. F., Woodhead, J. S. T., Zeng, N., Blenkiron, C., Merry, T. L., Cameron-Smith, D., et al. (2018). Circulatory exosomal miRNA following intense exercise is unrelated to muscle and plasma miRNA abundances. *Am. J. Physiol. Endocrinol. Metab*. 315, E723–E733. doi: 10.1152/ajpendo.00138.2018

Edwards, H. V., Cameron, R. T., and Baillie, G. S. (2011). The emerging role of HSP20 as a multifunctional protective agent. *Cell Signal*. 23, 1447–1454. doi: 10.1016/j.cellsig.2011.05.009

Esposito, K., Chiodini, P., Colao, A., Lenzi, A., and Giugliano, D. (2012). Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. *Diabetes Care* 35, 2402–2411. doi: 10.2337/dc12-0336

Estell, E. G., Le, P. T., Vegting, Y., Kim, H., Wrann, C., Bouxsein, M. L., et al. (2020). Irisin directly stimulates osteoclastogenesis and bone resorption *in vitro* and *in vivo*. *Elife* 9:e58172. doi: 10.7554/eLife.58172

Febbraio, M. A., and Pedersen, B. K. (2005). Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? *Exerc. Sport Sci. Rev*. 33, 114–119. doi: 10.1097/00003677-200507000-00003

Fedele, C., Singh, A., Zerlanko, B. J., Iozzo, R. V., and Languino, L. R. (2015). The αvβ6 integrin is transferred intercellularly via exosomes. *J. Biol. Chem*. 290, 4545–4551. doi: 10.1074/jbc.C114.617662

Festa, A., D'Agostino, R., Howard, G., Mykkänen, L., Tracy, R. P., and Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation* 102, 42–47. doi: 10.1161/01.CIR.102.1.42

Forterre, A., Jalabert, A., Berger, E., Baudet, M., Chikh, K., Errazuriz, E., et al. (2014b). Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? *PLoS ONE* 9:e84153. doi: 10.1371/journal.pone.0084153

Forterre, A., Jalabert, A., Chikh, K., Pesenti, S., Euthine, V., Granjon, A., et al. (2014a). Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell Cycle* 13, 78–89. doi: 10.4161/cc.26808

Frenette, P. S., Mayadas, T. N., Rayburn, H., Hynes, R. O., and Wagner, D. D. (1996). Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. *Cell* 84, 563–574. doi: 10.1016/S0092-8674(00)81032-6

Frühbeis, C., Fröhlich, D., Kuo, W. P., Amphornrat, J., Thilemann, S., Saab, A. S., et al. (2013). Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. *PLoS Biol*. 11:e1001604. doi: 10.1371/journal.pbio.1001604

Frühbeis, C., Helmig, S., Tug, S., Simon, P., and Krämer-Albers, E. M. (2015). Physical exercise induces rapid release of small extracellular vesicles into the circulation. *J. Extracell. Vesicles* 4:28239. doi: 10.3402/jev.v4.28239

Fulzele, S., Mendhe, B., Khayrullin, A., Johnson, M., Kaiser, H., Liu, Y., et al. (2019). Muscle-derived miR-34a increases with age in circulating extracellular vesicles and induces senescence of bone marrow stem cells. *Aging* 11, 1791–1803. doi: 10.18632/aging.101874

Gaggini, M., Cabiati, M., Del Turco, S., Navarra, T., De Simone, P., Filipponi, F., et al. (2017). Increased FNDC5/Irisin expression in human hepatocellular carcinoma. *Peptides* 88, 62–66. doi: 10.1016/j.peptides.2016.12.014

Gannon, N. P., Vaughan, R. A., Garcia-Smith, R., Bisoffi, M., and Trujillo, K. A. (2015). Effects of the exercise-inducible myokine irisin on malignant and non-malignant breast epithelial cell behavior *in vitro*. *Int. J. Cancer*. 136, E197–E202. doi: 10.1002/ijc.29142

Garcia, N. A., Moncayo-Arlandi, J., Sepulveda, P., and Diez-Juan, A. (2016). Cardiomyocyte exosomes regulate glycolytic flux in endothelium by direct transfer of GLUT transporters and glycolytic enzymes. *Cardiovasc. Res*. 109, 397–408. doi: 10.1093/cvr/cvv260

Garcia, N. A., Ontoria-Oviedo, I., González-King, H., Diez-Juan, A., and Sepúlveda, P. (2015). Glucose starvation in cardiomyocytes enhances exosome secretion and promotes angiogenesis in endothelial cells. *PLoS ONE* 10:e0138849. doi: 10.1371/journal.pone.0138849

Garzón-Muvdi, T., and Quiñones-Hinojosa, A. (2009). Neural stem cell niches and homing: recruitment and integration into functional tissues. *ILAR J*. 51, 3–23. doi: 10.1093/ilar.51.1.3

Golbidi, S., Mesdaghinia, A., and Laher, I. (2012). Exercise in the metabolic syndrome. *Oxid. Med. Cell Longev*. 2012:349710. doi: 10.1155/2012/349710

Gupta, S., and Knowlton, A. A. (2007). HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *Am. J. Physiol. Heart Circ. Physiol*. 292, H3052–H3056. doi: 10.1152/ajpheart.01355.2006

Gustat, J., Srinivasan, S. R., Elkasabany, A., and Berenson, G. S. (2002). Relation of self-rated measures of physical activity to multiple risk factors of insulin resistance syndrome in young adults: the Bogalusa Heart Study. *J. Clin. Epidemiol*. 55, 997–1006. doi: 10.1016/S0895-4356(02)00427-4

Haeger, A., Alexander, S., Vullings, M., Kaiser, F. M. P., Veelken, C., Flucke, U., et al. (2020). Collective cancer invasion forms an integrin-dependent radioresistant niche. *J. Exp. Med*. 217:e20181184. doi: 10.1084/jem.20181184

Hagiwara, K., Ochiya, T., and Kosaka, N. (2014). A paradigm shift for extracellular vesicles as small RNA carriers: from cellular waste elimination to therapeutic applications. *Drug Deliv. Transl. Res*. 4, 31–37. doi: 10.1007/s13346-013-0180-9

Harvey, A. E., Lashinger, L. M., and Hursting, S. D. (2011). The growing challenge of obesity and cancer: an inflammatory issue. *Ann. N. Y. Acad. Sci*. 1229, 45–52. doi: 10.1111/j.1749-6632.2011.06096.x

Henne, W. M., Stenmark, H., and Emr, S. D. (2013). Molecular mechanisms of the membrane sculpting ESCRT pathway. *Cold Spring Harb. Perspect. Biol*. 5:a016766. doi: 10.1101/cshperspect.a016766

Heo, J., Yang, H. C., Rhee, W. J., and Kang, H. (2020). Vascular smooth muscle cell-derived exosomal microRNAs regulate endothelial cell migration under PDGF stimulation. *Cells* 9:639. doi: 10.3390/cells9030639

Hirata, Y., Furuhashi, K., Ishii, H., Li, H. W., Pinho, S., Ding, L., et al. (2018). CD150(high) bone marrow tregs maintain hematopoietic stem cell quiescence and immune privilege via adenosine. *Cell Stem Cell* 22, 445–453 e5. doi: 10.1016/j.stem.2018.01.017

Hoshino, A., Costa-Silva, B., Shen, T. L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., et al. (2015). Tumour exosome integrins determine organotropic metastasis. *Nature* 527, 329–335. doi: 10.1038/nature15756

Hu, M., Guo, G., Huang, Q., Cheng, C., Xu, R., Li, A., et al. (2018). The harsh microenvironment in infarcted heart accelerates transplanted bone marrow mesenchymal stem cells injury: the role of injured cardiomyocytes-derived exosomes. *Cell Death Dis*. 9:357. doi: 10.1038/s41419-018-0392-5

Hugel, B., Martínez, M. C., Kunzelmann, C., and Freyssinet, J. M. (2005). Membrane microparticles: two sides of the coin. *Physiology* 20, 22–27. doi: 10.1152/physiol.00029.2004

Huttenlocher, A., and Horwitz, A. R. (2011). Integrins in cell migration. *Cold Spring Harb. Perspect. Biol*. 3:a005074. doi: 10.1101/cshperspect.a005074

Imitola, J., Raddassi, K., Park, K. I., Mueller, F. J., Nieto, M., Teng, Y. D., et al. (2004). Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc. Natl. Acad. Sci. U.S.A*. 101, 18117–18122. doi: 10.1073/pnas.0408258102

Isles, H. M., Herman, K. D., Robertson, A. L., Loynes, C. A., Prince, L. R., Elks, P. M., et al. (2019). The CXCL12/CXCR4 signaling axis retains neutrophils at inflammatory sites in zebrafish. *Front. Immunol*. 10:1784. doi: 10.3389/fimmu.2019.01784

Jalabert, A., Vial, G., Guay, C., Wiklander, O. P., Nordin, J. Z., Aswad, H., et al. (2016). Exosome-like vesicles released from lipid-induced insulin-resistant muscles modulate gene expression and proliferation of beta recipient cells in mice. *Diabetologia* 59, 1049–1058. doi: 10.1007/s00125-016-3882-y

Kalluri, R., and LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. *Science* 367:eaau6977. doi: 10.1126/science.aau6977

Kalra, H., Drummen, G. P., and Mathivanan, S. (2016). Focus on extracellular vesicles: introducing the next small big thing. *Int. J. Mol. Sci*. 17:170. doi: 10.3390/ijms17020170

Kapustin, A. N., Chatrou, M. L., Drozdov, I., Zheng, Y., Davidson, S. M., Soong, D., et al. (2015). Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. *Circ. Res*. 116, 1312–1323. doi: 10.1161/CIRCRESAHA.116.305012

Kapustin, A. N., Davies, J. D., Reynolds, J. L., McNair, R., Jones, G. T., Sidibe, A., et al. (2011). Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circ. Res*. 109, e1–e12. doi: 10.1161/CIRCRESAHA.110.238808

Kapustin, A. N., and Shanahan, C. M. (2016). Emerging roles for vascular smooth muscle cell exosomes in calcification and coagulation. *J. Physiol*. 594, 2905–2914. doi: 10.1113/JP271340

Kawai, T., and Malech, H. L. (2009). WHIM syndrome: congenital immune deficiency disease. *Curr. Opin. Hematol*. 16, 20–26. doi: 10.1097/MOH.0b013e32831ac557

Kim, H., Wrann, C. D., Jedrychowski, M., Vidoni, S., Kitase, Y., Nagano, K., et al. (2018). Irisin mediates effects on bone and fat via αV integrin receptors. *Cell* 175, 1756–1768.e17. doi: 10.1016/j.cell.2018.10.025

Kim, S., Lee, M. J., Choi, J. Y., Park, D. H., Kwak, H. B., Moon, S., et al. (2018). Roles of exosome-like vesicles released from inflammatory C2C12 myotubes: regulation of myocyte differentiation and myokine expression. *Cell Physiol. Biochem*. 48, 1829–1842. doi: 10.1159/000492505

Kong, G., Jiang, Y., Sun, X., Cao, Z., Zhang, G., Zhao, Z., et al. (2017). Irisin reverses the IL-6 induced epithelial-mesenchymal transition in osteosarcoma cell migration and invasion through the STAT3/Snail signaling pathway. *Oncol. Rep*. 38, 2647–2656. doi: 10.3892/or.2017.5973

Korta, P., Pocheć, E., and Mazur-Biały, A. (2019). Irisin as a multifunctional protein: implications for health and certain diseases. *Medicina* 55:485. doi: 10.3390/medicina55080485

Krishn, S. R., Singh, A., Bowler, N., Duffy, A. N., Friedman, A., Fedele, C., et al. (2019). Prostate cancer sheds the αvβ3 integrin *in vivo* through exosomes. *Matrix Biol*. 77, 41–57. doi: 10.1016/j.matbio.2018.08.004

Lauritzen, H. P., Brandauer, J., Schjerling, P., Koh, H. J., Treebak, J. T., Hirshman, M. F., et al. (2013). Contraction and AICAR stimulate IL-6 vesicle depletion from skeletal muscle fibers *in vivo*. *Diabetes* 62, 3081–3092. doi: 10.2337/db12-1261

Leal, L. G., Lopes, M. A., and Batista, M. L. (2018). Physical exercise-induced myokines and muscle-adipose tissue crosstalk: a review of current knowledge and the implications for health and metabolic diseases. *Front. Physiol*. 9:1307. doi: 10.3389/fphys.2018.01307

Lee, Y., El Andaloussi, S., and Wood, M. J. (2012). Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum. Mol. Genet*. 21, R125–R134. doi: 10.1093/hmg/dds317

Li, C., Zheng, X., Li, W., Bai, F., Lyu, J., and Meng, Q. H. (2018). Serum miR-486-5p as a diagnostic marker in cervical cancer: with investigation of potential mechanisms. *BMC Cancer* 18:61. doi: 10.1186/s12885-017-3753-z

Liesveld, J. L., Sharma, N., and Aljitawi, O. S. (2020). Stem cell homing: from physiology to therapeutics. *Stem Cells* 38, 1241–1253. doi: 10.1002/stem.3242

Lucas, D. (2019). Leukocyte trafficking and regulation of murine hematopoietic stem cells and their niches. *Front. Immunol*. 10:387. doi: 10.3389/fimmu.2019.00387

Madison, R. D., McGee, C., Rawson, R., and Robinson, G. A. (2014). Extracellular vesicles from a muscle cell line (C2C12) enhance cell survival and neurite outgrowth of a motor neuron cell line (NSC-34). *J. Extracell. Vesicles* 3:22865. doi: 10.3402/jev.v3.22865

Madison, R. D., and Robinson, G. A. (2019). Muscle-derived extracellular vesicles influence motor neuron regeneration accuracy. *Neuroscience* 419, 46–59. doi: 10.1016/j.neuroscience.2019.08.028

Mazur-Bialy, A. I., Bilski, J., Pochec, E., and Brzozowski, T. (2017a). New insight into the direct anti-inflammatory activity of a myokine irisin against proinflammatory activation of adipocytes. Implication for exercise in obesity. *J. Physiol. Pharmacol*. 68, 243–251. Available online at: http://www.jpp.krakow.pl/journal/archive/04_17/pdf/243_04_17

Mazur-Bialy, A. I., Pocheć, E., and Zarawski, M. (2017b). Anti-inflammatory properties of irisin, mediator of physical activity, are connected with TLR4/MyD88 signaling pathway activation. *Int. J. Mol. Sci*. 18:701. doi: 10.3390/ijms18040701

McCabe, N. P., De, S., Vasanji, A., Brainard, J., and Byzova, T. V. (2007). Prostate cancer specific integrin alphavbeta3 modulates bone metastatic growth and tissue remodeling. *Oncogene* 26, 6238–6243. doi: 10.1038/sj.onc.1210429

McCullough, J., Fisher, R. D., Whitby, F. G., Sundquist, W. I., and Hill, C. P. (2008). ALIX-CHMP4 interactions in the human ESCRT pathway. *Proc. Natl. Acad. Sci. U.S.A*. 105, 7687–7691. doi: 10.1073/pnas.0801567105

Mika, A., Macaluso, F., Barone, R., Di Felice, V., and Sledzinski, T. (2019). Effect of exercise on fatty acid metabolism and adipokine secretion in adipose tissue. *Front. Physiol*. 10:26. doi: 10.3389/fphys.2019.00026

Moore, K. A., and Lemischka, I. R. (2006). Stem cells and their niches. *Science* 311, 1880–1885. doi: 10.1126/science.1110542

Morelli, M. B., Shu, J., Sardu, C., Matarese, A., and Santulli, G. (2019). Cardiosomal microRNAs are essential in post-infarction myofibroblast phenoconversion. *Int. J. Mol. Sci*. 21:201. doi: 10.3390/ijms21010201

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., et al. (2009). ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr. Biol*. 19, 1875–1885. doi: 10.1016/j.cub.2009.09.059

Myint, P. K., Park, E. J., Gaowa, A., Kawamoto, E., and Shimaoka, M. (2020). Targeted remodeling of breast cancer and immune cell homing niches by exosomal integrins. *Diagn Pathol*. 15:38. doi: 10.1186/s13000-020-00959-3

Neven, K. Y., Nawrot, T. S., and Bollati, V. (2017). Extracellular vesicles: how the external and internal environment can shape cell-to-cell communication. *Curr. Environ. Health Rep*. 4, 30–37. doi: 10.1007/s40572-017-0130-7

New, S. E., Goettsch, C., Aikawa, M., Marchini, J. F., Shibasaki, M., Yabusaki, K., et al. (2013). Macrophage-derived matrix vesicles: an alternative novel mechanism for microcalcification in atherosclerotic plaques. *Circ. Res*. 113, 72–77. doi: 10.1161/CIRCRESAHA.113.301036

Nie, Y., Sato, Y., Garner, R. T., Kargl, C., Wang, C., Kuang, S., et al. (2019). Skeletal muscle-derived exosomes regulate endothelial cell functions via reactive oxygen species-activated nuclear factor-κB signalling. *Exp. Physiol*. 104, 1262–1273. doi: 10.1113/EP087396

Nielsen, A. R., Mounier, R., Plomgaard, P., Mortensen, O. H., Penkowa, M., Speerschneider, T., et al. (2007). Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. *J. Physiol*. 584, 305–312. doi: 10.1113/jphysiol.2007.139618

Oguri, Y., Shinoda, K., Kim, H., Alba, D. L., Bolus, W. R., Wang, Q., et al. (2020). CD81 controls beige fat progenitor cell growth and energy balance via FAK signaling. *Cell* 182, 563–577.e20. doi: 10.1016/j.cell.2020.06.021

Okada, T., Ngo, V. N., Ekland, E. H., Förster, R., Lipp, M., Littman, D. R., et al. (2002). Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J. Exp. Med*. 196, 65–75. doi: 10.1084/jem.20020201

Olsen, R. H., Krogh-Madsen, R., Thomsen, C., Booth, F. W., and Pedersen, B. K. (2008). Metabolic responses to reduced daily steps in healthy nonexercising men. *JAMA* 299, 1261–1263. doi: 10.1001/jama.299.11.1259

Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., et al. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat. Cell Biol.* 12, 19–30. doi: 10.1038/ncb2000

Pabst, O., Förster, R., Lipp, M., Engel, H., and Arnold, H. H. (2000). NKX2.3 is required for MAdCAM-1 expression and homing of lymphocytes in spleen and mucosa-associated lymphoid tissue. *EMBO J.* 19, 2015–2023. doi: 10.1093/emboj/19.9.2015

Panati, K., Narala, V. R., Narasimha, V. R., Derangula, M., Arva Tatireddigari, V. R. R., and Yeguvapalli, S. (2018). Expression, purification and biological characterisation of recombinant human irisin (12.5 kDa). *J. Genet. Eng. Biotechnol.* 16, 459–466. doi: 10.1016/j.jgeb.2018.06.007

Park, E. J., Appiah, M. G., Myint, P. K., Gaowa, A., Kawamoto, E., and Shimaoka, M. (2019a). Exosomes in sepsis and inflammatory tissue injury. *Curr. Pharm. Des.* 25, 4486–4495. doi: 10.2174/1381612825666191116125525

Park, E. J., Myint, P. K., Ito, A., Appiah, M. G., Darkwah, S., Kawamoto, E., et al. (2020). Integrin-ligand interactions in inflammation, cancer, and metabolic disease: insights into the multifaceted roles of an emerging ligand irisin. *Front. Cell Dev. Biol.* 8:588066. doi: 10.3389/fcell.2020.588066

Park, E. J., Prajuabjinda, O., Soe, Z. Y., Darkwah, S., Appiah, M. G., Kawamoto, E., et al. (2019b). Exosomal regulation of lymphocyte homing to the gut. *Blood Adv.* 3, 1–11. doi: 10.1182/bloodadvances.2018024877

Pollak, M. (2008). Insulin and insulin-like growth factor signalling in neoplasia. *Nat. Rev. Cancer* 8, 915–928. doi: 10.1038/nrc2536

Pols, M. S., and Klumperman, J. (2009). Trafficking and function of the tetraspanin CD63. *Exp. Cell Res.* 315, 1584–1592. doi: 10.1016/j.yexcr.2008.09.020

Prestoz, L., Relvas, J. B., Hopkins, K., Patel, S., Sowinski, P., Price, J., et al. (2001). Association between integrin-dependent migration capacity of neural stem cells *in vitro* and anatomical repair following transplantation. *Mol. Cell Neurosci.* 18, 473–484. doi: 10.1006/mcne.2001.1037

Pukajło, K., Kolackov, K., and Łaczmański, Ł., Daroszewski, J. (2015). Irisin–a new mediator of energy homeostasis. *Postepy Hig. Med. Dosw.* 69, 233–242. doi: 10.5604/17322693.1141097

Quinn, L. S., Strait-Bodey, L., Anderson, B. G., Argilés, J. M., and Havel, P. J. (2005). Interleukin-15 stimulates adiponectin secretion by 3T3-L1 adipocytes: evidence for a skeletal muscle-to-fat signaling pathway. *Cell Biol. Int.* 29, 449–457. doi: 10.1016/j.cellbi.2005.02.005

Rabiee, F., Lachinani, L., Ghaedi, S., Nasr-Esfahani, M. H., Megraw, T. L., and Ghaedi, K. (2020). New insights into the cellular activities of Fndc5/Irisin and its signaling pathways. *Cell Biosci.* 10:51. doi: 10.1186/s13578-020-00413-3

Regev-Rudzki, N., Wilson, D. W., Carvalho, T. G., Sisquella, X., Coleman, B. M., Rug, M., et al. (2013). Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell* 153, 1120–1133. doi: 10.1016/j.cell.2013.04.029

Reynolds, J. L., Joannides, A. J., Skepper, J. N., McNair, R., Schurgers, L. J., Proudfoot, D., et al. (2004). Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J. Am. Soc. Nephrol.* 15, 2857–2867. doi: 10.1097/01.ASN.0000141960.01035.28

Reynolds, J. L., Skepper, J. N., McNair, R., Kasama, T., Gupta, K., Weissberg, P. L., et al. (2005). Multifunctional roles for serum protein fetuin-a in inhibition of human vascular smooth muscle cell calcification. *J. Am. Soc. Nephrol.* 16, 2920–2930. doi: 10.1681/ASN.2004100895

Ribeiro, M. F., Zhu, H., Millard, R. W., and Fan, G. C. (2013). Exosomes function in pro- and anti-angiogenesis. *Curr. Angiogenes* 2, 54–59. doi: 10.2174/22115528113020020001

Rodríguez, A., Becerril, S., Ezquerro, S., Méndez-Giménez, L., and Frühbeck, G. (2017). Crosstalk between adipokines and myokines in fat browning. *Acta Physiol.* 219, 362–381. doi: 10.1111/apha.12686

Rome, S., Forterre, A., Mizgier, M. L., and Bouzakri, K. (2019). Skeletal muscle-released extracellular vesicles: state of the art. *Front. Physiol.* 10:929. doi: 10.3389/fphys.2019.00929

Sahin, A. O., and Buitenhuis, M. (2012). Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. *Cell Adh. Migr.* 6, 39–48. doi: 10.4161/cam.18975

Schippers, A., Kochut, A., Pabst, O., Frischmann, U., Clahsen, T., Tenbrock, K., et al. (2012). β7 integrin controls immunogenic and tolerogenic mucosal B cell responses. *Clin. Immunol.* 144, 87–97. doi: 10.1016/j.clim.2012.05.008

Schön, M. P., Arya, A., Murphy, E. A., Adams, C. M., Strauch, U. G., Agace, W. W., et al. (1999). Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J. Immunol.* 162, 6641–6649.

Schöneberg, J., Lee, I. H., Iwasa, J. H., and Hurley, J. H. (2017). Reverse-topology membrane scission by the ESCRT proteins. *Nat. Rev. Mol. Cell Biol.* 18, 5–17. doi: 10.1038/nrm.2016.121

Schurgers, L. J., Akbulut, A. C., Kaczor, D. M., Halder, M., Koenen, R. R., and Kramann, R. (2018). Initiation and propagation of vascular calcification is regulated by a concert of platelet- and smooth muscle cell-derived extracellular vesicles. *Front. Cardiovasc. Med.* 5:36. doi: 10.3389/fcvm.2018.00036

Schweitzer, K. M., Dräger, A. M., van der Valk, P., Thijsen, S. F., Zevenbergen, A., Theijsmeijer, A. P., et al. (1996). Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. *Am. J. Pathol.* 148, 165–175.

Seldin, M. M., and Wong, G. W. (2012). Regulation of tissue crosstalk by skeletal muscle-derived myonectin and other myokines. *Adipocyte* 1, 200–202. doi: 10.4161/adip.20877

Shao, L., Li, H., Chen, J., Song, H., Zhang, Y., Wu, F., et al. (2017). Irisin suppresses the migration, proliferation, and invasion of lung cancer cells via inhibition of epithelial-to-mesenchymal transition. *Biochem. Biophys. Res. Commun.* 485, 598–605. doi: 10.1016/j.bbrc.2016.12.084

Singh, A., Fedele, C., Lu, H., Nevalainen, M. T., Keen, J. H., and Languino, L. R. (2016). Exosome-mediated transfer of αvβ3 integrin from tumorigenic to nontumorigenic cells promotes a migratory phenotype. *Mol. Cancer Res.* 14, 1136–1146. doi: 10.1158/1541-7786.MCR-16-0058

Stahl, P. D., and Raposo, G. (2018). Exosomes and extracellular vesicles: the path forward. *Essays Biochem.* 62, 119–124. doi: 10.1042/EBC20170088

Streeter, P. R., Berg, E. L., Rouse, B. T., Bargatze, R. F., and Butcher, E. C. (1988). A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* 331, 41–46. doi: 10.1038/331041a0

Stuffers, S., Sem Wegner, C., Stenmark, H., and Brech, A. (2009). Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic* 10, 925–937. doi: 10.1111/j.1600-0854.2009.00920.x

Sung, B. H., Ketova, T., Hoshino, D., Zijlstra, A., and Weaver, A. M. (2015). Directional cell movement through tissues is controlled by exosome secretion. *Nat. Commun.* 6:7164. doi: 10.1038/ncomms8164

Takafuji, Y., Tatsumi, K., Ishida, M., Kawao, N., Okada, K., and Kaji, H. (2020a). Extracellular vesicles secreted from mouse muscle cells suppress osteoclast formation: roles of mitochondrial energy metabolism. *Bone* 134:115298. doi: 10.1016/j.bone.2020.115298

Takafuji, Y., Tatsumi, K., Kawao, N., Okada, K., Muratani, M., and Kaji, H. (2020b). MicroRNA-196a-5p in extracellular vesicles secreted from myoblasts suppresses osteoclast-like cell formation in mouse cells. *Calcif. Tissue Int.* doi: 10.1007/s00223-020-00772-6. [Epub ahead of print].

Teti, A., Migliaccio, S., and Baron, R. (2002). The role of the alphaVbeta3 integrin in the development of osteolytic bone metastases: a pharmacological target for alternative therapy? *Calcif. Tissue Int.* 71, 293–299. doi: 10.1007/s00223-001-2071-1

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 7:1535750. doi: 10.1080/20013078.2018.1535750

Tidball, J. G. (2017). Regulation of muscle growth and regeneration by the immune system. *Nat. Rev. Immunol.* 17, 165–178. doi: 10.1038/nri.2016.150

Tracy, E., Rowe, G., and LeBlanc, A. J. (2020). Cardiac tissue remodeling in healthy aging: the road to pathology. *Am. J. Physiol. Cell Physiol.* 319, C166–C182. doi: 10.1152/ajpcell.00021.2020

Tsuchiya, Y., Ando, D., Goto, K., Kiuchi, M., Yamakita, M., and Koyama, K. (2014). High-intensity exercise causes greater irisin response compared with low-intensity exercise under similar energy consumption. *Tohoku J. Exp. Med.* 233, 135–140. doi: 10.1620/tjem.233.135

Tsuchiya, Y., Ando, D., Takamatsu, K., and Goto, K. (2015). Resistance exercise induces a greater irisin response than endurance exercise. *Metabolism* 64, 1042–1050. doi: 10.1016/j.metabol.2015.05.010

Viaud, S., Théry, C., Ploix, S., Tursz, T., Lapierre, V., Lantz, O., et al. (2010). Dendritic cell-derived exosomes for cancer immunotherapy: what's next? *Cancer Res*. 70, 1281–1285. doi: 10.1158/0008-5472.CAN-09-3276

Waldenström, A., Gennebäck, N., Hellman, U., and Ronquist, G. (2012). Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. *PLoS ONE* 7:e34653. doi: 10.1371/journal.pone.0034653

Wang, S., Zeng, Y., Zhou, J. M., Nie, S. L., Peng, Q., Gong, J., et al. (2016). MicroRNA-1246 promotes growth and metastasis of colorectal cancer cells involving CCNG2 reduction. *Mol. Med. Rep*. 13, 273–280. doi: 10.3892/mmr.2015.4557

Wang, X., Huang, W., Liu, G., Cai, W., Millard, R. W., Wang, Y., et al. (2014). Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. *J. Mol. Cell Cardiol*. 74, 139–150. doi: 10.1016/j.yjmcc.2014.05.001

Wang, X., Morelli, M. B., Matarese, A., Sardu, C., and Santulli, G. (2020). Cardiomyocyte-derived exosomal microRNA-92a mediates post-ischemic myofibroblast activation both *in vitro* and *ex vivo*. *ESC Heart Fail*. 7, 284–288. doi: 10.1002/ehf2.12584

Wang, X. H., Qian, R. Z., Zhang, W., Chen, S. F., Jin, H. M., and Hu, R. M. (2009). MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. *Clin. Exp. Pharmacol. Physiol*. 36, 181–188. doi: 10.1111/j.1440-1681.2008.05057.x

Whitham, M., Parker, B. L., Friedrichsen, M., Hingst, J. R., Hjorth, M., Hughes, W. E., et al. (2018). Extracellular vesicles provide a means for tissue crosstalk during exercise. *Cell Metab*. 27, 237–251.e4. doi: 10.1016/j.cmet.2017.12.001

Wiklander, O. P. B., Brennan, M., Lötvall, J., Breakefield, X. O., and El Andaloussi, S. (2019). Advances in therapeutic applications of extracellular vesicles. *Sci. Transl. Med*. 11:eaav8521. doi: 10.1126/scitranslmed.aav8521

Willis, M. S., and Patterson, C. (2010). Hold me tight: role of the heat shock protein family of chaperones in cardiac disease. *Circulation* 122, 1740–1751. doi: 10.1161/CIRCULATIONAHA.110.942250

Wong, K. K., Engelman, J. A., and Cantley, L. C. (2010). Targeting the PI3K signaling pathway in cancer. *Curr. Opin. Genet. Dev*. 20, 87–90. doi: 10.1016/j.gde.2009.11.002

Wortzel, I., Dror, S., Kenific, C. M., and Lyden, D. (2019). Exosome-mediated metastasis: communication from a distance. *Dev. Cell* 49, 347–360. doi: 10.1016/j.devcel.2019.04.011

Xiong, X. Q., Geng, Z., Zhou, B., Zhang, F., Han, Y., Zhou, Y. B., et al. (2018). FNDC5 attenuates adipose tissue inflammation and insulin resistance via AMPK-mediated macrophage polarization in obesity. *Metabolism* 83, 31–41. doi: 10.1016/j.metabol.2018.01.013

Xu, Q., Cui, Y., Luan, J., Zhou, X., Li, H., and Han, J. (2018). Exosomes from C2C12 myoblasts enhance osteogenic differentiation of MC3T3-E1 pre-osteoblasts by delivering miR-27a-3p. *Biochem. Biophys. Res. Commun*. 498, 32–37. doi: 10.1016/j.bbrc.2018.02.144

Xu, X., Cao, L., Zhang, Y., Lian, H., Sun, Z., and Cui, Y. (2018). MicroRNA-1246 inhibits cell invasion and epithelial mesenchymal transition process by targeting CXCR4 in lung cancer cells. *Cancer Biomark*. 21, 251–260. doi: 10.3233/CBM-170317

Xuan, W., Wang, L., Xu, M., Weintraub, N. L., and Ashraf, M. (2019). miRNAs in extracellular vesicles from iPS-derived cardiac progenitor cells effectively reduce fibrosis and promote angiogenesis in infarcted heart. *Stem Cells Int*. 2019:3726392. doi: 10.1155/2019/3726392

Yamada, M., Kubo, H., Kobayashi, S., Ishizawa, K., He, M., Suzuki, T., et al. (2011). The increase in surface CXCR4 expression on lung extravascular neutrophils and its effects on neutrophils during endotoxin-induced lung injury. *Cell Mol. Immunol*. 8, 305–314. doi: 10.1038/cmi.2011.8

Yang, J., Yu, X., Xue, F., Li, Y., Liu, W., and Zhang, S. (2018). Exosomes derived from cardiomyocytes promote cardiac fibrosis via myocyte-fibroblast cross-talk. *Am. J. Transl. Res*. 10, 4350–4366.

Yu, D. W., Ge, P. P., Liu, A. L., Yu, X. Y., and Liu, T. T. (2019). HSP20-mediated cardiomyocyte exosomes improve cardiac function in mice with myocardial infarction by activating Akt signaling pathway. *Eur. Rev. Med. Pharmacol. Sci*. 23, 4873–4881. doi: 10.26355/eurrev_201906_18075

Yu, H., and Wang, Z. (2019). Cardiomyocyte-derived exosomes: biological functions and potential therapeutic implications. *Front. Physiol*. 10:1049. doi: 10.3389/fphys.2019.01049

Yuan, J., Liu, H., Gao, W., Zhang, L., Ye, Y., Yuan, L., et al. (2018). MicroRNA-378 suppresses myocardial fibrosis through a paracrine mechanism at the early stage of cardiac hypertrophy following mechanical stress. *Theranostics* 8, 2565–2582. doi: 10.7150/thno.22878

Yue, B., Yang, H., Wang, J., Ru, W., Wu, J., Huang, Y., et al. (2020). Exosome biogenesis, secretion and function of exosomal miRNAs in skeletal muscle myogenesis. *Cell Prolif*. 53:e12857. doi: 10.1111/cpr.12857

Zhang, X., Wang, X., Zhu, H., Kranias, E. G., Tang, Y., Peng, T., et al. (2012). Hsp20 functions as a novel cardiokine in promoting angiogenesis via activation of VEGFR2. *PLoS ONE* 7:e32765. doi: 10.1371/journal.pone.0032765

Zhang, Y., Li, R., Meng, Y., Li, S., Donelan, W., Zhao, Y., et al. (2014). Irisin stimulates browning of white adipocytes through mitogen-activated protein kinase p38 MAP kinase and ERK MAP kinase signaling. *Diabetes* 63, 514–525. doi: 10.2337/db13-1106

Zhang, Z. P., Zhang, X. F., Li, H., Liu, T. J., Zhao, Q. P., Huang, L. H., et al. (2018). Serum irisin associates with breast cancer to spinal metastasis. *Medicine* 97:e0524. doi: 10.1097/MD.0000000000010524

Zheng, B., Yin, W. N., Suzuki, T., Zhang, X. H., Zhang, Y., Song, L. L., et al. (2017). Exosome-mediated miR-155 transfer from smooth muscle cells to endothelial cells induces endothelial injury and promotes atherosclerosis. *Mol. Ther*. 25, 1279–1294. doi: 10.1016/j.ymthe.2017.03.031

Zou, L., Barnett, B., Safah, H., Larussa, V. F., Evdemon-Hogan, M., Mottram, P., et al. (2004). Bone marrow is a reservoir for $CD4^{+}CD25^{+}$ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res*. 64, 8451–8455. doi: 10.1158/0008-5472.CAN-04-1987

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 29 March 2021
doi: 10.3389/fcell.2021.633358

Exosomal miR-3682-3p Suppresses Angiogenesis by Targeting ANGPT1 via the RAS-MEK1/2-ERK1/2 Pathway in Hepatocellular Carcinoma

Shuang-Shuang Dong[1†], Dan-Dan Dong[2†], Zhang-Fu Yang[1], Gui-Qi Zhu[1], Dong-Mei Gao[1], Jie Chen[1], Yan Zhao[1] and Bin-Bin Liu[1]*

[1] Liver Cancer Institute, Zhongshan Hospital, Fudan University and Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Shanghai, China, [2] Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai, China

OPEN ACCESS

Edited by:
Lucas Treps,
VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Delphine Fradin,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Wen Yang,
Eastern Hepatobiliary Surgery Hospital, China

***Correspondence:**
Bin-Bin Liu
liu.binbin@zs-hospital.sh.cn

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 25 November 2020
Accepted: 04 March 2021
Published: 29 March 2021

Citation:
Dong S-S, Dong D-D, Yang Z-F, Zhu G-Q, Gao D-M, Chen J, Zhao Y and Liu B-B (2021) Exosomal miR-3682-3p Suppresses Angiogenesis by Targeting ANGPT1 via the RAS-MEK1/2-ERK1/2 Pathway in Hepatocellular Carcinoma. Front. Cell Dev. Biol. 9:633358. doi: 10.3389/fcell.2021.633358

Background: Angiogenesis is a crucial process in tumorigenesis and development. The role of exosomes derived from hepatocellular carcinoma (HCC) cells in angiogenesis has not been clearly elucidated.

Methods and Results: Exosomes were isolated from HCC cell lines (HCCLM3, MHCC97L, and PLC/RFP/5) by ultracentrifugation and identified by nano transmission electron microscopy (TEM), NanoSight analysis and western blotting, respectively. *In vitro* and *in vivo* analyses showed that exosomes isolated from highly metastatic HCC cells enhanced the migration, invasion and tube formation of human umbilical vein endothelial cells (HUVECs) compared to exosomes derived from poorly metastatic HCC cells. In addition, microarray analysis of HCC-Exos was conducted to identify potential functional molecules, and miR-3682-3p expression was found to be significantly downregulated in exosomes isolated from highly metastatic HCC cells. By *in vitro* gain-of-function experiments, we found that HCC cells secreted exosomal miR-3682-3p, which negatively regulates angiopoietin-1 (ANGPT1), and this led to inhibition of RAS-MEK1/2-ERK1/2 signaling in endothelial cells and eventually impaired angiogenesis.

Conclusion: Our study elucidates that exosomal miR-3682-3p attenuates angiogenesis by targeting ANGPT1 through RAS-MEK1/2-ERK1/2 signaling and provides novel potential targets for liver cancer therapy.

Keywords: HCC, exosomes, angiogenesis, miR-3682-3p, ANGPT1

INTRODUCTION

Liver cancer is one of the most common malignancies, ranking sixth globally in incidence and fourth in mortality (Bray et al., 2018). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for approximately 750,000 deaths worldwide each year, and more than 50% of the cases occur in China (Siegel et al., 2018). Despite progress in treatment, such

as surgical resection, radiofrequency thermal ablation, liver transplantation, and adjuvant therapy, the prognosis of HCC patients remains poor due to high recurrence and metastasis rates (Zhu et al., 2019). Therefore, identification of novel therapeutic targets and further exploration of the molecular mechanisms underlying HCC are urgently needed.

Exosomes (Exos) are lipid bilayer-enclosed vesicles with a diameter of 30–200 nm (Pegtel and Gould, 2019), and they are secreted by almost all cells. Specific proteins are highly expressed in Exos, such as Alix, CD9, CD81, and TSG101, which are usually used as markers for the identification of exosomes (Pfeffer, 2010). Increasing evidence has demonstrated that exosomes play important roles in the regulation of the tumor microenvironment via the promotion of angiogenesis, signaling pathway activation, tumorigenesis, and metastasis (Taylor and Gercel-Taylor, 2011). Exosomes contain many functional molecules, such as proteins, DNA, RNA, and lipids, and play important roles in intercellular material and information transmission.

Angiogenesis, the generation of new blood vessels, is one of the hallmarks of cancer because it plays essential roles in tumor cell growth, invasion, recurrence, and metastasis by supplying oxygen and nutrients as well as transporting carbon dioxide and metabolic wastes (Hanahan and Weinberg, 2011). It has been widely shown that active angiogenesis is responsible for the rapid growth of tumor cells and poor prognosis of cancer patients (Fang et al., 2011). Although the mechanism of tumor angiogenesis has been studied for decades, the detailed mechanism and crosstalk between effector and recipient cells have not been well elucidated. The most well-studied molecule implicated in tumor angiogenesis is vascular endothelial growth factor (VEGF) (Goel and Mercurio, 2013); however, other involved molecules still require further study. Angiopoietin-1 (ANGPT1) is a member of the human ANGPT-TIE protein family, which includes three ligands (ANGPT1, ANGPT2, and ANGPT4) and two receptors (TIE1 and TIE2). ANGPT1 is considered to be the most important mediator of the VEGF-independent proangiogenic signaling pathway. Extensive investigations into its roles in the process of tumor angiogenesis are still ongoing.

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs of 18-24 nucleotides that are implicated in the posttranscriptional regulation of gene expression (Ediriweera and Cho, 2019). They bind to complementary sites within the 3′ untranslated region (UTR) or open reading frame to target messenger RNAs (mRNAs), leading to downregulation of protein expression or degradation of the target mRNA (Iwakawa and Tomari, 2013). Emerging evidence indicates that miRNAs can be selectively packed into extracellular vesicles, particularly exosomes, secreted into the extracellular fluid, and delivered into recipient cells to affect a variety of biological processes, such as differentiation, proliferation, and apoptosis (De Bem et al., 2017). However, the relation between angiogenesis and HCC cell-derived exosomal miRNAs has not been clearly elucidated.

In our study, we found that HCC-derived exosomes promoted angiogenesis *in vitro* and *in vivo*. Furthermore, we examined the exosomal miRNA expression profiles of three liver cancer cell lines with different metastatic potential through a microarray assay and focused on the candidate miR-3682-3p, which had lower expression in highly metastatic liver cancer cells than in poorly metastatic liver cancer cells. In terms of mechanism, we found that miR-3682-3p could suppress HUVEC angiogenesis, migration, and invasion by targeting ANGPT1 through the RAS-MEK1/2-ERK1/2 pathway. Our study illuminates a new molecular mechanism of angiogenesis and offers new potential therapeutic targets in tumor angiogenesis.

MATERIALS AND METHODS

Cell Lines and Culture

Human umbilical vein endothelial cells (HUVECs) and the human liver cancer cell line PLC/RFP/5 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). HUVECs were maintained in endothelial cell medium (ScienCell, United States). The HCCLM3 and MHCC97L cell lines, which were obtained from the Liver Cancer Institute of Zhongshan Hospital (Shanghai, China), as well as the PLC/RFP/5 cell line were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, United States). All cell lines were cultured in a humidified incubator containing 5% CO_2 at 37°C.

Extraction of Exosomes From Cell Culture Medium

All human liver cancer cell lines (HCCLM3, MHCC97L, and PLC/RFP/5) were plated in 10-cm plates and cultured to 70% confluence at equal numbers. The cells were then washed with serum-free DMEM twice and refreshed with 10 mL DMEM. The conditioned medium was harvested 48 h later and filtered through 0.22-μm filters (Millipore, United States). The supernatants were subjected to centrifugation for 30 min at 2,000 × *g* and 4°C and then for 20 min at 1,000 × *g* and 4°C to remove cell debris and ultracentrifuged at 110,000 × *g* and 4°C for 70 min to collect extracellular vesicles. The pellets were washed twice with 1 × PBS, ultracentrifuged at 110,000 × *g* for 70 min in a Beckman Optima L-100XP ultracentrifuge using a SW 32Ti rotor (Beckman Coulter, Germany), dissolved in 50–100 μl 1 × PBS and stored at −80°C.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed to evaluate the morphology of isolated exosomes as previously described (Thery et al., 2006). Samples were fixed with 2% paraformaldehyde for 2 h at room temperature, loaded on film copper-mesh electron microscopy grids, and negatively stained with 2% uranyl acetate for 2 min. Images were captured with a Tecnai G2 transmission electron microscope (FEI Company, United States) operating at 120 kV.

Nanoparticle Tracking Analysis

The size and number of isolated exosomes were assessed with a NanoSight NS 300 (NanoSight, Malvern, PA, United Kingdom) using NTA 3.3 software, as described elsewhere (Hazan-Halevy et al., 2015). In brief, samples were diluted with 1× PBS and measured at a concentration between 2×10^8 and 7×10^8 particles/ml in triplicate.

Interactions Between HUVECs and Exosomes

Human umbilical vein endothelial cells were incubated in complete medium with exosomes pretreated with DiO (Beyotime, China) for 8 h at 37°C, washed twice with 1× PBS and then fixed in 4% formaldehyde for 10 min. Next, the cells were costained with DAPI (Thermo Fisher Scientific, United States) for 15 min and observed using a Zeiss SP2 confocal system (Leica, Germany).

Western Blot Analysis

Exosomes and cells were lysed in RIPA buffer containing 1% protease inhibitor PMSF, and their protein concentrations were determined by the BCA protein assay (Beyotime, China). The same amounts of total lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels at 80 V for 0.5 h followed by 120 V for 1 h and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, United States) at 300 mA for 1.5 h. The surface markers CD9 (ab92726, 1:500), CD81 (ab109201, 1:1000), Alix (ab186429, 1:1000), and TSG101 (ab125011, 1:1000) were used to identify exosomes. In addition, the following antibodies were used as the primary antibody: anti-RAS (#3339, CST, 1:1000), anti-MEK1/2 (ab178876, 1:2000), anti-p-MEK1/2 (Ser217/221) (#9154, CST, 1:1000), anti-ERK1/2 (Thr202/Tyr204) (#4370, CST, 1:2000), anti-p-ERK1/2 (#4695, CST, 1:1000), and anti-GAPDH (60004-1-Ig, Proteintech). Blots were detected by enhanced chemiluminescence after incubation with the secondary antibody.

Microarray Analysis of Exosomal miRNAs

Total RNA was extracted from HCC-derived exosomes using TRIzol reagent (Invitrogen, United States), and exosomal miRNA microarray analysis was conducted using Six Agilent Human miRNA 8*60K (Agilent Technologies, United States) at Wayen Corporation (Shanghai, China). Exosomal miRNAs were labeled and hybridized using the miRNA Complete Labeling and Hyb Kit (Agilent Technologies, United States) according to the manufacturer's instructions. The microarray slides were scanned with an Agilent Microarray Scanner (Agilent Technologies, United States), and microarray images were analyzed using Agilent Feature Extraction software v10.7.

Capillary Tube Formation Assay

For the capillary tube formation assay, 50 μl Matrigel (BD Matrigel 356234) was added to each well of 96-well plates and allowed to polymerize at 37°C for 30 min. Then, HUVECs were pretreated with 5 μg HCC-Exos for 24 h, resuspended in FBS-free medium and seeded at the bottom of each well at a density of 2×10^4 cells/well. After culturing for 6 h, the HUVECs were examined for capillary-like structure formation using an inverted phase-contrast microscope. The branch points which represent the degree of angiogenesis *in vitro*, were scanned and counted in five random microscopic fields (100×) using ImageJ software.

Cell Migration and Invasion Assays

Human umbilical vein endothelial cell migration and invasion were analyzed using transwell inserts (8-μm pore size; Corning) according to the manufacturer's protocol. In brief, HUVECs were plated in the upper chamber, which was uncoated (1×10^5 cells in 200 μl serum-free DMEM for the migration assay) or coated with 100 μl of Matrigel (BD Matrigel 356234) diluted 8× in DMEM (2×10^5 cells in 200 μl serum-free DMEM for the invasion assay), and DMEM containing 10% FBS (500 μl) was added to the lower chamber as a chemoattractant. Subsequently, the HUVECs were treated with HCC-Exos or 1× PBS. After 24 h of incubation at 37°C and 5% CO_2, the cells on the upper side of the membrane were removed with cotton swabs. Following this, the migrated or invaded cells on the underside of the membrane were fixed in 4% paraformaldehyde for 20 min at room temperature and then stained with 0.1% crystal violet for 10 min. Five random fields were imaged and counted. The experiments were performed in triplicate independently.

In vivo Matrigel Plug Assay

To examine the roles of HCC-Exos in angiogenesis *in vivo*, 12 six-week-old BALB/c nude mice were injected subcutaneously with 500 μl Matrigel-reduced growth factor (BD Matrigel, 356230) containing HCCLM3, MHCC97L or PLC/RFP/5 Exos (100 μg) or 500 μl 1× PBS as a control. The mice were sacrificed, and the Matrigel plugs were harvested on day 22 after initial injection. All animal experimental procedures were conducted under guidelines approved by the Animal Care Committee of Fudan University (Shanghai, China).

Histology

Matrigel plugs were fixed in 4% formaldehyde, embedded in paraffin, cut transversely into blocks and then sectioned at 5-μm thickness. Paraffin sections of Matrigel plugs were stained with hematoxylin and eosin (H&E). Sections were subjected to rehydration and antigen retrieval, blocked in 5% BSA and then incubated with a primary antibody against CD31 (ab182981) overnight, followed by incubation with a secondary antibody for 30 min and staining with DAB peroxidase substrate (Maxim, #DAB4033). Cells with brown granules in the cytomembrane were deemed positive staining cells.

Lentivirus and RNA Oligonucleotides

A miR-3682-3p mimic and mimic negative control were purchased from Genomeditech (Shanghai, China) and transfected into HUVECs using Lipofectamine 2000 reagent (Invitrogen, United States). In addition, to overexpress ANGPT1, lentiviral vectors encoding ANGPT1 were constructed using the GV492 vector (GeneChem Inc., Shanghai, China) based on the GenBank information for ANGPT1 (NM_001146). The empty vector (LV-vector) was used as a negative control. HUVECs were transfected with a lentivirus to establish a stable ANGPT1-overexpressing cell line using polybrene and subsequently selected with puromycin for 1 week following the instructions of the manufacturer.

miR-3682-3p Target Prediction

We used the TargetScan database[1] to predict and analyze the target genes of miRNA-3682-3p, and predicted binding sites were found to be at positions 852-859 and 2321-2328 in the 3′-UTR of the ANGPT1 mRNA transcript.

Luciferase Reporter Assay

For luciferase activity assays, HEK293T cells were seeded in a 96-well plate and cultured to 70% confluence. The cells were cotransfected with miR-3682-3p or NC duplex, pRL-CMV (expressing Renilla luciferase) and a firefly luciferase activity plasmid that contained either a mutant 3′-UTR of the target gene or the wild-type 3′-UTR using Lipofectamine 2000 reagent (Invitrogen, United States). Luciferase activity was detected 48 h after transfection using a dual-luciferase reporter assay kit (Promega, United States).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad, United States). Each experiment was performed at least in triplicate, and all data are presented as the mean ± standard error. Two groups were compared by Student's *t*-test, and multiple groups were analyzed using one-way ANOVA.$p < 0.05$ was considered significant.

RESULTS

Isolation and Identification of HCC-Derived Exosomes

Exosomes were isolated from the culture supernatants of the HCC cell lines HCCLM3, MHCC97L, and PLC/RFP/5 by the differential ultracentrifugation method, as shown in **Figure 1D**. TEM was used to observe the morphology and structure of isolated particles, which exhibited a round or cup-shaped morphology (**Figure 1A**). The characteristic exosomal markers TSG101, Alix, CD9, and CD81 were detected by western blotting (**Figure 1B**). Nanoparticle tracking analysis (NTA) was performed to analyze the size and distribution of particles obtained from HCCLM3, MHCC97L, and PLC/RFP/5 cells, and the results indicated that the mean diameter of the derived particles were 129, 154, and 139 nm, respectively (**Figure 1C**). The above results indicated that the particles we isolated were exosomes.

HCC-Derived Exosomes Were Internalized by Endothelial Cells

To explore whether HCC-secreted exosomes can interact with endothelial cells, HCC-Exos were fluorescently labeled with DiO (Beyotime, Shanghai, China) and then incubated with HUVECs. After a 6-h incubation, the HUVECs were labeled with DAPI (Thermo Fisher Scientific, United States) and captured using a confocal microscope. The images showed that DiO-labeled HCC-Exos were internalized into the cytoplasm of HUVECs, indicating that HCC-Exos could be absorbed by endothelial cells (**Figure 2A**).

HCC-Derived Exosomes Promote Endothelial Cell Tube Formation, Migration, and Invasion *in vitro*

Many studies have indicated that tumor-derived exosomes can act as cargo carriers to transport signals regulating the tumor microenvironment. To explore the biological functions of HCC-derived exosomes in tumor angiogenesis, a hallmark of invasive tumor growth and metastasis, HUVECs were incubated with different kinds of collected HCC-Exos at equal amounts. In a capillary tube formation assay, the number of nodes formed by HUVECs incubated with exosomes obtained from HCCLM3, MHCC97L, or PLC/RFP/5 cells were significantly increased compared to those of control-treated HUVECs, which were incubated with an equal volume of 1× PBS (**Figures 2B,E**). A transwell migration assay and subsequent manual counting indicated that the number of migrated HUVECs significantly increased when the cells were incubated with HCC-secreted exosomes (**Figure 2C**), as shown in **Figure 2F**. Meanwhile, the number of invaded control cells was dramatically decreased, as measured by the transwell invasion assays (**Figures 2D,G**). Hence, we concluded that exosomes purified from human liver cancer lines promote endothelial cell tube formation, migration, and invasion. In addition, we also found that this effect increases with increasing metastatic potential for HCC cells.

HCC-Derived Exosomes Promote Angiogenesis *in vivo*

To further evaluate the effects of HCC-derived exosomes on proangiogenic function *in vivo*, Matrigel containing 100 μg exosomes derived from HCCLM3, MHCC97L, or PLC/RFP5 cells or an equivalent amount of 1× PBS as a control was injected subcutaneously into BALB/c nude mice (n = 3 per group). The implanted Matrigel plugs were harvested 21 days after injection (**Figure 3A**). Formaldehyde-fixed, paraffin-embedded Matrigel plug sections were subjected to histological examination (H&E staining), and the results indicated that there were more endothelial cells and blood vessels in the HCC-Exo groups than in the control group (**Figure 3B**). Immunohistochemistry was conducted to determine the effect of HCC-Exos on angiogenesis by staining for the vascular marker CD31. The HCC-Exo groups showed significantly higher capillary density than the control group (**Figure 3C**), indicating a potential role for HCC-Exos in angiogenesis. Moreover, the exosomes derived from HCCLM3/MHCC97L cells, which are highly invasive and have a metastatic potential, had a more significant effect on angiogenesis *in vivo* than those derived from PLC/RFP/5 cells, which are poorly invasive and have a low metastatic potential, which was consistent with the results of the *in vitro* experiments. The *in vivo* Matrigel plug assays revealed that HCC-Exos promote angiogenesis and that this effect becomes more obvious as the invasiveness and metastatic potential of the source HCC cells increase.

[1] http://www.targetscan.org/vert_72/

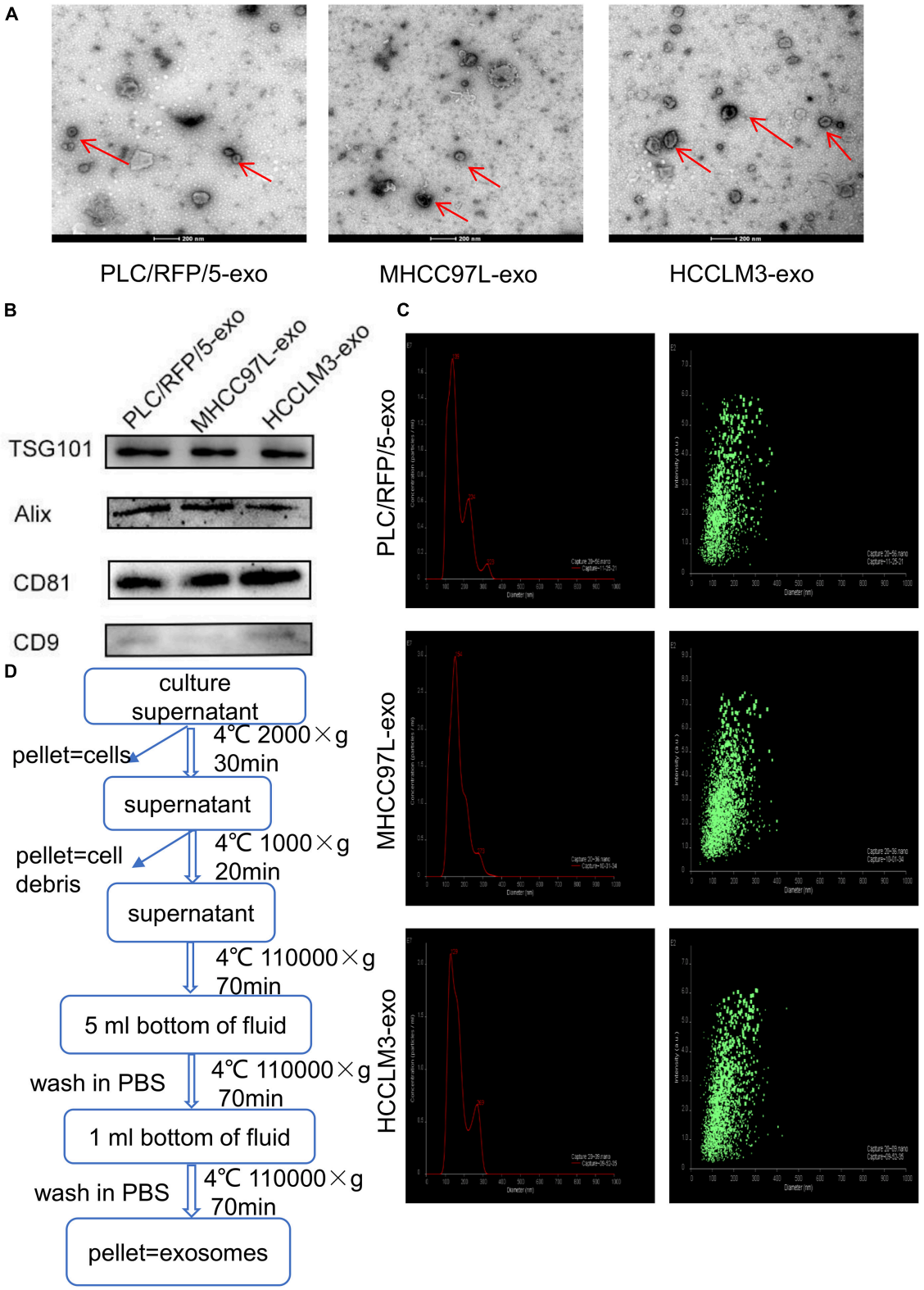


FIGURE 1 | Characterization of exosomes derived from liver cancer cells. **(A)** Transmission electron microscopy analysis of exosomes (Exos) derived from HCC cell lines (HCCLM3, MHCC97L, and PLC/RFP/5). Scale bar, 200 nm. **(B)** Immunoblotting assay of exosomal biomarkers in different liver cancer cells. **(C)** Mean diameter and size distribution of HCC-Exos was detected by Nanoparticle tracking analysis. **(D)** Strategy for exosomes purifcation from the HCC cell lines culture supernatants based on differential centrifugations.

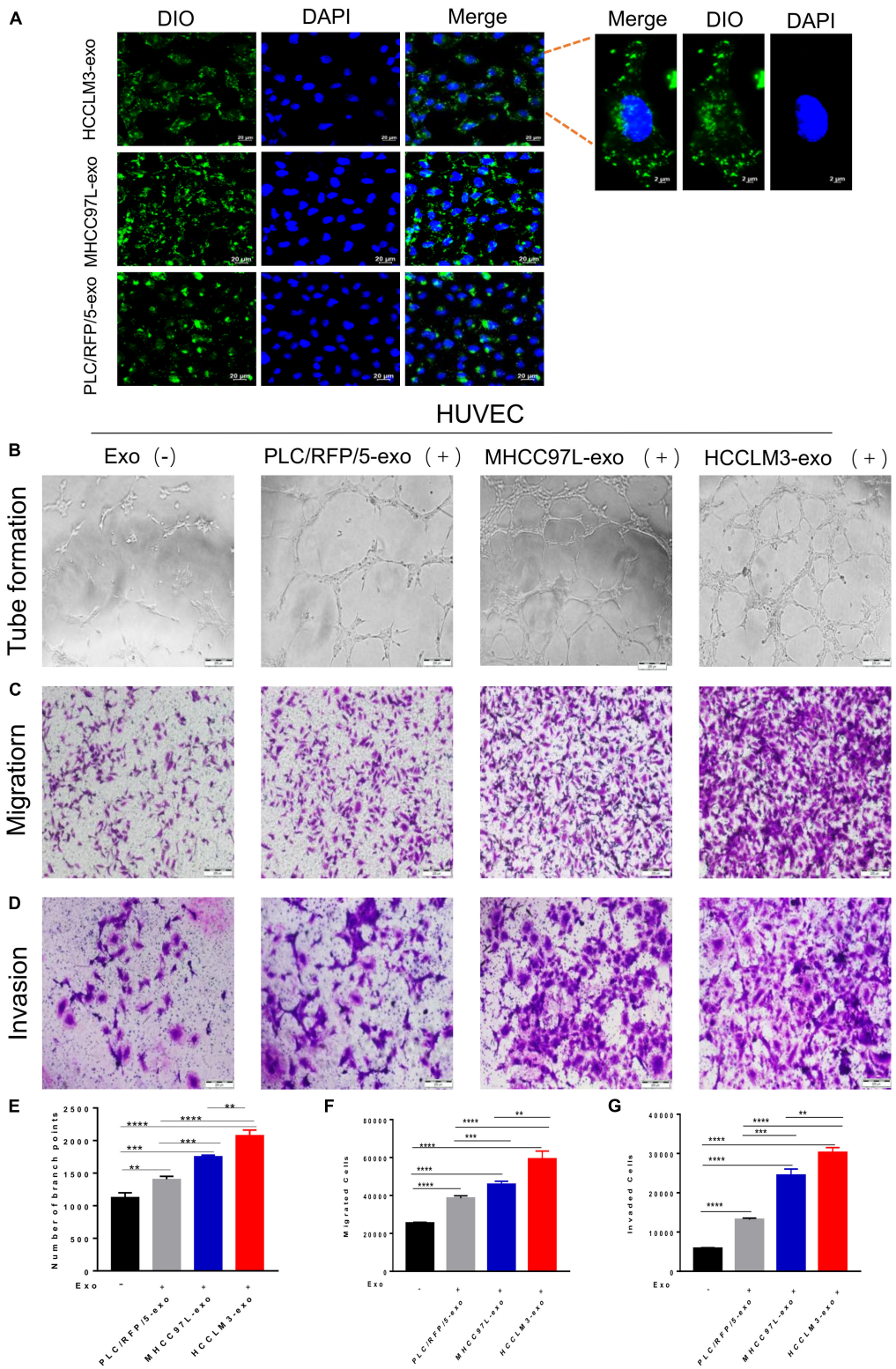


FIGURE 2 | HCC exosomes promoted HUVECs tube formation, migration, and invasion *in vitro* and this effect increases with increasing metastatic potential of HCC cells. **(A)** Confocal imaging showed the delivery of Dio-labeled exosomes (green) to DAPI-labeled HUVECs (blue) and representative images were presented. Scale bar, 20 μm. **(B–D)** Tube formation assays **(B)**, migration assays **(C)**, and invasion assays **(D)** of HUVECs treated with equal quantities of exosomes derived from different liver cancer cells or blank control. Scale bar, 200 μm. **(E–G)** Each experiment was performed three times independently and results were analyzed with ImageJ software. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

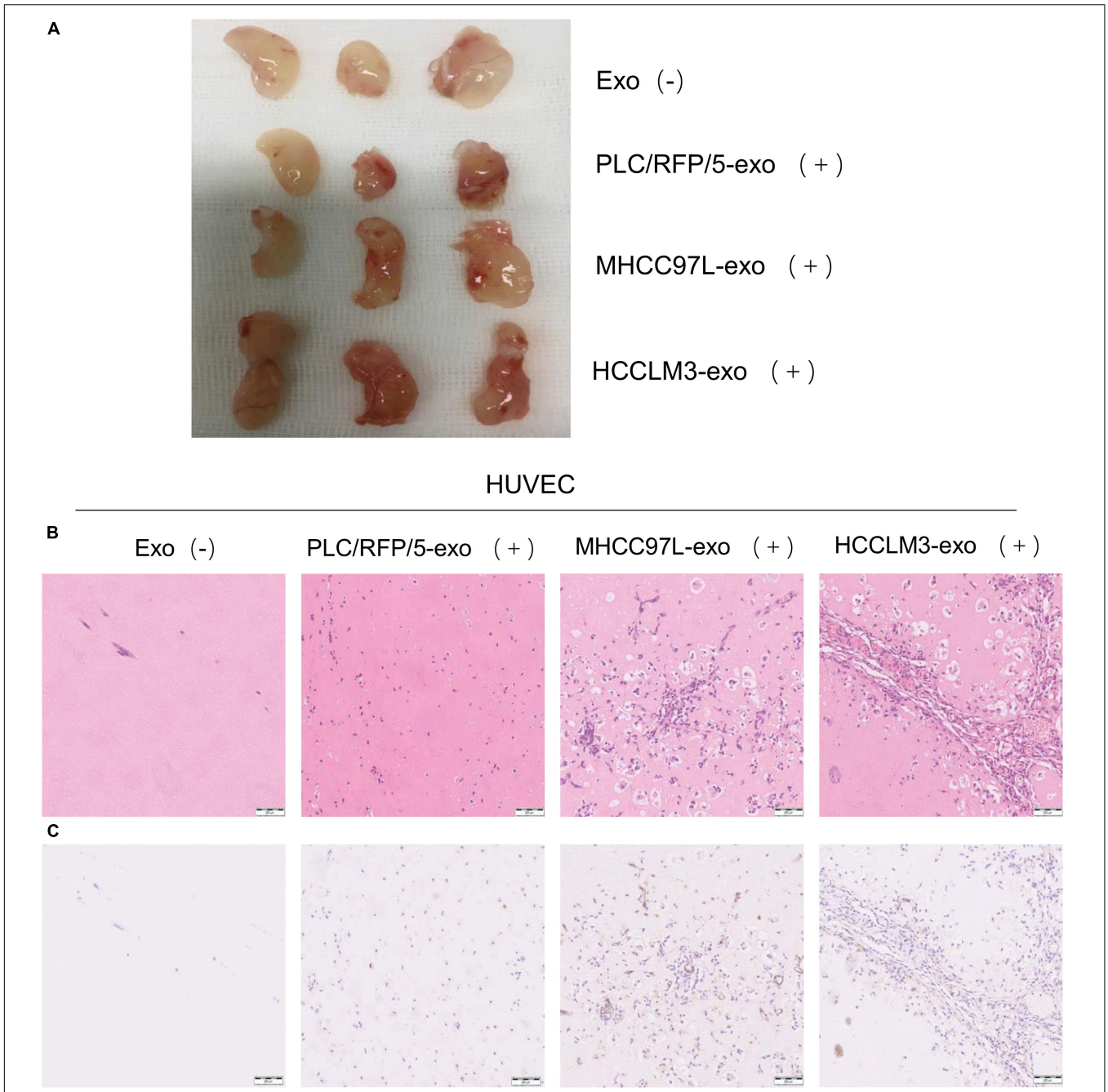


FIGURE 3 | HCC derived exosomes promote angiogenesis *in vivo*. **(A)** Matrigel plug assay performed by subcutaneous injection of Matrigel mixed with/without HCC exosomes (100 μg) in 12 BALB/c nude mice (n = 3/group). **(B)** Representative of H&E staining of matrigel plug: PBS; PLC/RFP/5 EXOs; MHCC97L EXOs; and HCCLM3 EXOs. (×100), scale bar, 200 μm. **(C)** Matrigel plug were formalin-fixed, paraffin-embedded, sliced and stained with CD31 and representative images of each group were presented.

Exosomal miR-3682-3p Attenuated Endothelial Cell Tube Formation, Migration, and Invasion

We next explored how HCC-derived exosomes promote angiogenesis *in vitro* and *in vivo*. To identify the proangiogenic components in exosomes, we performed miRNA microarray analysis of HCC-Exos because previous studies indicated that miRNAs are abundant in exosomes and have significant roles in cell–cell communication (Valadi et al., 2007). The differentially expressed miRNAs were compared and are shown in a heat map (**Figure 4**). In this study, a fold change >2 and p-value < 0.001 were applied to select proangiogenic miRNAs. MiR-3682-3p was chosen for further analysis because the expression of

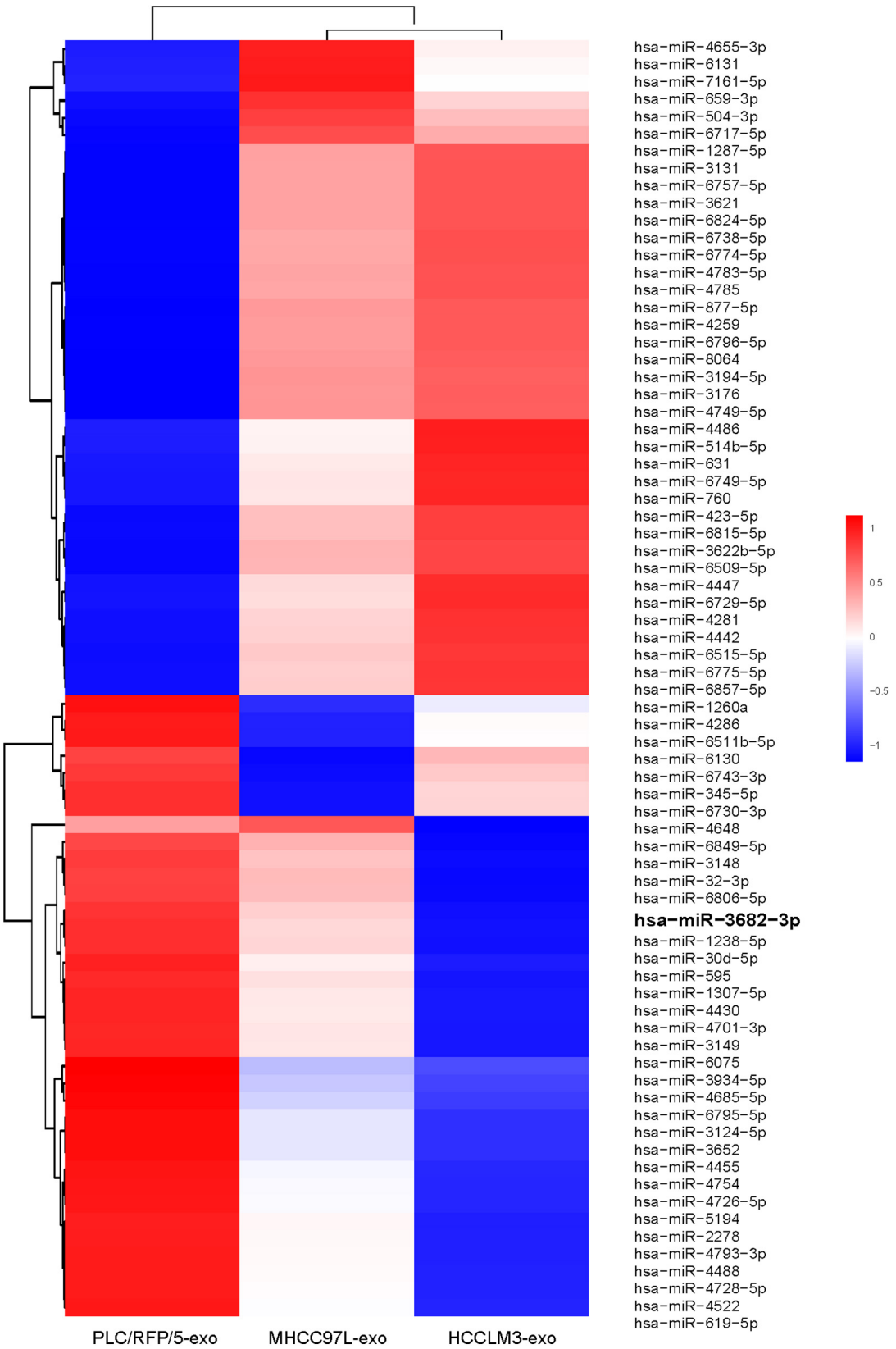


FIGURE 4 | Microarray analysis of exosomal miRNAs from different liver cancer cells were presented in a heatmap.

miR-3682-3p significantly decreased as the invasiveness and metastatic potential of HCC cell lines increased and because this miRNA has not been implicated in the suppression of angiogenesis. We verified the microarray results by qPCR (**Figure 5A**), and we also found that miR-3682-3p expression in 8 frozen HCC tissue sections was generally decreased compared with that in corresponding tumor-adjacent normal tissue samples (**Figure 5B**).

Human umbilical vein endothelial cells were infected with a miR-3682-3p mimic or negative control to explore the role of exosomal miR-3682-3p in angiogenesis. First, we found that miR-3682-3p inhibited endothelial cell tube formation compared with the negative control by performing capillary tube formation assays with HUVECs (**Figure 5C**). Second, migration assays were performed, and the results showed that fewer HUVECs migrated in the miR-3682-3p mimic group than in the negative control group (**Figure 5D**). Additionally, Transwell invasion assays confirmed that increased expression of miR-3682-3p could remarkably attenuate the invasive ability of HUVECs (**Figure 5E**). Quantification of the results of the tube formation assays, migration assays, and invasion assays are also shown in this figure. These data indicate that exosomal miR-3682-3p attenuates the tube formation, migration, and invasion of endothelial cells.

Exosomal miR-3682-3p Attenuated Endothelial Cell Tube Formation, Migration, and Invasion by Targeting ANGPT1

MicroRNAs have emerged as important regulators of diverse biological processes, but the regulatory mechanism of HCC exosomal miRNA-mediated angiogenesis remains unclear. We next explored how HCC-secreted miR-3682-3p attenuate the tube formation, migration, and invasion of endothelial cells. MicroRNAs, as a category of endogenous non-coding RNAs with a length of 22–24 nucleotides, can regulate target gene expression at the posttranscriptional level. The putative targets of miR-3682-3p were predicted using the TargetScan databases, which showed that there are two potential binding sites for miR-3682-3p in the ANGPT1 transcript (**Figure 6A**). For this reason, we wondered whether ANGPT1, which has not been reported as a target gene of miR-3682-3p, affects angiogenesis, and we performed further research to address this question. Luciferase reporter assays were conducted to verify whether the ANGPT1 gene is a direct target gene of miR-3682-3p. The 3′-UTR of the ANGPT1 transcript sequence containing the predicted miR-3682-3p binding sites was cloned into a plasmid vector, and the plasmid vector was transfected into HEK293T cells. Luciferase activity results revealed that cotransfection of miR-3682-3p significantly inhibited the activity of the firefly luciferase reporter carrying the wild-type 3′-UTR of the ANGPT1 transcript, whereas this effect was abrogated when the predicted binding sites in the 3′-UTR were mutated (**Figure 6D**). Immunofluorescence staining confirmed that ANGPT1 gene expression was markedly downregulated after HUVECs were transfected with the miR-3682-3p mimic compared with the negative control (**Figure 6B**). In addition, ANGPT1 mRNA expression in HCC patients from the TCGA database was analyzed, and we found that this expression was generally increased in tumor tissues compared with tumor-adjacent normal tissues (**Figure 6C**). To investigate the relationship of miR-3682-3p and ANGPT1 in HCC patients, total RNAs were first extracted from 16 HCC patients randomly and then the level of miR-3682-3p and ANGPT1 was analyzed by RT-PCR. There existed a significant negative correlation between miR-3682-3p and ANGPT1 in HCC tissues, which was in line previous observations ($r = -0.5378$, $p = 0.0317$; **Figure 6E**). Tube formation, migration, and invasion assays were conducted, and the results indicated that ANGPT1 enhanced the tube formation, migration, and invasion of endothelial cells *in vitro* (**Figure 6F**). Overall, these data revealed that exosomal miR-3682-3p secreted from HCC cells could inhibit tube formation, invasion and migration by targeting ANGPT1 in endothelial cells.

Exosomal miR-3682-3p Suppresses ANGPT1 Expression via RAS-MEK1/2-ERK1/2 Signaling

The RAS-MEK1/2-ERK1/2 pathway is a pivotal intracellular signaling pathway that regulates many biological functions and cellular processes, including proliferation, migration, invasion, differentiation, apoptosis, and metabolism (Xu et al., 2017). Hyperactivation of the RAS-MEK1/2-ERK1/2 signaling pathway is regarded as a hallmark driving angiogenesis and tumorigenesis in various human cancers (Peng and Gassama-Diagne, 2017). In view of this, we examined whether miR-3682-3p and ANGPT1 modulation affect three endogenous targets of RAS-MEK1/2-ERK1/2 signaling in HUVECs. We found that pathway activation was suppressed by the miR-3682-3p mimic, as demonstrated by significant reductions in the levels of the activated forms of RAS, p-MEK1/2, and p-ERK1/2, and this effect could be rescued by co-overexpression of ANGPT1. In addition, overexpression of ANGPT1 also activated the RAS-MEK1/2-ERK1/2 pathway in HUVECs. These findings imply that miR-3682-3p may suppress angiogenesis by repressing ANGPT1 gene expression via RAS-MEK1/2-ERK1/2 signaling in HUVECs (**Figures 7A,B**).

DISCUSSION

Accumulating evidence indicates that exosomes play important roles in cell–cell communication under physiological and pathophysiological conditions (Sahoo and Losordo, 2014; Sluijter et al., 2014). Multiple stromal cells, including endothelial cells, fibroblasts, and macrophages, which contribute to the generation of metastatic tumor microenvironments, can be influenced by the horizontal transfer of functional molecules, such as miRNAs and other bioactive molecules in exosomes (Skog et al., 2008; Joyce and Pollard, 2009; Lima et al., 2009). Fang et al. (2018b) demonstrated that HCC cell-derived exosomes could deliver miR-1247-3p into fibroblasts and thereby convert normal fibroblasts into cancer-associated fibroblasts to promote the lung metastasis of liver cancer. Data from Liu et al. (2019) clearly showed that exosomal miR-192-5p played a significant role in inducing macrophage polarization toward a proinflammatory

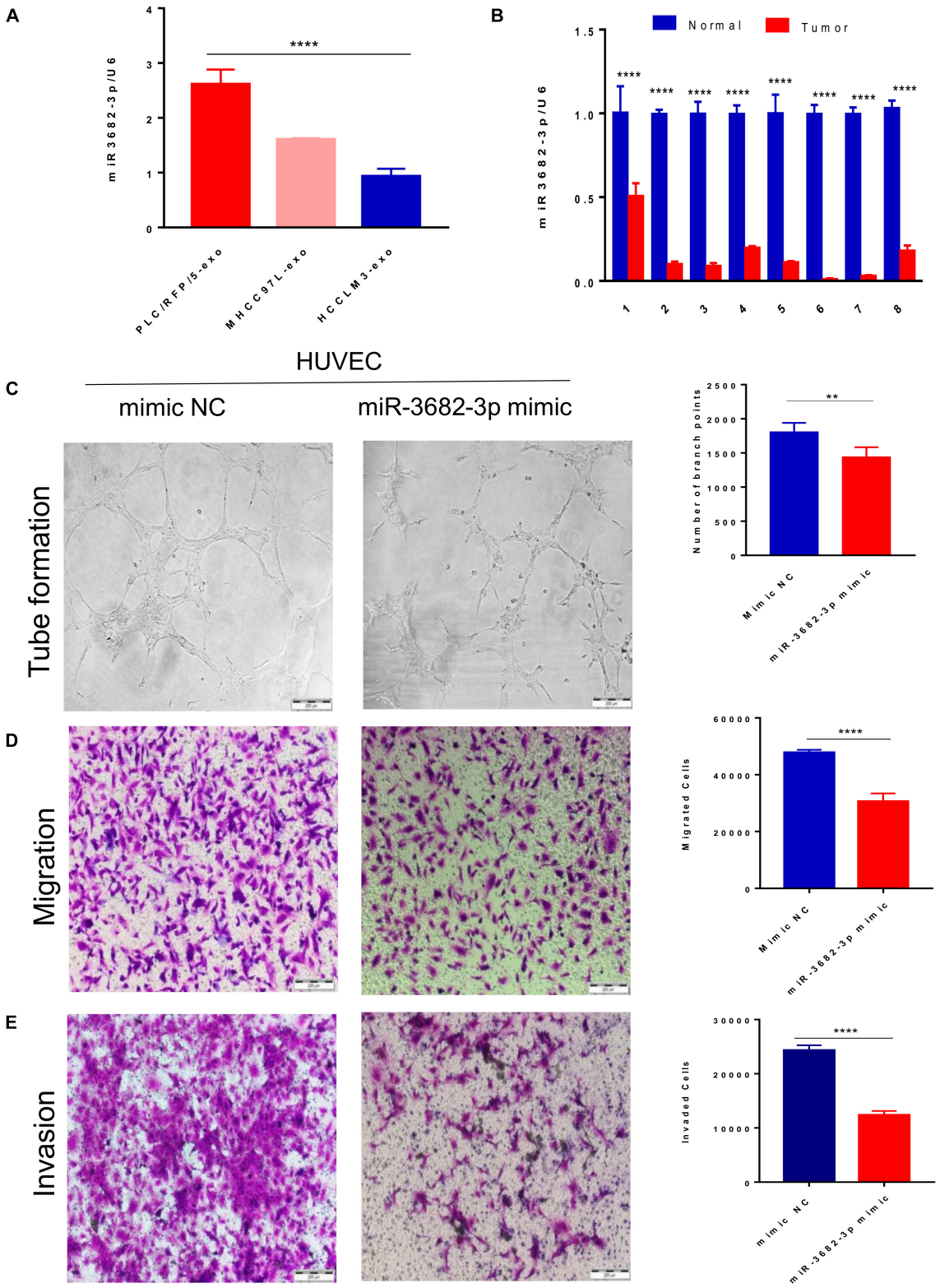


FIGURE 5 | Exosomal miR-3682-3p secreted by liver cancer cells suppressed HUVECs angiogenesis. **(A)** The microarray results were verified by qPCR. **(B)** The miR-3682-3p expressions in 8 frozen HCC tissues were generally decreased as compared with their corresponding normal tumor–adjacent tissues. **(C–E)** Tube formation assays **(C)**, migration assays **(D)**, and invasion assays **(E)** of HUVECs transfected with miR-3682-3p-mimic or negative control. Scale bar, 200 μm. (*$p < 0.05$; **i < 0.01; ***$p < 0.001$; ****$p < 0.0001$.)

A **Poorly conserved**

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 852-859 of ANGPT1 3' UTR hsa-miR-3682-3p	5' ...AAAGAAUGUAAAAUAUAUCAUCA... 3' GAUGGAGGUGGACAUAGUAGU	8mer	-0.34	98	-0.22	0	N/A
Position 2321-2328 of ANGPT1 3' UTR hsa-miR-3682-3p	5' ...AUGCAAAGUUCUUCAUAUCAUCA... 3' GAUGGAGGUGGACAUAGUAGU	8mer	-0.34	98	-0.22	0	N/A

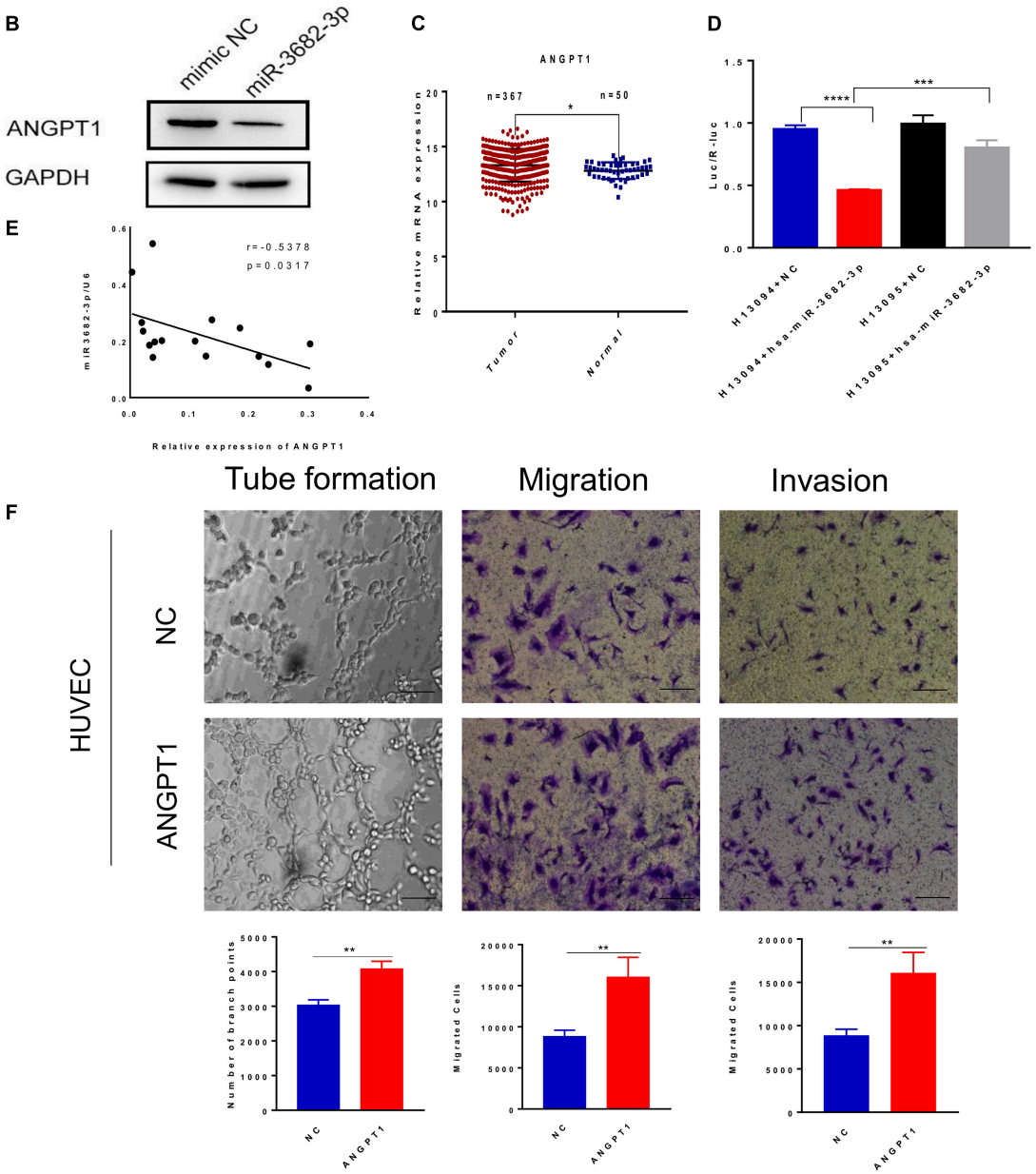


FIGURE 6 | Exosomal miR-3682-3p downregulated the expression of ANGPT1. **(A)**There were two potential binding sites for miR-3682-3p in the ANGPT1 transcript within its 3′–UTR (position 852-859 and 2321-2328). **(B)** The protein levels of ANGPT1 were determined by western blot. **(C)** ANGPT1 mRNA expression in hepatocellular carcinoma patients from the TCGA database. **(D)** miR–3682–3p significantly suppressed the luciferase activity of the ANGPT1–WT but had no obvious effects on the luciferase activity of ANGPT1-MUT. **(E)** Negative correlation between miR-3682-3p and ANGPT1 mRNA in 16 HCC tissues. **(F)** Tube formation assays, migration assays, and invasion assays of HUVECs transfected with ANGPT1-OE lentivirus or negative control. Scale bar, 200 μm. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

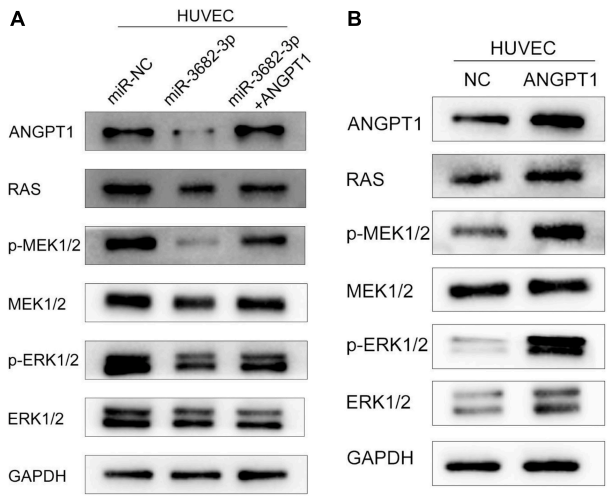


FIGURE 7 | The hepatocellular carcinoma exosomal-miR-3682-3p suppressed HUVECs via ANGPT1-RAS-MEK1/2-ERK1/2 signaling axis. **(A)** Exosomal-miR-3682-3p suppressd the expression of p-MEK1/2, p-ERK1/2, and ANGPT1 and this effet can be counteract by ANGPT1. **(B)** Western blot assays showed that overexpression of ANGPT1 also activated the RAS-MEK1/2-ERK1/2 pathway in HUVECs.

phenotype and activating hepatic inflammation. Wang et al. (2018) revealed that tumor-derived exosomes affected the $PD1^{+}$ macrophage population in human gastric cancer. However, there are few studies addressing the mechanism involving exosomes in tumor angiogenesis. Thus, it is necessary to study the interaction between tumors and endothelial cells mediated by exosomes.

In our study, we found that exosomes purified from HCC cell lines (HCCLM3, MHCC97L, and PLC/RFP/5) could be delivered into endothelial cells and subsequently accelerate endothelial cell tube formation, migration, and invasion. *In vivo* Matrigel plug assays showed that more vessels were detected in the HCC-Exos groups than in the control group by analysis of Matrigel plug sections through immunohistochemical staining for the vascular marker CD31 and H&E staining. Furthermore, this effect was enhanced with increases in the invasiveness and metastatic potential of HCC cells. It is implied that HCC-secreted exosomes play an important role in angiogenesis.

To determine the functional contents and molecular mechanisms of HCC-derived exosomes, we analyzed the miRNA profiles of exosomes isolated from three different HCC cell lines using microarray analysis. We found that miR-3682-3p expression in poorly metastatic cell lines was significantly higher than that in highly metastatic cell lines. Additionally, miR-3682-3p expression in 8 frozen HCC tissue samples was generally lower than that in corresponding tumor-adjacent normal tissue samples. Then, we performed a series of functional experiments *in vitro* to assess the role of miR-3682-3p in tumor angiogenesis. The results showed that miR-3682-3p significantly suppressed tube formation, migration, and invasion of endothelial cells. Many previous studies have reported that miRNAs participate in the promotion of angiogenesis by regulating the expression of tumor promoters. For instance, Zeng et al. (2018) revealed that exosomal miR-25-3p regulated the expression of VEGFR2, ZO-1, occludin, and Claudin5 in endothelial cells by targeting KLF2 and KLF4, consequently promoting vascular permeability and angiogenesis. Lin et al. (2018) reported that exosomal miR-210 secreted from HCC cells could be delivered into endothelial cells and then directly inhibit the expression of SMAD4 and STAT6, resulting in enhanced angiogenesis. In addition, Fang et al. (2018a) found that exosomal miR-103 increased vascular permeability and promoted metastasis by targeting the junction protein p120 in HCC. In our study, we used the TargetScan database to predict the downstream regulators of miR-3682-3p and observed that the 3′-UTR of the ANGPT1 transcript exhibited two putative binding sites for miR-3682-3p at positions 852-859 and 2321-2328. Through a dual-luciferase assay and gain-of-function analysis, ANGPT1 was identified to be the direct target gene of miR-3682-3p, and ANGPT1 expression levels could be negatively regulated by miR-3682-3p. Functional experiments further revealed that ANGPT1 could enhance the tube formation, migration, and invasion of endothelial cells *in vitro*. In addition, Suri et al. (1998) showed that transgenic overexpression of angiopoietin-1 in the skin of mice produced larger, more numerous, and more highly branched vessels. That study corroborates our findings; thus, we hypothesized that miR-3682-3p suppresses angiogenesis by inhibiting the expression of ANGPT1.

Most proangiogenic factors activate the RAS-MEK1/2-ERK1/2 cascade, a signaling pathway that promotes angiogenesis by triggering the proliferation, migration, and invasion of endothelial cells (Mavria et al., 2006). MEK1/2-ERK1/2 inhibition is a prospective therapeutic approach for preventing angiogenesis and tumor growth and metastasis (Gourlaouen et al., 2013). We wondered whether miR-3682-3p performs its biological function by targeting ANGPT1 through the RAS-MEK1/2-ERK1/2 signaling pathway. Our experiments confirmed that miR-3682-3p inhibited the pathway by suppressing the expression of ANGPT1, RAS, p-MEK1/2, and p-ERK1/2. Moreover, we also found that ANGPT1 activated the RAS-MEK1/2-ERK1/2 pathway by western blot assays.

CONCLUSION

In conclusion, the present study suggests that HCC-derived exosomal miR-3682-3p suppresses the expression of ANGPT1 and inhibits the RAS-MEK1/2-ERK1/2 signaling pathway in endothelial cells during angiogenesis. Our findings elucidate a new molecular mechanism underlying the crosstalk between HCC cells and endothelial cells mediated by exosomes. These new findings may provide insights for developing efficient therapeutic strategies to treat HCC by focusing on tumor angiogenesis.

STANDARD BIOSECURITY AND INSTITUTIONAL SAFETY PROCEDURES STATEMENT

We adhered to standard biosecurity and institutional safety procedures of Fudan University.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of Fudan University (Shanghai, China).

AUTHOR CONTRIBUTIONS

B-BL contributed the study concept and critical design of this study. S-SD, D-DD, Z-FY, G-QZ, D-MG, JC, and YZ conducted the cell experiments. S-SD and D-DD analyzed and interpreted the data. B-BL, S-SD, and D-DD fulfill the initial manuscript. B-BL critically reviewed and revised the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The experimental consumables and reagents are provided by the Research and Development Fund of Zhongshan Hospital (181) affiliated to Fudan University; Fees for public access to publications are provided by Fudan University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.633358/full#supplementary-material

REFERENCES

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

De Bem, T. H. C., da Silveira, J. C., and Sampaio, R. V. (2017). Low levels of exosomal-miRNAs in maternal blood are associated with early pregnancy loss in cloned cattle. *Sci. Rep.* 7:14319. doi: 10.1038/s41598-017-14616-1

Ediriweera, M. K., and Cho, S. K. (2019). Targeting miRNAs by histone deacetylase inhibitors (HDACi): Rationalizing epigenetics-based therapies for breast cancer. *Pharmacol. Ther.* 206:107437. doi: 10.1016/j.pharmthera.2019.107437

Fang, J. H., Zhang, Z. J., Shang, L. R., Luo, Y. W., Lin, Y. F., Yuan, Y., et al. (2018a). Hepatoma cell-secreted exosomal microRNA-103 increases vascular permeability and promotes metastasis by targeting junction proteins. *Hepatology* 68, 1459–1475. doi: 10.1002/hep.29920

Fang, J. H., Zhou, H. C., Zeng, C., Yang, J., Liu, Y., Huang, X., et al. (2011). MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. *Hepatology* 54, 1729–1740. doi: 10.1002/hep.24577

Fang, T., Lv, H., Lv, G., Li, T., Wang, C., Han, Q., et al. (2018b). Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nat. Commun.* 9:191. doi: 10.1038/s41467-017-02583-0

Goel, H. L., and Mercurio, A. M. (2013). VEGF targets the tumour cell. *Nat. Rev. Cancer* 13, 871–882. doi: 10.1038/nrc3627

Gourlaouen, M., Welti, J. C., Vasudev, N. S., and Reynolds, A. R. (2013). Essential role for endocytosis in the growth factor-stimulated activation of ERK1/2 in endothelial cells. *J. Biol. Chem.* 288, 7467–7480. doi: 10.1074/jbc.M112.446401

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hazan-Halevy, I., Rosenblum, D., Weinstein, S., Bairey, O., Raanani, P., and Peer, D. (2015). Cell-specific uptake of mantle cell lymphoma-derived exosomes by malignant and non-malignant B-lymphocytes. *Cancer Lett.* 364, 59–69. doi: 10.1016/j.canlet.2015.04.026

Iwakawa, H. O., and Tomari, Y. (2013). Molecular insights into microRNA-mediated translational repression in plants. *Mol. Cell* 52, 591–601. doi: 10.1016/j.molcel.2013.10.033

Joyce, J. A., and Pollard, J. W. (2009). Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 9, 239–252. doi: 10.1038/nrc2618

Lima, L. G., Chammas, R., Monteiro, R. Q., Moreira, M. E., and Barcinski, M. A. (2009). Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. *Cancer Lett.* 283, 168–175. doi: 10.1016/j.canlet.2009.03.041

Lin, X. J., Fang, J. H., Yang, X. J., Zhang, C., Yuan, Y., Zheng, L., et al. (2018). Hepatocellular Carcinoma Cell-Secreted Exosomal MicroRNA-210 Promotes Angiogenesis In Vitro and In Vivo. *Mol. Ther. Nucleic Acids* 11, 243–252. doi: 10.1016/j.omtn.2018.02.014

Liu, X. L., Pan, Q., Cao, H. X., Xin, F. Z., Zhao, Z. H., Yang, R. X., et al. (2019). Lipotoxic Hepatocyte-Derived Exosomal miR-192-5p Activates Macrophages via Rictor/Akt/FoxO1 Signaling in NAFLD. *Hepatology* 72, 454–469. doi: 10.1002/hep.31050

Mavria, G., Vercoulen, Y., Yeo, M., Paterson, H., Karasarides, M., Marais, R., et al. (2006). ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell* 9, 33–44. doi: 10.1016/j.ccr.2005.12.021

Pegtel, D. M., and Gould, S. J. (2019). Exosomes. *Annu. Rev. Biochem.* 88, 487–514. doi: 10.1146/annurev-biochem-013118-111902

Peng, J., and Gassama-Diagne, A. (2017). Apicobasal polarity and Ras/Raf/MEK/ERK signalling in cancer. *Gut* 66, 986–987. doi: 10.1136/gutjnl-2016-312986

Pfeffer, S. R. (2010). Two Rabs for exosome release. *Nat. Cell Biol.* 12, 3–4. doi: 10.1038/ncb0110-3

Sahoo, S., and Losordo, D. W. (2014). Exosomes and cardiac repair after myocardial infarction. *Circ. Res.* 114, 333–344. doi: 10.1161/circresaha.114.300639

Siegel, R. L., Miller, K. D., and Jemal, A. (2018). Cancer statistics, 2018. *CA Cancer J. Clin.* 68, 7–30. doi: 10.3322/caac.21442

Skog, J., Wurdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., et al. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476. doi: 10.1038/ncb1800

Sluijter, J. P., Verhage, V., Deddens, J. C., van den Akker, F., and Doevendans, P. A. (2014). Microvesicles and exosomes for intracardiac communication. *Cardiovasc. Res.* 102, 302–311. doi: 10.1093/cvr/cvu022

Suri, C., McClain, J., Thurston, G., McDonald, D. M., Zhou, H., Oldmixon, E. H., et al. (1998). Increased vascularization in mice overexpressing angiopoietin-1. *Science* 282, 468–471. doi: 10.1126/science.282.5388.468

Taylor, D. D., and Gercel-Taylor, C. (2011). Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Semin. Immunopathol.* 33, 441–454. doi: 10.1007/s00281-010-0234-8

Thery, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell Biol.* 2006:cb0322s30. doi: 10.1002/0471143030.cb0322s30

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., and Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Wang, F., Li, B., Wei, Y., Zhao, Y., Wang, L., Zhang, P., et al. (2018). Tumor-derived exosomes induce PD1(+) macrophage population in human gastric cancer that promotes disease progression. *Oncogenesis* 7:41. doi: 10.1038/s41389-018-0049-3

Xu, J., Pfarr, N., Endris, V., Mai, E. K., Md Hanafiah, N. H., Lehners, N., et al. (2017). Molecular signaling in multiple myeloma: association of RAS/RAF mutations and MEK/ERK pathway activation. *Oncogenesis* 6:e337. doi: 10.1038/oncsis.2017.36

Zeng, Z., Li, Y., Pan, Y., Lan, X., Song, F., Sun, J., et al. (2018). Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis. *Nat. Commun.* 9:5395. doi: 10.1038/s41467-018-07810-w

Zhu, M. X., Wei, C. Y., Zhang, P. F., Gao, D. M., Chen, J., Zhao, Y., et al. (2019). Elevated TRIP13 drives the AKT/mTOR pathway to induce the progression of hepatocellular carcinoma via interacting with ACTN4. *J. Exp. Clin. Cancer Res.* 38:409. doi: 10.1186/s13046-019-1401-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 12 April 2021
doi: 10.3389/fcell.2021.612774

Different Forms of Tumor Vascularization and Their Clinical Implications Focusing on Vessel Co-option in Colorectal Cancer Liver Metastases

*Gwendolyn Haas, Shuang Fan, Michael Ghadimi, Tiago De Oliveira and Lena-Christin Conradi**

Department of General, Visceral and Pediatric Surgery, University Medical Center Göttingen, Göttingen, Germany

OPEN ACCESS

Edited by:
Lucas Treps,
VIB KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Peter Vermeulen,
GZA, Belgium
Robert Kerbel,
Sunnybrook Research Institute (SRI), Canada

****Correspondence:***
Lena-Christin Conradi
lena.conradi@med.uni-goettingen.de

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *30 September 2020*
Accepted: *15 March 2021*
Published: *12 April 2021*

Citation:
Haas G, Fan S, Ghadimi M, De Oliveira T and Conradi L-C (2021) Different Forms of Tumor Vascularization and Their Clinical Implications Focusing on Vessel Co-option in Colorectal Cancer Liver Metastases. Front. Cell Dev. Biol. 9:612774. doi: 10.3389/fcell.2021.612774

In modern anti-cancer therapy of metastatic colorectal cancer (mCRC) the anti-angiogenic treatment targeting sprouting angiogenesis is firmly established for more than a decade. However, its clinical benefits still remain limited. As liver metastases (LM) represent the most common metastatic site of colorectal cancer and affect approximately one-quarter of the patients diagnosed with this malignancy, its treatment is an essential aspect for patients' prognosis. Especially in the perioperative setting, the application of anti-angiogenic drugs represents a therapeutic option that may be used in case of high-risk or borderline resectable colorectal cancer liver metastases (CRCLM) in order to achieve secondary resectability. Regarding CRCLM, one reason for the limitations of anti-angiogenic treatment may be represented by vessel co-option (VCO), which is an alternative mechanism of blood supply that differs fundamentally from the well-known sprouting angiogenesis and occurs in a significant fraction of CRCLM. In this scenario, tumor cells hijack pre-existing mature vessels of the host organ independently from stimulating new vessels formation. This represents an escape mechanism from common anti-angiogenic anti-cancer treatments, as they primarily target the main trigger of sprouting angiogenesis, the vascular endothelial growth factor A. Moreover, the mechanism of blood supply in CRCLM can be deduced from their phenotypic histopathological growth pattern (HGP). For that, a specific guideline has already been implemented. These HGP vary not only regarding their blood supply, but also concerning their tumor microenvironment (TME), as notable differences in immune cell infiltration and desmoplastic reaction surrounding the CRCLM can be observed. The latter actually serves as one of the central criteria for the classification of the HGP. Regarding the clinically relevant effects of the HGP, it is still a topic of research whether the VCO-subgroup of CRCLM results in an impaired treatment response to anti-angiogenic treatment when compared to an angiogenic subgroup. However, it is well-proved, that VCO in CRCLM generally relates to an inferior survival compared to the angiogenic subgroup. Altogether the different types of blood supply

result in a relevant influence on the patients' prognosis. This reinforces the need of an extended understanding of the underlying mechanisms of VCO in CRCLM with the aim to generate more comprehensive approaches which can target tumor vessels alternatively or even other components of the TME. This review aims to augment the current state of knowledge on VCO in CRCLM and other tumor entities and its impact on anti-angiogenic anti-cancer therapy.

Keywords: angiogenesis, colorectal cancer, vessel co-option, liver metastasases, histopathological growth patterns, lung metastasis, brain metastasis

ROLE OF TUMOR VESSELS AND ASSOCIATED RESISTANCE MECHANISMS

Tumor vessels can be very heterogeneous in their characteristics and mode of formation, depending on the tumor entity and its host tissue. The tumor vasculature is a well-established therapeutic target in addition to classical anti-tumor systemic therapy approaches. For targeting the tumor vasculature as an anti-cancer treatment, the most common modes of blood vessel development should be considered and will be described in this section.

Sprouting Angiogenesis

Sprouting angiogenesis is presumably the best characterized way of how tumors secure their blood and nutrients supply. Basically, this mechanism comprises the proliferation and migration of endothelial cells for generating a new immature vessel growing from a mature one (Hanahan and Folkman, 1996; **Figure 1A**). Thereby, the basal lamina of the mature original vessel becomes discontinuous, evolves various layers, or even completely fades. Next, endothelial cells and pericytes start proliferating, accompanied by endothelial cell migration (Paku and Paweletz, 1991). The key player driving this process is vascular endothelial growth factor (VEGF), which promotes sprouting angiogenesis in physiological situations during development and growth of normal tissues (e.g., muscles) as well as malignant tumors (Melincovici et al., 2018).

Vasculogenesis

In the event of vasculogenesis, the release of cytokines and chemokines recruits endothelial and hematopoietic progenitor cells from the circulating blood stream to help generate new vessels (Rafii et al., 2008; **Figure 1B**). There is evidence that the endothelial progenitor cells can also express different growth factors and probably promote simultaneously sprouting angiogenesis (Urbich and Dimmeler, 2004). The mechanism of vasculogenesis is well-known to take place during embryonic development (Risau and Flamme, 1995), wound healing, after ischemic events, and for tumor growth (Rafii et al., 2008).

Intussusceptive Angiogenesis

During intussusceptive or non-sprouting angiogenesis, new blood vessels are generated by splitting and remodeling of pre-existing ones (Patan et al., 1996; Burri et al., 2004; Mentzer and Konerding, 2014). Its hallmark is the formation of intraluminal pillars at the beginning of the process, which will be further invaded by pericytes and split up giving rise to two new capillaries (Karthik et al., 2018; **Figure 1C**). This mechanism provides an enlargement of the vessel surface, which improves gas and nutrient exchange of the pre-existing vasculature (Burri et al., 2004). Factors like hemodynamic conditions, oxygen levels, and pro-angiogenic molecules are being discussed to contribute to its initiation (Djonov and Makanya, 2005). Earlier studies suggested that angiopoetins I and II, platelet-derived growth factor beta polypeptide, and the fibroblast growth factor contribute to the regulation of intussusceptive angiogenesis (De Spiegelaere et al., 2012). Interestingly, VEGF appears to have a subordinated role in this process and its accurate contribution to intussusceptive angiogenesis is still not fully understood (De Spiegelaere et al., 2012). Nevertheless, intussusceptive angiogenesis can be considered a "complementary method" to sprouting angiogenesis (Karthik et al., 2018) and has been described to occur physiologically in some organs, during tissue repair and in several tumors (Hillen and Griffioen, 2007; Ribatti and Djonov, 2012).

Vascular Mimicry

Vascular mimicry describes the capacity of cancer cells to form structures that are similar to regular vessels by themselves, independently from common endothelial cells (**Figure 1D**). In this scenario the tumor cells even adopt endothelial features (Maniotis et al., 1999). Some of its molecular drivers, such as vascular endothelial cadherin, VEGF-receptor 1, metalloproteases, hypoxia-inducible factor 1α, and others have already been identified (Qiao et al., 2015), but the exact underlying mechanism for the conversion of cancer cells into endothelial-like cells still needs to be investigated further (Qian et al., 2016). The phenomenon of vascular mimicry occurs in a variety of tumor entities, including melanoma, osteosarcoma, Ewing sarcoma, ovarian cancer, breast cancer, prostate cancer, lung cancer, head and neck tumors, malignancies of the gastrointestinal tract, e.g., esophageal, gastric, hepatocellular, and colorectal cancer, malignancies of the gallbladder, and in several intracranial tumors, such as glioblastoma, astrocytoma, and non-functioning pituitary adenoma (Qiao et al., 2015; Ge and Luo, 2018).

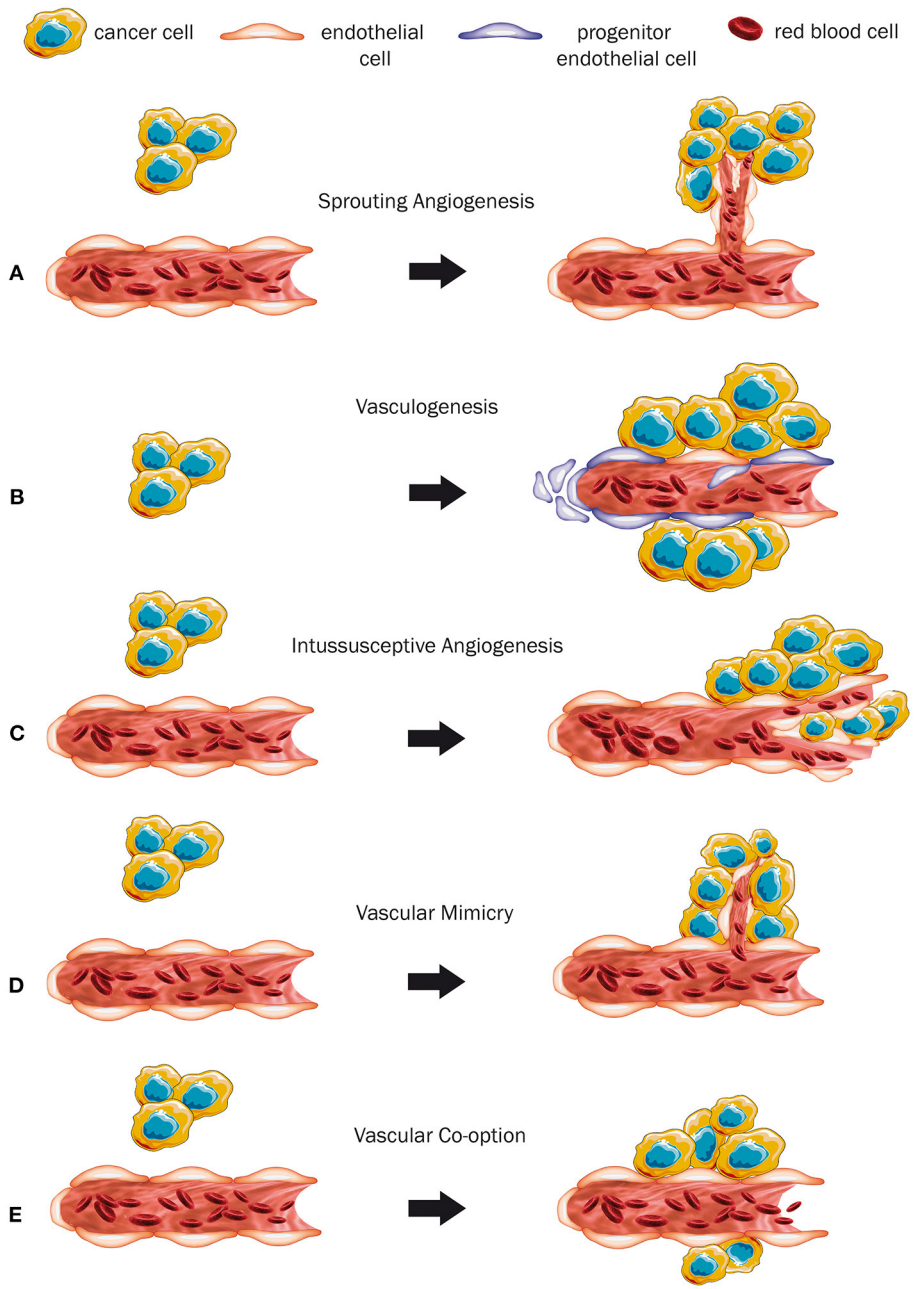


FIGURE 1 | Modes of vascularization **(A–E)**. **(A)** The mechanism of sprouting angiogenesis. As the tumor grows, sprouting of new immature vessels from a mature preexisting vessel is induced. **(B)** Vasculogenesis, a mechanism where a completely new vessel is formed by progenitor cells. **(C)** Intussusceptive angiogenesis. Hereby, a pre-existing vessel is promoted to split itself into two new ones. **(D)** Vascular mimicry. The tumor cells form a vessel-like structure themselves. **(E)** Co-option of a pre-existing mature blood vessel by expanding tumor cells.

Vessel Co-option

Vessel co-option (VCO) represents an entirely different mechanism of acquiring blood supply without the need of generating new vessels. Instead, tumor cells hijack pre-existing mature vessels of the host organ (Latacz et al., 2020a; **Figure 1E**). This phenomenon is mostly observed in highly vascularized organs like the brain, the lungs, and the liver, which provide an opulent supply of nutrients and oxygen (Donnem et al., 2013). The identification of VCO is often feasible through common light microscopy and basic histological analysis (Donnem et al., 2013). Vessel co-opting tumors tend to grow less destructive (Pezzella et al., 1997), can show a close contact to the surrounding tissue (Vermeulen et al., 2001), and grow in an infiltrative manner (van Dam et al., 2017; Kuczynski

et al., 2019). Kuczynski et al. (2019) conflated histopathological key characteristics of VCO. As tumors grow along the pre-existing vessels, they tend to mimic the histological morphology of the host organ, rather than destroying the surrounding tissue (van Dam et al., 2017; Kuczynski et al., 2019). Also, the morphology and structure of the co-opted vessels might remain unaltered to some extent. However, this latter aspect is a relatively frail criterion, as vessel alterations can occur nevertheless (Kuczynski et al., 2019). In some malignancies, e.g., CRCLM and lung metastases, VCO is directly related to specific growth patterns of the tumor, so that the occurrence of VCO can be simply deduced from morphological aspects of the tumor (Pezzella et al., 1997; Bridgeman et al., 2017; van Dam et al., 2017). Immunohistochemical analysis allows the differentiation between mature and immature vessels, which helps to distinguish between VCO and sprouting angiogenesis. The observed level of smooth muscle actin, which serves as a pericyte marker, can indicate the pericyte coverage of the vessels (Donnem et al., 2013; Lazaris et al., 2018). Immuno-co-staining for endothelial and proliferation markers, such as CD34 and Ki67, can be used to identify proliferating vessels (Lazaris et al., 2018). Thus, a considerable layer of pericytes and the lack of proliferating cells can indicate the presence of VCO. Additionally, an increased micro-vessel density is often used as a supportive criterion to describe the presence of angiogenesis, and is often misunderstood as a reliable indicator for the absence of VCO (Kuczynski et al., 2019). Conversely, a level of microvascular density which resembles the one in the surrounding tissue can indirectly indicate the absence of angiogenesis (Donnem et al., 2013). Nevertheless, also increased levels of microvascular density were observed in vessel co-opting tumors (Lazaris et al., 2018), reinforcing that the micro-vessel density is an inconsistent criterion, and therefore it should be used as a supportive element, rather than as a defining one (Donnem et al., 2013; Kuczynski et al., 2019).

A related mechanism used by cancer cells to benefit from pre-existing, but also from newly formed vessels, is the process of pericytic mimicry (PM) (Lugassy et al., 2020). The histopathological counterpart of the mechanism of this phenomenon is angiotropism (Lugassy et al., 2020), which is characterized by the localization of the cancer cells in a pericytic location, migrating along the abluminal side of vessels (Barnhill and Lugassy, 2004; Lugassy et al., 2020). This spatial proximity to vessels naturally blends PM to VCO (Lugassy et al., 2020). Although PM represents the migratory process, whereas vessel co-opting cells are more invading (Lugassy et al., 2020), there is evidence that both processes are at least closely interlinked or might even be identical (Bentolila et al., 2016; Barnhill et al., 2018). In a murine model it was shown that the implantation of melanoma cells in the brain resulted in melanoma cells spreading on the abluminal surface of pre-existing vessels (Bentolila et al., 2016). Thus, characteristics of VCO and PM were found coincidentally (Bentolila et al., 2016). However, in the event of VCO, an intravascular metastatic process might be commonly expected (Bentolila et al., 2016). With regard to the exclusively extraluminal migration process of PM (Lugassy et al., 2020), and considering it as an alternative way of metastasis formation (Bentolila et al., 2016), it would be highly interesting to investigate further whether metastases that are known for VCO, that are known for VCO, e.g., vessel co-opting CRCLM, reached the side, reached the side of metastases via an intravascular or an extravascular route.

So far, the exact underlying mechanisms of VCO are still not fully understood. To date, despite the previously described tumor cell invasion, other variables such as cell adhesion mechanisms seem to play a role in VCO (Kuczynski et al., 2019). In brain metastases, it was shown that the L1 cell adhesion molecule (L1CAM), an axon pathfinding molecule, is involved in the process of VCO (Valiente et al., 2014). Previous analyses, obtained through a murine model for brain metastases, also pointed out the crucial role of cell adhesion in VCO and showed that the contractile cytoskeleton of the tumor cells contributes to VCO establishment (Dome et al., 2003). Other cell adhesion molecules such as, β1-integrin and α3-integrin have also been associated with VCO in brain metastases *in vivo* (Carbonell et al., 2009; Bugyik et al., 2011). Following this rationale, Frentzas et al. (2016) were able to prove *in vivo* that the knockdown of actin-related protein complex 2/3, which is involved in cancer cell motility and invasion (Otsubo et al., 2004), has the potential to suppress VCO (Frentzas et al., 2016). Therefore, the currently available data suggest that cancer cell motility might also directly contribute to VCO. Recently, comparing mainly angiogenic CRCLM with mainly vessel co-opting CRCLM, a higher expression of lysyl oxidase-like 4 protein (LOXL4) was observed in neutrophils near the vessel co-opting CRCLM (Palmieri et al., 2020). Lysyl oxidases are physiologically involved in crosslinking of collagen and elastin (Lucero and Kagan, 2006), but in tumors, they are involved in malignant processes, such as epithelial-to-mesenchymal transition and promotion of metastases through remodeling of the tumor microenvironment (TME) (Xiao and Ge, 2012). This association of a high expression of LOXL4 in neutrophils near vessel co-opting CRCLM in contrast to a lower expression of LOXL4 in neutrophils close to angiogenic CRCLM might emphasize the importance of the interplay between TME and tumor cells.

Resistance to Anti-angiogenic Treatment

Since the Food and Drug Administration (FDA) approved bevacizumab for approval of bevacizumab for metastatic colorectal cancer (mCRC) in 2004 (STN-125085/0), the anti-angiogenic approach in anti-cancer therapies has been well-established and further extended (Garcia et al., 2020). As angiogenesis represents one of the hallmarks of cancer (Hanahan and Weinberg, 2000), the anti-angiogenic treatment was expected to show an undeniable efficacy in anti-cancer treatment. Knowing the different kinds of blood supply, it remains obvious that targeting sprouting angiogenesis cannot inhibit all types of tumor growth patterns. In experimental settings it has been shown that vascular mimicry seems to resist anti-angiogenic agents, such as anginex, TNP-470, endostatin, bevacizumab, and vatanib (van der Schaft et al., 2004; Angara et al., 2018). Moreover, there are indications that further tumor progression after inhibition of angiogenesis might be linked to the formation of vascular mimicry (Xu et al., 2012).

Recently, *in vitro* data highlighted the association of tumor stiffness with an impaired response to anti-angiogenic treatment in CRCLM, whereas softer tumor tissues seem to respond better to anti-angiogenic treatment (Shen et al., 2020). The key players of this process in CRCLM are metastases-associated fibroblasts (Shen et al., 2020), and therefore matrix-related components of the CRCLM TME. This indicates an important role of TME alterations in CRCLM on clinical effects of anti-angiogenic treatment. Moreover, VCO represents a way of blood supply, which should be unaffected by anti-angiogenic anti-cancer treatment approaches (Bergers and Hanahan, 2008). Various studies have already confirmed this hypothesis by showing that VCO serves as resistance mechanism to anti-angiogenic agents in several cancer entities, such as glioblastoma, cerebral melanoma metastases, hepatocellular carcinoma, lung metastases, and CRCLM (Rubenstein et al., 2000; Leenders et al., 2004; Keunen et al., 2011; Frentzas et al., 2016; Kuczynski et al., 2016; Bridgeman et al., 2017). In a recent review, Kuczynski and Reynolds (2020) comprehensively summarize the role of VCO as a resistance mechanism to anti-angiogenic therapy. Based on the available current knowledge, the development of new experimental models (*in vitro*, *in vivo*, or mathematical) and possible clinical therapeutic guidelines, such as the ones suggested by Voutouri et al. (2019), are imperative to a better understanding of anti-angiogenic therapy and its relationship with different types of blood supply.

THE TUMORS' BORDER DEFINES THE HISTOPATHOLOGICAL GROWTH PATTERN

As previously mentioned, in many tumor entities the occurrence of VCO can be deduced from the evaluation of its morphologic criteria (Kuczynski et al., 2019). In CRCLM the mode of blood supply used by the cancer cells comes in association with a specific morphological appearance. A pivotal aspect consists in the border of the CRCLM and its relation to the surrounding liver tissue, when analyzed under the light microscope by haematoxylin and eosin stainings (van Dam et al., 2017). These morphologic characteristics are classified as HGP, which, which can be understood as a clearly differing TME between the different HGP. In human CRCLM, three main HGP are observed and well-defined according to the international consensus guideline (van Dam et al., 2017).

Desmoplastic HGP

In case of a desmoplastic HGP, the CRCLM tissue is surrounded by a rim, composed frequently by fibroblasts and immune cells, which are generally lymphocytes. Also, a ductular reaction, due to increased bile ducts proliferation, can be observed (Vermeulen et al., 2001; Nielsen et al., 2014; van Dam et al., 2017). The desmoplastic rim is the main feature of this HGP, preventing direct contact between tumor cells and the adjacent hepatocytes (Vermeulen et al., 2001; van Dam et al., 2017; **Figures 2A**, **3A**). Of crucial relevance, different independent studies already showed that desmoplastic CRCLM ensure their blood supply via sprouting angiogenesis (Vermeulen et al., 2001; Stessels et al., 2004; Lazaris et al., 2018).

Replacement HGP

The replacement HGP differentiates sharply from the previously described desmoplastic HGP. In this type of HGP, when expanding, tumor cells grow within the liver cell plates while replacing the pre-existing hepatocytes (Vermeulen et al., 2001; Stessels et al., 2004; van Dam et al., 2017). Thus, a close cell–cell contact between tumor cells and hepatocytes is included in the definition of the replacement HGP (van Dam et al., 2017). A key consequence of this HGP consists in the utilization of VCO to ensure their blood supply. Hereby, VCO represents the predominant way of blood supply, as the sinusoidal vessels are co-opted by the tumor cells (Vermeulen et al., 2001; Stessels et al., 2004; Frentzas et al., 2016; van Dam et al., 2017; **Figures 2B**, **3B**).

Pushing HGP

The pushing HGP is characterized by the CRCLM pushing away the surrounding normal tissue (Vermeulen et al., 2001; Stessels et al., 2004; van Dam et al., 2017). Although there is explicitly no spatial segregation between CRCLM and liver tissue, there is no direct contact between the CRCLM cells and the hepatocytes (van Dam et al., 2017). In spite of this lack of a boundary, the tumor cells do not infiltrate the liver tissue (van Dam et al., 2017). Just like in the desmoplastic HGP, the CRCLM with pushing HGP also obtain blood and nutrients via sprouting angiogenesis (Vermeulen et al., 2001; Eefsen et al., 2012; Van den Eynden et al., 2013; van Dam et al., 2017).

The Tumor Microenvironment of HGP Is Characterized by Different Levels of Immune Cell Infiltration

For almost two decades, the differences in immune cell infiltration between different HGP is recognized as an important feature which is further studied in current research (Vermeulen et al., 2001). Generally, among the common HGP the immune cell infiltration is most dense in desmoplastic CRCLM, followed by pushing CRCLM and lastly by replacement CRCLM (Vermeulen et al., 2001; Nielsen et al., 2014; van Dam et al., 2017).

Since 2017, when the definition of different immune phenotypes of tumors was suggested, tumors can be classified according to their immune infiltration level as either an (i) immune-desert type, an (ii) immune-excluded type, or an (iii) inflamed type (Chen and Mellman, 2017).

Stremitzer et al. (2020) were able to show a significant association of the desmoplastic HGP with the inflamed immune phenotype, in a group of patients with CRCLM that were preoperatively treated with chemotherapy and bevacizumab. This inflamed immune phenotype was defined by the presence of a considerable infiltration of CD8 positive immune cells (Stremitzer et al., 2020; **Figures 2A**, **3A**). In contrast to the desmoplastic HGP, the replacement HGP shows a far reduced association with CD8 positive immune cells, when comparing those two HGP within one mixed metastasis that displayed both, replacement and desmoplastic growth (van Dam et al., 2018).

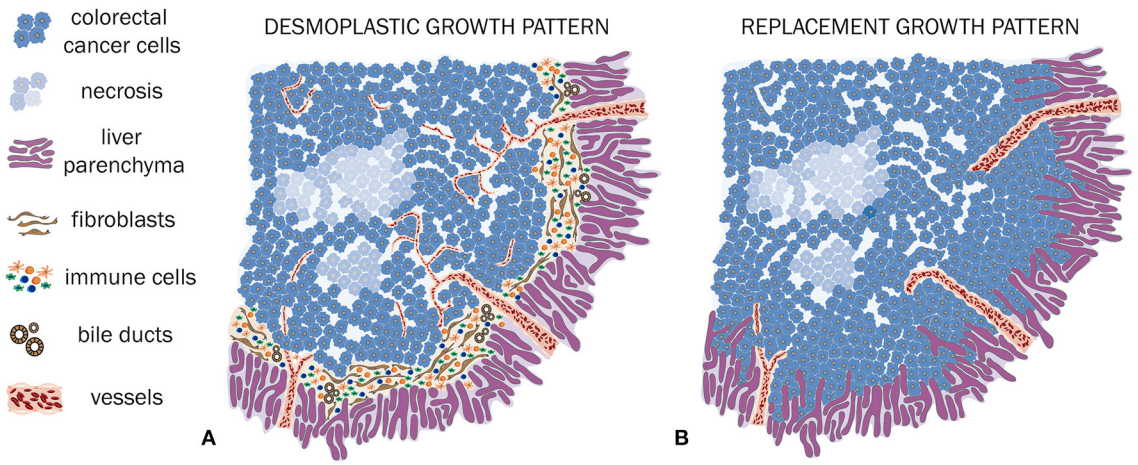


FIGURE 2 | HGP of CRCLM **(A,B)**. **(A)** Schematic illustration of a desmoplastic CRCLM. Hereby, the defining desmoplastic rim between tumor and liver tissue is clearly visible. A dense immune cell infiltration is surrounding and also invading the metastasis. Within the desmoplastic rim, the ductular reaction is illustrated. The ingrowing vessels demonstrate sprouting angiogenesis, which is characteristic for desmoplastic CRCLM. **(B)** Schematic illustration of replacement CRCLM. This type of metastases is characterized by tumor cells, mimicking the liver architecture, co-option of mature pre-existing vessels, and absence of an inflammatory and fibroblastic rim.

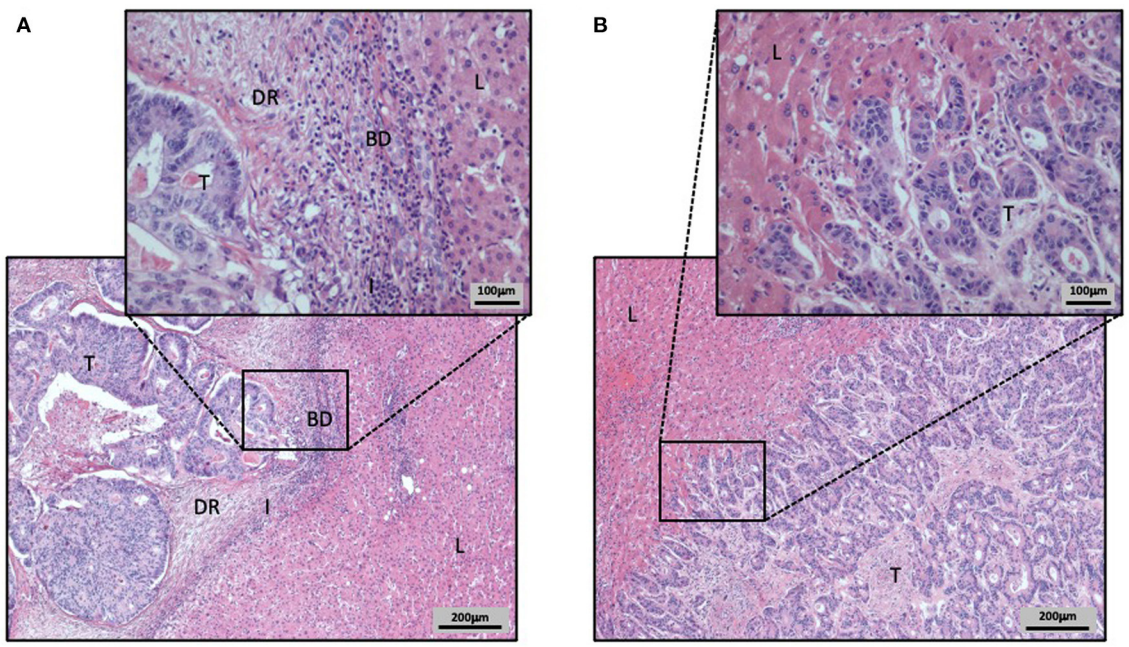


FIGURE 3 | Hematoxylin and Eosin stained CRCLM with different HGP **(A,B)**. Liver tissue is labeled as L, tumor tissue as T, ductular reaction of bile ducts as BD, the immune cell infiltration as I, and the desmoplastic rim as DR. **(A)** CRCLM with desmoplastic HGP. The CRCLM is differentiated from the surrounding liver tissue by the HGP defining desmoplastic rim. Additionally, in the desmoplastic rim the ductular reaction, composed by proliferating bile ducts, and the inflammatory infiltration are visible. **(B)** CRCLM with replacement HGP. The close cell–cell contact of tumor cells and hepatocytes is clearly visible, as the tumor cells invade the liver cell plates.

Thus, the replacement HGP often tends to exhibit the desert type of immune phenotype (van Dam et al., 2018; **Figures 2B**, **3B**).

Interestingly, in CRCLM that mainly express the vessel co-opting replacement HGP, a higher level of LOXL4-positive neutrophils has been observed in their TME when compared to angiogenic, desmoplastic HGP (Palmieri et al., 2020). Nevertheless, also in CRCLM with a predominantly desmoplastic HGP, LOXL4-positive neutrophils can be observed, but interestingly, they tend to concentrate in regions with pervasive areas of replacement HGP

(Palmieri et al., 2020). These findings emphasize the relevant interplay between the biology of CRCLM and how the HGP might define their TME. Additionally, LOXL4-expressing neutrophils might represent a possible biomarker for the replacement HGP (Palmieri et al., 2020).

As previously highlighted by van Dam et al. (2018), it is very likely that the immunologic TME and its tumor blood supply are conditionally related. As VEGF, the main mediator of sprouting angiogenesis, promotes not only angiogenesis, but also immunosuppression (Motz and Coukos, 2011), there might be an indication that the desmoplastic angiogenic HGP is related to an immunosuppressive TME, which is supported by the presence of tumor surrounding immune cells that are not penetrating the tumor (van Dam et al., 2018). Nevertheless, inflamed immune type tumors have been described in desmoplastic CRCLM (Stremitzer et al., 2020), alerting to controversial and ambiguous scenarios. Last, it is important to recognize that the hepatic sinusoidal endothelial cells, which surround the co-opted vessels in the liver, strongly contribute to tolerant immune responses (Knolle and Gerken, 2000; van Dam et al., 2018), rather than induction of immunity, as the constant contact with ingested and systemic circulating antigens could trigger severe and deleterious outcomes in the organ. Taken together, the immunologic status of the different HGP in CRCLM is still a controversial topic which requires further research.

Potential Drivers for the Different HGP in CRCLM

To date, it is not yet elucidated which molecular drivers support CRCLM growth in different HGP (van Dam et al., 2017). One hypothesis consists in the assumption that the way how the liver reacts to damage has an impact on the expression of the different HGP (van Dam et al., 2017, 2018). This hypothesis points out to the interaction between tumor cells and their microenvironment. Both, desmoplastic CRCLM and liver fibrosis show ductular reactions (van Dam et al., 2018; Sato et al., 2019). Therefore, the desmoplastic HGP resembles a fibrotic reaction of the liver to damage (van Dam et al., 2018). In contrast to that, the replacement HGP shows some parallels with liver regeneration as reaction to liver damage, since the tumor cells replace the hepatocytes within the pre-existing liver cell plates. This resembles the physiological regeneration in the liver, where new hepatocytes also grow in the pre-existing plates without generating new ones (van Dam et al., 2018; Sato et al., 2019).

Another hypothesis emphasizes the expression of adhesion molecules, including L1CAM, β1-integrin, and α3-integrin as key players in VCO in several brain tumors (Dome et al., 2003; Carbonell et al., 2009; Bugyik et al., 2011; Valiente et al., 2014). These findings, deduced from brain tumors, could potentially also play a role in the vessel co-opting replacement HGP in CRCLM. Furthermore, tumor cell motility represents a crucial feature in VCO, as shown by Frentzas et al. (2016). The knockdown of the actin related protein complex 2/3 effectively inhibited VCO, suggesting that cell motility might play an essential role in the promotion of this mode of vascularization (Frentzas et al., 2016).

CLINICAL IMPLICATIONS OF DIFFERENT HGP

The HGP and its TME composition are not only questions of academic interest, but they also have relevant clinical implications. In CRCLM, different HGP come along with different survival prognosis.

Generally, patients with desmoplastic CRCLM tend to have an improved overall survival (OS) when compared to other HGP (Nyström et al., 2012; Nielsen et al., 2014; Siriwardana et al., 2016; Galjart et al., 2019). Especially the pure desmoplastic HGP is associated with a favorable prognosis, whereas the presence of any amount of non-desmoplastic HGP impairs the prognosis (Galjart et al., 2019). Conversely, impaired OS was described for the replacement HGP in comparison to other HGP (Nielsen et al., 2014). Whilst the amount of desmoplastic HGP increases after the application of pre-operative chemotherapy, the survival benefit for patients with this HGP fades (Galjart et al., 2019). This has been shown by Galjart et al. (2019) in one analysis based on a cohort of patients with CRCLM. However, in a chemo-naive subgroup, pure desmoplastic HGP was significantly associated with improved OS and progression-free survival in multivariate analyses, in a pre-treated subgroup this survival benefit remained only significant in univariate analysis, but not in multivariate analysis (Galjart et al., 2019). Regarding the pushing HGP in CRCLM, different trends were described. While an intermediate survival between desmoplastic and replacement CRCLM was shown by Nielsen et al. (2014), other analyses pointed out to an association with a generally impaired OS (Falcao et al., 2018) or even identified pushing HGP as a prognostic marker for poor survival (Van den Eynden et al., 2012).

Additionally, the HGP can have clinical impact on disease progression after resection of the CRCLM. Firstly, when the resected CRCLM shows a desmoplastic HGP, the recurrence-free survival is improved (Eefsen et al., 2015), whereas the replacement HGP has been identified as an independent risk factor for both, intrahepatic and overall recurrences (Pinheiro et al., 2014).

Secondly, concerning local recurrences after CRCLM resection, patients with previously desmoplastic CRCLM tend to have generally a lower rate of recurrences than other HGP (Lunevicius et al., 2001). Moreover, in case of recurrence, patients with previously desmoplastic CRCLM tend to recur restricted to the liver, whilst patients, with previously non-desmoplastic CRCLM tend to recur more often in multiple locations and organs (Nierop et al., 2019).

Likewise, further studies investigated the prognostic value of HGP in LM derived from other primaries. In a cohort of patients with uveal melanoma LM, a predominant desmoplastic HGP was significantly associated with an improved OS compared to a predominant replacement HGP (Barnhill et al., 2018). Similar results were obtained from one analysis performed on patients with LM derived from cutaneous melanoma.

Hereby, a predominantly desmoplastic HGP was also associated with a significantly improved OS compared to predominantly replacement HGP or mixed HGP (Barnhill et al., 2020). Moreover, this favorable prognostic effect of the desmoplastic HGP was even stronger when pure desmoplastic HGP were compared to any amount of replacement HGP (Barnhill et al., 2020), which is similar to the previously described results that Galjart et al. (2019) reported in a cohort of patients with CRCLM. In another analysis focused on HGP in LM derived from breast cancer, LM displaying any amount of desmoplastic HGP were associated with significantly improved progression free survival and OS when compared to LM with a pure replacement HGP (Bohlok et al., 2020). Last, recent analysis performed with patients diagnosed with pancreatic cancer LM showed that a predominant replacement HGP in these LM represents an independent factor for a poor prognosis (Watanabe et al., 2020). When compared to non-predominantly replacement HGP, the predominant replacement HGP was significantly associated with impaired OS (Watanabe et al., 2020). Limitations of this latter analysis might consist in the usage of LM-needle biopsies for scoring the HGP. However, by analyzing also excisional biopsies, the authors were able to show that most of the LM derived from pancreatic cancer express a homogenous HGP, which was defined as the expression of a specific HGP in >80% of the tumor-liver-interface (Watanabe et al., 2020). Thus, the usage of needle biopsies might represent an adequate method for HGP assessment in the specific context of prognostic relevance of HGP in pancreatic LM (Watanabe et al., 2020).

Taken together, these findings reveal that the different HGP are importantly related to the clinical outcome with a considerable impact on patients' further prognosis. Importantly, the prognostic impact and the clinical relevance of the HGP displayed by the LM was shown for different primary tumor entities.

Anti-angiogenic Approach

Targeting sprouting angiogenesis in CRCLM is well-established since 2004, when the monoclonal antibody bevacizumab, which inhibits sprouting angiogenesis through VEGF inhibition, was approved by the FDA for usage in combination with chemotherapy in mCRC (Hurwitz et al., 2004). To date, the range of anti-angiogenic agents that are approved by the FDA for treatment of mCRC in specific settings is enlarged by further anti-angiogenic agents, such as aflibercept, ramucirumab, and regorafenib (Ciombor and Bekaii-Saab, 2018). Although all of the approved anti-angiogenic agents inhibit sprouting angiogenesis, they differ regarding their exact pharmacological mode of action, their usage in specific settings and combinations with or without chemotherapy and by their clinical effects on the patients' survival (Lopez et al., 2019). Bevacizumab was shown to effectively improve the OS of patients diagnosed with mCRC when combined with chemotherapy, compared to chemotherapy and placebo (median survival 20.3 vs. 15.6 months; Hurwitz et al., 2004). Aflibercept is a recombinant fusion protein that prevents VEGF binding its receptor with high affinity (Van Cutsem et al., 2012). Hence, it is also known as a VEGF Trap (Van Cutsem et al., 2012). In combination with chemotherapy, aflibercept also improves the OS of patients diagnosed with mCRC, when compared to chemotherapy and placebo (median survival 13.5 vs. 12.06 months; Van Cutsem et al., 2012). Ramucirumab is a monoclonal antibody, which specifically binds the extracellular domain of VEGF-receptor-2 preventing the binding of an endogenous ligand (Tabernero et al., 2015). In a second-line setting of patients diagnosed with mCRC, the application of ramucirumab in combination with chemotherapy showed an improved OS when compared to chemotherapy and placebo (median survival 13.3 vs. 11.7 months; Tabernero et al., 2015). The small molecule multi-kinase inhibitor regorafenib inhibits different kinases involved in tumor angiogenesis, kinases involved in oncogenesis, and kinases involved in signalling pathways in the TME (Grothey et al., 2013; Li et al., 2015). Its anti-angiogenic effect is based on the inhibition of VEGF-receptors-1–3 (Grothey et al., 2013; Li et al., 2015). In a cohort of patients with mCRC, who received at least two treatment lines before, the application of regorafenib resulted in improved OS when compared with the placebo group (median survival 8.8 vs. 6.3 months; Li et al., 2015). Also, in a multicenter based study of patients with mCRC that failed standard therapeutic regimes, the application of regorafenib compared to placebo prolonged the OS (median survival 6.4 vs. 5.0 months; Grothey et al., 2013). Altogether, anti-angiogenic treatment strategies represent an important and effective tool to improve the prognosis of patients diagnosed with mCRC in specific settings (Lopez et al., 2019).

Nevertheless, besides the described specific anti-angiogenic agents, other agents targeting the epidermal growth-factor are approved by FDA (Ciombor and Bekaii-Saab, 2018).

Thus, the mechanism of sprouting angiogenesis is already being targeted by several established therapeutic agents. However, the prolongation of the survival enabled by anti-angiogenic agents usually still remains limited to months or even weeks (Hurwitz et al., 2004; Van Cutsem et al., 2012; Grothey et al., 2013; Li et al., 2015; Tabernero et al., 2015). Unfortunately, other types of tumor vascularization than sprouting angiogenesis may still remain unaffected. Therefore, it is of crucial interest to investigate whether targeting other modes of tumor vascularization exclusively or additionally to anti-angiogenic treatment might have the potential to further improve patients' survival.

Anti-VCO Approaches?

The fact that vessel co-opting CRCLM are not only less responsive to anti-angiogenic therapies than angiogenic CRCLM, but that they might also occur as a form of acquired resistance after the application of anti-angiogenic treatment, has been suggested by Frentzas et al. (2016). Generally, VCO represents an effective resistance mechanism to anti-angiogenic therapies not only in CRCLM but also in many different malignancies (Kuczynski and Reynolds, 2020). Therefore, VCO as an important tumor characteristic might be considered in the future when investigating or planning therapeutic strategies (Kuczynski and Reynolds, 2020). This highlights the necessity of an alternative therapeutic option for targeting vessel co-opting CRCLM, which to date remains unchallenged. As mentioned,

in a LM mouse model, it has been demonstrated that the knockdown of the actin related protein complex 2/3 results in suppression of VCO, suggesting that cell motility represents a potential effective target (Frentzas et al., 2016). Other potential therapeutic targets, that were considered in other vessel co-opting tumor entities than CRCLM, affect cell adhesion, the expression of endothelial markers, the angiopoietin signalling and immune checkpoint inhibition (Kuczynski et al., 2019). Importantly, as VCO seems to define the tumor biology and its microenvironment in different tumor entities, new therapeutic approaches targeting the mechanism of VCO may not be limited to specific tumor types, but also represent a therapeutic option for several other vessel co-opting malignancies.

Approaches to Assess the Histopathological Growth Pattern Prior to Resection

In order to achieve clinical benefit from the knowledge about the HGP and thus the biology of the CRCLM, it is paramount to find a reliable clinically available method to identify different HGP.

As Latacz et al. elaborated, several analyses have paved the way for medical imaging based in HGP assessment (Latacz et al., 2020b). Already two decades ago, the association of the thickness of the peritumoral border around LM was associated with enhanced perilesional gadolinium-uptake in magnetic resonance imaging (Semelka et al., 2000). However, limitations of this study include a small number of patients and varying primary tumors (Semelka et al., 2000).

In a retrospective study, the pre-operative identification of the predominant HGP of CRCLM was successfully performed by using a radiomics model, based on contrast-enhanced multidetector computer tomography (Cheng et al., 2019). Interestingly, an enhancement of the tumors rim was significantly stronger associated with the presence of a predominantly desmoplastic HGP (Cheng et al., 2019). Additionally, to the detection of the HGP in CRCLM (Cheng et al., 2019), also the response to treatment can be deduced from imaging (Chun et al., 2009). In a cohort of patients with CRCLM, who underwent a pre-treatment with bevacizumab and chemotherapy, a clear association of the morphological response in contrast-enhanced computer tomography scans with the pathological response to the treatment has been identified (Chun et al., 2009). The morphological response was assessed by rim enhancement, overall attenuation, and characteristics of the tumor liver interface (Chun et al., 2009). Recently, Han et al. (2020) showed that also a magnetic resonance based radiomics model is able to predict the predominant HGP in CRCLM. Especially radiomics of the tumor-liver-interface, combined with clinical characteristics provide a high diagnostic accuracy (Han et al., 2020). Thus, these findings indicate the possibility to deduce the pathological response of CRCLM from a non-invasive imaging method.

Besides imaging, another non-invasive strategy to achieve further information about tumor characteristics consists in the analysis of liquid biopsies (Poulet et al., 2019). In a cohort of patients, who received bevacizumab treatment, it was shown that several microRNAs found in extracellular vesicles, are potential predictive biomarkers for patients' prognosis (de Miguel Perez et al., 2020). However, in this study an association with histological aspects is lacking. An additional promising biomarker in liquid biopsy analysis is the expression of LOXL4 in circulating neutrophils, which seems to be higher in patients suffering from CRCLM than in healthy subjects (Palmieri et al., 2020). Nevertheless, a clear association with its expression and different HGP must be further characterized (Palmieri et al., 2020).

Furthermore, the measurement of collagen combined with the conventional measurement of the carcinoembryonic antigen (CEA) provides an improved non-invasive and non-imaging detection of CRCLM (Nyström et al., 2015; Lalmahomed et al., 2016; van Huizen et al., 2020). For instance, the assessment of both, circulatory collagen type IV and CEA in blood samples, results in a more sensitive detection of CRCLM (Nyström et al., 2015). Additionally, enhancement of both markers is related to impaired survival (Nyström et al., 2015). Although the assessment of collagen type IV improves the diagnostic of CRCLM, a relationship of the preoperative collagen type IV levels with the expression of specific HGP in the CRCLM was not found (Nyström et al., 2015). Besides blood analysis, also urine markers have the potential to effectively contribute to the detection of CRCLM (Lalmahomed et al., 2016; van Huizen et al., 2020). The combination of the CEA level in serum combined with specific collagen markers in urine provide a detection of CRCLM with a high diagnostic accuracy (Lalmahomed et al., 2016; van Huizen et al., 2020). However, hereby the expressed HGP in the diagnosed CRCLM was not investigated (Lalmahomed et al., 2016; van Huizen et al., 2020).

ROLE OF VESSEL CO-OPTION IN DIFFERENT TUMOR ENTITIES

Vessel Co-option in Lung Tumors

Like many other tumors, the development and metastasis of lung tumors requires adequate nutrients supplied by blood vessels. For many years, angiogenesis has been considered to be crucial for tumor formation (Hanahan and Folkman, 1996; Kuczynski et al., 2019). A variety of targeted drugs, are clinically used for targeting angiogenesis, including the VEGF antibody bevacizumab (Sandler et al., 2006), the VEGF-recepotor-2 antibody ramucirumab (Garon et al., 2014), and nintedanib (Reck et al., 2014), a tyrosine kinase inhibitor with activity against VEGF-receptors (Janning and Loges, 2018). However, the clinical benefit from anti-angiogenic treatment of lung tumors is not yet as satisfactory as expected (Janning and Loges, 2018). In 1996, Pezzella et al. reported the occurrence of VCO in several primary and secondary lung tumors (Pezzella et al., 1996, 1997). This finding led to the hypothesis that the angiogenesis-independent growth might affect the efficacy of anti-angiogenic treatment strategies (Pezzella et al., 1997). In this section, we discuss specific examples of VCO in primary and secondary lung tumors and consider the clinical significance of this alternative tumor blood supply.

Primary Lung Cancer

Pezzella et al. (1996) proposed that in the lung, which provides an optimal vascular bed around empty spaces **Figures 4A,B** primary lung cancers and metastases to the lungs could develop without producing either new vessels or any tumor stroma. Additionally, they observed that primary lung tumors, using local blood vessels of normal lung tissue, tend to be more aggressive (Pezzella et al., 1996). Generally, different HGP can be distinguished in primary lung tumors (Kuczynski et al., 2019). In 1997, Pezzella et al. described four different tumor HGP in the lung (Pezzella et al., 1997). The (i) papillary, the (ii) basal, and the (iii) diffuse HGP are characterized by destroying the normal lung tissue and the interstitium and are accompanied by newly formed blood vessels (Pezzella et al., 1997). Conversely, the (iv) alveolar HGP, growing within the alveoli (**Figure 4C**), uses VCO for ensuring its blood supply (Pezzella et al., 1997), respects the structure of the pre-existing alveoli, and grows without destroying the lung parenchyma (Pezzella et al., 1997). Yet, another classification, suggested by Sardari Nia et al. (2008), distinguishes between three different HGP with respect to their mechanism of blood supply. Hereby, the (i) destructive HGP, which resembles the previously mentioned basal and diffuse HGP (Kuczynski et al., 2019), is characterized by angiogenic growth, the (ii) papillary HGP is known for using both, angiogenic and vessel co-opting growth, whereas the (iii) alveolar HGP is defined by angiogenesis-independent growth applying VCO (Sardari Nia et al., 2008; **Figure 4C**). Generally, the vessel co-opting alveolar HGP is characterized by cancer cells, filling the air gap of the alveoli and co-opting the pre-existing vessels (Pezzella et al., 1997; Sardari Nia et al., 2008; Donnem et al., 2018; **Figure 4C**). Additionally, another established HGP of primary lung cancer consists in the lepidic HGP (Travis et al., 2011; Kuczynski et al., 2019; **Figure 4D**). Hereby, the cancer cells spread along preexisting alveolar walls (Travis et al., 2011; Kuczynski et al., 2019; Nakamura et al., 2020), which allows them to co-opt alveolar capillaries (Kuczynski et al., 2019). In contrast to the alveolar HGP, the lepidic HGP is characterized by preserving some space within the alveoli, instead of completely filling the airspace (Kuczynski et al., 2019). In 2015, the World Health Organization added a new specific invasive pattern of lung tumors, named "spread through air spaces" characterized by cancer cells invading the alveoli (Travis et al., 2015). Recently, it has been shown that in this scenario, tumor cells co-opt pre-existing capillaries while attaching to the alveolar walls (Yagi et al., 2020). Additionally, researchers have shown that this newly defined pattern of lung cancer invasion was associated with aggressive clinical pathologic factors (Kadota et al., 2015; Lu et al., 2017).

Lung Metastases

In addition to primary lung tumors, also in metastatic pulmonary lesions, different HGP are associated with either angiogenic or vessel co-opting blood supply (Bridgeman et al., 2017; Kuczynski et al., 2019). In one of the characterized HGP, called pushing or destructive HGP, the blood supply is obtained via angiogenesis. In contrast to that, four other HGP are characterized by obtaining their blood supply through VCO. The latter ones include the (i) alveolar HGP (**Figure 4C**), the (ii) lepidic HGP (**Figure 4D**), the (iii) interstitial HGP (**Figure 4E**), and the (iv) perivascular cuffing HGP (**Figure 4F**) (Kuczynski et al., 2019). The evaluation of HGP in human lung metastases, derived from colorectal, renal, and breast cancer primaries confirmed the regular occurrence of VCO in their lung metastases (Bridgeman et al., 2017). Interestingly, it must be acknowledged that eventually both VCO and angiogenesis may occur inside the same metastatic lesion (Bridgeman et al., 2017). It was further hypothesized that in case of alveolar and interstitial HGP, it is conceivable that newly sprouting vessels can originate from co-opted ones as long as the co-opted vessels are located in the center of the metastatic lesion (Bridgeman et al., 2017). Nevertheless, new studies are still necessary to elucidate which are the molecular signals involved in these vascular-cancer cell interactions and which are the factors determining vascular blood supply patterns.

Vessel Co-option in Brain Tumors

Likewise, in brain tumors VCO is a relevant mechanism of blood supply besides angiogenesis (Seano and Jain, 2020). The occurrence of brain tumors, growing independently from angiogenesis has been described already long ago (Holash et al., 1999). In the following, we will discuss primary and secondary brain tumors in the context of VCO.

Primary Glioma and Their Vascularization

Glioblastoma is the most common malignant tumor in the brain (Ostrom et al., 2018) and although this tumor entity rarely forms distant metastases, it is one of the most invasive and aggressive intracranial malignancies (Agnihotri et al., 2013). It has been already shown that besides angiogenesis, VCO is a relevant mechanism of blood supply in glioblastoma (Seano and Jain, 2020) and that glioblastoma cells can grow independently from angiogenesis (Holash et al., 1999; Seano and Jain, 2020). Previously, an *in vivo* rat model, showed that anti-VEGF treatment leads to slower growth of intracranial glioblastoma tumors, however their histological evaluation revealed an increased invasive tumor growth and co-option of pre-existing vessels, indicating acquired strong resistance mechanisms to anti-angiogenic therapy (Rubenstein et al., 2000). Additionally, in an angiogenic xenograft mouse model, bearing glioblastoma cells, treatment with the anti-angiogenic agent bevacizumab resulted in promoted tumor cell infiltration, suggesting that VCO could represent an escape mechanism from anti-angiogenic treatment (de Groot et al., 2010). Moreover, analysis performed with human brain tissue from autopsies further elucidated the impact of anti-angiogenic treatment on the vascular structure of recurrent glioblastoma (di Tomaso et al., 2011). Subjects who received cediranib, a pan-VEGF-receptor tyrosine kinase inhibitor, showed a vessel structure more similar to the tumor-free brain tissues without indication of further angiogenesis, when compared to the subgroup who did not receive anti-angiogenic therapy (di Tomaso et al., 2011). Therefore, this switch in growth might represent a clinically relevant acquired resistance mechanism following anti-angiogenic therapy (di Tomaso et al., 2011), which potentially involves VCO (Kuczynski et al., 2019). Further evidence for VCO

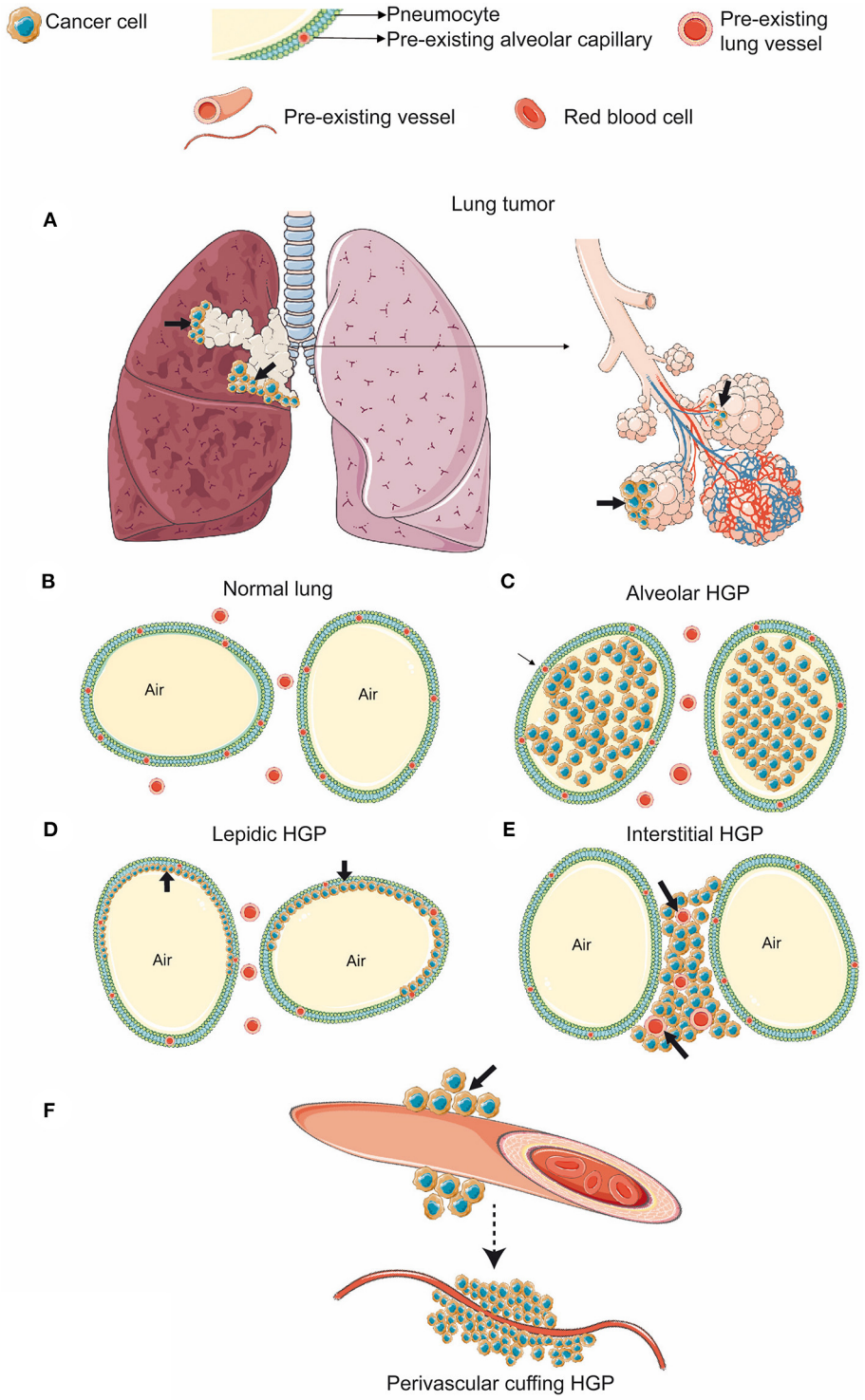


FIGURE 4 | HGP in primary lung cancer **(A–F)**. **(A)** Cancer cells growing in the bronchus of the lung (black arrows). **(B)** In the normal alveolar parenchyma, the lung alveolar spaces are filled by air and are delimited by the alveolar walls. **(C)** Alveolar HGP. Cancer cells fill the alveolar air space. **(D)** Lepidic HGP. Cancer cells spread along the alveolar walls, but preserve some air space by not filling the alveoli completely. **(E)** Interstitial HGP. Cancer cells grow between the alveoli, but do not enter the alveolar space. **(F)** Perivascular cuffing HGP. Cancer cells grow cuff-like around blood vessels. (Figure created with vectors from Servier Medical Art. https://smart.servier.com/).

acting as a resistance mechanism to anti-angiogenic treatment was found in one analysis based on tumor tissue from three patients, who were diagnosed with recurrent glioma despite anti-angiogenic therapy with bevacizumab, and on a murine model for glioma also treated with bevacizumab (de Groot et al., 2010). In this analysis, histological examination of the tissue obtained from the previously described three patients revealed tumor vessel normalization without evidence for perivascular invasion, whereas in the murine tissue samples, perivascular invasion was clearly present, indicating that VCO may be involved in clinically relevant resistance mechanisms to anti-angiogenic therapy in recurrent glioma (de Groot et al., 2010). At the cellular and molecular levels, some new insights on glioblastoma and its vascular nature have already been reported. Caspani et al. (2014) precisely evaluated the steps of VCO in glioblastoma, demonstrating that once cancer cells reach the blood vessels, they produce flectopodia, which can alter the contractility of the pericytes. Moreover, the authors have also shown that cancer cells can fuse with pericytes, a process mediated by CDC42, to create new hybrid cell types (Caspani et al., 2014; **Figure 5**). Also, Watkins et al. (2014) described in detail that glioblastoma cells place themselves under astrocytic end-feet interrupting coupling of astrocytes to blood vessels (**Figure 5**). Recently, new molecular pieces of evidence have been reported on glioblastomas and VCO interactions. Griveau et al. (2018) showed that WNT7-induced Oligo2^{+}-oligodendrocyte precursor-like cells are able to invade the brain parenchyma in a VCO-manner without disrupting the adjacent vasculature. Crucially, they observed that Oligo2^{-}-cancer cells grow as perivascular clusters, directly affecting the blood brain barrier and triggering an immune cell activation (Griveau et al., 2018). These findings strongly support the idea that specific molecular traits are involved in tumors vascular choices. Moreover, they suggested that WNT signalling might be directly involved in this process.

Brain Metastases

Investigations in the field of brain metastases based on analysis of human tissue samples and different *in vivo* models revealed that brain metastases use VCO to achieve blood supply (Kienast et al., 2010; Bugyik et al., 2011; Berghoff et al., 2013; Siam et al., 2015; Bentolila et al., 2016). In case of VCO in brain metastases, it can be understood as an alternative to neovascularization, and probably plays a crucial role in transporting nutrients and oxygen (Carbonell et al., 2009). In a murine model of brain metastases, it was shown that the expression of the adhesion molecule β1-integrin by cancer cells is required for an adequate adhesion to the vascular basement membrane of vessels (Carbonell et al., 2009). This molecular connection allows the cancer cells to co-opt the particular vessel (Carbonell et al., 2009). In 2014, Valiente et al. proposed a mechanism for VCO in brain metastases (Valiente et al., 2014). In that, one important driver for VCO in brain metastasis is the adhesion molecule L1CAM which allows the metastatic cells to spread on the capillaries and to co-opt them (Valiente et al., 2014). However, the enzyme plasmin has the power to inhibit this metastatic process, as it is involved in the inactivation of L1CAM and in the mobilization of FAS ligand (Valiente et al., 2014). By secreting plasminogen activator inhibitory serpins, brain metastatic cells prevent the formation of plasmin (Valiente et al., 2014). Thus, the expression of plasminogen activator inhibitory serpins allows the metastatic cells to continue benefiting from L1CAM spreading along capillaries and avoid FAS induced cell death mediated by plasmin (Valiente et al., 2014; **Figure 6**).

Vessel Co-option at Other Tumor Sites Than Lung and Brain

Vessel co-option is also known to occur in other tissues, including tumors in the skin and in lymph nodes (Naresh et al., 2001; Dome et al., 2002). Melanoma tumor cells of the skin fuse with the surrounding normal blood vessels to obtain nutrients and support their own growth and survival (Dome et al., 2002). Lymph nodes have a dense network of blood vessels, which can provide ideal nutrition for tumor cells, so lymph node metastases are able to exploit preexisting vessels (Naresh et al., 2001). The currently clinically approved angiogenesis inhibitors are not active during early cancer progression in the lymph node, suggesting that inhibitors of sprouting angiogenesis will not be able to treat or to prevent lymph node metastases effectively (Jeong et al., 2015). However, lymph node tumors do not grow completely independent from sprouting angiogenesis (Vermeulen et al., 2002). Instead of that, lymph node tumors seem to grow in different grades of angiogenesis-independency depending on their actual growth patterns (Vermeulen et al., 2002). Renal cell carcinomas also use VCO, which might be driven by an epithelial-to-mesenchymal-like invasive process (Bridgeman et al., 2017).

DISCUSSION

The widely varying diversity of the TME is a topic of current research. As the different types of blood supply have clear clinical implications, an improved understanding is urgently needed. Not only in CRCLM, but also in many other tumor entities, VCO was identified as an underlying mechanism of blood supply that is often associated with a specific morphology and a poor prognosis (van Dam et al., 2017).

Although the inhibition of sprouting angiogenesis in CRCLM is for more than a dozen years a well-established therapeutic anti-cancer strategy (Hurwitz et al., 2004), to date there is no equivalent approach for targeting VCO (Kuczynski et al., 2019). Moreover, the occurrence of the vessel co-opting replacement HGP in CRCLM shows a considerable association with impaired survival (Fernandez Moro et al., 2018), indicating that VCO and TME play a crucial role in disease progression. As Frentzas et al. (2016) showed, replacement HGP tend to occur more frequently after anti-angiogenic treatment. This could implicate that VCO might represent a mode of acquired resistance to anti-angiogenic approaches. Consequently, targeting VCO could also prolong patients' survival. Another potential strategy in vessel co-opting CRCLM might consist in targeting cancer cell motility (Frentzas et al., 2016).

Stremitzer et al. (2020) showed a considerable association between the immunologic TME and patient survival. An

inflamed TME in CRCLM is associated with improved recurrence-free survival and shows a trend for an improved OS after resection (Stremitzer et al., 2020). In contrast to that, immune cell infiltration is nearly not existing at all in replacement HGP (van Dam et al., 2017) and this HGP could even be described as a desert immune phenotype (van Dam et al., 2018). Thus, promotion of immune cell infiltration in replacement CRCLM could also result in an improved survival.

Interestingly, the desert immune phenotype observed in replacement HGP (van Dam et al., 2018) shows a higher level of LOXL4-positive neutrophils than desmoplastic HGP (Palmieri et al., 2020), which emphasizes the role of rare immune cells in the replacement HGP. Nevertheless, also in the desmoplastic HGP LOXL4-positive neutrophils were observed, but they were mainly concentrated on areas, where replacement HGP pervades and might indicate the conversion of desmoplastic to replacement HGP (Palmieri et al., 2020). Taken these results together, it would be interesting to investigate whether LOXL4 expression plays a role in acquired resistance.

Besides therapeutic challenges, a pre-operative diagnosis of the exact HGP in CRCLM is still an essential topic of research to achieve the maximum clinical benefit for CRCLM patients. As previously described, multi-detector computer tomography, and also radiation-free magnet resonance based radiomics seem to be promising tools to assess the predominant HGP (Cheng et al., 2019; Han et al., 2020). However, in both techniques the predominant HGP was classified as such, when >50% of the tumor border displayed the specific HGP (Cheng et al., 2019; Han et al., 2020). Referring to the study of Galjart et al. (2019), the presence of any non-desmoplastic HGP in CRCLM impairs the patients' prognosis. Therefore, further research on imaging methods is required to achieve an optimized diagnostic accuracy with a precise prediction of the HGP. Fortunately, new findings about imaging methods combined with a detailed analysis of the HGP in CRCLM can be expected, as a prospective study, addressing this issue, is currently ongoing (Latacz et al., 2020b).

As enhanced imaging contrast in the portal venous phase seems to be a reliable characteristic of the HGP in computer tomography based radiomics (Cheng et al., 2019), the analysis of enhancement characteristics in ultrasound images could represent another potential method for rough prediction of HGP

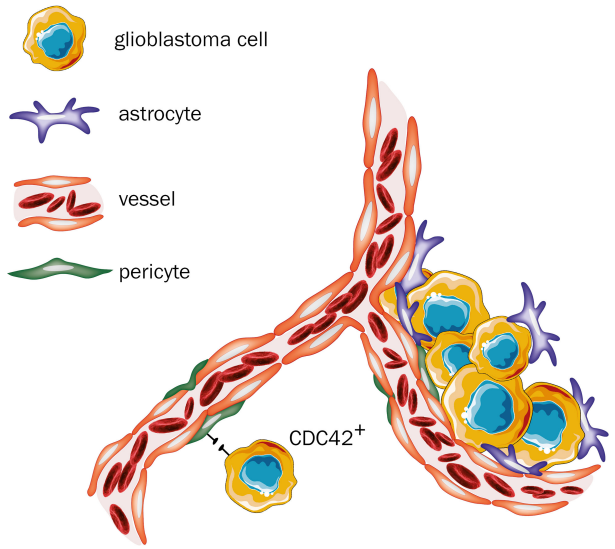


FIGURE 5 | Glioblastoma—primary tumor. Once cancer cells reach the blood vessels, two outcomes are feasible: (i) neoplastic cells produce flectopodia and fuse with pericytes through CDC42-mediated processes to produce hybrid cell types. However, when CDC42 activity is blocked, fusion is abrogated, and pericytes might gain anti-tumor activities (assuming a peripheral-derived macrophage-like phenotype). (ii) tumor cells place themselves between astrocytes and pericytes, thereby blocking their physiological interactions and changing their normal functions (Figure created with vectors from Servier Medical Art. https://smart.servier.com/).

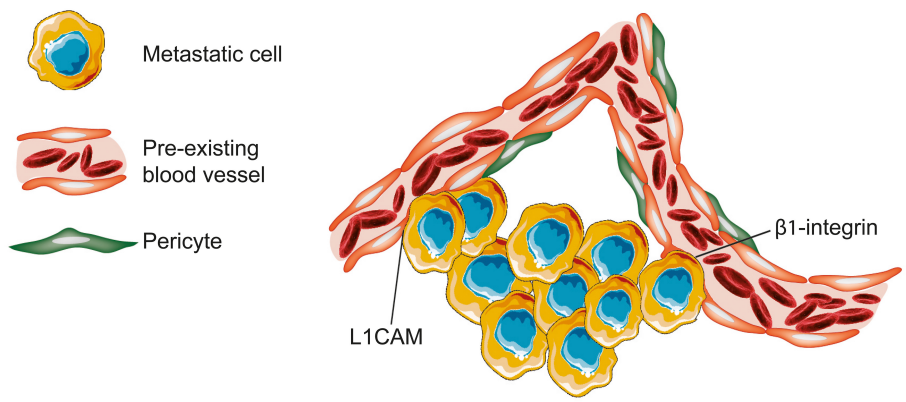


FIGURE 6 | Brain—metastases. In brain metastases, tumor cells can attach to the vascular basement membrane via β1-integrin. The resultant close spatial proximity to vessels allows then the metastatic cells to co-opt the pre-existing blood vessel. In the presented model, non-angiogenic metastatic cells infiltrating the brain tissue are characterized by secretion of neuroserine protease inhibitors and expression of L1CAM. Neuroserpin blocks plasminogen activator and prevents the formation of plasmin. The inhibition of plasmin release into the microenvironment prevents both the death of cancer cells by FAS ligand and the further blockade of L1CAM (Figure created with vectors from Servier Medical Art. https://smart.servier.com/).

in CRCLM with a radiation-free and easily accessible method. However, also in this scenario, a rough estimation might be insufficient, since the presence of any non-desmoplastic growth seems to affect the patients' prognosis, as previously described (Galjart et al., 2019).

Further exploration of liquid biopsy data might represent a promising perspective for HGP diagnosis and guidance for further therapeutical decisions (de Miguel Perez et al., 2020; Palmieri et al., 2020). Yet, the extension of the predictive value of this new method needs to be closer investigated.

Co-opting sinusoidal vessels directly implicate the access to blood enriched with nutrients. Consequently, co-opting CRCLM cells might be pre-disposed to altered metabolic states. Thus, it is well-conceivable that these cells might be prone to a metabolic switch toward glycolysis. The Warburg's effect is known for increased glucose uptake and increased lactate production, despite of sufficient oxygen supply, which leads to the promotion of cancer development (Liberti and Locasale, 2016). Both, the facilitated access to glucose, while co-opting the nutrient enriched sinusoids, as well as the decreased survival of patients with replacement CRCLM, could indicate a metabolic switch in direction of the Warburg's effect. Consequently, this would open a new field of therapeutic approaches for replacement CRCLM by targeting the cancer cell metabolism, a domain which absolutely has strong therapeutic potential (Luengo et al., 2017). Unfortunately, the metabolic status of CRCLM with differing HGP still remains to be accurately evaluated.

Considering the mechanisms of PM and the associated histopathological marker angiotropism, it would be highly interesting to elucidate further its relationship with VCO. Since in case of angiotropism tumor cells are localized on the abluminal side of vessels in a pericyte location (Barnhill and Lugassy, 2004; Lugassy et al., 2020), this spatial localization consecutively blends PM to VCO (Lugassy et al., 2020). Additionally, based on a mouse model, the close relationship of PM and VCO was described (Bentolila et al., 2016). However, angiotropism also subsumes a completely extraluminal migratory process along vessels (Lugassy et al., 2020), whereas in case of vessel co-opting metastases, one might simply assume an intravasal way of metastasis formation (Bentolila et al., 2016). However, the mechanism of angiotropism or PM should be considered as an alternative way of metastasis formation (Bentolila et al., 2016). Thus, additional analysis with a focus on the exact mechanisms of metastasis formation are required to elucidate whether metastases that are known to obtain their blood via VCO, arise from an extraluminal migration process or via the commonly expected intravasal dissemination process.

To summarize, TME and VCO play a key role in the biology of many different tumors. A direct association between the TME and clinical aspects has been clearly shown by several studies. Researchers have been able to point out important attributes of VCO in different tumor entities. However, the many underlying mechanisms are not fully elucidated. Thus, an extended understanding of VCO is absolutely required to improve not only the therapeutic strategies for patients with CRCLM, but also for patients suffering from other solid malignancies.

AUTHOR CONTRIBUTIONS

GH and SF drafted the manuscript. TD and L-CC conceptualized the review article. TD, SF, and GH designed the figures. All authors contributed to the article and approved the submitted version.

REFERENCES

Agnihotri, S., Burrell, K. E., Wolf, A., Jalali, S., Hawkins, C., Rutka, J. T., et al. (2013). Glioblastoma, a brief review of history, molecular genetics, animal models and novel therapeutic strategies. *Arch. Immunol. Ther. Exp*. 61, 25–41. doi: 10.1007/s00005-012-0203-0

Angara, K., Borin, T. F., Rashid, M. H., Lebedyeva, I., Ara, R., Lin, P. C., et al. (2018). CXCR2-expressing tumor cells drive vascular mimicry in antiangiogenic therapy-resistant glioblastoma. *Neoplasia* 20, 1070–1082. doi: 10.1016/j.neo.2018.08.011

Barnhill, R., van Dam, P. J., Vermeulen, P., Champenois, G., Nicolas, A., Rawson, R. V., et al. (2020). Replacement and desmoplastic histopathological growth patterns in cutaneous melanoma liver metastases: frequency, characteristics, and robust prognostic value. *J. Pathol. Clin. Res*. 6, 195–206. doi: 10.1002/cjp2.161

Barnhill, R., Vermeulen, P., Daelemans, S., van Dam, P. J., Roman-Roman, S., Servois, V., et al. (2018). Replacement and desmoplastic histopathological growth patterns: a pilot study of prediction of outcome in patients with uveal melanoma liver metastases. *J. Pathol. Clin. Res*. 4, 227–240. doi: 10.1002/cjp2.105

Barnhill, R. L., and Lugassy, C. (2004). Angiotropic malignant melanoma and extravascular migratory metastasis: description of 36 cases with emphasis on a new mechanism of tumour spread. *Pathology* 36, 485–490. doi: 10.1080/00313020412331282708

Bentolila, L. A., Prakash, R., Mihic-Probst, D., Wadehra, M., Kleinman, H. K., Carmichael, T. S., et al. (2016). Imaging of angiotropism/vascular co-option in a murine model of brain melanoma: implications for melanoma progression along extravascular pathways. *Sci. Rep*. 6:23834. doi: 10.1038/srep23834

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Berghoff, A. S., Rajky, O., Winkler, F., Bartsch, R., Furtner, J., Hainfellner, J. A., et al. (2013). Invasion patterns in brain metastases of solid cancers. *Neurooncology* 15, 1664–1672. doi: 10.1093/neuonc/not112

Bohlok, A., Vermeulen, P., Leduc, S., Latacz, E., Botzenhart, L., Richard, F., et al. (2020). Association between the histopathological growth patterns of liver metastases and survival after hepatic surgery in breast cancer patients. *NPJ Breast Cancer* 6:64. doi: 10.1038/s41523-020-00209-1

Bridgeman, V. L., Vermeulen, P. B., Foo, S., Bilecz, A., Daley, F., Kostaras, E., et al. (2017). Vessel co-option is common in human lung metastases and mediates resistance to anti-angiogenic therapy in preclinical lung metastasis models. *J. Pathol*. 241, 362–374. doi: 10.1002/path.4845

Bugyik, E., Dezso, K., Reiniger, L., Laszlo, V., Tovari, J., Timar, J., et al. (2011). Lack of angiogenesis in experimental brain metastases. *J. Neuropathol. Exp. Neurol*. 70, 979–991. doi: 10.1097/NEN.0b013e318233afd7

Burri, P. H., Hlushchuk, R., and Djonov, V. (2004). Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev. Dyn*. 231, 474–488. doi: 10.1002/dvdy.20184

Carbonell, W. S., Ansorge, O., Sibson, N., and Muschel, R. (2009). The vascular basement membrane as "soil" in brain metastasis. *PLoS ONE* 4:e5857. doi: 10.1371/journal.pone.0005857

Caspani, E. M., Crossley, P. H., Redondo-Garcia, C., and Martinez, S. (2014). Glioblastoma: a pathogenic crosstalk between tumor cells and pericytes. *PLoS ONE* 9:e101402. doi: 10.1371/journal.pone.0101402

Chen, D. S., and Mellman, I. (2017). Elements of cancer immunity and the cancer-immune set point. *Nature* 541, 321–330. doi: 10.1038/nature21349

Cheng, J., Wei, J., Tong, T., Sheng, W., Zhang, Y., Han, Y., et al. (2019). Prediction of histopathologic growth patterns of colorectal liver metastases with a noninvasive imaging method. *Ann. Surg. Oncol.* 26, 4587–4598. doi: 10.1245/s10434-019-07910-x

Chun, Y. S., Vauthey, J. N., Boonsirikamchai, P., Maru, D. M., Kopetz, S., Palavecino, M., et al. (2009). Association of computed tomography morphologic criteria with pathologic response and survival in patients treated with bevacizumab for colorectal liver metastases. *JAMA* 302, 2338–2344. doi: 10.1001/jama.2009.1755

Ciombor, K. K., and Bekaii-Saab, T. (2018). A comprehensive review of sequencing and combination strategies of targeted agents in metastatic colorectal cancer. *Oncologist* 23, 25–34. doi: 10.1634/theoncologist.2017-0203

de Groot, J. F., Fuller, G., Kumar, A. J., Piao, Y., Eterovic, K., Ji, Y., et al. (2010). Tumor invasion after treatment of glioblastoma with bevacizumab: radiographic and pathologic correlation in humans and mice. *Neurooncology* 12, 233–242. doi: 10.1093/neuonc/nop027

de Miguel Perez, D., Rodriguez Martinez, A., Ortigosa Palomo, A., Delgado Urena, M., Garcia Puche, J. L., Robles Remacho, A., et al. (2020). Extracellular vesicle-miRNAs as liquid biopsy biomarkers for disease identification and prognosis in metastatic colorectal cancer patients. *Sci. Rep.* 10:3974. doi: 10.1038/s41598-020-60212-1

De Spiegelaere, W., Casteleyn, C., Van den Broeck, W., Plendl, J., Bahramsoltani, M., Simoens, P., et al. (2012). Intussusceptive angiogenesis: a biologically relevant form of angiogenesis. *J. Vasc. Res.* 49, 390–404. doi: 10.1159/000338278

di Tomaso, E., Snuderl, M., Kamoun, W. S., Duda, D. G., Auluck, P. K., Fazlollahi, L., et al. (2011). Glioblastoma recurrence after cediranib therapy in patients: lack of "rebound" revascularization as mode of escape. *Cancer Res.* 71, 19–28. doi: 10.1158/0008-5472.CAN-10-2602

Djonov, V., and Makanya, A. N. (2005). "New insights into intussusceptive angiogenesis," in *Mechanisms of Angiogenesis. Experientia Supplementum*, eds M. Clauss and G. Breier (Basel: Birkhäuser), 17–33. doi: 10.1007/3-7643-7311-3_2

Dome, B., Paku, S., Somlai, B., and Timar, J. (2002). Vascularization of cutaneous melanoma involves vessel co-option and has clinical significance. *J. Pathol.* 197, 355–362. doi: 10.1002/path.1124

Dome, B., Timar, J., and Paku, S. (2003). A novel concept of glomeruloid body formation in experimental cerebral metastases. *J. Neuropathol. Exp. Neurol.* 62, 655–661. doi: 10.1093/jnen/62.6.655

Donnem, T., Hu, J., Ferguson, M., Adighibe, O., Snell, C., Harris, A. L., et al. (2013). Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? *Cancer Med.* 2, 427–436. doi: 10.1002/cam4.105

Donnem, T., Reynolds, A. R., Kuczynski, E. A., Gatter, K., Vermeulen, P. B., Kerbel, R. S., et al. (2018). Non-angiogenic tumours and their influence on cancer biology. *Nat. Rev. Cancer* 18, 323–336. doi: 10.1038/nrc.2018.14

Eefsen, R. L., Van den Eynden, G. G., Hoyer-Hansen, G., Brodt, P., Laerum, O. D., Vermeulen, P. B., et al. (2012). Histopathological growth pattern, proteolysis and angiogenesis in chemonaive patients resected for multiple colorectal liver metastases. *J. Oncol.* 2012:907971. doi: 10.1155/2012/907971

Eefsen, R. L., Vermeulen, P. B., Christensen, I. J., Laerum, O. D., Mogensen, M. B., Rolff, H. C., et al. (2015). Growth pattern of colorectal liver metastasis as a marker of recurrence risk. *Clin. Exp. Metastasis* 32, 369–381. doi: 10.1007/s10585-015-9715-4

Falcao, D., Alexandrino, H., Caetano Oliveira, R., Martins, J., Ferreira, L., Martins, R., et al. (2018). Histopathologic patterns as markers of prognosis in patients undergoing hepatectomy for colorectal cancer liver metastases - pushing growth as an independent risk factor for decreased survival. *Eur. J. Surg. Oncol.* 44, 1212–1219. doi: 10.1016/j.ejso.2018.03.023

Fernandez Moro, C., Bozoky, B., and Gerling, M. (2018). Growth patterns of colorectal cancer liver metastases and their impact on prognosis: a systematic review. *BMJ Open Gastroenterol.* 5:e000217. doi: 10.1136/bmjgast-2018-000217

Frentzas, S., Simoneau, E., Bridgeman, V. L., Vermeulen, P. B., Foo, S., Kostaras, E., et al. (2016). Vessel co-option mediates resistance to anti-angiogenic therapy in liver metastases. *Nat. Med.* 22, 1294–1302. doi: 10.1038/nm.4197

Galjart, B., Nierop, P. M. H., van der Stok, E. P., van den Braak, R., Hoppener, D. J., Daelemans, S., et al. (2019). Angiogenic desmoplastic histopathological growth pattern as a prognostic marker of good outcome in patients with colorectal liver metastases. *Angiogenesis* 22, 355–368. doi: 10.1007/s10456-019-09661-5

Garcia, J., Hurwitz, H. I., Sandler, A. B., Miles, D., Coleman, R. L., Deurloo, R., et al. (2020). Bevacizumab (Avastin(R)) in cancer treatment: a review of 15 years of clinical experience and future outlook. *Cancer Treat. Rev.* 86:102017. doi: 10.1016/j.ctrv.2020.102017

Garon, E. B., Ciuleanu, T. E., Arrieta, O., Prabhash, K., Syrigos, K. N., Goksel, T., et al. (2014). Ramucirumab plus docetaxel versus placebo plus docetaxel for second-line treatment of stage IV non-small-cell lung cancer after disease progression on platinum-based therapy (REVEL): a multicentre, double-blind, randomised phase 3 trial. *Lancet* 384, 665–673. doi: 10.1016/S0140-6736(14)60845-X

Ge, H., and Luo, H. (2018). Overview of advances in vasculogenic mimicry - a potential target for tumor therapy. *Cancer Manag. Res.* 10, 2429–2437. doi: 10.2147/CMAR.S164675

Griveau, A., Seano, G., Shelton, S. J., Kupp, R., Jahangiri, A., Obernier, K., et al. (2018). A glial signature and Wnt7 signaling regulate glioma-vascular interactions and tumor microenvironment. *Cancer Cell* 33, 874.e7–889.e7. doi: 10.1016/j.ccell.2018.03.020

Grothey, A., Van Cutsem, E., Sobrero, A., Siena, S., Falcone, A., Ychou, M., et al. (2013). Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 381, 303–312. doi: 10.1016/S0140-6736(12)61900-X

Han, Y., Chai, F., Wei, J., Yue, Y., Cheng, J., Gu, D., et al. (2020). Identification of predominant histopathological growth patterns of colorectal liver metastasis by multi-habitat and multi-sequence based radiomics analysis. *Front. Oncol.* 10:1363. doi: 10.3389/fonc.2020.01363

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364. doi: 10.1016/S0092-8674(00)80108-7

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57–70. doi: 10.1016/S0092-8674(00)81683-9

Hillen, F., and Griffioen, A. W. (2007). Tumour vascularization: sprouting angiogenesis and beyond. *Cancer Metastasis Rev.* 26, 489–502. doi: 10.1007/s10555-007-9094-7

Holash, J., Wiegand, S. J., and Yancopoulos, G. D. (1999). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 18, 5356–5362. doi: 10.1038/sj.onc.1203035

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., et al. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* 350, 2335–2342. doi: 10.1056/NEJMoa032691

Janning, M., and Loges, S. (2018). Anti-angiogenics: their value in lung cancer therapy. *Oncol Res Treat.* 41, 172–180. doi: 10.1159/000488119

Jeong, H. S., Jones, D., Liao, S., Wattson, D. A., Cui, C. H., Duda, D. G., et al. (2015). Investigation of the lack of angiogenesis in the formation of lymph node metastases. *J. Natl. Cancer Inst.* 107:djv155. doi: 10.1093/jnci/djv155

Kadota, K., Nitadori, J. I., Sima, C. S., Ujiie, H., Rizk, N. P., Jones, D. R., et al. (2015). Tumor spread through air spaces is an important pattern of invasion and impacts the frequency and location of recurrences after limited resection for small stage I lung adenocarcinomas. *J. Thorac. Oncol.* 10, 806–814. doi: 10.1097/JTO.0000000000000486

Karthik, S., Djukic, T., Kim, J. D., Zuber, B., Makanya, A., Odriozola, A., et al. (2018). Synergistic interaction of sprouting and intussusceptive angiogenesis during zebrafish caudal vein plexus development. *Sci. Rep.* 8:9840. doi: 10.1038/s41598-018-27791-6

Keunen, O., Johansson, M., Oudin, A., Sanzey, M., Rahim, S. A., Fack, F., et al. (2011). Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3749–3754. doi: 10.1073/pnas.1014480108

Kienast, Y., von Baumgarten, L., Fuhrmann, M., Klinkert, W. E., Goldbrunner, R., Herms, J., et al. (2010). Real-time imaging reveals the single steps of brain metastasis formation. *Nat. Med.* 16, 116–122. doi: 10.1038/nm.2072

Knolle, P. A., and Gerken, G. (2000). Local control of the immune response in the liver. *Immunol. Rev.* 174, 21–34. doi: 10.1034/j.1600-0528.2002.017408.x

Kuczynski, E. A., and Reynolds, A. R. (2020). Vessel co-option and resistance to anti-angiogenic therapy. *Angiogenesis* 23, 55–74. doi: 10.1007/s10456-019-09698-6

Kuczynski, E. A., Vermeulen, P. B., Pezzella, F., Kerbel, R. S., and Reynolds, A. R. (2019). Vessel co-option in cancer. *Nat. Rev. Clin. Oncol.* 16, 469–493. doi: 10.1038/s41571-019-0181-9

Kuczynski, E. A., Yin, M., Bar-Zion, A., Lee, C. R., Butz, H., Man, S., et al. (2016). Co-option of liver vessels and not sprouting angiogenesis drives acquired sorafenib resistance in hepatocellular carcinoma. *J. Natl. Cancer Inst.* 108:djw030. doi: 10.1093/jnci/djw030

Lalmahomed, Z. S., Broker, M. E., van Huizen, N. A., Coebergh van den Braak, R. R., Dekker, L. J., Rizopoulos, D., et al. (2016). Hydroxylated collagen peptide in urine as biomarker for detecting colorectal liver metastases. *Am. J. Cancer Res.* 6, 321–330.

Latacz, E., Caspani, E., Barnhill, R., Lugassy, C., Verhoef, C., Grunhagen, D., et al. (2020a). Pathological features of vessel co-option versus sprouting angiogenesis. *Angiogenesis* 23, 43–54. doi: 10.1007/s10456-019-09690-0

Latacz, E., van Dam, P. J., Vanhove, C., Llado, L., Descamps, B., Ruiz, N., et al. (2020b). Can medical imaging identify the histopathological growth patterns of liver metastases? *Semin. Cancer Biol.* doi: 10.1016/j.semcancer.2020.07.002. [Epub ahead of print].

Lazaris, A., Amri, A., Petrillo, S. K., Zoroquiain, P., Ibrahim, N., Salman, A., et al. (2018). Vascularization of colorectal carcinoma liver metastasis: insight into stratification of patients for anti-angiogenic therapies. *J Pathol Clin Res.* 4, 184–192. doi: 10.1002/cjp2.100

Leenders, W. P., Kusters, B., Verrijp, K., Maass, C., Wesseling, P., Heerschap, A., et al. (2004). Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option. *Clin. Cancer Res.* 6222–6230. doi: 10.1158/1078-0432.CCR-04-0823

Li, J., Qin, S., Xu, R., Yau, T. C., Ma, B., Pan, H., et al. (2015). Regorafenib plus best supportive care versus placebo plus best supportive care in Asian patients with previously treated metastatic colorectal cancer (CONCUR): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* 16, 619–629. doi: 10.1016/S1470-2045(15)70156-7

Liberti, M. V., and Locasale, J. W. (2016). The Warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.* 41, 211–218. doi: 10.1016/j.tibs.2015.12.001

Lopez, A., Harada, K., Vasilakopoulou, M., Shanbhag, N., and Ajani, J. A. (2019). Targeting angiogenesis in colorectal carcinoma. *Drugs* 79, 63–74. doi: 10.1007/s40265-018-1037-9

Lu, S., Tan, K. S., Kadota, K., Eguchi, T., Bains, S., Rekhtman, N., et al. (2017). Spread Through Air Spaces (STAS) is an independent predictor of recurrence and lung cancer-specific death in squamous cell carcinoma. *J. Thorac. Oncol.* 12, 223–234. doi: 10.1016/j.jtho.2016.09.129

Lucero, H. A., and Kagan, H. M. (2006). Lysyl oxidase: an oxidative enzyme and effector of cell function. *Cell. Mol. Life Sci.* 63, 2304–2316. doi: 10.1007/s00018-006-6149-9

Luengo, A., Gui, D. Y., and Vander Heiden, M. G. (2017). Targeting metabolism for cancer therapy. *Cell Chem Biol.* 24, 1161–1180. doi: 10.1016/j.chembiol.2017.08.028

Lugassy, C., Kleinman, H. K., Vermeulen, P. B., and Barnhill, R. L. (2020). Angiotropism, pericytic mimicry and extravascular migratory metastasis: an embryogenesis-derived program of tumor spread. *Angiogenesis* 23, 27–41. doi: 10.1007/s10456-019-09695-9

Lunevicius, R., Nakanishi, H., Ito, S., Kozaki, K., Kato, T., Tatematsu, M., et al. (2001). Clinicopathological significance of fibrotic capsule formation around liver metastasis from colorectal cancer. *J. Cancer Res. Clin. Oncol.* 127, 193–199. doi: 10.1007/s004320000199

Maniotis, A. J., Folberg, R., Hess, A., Seftor, E. A., Gardner, L. M., Pe'er, J., et al. (1999). Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry. *Am. J. Pathol.* 155, 739–752. doi: 10.1016/S0002-9440(10)65173-5

Melincovici, C. S., Bosca, A. B., Susman, S., Marginean, M., Mihu, C., Istrate, M., et al. (2018). Vascular endothelial growth factor (VEGF) - key factor in normal and pathological angiogenesis. *Rom. J. Morphol. Embryol.* 59, 455–467.

Mentzer, S. J., and Konerding, M. A. (2014). Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. *Angiogenesis* 17, 499–509. doi: 10.1007/s10456-014-9428-3

Motz, G. T., and Coukos, G. (2011). The parallel lives of angiogenesis and immunosuppression: cancer and other tales. *Nat. Rev. Immunol.* 11, 702–711. doi: 10.1038/nri3064

Nakamura, S., Mori, K., Iwano, S., Kawaguchi, K., Fukui, T., Hakiri, S., et al. (2020). Micro-computed tomography images of lung adenocarcinoma: detection of lepidic growth patterns. *Nagoya J. Med. Sci.* 82, 25–31. doi: 10.18999/nagjms.82.1.25

Naresh, K. N., Nerurkar, A. Y., and Borges, A. M. (2001). Angiogenesis is redundant for tumour growth in lymph node metastases. *Histopathology* 38, 466–470. doi: 10.1046/j.1365-2559.2001.01061.x

Nielsen, K., Rolff, H. C., Eefsen, R. L., and Vainer, B. (2014). The morphological growth patterns of colorectal liver metastases are prognostic for overall survival. *Mod. Pathol.* 27, 1641–1648. doi: 10.1038/modpathol.2014.4

Nierop, P. M. H., Galjart, B., Hoppener, D. J., van der Stok, E. P., Coebergh van den Braak, R. R. J., Vermeulen, P. B., et al. (2019). Salvage treatment for recurrences after first resection of colorectal liver metastases: the impact of histopathological growth patterns. *Clin. Exp. Metastasis* 36, 109–118. doi: 10.1007/s10585-019-09960-7

Nyström, H., Naredi, P., Berglund, A., Palmqvist, R., Tavelin, B., and Sund, M. (2012). Liver-metastatic potential of colorectal cancer is related to the stromal composition of the tumour. *Anticancer Res.* 32, 5183–5191.

Nyström, H., Tavelin, B., Bjorklund, M., Naredi, P., and Sund, M. (2015). Improved tumour marker sensitivity in detecting colorectal liver metastases by combined type IV collagen and CEA measurement. *Tumour Biol.* 36, 9839–9847. doi: 10.1007/s13277-015-3729-z

Ostrom, Q. T., Gittleman, H., Truitt, G., Boscia, A., Kruchko, C., and Barnholtz-Sloan, J. S. (2018). CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2011–2015. *Neuro Oncol.* 20(Suppl 4), iv1–iv86. doi: 10.1093/neuonc/noy131

Otsubo, T., Iwaya, K., Mukai, Y., Mizokami, Y., Serizawa, H., Matsuoka, T., et al. (2004). Involvement of Arp2/3 complex in the process of colorectal carcinogenesis. *Mod. Pathol.* 17, 461–467. doi: 10.1038/modpathol.3800062

Paku, S., and Paweletz, N. (1991). First steps of tumor-related angiogenesis. *Lab. Invest.* 65, 334–346.

Palmieri, V., Lazaris, A., Mayer, T. Z., Petrillo, S. K., Alamri, H., Rada, M., et al. (2020). Neutrophils expressing lysyl oxidase-like 4 protein are present in colorectal cancer liver metastases resistant to anti-angiogenic therapy. *J. Pathol.* 251, 213–223. doi: 10.1002/path.5449

Patan, S., Munn, L. L., and Jain, R. K. (1996). Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: a novel mechanism of tumor angiogenesis. *Microvasc. Res.* 51, 260–272. doi: 10.1006/mvre.1996.0025

Pezzella, F., Di Bacco, A., Andreola, S., Nicholson, A. G., Pastorino, U., and Harris, A. L. (1996). Angiogenesis in primary lung cancer and lung secondaries. *Eur. J. Cancer* 32A, 2494–2500. doi: 10.1016/S0959-8049(96)00377-2

Pezzella, F., Pastorino, U., Tagliabue, E., Andreola, S., Sozzi, G., Gasparini, G., et al. (1997). Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. *Am. J. Pathol.* 151, 1417–1423.

Pinheiro, R. S., Herman, P., Lupinacci, R. M., Lai, Q., Mello, E. S., Coelho, F. F., et al. (2014). Tumor growth pattern as predictor of colorectal liver metastasis recurrence. *Am. J. Surg.* 207, 493–498. doi: 10.1016/j.amjsurg.2013.05.015

Poulet, G., Massias, J., and Taly, V. (2019). Liquid biopsy: general concepts. *Acta Cytol.* 63, 449–455. doi: 10.1159/000499337

Qian, C. N., Tan, M. H., Yang, J. P., and Cao, Y. (2016). Revisiting tumor angiogenesis: vessel co-option, vessel remodeling, and cancer cell-derived vasculature formation. *Chin. J. Cancer* 35:10. doi: 10.1186/s40880-015-0070-2

Qiao, L., Liang, N., Zhang, J., Xie, J., Liu, F., Xu, D., et al. (2015). Advanced research on vasculogenic mimicry in cancer. *J. Cell. Mol. Med.* 19, 315–326. doi: 10.1111/jcmm.12496

Rafii, D. C., Psaila, B., Butler, J., Jin, D. K., and Lyden, D. (2008). Regulation of vasculogenesis by platelet-mediated recruitment of bone marrow-derived cells. *Arterioscler. Thromb. Vasc. Biol.* 28, 217–222. doi: 10.1161/ATVBAHA.107.151159

Reck, M., Kaiser, R., Mellemgaard, A., Douillard, J. Y., Orlov, S., Krzakowski, M., et al. (2014). Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): a phase 3, double-blind, randomised controlled trial. *Lancet Oncol.* 15, 143–155. doi: 10.1016/S1470-2045(13)70586-2

Ribatti, D., and Djonov, V. (2012). Intussusceptive microvascular growth in tumors. *Cancer Lett.* 316, 126–131. doi: 10.1016/j.canlet.2011.10.040

Risau, W., and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* 11, 73–91. doi: 10.1146/annurev.cb.11.110195.000445

Rubenstein, J. L., Kim, J., Ozawa, T., Zhang, M., Westphal, M., Deen, D. F., et al. (2000). Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. *Neoplasia* 2, 306–314. doi: 10.1038/sj.neo.7900102

Sandler, A., Gray, R., Perry, M. C., Brahmer, J., Schiller, J. H., Dowlati, A., et al. (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N. Engl. J. Med.* 355, 2542–2550. doi: 10.1056/NEJMoa061884

Sardari Nia, P., Colpaert, C., Vermeulen, P., Weyler, J., Pezzella, F., Van Schil, P., et al. (2008). Different growth patterns of non-small cell lung cancer represent distinct biologic subtypes. *Ann. Thorac. Surg.* 85, 395–405. doi: 10.1016/j.athoracsur.2007.08.054

Sato, K., Marzioni, M., Meng, F., Francis, H., Glaser, S., and Alpini, G. (2019). Ductular reaction in liver diseases: pathological mechanisms and translational significances. *Hepatology* 69, 420–430. doi: 10.1002/hep.30150

Seano, G., and Jain, R. K. (2020). Vessel co-option in glioblastoma: emerging insights and opportunities. *Angiogenesis* 23, 9–16. doi: 10.1007/s10456-019-09691-z

Semelka, R. C., Hussain, S. M., Marcos, H. B., and Woosley, J. T. (2000). Perilesional enhancement of hepatic metastases: correlation between MR imaging and histopathologic findings-initial observations. *Radiology* 215, 89–94. doi: 10.1148/radiology.215.1.r00mr2989

Shen, Y., Wang, X., Lu, J., Salfenmoser, M., Wirsik, N. M., Schleussner, N., et al. (2020). Reduction of liver metastasis stiffness improves response to bevacizumab in metastatic colorectal cancer. *Cancer Cell.* 37, 800.e7–817.e7. doi: 10.1016/j.ccell.2020.05.005

Siam, L., Bleckmann, A., Chaung, H. N., Mohr, A., Klemm, F., Barrantes-Freer, A., et al. (2015). The metastatic infiltration at the metastasis/brain parenchyma-interface is very heterogeneous and has a significant impact on survival in a prospective study. *Oncotarget* 6, 29254–29267. doi: 10.18632/oncotarget.4201

Siriwardana, P. N., Luong, T. V., Watkins, J., Turley, H., Ghazaley, M., Gatter, K., et al. (2016). Biological and prognostic significance of the morphological types and vascular patterns in Colorectal Liver Metastases (CRLM): looking beyond the tumor margin. *Medicine* 95:e2924. doi: 10.1097/MD.0000000000002924

Stessels, F., Van den Eynden, G., Van der Auwera, I., Salgado, R., Van den Heuvel, E., Harris, A. L., et al. (2004). Breast adenocarcinoma liver metastases, in contrast to colorectal cancer liver metastases, display a non-angiogenic growth pattern that preserves the stroma and lacks hypoxia. *Br. J. Cancer* 90, 1429–1436. doi: 10.1038/sj.bjc.6601727

Stremitzer, S., Vermeulen, P., Graver, S., Kockx, M., Dirix, L., Yang, D., et al. (2020). Immune phenotype and histopathological growth pattern in patients with colorectal liver metastases. *Br. J. Cancer* 122, 1518–1524. doi: 10.1038/s41416-020-0812-z

Tabernero, J., Yoshino, T., Cohn, A. L., Obermannova, R., Bodoky, G., Garcia-Carbonero, R., et al. (2015). Ramucirumab versus placebo in combination with second-line FOLFIRI in patients with metastatic colorectal carcinoma that progressed during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (RAISE): a randomised, double-blind, multicentre, phase 3 study. *Lancet Oncol.* 16, 499–508. doi: 10.1016/S1470-2045(15)70127-0

Travis, W. D., Brambilla, E., Nicholson, A. G., Yatabe, Y., Austin, J. H. M., Beasley, M. B., et al. (2015). The 2015 World Health Organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. *J. Thorac. Oncol.* 10, 1243–1260. doi: 10.1097/JTO.0000000000000630

Travis, W. D., Brambilla, E., Noguchi, M., Nicholson, A. G., Geisinger, K. R., Yatabe, Y., et al. (2011). International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. *J. Thorac. Oncol.* 6, 244–285. doi: 10.1097/JTO.0b013e318206a221

Urbich, C., and Dimmeler, S. (2004). Endothelial progenitor cells: characterization and role in vascular biology. *Circ. Res.* 95, 343–353. doi: 10.1161/01.RES.0000137877.89448.78

Valiente, M., Obenauf, A. C., Jin, X., Chen, Q., Zhang, X. H., Lee, D. J., et al. (2014). Serpins promote cancer cell survival and vascular co-option in brain metastasis. *Cell* 156, 1002–1016. doi: 10.1016/j.cell.2014.01.040

Van Cutsem, E., Tabernero, J., Lakomy, R., Prenen, H., Prausova, J., Macarulla, T., et al. (2012). Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. *J. Clin. Oncol.* 30, 3499–3506. doi: 10.1200/JCO.2012.42.8201

van Dam, P. J., Daelemans, S., Ross, E., Waumans, Y., Van Laere, S., Latacz, E., et al. (2018). Histopathological growth patterns as a candidate biomarker for immunomodulatory therapy. *Semin. Cancer Biol.* 52(Pt 2), 86–93. doi: 10.1016/j.semcancer.2018.01.009

van Dam, P. J., van der Stok, E. P., Teuwen, L. A., Van den Eynden, G. G., Illemann, M., Frentzas, S., et al. (2017). International consensus guidelines for scoring the histopathological growth patterns of liver metastasis. *Br. J. Cancer* 117, 1427–1441. doi: 10.1038/bjc.2017.334

Van den Eynden, G. G., Bird, N. C., Majeed, A. W., Van Laere, S., Dirix, L. Y., and Vermeulen, P. B. (2012). The histological growth pattern of colorectal cancer liver metastases has prognostic value. *Clin. Exp. Metastasis* 29, 541–549. doi: 10.1007/s10585-012-9469-1

Van den Eynden, G. G., Majeed, A. W., Illemann, M., Vermeulen, P. B., Bird, N. C., Hoyer-Hansen, G., et al. (2013). The multifaceted role of the microenvironment in liver metastasis: biology and clinical implications. *Cancer Res.* 73, 2031–2043. doi: 10.1158/0008-5472.CAN-12-3931

van der Schaft, D. W., Seftor, R. E., Seftor, E. A., Hess, A. R., Gruman, L. M., Kirschmann, D. A., et al. (2004). Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells. *J. Natl. Cancer Inst.* 96, 1473–1477. doi: 10.1093/jnci/djh267

van Huizen, N. A., van Rosmalen, J., Dekker, L. J. M., Coebergh van den Braak, R. R. J., Verhoef, C., IJzermans, J. N. M. et al. (2020). Identification of a Collagen Marker in Urine Improves the Detection of Colorectal Liver Metastases. *J. Proteome Res.* 19, 153–160. doi: 10.1021/acs.jproteome.9b00474

Vermeulen, P. B., Colpaert, C., Salgado, R., Royers, R., Hellemans, H., Van Den Heuvel, E., et al. (2001). Liver metastases from colorectal adenocarcinomas grow in three patterns with different angiogenesis and desmoplasia. *J. Pathol.* 195, 336–342. doi: 10.1002/path.966

Vermeulen, P. B., Sardari Nia, P., Colpaert, C., Dirix, L. Y., and Van Marck, E. (2002). Lack of angiogenesis in lymph node metastases of carcinomas is growth pattern-dependent. *Histopathology* 40, 105–107. doi: 10.1046/j.1365-2559.2002.1340c.x

Voutouri, C., Kirkpatrick, N. D., Chung, E., Mpekris, F., Baish, J. W., Munn, L. L., et al. (2019). Experimental and computational analyses reveal dynamics of tumor vessel cooption and optimal treatment strategies. *Proc. Natl. Acad. Sci. U.S.A.* 116, 2662–2671. doi: 10.1073/pnas.1818322116

Watanabe, K., Mitsunaga, S., Kojima, M., Suzuki, H., Irisawa, A., Takahashi, H., et al. (2020). The "histological replacement growth pattern" represents aggressive invasive behavior in liver metastasis from pancreatic cancer. *Cancer Med.* 9, 3130–3141. doi: 10.1002/cam4.2954

Watkins, S., Robel, S., Kimbrough, I. F., Robert, S. M., Ellis-Davies, G., and Sontheimer, H. (2014). Disruption of astrocyte-vascular coupling and the blood-brain barrier by invading glioma cells. *Nat. Commun.* 5:4196. doi: 10.1038/ncomms5196

Xiao, Q., and Ge, G. (2012). Lysyl oxidase, extracellular matrix remodeling and cancer metastasis. *Cancer Microenviron.* 5, 261–273. doi: 10.1007/s12307-012-0105-z

Xu, Y., Li, Q., Li, X. Y., Yang, Q. Y., Xu, W. W., and Liu, G. L. (2012). Short-term anti-vascular endothelial growth factor treatment elicits vasculogenic mimicry formation of tumors to accelerate metastasis. *J. Exp. Clin. Cancer Res.* 31:16. doi: 10.1186/1756-9966-31-16

Yagi, Y., Aly, R. G., Tabata, K., Barlas, A., Rekhtman, N., Eguchi, T., et al. (2020). Three-dimensional histologic, immunohistochemical, and multiplex immunofluorescence analyses of dynamic vessel co-option of spread through air spaces in lung adenocarcinoma. *J. Thorac. Oncol.* 15, 589–600. doi: 10.1016/j.jtho.2019.12.112

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors L-CC.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 14 April 2021
doi: 10.3389/fcell.2021.652544

Targeting the Urotensin II/UT G Protein-Coupled Receptor to Counteract Angiogenesis and Mesenchymal Hypoxia/Necrosis in Glioblastoma

Vadim Le Joncour[1], Pierre-Olivier Guichet[1], Kleouforo-Paul Dembélé[1], Alexandre Mutel[1], Daniele Campisi[1], Nicolas Perzo[1], Laurence Desrues[1], Romain Modzelewski[2], Pierre-Olivier Couraud[3], Jérôme Honnorat[4,5,6], François-Xavier Ferracci[1,7], Florent Marguet[8], Annie Laquerrière[8], Pierre Vera[2], Pierre Bohn[2], Olivier Langlois[1,7], Fabrice Morin[1], Pierrick Gandolfo[1†] and Hélène Castel[1†]*

[1] UNIROUEN, INSERM U1239, DC2N, Institute for Research and Innovation in Biomedicine (IRIB), Normandie Rouen Université, Rouen, France, [2] EA 4108, Laboratoire d'Informatique, de Traitement de l'Information et des Systèmes (LITIS), University of Rouen, Mont-Saint-Aignan, France, [3] Université de Paris, Institut Cochin, Inserm U1016, CNRS UMR 8104, Paris, France, [4] Neuro-Oncology Department, Hospices Civils de Lyon, Hôpital Neurologique, Bron, France, [5] Institute NeuroMyoGéne, INSERM U1217/CNRS UMR 5310, Lyon, France, [6] University Claude Bernard Lyon 1, Université de Lyon, Lyon, France, [7] Neurosurgery Service, Rouen CHU Hospital, Rouen, France, [8] Anathomocytopathology Service, Rouen CHU Hospital, Rouen, France

Glioblastomas (GBMs) are the most common primary brain tumors characterized by strong invasiveness and angiogenesis. GBM cells and microenvironment secrete angiogenic factors and also express chemoattractant G protein-coupled receptors (GPCRs) to their advantage. We investigated the role of the vasoactive peptide urotensin II (UII) and its receptor UT on GBM angiogenesis and tested potential ligand/therapeutic options based on this system. On glioma patient samples, the expression of UII and UT increased with the grade with marked expression in the vascular and peri-necrotic mesenchymal hypoxic areas being correlated with vascular density. *In vitro* human UII stimulated human endothelial HUV-EC-C and hCMEC/D3 cell motility and tubulogenesis. In mouse-transplanted Matrigel sponges, mouse (mUII) and human UII markedly stimulated invasion by macrophages, endothelial, and smooth muscle cells. In U87 GBM xenografts expressing UII and UT in the glial and vascular compartments, UII accelerated tumor development, favored hypoxia and necrosis associated with increased proliferation (Ki67), and induced metalloproteinase (MMP)-2 and -9 expression in Nude mice. UII also promoted a "tortuous" vascular collagen-IV expressing network and integrin expression mainly in the vascular compartment. GBM angiogenesis and integrin αvβ3 were confirmed by *in vivo* ^{99m}Tc-RGD tracer imaging and tumoral capture in the non-necrotic area of U87 xenografts in Nude mice. Peptide analogs of UII and UT antagonist were also tested as potential tumor repressor. Urotensin II-related peptide URP inhibited angiogenesis *in vitro* and failed

OPEN ACCESS

Edited by:
Lucas Treps,
VIB-KU Leuven Center for Cancer Biology, Belgium

Reviewed by:
Michele Caraglia,
University of Campania Luigi Vanvitelli, Italy
Yu Gan,
Shanghai Cancer Institute, China

***Correspondence:**
Hélène Castel
helene.castel@univ-rouen.fr

[†]These authors share last authorship

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 12 January 2021
Accepted: 11 March 2021
Published: 14 April 2021

Citation:
Le Joncour V, Guichet P-O, Dembélé K-P, Mutel A, Campisi D, Perzo N, Desrues L, Modzelewski R, Couraud P-O, Honnorat J, Ferracci F-X, Marguet F, Laquerrière A, Vera P, Bohn P, Langlois O, Morin F, Gandolfo P and Castel H (2021) Targeting the Urotensin II/UT G Protein-Coupled Receptor to Counteract Angiogenesis and Mesenchymal Hypoxia/Necrosis in Glioblastoma. Front. Cell Dev. Biol. 9:652544. doi: 10.3389/fcell.2021.652544

to attract vascular and inflammatory components in Matrigel *in vivo*. Interestingly, the UT antagonist/biased ligand urantide and the non-peptide UT antagonist palosuran prevented UII-induced tubulogenesis *in vitro* and significantly delayed tumor growth *in vivo.* Urantide drastically prevented endogenous and UII-induced GBM angiogenesis, MMP, and integrin activations, associated with GBM tumoral growth. These findings show that UII induces GBM aggressiveness with necrosis and angiogenesis through integrin activation, a mesenchymal behavior that can be targeted by UT biased ligands/antagonists.

Keywords: glioblastoma, urotensin II, UT receptor, angiogenesis, necrosis, biased ligand

INTRODUCTION

Malignant gliomas and mainly glioblastomas (GBMs) are the most common group of primary brain tumors with an incidence of 8.9 cases per 100,000 persons/year in the United States (CBTRUS 2008–2012). According to the WHO classification of 2016, three major diagnostic subtypes of gliomas are individualized in three groups: (i) IDH-mutant, (ii) IDH-wildtype for most comprising GBM, and (iii) IDH not otherwise specified (Louis et al., 2016). GBMs are also characterized by common histopathological features including heterogeneity with regional high abnormal vascularized networks with necrotic foci, surrounded by hyper-cellular areas of "pseudopalisading" cells (Karsy et al., 2012; Alifieris and Trafalis, 2015), correlated with response to treatment (Stupp et al., 2005; Wen and Brandes, 2009). This GBM inter- and intra- heterogeneity was highlighted by the identification of major transcriptomic subgroups including the proneural (PN), neural (N), classical (CL), and mesenchymal (MES) GBM subgroups (Verhaak et al., 2010), potentially recapitulated within a GBM resection fragment (Wick and Kessler, 2018), strongly supporting the heterogeneous and constantly metamorphosing nature of GBM (Aubry et al., 2015).

The pronounced vascularization of GBM exhibits aberrant, malfunctioning, and leaky features resulting in vasogenic edema and increased tissue hypoxia sustaining the increased tumor malignancy (Takano, 2012; Bougnaud et al., 2016; Cavazos and Brenner, 2016). Angiogenic regulators are secreted by GBM cells but also infiltrating myeloid cells such as tumor-associated macrophages (TAMs) and Tie-expressing monocytes via ligands expressed by the tumor and/or stromal cells and targets present at the endothelial level (Eelen et al., 2020). The well-known anti-human VEGFA antibody bevacizumab has been shown not only to reduce tumor edema, angiogenesis, and disease burden but also to provoke adaptive escape mechanisms involving hypoxia by pruning tumor blood vessels, switching to a glycolytic metabolism, neo-vascularization, and/or infiltrative tumor growth (Norden et al., 2008; Eelen et al., 2020). Tumor relapse is associated with cell invasion of brain parenchyma likely involving chemotactic factors (Xie et al., 2014), such as cytokines, growth factors, chemokines, or vasoactive peptides activating G protein-coupled receptors (GPCRs) (Hembruff and Cheng, 2009; Borsig et al., 2014). Glioma cells but also macrophages are a major source of chemoattractants and they express one or more chemoattractant GPCRs to their advantage (Cherry and Stella, 2014; Gagliardi et al., 2014; Zhou et al., 2014), allowing leukocyte attraction or exacerbating angiogenesis (Glass and Synowitz, 2014). Some vasoactive peptides and their GPCRs, conventionally involved in the angiogenic and inflammatory processes, constitute promising therapeutic targets in angiogenic and invasive tumors. The selective blockade of the angiotensin II (Ang-II) receptor AT1 by losartan inhibits the development of murine glioma and decreases tumor neo-angiogenesis (Rivera et al., 2001; Arrieta et al., 2005). The antagonism of endothelin-1 (ET-1) receptor by atrasentan in a phase I clinical study showed partial responses, lasting stabilization, or even absence of progression of GBM (Phuphanich et al., 2008). The immunoneutralization of adrenomedullin (ADM) caused a dramatic inhibition of prostate and GBM tumor development in Nude mice, accompanied by a drastic diminution of intratumoral vascular density (Kaafarani et al., 2009; Metellus et al., 2011).

Human urotensin II (UII) (Coulouarn et al., 1998) and its peptide paralog UII-related peptide (URP) (Sugo et al., 2003) are cyclic neuropeptides of 11 and 8 amino acids in human, respectively, and exhibit a fully conserved C-terminal cyclic hexapeptide (CFWKYC) core that plays a major role in biological activity (Brulé et al., 2014; **Table 1**). UII and URP are considered the most potent endogenous vasoactive molecules known so far acting through a common GPCR called UT, being evolutionary linked to chemokine GPCRs (Ames et al., 1999; Brulé et al., 2014; Castel et al., 2017).

TABLE 1 | Names, sequences, and characteristics of the different urotensinergic peptide and non-peptide ligands used in the study.

UT ligand	Known function	Sequence
Human UII (UII)	Endogenous agonist	HTPD[CFWKYC]V
Murine UII (mUII)		pEHGAAPE[CFWLYC]I
UII-related peptide (URP)		HA[CFWKYC]V
UII_{4-11}	Synthetic agonist	HD[CFWKYC]V
Urantide	Peptide antagonist/biased ligand	D[*Pen*-F_DW-*Orn*-YC]V
Palosuran	Non-peptide antagonist	1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt)

Cyclic core domain (disulfide bonds between two C residues) is represented by brackets. Unnatural amino acids: Orn, ornithine; Pen, penicillamine.

First studies established that the urotensinergic system is involved in migration of rat fibroblasts (Zhang et al., 2008), rat endothelial progenitor cells (Xu et al., 2009), human monocytes (Segain et al., 2007), and *in vitro* angiogenesis (Spinazzi et al., 2006), therefore stressing on the potential chemokine and pro-angiogenic status of UII in cancer. Indeed, UT receptor was shown to regulate migration of prostatic (Grieco et al., 2011), bladder (Franco et al., 2014), and colon (Federico et al., 2014) cancer cell lines. More recently, we and others proposed UII as an original chemokine (Castel et al., 2017; Sun and Liu, 2019), stimulating lung or colorectal cancer cell proliferation and metalloproteinase-9 (MMP-9) activation (Zhou et al., 2012). In the central nervous system (CNS), UT was detected in the vascular compartment (Clavier et al., 2018), in astroglial processes, and in human native astrocytes and GBM cell lines (Castel et al., 2006; Jarry et al., 2010; Desrues et al., 2012). In GBM cells, UII induces chemotactic migration/adhesion *via* G13/Rho/ROCK/actin polymerization and partially the Gi/o/PI3K pathways involving inhibition of the autophagy process (Lecointre et al., 2015; Coly et al., 2016). The chemoattractant activity of the UII-UT system may play multiple roles during glioma invasion or on microenvironmental cells leading to angiogenesis. In the present study, we characterized the expression of UII peptides and their receptor UT in a series of human glioma biopsies compared with normal brain tissue by immunohistochemistry and from transcriptome array analyses of gliomas from The Cancer Genome Atlas (TCGA) database as well as by quantitative-PCR (qPCR) in glioma cell lines. We found marked increased expression of UII and UT in high malignancy gliomas associated with the vascular component in GBM. The native UII and no other urotensin analog stimulates endothelial tube formation *in vitro* and promotes the *in vivo* recruitment of pro-angiogenic cells in matrices' sponges. In heterotopic U87 GBM cells xenografted in Nude mice, intratumoral injections of UII accelerated proliferation and GBM growth and exacerbated abnormal angiogenesis associated with hypoxia and necrotic features confirmed by *in vivo* ^{99m}Tc-RGD tracer imaging and tumoral capture in non-necrotic areas. The UT antagonist/biased ligand urantide and the non-peptide UT antagonist palosuran prevented UII-induced tubulogenesis *in vitro* and significantly delayed tumor growth *in vivo*. Urantide drastically prevented UII-induced GBM angiogenesis, MMP-2 and MMP-9 expression, and integrin activation associated with GBM growth. The specific blockade of UT receptor signaling should constitute a new multicellular targeting option in the therapeutic arsenal against GBM.

MATERIALS AND METHODS

Tumor Patient Samples

Non-tumoral brain tissues and brain glioma tumors were obtained from patients cared at the Rouen CHU Hospital in France from 2008 to 2014. Sixty-six tumors were taken from the Center of Biological Resources located in the Department of Pathology, Rouen University Hospital. The selection criteria were a diagnosis of glial tumor, surgical resection or biopsy, treated by surgery alone, surgery plus radiotherapy, surgery plus radiotherapy and Temozolomide chemotherapy, or surgery plus radiotherapy and PCV (Procarbazine, Lomustine, and Vincristine) chemotherapy (Stupp protocol), and patients with completed clinical information (see **Table 2** for detailed information). All patients provided written informed consent for the study. The age of patients, their clinical outcomes, and tumor histopathologic classifications were typical of the category of adult with diffuse glioma (Verhaak et al., 2010; Brennan et al., 2013).

Immunohistochemistry

Four-micrometer sections were obtained from formalin-fixed paraffin-embedded tissues. Before antibody staining, heat-induced antigen retrieval was performed in a sodium citrate buffer solution at 95°C for 45 min. Immunohistochemistry was performed using Envision GI2 Doublestain System Rabbit/Mouse kit (Dako, K5361) according to the manufacturer's protocol. Primary antibodies against Urotensin II (Sigma-Aldrich, HPA017000, 1:50), urotensin II receptor (Santa Cruz, sc20940, 1:50), CD34 (Novocastra, RTU-END, 1:1), CA9 (Abcam, ab15086, 1:1000), and α-SMA (Sigma-Aldrich, A2547, 1:500) were used and incubated overnight at 4°C. Rabbit polyclonal and mouse monoclonal antibodies were revealed with DAB and Permanent red, respectively. Nuclei were counterstained with hematoxylin. For each tumor series, a consecutive section was stained using hematoxylin–eosin (H&E) for structural information.

Scoring of UII/UT Staining in Human Biopsies

Tissue samples were independently scored by two pathologists (AL and FM) and one scientist (POG). The percentage of positive cells (P) was determined as follows: $P = 0$, no positive cells; $P = 1$, <10% of positive cells; $P = 2$, 11–50% of positive cells, $P = 3$, >50% of positive cells. Semi-quantitative evaluation of immunolabeling intensity (I) was determined as follows: $I = 0$, no staining; $I = 1$, weak expression; $I = 2$, moderate expression; $I = 3$, strong expression. The score was expressed as the sum of (P + I) comprised between 0 and 6. For each sample, we scored the expression of UII and UT in three different tumor locations: parenchyma, vascular, and perinecrotic components. The total score was the sum of each score.

Culture of Cell Lines

The human glioma SW1088 and U87 cell lines were obtained from ATCC (LGC Standards, Molsheim, France) and 8MG, 42MG, and U251 cell lines were provided by Pr. J. Honnorat (CRNL, Lyon, France). Culture media components were purchased from Life Technologies (Thermo Fisher Scientific, Saint-Aubin, France) or Lonza (Levallois-Perret, France). U87 cells were maintained in minimum essential medium (DMEM) supplemented with sodium pyruvate (1%), non-essential amino acids (1%), and antibiotics (1%) all from Fischer Scientific (Illkirch, France) and heat-inactivated fetal bovine serum (FBS, 10%). Human umbilical vein (HUV-EC-C, from ATCC) and human cerebral microvascular (hCMEC/D3) (Weksler et al., 2005, 2013) endothelial cell lines were maintained in endothelial

TABLE 2 | Clinico-demographical parameters of the different glioma groups studied retrospectively and prospectively.

CNS tissues	Grade WHO 2016	Grade WHO 2008	Subtype	*N*	Median age	Gender (M/F)	Localization	Treatment
Gliomas	**I**		Pilocytic astrocytoma	**8**	16 (2–25)	4/4	CH (*n* = 6), V (*n* = 2)	CR
	II			**13**	36 (26–60)	8/5	SC (*n* = 11), BG (*n* = 1), BS (*n* = 1)	CR alone (*n* = 4), T (*n* = 5), RT (*n* = 4)
		AII	Diffuse astrocytoma, IDH1-mutant	3				
		AII	Diffuse astrocytoma, IDH1-wildtype	3				
		OII	Oligodendroglioma, IDH1-mutant and 1p/19q-codeleted	3				
		OIII	Oligodendroglioma, IDH1-wildtype and 1p/19q-codeleted	1				
		OAII	Oligoastrocytoma, NOS	3				
	III			**15**	52 (24–71)	11/4	SC (*n* = 15)	CR alone (*n* = 1), STUPP (*n* = 6), RT + PCV (*n* = 1), RT alone (*n* = 6), T alone (*n* = 1)
		AIII	Anaplastic astrocytoma, IDH1-mutant	1				
		AIII	Anaplastic astrocytoma, IDH1-wildtype	6				
		OIII	Anaplastic oligodendroglioma, IDH1-mutant and 1p/19q-codeleted	4				
		OAIII	Anaplastic oligoastrocytoma, NOS	4				
	IV			**24**	56 (23–75)	16/8	SC (*n* = 23), M (*n* = 1)	STUPP (*n* = 23), RT alone (*n* = 1)
		GBM	Glioblastoma, IDH1-mutant	5				
		GBM	Glioblastoma, IDH1-wildtype	19				
Non-tumoral				**6**	45 (32–49)	1/4	–	–
			Hippocampal sclerosis	4				
			Focal cortical dysplasia	1				
			Normal cerebellum	1				

*Gradings are indicated along histopathological classification (see **Figure 1**) and according to the WHO classification 2016 from last information available in 2017. BG, basal ganglia; BS, brainstem; CH, cerebellar hemisphere; CR, chirurgical resection; IDH1mut, IDH1(R132H) positive (immunochemistry); IDH1wt, IDH1(R132H) negative (immunochemistry); M, medullary; N, number of samples; NOS, not otherwise specified; PCV, procarbazine, lomustine (CCNU) and vincristine; RT, radiotherapy; SC, supratentorial cortex; STUPP, therapy protocol involving radiotherapy plus concomitant and adjuvant Temozolomide; T, Temozolomide; V, vermis. Bold values correspond to the total of samples per/grade in the table.*

basal medium-2 (Lonza, Bâle, Switzerland) containing chemically defined concentrated lipids (1%, Fischer Scientific), HEPES (1%, Fischer Scientific), hydroxycortisone (0.2 μg/ml, Sigma-Aldrich), bFGF-2 (50 ng/ml, Eurobio Abcys, Courtaboeuf Les Ulis, France), ascorbic acid (1 μg/ml, Fischer Scientific), and gold-defined FBS (5%, PAA Laboratories). When cultures reached 90% of confluence, cells were harvested and prepared for *in vitro* experiments according to the following procedures or were just rinsed in PBS for transplantation in Nude mice.

Vascular Characteristic of Malignant Glioma Patient Samples

For each patient sampled tumor, at least three microphotographs were taken (Nikon 1 widefield microscope) from slices stained with an anti-CD34, and saved as 2560 × 1920 pixels pictures. Three different vascular properties were analyzed on vascular CD34+ structures with vascular histological characteristics by using ImageJ software (NIH, Bethesda, MD, United States). First, the vascular density was quantified by manually isolating each microvessel in the field and summed, and then microvessel coverage area was normalized to the whole tumoral area. Vessel diameter was quantified from transversal vessel sections, after manual selections, and the highest radius value was used for the quantification by using ImageJ shape descriptors feature. Vessel circularity was quantified on transversal sections to estimate vessel irregularities, after manual selections, and measured by using the following equation:

$$\frac{4\pi[Area]}{[Perimeter]^2}$$

Quantitative Real-Time PCR Analysis

Total RNA was isolated from tumor cell line extracts using TRIzol reagent according to the manufacturer's protocol (Sigma-Aldrich, Saint-Quentin-Fallavier, France). First-strand cDNA were transcribed from 1 μg of total RNA in quadruplicates using the Superscript II reverse transcriptase (random primers method, Promega, Charbonnières-les-bains, France). For quantitative RT-PCR, cDNA amplification was monitored using SYBR Green (Thermo Fisher Scientific, Illkirch, France) chemistry on the real-time PCR system (QuantStudio3, Applied Biosystems, Zug, Switzerland). Primers were designed by retrieving nucleotide sequence from NCBI gene database and using the Primer3Plus program[1]. The specific primers targeting UTS2 (encoding UII), UTS2D (encoding URP), UTS2R (encoding UT), and MMP9 and ITGAV (encoding integrin αv) mRNA were synthesized by Eurofins genomics (Les Ulis, France), and their sequences are listed in **Supplementary Table 1**. The conditions for PCR reactions were 45 cycles, 95°C/1 s, 60°C/20 s, a step specific for UII mRNA (73°C/5 s), and 95°C/1 s using the primers specified in **Supplementary Table 1**. Minus-reverse transcription ("-RT") controls were systematically performed, and the quality of PCR products was evaluated by generating a melting curve, which was also used to verify the absence of PCR artifacts (primer dimers) or non-specific PCR products. Samples were amplified in triplicates (three different culture flasks and three different RT) and relative mRNA copy levels were determined using the comparative ΔΔCt method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ubiquitin C (UBC) transcript levels were used as a reference to control mRNA levels and stability within each cell line. Results were analyzed by using the Quantstudio design and analysis software (Applied Biosystems) and are expressed as mean of gene of interest expression relative to GAPDH or UBC reference gene.

Tumor Data Base Analysis

The clinical and molecular data on low-grade glioma and GBM samples for this study were downloaded from The Cancer Genome Atlas database[2] firstly in 2014, and the molecular information was again downloaded in February 2016. The clinical information used was the overall patient survival. The molecular data used were mRNA expression levels of UTS2, UTS2D, and UTS2R obtained from RNA-seq information by means of Illumina TruSeq Kit Paired-end Sequencing on Illumina HiSeq2000 (Cancer Genome Atlas Research Network, 2008), and the gene expression level within the GBM subgroups of the Verhaak classification was obtained from different microarray platforms previously processed and summarized (Verhaak et al., 2010; Brennan et al., 2013). Detection of somatic variants (IDHmut) from the TCGA Whole-Exome Sequencing and RNA-Seq data was done using RADIA. RADIA is a computational method combining the patient-matched normal and tumor DNA with the tumor RNA (Lecointre et al., 2015; Coly et al., 2016). The mRNA levels were represented as "normalized count" corresponding to a transformation of the "raw_count." For each gene of interest, all "raw_count" values were divided by the 75th percentile of the column patient (after removing zeros) and multiplied by 1,000. For the gene-level survival analysis, we divided the corresponding cohorts (all glioma) by the mean gene expression level (low or high) of UTS2, UTS2D, or UTS2R. We then used a log rank (Mantel–Cox) test to compare the survival durations between the two groups.

Western Blot of Pre-Pro UII and UT

Glioblastoma cell lines and hCMEC/D3 cells were treated during 24 h in the absence of FBS and in the absence or the presence of UII (10^{-9} M). Cell lysates (20 μg total proteins) were prepared in ice-cold Lysis Buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% SDS), mixed with Laemmli buffer and loaded onto 4–12% polyacrylamide gels (CliniSciences, Nanterre, France). Proteins were transferred onto PVDF membranes, blocked with 5% non-fat milk and 5% bovine serum albumin, and incubated with the anti-pre-pro UII (Sigma, HPA-01700), UT (H-90) (Santa-Cruz, sc-20940), or β-tubulin (Santa-Cruz, sc-9104) overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibodies (Santa-Cruz) for 2 h at room temperature. Immunoreactive bands were visualized by using the ECL Western blotting substrate (GE Healthcare, Aulnay-sous-Bois, France) and their molecular weight were determined by using PageRuler Plus prestained protein ladder (10–250 kDa) markers (Fisher Scientific).

Cell Migration Assay

Transwell membranes (24 wells, Corning, Fisher Scientific) with 8-μm pores were coated with 25 μg/ml collagen I (Invitrogen) (4°C, 12 h). Endothelial and glioma cells (5×10^4) suspended in 100 μl of culture medium (with 1% gold-defined FBS for endothelial cells) were added onto the upper chamber of the Transwell and UT ligands (600 μl) were added in the bottom chamber. After 24-h incubation at 37°C, cells were rinsed with PBS and the non-migrating cells were removed using a cotton swab from the upper chamber. Migrating cells were fixed in successive methanol baths and stained with H&E, and then membranes were mounted on glass slides with Mowiol (Calbiochem, Molsheim, France). Random phase contrast images were acquired using a digital camera (Nikon D-600), and image analysis was carried out using *ImageJ*'s Cell Counter plugin (NIH, Bethesda, United States).

In vitro Tubulogenesis Assay

Growth factor-reduced Matrigel (Beckon Dickinson, Le Pont-de-Claix, France) was thawed on ice overnight and spread homogeneously (100 μl) in 24-well plates for 30 min at 37°C to allow Matrigel polymerization. Endothelial cells (5×10^4 cells/cm^2) were seeded in basal medium and incubated for 24 h in the absence or the presence of UT agonists (Phoenix Pharmaceuticals, Inc) (UII, mUII, URP, UII_{4-11}, **Table 1**) at 10^{-12} to 10^{-7} M, the UT antagonist/biased ligand urantide (Peptides International, Louisville, KY, United States, **Table 1**), or the UT antagonist palosuran (Actelion Pharmaceuticals, Allschwil, Switzerland, **Table 1**) from 10^{-10} to 10^{-7} M. Phase contrast image analysis was carried out using *ImageJ* software.

[1]http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/

[2]https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga

Animal Studies

All procedures were performed in accordance with the French Ethical Committee as well as the guidelines of European Parliament directive 2010/63/EU and the Council for the Protection of Animals Used for Scientific Purposes. This project was approved by the "Comité d'Ethique NOrmandie en Matière d'EXpérimentation Animale" CENOMEXA under the National Committee on Animal Experimentation and received the following number N/13-11-12/36/11-17. Animal manipulations were carried out under the supervision of an authorized investigator (H. Castel; authorization no. 76.98 from the Ministère de l'Alimentation, de l'Agriculture et de la Pêche and surgery agreement). For the tumorigenesis studies, 6-week-old male athymic *Swiss* (*nu/nu*) mice (Charles River Laboratories, L'Arbresle, France) were used. For the *in vivo* chemoattraction assay, 3- to 6-week-old male C57Bl/6 mice (Janvier Labs, Saint-Berthevin, France) were used. Mice were housed in sterile cages, in a temperature-controlled room with a 12-h light/12-h dark schedule and fed with autoclaved food and water *ad libitum*.

In vivo Chemoattraction Assay

Male C57B/l6 mice were injected subcutaneously above the pelvis area with 200 μl of liquid growth factor-reduced Matrigel combined either with EG-VEGF (Miltenyi Biotechnologies, Paris, France) or with human recombinant EGF (R&D Systems, Metz, France) (500 ng/ml, each), UT agonists (UII, mUII, URP, and UII_{4-11}) at 50 ng/ml, UT antagonists/biased ligand palosuran or urantide at 1 μg/ml, or alone as a negative control. Subcutaneous plug incubation continued for 21 days or until mice had to be sacrificed following institutional ethical guidelines criteria. Matrigel plugs were resected and immediately frozen in a −40°C isopentane solution until immunohistochemistry.

Xenograft Studies

U87 cells were injected subcutaneously into the right flank (3×10^7) of 6-week-old male athymic mice. Mice were randomized into treatment groups after tumors were developed (mean s.c. volume = 100 mm^3, 400–600 mm^3). UT agonists (UII, mUII, URP, and UII_{4-11}; 2.9 ng/kg, each) or UT antagonists/biased ligand (palosuran, 29 ng/kg; urantide 290 ng/kg) or saline solution (vehicle) was administered intratumorally every day. Subcutaneous tumor volumes were measured with a caliper every 2 days, by using the following formula: width × length × height × 0.52. Treatment continued until mice had to be sacrificed (i.e., 2-cm^3 maximal tumor dimension, cutaneous ulceration, or symptoms) or study end. In some experiments, 30 min before euthanasia, pimonidazole (60 mg/kg, Hypoxyprobe^{TM}-1 Omni Kit, Burlington, United States) was administered intravenously, and glioma tissues were collected and immediately frozen in a −40°C isopentane solution until immunohistochemistry.

Animal Immunohistochemistry

Cryostat sections of Matrigel plugs (10 μm) were processed in a Leica CM1900 cryostat, or xenograft sections (9 μm) were processed in a Leica CM1950 cryostat, mounted directly on slides, and then fixed in 4% paraformaldehyde. Non-specific binding sites were blocked with normal serum (10%) from the animal source of the appropriate corresponding secondary antibody. Immunolabeling was conducted using primary antibodies against UT receptor, F4/80 (Santa Cruz Biotechnologies, Heidelberg, Germany), collagen-IV (Coll-IV, Merck-Millipore, Molsheim, France), α-smooth muscle cell actin (α-SMA) and Prestige-Graded UII (Sigma-Aldrich, Saint-Quentin Fallavier, France), or α_V integrin, MMP-2 and MMP-9, and CD31 and CD34 (Abcam, Paris, France), diluted according to the manufacturer's technical specifications in the incubation media [0.1 M Tris (pH 7.5), 0.15 M NaCl, 10% normal serum, and 0.03% Triton X-100] and then incubated overnight at 4°C. Signal amplification utilized fluorochrome (Alexa 488 or Alexa 594)-conjugated secondary antibodies (Life Technologies, Saint Aubin, France), and cell nuclei DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Control sections were incubated in the absence of the primary antibodies.

Micro-SPECT Imaging and ^{99m}Tc RGD Radioligand

Cyclo(RGD_DY[K-HYNIC]) (HYNIC-RGD) was synthesized on a solid support using standard solid-phase synthesis and was purchased from the PolyPeptide Group (Strasbourg, France). The labeling method following the previously described protocol (Becker et al., 2015) is more detailed in the **Supplementary Material**, and chemical structures are shown in **Supplementary Figure 6A**. Other reagents were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). $Na^{99m}TcO_4$ was obtained by elution, with 0.9% saline, from a commercial $^{99}Mo/^{99m}Tc$ generator (Ultra-TechneKow; Covidien, Petten, Netherlands). The RGD-derived peptide purity was greater than 95% as analyzed by reverse-phase high-performance liquid chromatography and mass spectroscopy, and the net peptide content was estimated by elemental analysis.

SPECT imaging was performed using a small animal imaging system with parallel collimators and a 140 keV ± 10% photopeak energy window (Triumph SPECT/CT; GMI, Los Angeles, CA, United States) and GE Healthcare (Fairfield, CT, United States). U87-xenografted Nude mice were injected through the tail vein with ∼30 MBq (in about 0.1 ml) of [^{99m}Tc]-HYNIC-RGD under continuous isoflurane anesthesia (2.5% in O_2, 1 L/min). [^{99m}Tc]-HYNIC-RGD dynamic imaging was carried out 1 h after injection and planar images were recorded every minute. [^{99m}Tc]-HYNIC-RGD SPECT imaging was carried out 2 h after injection. Treatment of the dynamic imaging is detailed in **Supplementary Methods**.

Ex vivo Micro-SPECT Imaging of Tumors

In tumor samples examined *ex vivo*, 30 MBq was precisely measured and diluted by a factor of 1000 to obtain a dose calibrator. Background counts were subtracted, and the radioactive decay was corrected to the time of injection. The radioactivity concentration that had accumulated in the tissue samples over the 2-h period following the [^{99m}Tc]HYNIC-RGD injection was expressed as a percentage of the injected

dose (%ID) and normalized per gram of tissue (%ID/g). For *ex vivo* biodistribution, samples of various tissues were collected 120 min after injection and weighed, and radioactivity was measured using an automatic gamma counter (1470–001 Wizard Gamma Counter; PerkinElmer and Wallac, Turku, Finland) calibrated with a gamma peak calibrator (^{129}I). A ^{99m}Tc sample was measured using an activity calibrator (MEDI404; Medisystem, Guyancourt, France). Detailed methods of visualization and quantification of radioactivity were provided in the **Supplementary Methods**.

Statistical Analyses

Data were expressed as mean ± SEM and GraphPad Prism (version 5; GraphPad Software, Inc., La Jolla, United States) was used for statistical analyses. Student *t*-test was used for parametric comparisons between paired variables, the Mann–Whitney *U* test was used for non-parametric pairwise comparisons, multivariate analyses were done with ANOVA with *post hoc* Dunnett (*in vitro* analyses) or Bonferroni tests (e.g., *in vivo* studies) as appropriate, the vascular property multivariate analyses were done with Kruskal–Wallis and Dunn's post-test, and survival curves were generated by the Kaplan–Meier method. Correlation is considered significant when $P < 0.05$, and r represents the Pearson correlation coefficient. All reported *P*-values were two-sided and considered to be statistically significant at $P < 0.05$.

RESULTS

UII and UT Expression in Patient Gliomas and Human Glioma Cell Lines

The UII/UT system was first characterized on different glial tumors from patient tumor samples obtained after a first tumor resection or a small size biopsy (tumor bank of Haute-Normandie, France) by immunohistochemistry. These glial tumors were presented according to WHO 2008 histopathological classification, ranging from astrocytoma (AII/III), oligodendroglioma (OII/III), or oligoastrocytoma (OAII/III) to highly aggressive glioblastoma multiform (GBM, IV) based on morphological features and associated with a very poor prognosis (Xu et al., 2015). Since 2016, grade II diffuse and grade II anasplatic astrocytoma have been divided into mutated and wild-type IDH1/2 even if the great majority falls into IDHmut; astrocytomas, grade II oligodendroglioma are IDHmut and 1p/19q codeleted while GBMs are most often represented by GBM IDHwt (90% of cases) (Louis et al., 2016). Sixty patients were included retrospectively and prospectively, and tumor grades and molecular signatures were described according to the WHO 2016 classification in **Table 2**. The anatomic sites of the tumors were supratentorial cortex (49/60, 81%), basal ganglia (1/60, 2%), brainstem (1/60, 2%), intramedullary (1/60, 2%), cerebellar hemisphere (6/60, 10%), and vermis (2/60, 3%) (**Table 2**). Immunohistochemical analysis showed a higher expression of the UII/UT couple in astrocytomas (AII/III) compared with oligodendrogliomas (OII/III) when positive cells were >50% (**Figure 1A**). In GBMs, strong localized expression of UII and UT was observed in vascular and perinecrotic areas, in immunoreactive zones for respectively, the mesenchymal marker α-SMA-positive cells and for the carbonic anhydrase 9 (CA9) (**Figure 1B**), suggesting links between tumoral pericytes and hypoxia. From consecutive sections of non-tumor CNS tissue, the UII and UT immunostaining appeared in gray matter (GM), in particular in neurons instead of oligodendrocytes (peri-neuronal satellitosis) and in the white matter (WM) (**Figure 1C**). Score analysis of the UII and UT staining indicated a gradual increased expression with the grade, from grade I (PA) to grade IV (GBM), and also from OII/OIII, OAII/III, and AII/III (**Figures 1A,D**) reaching significant scores in GBM compared with PA (**Figure 1D**). As shown in **Figure 1E**, the vascular density and diameter were significantly increased in anaplastic astrocytoma and GBM while vessel defaults in circularity were shown altered in GBM. The expression levels of UII and UT were significantly correlated with increased vascular density, mainly in high-grade tumors (**Figure 1E**) and UII was also correlated with UT (**Figure 1F**) (Pearson coefficient, $P < 0.001$). Progression-free survival curves of 20 patients with primary GBM indicated that high expression of the urotensinergic components were significantly correlated with early recurrence (UII, score > 11.5/18; UT, score > 12.5/18) and were correlated with a median survival of 224 days when any of the UII and UT are highly expressed (**Figure 1F**). Expression mRNA level of UTS2 encoding UII, UTS2R encoding UT, and UTS2D encoding URP was also tested from TCGA (**Figure 2**). For each gene, profiles of normalized expression were extracted from 66 glioma samples presented in **Figure 2A** according to the grading classification. UTS2 was found to be significantly more expressed in astrocytoma and mostly GBM, suggesting an association with high-grade gliomas. As observed in the histopathological classification, UTS2R and UTS2D were equally expressed in IDHwt or IDHmut tumors, whereas UTS2 displayed a higher expression in IDHwt gliomas, suggesting an association with malignancy (**Figure 2B**). Within the four groups of GBM along with the Verhaak classification (Verhaak et al., 2010), UTS2R (UT) and UTS2D (URP) were expressed in all groups of GBM, but importantly, UTS2 (UII) exhibited a net higher expression in the mesenchymal subgroup, suggesting that UII may be associated with glioma prognosis (**Figure 2C**). UTS2R and UTS2 mRNA levels from TCGA showed that patients having high UTS2R or UTS2 expression exhibit significantly lower mean survival probability, while no difference was observed for UTS2D (**Figure 2D**). These data establish a relationship between UII, and also UT expression in GBM, mainly with the hypoxic/vascularized state of the more aggressive GBM. QPCR analyses of mRNAs encoding UII, URP, and UT in the SW1088 (anaplastic astrocytoma) and in U87, U251, 8MG, and 42MG GBM cell lines here established that expression levels were non-homogeneous and higher for UTS2R in 8MG and 42MG, UTS2 in U87, and UTS2D in SW1088 (**Figure 3A**). To verify a possible autocrine/paracrine mechanism in which UII may in turn induce UTS2 or UTS2R expression encoding UII and UT, we evaluate 24-h GBM cells as well as a human EC line hCMEC/D3 exposed to UII (10^{-9} M). UII stimulated the expression of UII at least in part by U87 and 8MG as confirmed by the qPCR and Western blot

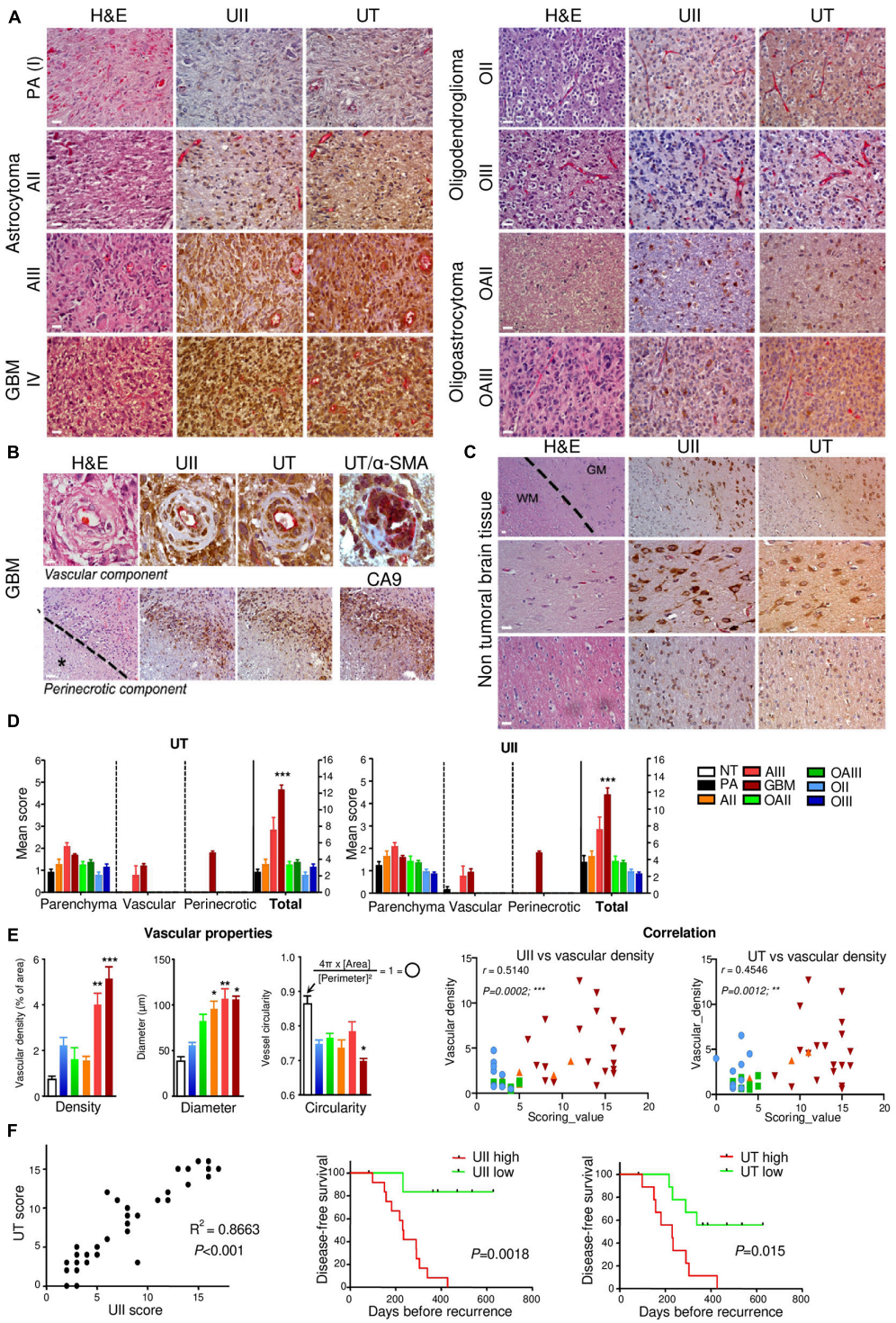


FIGURE 1 | Characterization of UII and UT expression in non-tumoral and glioma samples. **(A)** UII and UT co-labeling (brown) with anti-CD34 immunoreactivity (pink) on consecutive sections from different grades of astrocytomas [PA (I), AII, and AIII], oligodendrogliomas (OII and OIII), and oligoastrocytomas (OAII and OAIII) along the histopathological classification showed a more intense staining in AIII and GBM tumors (left panels) compared with O and mixed OA tumors (right panels). **(B)** UII and UT immunohistochemical co-expression with anti-CD34 structures on consecutive sections of GBM samples revealed staining in vascular cells of hyperplasic vessels (upper panels) and in pseudopalisading cells around areas of necrosis (lower panels). Co-expression of UT (brown) with α-SMA (pink) (upper right panel) in smooth muscle/pericyte vascular components. CA9 staining (brown) highlights hypoxic environment around necrotic areas (lower right panel) exhibiting immunoreactivity for UII and UT. The black star and the dotted line in the lower panels indicate necrosis location. **(C)** UII and UT expression on consecutive sections of three different non-tumoral tissues showed a strong staining in the gray matter (GM) especially in neurons (upper and middle panels) compared to

(Continued)

FIGURE 1 | Continued
oligodendrocytes from the GM (peri-neuronal satellitosis) and in the white matter (WM) (upper and lower panels). The dotted line delineates the frontier between GM and WM. **(D)** Quantification of experiments presented in **(A,B)** and represented as the mean ± SEM of UT (left panel) and UII (right panel) scores (see section "Materials and Methods"). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (one-way ANOVA and multiple comparison test with Tukey's correction). **(E)** Vascular density, diameter, and circularity quantified from NT and malignant glioma (AII/OII to GBM) samples based on the CD34-positive structures and histological characteristics from, at least in part, three different images per patient tumor slice. *Left*, Histograms of mean ± SEM (Kruskal–Wallis and Dunn's post-test: $***P < 0.001$). *Right*, Scatter plot of the correlation between UII scoring value versus vascular density (left) and UT scoring value versus vascular density (right). Score correlation in glioma showed a significant correlation between UII and UT and vascular density more particular within AIII and GBM samples. **(F)** Total score correlation in glioma samples showed a significant correlation between UII and its receptor UT. Disease-free survival curves of 20 primary GBM for UII (left panel) and UT (right panel). In **(E,F)**, the correlation is considered significant when $P < 0.05$, r represents the Pearson correlation coefficient. H/E: Hematoxylin–Eosin staining. Scale bar = 10 μm for **(A,B)**, and (**C**, middle and lower panels), and 20 μm for (**C**, upper panel).

analysis, but not in EC constitutively expressing UII and UT (**Figures 3B,C**).

UT Mediates Motility of GBMs and Endothelial Cells and Stimulates Tubulogenesis *in vitro*

To examine the role of UII on glioma tumorigenesis, we used U87 GBM cells, endogenously expressing UII and functional UT (**Figure 4A**). We confirmed that UT activation by UII (10^{-9} and 10^{-8} M) leads to chemotactic migration in Boyden's chamber assay (**Figure 4B**) and did not alter cell density after a 48-h treatment (**Supplementary Figure 1A**). We confirmed that hCMEC/D3 expressed UT at the plasma membrane and UII in the cytoplasmic and perinuclear compartments (**Figure 4A**). UII (10^{-10} and 10^{-9} M) stimulated hCMEC/D3 cell migration (**Figure 4B**) with no measurable effects on hCMEC/D3 or HU-VEC-C cell density/proliferation (**Supplementary Figures 1B,C**). These observations were suggestive of proinvasive and angiogenic capacities of the UII/UT system. We then tested the ability of the UT endogenous agonists UII and URP, the shorter synthetic peptide analog referred to as a full agonist UII_{4-11}, the UT antagonist/biased ligand urantide (Camarda et al., 2004; Brulé et al., 2014), and the UT antagonist palosuran (Clozel et al., 2004; **Table 1**) to promote tubular organization of hCMEC/D3 and HUV-EC-C cells (**Figure 4C**). Only UII was able to enhance the endothelial network complexity in both hCMEC/D3 and HUV-EC-C, whereas URP and UII_{4-11} (10^{-7} M) disrupted EC associations (**Figure 4D**). The proangiogenic properties of UII were mainly relayed by EC collective motility, as demonstrated by significant increased amount of endothelial junctions and branches, resulting in a much more intensified and complex tubular structure of hCMEC/D3 and HUV-EC-C (**Figures 4C,D**) and increased segments and polygons of hCMEC/D3 (**Figure 4D**). Urantide (10^{-6} M) drastically counteracted these endothelial associations and branch length mainly in hCMEC/D3, whereas palosuran (10^{-6} M) did not significantly modify basal tubulogenesis (**Figure 4D**). Urantide and palosuran systematically prevented UII-induced tubulogenic effects (**Figure 4D**).

Chemoattractant Function of UII Toward Proangiogenic Cells *in vivo*

The potential proangiogenic properties of the urotensinergic system were then investigated *in vivo*, *via* Matrigel plugs subcutaneously implanted in C57B/l6 mice. To validate the assay, liquid Matrigel containing either EG-VEGF or EGF (500 ng/ml, each) was injected in mice for 3 weeks. Resected sponges revealed blood drops or fully formed blood vessels (**Figure 5A**) and immunopositive cells for the macrophage F4/80, endothelial basal membrane collagen-IV (COLL-IV), and α-SMA markers (**Figure 5A**). Matrigel sponges containing UII or the murine sequence of the peptide (mUII) (50 ng/ml, each) exhibited similar hemorrhagic drops (**Figure 5B**). Quantification showed a marked stimulation of macrophage invasion and of capillary-like structures, formed by the association of endothelial and smooth muscle cells (**Figure 5B**). URP and UII_{4-11} (50 ng/ml, each) or urantide and palosuran (50 ng/ml, each) failed to chemoattract cells into the plugs (**Figure 5B**). These results indicate that the urotensinergic system is involved in angiogenesis *in vivo* and that available drugs may constitute anti-angiogenic compounds targeting UT.

The Urotensinergic System Is Required for GBM Growth Interfering With Mouse Survival

To investigate the involvement of the urotensinergic system in angiogenesis and tumorigenic process in GBM, daily intratumoral injections of UII on U87 xenograft tumors were performed when tumors reached ≈100 mm^3. Exogenous administration of UII (2.9 ng/kg) significantly accelerated tumor growth (**Figures 6A,D**) without affecting the body weight (**Supplementary Figure 2**). The median survival of animals receiving intratumoral injections of UII decreased compared with vehicle (**Figures 6A,D**). Vehicle or UII-tumor xenografts expressed UII and mostly UT in perinecrotic areas (pseudopalisadic area) and in vascular components (**Figure 6B**). URP (2.9 ng/kg) and UII_{4-11} (2.9 ng/kg) did not significantly modify GBM tumorigenic growth as well as mouse median survival (**Figure 6C**).

Animals receiving intratumoral administration of palosuran featured diminished tumor growth but no significant extended survival (**Figure 6D**, $P = 0.2922$). Co-administered with UII, palosuran partially prevented the UII-evoked stimulation of tumor growth (**Figure 6D**). The biased UT ligand urantide showed a strong inhibitory effect on tumor growth by maintaining significantly smaller tumor volumes for an extended period (**Figure 6D**), whereas the animal survival was markedly increased. When added at a 10-fold higher dose (**Supplementary Figure 3A**), or when

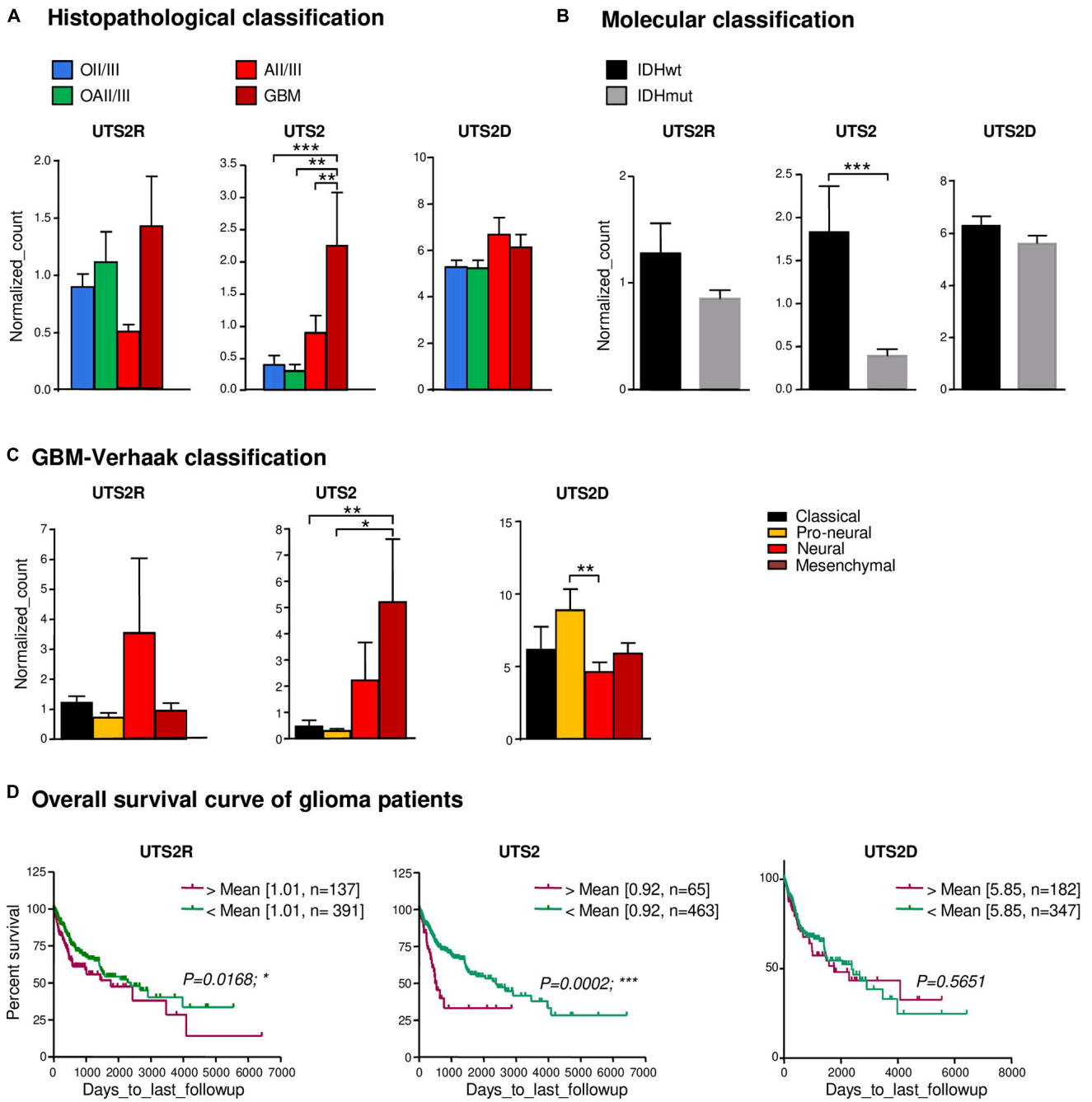


FIGURE 2 | Gene expression of UTS2, UTS2R, and UTS2D and patient survival in gliomas. **(A)** UTS2R, UTS2, and UTS2D are expressed in all histological OII/III, OAII/III, AII/III, and GBM classes and according to the glioma tumor grade, $n = 66$ tumors of the TCGA database. Histograms show gene expression from RNA-seq analysis. The data are from the following cohorts: OII/III, OAII/III, AII/III, and GBM. Of all glioma tissues, UTS2 expression exhibits significant higher levels in GBM compared to others ($P < 0.0001$). **(B,C)** Based on mRNA information on the subgroup classification of GBM from Verhaak et al. (2010) available in TCGA, neural, mesenchymal, and pro-neural groups expressed higher levels of UTS2R, UTS2, and UTS2D, respectively. **(D)** Kaplan–Meier statistical analysis of the prognostic significance of UTS2R, UTS2, and UTS2D. The overall survival period of glioma patients with UTS2R and UTS2 high expression was shorter than that of GBM patients with low expression. Green line, low expression; red line, high expression. *N* indicates the number of samples in each low or high expression group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

tumors reached a large volume (>500 mm^3, **Supplementary Figure 3B**), urantide exhibited a significant anti-tumoral effect. Also, UII-treated xenografts expressed UII and UT in GBM and in vascular components (**Figure 6E**), while urantide prevented UII-promoting UII and UT expression (**Figure 6E**).

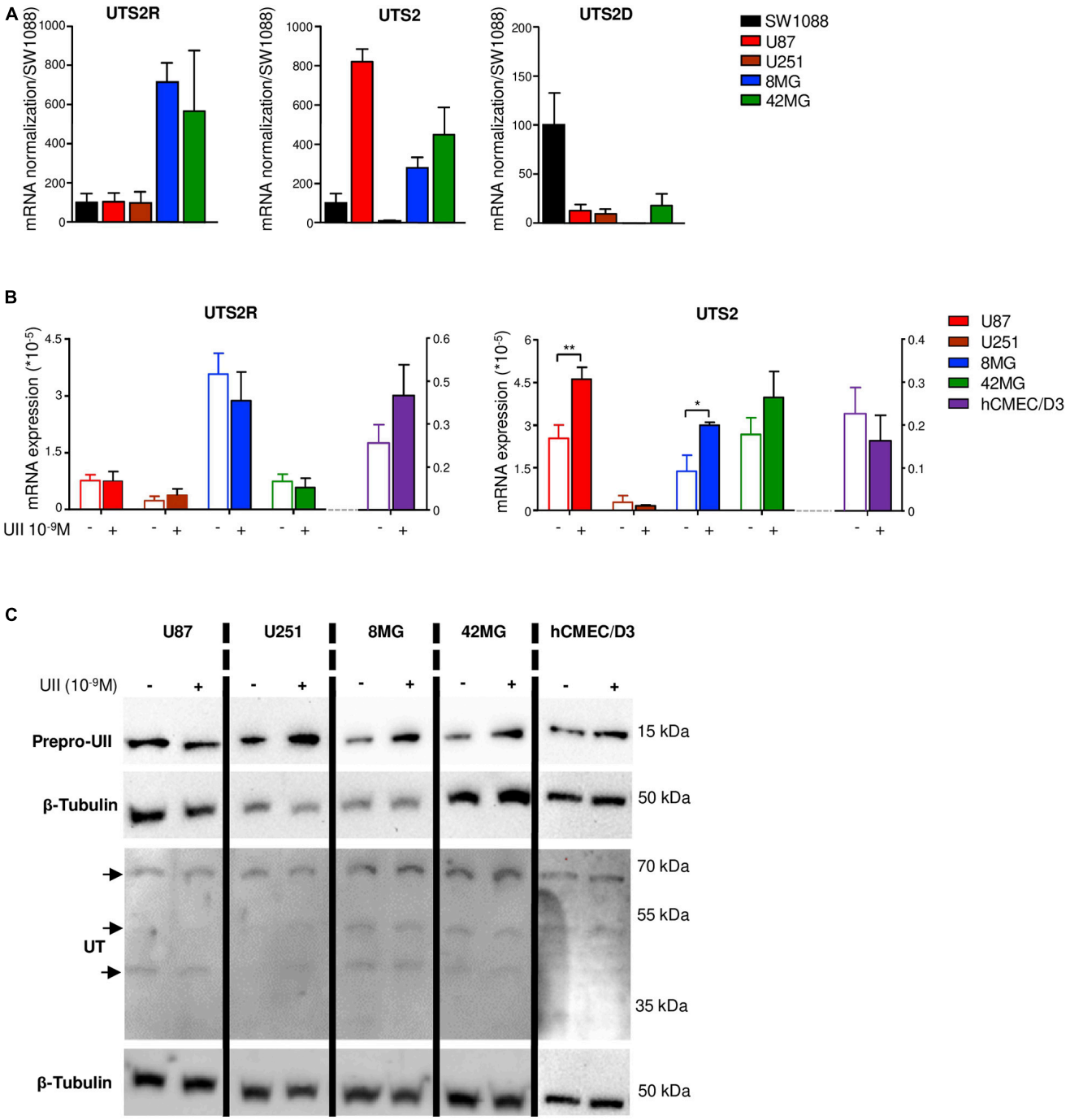


FIGURE 3 | Gene expression of UTS2, UTS2R, and UTS2D in glioma and hCMEC/D3 cell lines. **(A)** UTS2R, UTS2, and UTS2D gene expression were analyzed by qPCR in gliomas. UTS2R, UTS2, and UTS2D mRNA expressions are presented as $\Delta\Delta$Ct, related to the GAPDH gene expression in the anaplastic astrocytoma cell line SW1088 and in U87, U251, 8MG, and 42MG GBM cell lines. Data were expressed as mean ± SEM of three independent cultures and normalized to mRNA first to the housekeeping gene GAPDH and then to levels of gene of interest in SW1088. **(B)** Effect of UII on UTS2 and UTS2R gene expression in U87, U251, 8MG, and 42MG GBM cell lines and the endothelial hCMEC/D3 cell line. Cells were treated by UII (10^{-9} M, 24 h) in the absence of FBS. UTS2R and UTS2 mRNA expressions were expressed as mean ± SEM of $\Delta\Delta$Ct from three independent cultures normalized to the housekeeping gene UBC. Statistical significance of treatments vs. control condition was assessed with Mann–Whitney test. *$P < 0.05$; **$P < 0.01$. **(C)** Representative example of the effect of UII on prepro-UII and UT protein expression detected by Western blot of U87, U251, 8MG, and 42MG or hCMEC/D3 cells exposed to UII (10^{-9} M, 24 h) in the absence of FBS. For UT, a doublet of bands (~42–53 kDa) and a higher molecular mass (~70 kDa) correspond to human UT as previously shown (Lecointre et al., 2015, **Supplementary Material**). β-tubulin antibody was used as protein loading control.

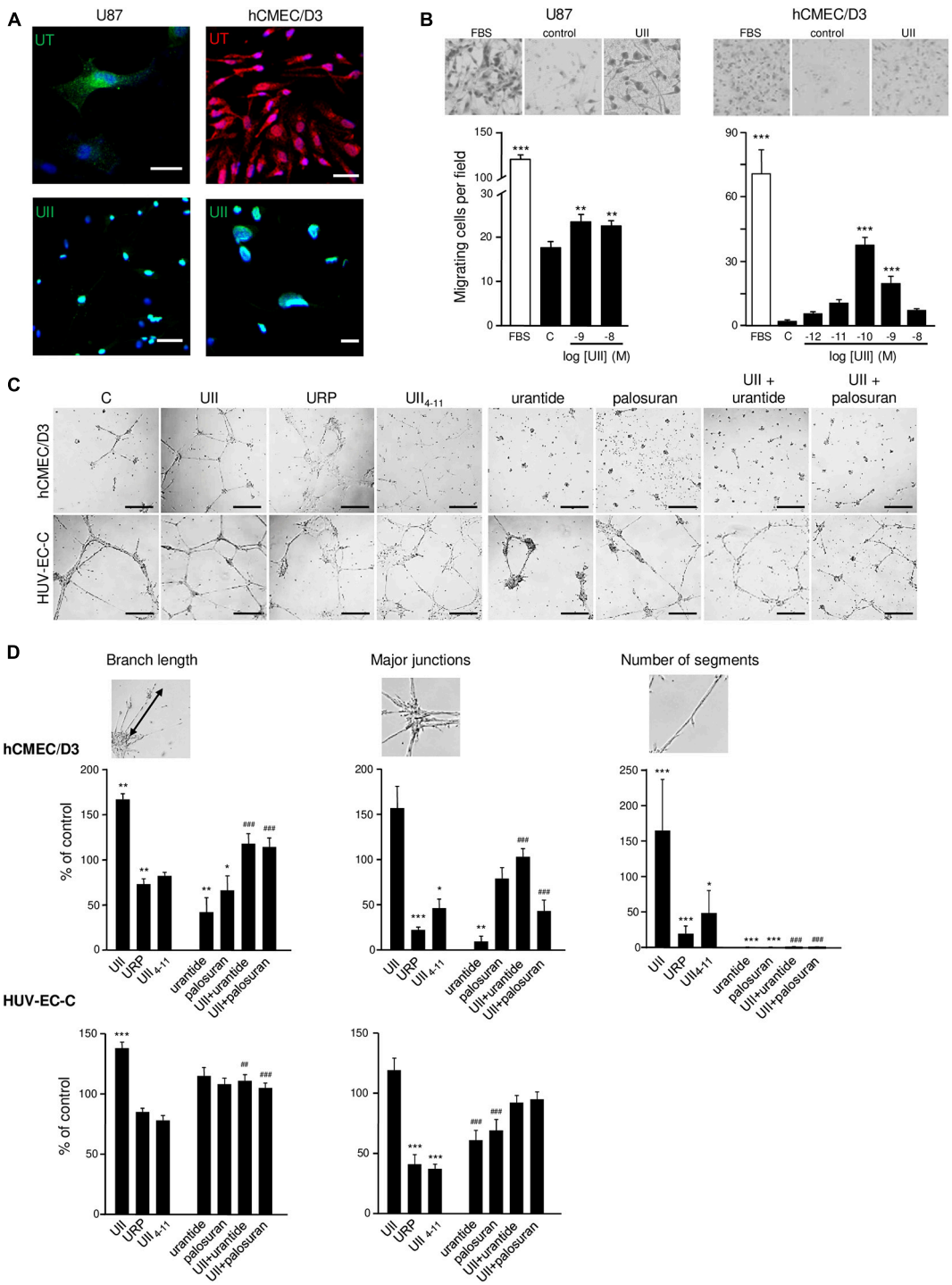


FIGURE 4 | *In vitro* effect of urotensinergic ligands on human glioma and endothelial cell migration and tubulogenesis. **(A)** Microphotographs of UT and UII staining on the GBM cell line U87 and EC hCMEC/D3 from human origins. Scale bar, 20 μm. **(B)** Representative fields (*Top panel*) of migrating U87 and hCMEC/D3 cells quantified from the Boyden chamber assay and quantification of U87 (*Left*) and hCMEC/D3 (*Right*) cell number in the absence or the presence of FBS 10% or UII (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, or 10^{-8} M). **(C)** Microphotographs of the tubulogenesis assay illustrating *in vitro* properties of UT agonists (UII, URP, and UII_{4-11}), biased ligand (urantide), or antagonist (palosuran) on angiogenic features of hCMEC/D3 and HUV-EC-C cells. Scale bars, 400 μm. **(D)** Data are presented as the% of control ± SEM from six independent experiments, and branch length, major junctions, and segment number were quantified and compared to control conditions. Statistical significance of treatments vs. control condition was assessed with one-way ANOVA with Dunnett *post hoc* test. *ns, non-significant;* $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical significance of co-treatments (UII + urantide; UII + palosuran) vs. UII alone was conducted with Student *t*-test. $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

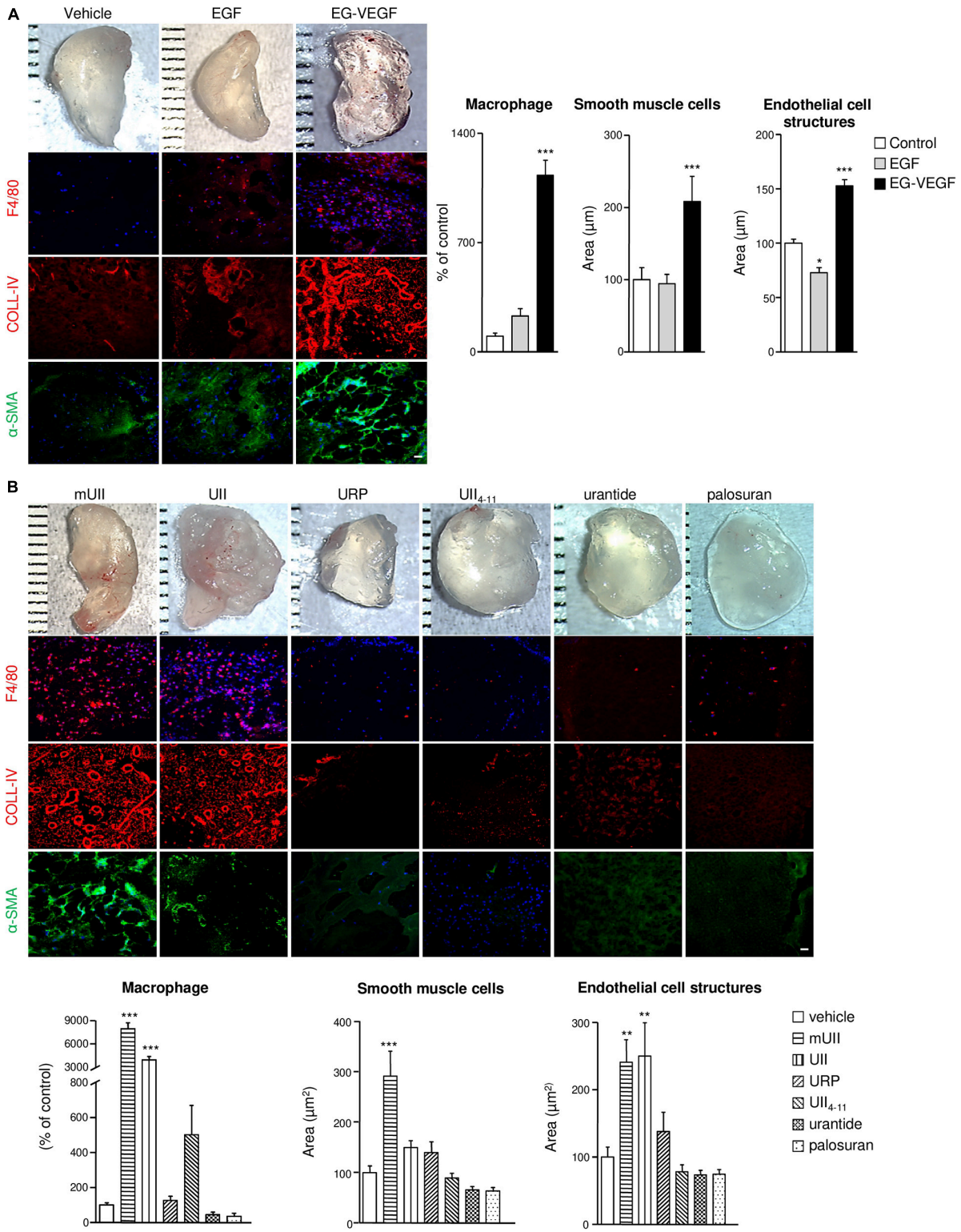


FIGURE 5 | *In vivo* chemoattractant effects of the urotensinergic ligands toward pro-angiogenic cells in Matrigel plugs. **(A,B)** Macrophotographs of resected Matrigel plugs (200 μl) containing vehicle (PBS), EGF (500 ng), EG-VEGF (500 ng) **(A)** or UII, mUII, URP, UII_{4-11} (50 ng, each), urantide (50 ng), or palosuran (50 ng) **(B)** 3 weeks after implantation in C57B/l6 mice. Matrigel invasion was detected by immunohistochemical analysis of macrophages (F4/80, red), EC matrix collagen-IV (COLL-IV, red), and smooth muscle cells (α-SMA, green). Scale bars, 40 μm. Quantification of macrophage number or smooth muscle cell and EC structure areas. Data are presented as percentage of control ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ from at least four independent experiments. Statistical significance is given by one-way ANOVA with Dunnett *post hoc* test vs. control.

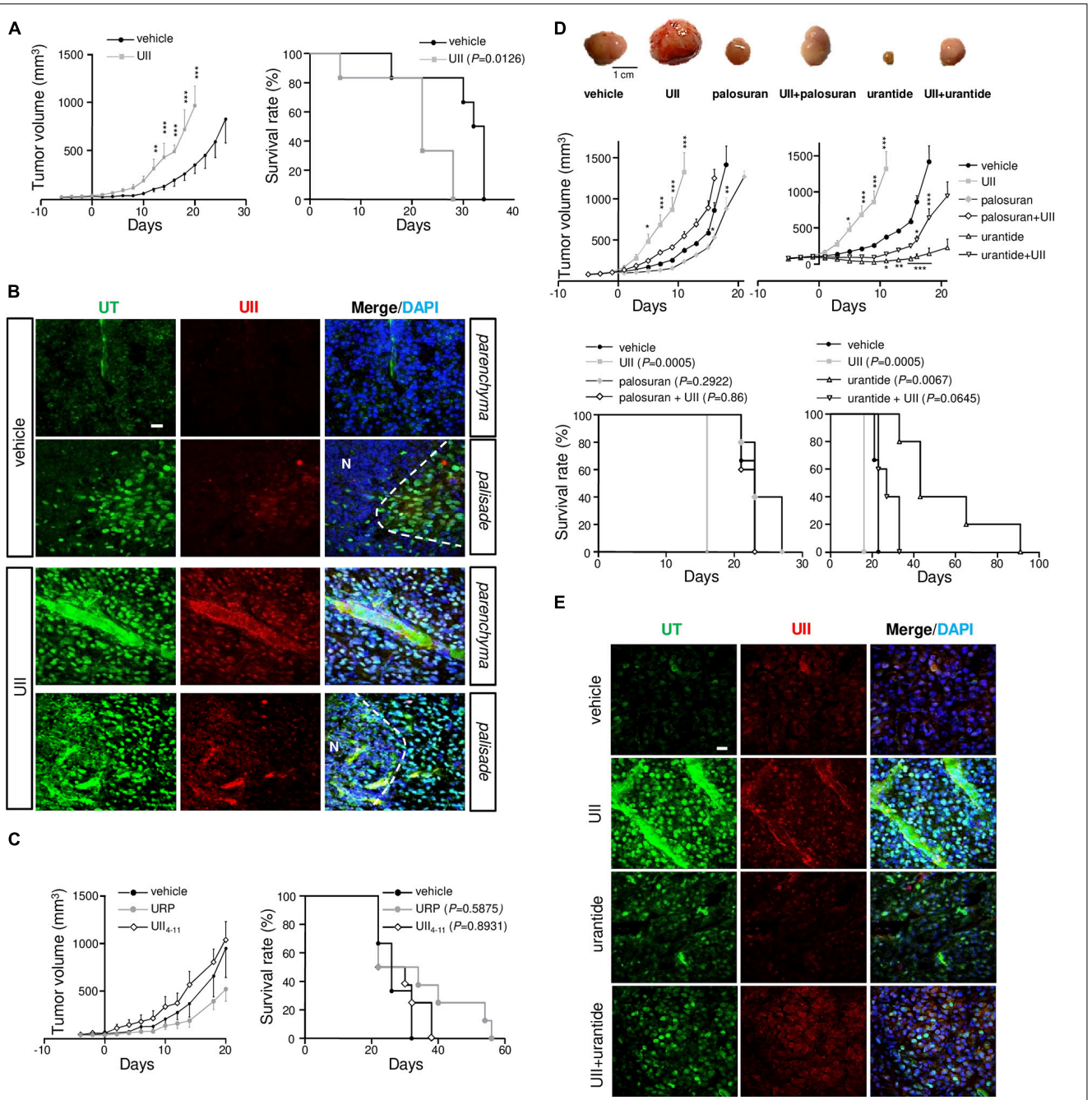


FIGURE 6 | Effects of the urotensinergic system on glioma development *in vivo*. **(A)** Tumor growth (*left*) and survival (*right*) of *Swiss Nude* mice transplanted with U87 cells. When tumor reached 100 mm^3, intratumoral injections of vehicle (saline) or UII (0.1 μg) were performed daily and tumor growth or median survival of treated mice was measured. $^{**}P < 0.01$; $^{***}P < 0.001$. Median survival: vehicle (n = 7), 33 days; UII (n = 7), 22 days (P = 0.0126). **(B)** Microphotographs of UT (green) and UII (red) expression in vehicle (PBS)- and UII-treated U87 xenografts. UII and UT were expressed in tumor parenchyma, in perinecrotic (N) areas and in vascular structures. **(C)** Tumor growth (*Left*) and mice survival (*Right*) during treatments with vehicle, URP, or UII_{4-11} (0.1 μg, each) of xenografted U87. Median survival: vehicle (n = 4), 22 days; URP (n = 4), 28 days (P = 0.5875); UII_{4-11} (n = 4), 26 days (P = 0.8931). **(D)** Top panel, typical gallery of representative example of U87 xenografts treated with vehicle, UII (0.1 μg), palosuran (1 μg), UII + palosuran (0.1/1 μg), urantide (1 μg), and UII + urantide (0.1/1 μg). Middle panel, tumor growth during the different treatments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Bottom panel, mice survival during the different treatments. Median survival: vehicle (n = 10), 23 days; UII (n = 10), 16 days (P = 0.0005); palosuran (n = 10), 23 days (P = 0.2922), UII + palosuran (n = 9), 23 days (P = 0.86), urantide (n = 10), 43 days (P = 0.0067), UII + urantide (n = 10), 27 days (P = 0.0645). **(E)** Immunolabeling of UT (green) and UII (red), and tumor distribution in vehicle, UII, urantide, and UII + urantide-treated U87 xenografts. Data presented as mean ± SEM. Animal survival was analyzed with Kaplan–Meier method, using the log-rank test for comparison. Statistical significance for tumor growth in **(A,C,D)** was given by using two-way ANOVA with Bonferroni *post hoc* test comparison with vehicle. Cell *nuclei* stained with DAPI (blue). Scale bars: 20 μm.

The Urotensinergic System Is Involved in GBM Cell Proliferation, Necrosis, and MMP-2/9 Activation *in vivo*

We next tested whether U87 xenograft treatments result in UT-regulating GBM proliferation. After a 15-day treatment, UII significantly increased the proliferation index, measured by the immunostaining of the proliferation marker Ki67 (**Figure 7A**). Palosuran and urantide significantly diminished endogenous cell proliferation by themselves, and UII-induced proliferation was also prevented by palosuran or urantide (**Figure 7A**, *bottom left*). Accordingly, a significant correlation (Pearson's $r = 0.6579$, $R^2 = 0.4328$, $P < 0.0001$) between the proliferation index and tumor volume was found at 15 days (**Figure 7A**, bottom right).

Rapid proliferation within glioma outstrips their blood supply likely leading to intratumoral necrosis and induction of hypoxia likely involving HIF-1α and HIF-2α overexpression (**Supplementary Figure 4**). We then investigated the distribution of the hypoxic marker pimonidazole in U87 xenografts after a 15-day treatment or when tumors reached 1,000 mm^3. In UII-treated tumors, the size of hypoxic areas (green) was strongly increased, indicating the hypoxic status of UII-treated tumors (**Figure 7B**). After urantide treatment, a similar tendency can be observed without significance. In GBM, the sustained hypoxia is classically followed by necrosis. The size of necrotic area was quantified after H&E coloration on 15-day-treated and on large tumors ($>$1,000 mm^3). UII strongly increased the size of necrotic areas (**Figure 7C**), whereas urantide evoked a significant decrease of necrosis at day 15, and finally showed a necrotic index resembling those of the UII-treated tumors after tumor relapse (**Figure 7C**).

In UII-treated tumors, capillaries appeared co-stained with anti-UII and anti-CD31, suggesting that endothelial compartment also serve as a reservoir of UII production (**Figure 8A**, left panel). UT was highly expressed in both glioma and EC, but not in α-SMA-positive structures (**Figure 8A**, right panel). The angiogenic status of xenografts under UII, urantide, or palosuran treatment after 15 days of daily injections and when tumors have reached 1,000 to 1,500 mm^3 was investigated. At 15 days, exogenous UII strongly altered the blood vessel architecture, with tortuous and disorganized vascular networks (**Figure 8B**, left). Palosuran or urantide markedly diminished the blood vessel density (**Figure 8B**, left). In $>$1,000 mm^3 tumors, UII maintained a strong angiogenic status whereas palosuran but mostly urantide decreased the basal vascular density and number of branches (**Figure 8B**, right), only urantide being able to reduce capillary-like structure diameter (**Figure 8B**, right). These suggest that urantide acts as a potent antagonist of the murine UT expressed on vascular components of host mice, to evoke a strong angiogenic activity.

Glioblastoma growth, hypoxia, and angiogenesis have been shown to be associated with mesenchymal marker expression including MMPs, and more especially MMP-2 during blood vessel reorganization and MMP-9 during invasion (Xu et al., 2015). As illustrated in **Figure 9A**, MMP-2 was mainly localized in large capillary-like structures and showed significant higher surface covered after treatment with UII. The proinvasive MMP-9 is also observed in large blood vessels and surrounding tumor cells, with intense immunofluorescence in UII-treated xenografts (**Figure 9B**). A similar trend was found *in vitro*, in hCMEC/D3 with higher MMP9 gene expression upon UII treatment (**Supplementary Figure 5**). Palosuran and, more efficiently, urantide markedly prevented MMP-2 and MMP-9 expression whereas urantide significantly counteracted the UII-evoked MMP-2 and MMP-9 activations (**Figures 9A,B**). These observations suggest endogenous UII-induced hypoxic and proliferative mechanisms in GBM, likely stimulating mesenchymal phenotype associated with angiogenic features.

UII Stimulates αvβ Integrin Expression and ^{99m}Tc-RGD Binding in U87 GBM Xenografts

Angiogenesis and mesenchymal phenotype are accompanied by integrin reactivation. At 15 days, it was observed that αv-immunolabeled structures expressed the EC progenitor CD34 marker, more specifically in UII-treated tumors (**Figures 10A,B**, left). *In vitro*, UII favored the expression of the ITGAV gene mRNA encoding alphav integrin from at least in part U87 and 8MG GBM cells (**Supplementary Figure 5**). The αvβ3 integrins were previously investigated as targets for direct molecular imaging of tumor angiogenesis with SPECT as they are considered as a key marker of activated EC (Schnell et al., 2009). Here, we used a ^{99}Technetium tracer containing the RGD (arginine, glycine, aspartate, ^{99}Tc-RGD) binding motif already validated (Rylova et al., 2014) to image αvβ3 expression in GBM *in vivo* (**Supplementary Material** and **Supplementary Figure 6**). In U87 xenografts, UII stimulated tumor growth (**Figures 10B,C**) but paradoxically provoked a decrease of RGD tumor uptake after 15 days of treatment (**Figure 10C**). The *ex vivo* γ-ray quantification of the total tumor showed ^{99}Tc-RGD capture enhanced in UII- and reduced in urantide-treated tumors, respectively, mainly due to the tumor size. From merged H&E staining and the ^{99}Tc-RGD distribution, it was observable that 10% of the control tumors and whole UII-treated tumors were necrotic, respectively. Analysis of the ^{99}Tc-RGD capture in the tumor (T), the necrotic tumor (NT), or the non-necrotic tumor (NNT) revealed that UII-treated tumors exhibited more RGD binding around the necrotic area (**Figure 10D**), suggesting increase of integrin expression and potentially tumor angiogenesis.

DISCUSSION

Angiogenesis has been shown to play a key role in the multi-step formation of GBM and results from a complex multicellular communication between glioma, endothelial, inflammatory, and/or reactive glial cells (Hanahan and Coussens, 2012). However, anti-angiogenics including bevacizumab can prolong the progression-free survival in patient with recurrent GBM (Lai et al., 2011) but led to a dramatic progression likely *via* exacerbated hypoxic and mesenchymal acquired status (Bergers and Hanahan, 2008; Keunen et al., 2011). Therefore, targeting vasoactive peptide receptor behaving as angiogenic chemokines involved in angiogenesis, inflammatory cell attraction, and

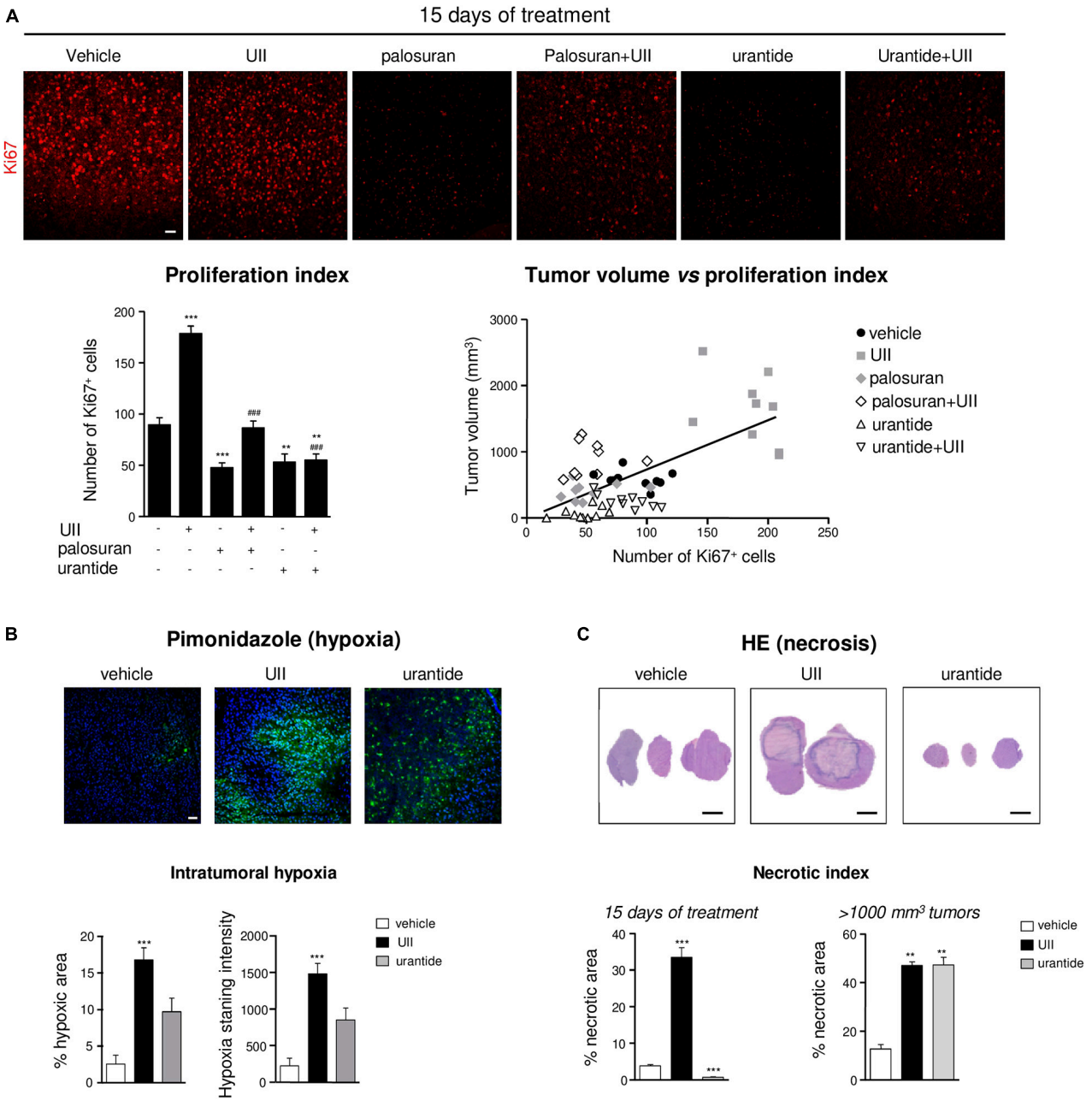


FIGURE 7 | Effects of the urotensinergic system on cell proliferation, hypoxia, and necrosis in U87 GBM. **(A)** Representative microscopic fields (*Top panel*) of U87 xenografts immunostained with an antibody directed against the proliferation marker Ki67 (red). Bottom left, quantification of the number of proliferative cells ($Ki67^+$) after 15 days of daily intratumoral injections of the UT ligands; bottom right, linear regression of tumor volume vs. proliferation index. Statistical significance of vehicle versus UII, palosuran, urantide, or co-treatments at day 15. $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical significance of UII versus co-treatments at day 15. $^{###}P < 0.001$. A significant positive correlation was given by the Pearson coefficient correlation $r = 0.6579$ ($P < 0.0001$). Scale bars: 40 μm. **(B)** Top, microscopic fields of U87 xenografts of tumoral hypoxia revealed by pimonidazole labeling (green) after 15 days of daily intratumoral injections of vehicle, UII (0.1 μg), or urantide (1 μg). Bottom, quantification of pimonidazole stained area (*Left*) and intensity (*Right*). Scale bars: 40 μm. $n = 4$ in each treatment groups. $^{***}P < 0.001$. **(C)** Top, H&E necrotic area coloration after 15 days of daily intratumoral injections of vehicle, UII (0.1 μg), or urantide (1 μg). Bottom, quantification of the necrotic area after 15 days (*left*) or after tumors reached 1000 mm^3 (*Right*). Scale bars: 2 mm. $n = 6$ in each treatment groups. $^{**}P < 0.01$; $^{***}P < 0.001$.

invasion of GBM cell lines should constitute an original and promising target system. Here, we report that the peptide UII and its receptor UT, expressed in malignant high-grade gliomas, promote angiogenesis and tumor vascular abnormal phenotype *via* up-regulation of mesenchymal factors including αvβ integrins and MMP-9, accompanying tumor growth, proliferation, and hypoxia/necrosis. This UII/UT system expressed in the U87-xenografted GBM model is shown to be efficiently targeted by

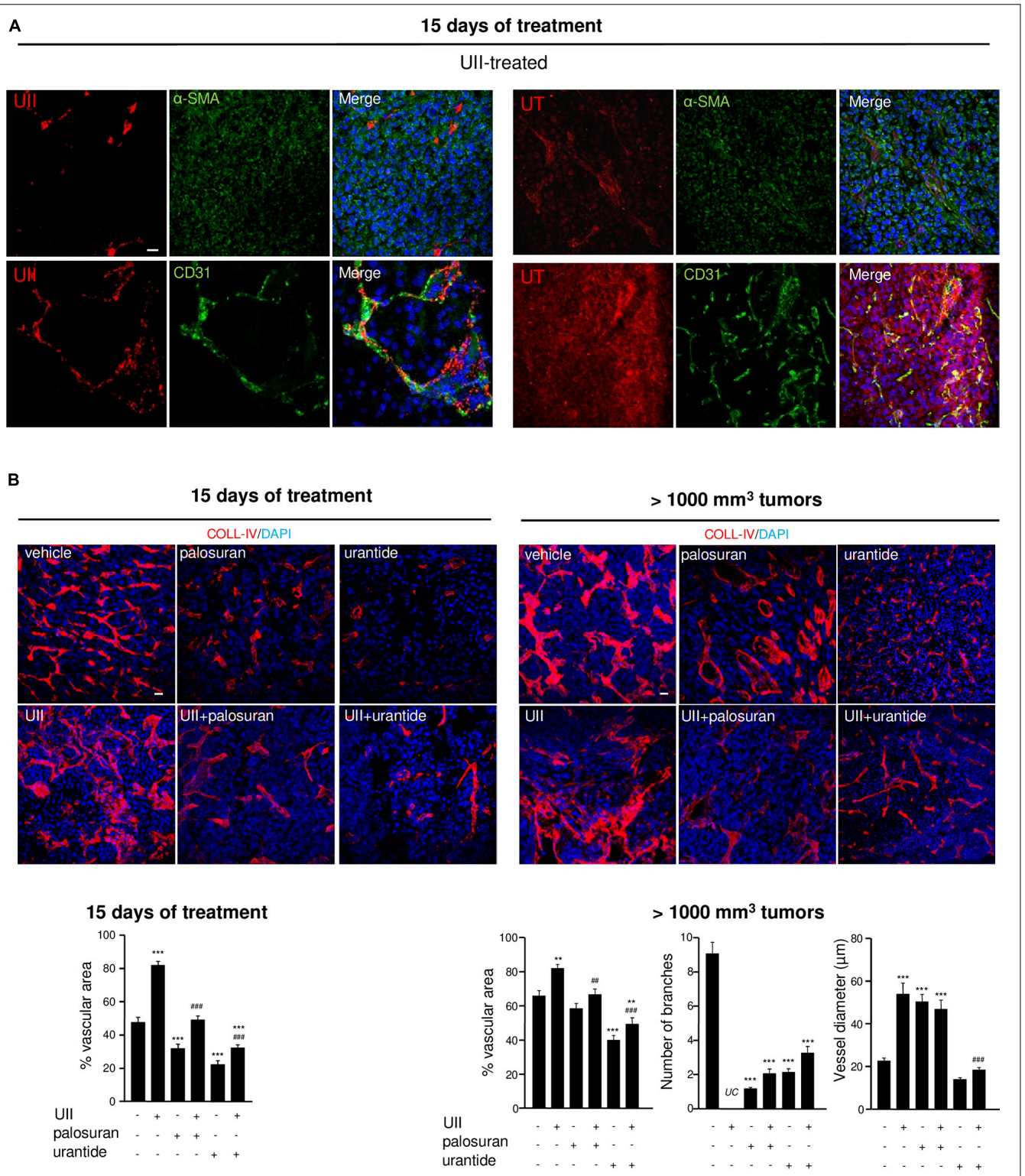


FIGURE 8 | Effects of the urotensinergic system on angiogenesis in U87 GBM. **(A)** UII (Top) and UT (Bottom) (red, each) immunostainings co-localized with VSMC and EC detected with anti-αSMA and anti-CD31 (green), respectively, in U87 xenografts. **(B)** COLL-IV (red) labeling in U87 tumors after 15 days of different treatments (*left panel*) or after tumors reach 1000 mm^3 (*right panel*). Quantification of intratumoral angiogenesis (vascular area) after 15 days of treatments or after tumors reach 1000 mm^3 (vascular area, number of branches and vessel diameter) as percentage of control ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$ vehicle vs. treatments; $^{\#\#}P < 0.05$; $^{\#\#\#}P < 0.001$; UII versus UII + palosuran or UII + urantide, from at least four different tumors in each group. Statistical significance was given by one-way ANOVA followed by Dunnett *post hoc* test. *UC*, uncountable.

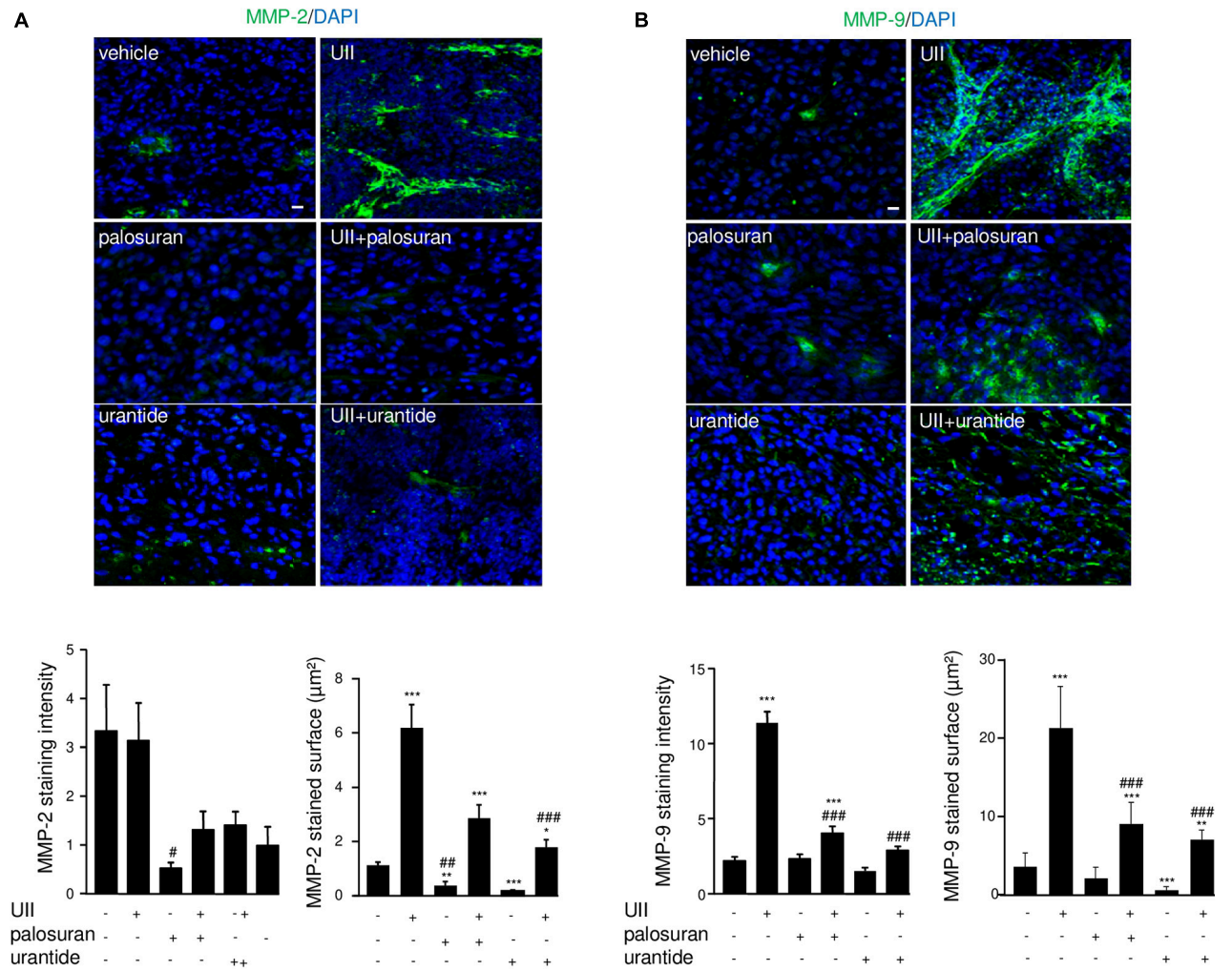


FIGURE 9 | Effects of the urotensinergic system on metalloprotease activation in U87 GBM. **(A,B)** MMP-2 (green) and quantification of labeled intensity and surface **(A)** or MMP-9 staining (green) and quantification **(B)** of labeled intensity and surface. Statistical significance was given by one-way ANOVA followed by Dunnett *post hoc* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vehicle *versus* treatments. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ UII vs treatments. Cell *nuclei* stained with DAPI (blue). Scale bars: 20 µm.

biased/antagonist UT ligands leading to tumor growth inhibition by, at least in part, reducing angiogenesis.

From TCGA, we show that UTS2 encoding UII is more expressed in high-grade astrocytic gliomas including GBMs and, within GBM, appears to be upregulated in the mesenchymal subclass of the Verhaak classification. A significant positive correlation between UT and UII expression in glioma samples also suggest paracrine/autocrine mechanisms within the GBM tumor bulk. Interestingly, glioma patients expressing the highest levels of UT and mainly UII mRNA had significantly shorter survival durations than cases with lower expressions; this prognostic cue can be associated with the strong expression of UII and UT in the hypoxic/vascular area of GBM samples, the positive correlation found between UII and UT expression, and the density of the abnormal vascularization. Indeed, inflammatory processes and/or hypoxic microenvironment within the tumor or neighboring normal tissues likely result in secretion by GBM cells of immunomodulatory cytokines and other factors such as CSF1 (colony-stimulating factor 1), C-C/CXC motif chemokines, or GDNF (glial cell-derived neurotrophic factor), which polarize TAMs toward an immunosuppressive M2 phenotype (Chang et al., 2016; Chen et al., 2017; Wang et al., 2019). TAM may in turn induce growth factor release triggering specific transcription factors important for mesenchymal transition associated with angiogenesis (Bogoch et al., 2017). Indeed, mesenchymal GBMs are characterized by expression of chemokine ligand/GPCRs such CXCL12/CXCR4, thrombin/PAR-1, or IL-8/CXCR2-associated signaling stimulating angiogenesis (Rollins, 2006; Albini et al., 2018) or factors supporting tissue remodeling and angiogenesis such as MMP-2 or MMP-9 (Gabrusiewicz et al., 2011). While GBM expressed UII and UT more specifically in mesenchymal tumor areas, the previous description of UT expression in a majority of monocytes/macrophages and the

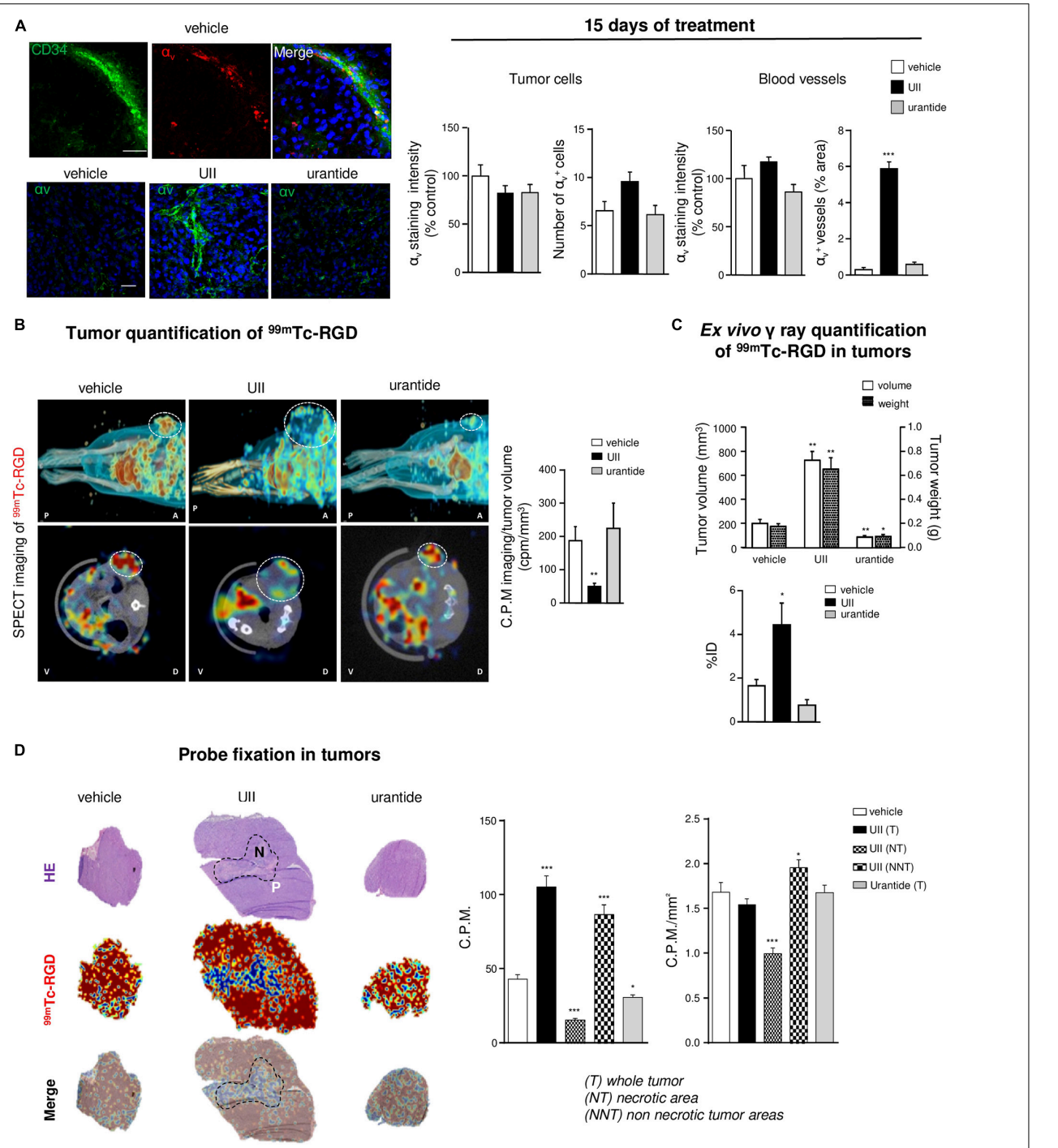


FIGURE 10 | *In vivo* imaging of the urotensinergic system-associated integrin expression and angiogenesis. **(A)** *Left*, U87 xenografts neo-vessels stained with antibodies directed against the endothelial precursor marker CD34 (green) and α_V integrins (red) after 15 days when daily treated with vehicle (*top panel*) or α_V integrins (green) in vehicle-, UII (0.1 μg)-, or urantide (1 μg)-treated xenograft after 15 days treatment. *Right*, Quantification of α_V staining in tumor xenografts after 15 days of different treatments in tumor parenchyma and vascular compartment. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; vehicle vs. treatments. Cell *nuclei* stained with DAPI (blue). Scale bars: 20 μm. **(B)** Representative MicroSPECT imaging of the ^{99m}Tc-RGD integrin ligand (red shades) binding in living tumors (circled by white dots), in antero-posterior (*A-P, top*), or dorso-ventral (V-D, *bottom*) positions and quantification of tracer incorporation in tumors (*right*) after 15 days of treatment. **(C)** Quantification of tumoral volume/weight and ^{99m}Tc-RGD γ ray emission in resected tumors, after 15 days of treatment (left, $n = 6$ in each group of treatment). ID, injected dose. **(D)** *Left*, representative fields of necrotic staining with H&E (*top*), ^{99m}Tc-RGD tracer binding (*middle*), and merged pictures (*bottom*) from consecutive slices of tumors acquired by β imaging. Scale bar: 2 mm. Right, Quantification of ^{99m}Tc-RGD in entire histological tumor sections (tumor, T) or when discriminating necrotic tumor areas (NT) in UII-treated tumors (non-necrotic tumor, NNT). $n = 6$ in each treatment groups. $^*P < 0.05$; $^{***}P < 0.001$. Statistical significance in **(A–D)** experiments was given by one-way ANOVA with Dunnett *post hoc* test comparison with vehicle.

UII-induced TAM infiltration promoting an inflammatory environment in lung cancer (Segain et al., 2007; Zhou et al., 2012) strongly support a key role of the UII/UT system in hypoxia/inflammation-induced mesenchymal transition, TAM infiltration, angiogenesis, and, as a consequence, resistance to treatment.

The pro-inflammatory cytokine function of the UII/UT system at the vascular compartment has been suggested by the UII-induced synthesis of pro-thrombotic and inflammatory markers such as PAI-1, in cultured smooth muscle cells or EC (Djordjevic et al., 2005; Cirillo et al., 2008). Here, we confirmed the expression of UT and UII by glioma cells and showed that UII induces chemotactic migration and tubulogenesis of human hCMEC/D3 and HUV-EC-C, without main impact on cell proliferation. The observed chemoattractant effects of UII on brain and umbilical ECs are in a good agreement with the first study of Spinazzi et al. (2006) establishing *in vivo* tubulogenic properties and sustains a UII-induced initiation of vessel sprouting during co-optation. These angiogenic properties were validated *in vivo* by means of the matrigel plugs, in which angiogenic compounds such as EG-VEGF allowed penetration by host cells and formation of new blood vessels. Matrigels containing urotensinergic ligands chemoattract and capture host cells, e.g., macrophages, vascular smooth muscle cells, or EC (Matsusaka and Wakabayashi, 2006; Segain et al., 2007; Park et al., 2013) signs of pro-inflammatory and pro-angiogenic properties of UII toward murine cells. Interestingly, the presupposed UT agonists URP or the short sequence peptide UII_{4-11} failed to promote recruitment of vascular cells *in vitro* (**Table 1**). Also, we describe the angiogenic potential of UT on EC *in vivo* when activated by UII but not URP or $hUII_{4-11}$. Originally observed here, URP and shorter UII-derived sequences exhibit antagonistic/inhibitory properties on tubulogenesis *in vitro,* suggesting a potential endogenous biased activity of URP on UT, antagonizing UII-induced migratory function. Some distinct activities on vascular and glial functions (Prosser et al., 2008; Hirose et al., 2009; Jarry et al., 2010) would at least in part reside in the UII-mediating long-lasting effects through insurmountable binding on UT, whereas URP usually induces transient responses (Hirose et al., 2009; Desrues et al., 2012). Both URP and $hUII_{4-11}$ were used as lead sequence for the design of UT synthetic drugs including antagonists. The first "peptide antagonists" of rodent UT were obtained by focusing on Lys8 of UII to develop urantide (**Table 1**; Patacchini et al., 2003) exhibiting on CHO or HEK cells expressing recombinant human UT residual agonist (Camarda et al., 2004) or biased activity (Brulé et al., 2014). Here, when urantide was directly tested on EC-forming tubulogenesis, a marked constitutive inhibition is observed whereas the antagonist palosuran (**Table 1**) remained inactive on tube formation, both preventing UII-induced angiogenesis. The urantide-inhibitory function raises the possibility of a biased function negatively regulating the UT-signaling cascades, e.g., partially Gq and internalization as previously shown (Brulé et al., 2014), specifically involved in tubulogenesis. Diebold et al. (2012) demonstrated a NOX2-containing NADPH oxidase as a source of ROS responsible for UII-induced angiogenesis (Patacchini et al., 2003), supporting the idea that UII recruits NOX2 through a mechanism involving at least in part Gq and/or internalization of UT. To dissect the involvement of the urotensinergic system in GBM growth and angiogenesis, U87 heterotopic xenografts in *Nude* mice were injected with UT "agonists" (UII or URP or $hUII_{4-11}$), biased ligand (urantide) or antagonist (palosuran) alone, or in combination with UII. UII dramatically accelerates GBM growth, correlated with a significant reduced animal survival, while URP or $hUII_{4-11}$ remained inactive at the same tested doses and/or exhibit tumor repressor function at a higher dose, reinforcing the notion that URP may be a naturally produced biased/competitive ligand of UT. In the same line, the short peptide urantide drastically inhibited GBM growth and promoted long-lasting survival. By comparison, the most well-studied primate UT antagonist palosuran only delayed gliomagenesis and animal death. It can be explained by the low antagonist behavior of palosuran toward rodent UT present in host cells, compared with the antagonist activity of urantide toward murine UT (Patacchini et al., 2003) combined with its biased activity on human UT (Brulé et al., 2014). As recently validated with the design of a radiolabeled DOTA-urantide binding UT injectable *in vivo* (Poret et al., 2020), UT expressed in GBM cells as well as in vascular/myeloid compartments would constitute a key pharmacological target for the design of therapeutic molecules based on the structure of urantide.

Detection of the key markers of the high-grade GBM as well as measurement of the proliferation index and hypoxic–necrotic areas provide cues of the impact of the urotensinergic system on glioma malignancy. We show that UII enhanced cell proliferation within GBM tumors, whereas palosuran and urantide inhibited this mitogenic activity, even in the presence of exogenous UII. This UII-promoting mitogenic mechanism should not result from a direct activation of glioma cells (Lecointre et al., 2015). However, anti-angiogenic treatment leads to tumoral hypoxic features and mesenchymal phenotype associated with increased proliferative capacity (Xu et al., 2015). Here, UII similarly exacerbates hypoxia surrounding large central necrotic areas, likely mimicking the adverse events of anti-angiogenics. Despite a report suggesting that UT relays activation of HIF-1α through NOX2 in endothelial cells controlling tubulogenesis *in vitro* (Diebold et al., 2012), we did not observe UII-evoked induction of HIF-1/2 in normo- or hypoxic conditions in GBM or EC *in vitro*. UII may rather configure the tumor microenvironment composed of endothelial, perivascular, and/or inflammatory cells, promoting tumor progression by mediating hypoxia, abnormal angiogenesis, desmoplasia, and/or mesenchymal characteristics. The mesenchymal components including MMP-2 and MMP-9 can contribute to tumor invasion through breaking down of basement membrane including Coll-IV (Fan et al., 2012). In agreement, the production of MMP-9 after UII treatment of lung adenocarcinoma in mice (Zhou et al., 2012) was previously described. We show that UII treatment of GBM xenografts evoked MMP-2 and MMP-9 overexpression mainly in the vascular and glioma cell compartments, respectively, and that UII stimulates MMP9 gene expression in hCMEC/D3 *in vitro*. Again, palosuran and urantide diminished and prevented UII-evoked MMPs expression, indicating that the urotensinergic system activates the vascular network during tumor progression.

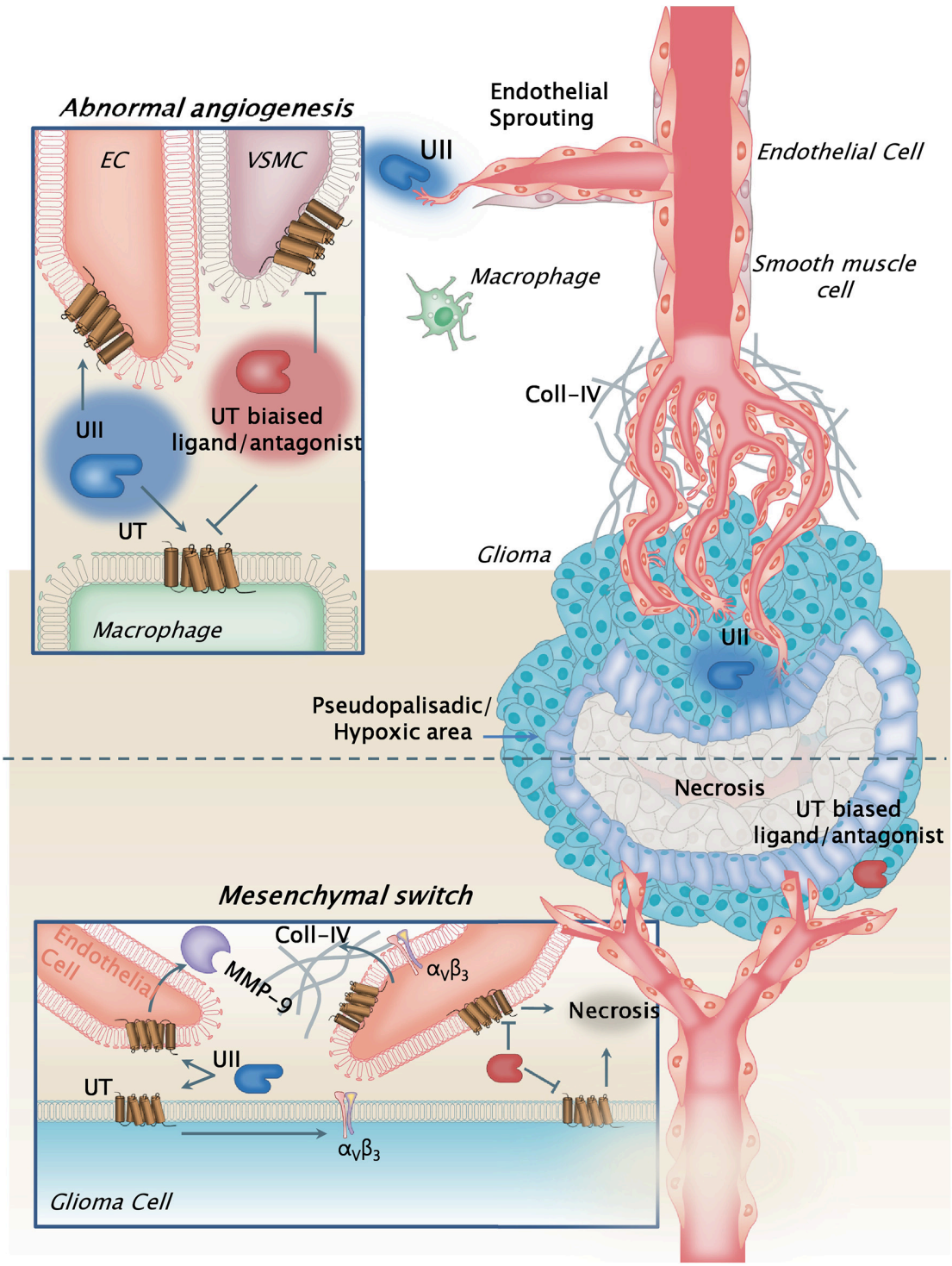


FIGURE 11 | Schematic model illustrating the pleiotrope functions of the urotensinergic system during GBM malignancy. The UT receptor when expressed at both the tumoral and vascular compartments, and activated by UII through endogenous release by tumor cells, relayed accelerated tumor growth and proliferation, hypoxia, and necrosis, leading to exacerbation of the abnormal and tortuous vascularization. These processes are accompanied by metalloprotease (MMPs) release such as MMP-9 by the endothelial compartment likely degrading extracellular matrix, and by increased expression of αv(β3) integrins at least in part by GBM cells. The hypothesis of a contributing mechanism of a UII-induced macrophage tumor invasion can also be proposed, as potential cell partners in necrosis and angiogenesis. UT receptor antagonist palosuran or the biased ligand urantide would constitute a new original strategy to prevent glioma malignancy. Here, the biased ligand urantide exhibits a better multicellular anti-UT activity than palosuran both repressing angiogenesis and tumor growth, suggesting a new avenue for GBM treatment targeting the urotensinergic system.

Extracellular Coll-IV and membrane α_V integrins are known to be scaffolding adhesion molecules for angiogenesis and tumoral infiltration correlated with progression of numerous cancers (Mammoto et al., 2013). We describe and quantify α_V integrin patterns in glioma cells or vessels and establish a marked increased expression in endothelium and glioma cells during the course of GBM malignancy when treated with UII. These αvβ3 integrins were previously investigated as targets for molecular SPECT imaging of tumor angiogenesis *in vivo* while they are also expressed by glioma cells (Schnell et al., 2009). We observe increased GBM uptake of ^{99}Tc-RGD in the periphery of the tumor surrounding the necrotic area after UII treatment. However, *in vivo* radio-counting showed decreased incorporation within the tumor. As recently previously proposed for bevacizumab-treated GBM in mice (Rylova et al., 2014), delayed necrosis explains the decrease of RGD uptake *in vivo*. We believe UII sets up tortuous angiogenesis with enhanced integrin expression, associated with chaotic blood flow and/or interstitial pressure reducing perfusion, thereby confining RGD probe capture to the tumor peripheral ring.

Altogether, our study emphasizes that UII and its receptor UT are more expressed in GBMs than in low-grade gliomas, specifically in vascular and hypoxia mesenchymal areas, and play a major role in gliomagenesis *via*, at least in part, angiogenesis, promoting MMP-2/-9 and α_V integrin expression within the tumor bulk. Because UT may also relay glioma cell migration, this work shows the interesting opportunity provided by new class of vasoactive and chemokine peptide receptor targets controlling multicellular functions within the GBM microenvironment and involved in the mesenchymal transformation (**Figure 11**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the "Comité d'Ethique NOrmandie en Matière d'EXpérimentation Animale" CENOMEXA under the National Committee on Animal Experimentation, and received the following number N/13-11-12/36/11-17.

AUTHOR CONTRIBUTIONS

VLJ performed main experiments, quantifications and analyses on glioma cells and endothelial cells *in vitro* (cell migration, tubulogenesis) and *in vivo* (matrigel plugs, tumor xenografts and ligand injections, animal immunohistochemistry, MicroSpect imaging). P-OG and K-PD carried out immunohistochemistry, analysis, scoring from patient clinical information, and quantitative PCR on glioma biopsies and glioma cell lines. AM analyzed *in silico* mRNA expression levels from the TCGA database and vascular characteristics from glioma patient biopsies. NP, DC, and LD maintained culture of glioma and endothelial cell lines, and performed cell proliferation studies, and Western blot for hypoxic marker expression. P-OC established and provided the hCMEC/D3 human microendothelial cell line. JH contributed to the studies on the GBM cell xenografts and intratumoral administration of urantide. F-XF, OL, AL, and FMa provided GBM biopsies and all clinical information from the Haute-Normandie tumorbank, France. RM, PB, and PV established the angiogenic RGD tracer and allowed the *in vivo* microSPECT imaging and analysis. FMo contributed to the analysis of the data and provided critical comments. PG and HC supervised the study, provided all critical analyses, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by INSERM, Géfluc, TC2N network, Ligue Régionale Contre le Cancer, the Regional Council of Normandy and the European Community FEDER program (Europe gets involved in regional development through the ERDF program) (Grants DO-IT, PACT-PACT-PACBS and PHEDERCPG), Institute on Innovative and Biomedical Research (IRIB), and University of Rouen. VLJ, P-OG, K-PD, AM, and DC were recipients of a fellowship from the Région Normandie or French ministry.

ACKNOWLEDGMENTS

We gratefully acknowledge the PRIMACEN platform (University of Rouen, France) for imaging equipment and Mr. Arnaud Arabo and Mrs. Huguette Lemonnier for skillful technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.652544/full#supplementary-material

REFERENCES

Albini, A., Bruno, A., Noonan, D. M., and Mortara, L. (2018). Contribution to tumor angiogenesis from innate immune cells within the tumor microenvironment: implications for immunotherapy. *Front. Immunol.* 9:527. doi: 10.3389/fimmu.2018.00527

Alifieris, C., and Trafalis, D. T. (2015). Glioblastoma multiforme: pathogenesis and treatment. *Pharmacol. Ther.* 152, 63–82. doi: 10.1016/j.pharmthera.2015.05.005

Ames, R. S., Sarau, H. M., Chambers, J. K., Willette, R. N., Aiyar, N. V., Romanic, A. M., et al. (1999). Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 401, 282–286. doi: 10.1038/45809

Arrieta, O., Guevara, P., Escobar, E., García-Navarrete, R., Pineda, B., and Sotelo, J. (2005). Blockage of angiotensin II type I receptor decreases the synthesis of growth factors and induces apoptosis in C6 cultured cells and C6 rat glioma. *Br. J. Cancer* 92, 1247–1252. doi: 10.1038/sj.bjc.6602483

Aubry, M., de Tayrac, M., Etcheverry, A., Clavreul, A., Saikali, S., Menei, P., et al. (2015). From the core to beyond the margin: a genomic picture of glioblastoma intratumor heterogeneity. *Oncotarget* 6, 12094–12109. doi: 10.18632/oncotarget.3297

Becker, S., Bohn, P., Bouyeure-Petit, A.-C., Modzelewski, R., Gensanne, D., Picquenot, J.-M., et al. (2015). Bevacizumab enhances efficiency of radiotherapy in a lung adenocarcinoma rodent model: role of αvβ3 imaging in determining optimal window. *Nucl. Med. Biol.* 42, 923–930. doi: 10.1016/j.nucmedbio.2015.08.002

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Bogoch, Y., Friedlander-Malik, G., Lupu, L., Bondar, E., Zohar, N., Langier, S., et al. (2017). Augmented expression of RUNX1 deregulates the global gene expression of U87 glioblastoma multiforme cells and inhibits tumor growth in mice. *Tumour Biol.* 39:1010428317698357. doi: 10.1177/1010428317698357

Borsig, L., Wolf, M. J., Roblek, M., Lorentzen, A., and Heikenwalder, M. (2014). Inflammatory chemokines and metastasis–tracing the accessory. *Oncogene* 33, 3217–3224. doi: 10.1038/onc.2013.272

Bougnaud, S., Golebiewska, A., Oudin, A., Keunen, O., Harter, P. N., Mäder, L., et al. (2016). Molecular crosstalk between tumour and brain parenchyma instructs histopathological features in glioblastoma. *Oncotarget* 7, 31955–31971. doi: 10.18632/oncotarget.7454

Brennan, C. W., Verhaak, R. G. W., McKenna, A., Campos, B., Noushmehr, H., Salama, S. R., et al. (2013). The somatic genomic landscape of glioblastoma. *Cell* 155, 462–477. doi: 10.1016/j.cell.2013.09.034

Brulé, C., Perzo, N., Joubert, J.-E., Sainsily, X., Leduc, R., Castel, H., et al. (2014). Biased signaling regulates the pleiotropic effects of the urotensin II receptor to modulate its cellular behaviors. *FASEB J.* 28, 5148–5162. doi: 10.1096/fj.14-249771

Camarda, V., Song, W., Marzola, E., Spagnol, M., Guerrini, R., Salvadori, S., et al. (2004). Urantide mimics urotensin-II induced calcium release in cells expressing recombinant UT receptors. *Eur. J. Pharmacol.* 498, 83–86. doi: 10.1016/j.ejphar.2004.07.089

Cancer Genome Atlas Research Network. (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455, 1061–1068. doi: 10.1038/nature07385

Castel, H., Desrues, L., Joubert, J.-E., Tonon, M.-C., Prézeau, L., Chabbert, M., et al. (2017). The G protein-coupled receptor UT of the neuropeptide urotensin II displays structural and functional chemokine features. *Front. Endocrinol.* 8:76. doi: 10.3389/fendo.2017.00076

Castel, H., Diallo, M., Chatenet, D., Leprince, J., Desrues, L., Schouft, M.-T., et al. (2006). Biochemical and functional characterization of high-affinity urotensin II receptors in rat cortical astrocytes. *J. Neurochem.* 99, 582–595. doi: 10.1111/j.1471-4159.2006.04130.x

Cavazos, D. A., and Brenner, A. J. (2016). Hypoxia in astrocytic tumors and implications for therapy. *Neurobiol. Dis.* 85, 227–233. doi: 10.1016/j.nbd.2015.06.007

Chang, A. L., Miska, J., Wainwright, D. A., Dey, M., Rivetta, C. V., Yu, D., et al. (2016). CCL2 produced by the glioma microenvironment is essential for the recruitment of regulatory T cells and myeloid-derived suppressor cells. *Cancer Res.* 76, 5671–5682. doi: 10.1158/0008-5472.CAN-16-0144

Chen, Z., Feng, X., Herting, C. J., Garcia, V. A., Nie, K., Pong, W. W., et al. (2017). Cellular and molecular identity of tumor-associated macrophages in glioblastoma. *Cancer Res.* 77, 2266–2278. doi: 10.1158/0008-5472.CAN-16-2310

Cherry, A. E., and Stella, N. (2014). G protein-coupled receptors as oncogenic signals in glioma: emerging therapeutic avenues. *Neuroscience* 278, 222–236. doi: 10.1016/j.neuroscience.2014.08.015

Cirillo, P., De Rosa, S., Pacileo, M., Gargiulo, A., Angri, V., Fiorentino, I., et al. (2008). Human urotensin II induces tissue factor and cellular adhesion molecules expression in human coronary endothelial cells: an emerging role for urotensin II in cardiovascular disease. *J. Thromb. Haemost.* 6, 726–736. doi: 10.1111/j.1538-7836.2008.02923.x

Clavier, T., Mutel, A., Desrues, L., Lefevre-Scelles, A., Gastaldi, G., Amki, M. E., et al. (2018). Association between vasoactive peptide urotensin II in plasma and cerebral vasospasm after aneurysmal subarachnoid hemorrhage: a potential therapeutic target. *J. Neurosurg.* doi: 10.3171/2018.4.JNS172313 Online ahead of print

Clozel, M., Binkert, C., Birker-Robaczewska, M., Boukhadra, C., Ding, S.-S., Fischli, W., et al. (2004). Pharmacology of the urotensin-II receptor antagonist palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt): first demonstration of a pathophysiological role of the urotensin System. *J. Pharmacol. Exp. Ther.* 311, 204–212. doi: 10.1124/jpet.104.068320

Coly, P.-M., Perzo, N., Le Joncour, V., Lecointre, C., Schouft, M.-T., Desrues, L., et al. (2016). Chemotactic G protein-coupled receptors control cell migration by repressing autophagosome biogenesis. *Autophagy* 12, 2344–2362. doi: 10.1080/15548627.2016.1235125

Coulouarn, Y., Lihrmann, I., Jegou, S., Anouar, Y., Tostivint, H., Beauvillain, J. C., et al. (1998). Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* 95, 15803–15808.

Desrues, L., Lefebvre, T., Lecointre, C., Schouft, M.-T., Leprince, J., Compère, V., et al. (2012). Down-regulation of GABA(A) receptor via promiscuity with the vasoactive peptide urotensin II receptor. Potential involvement in astrocyte plasticity. *PLoS One* 7:e36319. doi: 10.1371/journal.pone.0036319

Diebold, I., Petry, A., Sabrane, K., Djordjevic, T., Hess, J., and Görlach, A. (2012). The HIF1 target gene NOX2 promotes angiogenesis through urotensin-II. *J. Cell Sci.* 125, 956–964. doi: 10.1242/jcs.094060

Djordjevic, T., BelAiba, R. S., Bonello, S., Pfeilschifter, J., Hess, J., and Görlach, A. (2005). Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 25, 519–525. doi: 10.1161/01.ATV.0000154279.98244.eb

Eelen, G., Treps, L., Li, X., and Carmeliet, P. (2020). Basic and therapeutic aspects of angiogenesis updated. *Circ. Res.* 127, 310–329. doi: 10.1161/CIRCRESAHA.120.316851

Fan, H.-X., Li, H.-X., Chen, D., Gao, Z.-X., and Zheng, J.-H. (2012). Changes in the expression of MMP2, MMP9, and ColIV in stromal cells in oral squamous tongue cell carcinoma: relationships and prognostic implications. *J. Exp. Clin. Cancer Res.* 31:90. doi: 10.1186/1756-9966-31-90

Federico, A., Zappavigna, S., Romano, M., Grieco, P., Luce, A., Marra, M., et al. (2014). Urotensin-II receptor is over-expressed in colon cancer cell lines and in colon carcinoma in humans. *Eur. J. Clin. Invest.* 44, 285–294. doi: 10.1111/eci.12231

Franco, R., Zappavigna, S., Gigantino, V., Luce, A., Cantile, M., Cerrone, M., et al. (2014). Urotensin II receptor determines prognosis of bladder cancer regulating cell motility/invasion. *J. Exp. Clin. Cancer Res.* 33:48. doi: 10.1186/1756-9966-33-48

Gabrusiewicz, K., Ellert-Miklaszewska, A., Lipko, M., Sielska, M., Frankowska, M., and Kaminska, B. (2011). Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas. *PLoS One* 6:e23902. doi: 10.1371/journal.pone.0023902

Gagliardi, F., Narayanan, A., Reni, M., Franzin, A., Mazza, E., Boari, N., et al. (2014). The role of CXCR4 in highly malignant human gliomas biology: current knowledge and future directions. *Glia* 62, 1015–1023. doi: 10.1002/glia.22669

Glass, R., and Synowitz, M. (2014). CNS macrophages and peripheral myeloid cells in brain tumours. *Acta Neuropathol.* 128, 347–362. doi: 10.1007/s00401-014-1274-2

Grieco, P., Franco, R., Bozzuto, G., Toccacieli, L., Sgambato, A., Marra, M., et al. (2011). Urotensin II receptor predicts the clinical outcome of prostate cancer patients and is involved in the regulation of motility of prostate adenocarcinoma cells. *J. Cell. Biochem.* 112, 341–353. doi: 10.1002/jcb.22933

Hanahan, D., and Coussens, L. M. (2012). Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 21, 309–322. doi: 10.1016/j.ccr.2012.02.022

Hembruff, S. L., and Cheng, N. (2009). Chemokine signaling in cancer: implications on the tumor microenvironment and therapeutic targeting. *Cancer Ther.* 7, 254–267.

Hirose, T., Takahashi, K., Mori, N., Nakayama, T., Kikuya, M., Ohkubo, T., et al. (2009). Increased expression of urotensin II, urotensin II-related peptide and urotensin II receptor mRNAs in the cardiovascular organs of hypertensive rats: comparison with endothelin-1. *Peptides* 30, 1124–1129. doi: 10.1016/j.peptides.2009.02.009

Jarry, M., Diallo, M., Lecointre, C., Desrues, L., Tokay, T., Chatenet, D., et al. (2010). The vasoactive peptides urotensin II and urotensin II-related peptide regulate astrocyte activity through common and distinct mechanisms: involvement in cell proliferation. *Biochem. J.* 428, 113–124. doi: 10.1042/BJ20090867

Kaafarani, I., Fernandez-Sauze, S., Berenguer, C., Chinot, O., Delfino, C., Dussert, C., et al. (2009). Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibits tumor angiogenesis and suppresses growth of human tumor xenografts in mice. *FASEB J.* 23, 3424–3435. doi: 10.1096/fj.08-127852

Karsy, M., Gelbman, M., Shah, P., Balumbu, O., Moy, F., and Arslan, E. (2012). Established and emerging variants of glioblastoma multiforme: review of morphological and molecular features. *Folia Neuropathol.* 50, 301–321.

Keunen, O., Johansson, M., Oudin, A., Sanzey, M., Rahim, S. A. A., Fack, F., et al. (2011). Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3749–3754. doi: 10.1073/pnas.1014480108

Lai, A., Tran, A., Nghiemphu, P. L., Pope, W. B., Solis, O. E., Selch, M., et al. (2011). Phase II study of bevacizumab plus temozolomide during and after radiation therapy for patients with newly diagnosed glioblastoma multiforme. *J. Clin. Oncol.* 29, 142–148. doi: 10.1200/JCO.2010.30.2729

Lecointre, C., Desrues, L., Joubert, J. E., Perzo, N., Guichet, P.-O., Le Joncour, V., et al. (2015). Signaling switch of the urotensin II vasosactive peptide GPCR: prototypic chemotaxic mechanism in glioma. *Oncogene* 34, 5080–5094. doi: 10.1038/onc.2014.433

Louis, D. N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W. K., et al. (2016). The 2016 world health organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol.* 131, 803–820. doi: 10.1007/s00401-016-1545-1

Mammoto, T., Jiang, A., Jiang, E., Panigrahy, D., Kieran, M. W., and Mammoto, A. (2013). Role of collagen matrix in tumor angiogenesis and glioblastoma multiforme progression. *Am. J. Pathol.* 183, 1293–1305. doi: 10.1016/j.ajpath.2013.06.026

Matsusaka, S., and Wakabayashi, I. (2006). Enhancement of vascular smooth muscle cell migration by urotensin II. *Naunyn. Schmiedebergs Arch. Pharmacol.* 373, 381–386. doi: 10.1007/s00210-006-0086-x

Metellus, P., Voutsinos-Porche, B., Nanni-Metellus, I., Colin, C., Fina, F., Berenguer, C., et al. (2011). Adrenomedullin expression and regulation in human glioblastoma, cultured human glioblastoma cell lines and pilocytic astrocytoma. *Eur. J. Cancer* 47, 1727–1735. doi: 10.1016/j.ejca.2011.02.021

Norden, A. D., Drappatz, J., and Wen, P. Y. (2008). Novel anti-angiogenic therapies for malignant gliomas. *Lancet Neurol.* 7, 1152–1160. doi: 10.1016/S1474-4422(08)70260-6

Park, S. L., Lee, B. K., Kim, Y.-A., Lee, B. H., and Jung, Y.-S. (2013). Inhibitory effect of an urotensin II receptor antagonist on proinflammatory activation induced by urotensin II in human vascular endothelial cells. *Biomol. Ther.* 21, 277–283. doi: 10.4062/biomolther.2013.051

Patacchini, C., Masci, S., D'Ovidio, R., and Lafiandra, D. (2003). Heterologous expression and purification of native and mutated low molecular mass glutenin subunits from durum wheat. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* 786, 215–220.

Phuphanich, S., Carson, K. A., Grossman, S. A., Lesser, G., Olson, J., Mikkelsen, T., et al. (2008). Phase I safety study of escalating doses of atrasentan in adults with recurrent malignant glioma. *Neuro Oncol.* 10, 617–623. doi: 10.1215/15228517-2008-013

Poret, B., Desrues, L., Bonin, M.-A., Pedard, M., Dubois, M., Leduc, R., et al. (2020). Development of novel 111-In-labelled DOTA urotensin II analogues for targeting the UT receptor overexpressed in solid tumours. *Biomolecules* 10:471. doi: 10.3390/biom10030471

Prosser, H. C. G., Forster, M. E., Richards, A. M., and Pemberton, C. J. (2008). Urotensin II and urotensin II-related peptide (URP) in cardiac ischemia-reperfusion injury. *Peptides* 29, 770–777. doi: 10.1016/j.peptides.2007.08.013

Rivera, E., Arrieta, O., Guevara, P., Duarte-Rojo, A., and Sotelo, J. (2001). AT1 receptor is present in glioma cells; its blockage reduces the growth of rat glioma. *Br. J. Cancer* 85, 1396–1399. doi: 10.1054/bjoc.2001.2102

Rollins, B. J. (2006). Inflammatory chemokines in cancer growth and progression. *Eur. J. Cancer* 42, 760–767. doi: 10.1016/j.ejca.2006.01.002

Rylova, S. N., Barnucz, E., Fani, M., Braun, F., Werner, M., Lassmann, S., et al. (2014). Does imaging αvβ3 integrin expression with PET detect changes in angiogenesis during bevacizumab therapy? *J. Nucl. Med.* 55, 1878–1884. doi: 10.2967/jnumed.114.137570

Schnell, O., Krebs, B., Carlsen, J., Miederer, I., Goetz, C., Goldbrunner, R. H., et al. (2009). Imaging of integrin alpha(v)beta(3) expression in patients with malignant glioma by [18F] Galacto-RGD positron emission tomography. *Neuro Oncol.* 11, 861–870. doi: 10.1215/15228517-2009-024

Segain, J.-P., Rolli-Derkinderen, M., Gervois, N., Raingeard de la Blétière, D., Loirand, G., and Pacaud, P. (2007). Urotensin II is a new chemotactic factor for UT receptor-expressing monocytes. *J. Immunol.* 179, 901–909.

Spinazzi, R., Albertin, G., Nico, B., Guidolin, D., Di Liddo, R., Rossi, G. P., et al. (2006). Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial cells, and urotensin-II via UT-R stimulates angiogenesis in vivo and in vitro. *Int. J. Mol. Med.* 18, 1107–1112.

Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J. B., et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* 352, 987–996. doi: 10.1056/NEJMoa043330

Sugo, T., Murakami, Y., Shimomura, Y., Harada, M., Abe, M., Ishibashi, Y., et al. (2003). Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain. *Biochem. Biophys. Res. Commun.* 310, 860–868.

Sun, S.-L., and Liu, L.-M. (2019). Urotensin II: an inflammatory cytokine. *J. Endocrinol.* doi: 10.1530/JOE-18-0505 Online ahead of print.

Takano, S. (2012). Glioblastoma angiogenesis: VEGF resistance solutions and new strategies based on molecular mechanisms of tumor vessel formation. *Brain Tumor Pathol.* 29, 73–86. doi: 10.1007/s10014-011-0077-6

Verhaak, R. G. W., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., et al. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17, 98–110. doi: 10.1016/j.ccr.2009.12.020

Wang, X., Guo, G., Guan, H., Yu, Y., Lu, J., and Yu, J. (2019). Challenges and potential of PD-1/PD-L1 checkpoint blockade immunotherapy for glioblastoma. *J. Exp. Clin. Cancer Res.* 38:87. doi: 10.1186/s13046-019-1085-3

Weksler, B., Romero, I. A., and Couraud, P.-O. (2013). The hCMEC/D3 cell line as a model of the human blood brain barrier. *Fluids Barriers CNS* 10:16. doi: 10.1186/2045-8118-10-16

Weksler, B. B., Subileau, E. A., Perrière, N., Charneau, P., Holloway, K., Leveque, M., et al. (2005). Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J.* 19, 1872–1874. doi: 10.1096/fj.04-3458fje

Wen, P. Y., and Brandes, A. A. (2009). Treatment of recurrent high-grade gliomas. *Curr. Opin. Neurol.* 22, 657–664. doi: 10.1097/WCO.0b013e32833229e3

Wick, W., and Kessler, T. (2018). Drug repositioning meets precision in glioblastoma. *Clin. Cancer Res.* 24, 256–258. doi: 10.1158/1078-0432.CCR-17-2989

Xie, Q., Mittal, S., and Berens, M. E. (2014). Targeting adaptive glioblastoma: an overview of proliferation and invasion. *Neuro Oncol.* 16, 1575–1584. doi: 10.1093/neuonc/nou147

Xu, H., Rahimpour, S., Nesvick, C. L., Zhang, X., Ma, J., Zhang, M., et al. (2015). Activation of hypoxia signaling induces phenotypic transformation of glioma cells: implications for bevacizumab antiangiogenic therapy. *Oncotarget* 6, 11882–11893. doi: 10.18632/oncotarget.3592

Xu, S., Jiang, H., Wu, B., Yang, J., and Chen, S. (2009). Urotensin II induces migration of endothelial progenitor cells via activation of the RhoA/Rho kinase pathway. *Tohoku J. Exp. Med.* 219, 283–288.

Zhang, Y.-G., Li, J., Li, Y.-G., and Wei, R.-H. (2008). Urotensin II induces phenotypic differentiation, migration, and collagen synthesis of adventitial fibroblasts from rat aorta. *J. Hypertens.* 26, 1119–1126. doi: 10.1097/HJH.0b013e3282fa1412

Zhou, C.-H., Wan, Y.-Y., Chu, X.-H., Song, Z., Xing, S.-H., Wu, Y.-Q., et al. (2012). Urotensin II contributes to the formation of lung adenocarcinoma inflammatory microenvironment through the NF-κB pathway in tumor-bearing nude mice. *Oncol. Lett.* 4, 1259–1263. doi: 10.3892/ol.2012.932

Zhou, J., Xiang, Y., Yoshimura, T., Chen, K., Gong, W., Huang, J., et al. (2014). The role of chemoattractant receptors in shaping the tumor microenvironment. *BioMed Res. Int.* 2014:751392. doi: 10.1155/2014/751392

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 06 September 2021
doi: 10.3389/fcell.2021.705697

LncRNA NEAT1 Promotes Gastric Cancer Progression Through miR-17-5p/TGFβR2 Axis Up-Regulated Angiogenesis

Yangwei Xu[1,2†], Yanyan Li[1,2†], Yue Qiu[1,2], Fei Sun[3], Guifang Zhu[4], Jingbo Sun[1,2], Guixing Cai[3], Wanmei Lin[1,3], Yun Fu[1,2], Hongmei Wu[1,2], Shanshan Jiang[1,2], Zhihui Wen[1,2], Feiyan Feng[1,2], Junjie Luo[1,2], Yuqin Yang[1,2] and Qingling Zhang[1,2]*

[1] Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China, [2] Department of Pathology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China, [3] Nanfang Hospital, First Clinical Medical School, Southern Medical University, Guangzhou, China, [4] Department of Pathology, Zhujiang Hospital, Southern Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Lucas Treps, INSERM U1232 Centre de Recherche en Cancérologie et Immunologie Nantes Angers (CRCINA), France

Reviewed by:
Macrina Beatriz Silva Cázares, Autonomous University of San Luis Potosí, Mexico
Pai-Sheng Chen, National Cheng Kung University, Taiwan

***Correspondence:**
Qingling Zhang
zqllc8@126.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 06 May 2021
Accepted: 17 August 2021
Published: 06 September 2021

Citation:
Xu Y, Li Y, Qiu Y, Sun F, Zhu G, Sun J, Cai G, Lin W, Fu Y, Wu H, Jiang S, Wen Z, Feng F, Luo J, Yang Y and Zhang Q (2021) LncRNA NEAT1 Promotes Gastric Cancer Progression Through miR-17-5p/TGFβR2 Axis Up-Regulated Angiogenesis. Front. Cell Dev. Biol. 9:705697. doi: 10.3389/fcell.2021.705697

Background: Long non-coding RNAs (lncRNAs) have been indicated to play critical roles in gastric cancer (GC) tumorigenesis and progression. However, their roles in GC remain to be further elucidated.

Methods: RT-qPCR and fluorescence *in situ* hybridzation (FISH) were conducted to detect the expression of lncRNA NEAT1 in GC tissues and cell lines. Gene Set Enrichment Analysis (GSEA) was performed to screen out potential phenotypes and pathways that NEAT1 may participate in. NEAT1-silenced AGS and MGC803 cells were constructed and a series of functional experiments to investigate the roles of NEAT1 in GC angiogenesis both *in vitro* and *in vivo*. RNA pull down and luciferase reporter assays were utilized to illustrate the mechanisms underlying the functions of NEAT1 in GC.

Results: We observed that NEAT1 was upregulated in most GC specimens and cell lines. NEAT1 high was correlated with poor prognosis of GC patients. *In vitro* experiments showed that NEAT1 promoted GC angiogenesis by enhancing proliferation, migration, and tube formation ability of endothelial cells. Mechanism researches revealed that NEAT1 could competitively sponge miR-17-5p which targeted TGFβR2 directly. Subsequently, activate TGFβ/Smad pathway by following with upregulation of a series of classical proangiogenic factors especially VEGF.

Conclusion: The study unveiled that the LncRNA NEAT1/miR-17-5p/TGFβR2 axis is a novel mechanism in GC angiogenesis. Disrupting this axis may be a potential strategy for GC treatment.

Keywords: LncRNA NEAT1, miR-17-5p, gastric cancer, TGFβR2, angiogenesis, progression

INTRODUCTION

Gastric cancer is the fifth most frequent malignancies and the fourth leading cause of cancer death globally. In spite of the remarkable progression in diagnoses and therapies, overall prognosis of GC patients remains dismal (Siegel et al., 2021). Thus, developing effective targeted therapies for successful intervention is vitally important. Excessive angiogenesis is widely believed to fuel tumor

proliferation and metastases, and is identified as a hallmark accounting for the poor prognosis of GC. Anti-angiogenic therapy has raised more and more interest due to its low response rate and the inevitable chemoresistance. However, the underlying molecular mechanisms that mediate GC angiogenesis have yet to be fully clarified.

Long-stranded non-coding RNAs are important members of the ncRNA family whose lengths exceed 200 nucleotides and have limited protein coding potential (Mercer et al., 2009). Growing evidences have indicated the critical roles that lncRNAs play in the tumorigenesis and progression of human cancers (Li et al., 2018). The lncRNA SATB2-AS1 suppresses colorectal cancer aggressiveness by repressing snail transcription and epithelial-mesenchymal transition (Wang et al., 2019). Dysregulation of lncRNAs result in aberrant expression of multiple genes through multiple mechanisms including the classical ceRNA mechanism. In this theory, lncRNAs decrease the expression of microRNAs (miRNAs) *via* sponging them, which lead to upregulation of miRNA targets. For instance, LncRNA LINC00662 promotes cell growth and metastasis by competitively binding with miR-340-5p to regulate CLDN8/IL22 co-expression and activating ERK signaling pathway in colorectal cancer (Cheng et al., 2020). LncRNA KCNQ1OT1 promotes cell proliferation and cisplatin resistance *via* sponging miR-211-5p to mediate the Ezrin/Fak/Src axis in tongue cancer (Zhang et al., 2018). Thus, it's of great value to elucidate the mechanisms underlying lncRNA-mediated cancer progression and exploring more effective therapeutic targets for tumor patients.

Long-stranded non-coding RNAs nuclear-enriched abundant transcript 1 (NEAT1) is a pivotal component of nuclear paraspeckles, which have been reported to exert extensive roles in cancer progression (Clemson et al., 2009). Accumulating evidences have revealed that NEAT1 is dysregulated and acts as an unfavorable prognostic factor in human cancers including gliomas (Zhen et al., 2016), colorectal cancer (Wu et al., 2015), liver cancer (Wang et al., 2017), gallbladder cancer (Yang et al., 2020), and gastric cancer (Wang et al., 2018). NEAT1 promotes tumorigenesis and progression *via* enhancing malignant phenotypes of cancer cells including migration, invasion, proliferation, and chemoresistance. However, the correlation between NEAT1 and tumor angiogenesis has hardly been reported. MiR-17-5p was widely reported to function in progression of multiple cancers such as GC (Song et al., 2020), colorectal cancer (Xu et al., 2019) and nasopharyngeal carcinoma (Duan et al., 2019). Nevertheless, its regulatory mechanisms and connection with NEAT1 in GC remain to be further elucidated.

In this study, based on bioinformatics analysis and a series of *in vitro* and *in vivo* experiments, we first proved that NEAT1 correlated with enhanced angiogenesis in GC. Next, we demonstrated that NEAT1 competitively sponged miR-17-5p, which subsequently upregulated Transforming growth factor-β receptor 2 (TGFβR2) expression and activated the TGFβ/Smad pathway. These results deepen our understanding on GC angiogenesis and may provide feasible therapeutic strategies for GC patients.

MATERIALS AND METHODS

Clinical Samples and Cell Culture

Sixty-four cases of gastric cancer and matched adjacent normal gastric tissue samples were obtained from patients undergoing surgical treatment at Nanfang Hospital, Southern Medical University. No chemotherapy or radiation therapy was administered prior surgery. Both study protocol and informed consent were approved by the Ethical Committee of Nanfang Hospital.

Human normal gastric mucosal cells GES-1, Human gastric cancer cell lines (AGS, MGC803, SGC7901, MKN45 and HGC27), and Human umbilical vein endothelial cell line (HUVEC) were obtained from the Cell Bank of Chinese Academy of Medical Sciences (Shanghai, China). These cells were cultured in PPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Waltham, MA, United States), 100 IU/mL penicillin G and 100 μg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA, United States). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. All cell lines were routinely tested for mycoplasma and the results were negative.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was extracted using Trizol (Invitrogen, United States). To quantify the expression of NEAT1 and proangiogenic factors, the total RNA was subjected to polyadenylation and reverse transcription (RT) using a ThermoScript^{TM} RT-PCR System (Invitrogen). Real-time polymerase chain reaction (PCR) analysis was performed using an SYBR Green PCR master mix (Applied Biosystems, United States) on an ABI 7500HT system. GAPDH was used as an endogenous control. All samples were normalized to internal controls, and fold changes were calculated through relative quantification ($2^{-\Delta\Delta CT}$). For miRNA detection, reverse transcription was performed and expression of miRNA was measured by All-in-One^{TM} miRNA qRT-PCR Detection Kit (GeneCopoeia) according to the use manual. Genomic DNA (gDNA) was isolated from tissues or cultured cells according to easy pure genomic DNA kit (Transgen Biotech). The primers used are shown in **Supplementary Tables 2, 3**.

Western Blot

Cells were lysed in RIPA lysis buffer with protease inhibitor cocktail to extract total proteins. Before separating by SDS-PAGE gel and transferring onto the PVDF membrane, proteins were quantified by BCA protein assay kit (Pierce, KeyGEN BioTECH, China). Tris buffer containing 0.1% Tween-20 and 5% BSA was used to block the membrane at room temperature. Rabbit antibodies to GAPDH, VEGF (1:1000, Proteintech), and P-smad2, P-smad3, TGFβR2 (1:1000, CST) were used to incubate with the membrane overnight at 4°C, followed by HRP-conjugated secondary antibody treatment (anti-rabbit IgG/anti-mouse IgG, CST, 1:10000). After washed three times with PBST, the membrane was

developed with ECL substrate and imaged using the enhanced chemiluminescence detection system (Tennon5200, China) as described by the manufacturer.

Human Umbilical Vein Endothelial Cells (HUVEC) Tube Formation Assay

Growth factor reduced matrigel (BD Biosciences, United States) was prepared and kept on ice overnight until used. 50 μl matrigel was added to 96-well plate per well evenly and incubated at 37°C for at least 30 min. After digested with 0.25% trypsin and washed with PBS for three times, HUVEC cells (2×10^4 per well) were resuspended with 200 μl serum-free medium and seeded onto the prepared matrigel. Images were photographed after incubation at 37°C for 4 h. The capillary tubes were quantified under a 100× bright-field microscope, by measuring the total numbers of the completed tube structure. Each experiment was repeated three times.

Chicken Chorioallantoic Membrane (CAM) Assay

Chicken chorioallantoic membrane assay was performed as reported (Deng et al., 2019). Briefly, a window about 1.0 cm in diameter was opened in the eggshell to expose the CAM. A sterile rubber ring in 0.5 cm diameter was placed on the CAM and then 100 μl conditioned medium (CM) was added. The window was closed using a piece of steriled adhesive tape, and eggs were placed in a 37°C incubator with 80–90% relative humidity for 2–3 days. CAMs were fixed by stationary solution (methanol: acetone = 1:1) for 15 min before it was cut and harvested. Photos were taken by a digital camera (Cannon, Japan) and the effects of CM on angiogenesis were assessed through assessing the number of second- and third-order vessels.

HUVEC Cell Proliferation Assay

Cell proliferation was analyzed with EdU assays according to the manufacturer's instructions (KeyFluor488 Click-iT EdU Kit, keyGEN BioTECH, China). Cells cultured in 96-well plate were treated with 100 μL of medium containing 20 μM EdU and incubated at 37°C, 5% CO_2 for 2 h. After washed with PBS for three times, the cells were fixed with 4% paraformaldehyde for 30 min and incubated with 0.5% Triton-X-100 in PBS for 20 min. The nuclei were stained with DAPI for 10 min. The images of five randomly selected areas of each group were taken with a fluorescence microscope (Leica, Germany) and the proliferation rate was calculated.

HUVEC Cell Migration Assay

Transwell assay was performed to access migration ability of HUVEC. Firstly, 500 μl culture medium containing 20% FBS was added to the lower chamber and HUVEC cells (1×10^5) were trypsinized and resuspended in 100 μl serum-free medium. Then the resuspended cells were seeded onto the upper chamber and incubated at 37°C for 24 h. Migrated cells attached to the bottom surface of the insert were then fixed with methanol and stained with hematoxylin. Penetrated cells were quantified under a light microscope in five random visual fields (200×).

Cell migration was also analyzed with wound healing assay. HUVECs were seeded to a 6 well plate and a 1000 μl plastic pipette tip was used to scratch the monolayers. After washed with PBS for three times, the wounded cells were then cultured in serum-free medium for an additional 12 h and photographed under a fluorescence microscope. The distance between the edge of the scratch wound was measured before and after cell migration. The mean migration distance (μm) was calculated by subtracting the length after 12 h from that at 0 h. The result was expressed as a migration index, i.e., the distance migrated by treated cells compared with the distance migrated by control cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatants from AGS and MGC803 were collected and the cell number was counted. The supernatant was used to measure the total levels of extracellular VEGF by the human VEGF ELISA Kit (Enzyme-linked Biotechnology, China) according to the manufacturer's introductions. The cytokine expression level (pg/ml) per 1×10^5 cells was analyzed.

IHC, IF and FISH

Specimens of gastric cancer and matched adjacent normal gastric tissue samples were fixed with 4% formalin, paraffin embedded and sectioned (4 μm). The tissue sections were then deparaffinized and dehydrated followed by incubation in 3% hydrogen peroxide for 10 min. Slides were stained with primary antibodies against CD31 (1:100, ZSGB Bio) and Ki67 (1:100, ZSGB Bio) at 4°C overnight after blocking with 5% BSA in PBS for 1 h at RT. Corresponding secondary antibodies were used for 1 h at RT. Targeted molecules were detected following DAB staining for immunohistochemistry. Slides were finally counterstained with hematoxylin. Two independent investigators blinded to sample identify, one investigator performed the staining and another one analyzed the tissue section.

For immunofluorescent staining, tissue sections and cells were immunostained with primary antibodies against TGFβR2 (Proteintech, 1:200) and CD31 (1:100, Abcam) overnight at 4°C and subsequently incubated with fluorochrome conjugated antibodies. Finally, DAPI was added as nuclear counterstain. Images were captured using a fluorescence microscope or laser scanning confocal microscope (Nikon, Japan).

LncRNA NEAT1-FISH was conducted as previously described (Adriaens et al., 2016).

Biotinylated RNA Pull-Down Assay

Pull down assay was carried out as described (Hu et al., 2019). In brief, 1×10^7 cells were harvested, lysed and sonicated for detecting the LncRNA NEAT1 pull down miRNAs. The LncRNA NEAT1 probe was incubated with streptavidin magnetic beads (Beaver, china) for 2 h to generate probe-coated beads, then incubated with cell lysates. The products after incubation were then eluted with trizol, followed by qRT-PCR. For miR-17-5p pulled down, 1×10^7 cells were collected, lysed, sonicated and incubated with streptavidin magnetic beads after

transfected with biotinylated miR-17-5p mimics or mutant using lipofectamine2000 (Thermo Fisher, China), followed by washed, eluted and qRT-PCR.

Dual-Luciferase Reporter System Analysis

For NEAT1 and miRNA luciferase assay, the NEAT1 sequences containing wild-type or mutated miRNA binding sites were, respectively, synthesized and inserted into pmirGLO luciferase vector (Jikai, China). MiR-17-5p mimics were then co-transfected with the above mentioned vectors into cells using lipofectamine2000. After 48 h transfection, cells were harvested, lysed, and subjected to luciferase activity detection by the Luc-pair^{TM} Duo-Luciferase HS assay kit (GeneCopoeia, China). Relative luciferase activity was normalized to the Renilla luciferase internal control. Three independent experiments were performed.

Orthotopic Xenograft Tumor Mouse Model

The Orthotopic GC mouse models were constructed as described in our previous research (Li et al., 2020). Briefly, after anesthetizing the mice with ketamine (70 μg/kg), a small incision was made in the abdomen and to reveal the stomach. Then, 100 μl of a cell suspension (1×10^6 cells) was injected into the muscle tissue of the stomach. Then, the stomach position was reset, the wound was treated with penicillin, and the abdominal incision was closed. Mice with digestive symptoms or moribund appearance were sacrificed. The tumor-bearing stomachs and livers were sectioned for immunohistochemical or immunofluorescence analysis.

Statistical Analysis

Each experiment was performed at least three times. Statistical analyses were performed using Prism 7 (GraphPad Software, San Diego, CA, United States). Pearson's chi-squared ($\chi 2$) test and student's *t*-test were used to evaluate the significance of the differences among different groups. Survival curves were analyzed using the Kaplan-Meier method and assessed by log rank testing. All data were presented as the means ± standard deviation (SD).

RESULTS

LncRNA NEAT1 Is Up-Regulated in Gastric Cancer, Predicts Poor Prognosis and Positively Correlates With Angiogenesis

To identify the roles and molecular mechanisms of LncRNA NEAT1 in gastric cancer (GC), we analyzed the expression of NEAT1 in GC across the GEO database. In GSE66229 cohort, we found that NEAT1 was significantly highly expressed in GC compared with normal gastric tissues (**Figure 1A**). In GSE15459 cohort, NEAT1 expression was higher in stage III and IV GC tissues than that in stage I and II (**Figure 1B**). Consistently, RT-qPCR results confirmed that NEAT1 expression was higher in 64 GC tissues than that in the matched adjacent normal mucosal tissues collected from patients undergoing surgery in Nanfang hospital (**Figure 1C**). The clinical and pathological characteristics of these patients are shown in **Supplementary Table 1**. Statistical analysis revealed that high NEAT1 expression levels were correlated with advanced T stage and big tumor size in GC patients (**Supplementary Table 1**). To explore the potential prognostic and predictive values of NEAT1 in GC progression, we next conducted survival analyses in the GSE15459 dataset and the Kaplan-Meier Plotter database[1]. The results consistently indicated that NEAT1 up-regulation was significantly correlated with shorter OS in GC patients (**Figures 1D,E**). We also detected NEAT1 expression through RT-qPCR in GC cell lines and GES-1, a normal stomach mucosal cell line, and observed that NEAT1 was higher expressed in GC cell lines compare with GES-1 cell line (**Figure 1F**).

To explore the potential regulatory role of NEAT1 in GC, the Gene Set Enrichment Analysis (GSEA) was performed in the GSE15459 dataset with 200 gastric cancer samples. As were shown in **Figure 1G**, pathways related to angiogenesis, including the VEGF, PDGF, TGFβ, and angiopoietin pathway, were positively enriched in patients harboring high NEAT1 expression. We next detected the mRNA expression of classical proangiogenic factors including TGFβ1, PDGF-B, VEGF-C, HIF-1α, PGF, FGF2, DLL4, VEGF-A, and CXCL8. The results showed that NEAT1 knockdown in AGS and MGC803 cells markedly decreased the expression of aforementioned proangiogenic factors (**Figure 1H**). Taken together, these findings suggested that NEAT1 might play an essential role in promoting GC progression *via* regulation of angiogenesis.

Silencing NEAT1 Represses Tube Formation, Proliferation and Migration of Vascular Endothelial Cells

In order to investigate the biological behaviors of NEAT1 in GC cells, we firstly constructed two independent NEAT1 shRNA-expression lentivirus (shNEAT1-1 and shNEAT1-3). AGS and MGC803 cell lines with high endogenous NEAT1 expression were chosen to silence NEAT1. Transfection efficiency was confirmed by RT-qPCR (**Figure 2A**). To verify NEAT1's potential roles in GC angiogenesis, we firstly detected the secretion of VEGF, a key secretory protein in regulating tumor vascularization, in the culture medium (CM) of GC cells with different NEAT1 expression levels through ELISA assays. The ELISA results indicated that silencing NEAT1 significantly decreased the secretion of VEGF in both AGS and MGC803 cells (**Figure 2B**). Tube formation assay was also performed and the results showed that NEAT1 silenced CM dramatically inhibited tube formation of HUVECs (**Figure 2C**). Besides, transwell assay (**Figure 2D**), wound healing assay (**Figure 2E**) and EdU assay (**Figure 2F**) were used to assess the effects of CM on HUVEC migration and proliferation, as the migration and proliferation of

[1]http://kmplot.com/analysis/index.php?p=service

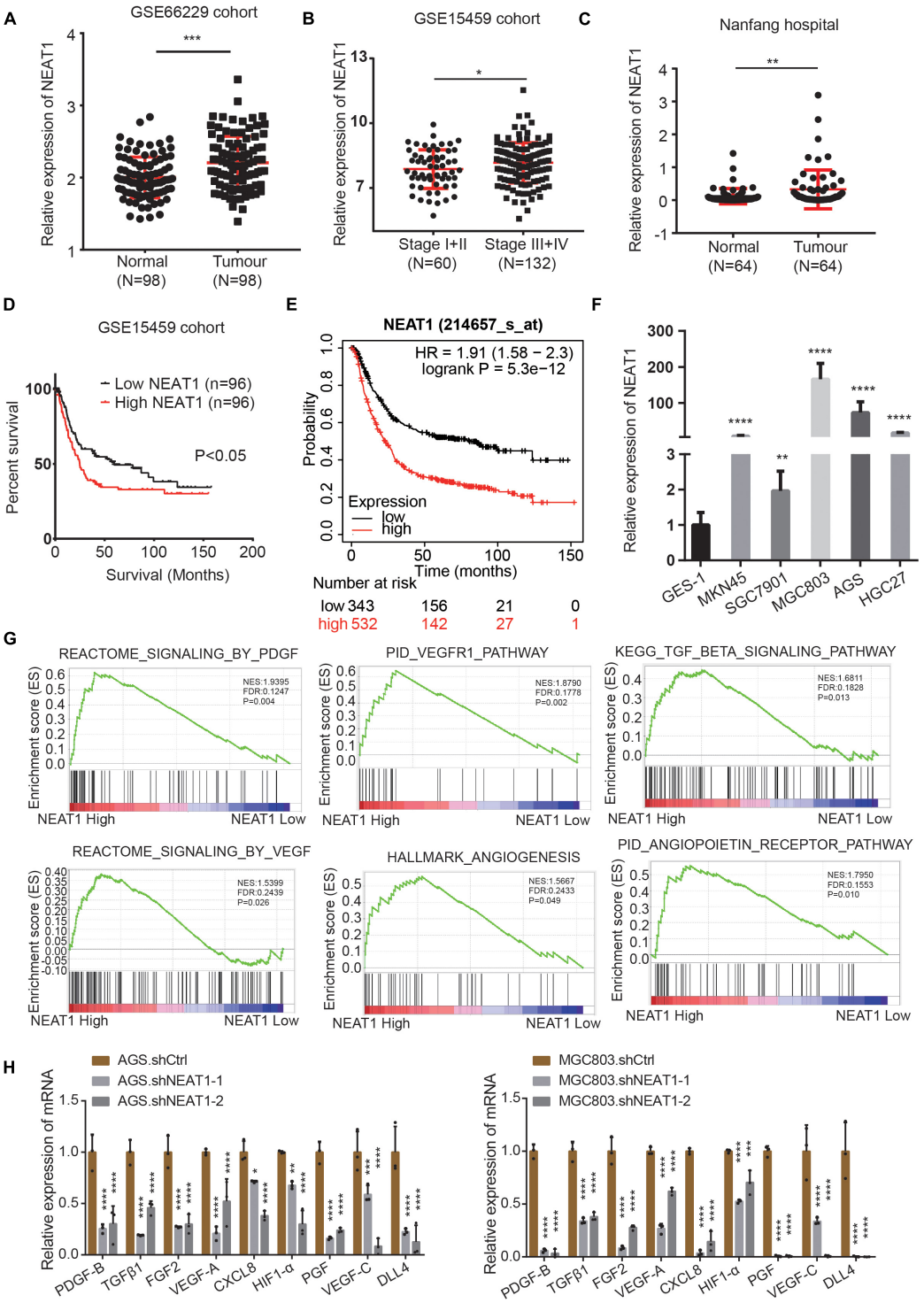


FIGURE 1 | LncRNA NEAT1 upregulation correlates with poor prognosis and angiogenesis in GC. **(A)** Scatter diagram represents the expression level of LncRNA NEAT1 in GC (n = 98) and normal gastric tissues (n = 98) derived from the GEO GSE66229 dataset. **(B)** NEAT1 expression level in GC tissues with TNM stage I + II (n = 60) and stage III + IV (n = 132). Data were derived from GSE15459 dataset. **(C)** Expression levels of NEAT1 were examined by RT-qPCR in 64 GC tissues and their pair-matched adjacent normal tissues from Nanfang hospital. **(D)** Kaplan-Meier analysis was used to assess the relation between NEAT1 expression level and overall survival in GC patients from GSE15459 cohort. **(E)** Kaplan-Meier plotter analysis of the correlation of NEAT1 expression level with overall survival of GC patients by the KM Plotter database. **(F)** The expression levels of NEAT1 in the GC cell lines and normal stomach mucosal cell line were determined by RT-qPCR. **(G)** GSEA validated angiogenesis-related pathways in high NEAT1 expression GC cohorts of GSE15459 dataset. **(H)** RT-qPCR detects the effects of silencing NEAT1 on the expression of classical proangiogenic factors in AGS and MGC803 cells (mean ± SD, n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

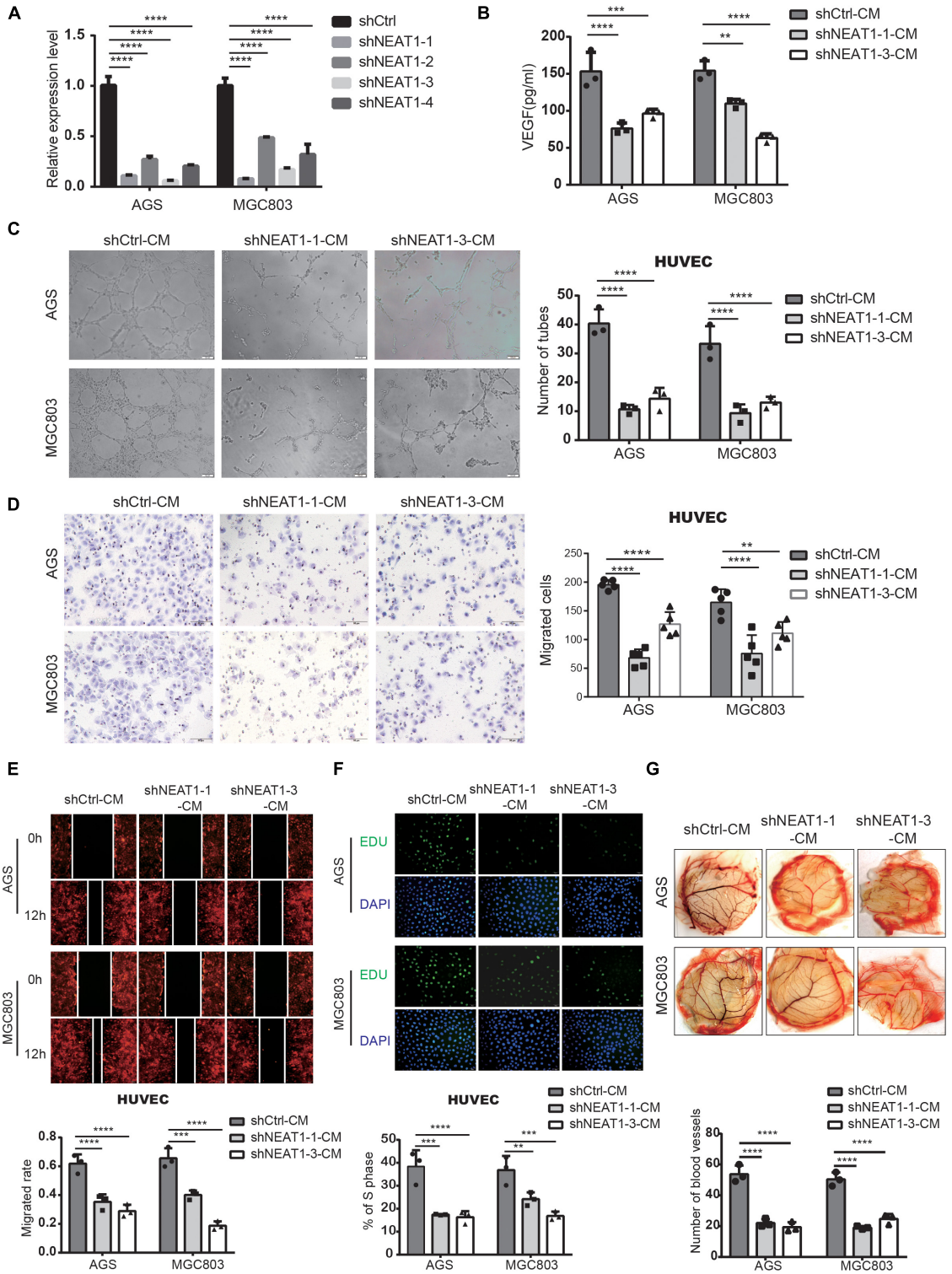


FIGURE 2 | Silencing of NEAT1 suppressed angiogenesis of GC cells. **(A)** RT-qPCR assay was used to verify the successful construction of NEAT1 knockdown GC cells. **(B)** The concentration of VEGF was detected in the culture medium of NEAT1-2 silenced AGS and MGC803 cells by ELISA assay (mean ± SD, $n = 3$). **(C)** Representative capillary tubule structures were shown for HUVECs treated with culture medium collected from the NEAT1 silenced cells. Scale bar represents 50 μm. **(D,E)** Transwell **(D)** and wound healing **(E)** assay were performed in HUVECs to detect the effect of CM treatment on cell migration. Scale bar represents 50 μm. **(F)** EdU assay was performed in HUVECs to detect the effect of CM treatment on cell proliferation. **(G)** Representative images of blood vessels formed in the CAM assay after CM treatment. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

endothelial cells are critical for angiogenesis. The results showed that CM of NEAT1 silenced cells represented markedly weaker promoting effect on the migration and proliferation of HUVECs compare with the control group. The proangiogenic effect of NEAT1 was further verified *in vivo* by using the chick embryo chorioallantoic membrane (CAM) assay. As shown in **Figure 2G**, CM of NEAT1 silenced cells represented markedly weaker promoting effect on vessels formation of CAM (**Figure 2G**). Collectively, these findings revealed that silencing NEAT1 in GC cells significantly inhibited angiogenesis by disrupting tube formation of endothelial cells.

LncRNA NEAT1 Upregulates TGFβR2 Expression by Sponging miR-17-5p as a ceRNA

Long non-coding RNAs most often function as competing endogenous RNAs (ceRNAs) to regulate downstream mRNAs by sponging miRNAs, suggesting that NEAT1 may exert its function in gastric cancer in a ceRNA manner. To elucidate the molecular mechanisms of NETA1 regulating angiogenesis in GC, bioinformatics analysis was conducted using starBase v2.0 to predict the miRNAs binding to NEAT1 and their potential binding sites. Ten miRNAs were identified as the potential targets of NEAT1 (**Supplementary Table 4**) and their interaction with NEAT1 was further verified through the RNA pulldown assay in both AGS (**Figure 3A**, left panel) and MGC803 cells (**Figure 3A**, right panel). What's more, among the miRNAs detected, miR-17-5p appeared to be the most significant miRNA pulled down by the LncRNA NEAT1 probe. Thus, we postulated that NEAT1 might regulate angiogenesis mainly by sponging miR-17-5p as a ceRNA. To further validate the interaction between NEAT1 and miR-17-5p, NEAT1-wt and NEAT1-mut dual luciferase reporter vectors were constructed (**Figure 3B**) and co-transfected with miR-17-5p or miR-NC into GC cells. Luciferase reporter assay demonstrated that upregulation of miR-17-5p relatively reduced the luciferase activity of NEAT1-wt vector, but didn't influence the luciferase activity of NEAT1-mut vector in AGS and MGC803 cell lines (**Figure 3C**). Additionally, RNA pull-down assay was implemented to determine whether miR-17-5p could directly bind to NEAT1. AGS and MGC803 cells were transfected with biotinylated miR-17-5p and then were harvested for biotin-based pull-down assays. As shown in **Figure 3D** by RT-qPCR, NEAT1 was pulled down by biotin-labeled miR-17-5p oligos but not the mutated oligos which disrupted base pairing between NEAT1 and miR-17-5p. Based on the above, our findings implied that miR-17-5p could directly bind to NEAT1.

To further elucidate the molecular mechanisms underlying the regulation of NEAT1/miR-17-5p axis, we examined miR-17-5p targets that were computationally predicted by the TargetScan algorithm. Among the high-scoring mRNA targets predicted for miR-17-5p, TGFβR2 was selected for further study. TGFβR2 and its downstream TGFβ/Smad signaling were recognized as one of the vital pathway in tumor angiogenesis (Oft et al., 1998; Flores-Pérez et al., 2016; Batlle et al., 2019). Besides, previous studies have demonstrated the interaction between miR-17-5p and TGFβR2 mRNA in non-small cell lung cancers (Li et al., 2017; Chen Y. et al., 2021). Herein, we aimed to examine whether NEAT1 regulates the expression of TGFβR2 in a miR-17-5p-dependent manner. Firstly, GC cells were transfected with miR-17-5p inhibitors, mimics and their counterpart control sequences. Transfection efficiency was confirmed through RT-qPCR (**Figure 3E**). Furthermore, the RT-qPCR results revealed that knocking down NEAT1 in AGS and MGC803 cells significantly upregulated the expression of miR-17-5p, accompanied with downregulation of TGFβR2 expression (**Figure 3F**). And we investigated that the expression of miR-17-5p was negatively associated with NEAT1expression in GC tissues (**Figure 3G**). Additionally, the effects of miR-17-5p on TGFβR2 expression were detected by western blot and the results indicated that the expression of miR-17-5p markedly negatively correlated with TGFβR2 expression in GC cells (**Figure 3H**). These results collectively proved that NEAT1 positively regulated TGFβR2 expression *via* sponging miR-17-5p in gastric cancer cells.

Repressing miR-17-5p Reverses Suppressive Effect of Silencing NEAT1 on Malignant Phenotypes of GC *in vitro*

Rescue experiments were conducted using miR-17-5p inhibitors to investigate whether NEAT1 promoted angiogenesis *via* the NEAT1/miR-17-5p/TGFβR2 axis. As shown in **Figure 4**, miR-17-5p inhibitor dramatically reversed the inhibitory effects of NEAT1 silencing on the tube formation ability (**Figure 4A**), migration (**Figures 4B,C**) and proliferation (**Figure 4D**) of HUVECs. Further ELISA assay indicated that inhibiting miR-17-5p markedly reversed the inhibitory effects of NEAT1 silencing on VEGF production (**Figure 4E**). Additionally, we conducted western blot assay to explored the effects of NEAT1/miR-17-5p/TGFβR2 axis on the TGFβ/Smad pathway. As expected, the results revealed that silencing NEAT1 in GC cells markedly decreased expression level of TGFβR2, P-Smad2, P-Smad3, and VEGF, pivotal indicators of TGF-β/smad pathway activity. Inhibiting miR-17-5p markedly reversed the inhibitory effects of NEAT1 silencing on the aforementioned proteins (**Figure 4F**). We also constructed the PPI (Protein-protein interaction) network for TGFβR2, TGFβ/Smad pathway-related proteins and the aforementioned proangiogenic factors. The results indicated that these proteins were closely associated with TGFβR2 alterations (**Figure 4G**). In conclusion, these findings indicated that NEAT1 promotes GC angiogenesis by regulating the miR-17-5p/TGFβR2 axis.

LncRNA NEAT1 Promotes GC Angiogenesis *via* the miR-17-5p/TGFβR2 Axis *in vivo*

To verify our *in vitro* findings, we established an *in vivo* xenograft model employing MGC803 cells in nude mice. The animal experiments were grouped as follows, shCtrl, shNEAT1, and shNEAT1 plus miR-17-5p-inhibitor. Results showed that tumor volume in the shNEAT1 group was dramatically smaller than those in the shCtrl group, and inhibiting miR-17-5p markedly reversed the inhibitory effects of NEAT1 silencing on the xenografts proliferation (**Figures 5A,B**). Consistent

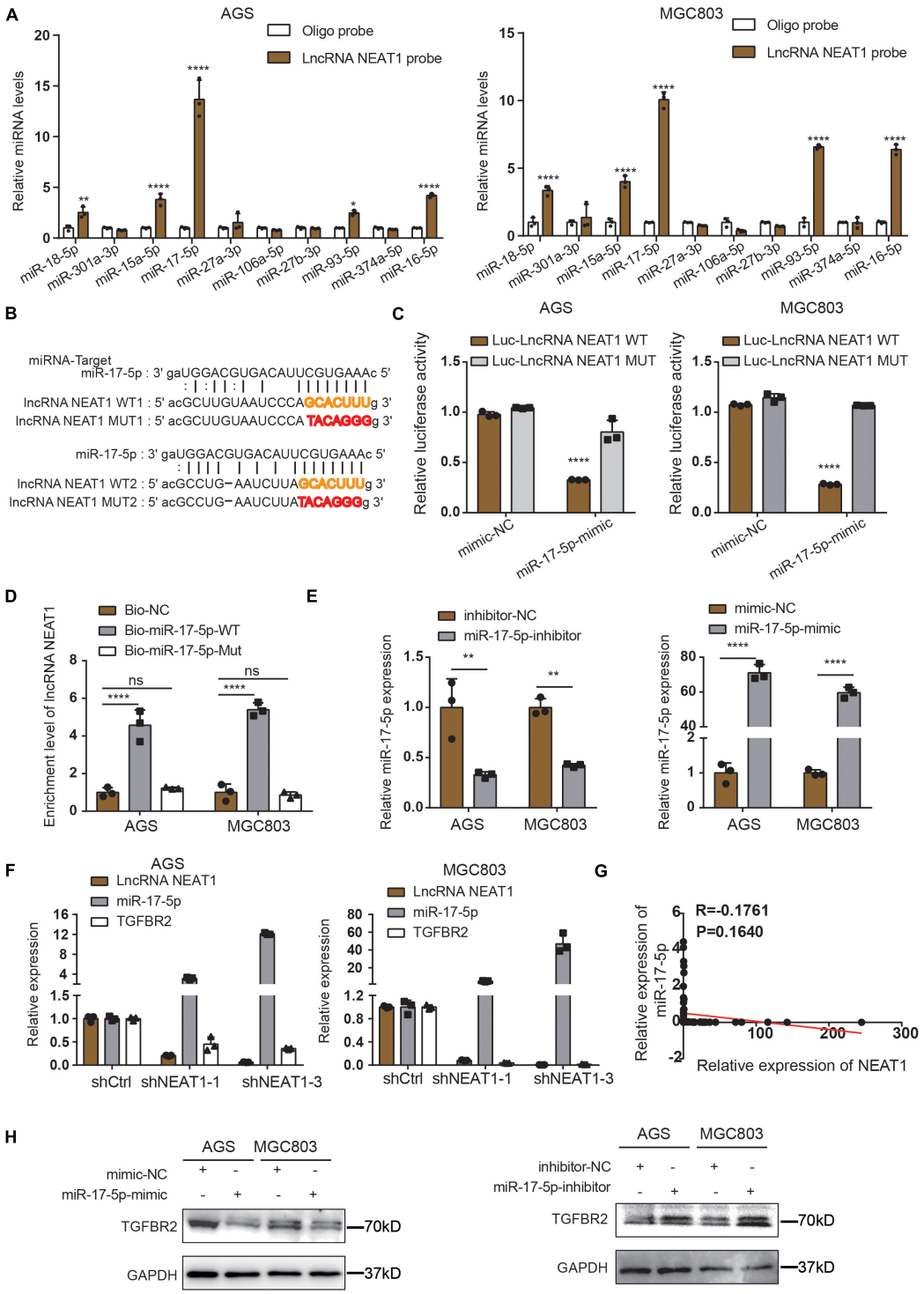


FIGURE 3 | NEAT1 regulates TGFBR2 expression by directly targeting miR-17-5p. **(A)** The expression levels of 10 candidate miRNAs were detected by RT-qPCR after RNA pulldown assay. **(B)** Predicted binding sites of miR-17-5p on LncRNA NEAT1 are shown. **(C)** Luciferase activity was conducted in AGS and MGC803 cells co-transfected with luciferase reporter containing LncRNA NEAT1 sequences with wild type and mutant binding site of miR-17-5p and the mimic of miR-17-5p or control. **(D)** Biotin-coupled miR-17-5p wild type (Bio-miR-17-5p-WT) or its mutant (Bio-miR-17-5p-Mut) captured relative expressions of LncRNA NEAT1 in the complex. Relative level of LncRNA NEAT1 was normalized to input. **(E)** Transfection efficiency of miR-17-5p inhibitor (left panel) and mimics (right panel) in AGS and MGC803 cells were validated by RT-qPCR assay. **(F)** Relative mRNA levels of miR-17-5p and TGFβR2 detected by RT-qPCR after knockdown NEAT1 in AGS and MGC803 cells. **(G)** Regression analysis of GC tissue showed a negative correlation between miR-17-5p and NEAT1 ($n = 64$). **(H)** Effects of miR-17-5p inhibitor (above panel) and mimics (below panel) on TGFβR2 expression in AGS and MGC803 cells were validated by western blot. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

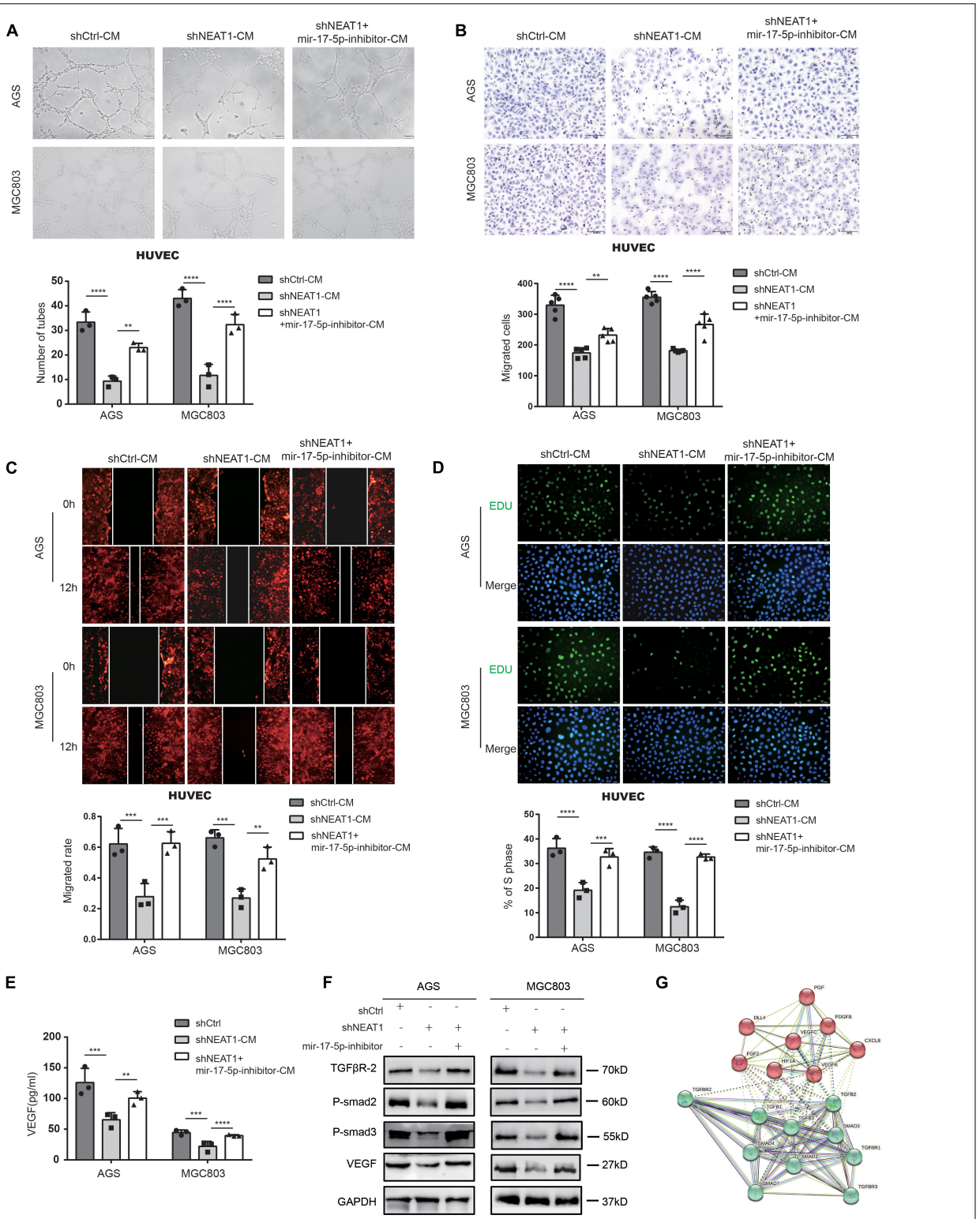


FIGURE 4 | Repressing miR-17-5p reverses suppressive effect of silencing NEAT1 on malignant phenotypes of GC *in vitro*. **(A)** Effects of miR-17-5p inhibitor on NEAT1 mediated GC angiogenesis through tube formation assay. **(B,C)** Effects of miR-17-5p inhibitor on NEAT1 mediated HUVEC migration through transwell **(B)** and wound healing assay **(C)**. **(D)** Effects of miR-17-5p inhibitor on NEAT1 mediated HUVEC proliferation through EdU assay. **(E)** Effects of miR-17-5p inhibitor on secretion changes of VEGF modulated by LncRNA NEAT1 through ELISA assay. **(F)** Effects of miR-17-5p inhibitor on expression changes of TGF-β/smad pathway proteins (P-smad2, P-smad3 and VEGF) modulated by LncRNA NEAT1 through western blot assay. **(G)** The PPI network of TGF-β/smad pathway-related proteins and classsical proangiogenic factors associated with TGFβR2 alterations in String analysis. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

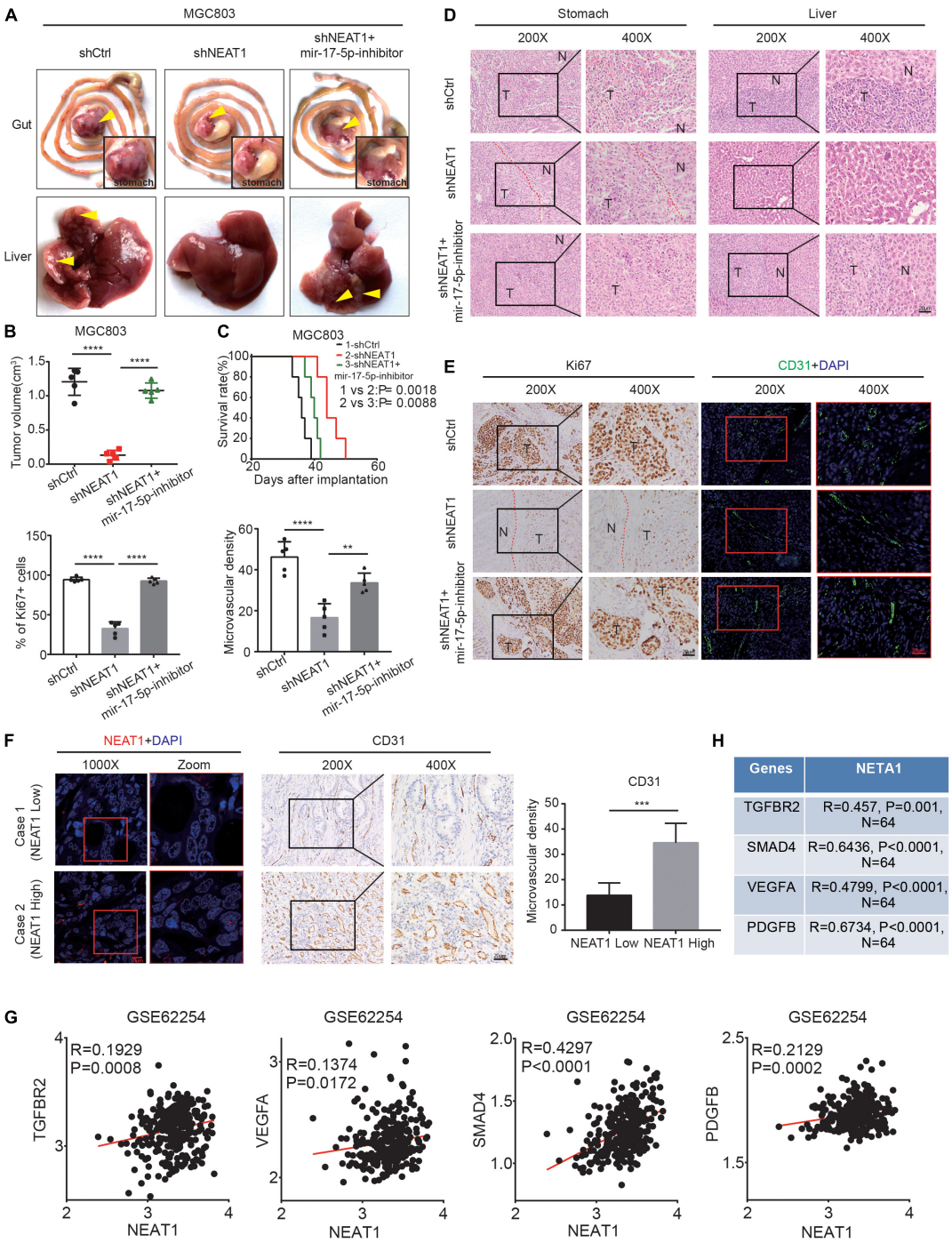


FIGURE 5 | LncRNA NEAT1 promotes GC angiogenesis *via* the miR-17-5p/TGFβR2 axis *in vivo*. **(A)** Gross of GC orthotopic tumors and corresponding livers. Representative images were shown. **(B)** Size analyses of the GC orthotopic tumors. **(C)** Kaplan-Meier survival analysis of mice bearing xenografts. $n = 5$ for each group. **(D)** H&E-stained paraffin-embedded tumor obtained from xenograft tumor **(E)** IHC and IF staining for ki67 and CD31 expression in xenograft tumor. **(F)** Representative images of GC patients' tumors with IHC and FISH staining. **(G)** Pearson correlation analysis was conducted to analyze the relation between LncRNA NEAT1, TGFβR2, VEGF, and Smad4 in GC GEO dataset. **(H)** Pearson correlation analysis was conducted to analyze the relation between LncRNA NEAT1, TGFβR2, VEGF, and Smad4 in GC tissues. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

results were observed after analysing the liver metastasis rate (**Supplementary Table 5**) and survival time (**Figure 5C**) among the three groups. In addition, HE staining showed that a more regular border was observed in the shNEAT1 group than the shNC group (**Figure 5D**). IHC and IF assays further revealed a significant decrease in expression levels of proliferation

marker Ki67 vessel marker CD31 in the shNEAT1 tumors versus the shCtrl tumors, respectively. These effects were rescued after blockade of miR-17-5p by miR-17-5p inhibitor (**Figure 5E**). Moreover, we collected 30 GC specimens as well as clinicopathological data. High and low NEAT1 expression were detected in GC specimens through FISH staining, respectively. As shown in **Figure 5F**, NEAT1 expression was positively correlated with CD31 expression (**Figure 5F**). Finally, a positive correlation between NEAT1 and TGFβR2, VEGF-A, Smad4, PDGFB was seen in GEO gastric cancer datasets GSE62254 (n = 200) (**Figure 5G**). The results obtained in the Nanfang group were consistent with the above results (**Figure 5H** and **Supplementary Figure 1A**). Therefore, these results further confirmed the vital role of LncRNA NEAT1/miR-17-5p/TGFβR2 signal axis in GC angiogenesis.

DISCUSSION

Excessive angiogenesis is an essential characteristic of cancer, which account for the tumor proliferation (Liu et al., 2019), invasion (Zeng et al., 2018), chemoresistance (Marti et al., 2015), etc., Emerging evidences have indicated that lncRNAs participate in regulating malignant phenotypes and act as critical clinical biomarkers for diagnosis and prognosis in multiple malignancies (Yang et al., 2019; Zhao et al., 2019; Gao et al., 2020; Shi et al., 2021). Although several angiogenesis-related lncRNAs have been identified in gastric cancers (Liu H. T. et al., 2020; Teng et al., 2021), effective anti-angiogenesis strategies in GC are still limited and the underlying mechanisms remain to be further disclosed.

Our study firstly identified lncRNA NEAT1 as a novel angiogenesis regulator in GC angiogenesis. NEAT1 was markedly upregulated in GC specimens and a higher NEAT1 expression was correlated with advanced stages and poor outcome in GC patients. Furthermore, functional experiments indicated that NEAT1 disruption inhibited angiogenesis both *in vitro* and *in vivo*. Numerous studies have demonstrated that lncRNAs regulate cellular processes in cancer cells by sponging specific miRNA as a ceRNA. For instance, lncRNA LINC00657 inhibites cervical cancer progression *via* sponging miR-20a-5p and targeting RUNX3 (Qin et al., 2021). LncRAN LINC01137 interacts with miR-22-3p to promote cell proliferation and invasion in oral squamous cell carcinoma (Du et al., 2021). Consistently, through the dual luciferase reporter assay and RNA pull-down assay, we found that the oncogenic function of NEAT1 in gastric cancer was modulated through its interaction with miR-17-5p, which has been identified as an oncogenic miRNA in a variety of tumor types. Silencing NEAT1 upregulated the expression level of miR-17-5p and in turn, inhibition of miR-17-5p reversed the inhibitory effects of NEAT1 silencing on GC angiogenesis both *in vitro* and *in vivo*.

Previous studies have revealed that miR-17-5p, a member of the miR-17-92 cluster, participates in comprehensive biological processes of cancer cells by regulating expression of its target mRNA at post-transcriptional level. For example, miR-17-5p directly targets RUNX3 and promotes proliferation and invasiveness in gastric cancer (Song et al., 2020). MiR-17-5p directly targets ERBB3 and suppresses postoperative metastasis of hepatocellular carcinoma (Liu D. L. et al., 2020). Herein, we identified TGFβR2 as a direct target of miR-17-5p in GC, which was consistent with previous studies (Li et al., 2017; Chen Y. et al., 2021). TGFβR2 protein expression was significantly negatively correlated with miR-17-5p in GC cell lines. Importantly, we further elucidated the involvement of LncRNA NEAT1

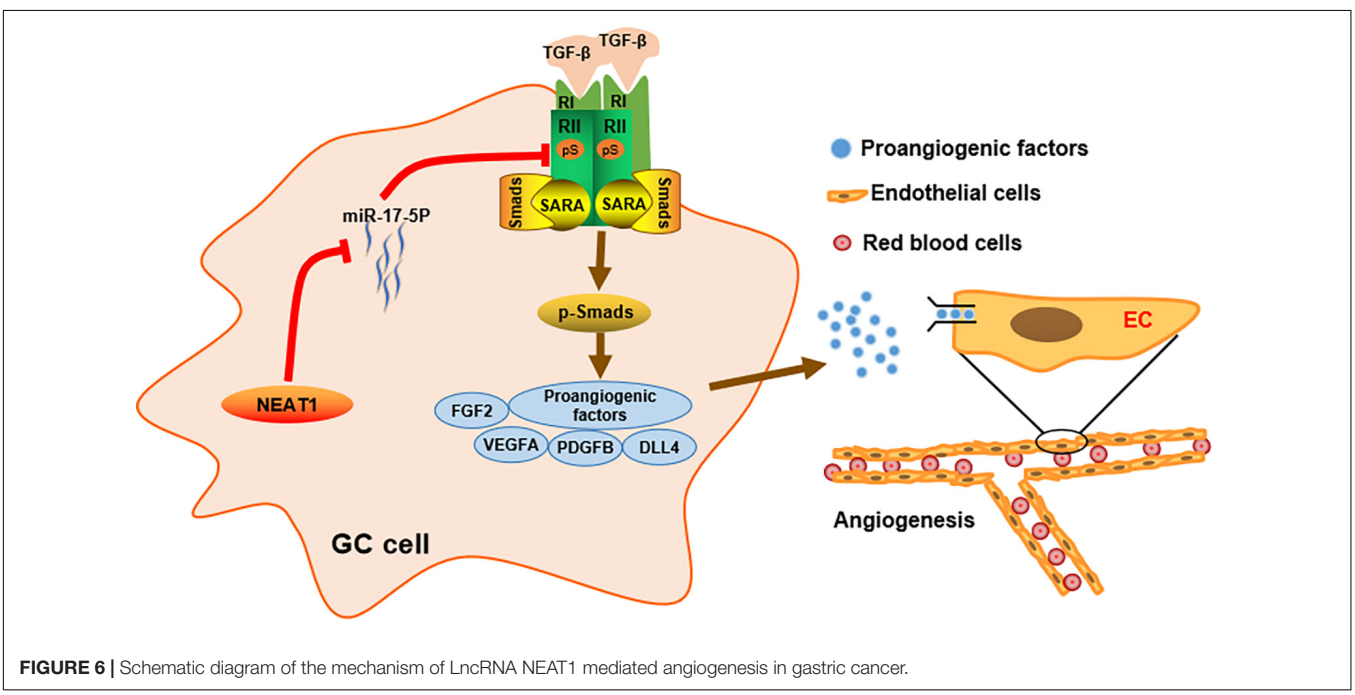


FIGURE 6 | Schematic diagram of the mechanism of LncRNA NEAT1 mediated angiogenesis in gastric cancer.

in the regulatory role of miR-17-5p in TGFβR2 expression. LncRNA NEAT1 sponges miR-17-5p and decreases its expression level, which subsequently activated the TGFβR2 expression and its downstream TGFβ/Smad pathway.

Transforming growth factor-β receptor 2 is located on the cell membrane and initiates the signal transduction by interacting with the TGFβ ligands (TGFβ-1, TGFβ-2, TGFβ-3). TGFβR2 phosphorylates the kinase domain of TGFβR1, which subsequently propagates the signal by phosphorylating Smad2 and Smad3. Phosphorylated- Smad2 and Smad3 polymerize with Smad4 to form the active transcriptome complex which translocate to nucleus and regulate extensive gene expression in both biological and pathological processes (Tsukazaki et al., 1998; De Caestecker et al., 2000). Activation of the TGFβ/Smad pathway have been recognized to upregulate numerous proangiogenic factors such as VEGF (Chruścik et al., 2018), FGF-2 (Yang et al., 2008), and TSP-4 (Muppala et al., 2017) in cancers. Among the aforementioned factors, VEGF was recognized to be the dominant angiogenesis regulator based on extensive researches (Leung et al., 1989; Carmeliet et al., 1996; Ferrara et al., 1996; Chen Z. et al., 2021). Herein, we demonstrated that LncRNA NEAT1 regulated TGFβR2 expression in a miR-17-5p-dependent manner. Silencing NEAT1 inhibited TGFβR2 expression as well as its downstream TGFβ signaling, accompanied with downregulation of a series of proangiogeneic factors especially VEGF. Our results are consistent with previous studies. However, further *in vitro* and *in vivo* experiments are required to validate the effects of TGFβR2 on LncRNA NEAT1-mediated angiogenesis in GC.

CONCLUSION

In conclusion, our study identified lncRNA NEAT1 as a novel driving factor in GC angiogenesis. We demonstrated *in vitro* and *in vivo* that lncRNA NEAT1 promoted TGFβR2 expression *via* competitively sponging miR-17-5p, thereby activated the TGFβ/Smad pathway and upregulated expression of proangiogenic factors especially VEGF. These findings suggest that the lncRNA NEAT1/miR-17-5p/TGFβR2 signal axis may provide promising targeting strategies for GC diagnosis and treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Nanfang Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care and Use Committee of Southern Medical University.

AUTHOR CONTRIBUTIONS

QZ led the study design and supervised this work. YX prepared the manuscript and analyzed the data. YX, YL, YQ, FS, GZ, JS, GC, WL, YF, and HW performed the experiments. SJ, ZW, FF, JL, and YY gave assistance in collecting tissue samples and animal experiments. All authors discussed the results and approved of the final version.

FUNDING

This project was supported by grants of the National Natural Science Foundation of China (Nos. 81772918 and 81972277), Guangdong Provincial Natural Science Foundation of China (No. 2017A030313896). Guangzhou Science & Technology Project (No. 201804010319). Key Project of Clinical Research of Southern Medical University (No. C1033401).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.705697/full#supplementary-material

REFERENCES

Adriaens, C., Standaert, L., Barra, J., Latil, M., Verfaillie, A., Kalev, P., et al. (2016). p53 induces formation of NEAT1 lncRNA-containing paraspeckles that modulate replication stress response and chemosensitivity. *Nat. Med.* 22, 861–868. doi: 10.1038/nm.4135

Batlle, R., Andrés, E., Gonzalez, L., Llonch, E., Igea, A., Gutierrez-Prat, N., et al. (2019). Regulation of tumor angiogenesis and mesenchymal-endothelial transition by p38α through TGF-β and JNK signaling. *Nat. Commun.* 10:3071.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380, 435–439. doi: 10.1038/380435a0

Chen, Y., Zhou, X., Huang, C., Li, L., Qin, Y., Tian, Z., et al. (2021). LncRNA PART1 promotes cell proliferation and progression in non-small-cell lung cancer cells via sponging miR-17-5p. *J. Cell. Biochem.* 122, 315–325. doi: 10.1002/jcb.29714

Chen, Z., Chen, Y., Li, Y., Lian, W., Zheng, K., Zhang, Y., et al. (2021). Prrx1 promotes stemness and angiogenesis via activating TGF-beta/smad pathway and upregulating proangiogenic factors in glioma. *Cell Death Dis.* 12:615.

Cheng, B., Rong, A., Zhou, Q., and Li, W. (2020). LncRNA LINC00662 promotes colon cancer tumor growth and metastasis by competitively binding with miR-340-5p to regulate CLDN8/IL22 co-expression and activating ERK signaling pathway. *J. Exp. Clin. Cancer Res.* 39:5.

Chruścik, A., Gopalan, V., and Lam, A. K. (2018). The clinical and biological roles of transforming growth factor beta in colon cancer stem cells: a systematic review. *Eur. J. Cell Biol.* 91, 15–22. doi: 10.1016/j.ejcb.2017.11.001

Clemson, C. M., Hutchinson, J. N., Sara, S. A., Ensminger, A. W., Fox, A. H., Chess, A., et al. (2009). An architectural role for a nuclear non-coding RNA: NEAT1 RNA is essential for the structure of paraspeckles. *Mol. Cell* 33, 717–726. doi: 10.1016/j.molcel.2009.01.026

De Caestecker, M. P., Piek, E., and Roberts, A. B. (2000). Role of transforming growth factor-beta signaling in cancer. *J. Natl. Cancer Inst.* 92, 1388–1402. doi: 10.1093/jnci/92.17.1388

Deng, F., Zhou, R., Lin, C., Yang, S., Wang, H., Li, W., et al. (2019). Tumor-secreted dickkopf2 accelerates aerobic glycolysis and promotes angiogenesis in colorectal cancer. *Theranostics* 9, 1001–1014. doi: 10.7150/thno.30056

Du, Y., Yang, H., Li, Y., Guo, W., Zhang, Y., Shen, H., et al. (2021). Long non-coding RNA LINC01137 contributes to oral squamous cell carcinoma development and is negatively regulated by miR-22-3p. *Cell. Oncol.* 44, 595–609. doi: 10.1007/s13402-021-00586-0

Duan, B., Shi, S., Yue, H., You, B., Shan, Y., Zhu, Z., et al. (2019). Exosomal miR-17-5p promotes angiogenesis in nasopharyngeal carcinoma via targeting BAMBI. *J. Cancer* 10, 6681–6692.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'shea, K. S., et al. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439–442. doi: 10.1038/380439a0

Flores-Pérez, A., Marchat, L. A., Rodríguez-Cuevas, S., Bautista-Piña, V., Hidalgo-Miranda, A., Ocampo, E. A., et al. (2016). Dual targeting of ANGPT1 and TGFBR2 genes by miR-204 controls angiogenesis in breast cancer. *Sci. Rep.* 6:34504.

Gao, Q., Zhou, R., Meng, Y., Duan, R., Wu, L., Li, R., et al. (2020). Long non-coding RNA CMPK2 promotes colorectal cancer progression by activating the FUBP3-c-Myc axis. *Oncogene* 39, 3926–3938. doi: 10.1038/s41388-020-1266-8

Hu, Y. P., Jin, Y. P., Wu, X. S., Yang, Y., Li, Y. S., Li, H. F., et al. (2019). LncRNA-HGBC stabilized by HuR promotes gallbladder cancer progression by regulating miR-502-3p/SET/AKT axis. *Mol. Cancer* 18:167.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306–1309. doi: 10.1126/science.2479986

Li, H., Zhou, H., Luo, J., and Huang, J. (2017). MicroRNA-17-5p inhibits proliferation and triggers apoptosis in non-small cell lung cancer by targeting transforming growth factor β receptor 2. *Exp. Ther. Med.* 13, 2715–2722. doi: 10.3892/etm.2017.4347

Li, J., Ye, D., Shen, P., Liu, X., Zhou, P., Zhu, G., et al. (2020). Mir-20a-5p induced WTX deficiency promotes gastric cancer progressions through regulating PI3K/AKT signaling pathway. *J. Exp. Clin. Cancer Res.* 39:212.

Li, Y., Li, L., Wang, Z., Pan, T., Sahni, N., Jin, X., et al. (2018). LncMAP: Pan-cancer atlas of long non-coding RNA-mediated transcriptional network perturbations. *Nucleic Acids Res.* 46, 1113–1123. doi: 10.1093/nar/gkx1311

Liu, D. L., Lu, L. L., Dong, L. L., Liu, Y., Bian, X. Y., Lian, B. F., et al. (2020). miR-17-5p and miR-20a-5p suppress postoperative metastasis of hepatocellular carcinoma via blocking HGF/ERBB3-NF-κB positive feedback loop. *Theranostics* 10, 3668–3683. doi: 10.7150/thno.41365

Liu, H. T., Ma, R. R., Lv, B. B., Zhang, H., Shi, D. B., Guo, X. Y., et al. (2020). LncRNA-HNF1A-AS1 functions as a competing endogenous RNA to activate PI3K/AKT signalling pathway by sponging miR-30b-3p in gastric cancer. *Br. J. Cancer* 122, 1825–1836. doi: 10.1038/s41416-020-0836-4

Liu, Y., Li, F., Yang, Y. T., Xu, X. D., Chen, J. S., Chen, T. L., et al. (2019). IGFBP2 promotes vasculogenic mimicry formation via regulating CD144 and MMP2 expression in glioma. *Oncogene* 38, 1815–1831. doi: 10.1038/s41388-018-0525-4

Marti, P., Stein, C., Blumer, T., Abraham, Y., Dill, M. T., Pikiolek, M., et al. (2015). YAP promotes proliferation, chemoresistance, and angiogenesis in human cholangiocarcinoma through TEAD transcription factors. *Hepatology* 62, 1497–1510. doi: 10.1002/hep.27992

Mercer, T. R., Dinger, M. E., and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10, 155–159.

Muppala, S., Xiao, R., Krukovets, I., Verbovetsky, D., Yendamuri, R., Habib, N., et al. (2017). Thrombospondin-4 mediates TGF-β-induced angiogenesis. *Oncogene* 36, 5189–5198. doi: 10.1038/onc.2017.140

Oft, M., Heider, K. H., and Beug, H. (1998). TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr. Biol.* 8, 1243–1252. doi: 10.1016/s0960-9822(07)00533-7

Qin, X., Zhou, M., Lv, H., Mao, X., Li, X., Guo, H., et al. (2021). Long non-coding RNA LINC00657 inhibits cervical cancer development by sponging miR-20a-5p and targeting RUNX3. *Cancer Lett.* 498, 130–141. doi: 10.1016/j.canlet.2020.10.044

Shi, L., Magee, P., Fassan, M., Sahoo, S., Leong, H. S., Lee, D., et al. (2021). A KRAS-responsive long non-coding RNA controls microRNA processing. *Nat. Commun.* 12:2038.

Siegel, R. L., Miller, K. D., Fuchs, H. E., and Jemal, A. (2021). Cancer Statistics, 2021. *CA Cancer J. Clin.* 71, 7–33.

Song, J., Liu, Y., Wang, T., Li, B., and Zhang, S. (2020). MiR-17-5p promotes cellular proliferation and invasiveness by targeting RUNX3 in gastric cancer. *Biomed. Pharmacother.* 128:110246. doi: 10.1016/j.biopha.2020.110246

Teng, F., Zhang, J. X., Chen, Y., Shen, X. D., Su, C., Guo, Y. J., et al. (2021). LncRNA NKX2-1-AS1 promotes tumor progression and angiogenesis via upregulation of SERPINE1 expression and activation of the VEGFR-2 signaling pathway in gastric cancer. *Mol. Oncol.* 15, 1234–1255.

Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* 95, 779–791. doi: 10.1016/s0092-8674(00)81701-8

Wang, C. L., Wang, D., Yan, B. Z., Fu, J. W., and Qin, L. (2018). Long non-coding RNA NEAT1 promotes viability and migration of gastric cancer cell lines through up-regulation of microRNA-17. *Eur. Rev. Med. Pharmacol. Sci.* 22, 4128–4137.

Wang, Y. Q., Jiang, D. M., Hu, S. S., Zhao, L., Wang, L., Yang, M. H., et al. (2019). SATB2-AS1 suppresses colorectal carcinoma aggressiveness by inhibiting SATB2-dependent Snail transcription and epithelial-mesenchymal transition. *Cancer Res.* 79, 3542–3556. doi: 10.1158/0008-5472.can-18-2900

Wang, Z., Zou, Q., Song, M., and Chen, J. (2017). NEAT1 promotes cell proliferation and invasion in hepatocellular carcinoma by negative regulating miR-613 expression. *Biomed. Pharmacother.* 94, 612–618. doi: 10.1016/j.biopha.2017.07.111

Wu, Y. C., Yang, L., Zhao, J., Li, C., Nie, J., Liu, F. Q., et al. (2015). Nuclear-enriched abundant transcript 1 as a diagnostic and prognostic biomarker in colorectal cancer. *Mol. Cancer* 14:191.

Xu, J., Meng, Q., Li, X., Yang, H., Xu, J., Gao, N., et al. (2019). Long non-coding RNA MIR17HG promotes colorectal cancer progression via miR-17-5p. *Cancer Res.* 79, 4882–4895. doi: 10.1158/0008-5472.can-18-3880

Yang, F., Strand, D. W., and Rowley, D. R. (2008). Fibroblast growth factor-2 mediates transforming growth factor-beta action in prostate cancer reactive stroma. *Oncogene* 27, 450–459. doi: 10.1038/sj.onc.1210663

Yang, F., Tang, Z., Duan, A., Yi, B., Shen, N., Bo, Z., et al. (2020). Long non-coding RNA NEAT1 upregulates survivin and facilitates gallbladder cancer progression by sponging microRNA-335. *Onco Targets Ther.* 13, 2357–2367. doi: 10.2147/ott.s236350

Yang, M. H., Zhao, L., Wang, L., Ou-Yang, W., Hu, S. S., Li, W. L., et al. (2019). Nuclear lncRNA HOXD-AS1 suppresses colorectal carcinoma growth and metastasis via inhibiting HOXD3-induced integrin beta3 transcriptional activating and MAPK/AKT signalling. *Mol. Cancer* 18:31.

Zeng, Z., Li, Y., Pan, Y., Lan, X., Song, F., Sun, J., et al. (2018). Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis. *Nat. Commun.* 9:5395.

Zhang, S., Ma, H., Zhang, D., Xie, S., Wang, W., Li, Q., et al. (2018). LncRNA KCNQ1OT1 regulates proliferation and cisplatin resistance in tongue cancer via miR-211-5p mediated Ezrin/Fak/Src signaling. *Cell Death Dis.* 9:742.

Zhao, L. N., Liu, Y., Zhang, J. B., Liu, Y., and Qi, Q. (2019). LncRNA SNHG14/miR-5590-3p/ZEB1 positive feedback loop promoted diffuse large B cell lymphoma progression and immune evasion through regulating PD-1/PD-L1 checkpoint. *Cell Death Dis.* 10:731.

Zhen, L., Yun-Hui, L., Hong-Yu, D., Jun, M., and Yi-Long, Y. (2016). Long non-coding RNA NEAT1 promotes glioma pathogenesis by regulating miR-449b-5p/c-Met axis. *Tumour Biol.* 37, 673–683. doi: 10.1007/s13277-015-3843-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 31 January 2022
doi: 10.3389/fcell.2021.657092

Analysis of 5-Methylcytosine Regulators and DNA Methylation-Driven Genes in Colon Cancer

Cheng Du[1,2,3†], XinLi Liu[4†], Mingwei Li[1,2,3], Yi Zhao[1,2,3], Jie Li[1,2,3], Zhikang Wen[1,2,3], Min Liu[1,2,3], Meina Yang[1,2,3], Boshi Fu[1,2,3] and Minjie Wei[1,2,3]**

[1]Department of Pharmacology, School of Pharmacy, China Medical University, Shenyang, China, [2]Liaoning Key Laboratory of Molecular Targeted Anti-Tumor Drug Development and Evaluation, Liaoning Cancer Immune Peptide Drug Engineering Technology Research Center, Shenyang, China, [3]Key Laboratory of Precision Diagnosis and Treatment of Gastrointestinal Tumors, Ministry of Education, China Medical University, Shenyang, China, [4]Department of Digestive Oncology, Cancer Hospital of China Medical University, Shenyang, China

OPEN ACCESS

Edited by:
Lucas Treps,
Centre de Recherche en Cancérologie et Immunologie Nantes Angers (CRCINA), France

Reviewed by:
Hamid Zand,
National Nutrition and Food Technology Research Institute, Iran
Baozhen Zhang,
Peking University Cancer Hospital, China

***Correspondence:**
Boshi Fu
fuboshi@whu.edu.cn
Minjie Wei
mjwei@cmu.edu.cn

†These authors have contributed equally to this work and share the first authorship

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 22 February 2021
Accepted: 17 December 2021
Published: 31 January 2022

Citation:
Du C, Liu X, Li M, Zhao Y, Li J, Wen Z, Liu M, Yang M, Fu B and Wei M (2022) Analysis of 5-Methylcytosine Regulators and DNA Methylation-Driven Genes in Colon Cancer. Front. Cell Dev. Biol. 9:657092. doi: 10.3389/fcell.2021.657092

Background: Epigenetic-driven events are important molecular mechanisms of carcinogenesis. The 5-methylcytosine (5mC) regulators play important roles in the methylation-driven gene expression. However, the effect of the 5mC regulators on the oncogenic pathways in colon cancer (CC) remains unclear. Also, the clinical value of such epigenetic-driven events needs further research.

Methods: The transcriptome and matching epigenetic data were obtained from The Cancer Genome Atlas dataset. The gene set variation analysis identified the oncogenic pathways adjusted by 5mC regulators. The "edgeR" and "methylmix" package identified the differential expression genes of DNA methylation-driven genes. The correlation between 5mC regulators or transcription factors and shortlisted genes was investigated by calculating the Spearman's rank correlation coefficient. Among them, the genes related to diagnosis were screened out based on differential gene expression in extracellular vesicles (EVs) by the "limma" package and histology by immunohistochemistry. Then, a risk signature was constructed by fitting the generalized linear model and validated by the receiver operating characteristic curve.

Results: MYC targets pathway and phosphatidylinositol-3-kinase–AKT–mammalian target of rapamycin signaling pathway were identified as the hallmark-related pathways associated with 5mC regulators. Also, the P53 pathway was subject to the influence of regulators' expression. A five methylation-driven gene signature (FIRRE, MYBL2, TGFBI, AXIN2, and SLC35D3) was developed as the biomarker for CC diagnosis. Meanwhile, those genes positively related to 5mC regulators and interacted with their relevant or transcription factors.

Abbreviations: AUC, area under the receiver operating characteristic curve; CC, colon cancer; CRC, colorectal cancer; CNAs, copy number alterations; DEGs, differential expression genes; EVs, extracellular vesicles; SCC, Spearman Correlation Coefficient; TFs, transcription factors; TCGA, The Cancer Genome Atlas; 5mC, 5-methylcytosine.

Conclusion: In general, 5mC regulators are positively related to each other and DNA methylation-driven genes, with the relationship of multiple active and inhibitory pathways related to cancer. Meanwhile, the signature (FIRRE, MYBL2, TGFBI, AXIN2, and SLC35D3) can prefigure prospective diagnosis in CC.

Keywords: 5mC regulators, methylation-driven gene, colon cancer, diagnosis, biomarker

INTRODUCTION

Colon cancer (CC) is one of the most common malignancies with heterogeneous incidence and mortality around the world (Siegel et al., 2021). During the last decade, the link between molecular events and clinical characteristics, including prognosis and therapeutic responses, has been at critical attention (Fearon, 2011). Such molecular characteristics are the major contributor to biomarkers for clinical incoming. Currently, the growing academic interest in multi-omics has been witnessed. To date, there remains a paucity of systematic analyses focusing specifically on methylation-driven events.

DNA methylation, one common epigenetic modification in mammalians (Suzuki and Bird, 2008), is regulated by DNA methylation regulators. Methylation-driven events were molecular events regulated by DNA methylation. DNA methylation at position C-5 of the cytosine loop [5-methylcytosine (5mC)] is the most common epigenetic event with the dynamic modification character (Bestor et al., 2015). The enzymes associated with hypermethylation or demethylation have been identified as the 5mC regulators. They are always grouped into three categories: "writers," "erasers," and "readers." "Writers" (DNA methyltransferases) can modify methylation on peculiar nucleotide bases in consort with "erasers" (DNA demethylase), eliminating these marks, and "readers" can recognize methylated DNA and provide the competence in recruiting other factors (Moore et al., 2013).

Poh et al. (2016) and Zhang and Xu (2017) illustrated that DNMT3A and DNMT3B are essential for *de novo* methylation of the genome to keep a cell's specific methylation profile and also for methylation of newly integrated retroviral sequences. "Erasers" [ten–eleven translocation (TET) family proteins] can remove DNA methylation and mediate the conversion of oxidized 5mC to 5-hydroxymethylcytosine in an α-ketoglutarate- and Fe(II)-dependent manner (Tahiliani et al., 2009). Screening of tumor suppressor genes in metastatic colorectal cancer (CRC) has revealed that methyl-binding protein 1 (MBD1) gene, compared with other genes that were screened for methylation levels, had the most downregulated messenger RNA (mRNA) expression and upregulated methylation levels in advanced CRC (Parry and Clarke, 2011). Some research has demonstrated that DNA methylation plays an important role in various biological processes (Luo et al., 2018), such as attaching transcriptional regulation (Poh et al., 2016) and functioning in diverse genomic partitions and developmental stages (Zhou et al., 2018). Li et al. (2020) proposed that upregulated writers (DNMT1, DNMT3A, and DNMT3B) facilitated hypermethylation and downregulation of BAD and INPPL1 in colitis-associated cancer. Xu et al. (2019) recommended TET2 (eraser) activity as a biomarker to predict the efficacy of anti-PD-1/PD-L1 treatment and patient response and the stimulation of TET2 activity as adjunct immunotherapy for CC. The MBD2 (reader) antigen only reacted with the serum of CC patients but not with the serum of normal blood donors (Martin et al., 2008). In summary, these results show that 5mC regulators play an important role in CC.

Specific epigenetic events also have been demonstrated as effective biomarkers in CC (Hao et al., 2017). Based on the DNA methylation level, Ren et al., 92016) unearthed three major subtypes (MCL1, MCL2, and MCL3), which is helpful to understand the subtypes of CC. Bai et al. (2020) put forward that circulating DNA and its methylation level can be regarded as new indicators for colitis cancer transformation (Jones and Baylin, 2007). Ginder and Williams (2018) reviewed the possibility of the MBD family as potential therapeutic targets. Recently, Chen et al. (2020) identified cross talk between m6A and 5mC regulators across 33 cancer types, which connect two different methods of epigenetic regulation.

Extracellular vesicles (EVs) include exosomes, microvesicles, which is formed by direct plasma membrane budding and range from 100 to 1,000 nm in diameter (Thery et al., 2018), and exosomes are nano-sized vesicles (30–150 nm) originating from the endocytic pathway (Kowal et al., 2016). All EVs enclose or expose on their surface a multitude of biomolecules, including RNA, lipids, proteins, and possibly DNA, that can be trafficked between cells as a means of intercellular communication at both paracrine and systemic levels. The versatility of EVs in cancer made them contribute to many of the hallmarks of cancer (Meehan and Vella, 2016), including cell proliferation and migration (Kim et al., 2018), angiogenesis (Webber et al., 2015), evasion of cell death (Wieckowski et al., 2009), and invasion and metastasis (Fong et al., 2015). EVs have emerged as novel players in the prevention and treatment of various human diseases. Li et al. (2021a) showed a circulating EV long RNA-based signature for prognostic assessment of pancreatic ductal adenocarcinoma. Recently, Wu et al. (2021) found a diagnosis protocol to realize ultrasensitive detection of urinary EVs and accurate classification of early urinary diseases *via* radiometric three-dimensional DNA machine combined with a machine learning algorithm. In recent years, people have taken advantage of the dynamic behaviors in cells to develop advanced drug delivery systems; EVs have great potential as drug delivery vectors (Chen et al., 2021). An adequate therapeutic system was developed for the treatment of autoimmune encephalomyelitis using EVs from modified neural stem cells with high expressed ligand platelet-derived growth factor subunit A and achieving locally targeted delivery (Xiao et al., 2022).

In this study, we investigated the interrelation of canonical 23 5mC regulators in CC. The somatic genomic alterations and copy number alterations (CNAs) of such 5mC regulators were profiled. Then, the effect of the expression of 5mC regulators on oncogenic pathways was analyzed by gene set variation analysis and visualized by the Cytoscape software. This study inquired about genes driven by DNA methylation and performed enrichment analysis to investigate the impact of DNA methylation on genes. The approach to preferably identifying the clinical relevance of these genes adopted for this study was analyzing the expression in EVs and histology. This study utilized prospective genes to assess the relationship between genes and transcription factors (TFs) and 5mC regulators.

MATERIALS AND METHODS

Data Collection

Multiple data of CC, including transcriptome expression, DNA methylation data analyzed by Illumina Human Methylation 450k, and mutation annotation format were acquired from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov). We collected 430 cancer samples and 40 normal tissue samples in the transcriptome dataset and 316 cancer samples and 38 normal tissue samples in the epigenetic dataset. The processed result analyzed by GISTIC2.0 of copy number was downloaded from the Xena platform (https://xenabrowser.net). GSE39582 was collected from the Gene Expression Omnibus database.

Collection and Analysis of 5-Methylcytosine Regulators

By reading pieces of literature published, 23 DNA methylation regulators were found to participate in the next research, including four writers, three erasers, together with 16 readers. Interconnection among DNA methylation regulators was resolved based on the analysis of the STRING database (http://www.stringdb.org/). The Shapiro–Wilk normality test was conducted to judge whether the expression of those genes conforms to normal distribution or not. The correlation analysis among DNA methylation regulators was performed with the R software.

Cancer-Related Pathways Analysis of 5-Methylcytosine Regulators

To find cancer hallmark-related pathway activity along with expression efficiency of regulators in CC, gene set variation analysis (Hanzelmann et al., 2013), a nonparametric, unsupervised method for assessing gene set enrichment variation through gene expression profile, was performed according to the package instruction. These pathways with $|\log_2 FC| > 0.2$ and p-value < 0.05 were regarded as significant enrichment pathways.

Relationship Among Genes

With a view to the evaluation of the correlation among genes, the Shapiro–Wilk normality test was conducted to judge whether the expression of genes conformed to normal distribution. Further to the result, the "corrplot" package (https://github.com/taiyun/corrplot) was performed to calculate the Spearman correlation coefficient and p-value by R software. Cytoscape software was performed to visualize the interactions between 5mC regulators and pathways.

Access to DNA Methylation-Driven Genes

The "edgeR" package (Robinson et al., 2010) in R software was used to normalize RNA sequencing data and then screened out the differential genes using the quasi-likelihood F test. We, as a result, selected the differential genes with $|\log_2 FC| > 2.0$ and p-value < 0.05 for further research. The "methylmix" package (Cedoz et al., 2018) was used to build a β-mixed model whose CorThreshold was set to 0.4. Samples from TCGA involving 462 cancer tissues and 41 normal tissues were applied to preparation for methylation-driven genes with |DM-values| > 0 (differential methylation values). Finally, genes in line with differential methylation expression filtered by the "limma" package (Ritchie et al., 2015) with a p-value < 0.05 and $|\log_2 FC| > 0.1$ were identified for the next investigation.

Visualization of DNA Methylation-Driven Genes

"OmicCircos" package (Hu et al., 2014) was used to map the multi-omics profile of DNA methylation-driven genes from CC in TCGA database screened by the β mixed model, including corresponding chromosomal location, bar charts of DNA methylation degree, and dot plots of transcriptome expression degree.

Gene Enrichment Analysis

The "ClusterProfiler" package (Yu et al., 2012) was used to perform gene set enrichment analysis of gene ontology (GSEA.GO) involving BP (Biological Process), CC (Cell Component) and MF (Molecular Function), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on DNA methylation-driven genes with the filter criteria of the cutoff p-value that was set to 0.05.

Clinical Relevance of 5-Methylcytosine Regulators

To deliver significant 5mC regulators of survival assessment in CC, The Human Protein Atlas (HPA) project database (https://www.proteinatlas.org/) was retrieved to find the best cutoff value of gene expression and draw Kaplan–Meier (KM) curves, based on 5-year survival condition. The drug–protein interaction information concerning regulators was collected with the "maftools" package (Mayakonda et al., 2018).

Clinical Significance Assessment of DNA Methylation-Driven Genes

To sort out significant genes that can be regarded as prospective diagnostic biomarkers of CC, we did the following tasks. The first

part was to find the optimal genes from the BBCancer database (http://bbcancer.renlab.org/), which should be apposite to the differential expression prerequisite in EVs or circulating tumor cells. Secondly, the "ROCR" package (Sing et al., 2005) using R statistical software was operated to calculate the area under the receiver operating characteristic curve (AUC) for them to measure and compare the model performance. Next, these genes conforming to the statistical result of p-value < 0.05 and AUC > 0.7, and the property of universal probes were worthy of further investigation on fitting generalized linear models using iteratively reweighted least-squares. Finally, the model based on iteratively reweighted least-squares could be concluded to construct a signature formula to help us predict CC. Also, the Z test was used to compare the AUC of biomarkers further. To find the optimal cutoff value, the point to be closest to the true-positive rate of (1) and the false-positive rate of (0) is acceptable, which is in the sense of equal trade-off between "sensitivity" and "specificity."

Immunohistochemistry-Based Expression Data of The Cancer Genome Atlas Samples

HPA is a systematic study to allow for a systematic exploration of the human proteome using antibody-based proteomics for multiple tissues and cell lines, which is accomplished by combining high-throughput generation of affinity-purified antibodies with protein profiling in a multitude of tissues and cells assembled in tissue microarrays. To compare candidates between pathology and normal immunohistochemistry-based expression data of TCGA samples, HPA was explored to collect and observe the immunohistochemistry results of colon adenocarcinoma tissue and colon normal tissue.

Discovery of Transcription Factors Corresponding to Genes

TFs of genes with clinical benefits were explored and visualized with a web tool NetworkAnalyst (http://www.networkanalyst.ca), which can analyze comprehensive gene expression profiling, referring to ENCODE (Encyclopedia of DNA Elements) whose prerequisite was that betweenness was set to 2.

Inherent Character of Genes in Colon Cancer

An inquiry into somatic genomic alterations and somatic mutation interactions was performed with the "Maftools" package to detect the results of somatic variants and significant pairs of genes, including 5mC regulators and the prospective diagnostic genes.

RESULTS

Oncogenic Pathways Regulated by 5-Methylcytosine Regulators

We curated a catalog of 23 5mC regulators according to the published research. They could fall into three main categories: writers (DNMT1, DNMT3A, DNMT3B, and DNMT3L), erasers (TET1, TET2, and TET3), and readers (MBD1, MBD2, MBD3, MBD4, MECP2, NEIL1, NTHL1, SMUG1, TDG, UHRF1, UHRF2, UNG, ZBTB33, ZBTB38, ZBTB4, and ZFP57). The gene expression data and matching methylation data were obtained from TCGA (all details were recorded in *Materials and Methods*). Analysis of global gene expression profiles identified the differential expression of these 5mC regulators. Among writers and readers, DNMT1, DNMT3B, UHRF1, and NTHL1 mRNA levels were markedly upregulated in CC patients, whereas the low expression of ZBTB4 was observed in CC patients (**Figure 1A**). Then, the correlation between the expression of individual regulators and hallmark pathways related to CC was analyzed by GSEA (**Figure 1B**; **Supplementary Table S4**). The first two genes, ZBTB4 and MECP2, were involved in most of the pathways, such as the inhibition of apical junction, hedgehog signaling, and NOTCH signaling pathways, and the activation of reactive oxygen species and bile acid metabolism pathways (**Figures 1B,C**). In addition, it can be seen that different writers, readers, or erasers were bound up with distinct hallmark pathway alterations, which meant disparate roles of 5mC regulators in the identical functional pathway (**Figures 1B,C**). The expression of ZBTB4, MBD1, and MECP2 positively affected the MYC targets pathway, contrary to DNMT3B and UNG. The positive impact of SMUG1 and UHRF2 on the phosphatidylinositol-3-kinase–AKT–mammalian target of rapamycin signaling pathway was also produced, in contrast to TDG and UNG. DNMT3B and ZBTB33 were positively associated with the P53 pathway, opposite of the influence of MBD2 and MBD1. In particular, DNMT3L, ZBTB38, ZBTB4, MECP2, and all erasers were negatively related to the transforming growth factor-β signaling pathway. DNMT3B, DNMT3A, and DNMT3L were involved in the inhibition of the Wnt/β-catenin signaling pathway.

Following that, we identified the 5mC regulators strongly related to the active or suppressive pathways with |SCC| > 0.5 and adjusted p-value < 0.05 (**Figure 1D**). Strong relativity was found between the DNA repair pathway and DNMT1, UNG, but there was no evidence that DNMT1 has a noticeable effect on DNA repair in this analysis (**Figures 1B,D**). The expression of DNMT1 and UNG strongly and negatively correlated with the E2F targets pathway (**Figures 1B,D**).

Interactions Among 5-Methylcytosine Regulators

The interactions among the 23 DNA methylation regulators produced highly correlated behaviors. We analyzed the interaction network among these regulators (**Figure 2A**) and calculated SCC among them after confirming the violation of normal distribution (**Figure 2B**). Interestingly, it turned out that DNA methyltransferases exerted synergistic efficacy as writers because of mutual binding action type, whereas proteins involved in DNA demethylation acted independently without binding behavior, but there was a significant positive correlation in writers or erasers due to large SCC (**Figures 2A,B**). Besides,

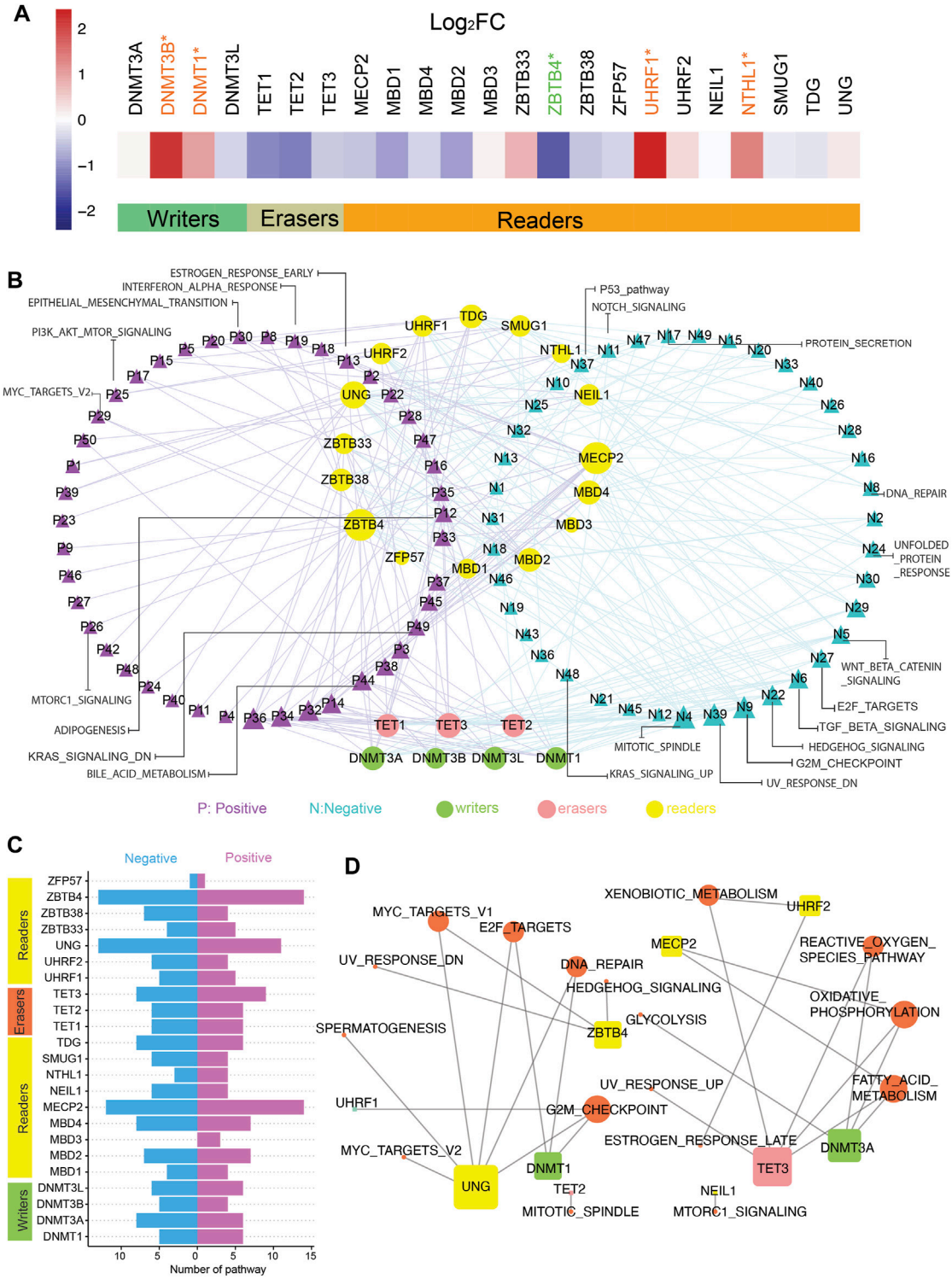


FIGURE 1 | Active and suppressive pathways of cancer are associated with 5mC regulators in colon cancer. **(A)** Gene expression of 5mC regulators in tumor and normal samples. *t*-test was used to evaluate differences between two groups, **p*-value < 0.05. **(B)** Network pictogram for hallmark-related pathways and 23 5mC regulators based on GSVA and correlation. Size of nodes is in proportion to number of related links. **(C)** Number of pathways correlated with individual 5mC regulators. (Right: positively correlated pathways; left: negatively correlated pathways.). **(D)** Pathways highly related with 23 5mC regulators (|SCC| > 0.5 and *p*-value < 0.05). Size of nodes is in proportion to number of related links.

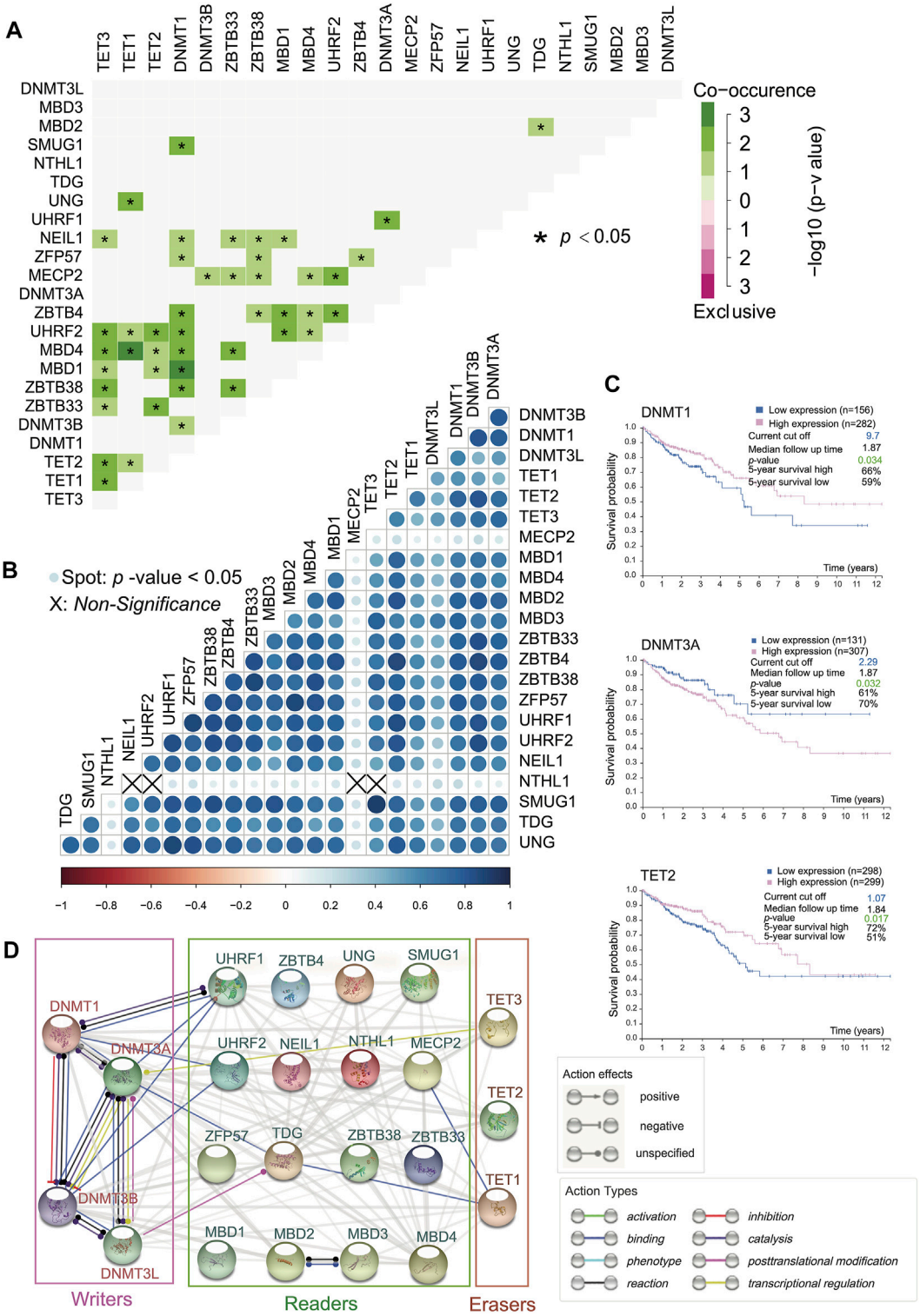


FIGURE 2 | Positive interaction generally among 5mC regulators and survival analysis. **(A)** Somatic mutation interactions among 23 5mC regulators. (*p-value < 0.05). DNMT3L and MBD3 are not related to other 5mC regulators. Other significant relationships are positive. **(B)** SCC regarding expression profile of 23 5mC regulators (Spot: p-value < 0.05). Writers are related to erasers and readers. **(C)** Overall survival curves of DNMT3A, DNMT1, and TET2 (p-value < 0.05) indicate that all of them are clinically significant. **(D)** Functional protein association network among 5mC regulators.

TABLE 1 | Emphasis on 5mC regulators and corresponding drugs.

Gene	Favorable prognosis	No. drug	Description	Name		
DNMT1	high expression	14	Inhibitor	Decitabine[a]	Azacitidine[a]	CHEMBL2349526
		—	—	Diethylstilbestrol	Hydroxyurea	Ifosfamide
		—	—	Zebularine	Arsenic Trioxide	Floxuridine
		—	—	Adriamycin	MG98	Mitoxantrone
		—	—	Cisplatin	FTI (farnesyltransferase Inhibitors)	—
DNMT3A	low expression	4	Inhibitor	Decitabine[a]	Azacitidine[a]	—
			—	Daunorubicin	Idarubicin	—
TET2	high expression	2	—	Decitabine[a]	Azacitidine[a]	—

[a]*Represents drugs targeted for DNMT1, DNMT3A, and TET2.*

there were complex network interactions and positive correlations between readers. Additionally, MBD4-TET1, DNMT1-TET2/MBD1, and UHRF1-UHRF2 had strong evidence of co-occurrence in somatic interactions as a result of the high odds ratio of the gene set and a p-value < 0.05. Moreover, it was apparent that there was a co-occurrence of genomic mutation between erasers with a p-value < 0.05 (**Figure 2A**). As could be seen from **Figure 2B**, NTHL1 did not correlate with TET3, UHRF2, NEIL1, and MECP2. MBD family members involved, ZBTB family members involved, and UHRF1, UHRF2, ZFP57, and NEIL1 were positively related to readers and erasers (**Figure 2B**). It was a special outpouring of attention to a wide range of action types that occurred among writers (**Figure 2D**). The TDG as a reader, due to its binding action type, seemed to be a bridge between DNMT3A (writer) and TET1 (eraser) (**Figure 2D**).

Clinical Significance of 5-Methylcytosine Regulators

The prevalent transcriptional expression alterations of 5mC regulators in CC may provide a vital perspective into clinical utility. To judge the effect of regulators on the overall survival of patients, survival analysis was performed simultaneously, and the optimal cutoff points were obtained from maximally selected rank statistics based on the HPA platform. Besides, target drugs of clinical regulators in clinical trials or markets were collected (**Table 1**). The result represented three genes with targeted drugs and survival differences, including DNMT3A, DNMT1, and TET2 (**Figure 2C**). After finding the optimal cutoff value of gene expression by the HPA website, the high expression group of DNMT1 and TET2 predicted better survival than the low expression of them with minor differences in the former and major differences in the latter, yet DNMT3A was the opposite of that result with little distinction. What stands out in **Table 1** is that DNMT3A, DNMT1, and TET2 all were affected by decitabine and azacitidine. It is worth mentioning that DNMT1 is a differentially expressed gene with the significant overall survival of patients.

Identification of DNA Methylation-Driven Genes

Based on the manipulation criteria from the methods pattern, 108 genes (**Supplementary Table S1**) were defined considering differential methylation level and differential RNA expression level (**Figure 3**). For the sake of apparent observation of DNA methylation and transcriptome expression differences of those genes screened, we displayed chromosomal location annotation of the corresponding genes selected, DNA methylation degree, and $\log_2$FC of transcriptome expression collected from TCGA database in a circos plot utilizing BioMart databases (**Figure 3**). As a whole, the DNA methylation degree tends to be negatively related to the expression level of the corresponding gene. The consequence demonstrated that these genes were distributed in chromosomes other than 5, 8, and Y chromosomes, and chromosome 1 contained the most genes. Those genes with diagnosis significance were highlighted in plum, including AXIN2, FIRRE, MYBL2, SLC35D3, and TGFBI.

Functional Enrichment Analysis of Methylation-Driven Genes

To further inquire about the function of DNA methylation-driven genes screened, we performed the GSEA.GO analysis (**Figures 4A–E**; **Supplementary Table S2**) and the KEGG pathway analysis (**Figure 4F**), which were finally selected per the relevant results with p.adjust < 0.05 (p-value after adjustment). The results indicated that these genes functioned as physiological regulatory effects were enriched in both BP and MF but not in CC, and only two pathways were enriched in KEGG pathways analysis. Transcription regulator activity and DNA-binding TFs activity were enriched in the MF (**Figure 4A**). In addition, the outcome enriched in BP mainly brought about the following characteristics: DNA-templated transcription and its regulation (**Figure 4B**), RNA biosynthetic process and its regulation, transcription by RNA polymerase II and its regulation (**Figure 4C**), regulation of gene expression (**Figure 4D**), and nucleic acid-templated transcription and its regulation (**Figure 4E**). Wnt signaling pathway and glycosaminoglycan biosynthesis with keratan sulfate were enriched as KEGG pathways (**Figure 4F**.).

Preliminary Screening for Clinical Evaluation of Methylation-Driven Genes

To dig out the application in clinical diagnosis, we initially assessed the level of gene expression in EVs (**Figure 5A**) *via* the BBCancer dataset depending on p.adjust < 0.05 and $|\log_2 FC| > 1.5$ (i.e., RNA expression degree) and immunohistochemistry *via* the HPA dataset (**Figure 5B**) (i.e., protein expression degree), and then related genes

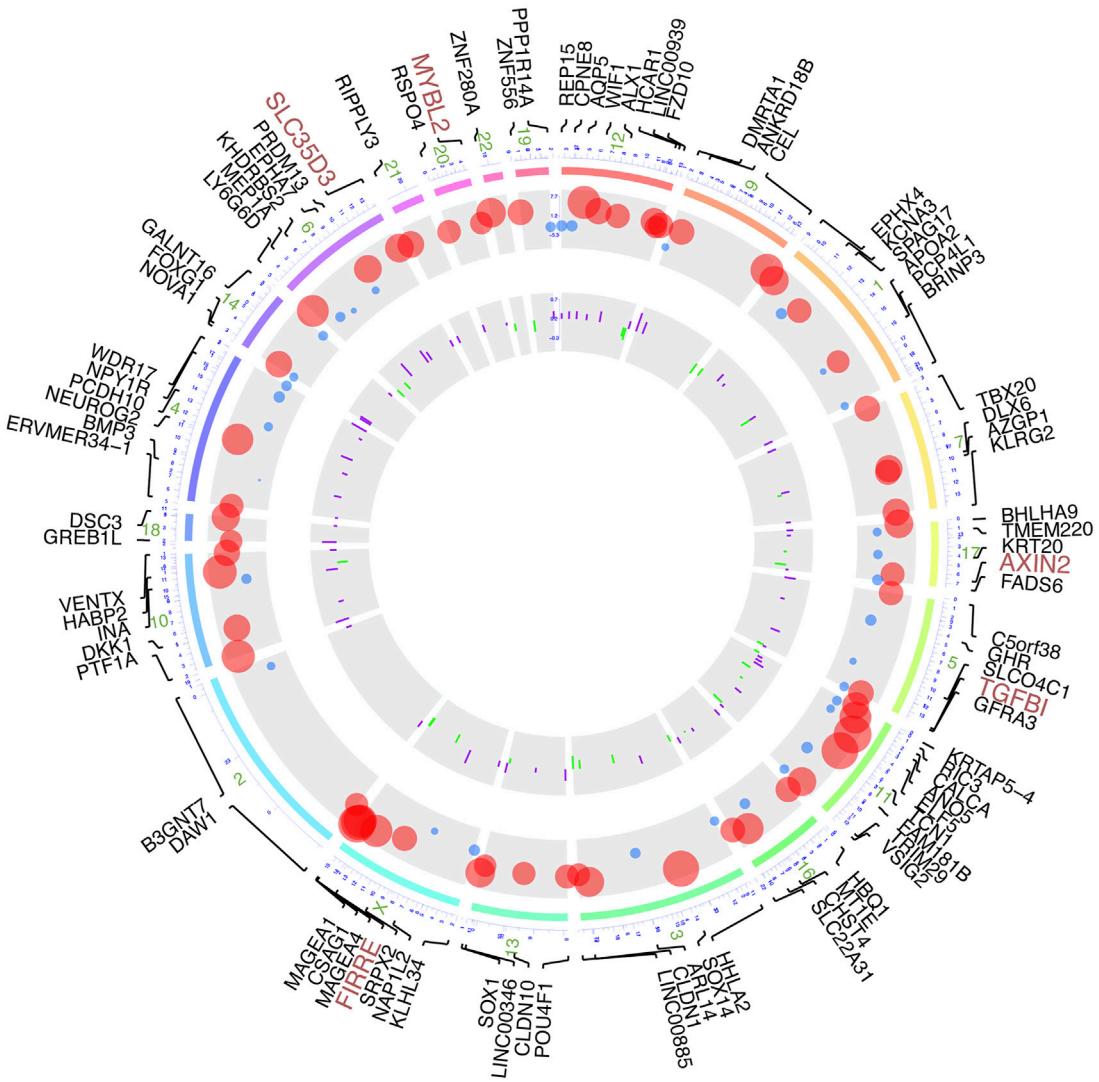


FIGURE 3 | Circos plot of DNA methylation-driven genes based on multi-omics. From outermost circle to inner circle, presentation on map is as follows: chromosome location with lines deriving from specific gene locus, log_2FC of transcriptome expression by dot plots with size of dots proportional to value and with red showing high expression and blue showing low expression, and log_2FC of DNA methylation by bar charts provided with purple indicating hypermethylation and green indicating hypomethylation.

was identified with the AUC of more than 0.7 (**Figure 6**). Concerning the proviso discussed, two long noncoding RNAs (FIRRE and LINC00346) and four protein-coding RNAs (AXIN2, MYBL2, TGFBI, and SLC35D3) were worthy of selection. FIRRE and LINC00346 had significant expression differences in EVs with $|log_2FC| > 10$ and $p.adjust < 0.05$, with other genes' expression having differences in EVs (**Figure 5A**). Immunohistochemical staining was performed for AXIN2, MYBL2, TGFBI, and SLC35D3, which represented that the part of cancer was deeper than that of normal (**Figure 5B**). All in all, the expression profile result of RNA and protein showed that all candidate genes were positively expressed in patients with CC. To identify the potential gene signature, AUC analysis was performed, dependent on a single gene. TCGA samples were divided into the training set and the test set (training set–test set = 7:3). The assessment result of a single gene indicated that for all genes, the test was close to the training. The values of the test set for FIRRE, MYBL2, TGFBI, LINC00346, SLC35D3, and AXIN2 were 0.833, 0.822, 0.956, 0.868, 0.782, and 0.771, respectively (**Figures 6A–F**). Furthermore, the values of the training set for FIRRE, MYBL2, TGFBI, LINC00346, SLC35D3, and AXIN2 were 0.910, 0.837, 0.931, 0.779, 0.781, and 0.808, respectively (**Figures 6A–F**).

Somatic Variants of 5-Methylcytosine Regulators and Preliminary Shortlisted Methylation-Driven Genes

Somatic mutation can be divided into three major types, point mutation, chromosomal mutation, and genomic mutation. Herein, we analyzed somatic genomic mutation and CNAs. We identified

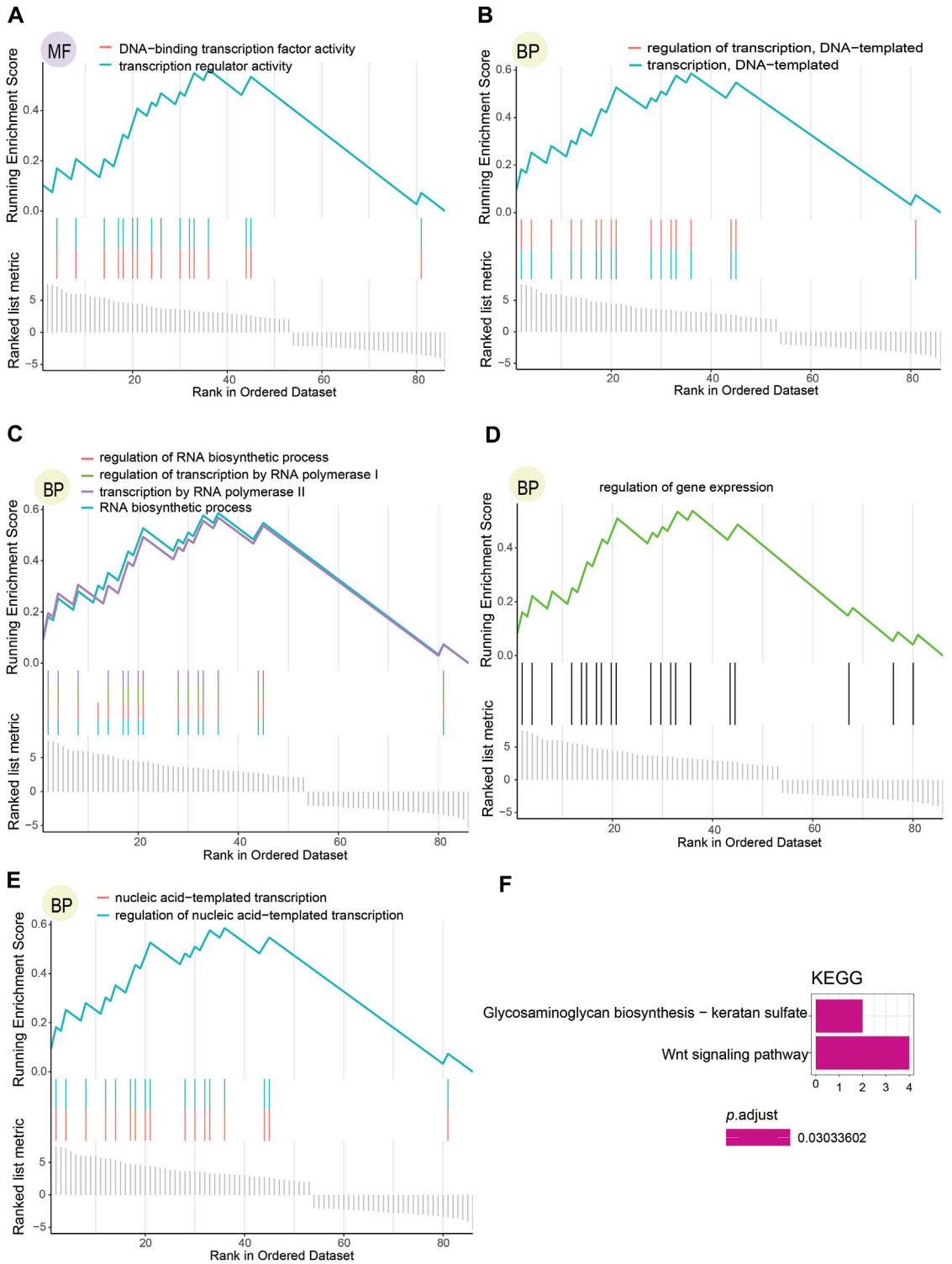


FIGURE 4 | Functional enrichment analysis results regarding DNA methylation-driven genes selected. **(A)** Consequence of molecular functions with *p*.adjust < 0.05. **(B–E)** Outcome of biological processes with *p*.adjust < 0.05. **(F)** Result of KEGG pathways with *p*.adjust < 0.05.

30.58% of patients who experienced somatic genomic mutations of 5mC regulators, comprising frameshift insertions or deletions, in-frame deletions, multi-hit, etc. (**Supplementary Figure S1A**). Despite a large number of patients in genomic mutation, only erasers (TET1, TET2, and TET3) were over 5% (**Supplementary Figure S1A**). As far as CNAs, multiple characters were observed among regulators. In the

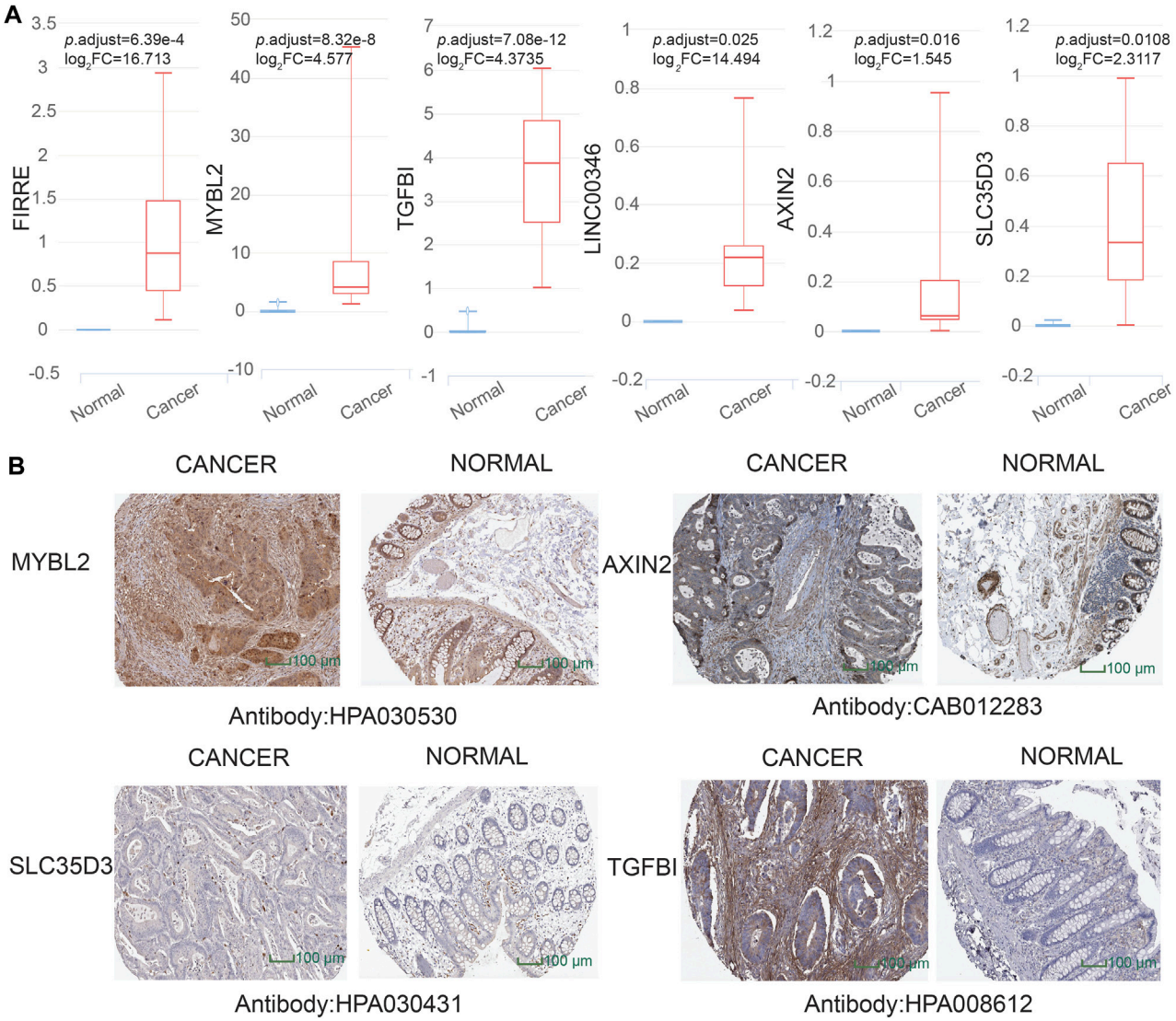


FIGURE 5 | Assessment of preliminary shortlisted candidate gene expression level in extracellular vesicles (EVs) and immunohistochemistry. **(A)** Highly expressed methylation-driven genes (FIRRE, MYBL2, TGFBI, LINC00346, AXIN2, and SLC35D3) in EVs for cancer samples. **(B)** Higher protein expression of immunohistochemistry (MYBL2, AXIN2, TGFBI, and SLC35D3) in cancer tissue than normal tissue from the Human Protein Atlas (HPA) project database (https://www.proteinatlas.org/).

readers, it indicated that the most frequent was DNMT3B, which occupied 73% amplification or gain among all regulators. DNMT3A (21%), DNMT1 (26%), and DNMT3L (35%) all had loss and gain in CNAs, notably with the highest frequency of loss in DNMT3L. For erasers, TET1 (25%) and TET2 (38%) mainly manifested loss, yet the gain of TET3 (20%) predominated. With the CNV rate of over 50% MBD1, MBD2, and ZBTB4, they focused on more loss and less gain. However, the gain of other readers was more than loss among patients (**Supplementary Figure S1C**).

Concerning somatic genome alteration of prospective diagnosis genes, AXIN2 altered in 7% of 399 samples, whereas the alteration rate of MYBL2, TGFBI, SLC35D3, FIRRE, and LINC00346 accounted for less than 5%. In CNAs of them, MYBL2 was the top first gene that occupied 72% for the alteration pattern of gain and amplification, and LINC00346 was the top second gene that occupied 61% for that of gain and amplification in the majority. TGFBI, SLC35D3, and AXIN2 were lower than 50%; moreover, there were no data of FIRRE (**Supplementary Figure S1**).

Function Analysis of Preliminary Shortlisted Genes

To further inquire about the function of shortlisted genes discussed earlier, GO analysis and KEGG analysis were performed, and all the significant results were displayed (**Supplementary Figures S2A–D**). The results of KEGG analysis showed that multiple cancer induction, particularly

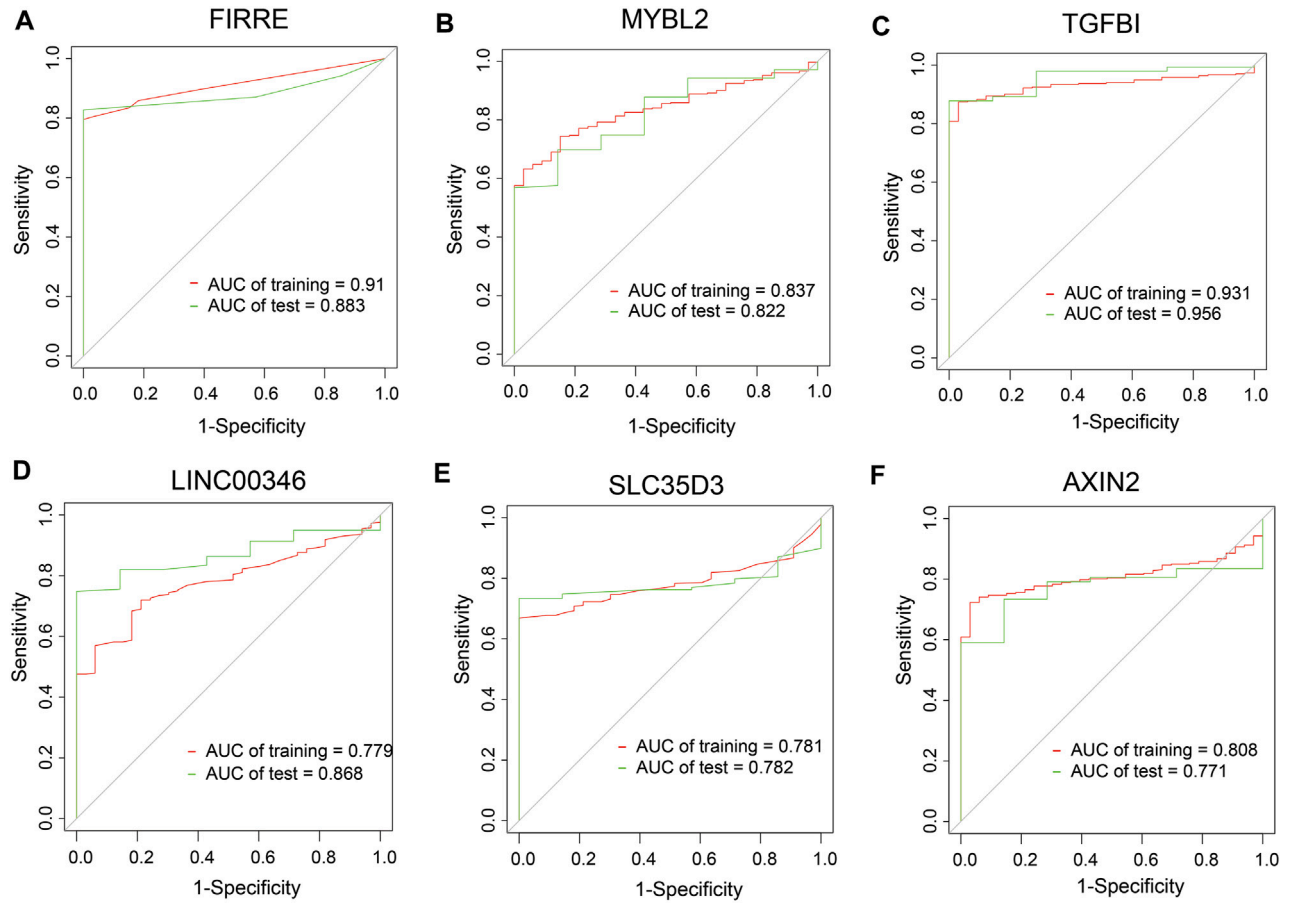


FIGURE 6 | Clinical performance assessment of preliminary shortlisted genes. **(A–F)** ROC curves of single genes, including FIRRE, MYBL2, TGFBI, LINC00346, SLC35D3, and AXIN2. AUC values are greater than 0.7.

CRC and signaling pathways including Wnt and Hippo, was enriched (**Supplementary Figure S2A**). In MF, different binding activities were witnessed, such as 1-SMAD binding and extracellular matrix binding (**Supplementary Figure S2B**). For CC, the consequent revealed the β-catenin destruction complex and basement membrane (**Supplementary Figure S2C**). As for BP, chondrocyte differentiation, cartilage development, and connective tissue development were enriched (**Supplementary Figure S2D**).

Interactions Between Preliminary Shortlisted Genes and Transcription Factors

TFs, as a direct interpretation of the genome, are one of the most significant components in performing DNA decoding sequences. Thus, we moved on to the further step to work out dependent molecular regulating the expression of shortlisted genes involving AXIN2, FIRRE, LINC00346, MYBL2, SLC35D3, and TGFBI. Further analysis shows that AXIN2, LINC00346, MYBL2, and TGFBI had a network with TFs (**Figure 7A**). Next, we screened out TFs, which implied they were able to be regarded as transcription activators such as MXD3 and SIN3A or transcriptional repressors such as GATA4, which embodied KLF9 and zinc finger protein consisting of ZNF24, ZNF580, GLIS2, ZNF341, etc. (**Figure 7A**). Only high expression of GATA4 ($log_2FC = 4.43$), together with low expression of KLF9 ($log_2FC = -2.11$), was geared to differential expression genes with $|log_2FC| > 1.5$ (**Figure 7B**; **Supplementary Table S3**). All genes were associated with their relevant TFs for a p-value < 0.05 (**Figures 7C–F**). There was a positive correlation between TGFBI and three TFs covering FOSL2, TFE3, and ZNF580, whereas a negative correlation to GATA4 (**Figure 7C**). AXIN2 was positively related to TFs except for GATA4 (**Figure 7D**). A strongly positive correlation was found between MYBL2 and TFs involving NRF1, RARA, MXD3, and ZNF341 (**Figure 7E**). LINC00346 was positively related to FOXA3, RAD21, GLIS2, NRF1, and DRAP1 (**Figure 7F**).

Evaluation and Verification of the Diagnostic Signature of Methylation-Driven Genes

Because EVs contain signal molecules such as protein, mRNA, microRNA, etc., they can often be used to reflect the physiological and pathological functional statuses of secreting cells, thereby

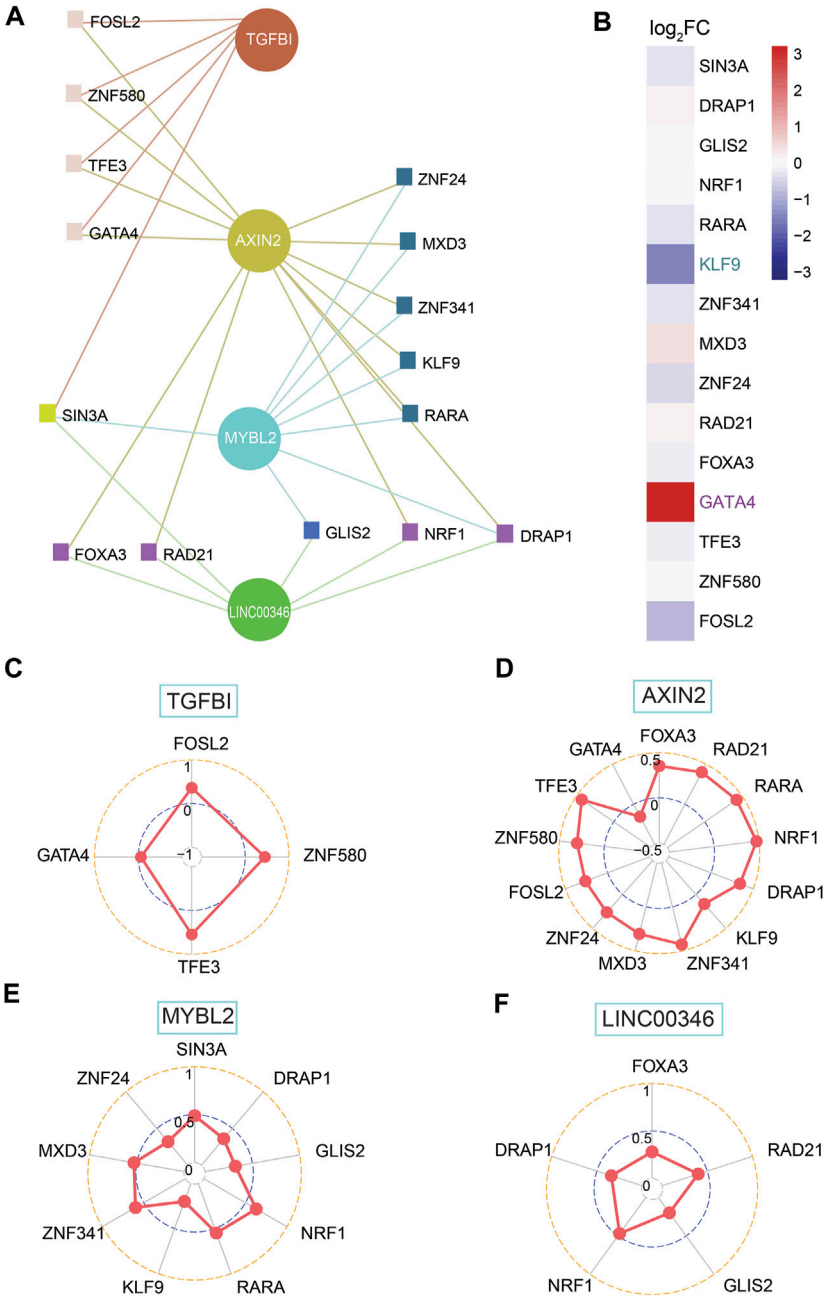


FIGURE 7 | Investigation of shortlisted genes and transcription factors (TFs) together with correlation among them. **(A)** Network of shortlisted genes and TFs. **(B)** Heatmap of log_2FC for RNA expression of 15 TFs (high expression: GATA4; low expression: KLF9, both of them is $|log_2FC| > 1.5$). **(C–F)** SCC between TFs and genes, including TGFBI, AXIN2, MYBL2, and LINC00346, based on network among them.

providing potential biomolecular markers, so the purification of exosomes from body fluids has a good effect. Clinical application prospects. The tumor will continuously release exosomes into the surrounding environment during the growth process, so searching for differentially expressed genes of EVs has potential clinical significance. After comparing gene expression differences in EVs based on the BBCancer database by the "limma" package and the property of universal probes, mixed genes comprising FIRRE, MYBL2, TGFBI, AXIN2, and SLC35D3 were selected. By fitting generalized linear model using iteratively reweighted least-squares (**Supplementary Table S5**) based on preliminary shortlisted genes, the conclusive diagnostic model formula (AIC = 134.88) is 0.9539 × FIRRE + 0.4983 × MYBL2 + 1.2050 × TGFBI − 0.3430 × AXIN2 + 0.1281 × SLC35D3. Surprisingly, the AUC values of the integration were 0.987 in the test set and 0.967 in the training set, which is larger than single

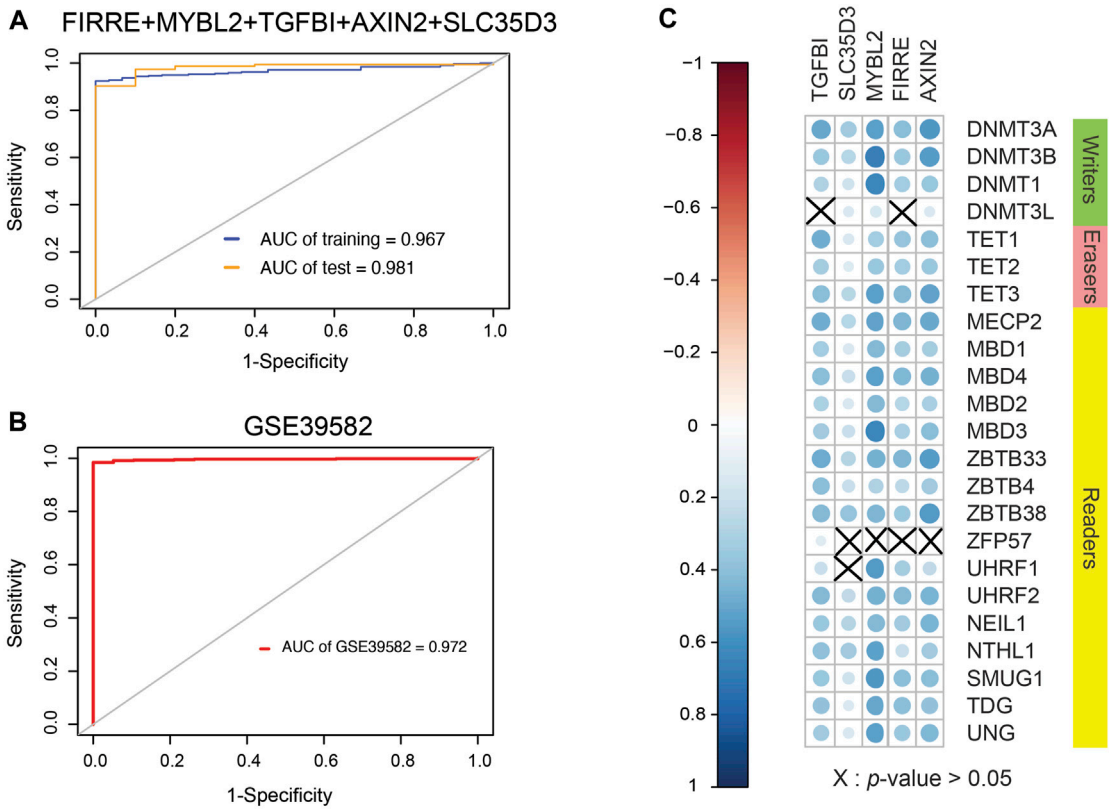


FIGURE 8 | Clinical performance assessment of signature and Spearman correlation coefficient between them and 5mC regulators. **(A)** ROC curves of five-gene signature involving FIRRE, MYBL2, TGFBI, AXIN2, and SLC35D3 (AUC of training set = 0.967, AUC of test set = 0.987). **(B)** ROC curves of diagnostic signature in GSE39582, which includes 19 normal cohorts and 566 cancer cohorts (AUC of GSE39582 = 0.972). **(C)** SCC between them and 5mC regulators. Size of nodes is inversely proportional to *p*-value. Erasers are related to genes from signature, and a part of writers and readers are related to these genes.

genes in TCGA database (**Figure 8A**). The optimal cutoff value is 16.2736569. To validate the model, GSE39582 was collected with 19 normal cohorts and 566 cancer cohorts. The AUC value of this model in the GSE39582 is 0.972, which supports the clinical significance of our model (**Figure 8B**).

Interactions Between Methylation-Driven Genes and 5-Methylcytosine Regulators

DNA methylation is a form of DNA chemical modification that can change the activity of a DNA piece without transforming the sequence and is regulated by DNA methylation regulators. Given inquiry into the relevance between 5mC regulators and genes selected, SCC was formulated (**Figure 8C**). As a consequence, a positive correlation was found between AXIN2 and regulators except for ZFP57. FIRRE was positively associated with regulators other than DNMT3L and ZFP57. There was a positive correlation between MYBL2 and regulators except for ZFP57. SLC35D3 showed a positive correlation with regulators excluding ZFP57 and UHRF1. TGFBI represented a positive interaction of regulators apart from DNMT3L and UHRF1. Generally, 5mC regulators interfere with the expression of DNA methylation-driven genes according to SCC.

DISCUSSION

Epigenetic processes are important mechanisms in the development of CC. The clinical value of epigenetic markers has been demonstrated. In this study, differential expression analysis of 5mC regulators between cancer and normal tissues was performed. The relationship between the regulators and hallmark-related pathways, which manifested that MYC targets pathway, phosphatidylinositol-3-kinase–AKT–mammalian target of rapamycin signaling pathway, and P53 pathway were subject to the influence of regulators' expression, was identified. Then, we investigated the interaction among 5mC regulators in CC in somatic interaction and expression interaction. Survival analysis indicated DNMT3A, DNMT1, and TET2 associated with the prognosis of the patients with CC. Furthermore, DNA methylation-driven genes in CC were all identified. In general, the degree of DNA methylation appears to be negatively correlated with the expression level of the corresponding DNA methylation-driven gene after observation of expression level in EVs and histology. We developed a five gene signature (FIRRE, MYBL2, TGFBI, AXIN2, and SLC35D3) for prospective CC diagnosing. Meanwhile, these genes are positively related to 5mC regulators and interact with their relevant TFs.

Validation of this model in EVs in the future will have important implications for CC diagnosis screening.

Abnormal DNA methylation usually occurs during carcinogenesis and has clinical value in human cancer. With the development of methylation sequencing technology, epigenetic changes are easily identified with the sequencing depth and accuracy, including methylation changes and methylation driver events detection. In 2020, a risk score prediction model based on the composition of differentially expressed genes driven by DNA methylation was identified and verified, which can accurately predict the prognosis of gastric cancer in clinical practice (Bai et al., 2020). A recent study constructed a predictive signature of 10-gene methylation in endometrial cancer (Li et al., 2021b). A group of methylation driver protein-coding genes and noncoding RNAs have been identified and experimentally confirmed in lung cancer that could be potential targets for epigenetic therapy by integrating methylation and mRNA expression profile data (Cai et al., 2021). Recently, the domestic team systematically demonstrated a multigene methylation detection method (ColonAiQ) that can realize early screening and recurrence prediction of CC, which is based on six markers for multisite blood detection (Sun et al., 2021). Polygene methylation is expected to become a new "tumor marker" to solve the problems of early screening and recurrence monitoring of cancer.

The long noncoding RNA FIRRE locus is associated with two interrelated features of the inactive X chromosome, namely being located near the nucleolus and maintaining H3K27me3 methylation (Yang et al., 2015). Also, there is a differential methylated region associated with multiple sclerosis in the FIRRE (Souren et al., 2019). The mutation and abnormality of AXIN2 (Axis inhibition protein 2) can lead to the occurrence of ampullary carcinoma (Hayata et al., 2021), endometrial cancer (Syed et al., 2020), and liver cancer (Abitbol et al., 2018) by adjusting the stability of beta-catenin. Ren et al. (2015) discovered that the downregulation of MYBL2 was able to suppress cell proliferation, regulate the cell cycle, and induce apoptosis, which may be involved in mediating aggressive CRC biology. TGFB1, the downstream protein of the transforming growth factor-β signaling, contributes to their metastatic potential and stromal cell independence directly in CRC cells (Chiavarina et al., 2021). Researchers identified SLC35D3 as a new biomarker with prognostic value (Olsson et al., 2020).

In conclusion, we investigated the functions of 5mC regulators in CC systematically. They are related to multiple active and inhibitory pathways associated with CC. Also, we identified the methylation-driven genes from RNA-seq data and matched methylation seq data. Based on such methylation-driven genes, a risk model for diagnosis in CC was developed. Our research would provide new insights into the epigenetic-driven events in CC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MW, BF, and CD conceived and designed the study. CD performed the data analysis and edited the paper. JL, ZW, MLi, MY, and MLiu helped to collect the data. YZ and XL helped to analyze the data. BF amended the paper and recommended the procedure. All authors read and approved the final manuscript.

FUNDING

This work was supported by the China Postdoctoral Science Foundation (grant no. 2019M661180) and the Special Foundation of China Postdoctoral Science Foundation (grant no. 2019T120225). This work was also supported by grants from the National Natural Science Foundation of China (nos. 31828005, 81872905, and 81673475), the National Natural Science Foundation of China and Liaoning joint fund key program (no. U1608281), Liaoning Revitalization Talents Program (no. XLYC1807155), and Shenyang S&T Projects (19-109-4-09).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.657092/full#supplementary-material

Additional file 1: | Supplementary Figure S1 Somatic genome alteration and CNAs of m5C regulators and shortlisted genes in CC.

Additional file 2: | Supplementary Figure S2 KEGG and GO analysis of shortlisted genes.

Additional file 3: | Supplementary Tables

REFERENCES

Abitbol, S., Dahmani, R., Coulouarn, C., Ragazzon, B., Mlecnik, B., Senni, N., et al. (2018). AXIN Deficiency in Human and Mouse Hepatocytes Induces Hepatocellular Carcinoma in the Absence of Beta-Catenin Activation. *J. Hepatol.* 68, 1203–1213. doi:10.1016/j.jhep.2017.12.018

Bai, Y., Wei, C., Zhong, Y., Zhang, Y., Long, J., Huang, S., et al. (2020). Development and Validation of a Prognostic Nomogram for Gastric Cancer Based on DNA Methylation-Driven Differentially Expressed Genes. *Int. J. Biol. Sci.* 16, 1153–1165. doi:10.7150/ijbs.41587

Bestor, T. H., Edwards, J. R., and Boulard, M. (2015). Notes on the Role of Dynamic DNA Methylation in Mammalian Development. *Proc. Natl. Acad. Sci. U S A.* 112, 6796–6799. doi:10.1073/pnas.1415301111

Cai, G., Cai, M., Feng, Z., Liu, R., Liang, L., Zhou, P., et al. (2021). A Multilocus Blood-Based Assay Targeting Circulating Tumor DNA Methylation Enables Early Detection and Early Relapse Prediction of Colorectal Cancer. *Gastroenterology* 161, 2053–2056. doi:10.1053/j.gastro.2021.08.054

Cedoz, P. L., Prunello, M., Brennan, K., and Gevaert, O. (2018). MethylMix 2.0: an R Package for Identifying DNA Methylation Genes. *Bioinformatics* 34, 3044–3046. doi:10.1093/bioinformatics/bty156

Chen, C., Zhang, Y., Chen, Z., Yang, H., and Gu, Z. (2021). Cellular Transformers for Targeted Therapy. *Adv. Drug Deliv. Rev.* 179, 114032. doi:10.1016/j.addr.2021.114032

Chen, Y. T., Shen, J. Y., Chen, D-P., Wu, C-F., Guo, R., Zhang, P-P., et al. (2020). Identification of Cross-Talk between M(6)A and 5mC Regulators Associated with Onco-Immunogenic Features and Prognosis across 33 Cancer Types. *J. Hematol. Oncol.* 13, 22. doi:10.1186/s13045-020-00854-w

Chiavarina, B., Costanza, B., Ronca, R., Blomme, A., Rezzola, S., Chiodelli, P., et al. (2021). Metastatic Colorectal Cancer Cells Maintain the TGFbeta Program and Use TGFBI to Fuel Angiogenesis. *Theranostics* 11, 1626–1640. doi:10.7150/thno.51507

Fearon, E. R. (2011). Molecular Genetics of Colorectal Cancer. *Annu. Rev. Pathol.* 6, 479–507. doi:10.1146/annurev-pathol-011110-130235

Fong, M. Y., Zhou, W., Liu, L., Alontaga, A. Y., Chandra, M., Ashby, J., et al. (2015). Breast-cancer-secreted miR-122 Reprograms Glucose Metabolism in Premetastatic Niche to Promote Metastasis. *Nat. Cell Biol* 17, 183–194. doi:10.1038/ncb3094

Ginder, G. D., and Williams, D. C., Jr. (2018). Readers of DNA Methylation, the MBD Family as Potential Therapeutic Targets. *Pharmacol. Ther.* 184, 98–111. doi:10.1016/j.pharmthera.2017.11.002

Hanzelmann, S., Castelo, R., and Guinney, J. (2013). GSVA: Gene Set Variation Analysis for Microarray and RNA-Seq Data. *BMC Bioinformatics* 14, 7. doi:10.1186/1471-2105-14-7

Hao, X., Luo, H., Krawczyk, M., Wei, W., Wang, W., Wang, J., et al. (2017). DNA Methylation Markers for Diagnosis and Prognosis of Common Cancers. *Proc. Natl. Acad. Sci. U S A.* 114, 7414–7419. doi:10.1073/pnas.1703577114

Hayata, Y., Nakagawa, H., Kurosaki, S., Kawamura, S., Matsushita, Y., Hayakawa, Y., et al. (2021). Axin2(+) Peribiliary Glands in the Periampullary Region Generate Biliary Epithelial Stem Cells that Give Rise to Ampullary Carcinoma. *Gastroenterology* 160, 2133–2148. doi:10.1053/j.gastro.2021.01.028

Hu, Y., Yan, C., Hsu, C-H., Chen, Q-R., Niu, K., Komatsoulis, G. A., et al. (2014). OmicCircos: A Simple-To-Use R Package for the Circular Visualization of Multidimensional Omics Data. *Cancer Inform.* 13, 13–20. doi:10.4137/CIN.S13495

Jones, P. A., and Baylin, S. B. (2007). The Epigenomics of Cancer. *Cell* 128, 683–692. doi:10.1016/j.cell.2007.01.029

Kim, J., Afshari, A., Sengupta, R., Sebastiano, V., Gupta, A., Kim, Y. H., et al. (2018). Replication Study: Melanoma Exosomes Educate Bone Marrow Progenitor Cells toward a Pro-metastatic Phenotype through. *MET. Elife.* 7. doi:10.7554/eLife.39944

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J. P., Primdal-Bengtson, B., et al. (2016). Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes. *Proc. Natl. Acad. Sci. U S A.* 113, E968–E977. doi:10.1073/pnas.1521230113

Li, J., Su, X., Dai, L., Chen, N., Fang, C., Dong, Z., et al. (2020). Temporal DNA Methylation Pattern and Targeted Therapy in Colitis-Associated Cancer. *Carcinogenesis* 41, 235–244. doi:10.1093/carcin/bgz199

Li, X., Yang, X., Fan, Y., Cheng, Y., Dong, Y., Zhou, J., et al. (2021). A Ten-Gene Methylation Signature as a Novel Biomarker for Improving Prediction of Prognosis and Indicating Gene Targets in Endometrial Cancer. *Genomics* 113, 2032–2044. doi:10.1016/j.ygeno.2021.04.035

Li, Y., Li, Y., Yu, S., Qian, L., Chen, K., Lai, H., et al. (2021). Circulating EVs Long RNA-Based Subtyping and Deconvolution Enable Prediction of Immunogenic Signatures and Clinical Outcome for PDAC. *Mol. Ther. Nucleic Acids* 26, 488–501. doi:10.1016/j.omtn.2021.08.017

Luo, C., Hajkova, P., and Ecker, J. R. (2018). Dynamic DNA Methylation: In the Right Place at the Right Time. *Science* 361, 1336–1340. doi:10.1126/science.aat6806

Martin, V., Jorgensen, H. F., Chaubert, A. S. B., Berger, J., Barr, H., Shaw, P., et al. (2008). MBD2-mediated Transcriptional Repression of the p14ARF Tumor Suppressor Gene in Human colon Cancer Cells. *Pathobiology* 75, 281–287. doi:10.1159/000151708

Mayakonda, A., Lin, D. C., Assenov, Y., Plass, C., and Koeffler, H. P. (2018). Maftools: Efficient and Comprehensive Analysis of Somatic Variants in Cancer. *Genome Res.* 28, 1747–1756. doi:10.1101/gr.239244.118

Meehan, K., and Vella, L. J. (2016). The Contribution of Tumour-Derived Exosomes to the Hallmarks of Cancer. *Crit. Rev. Clin. Lab. Sci.* 53, 121–131. doi:10.3109/10408363.2015.1092496

Moore, L. D., Le, T., and Fan, G. (2013). DNA Methylation and its Basic Function. *Neuropsychopharmacology* 38, 23–38. doi:10.1038/npp.2012.112

Olsson, L., Hammarstrom, M. L., Israelsson, A., Lindmark, G., and Hammarström, S. (2020). Allocating Colorectal Cancer Patients to Different Risk Categories by Using a Five-Biomarker mRNA Combination in Lymph Node Analysis. *PLoS One* 15, e0229007. doi:10.1371/journal.pone.0229007

Parry, L., and Clarke, A. R. (2011). The Roles of the Methyl-CpG Binding Proteins in Cancer. *Genes Cancer* 2, 618–630. doi:10.1177/1947601911418499

Poh, W. J., Wee, C. P. P., and Gao, Z. (2016). DNA Methyltransferase Activity Assays: Advances and Challenges. *Theranostics* 6, 369–391. doi:10.7150/thno.13438

Ren, F., Wang, L., Shen, X., Xiao, X., Liu, Z., Wei, P., et al. (2015). MYBL2 Is an Independent Prognostic Marker that Has Tumor-Promoting Functions in Colorectal Cancer. *Am. J. Cancer Res.* 5, 1542–1552.

Ren, Z., Wang, W., and Li, J. (2016). Identifying Molecular Subtypes in Human colon Cancer Using Gene Expression and DNA Methylation Microarray Data. *Int. J. Oncol.* 48, 690–702. doi:10.3892/ijo.2015.3263

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Sh, W., et al. (2015). Limma powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies. *Nucleic Acids Res.* 43, e47. doi:10.1093/nar/gkv007

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. *Bioinformatics* 26, 139–140. doi:10.1093/bioinformatics/btp616

Siegel, R. L., Miller, K. D., Fuchs, H. E., and Jemal, A. (2021). Cancer Statistics, 2021. *CA Cancer J. Clin.* 71, 7–33. doi:10.3322/caac.21654

Sing, T., Sander, O., Beerenwinkel, N., and Lengauer, T. (2005). ROCR: Visualizing Classifier Performance in R. *Bioinformatics* 21, 3940–3941. doi:10.1093/bioinformatics/bti623

Souren, N. Y., Gerdes, L. A., Lutsik, P., Gasparoni, G., Beltrán, E., Salhab, A., et al. (2019). DNA Methylation Signatures of Monozygotic Twins Clinically Discordant for Multiple Sclerosis. *Nat. Commun.* 10, 2094. doi:10.1038/s41467-019-09984-3

Sun, X., Yi, J., Yang, J., Han, Y., Qian, X., Liu, Y., et al. (2021). An Integrated Epigenomic-Transcriptomic Landscape of Lung Cancer Reveals Novel Methylation Driver Genes of Diagnostic and Therapeutic Relevance. *Theranostics* 11, 5346–5364. doi:10.7150/thno.58385

Suzuki, M. M., and Bird, A. (2008). DNA Methylation Landscapes: Provocative Insights from Epigenomics. *Nat. Rev. Genet.* 9, 465–476. doi:10.1038/nrg2341

Syed, S. M., Kumar, M., Ghosh, A., Tomasetig, F., Ali, A., Whan, R. M., et al. (2020). Endometrial Axin2(+) Cells Drive Epithelial Homeostasis, Regeneration, and Cancer Following Oncogenic Transformation. *Cell Stem Cell* 26, 64–80. doi:10.1016/j.stem.2019.11.012

Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., et al. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science* 324, 930–935. doi:10.1126/science.1170116

Thery, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell Vesicles* 7, 1535750. doi:10.1080/20013078.2018.1535750

Webber, J. P., Spary, L. K., Sanders, A. J., Chowdhury, R., Jiang, W. G., Steadman, R., et al. (2015). Differentiation of Tumour-Promoting Stromal Myofibroblasts by Cancer Exosomes. *Oncogene* 34, 290–302. doi:10.1038/onc.2013.560

Wieckowski, E. U., Visus, C., Szajnik, M., Szczepanski, M. J., Storkus, W. J., and Whiteside, T. L. (2009). Tumor-derived Microvesicles Promote Regulatory T Cell Expansion and Induce Apoptosis in Tumor-Reactive Activated CD8+ T Lymphocytes. *J. Immunol.* 183, 3720–3730. doi:10.4049/jimmunol.0900970

Wu, N., Zhang, X. Y., Xia, J., Li, X., Yang, T., and Wang, J-H. (2021). Ratiometric 3D DNA Machine Combined with Machine Learning Algorithm for Ultrasensitive and High-Precision Screening of Early Urinary Diseases. *ACS Nano* 15 (12), 19522–19534. doi:10.1021/acsnano.1c06429

Xiao, Y., Tian, J., Wu, W-C., Gaoa, Y-H., Guo, Y-X., Song, S-J., et al. (2022). Targeting central Nervous System Extracellular Vesicles Enhanced Triiodothyronine Remyelination Effect on Experimental Autoimmune Encephalomyelitis. *Bioact Mater.* 9, 373–384. doi:10.1016/j.bioactmat.2021.07.017

Xu, Y. P., Lv, L., Liu, Y., Smith, M. D., Li, W-C., Tan, X-M., et al. (2019). Tumor Suppressor TET2 Promotes Cancer Immunity and Immunotherapy Efficacy. *J. Clin. Invest.* 129, 4316–4331. doi:10.1172/JCI129317

Yang, F., Deng, X., Ma, W., Berletch, J. B., Rabaia, N., Wei, G., et al. (2015). The lncRNA Firre Anchors the Inactive X Chromosome to the Nucleolus by Binding CTCF and Maintains H3K27me3 Methylation. *Genome Biol.* 16, 52. doi:10.1186/s13059-015-0618-0

Yu, G., Wang, L. G., Han, Y., and He, Q-Y. (2012). clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *OMICS* 16, 284–287. doi:10.1089/omi.2011.0118

Zhang, W., and Xu, J. (2017). DNA Methyltransferases and Their Roles in Tumorigenesis. *Biomark Res.* 5, 1. doi:10.1186/s40364-017-0081-z

Zhou, W., Dinh, H. Q., Ramjan, Z., Weisenberger, D. J., Nicolet, C. M., Shen, H., et al. (2018). DNA Methylation Loss in Late-Replicating Domains Is Linked to Mitotic Cell Division. *Nat. Genet.* 50, 591–602. doi:10.1038/s41588-018-0073-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership