THE ROLE OF STEROID HORMONES AND GROWTH FACTORS IN CANCER

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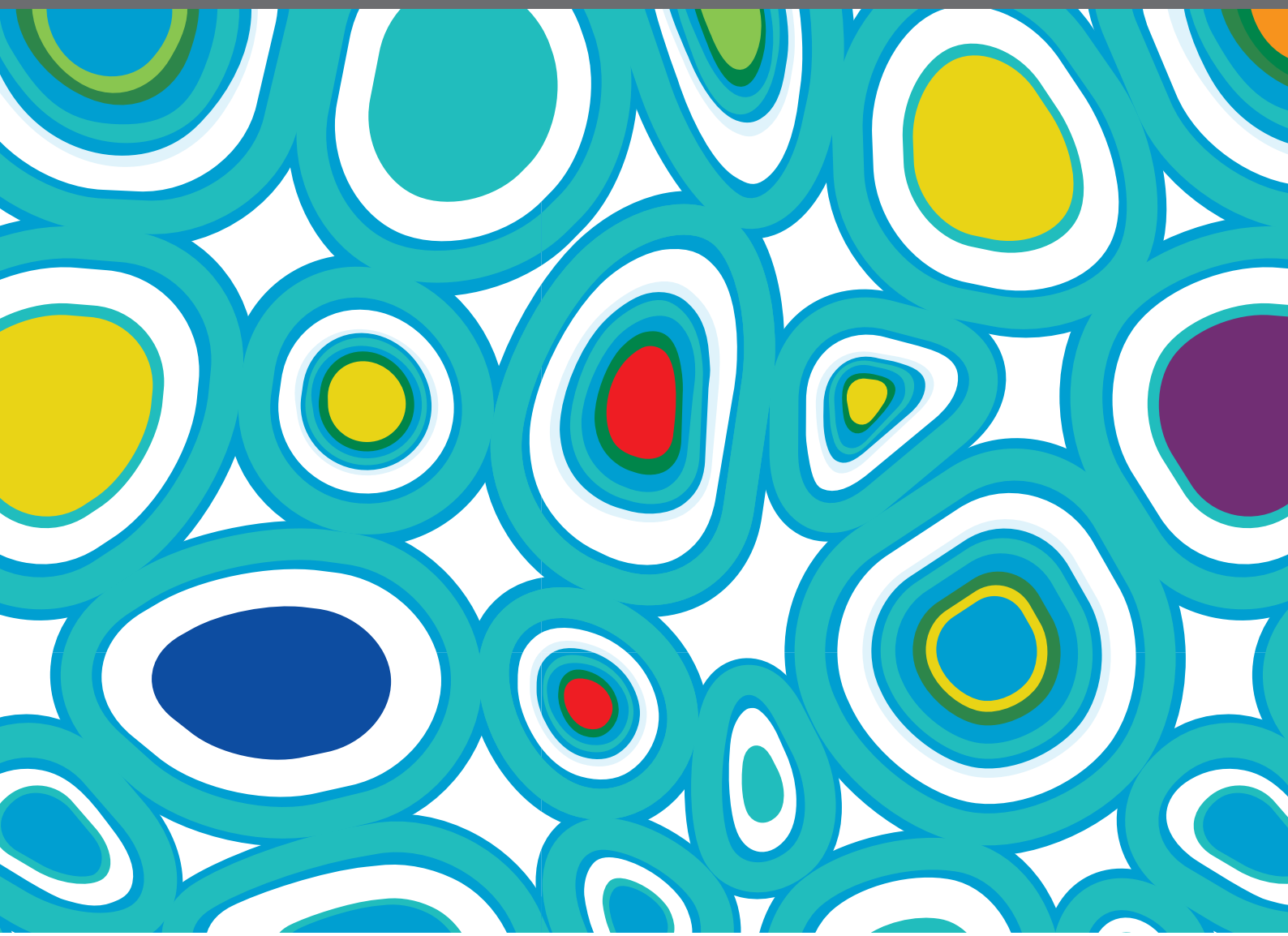
PUBLISHED IN: Frontiers in Cell and Developmental Biology and Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88976-806-6
DOI 10.3389/978-2-88976-806-6

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THE ROLE OF STEROID HORMONES AND GROWTH FACTORS IN CANCER

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Citation: Di Donato, M., Higgins, P. J., Schlaepfer, I. R., Cernera, G., eds. (2022). The Role of Steroid Hormones and Growth Factors in Cancer. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-806-6

Table of Contents

05 ***Editorial: The Role of Steroid Hormones and Growth Factors in Cancer***
Gustavo Cernera, Marzia Di Donato, Paul J. Higgins and Isabel R. Schlaepfer

08 ***FGFR2/STAT3 Signaling Pathway Involves in the Development of MMTV-Related Spontaneous Breast Cancer in TA2 Mice***
Jiaxing Du, Qi Zhao, Kai Liu, Zugui Li, Fangmei Fu, Kexin Zhang, Hao Zhang, Minying Zheng, Yongjie Zhao and Shiwu Zhang

20 ***Cryptorchidism and Testicular Tumor: Comprehensive Analysis of Common Clinical Features and Search of SNVs in the* KIT *and* AR *Genes***
Daniel Adrian Landero-Huerta, Rosa María Vigueras-Villaseñor, Emiy Yokoyama-Rebollar, Fabiola García-Andrade, Julio César Rojas-Castañeda, Luis Alonso Herrera-Montalvo, José Díaz-Chávez, Isidro Xavier Pérez-Añorve, Elena Aréchaga-Ocampo and Margarita Dolores Chávez-Saldaña

27 ***Recent Discoveries in the Androgen Receptor Pathway in Castration-Resistant Prostate Cancer***
Daisuke Obinata, Mitchell G. Lawrence, Kenichi Takayama, Nicholas Choo, Gail P. Risbridger, Satoru Takahashi and Satoshi Inoue

38 ***Androgen Receptor Signaling and Metabolic and Cellular Plasticity During Progression to Castration Resistant Prostate Cancer***
Takuma Uo, Cynthia C. Sprenger and Stephen R. Plymate

50 ***Epidermal Growth Factor Is Essential for the Maintenance of Novel Prostate Epithelial Cells Isolated From Patient-Derived Organoids***
Katia Cheaito, Hisham F. Bahmad, Hiba Jalloul, Ola Hadadeh, Hiba Msheik, Albert El-Hajj, Deborah Mukherji, Mohamed Al-Sayegh and Wassim Abou-Kheir

68 ***Dietary Energy Modulation and Autophagy: Exploiting Metabolic Vulnerabilities to Starve Cancer***
Alyssa J. Cozzo, Michael F. Coleman, Jane B. Pearce, Alexander J. Pfeil, Suhas K. Etigunta and Stephen D. Hursting

87 ***miR-9-5p as a Regulator of the Androgen Receptor Pathway in Breast Cancer Cell Lines***
Erika Bandini, Francesca Fanini, Ivan Vannini, Tania Rossi, Meropi Plousiou, Maria Maddalena Tumedei, Francesco Limarzi, Roberta Maltoni, Francesco Fabbri, Silvana Hrelia, William C. S. Cho and Muller Fabbri

100 ***Microenvironmental Determinants of Breast Cancer Metastasis: Focus on the Crucial Interplay Between Estrogen and Insulin/Insulin-Like Growth Factor Signaling***
Veronica Vella, Ernestina Marianna De Francesco, Rosamaria Lappano, Maria Grazia Muoio, Livia Manzella, Marcello Maggiolini and Antonino Belfiore

122 ***Functional Gene Expression Differentiation of the Notch Signaling Pathway in Female Reproductive Tract Tissues—A Comprehensive Review With Analysis***
Magdalena Orzechowska, Dorota Anusewicz and Andrzej K. Bednarek

156 ***Role of Fibroblast Growth Factor 23 (FGF23) and αKlotho in Cancer***
Franz Ewendt, Martina Feger and Michael Föller

172 ***Determinants and Functions of CAFs Secretome During Cancer Progression and Therapy***
Jenniffer Linares, Juan A. Marín-Jiménez, Jordi Badia-Ramentol and Alexandre Calon

191 ***Molecular Basis of LH Action on Breast Cancer Cell Migration and Invasion via Kinase and Scaffold Proteins***
Joselina Magali Mondaca, Ivonne Denise Uzair, Ana Carla Castro Guijarro, Marina Inés Flamini and Angel Matias Sanchez

204 ***Androgen-Driven Fusion Genes and Chimeric Transcripts in Prostate Cancer***
Mauro Scaravilli, Sonja Koivukoski and Leena Latonen

218 ***Growth Hormone/Insulin Growth Factor Axis in Sex Steroid Associated Disorders and Related Cancers***
Rachel Bleach, Mark Sherlock, Michael W. O'Reilly and Marie McIlroy

233 ***Role of the IGF-1 Axis in Overcoming Resistance in Breast Cancer***
Anna Ianza, Marianna Sirico, Ottavia Bernocchi and Daniele Generali

242 ***Androgen Receptor-Related Non-coding RNAs in Prostate Cancer***
Yongyong Yang, Kilia Y. Liu, Qi Liu and Qi Cao

260 ***Steroid Hormone Biosynthesis Metabolism Is Associated With Fatigue Related to Androgen Deprivation Therapy for Prostate Cancer***
Li Rebekah Feng, Jennifer J. Barb, Hannah Allen, Jeniece Regan and Leorey Saligan

274 ***Targeting the Nerve Growth Factor Signaling Impairs the Proliferative and Migratory Phenotype of Triple-Negative Breast Cancer Cells***
Marzia Di Donato, Giovanni Galasso, Pia Giovannelli, Antonio A. Sinisi, Antimo Migliaccio and Gabriella Castoria

289 ***C1GALT1, Negatively Regulated by miR-181d-5p, Promotes Tumor Progression via Upregulating RAC1 in Lung Adenocarcinoma***
Xiaoxia Dong, Yongyu Liu, Xinzhou Deng, Jun Shao, Shuangyue Tian, Shuang Chen, Rongxin Huang, Ziao Lin, Chunli Chen and Li Shen

305 ***Androgen Activity Is Associated With PD-L1 Downregulation in Thyroid Cancer***
Timmy J. O'Connell, Sina Dadafarin, Melanie Jones, Tomás Rodríguez, Anvita Gupta, Edward Shin, Augustine Moscatello, Codrin Iacob, Humayun Islam, Raj K. Tiwari and Jan Geliebter

315 ***To B (Bone Morphogenic Protein-2) or Not to B (Bone Morphogenic Protein-2): Mesenchymal Stem Cells May Explain the Protein's Role in Osteosarcomagenesis***
Chunfeng Xu, Mingjie Wang, Behrouz Zandieh-Doulabi, Wei Sun, Lingfei Wei and Yuelian Liu

frontiers | Frontiers in Cell and Developmental Biology

EDITORIAL
published: 12 April 2022
doi: 10.3389/fcell.2022.887529

Editorial: The Role of Steroid Hormones and Growth Factors in Cancer

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Keywords: steroid hormones and receptors, growth factors, cancer biology, cancer cells, therapeutic breakthroughs

OPEN ACCESS

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

Received: *01 March 2022*
Accepted: *28 March 2022*
Published: *12 April 2022*

Citation:
Cernera G, Di Donato M, Higgins PJ and Schlaepfer IR (2022) Editorial: The Role of Steroid Hormones and Growth Factors in Cancer. Front. Cell Dev. Biol. 10:887529. doi: 10.3389/fcell.2022.887529

Editorial on the Research Topic

The Role of Steroid Hormones and Growth Factors in Cancer

The relationship between steroid hormones (SHs), growth factors (GFs), their cognate receptors and the downstream signaling pathways lie at the center of cancer development, progression, and therapeutic resistance (Gao et al., 2002; Witsch et al., 2010). A plethora of direct and indirect mechanisms have been described that link SHs- and GFs-signaling to carcinogenesis; nonetheless, the complete picture remains unclear and the underlying mechanisms uncertain. The collection of papers in this Special Issue demonstrates the complexity of issues in this field and provides an update on the latest findings regarding SHs and GFs in cancer, with a focus on future therapeutic breakthroughs. Cancer was associated with soluble GFs for the first time in the 1950s (Waterfield et al., 1983). Later studies established that cells derived from different human tumors are not only stimulated by GFs, but they can also release GFs for an autocrine-regulation of cell proliferation and migration (Sporn and Roberts, 1985). In this context, high plasma levels of Insulin-like growth factor-1 (IGF-1) represent a risk factor for the development and recurrence of breast cancer (BC) in the general population and its receptor, IGF-IR, is overexpressed and hyper-phosphorylated in several subtypes of BCs. In this Special Issue, Lanza et al. describe in their interesting review the pathway underlying IGF-1/IGF-IR signaling and their co-protagonists that lead them to propose combinatorial therapies. In parallel, reducing the IGF1 circulating levels with dietary restrictions could exert anticancer effects by promoting apoptosis, inhibiting angiogenesis, and impairing the downstream engagement of the IGF1/IGF1R pathway. On this topic and with a broader discussion about dietary energy modulation and autophagy, nutritional deprivation of tumor cells as a therapeutic strategy is the subjected of the review by Cozzo et al. The insulin and insulin-like growth factor system (IIGFs) and estrogenic signaling intersect and have high impact in modulating the crosstalk between BC cells and its tumor microenvironment. The review by Vella et al. nicely describes how estrogen and the IIGFs impact stromal elements through soluble and non-soluble secreted molecules, which regulate ECM remodeling, neoangiogenesis, migration, and invasion. The authors open new perspectives: targeting the estrogen–IIGFs cross-talk in both cancer cells and the tumor microenvironment may well be an effective therapeutic option, particularly in patients affected by hyperinsulinemia due to insulin resistance.

BC is an eterogeneous disease and effective therapies are elusive for the more aggressive subtype, triple negative BC (TNBC). By using cellular and animal models, Du et al. describe the role of STAT3 in proliferation and invasiveness of MA-891 cells and the growth of TA2 xenografts and propose

STAT3 as a potential therapeutic target for patients affected by TNBC. Data presented by Di Donato et al. show that TNBC cells, expressing significant amounts of TrkA, release abundant quantities of biologically active Nerve Growth factor (NGF). NGF, through the TrkA/β1-integrin/FAK/Src complex, induces mitogenesis, cell migration and increases in multicellular spheroid size and the associated extracellular matrix (ECM). Similarly, as described by Cheaito et al., epidermal growth factor (EGF) is determinant for the growth of different patient-derived prostate cancer (PC) cells from 3D-organoids. GFs are short-range mediators, which can be exchanged between cancer cells and ECM (Lee et al., 1984) stromal cells [such as tumor microenvironment cells; (Kalluri, 2016; Di Donato et al., 2021)], non-malignant cells and inflammatory cells (Zamarron and Chen, 2011). This cross-talk has a key role in tumor progression (Witsch et al., 2010), angiogenesis (Ucuzian et al., 2010) and inflammation (Wahl et al., 1989). Carcinoma-associated fibroblasts (CAFs) are an heterogeneous and dynamic population that play a major role in the initiation and progression of variousl malignancies by remodeling the supporting stromal matrix into a dense, fibrotic structure while secreting factors that lead to the acquisition of cancer stem-like characteristics. CAFs communicate via autocrine or paracrine mechanisms as well as by release of extracellular vescicles (EVs) with other cellular types in the tumor microenvironment and their secretoma are a valuable source of biomarkers to improve patient selection and treatment follow-up. These and other CAFs attributes are described in Linares et al. intriguing review.

In this context, *fibroblast growth factor 23* (FGF23) is highly expressed in osteomalacia (TIO) and in the oncogenic hypophosphatemic TIO, but also detactable in other types of cancer. By activating the FGF receptor 1c/αKlotho, FGF23 promotes the bone-like microenvironment in phosphaturic mesenchymal tumor, mixed connective tissue variant (PMTMCT), enhancing the FGF23 production by the tumor cells and worsening TIO as assessed by Ewendt et al. In osteosarcoma (OS), the safe application of Bone Morphogenic Protein-2 (BMP-2) in clinical settings remains unclear. In their review, Xu et al. propose a low-dose and slow-release strategy of BMP-2 for bone regeneration protocols. They suggest reconsidering BMP-1 use in patients with bone metabolic diseases, since it might increase (if used at supra-physiological concentration) the occurrence of OS. It is important in this regard to remember that SHs and GFs signaling are often cooperative. In this regard, Bleach et al. describe the intersection between the IGF/IGF-1R axis and different SHs evident in normal growth and development, and extending to metabolism disorders and various endocrine-related cancers. The authors analyze this cross-talk also taking in consideration clinical trials targeting IGF in cancer. SHs have pivotal roles in the common hormone-dependent types of cancer, such as BC and PC. By using T-47D human BC cells, Magali Mondaca et al., demonstrate that after luteinizing hormone (LH) stimulation, the receptor LHR, trough non-genomic actions, recruites and activates a signalling cascade involving Src, FAK and paxillin, leading to an increased phosphorylation and translocation of N-WASP, which culminates in cell migration, invasion and cytoskeletal reorganization. Considering the available data in literature and analyses of the TCGA database, Orzechowska et al. analyzed genes responsive to Notch signaling in BC and in other female reproductive tract tissues, including ovary, cervix, and uterine endometrium and confirmed distinct expression profiles of Notch pathway members as well as their target genes in normal tissues compared their cancerous counterparts. Searching for new therapeutic targets based on specific Notch pathway profiles may be a promising strategy.

PC is the second leading cause of death in men and androgen-deprivation therapy (ADT) is the first treatment option. However, as detailed in the manuscript presented by Feng et al., this type of therapy is not without adverse effects. When ADT is employed for patients with non-metastatic localized PC an increase of fatigue is common and likely due to sleep-related impairment connected to alterations in steroid hormone biosynthesis.

The Androgen Receptor (AR) can interact with different proteins and its signaling in PC initiation or progression can be regulated by many non-coding RNAs that Yang et al. discuss as possible diagnostic biomarkers and therapeutic targets. Genetic rearrangements can promote the formation of fusion genes in which an androgen-regulated promoter is fused to a protein-coding area of a previously androgen-unaffected gene. The presence of these gene fusions is more frequent in PC than in other types of cancer. The role of pseudogenes and non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs), is the subject of the review by Scaravilli et al. A more troubling form of PC is the castration-resistant PC (CRPC), which occurs when PC develop resistance after first line therapies. Uo et al. shed light on mechanisms underlying the transition to a more metabolically aggressive PC phenotype. They explain in their review that two different metabolic and cellular adaptations are involved in PC progression. In the final stage, PC cells appear highly glycolytic, as determined by imaging with ^{18}F-fluorodoxyglucose (^{18}F-FDG), a tracer frequently used to assess tumor energetics. In addition, PC acquires a neuroendocrine phenotyope characterized by robust ^{18}F-FDG uptake and loss of AR signaling. At this stage, unfortunately, the commonly used therapies fail, but in the recent years novel compounds, using alternative approaches to target the AR pathway directly or indirectly, are available. Among them, as described in the intriguing review by Obinata et al., BET inhibitors targeting BET proteins that directly interact with the N-terminal domanin (NTD) of AR, or OCT1 targeting molecules, seem to be efficacious. The authors propose an in-depth discussion about CRPC. The expression and the utility of assessing single nucleotide variants of AR and KIT genes were also investigated in Mexican patients with isolated Cryptorchidism (CO) and testicular germ cell tumors (TGCT). By using this approach Landero-Huerta et al. identified clinical features and genetic variants that may support the early diagnosis of TGCT in pediatric patients with isolated CO.

The paper by O' Connell et al. describes the involvement of the AR in thyroid cancer where it inhibits NF-kB signaling and, by increasing the IkBα inhibitory subunit, negatively modulates the expression of the immune checkpoint molecule PD-L1. The AR has a recognized role also in BC. In this context, Bandini et al., propose the miRNA, miR-9-5p, as an inhibitor of AR expression, leading to an inverse correlation between miR-9-5p and AR in primary BC samples. The use of miR-9-5p inhibits proliferation of BC cells and revert the AR-downstream signaling. Dong et al. further describe in their paper, that miR-181d-5p negatively regulates Core 1 β 1, 3-galactosyltransferase 1 (C1GALT1), an enzyme highly expressed in lung adenocarcinoma (LUAD) and which promotes cell proliferation, migration, and invasion as well as tumor formation *in vivo*. In addition, they found a role for the axis miR-181d-5p/C1GALT1/RAC1 in the LUAD progression. Targeting C1GALT1 could be a potential therapeutic approach for fighting LUAD.

In conclusion, in this Special issue, we have invited leading research groups to contribute with Reviews and original Articles to enriche the knowledge on the role of SHs and GRs in cancer. The manuscripts here collected address some of the questions still pending and identify new challenges in this intricate field. It this the time to re-examine the molecular landscape of SHs and GFs and the possible intersection points in their signaling pathways and to consider the emerging molecular targets and the new drugs available.

AUTHOR CONTRIBUTIONS

MD, GC and PH contributed to the conception of the draft. MD and GC wrote the first draft. PH supervisor. All authors contributed to manuscript revision, read, and approved the submitted version.

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ORIGINAL RESEARCH
published: 05 May 2020
doi: 10.3389/fonc.2020.00652

FGFR2/STAT3 Signaling Pathway Involves in the Development of MMTV-Related Spontaneous Breast Cancer in TA2 Mice

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OPEN ACCESS

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

Received: 31 January 2020
Accepted: 07 April 2020
Published: 05 May 2020

Citation:
Du J, Zhao Q, Liu K, Li Z, Fu F, Zhang K, Zhang H, Zheng M, Zhao Y and Zhang S (2020) FGFR2/STAT3 Signaling Pathway Involves in the Development of MMTV-Related Spontaneous Breast Cancer in TA2 Mice. Front. Oncol. 10:652. doi: 10.3389/fonc.2020.00652

The Tientsin Albino 2 (TA2) mouse has a high incidence of spontaneous breast cancer (SBC) in the absence of external inducers or carcinogens. The initiation of SBC is related to mouse mammary tumor virus (MMTV) infection and pregnancy. Pathologic analysis showed that breast cancer cells in TA2 mice are triple negative. Our previous study confirmed that fibroblast growth factor receptor 2 (FGFR2) expression increased in SBC tissue compared to that in their corresponding normal breast tissues of TA2 mice. The present study focused on the function of the FGFR2/STAT3 signaling pathway in the initiation of SBC. In this study, the expression of FGF3, FGFR2, STAT3, p-STAT3^{Tyr705}, and p-STAT3^{Ser727} was detected in serum and normal mammary gland tissues of TA2 mice with different number of pregnancies and SBC. The proliferation, invasiveness, and migration abilities of MA-891 cells from TA2 SBC were compared before and after cryptotanshinone and Stattic treatment. Transient siRNA transfection was used to detect the invasiveness, and migration abilities to avoid the off-targets effects. Downstream protein expression of STAT3 was also detected in MA-891 cells and TA2 xenografts from MA-891 inoculation. In addition, STAT3 expression was analyzed in 139 cases of human breast cancer including 117 cases of non-triple negative breast cancer (non-TNBC) (group I) and 22 cases of triple-negative breast cancer (TNBC) (group II). Results of our study confirmed that MMTV-LTR amplification, and FGFR2, p-STAT3^{Tyr705}, p-STAT3^{Ser727} expression increased with the number of pregnancies in the breast tissue of TA2 mice and were the highest in SBC. Serum FGF3 expression of SBC was higher than it of TA2 mice with different number of pregnancies. After STAT3 was inhibited, the abilities of proliferation, invasiveness, and migration in MA-891 decreased and the expression levels of STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc in MA-891 and animal xenografts were also down-regulated. In human breast cancer, STAT3 expression was significantly higher in TNBC than that in non-TNBC. Our results showed that the FGFR2/STAT3 signaling pathway may be related to SBC initiation in TA2 mice. Inhibition of STAT3 can decrease proliferation, invasiveness, and migration in MA-891 cells and the growth of TA2 xenografts.

Keywords: tientsin albino 2, FGFR2/STAT3, triple-negative breast cancer, spontaneous breast cancer, MMTV

INTRODUCTION

Breast cancer is the most common malignant tumor and the leading cause of death in women. There were more 2.1 million breast cancer cases in 2018 which accounted for one-quarter of all cancers in women (1). Of the 185 countries included in these statistics, women in 154 countries were most commonly diagnosed with breast cancer (2). Tientsin albino 2 (TA2) mice, a model for spontaneous breast cancer (SBC) established by Tianjin Medical University, have a SBC morbidity rate above 84% (3). Our previous studies showed that the initiation of SBC in TA2 mice was associated with pregnancy status, pregnancy frequency, and mouse mammary tumor virus (MMTV) infection. MMTV is a retrovirus with a long terminal repeats (LTRs). It contains hormone-responsive elements (HRE), transcription enhancer factor-1 (TEF-1) family members, and a superantigen (SAG) coded by open reading frame (ORF) (4, 5). Hormone receptors (progestin, glucocorticoid receptors, and androgen receptors) can bind with the HRE in MMTV promoter to promote the expression of MMTV genes. Our previous studies confirmed that combined exogenous estradiol and progesterone treatment induces breast cancer initiation in TA2 mice without ovaries (4). As a retrovirus, MMTV can integrate its genome into TA2 mouse genome. Insertion of viral DNA within or near an oncogene changes the expression of that gene, leading to the initiation and development of cancer (6, 7). The human genome carries many endogenous retrovirus sequences similar to those of MMTV. Six hundred and sixty MMTV-like env sequences were reported in about 38% of human breast cancer tissues but not in normal breast tissues or other cancers (8). Our previous studies demonstrated that SBC in TA2 mice belongs to triple-negative breast cancer (TNBC), which is negative for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expression (5, 9, 10). TNBC is more likely to affect younger women and the patients' prognosis is poor (5). Moreover, recent studies have shown that signal transducer and activator of transcription 3 (STAT3) is often over-expressed in TNBC and is closely related to TNBC initiation, progression, metastasis, drug resistance and adverse survival outcomes (11–13).

Our previous study showed that fibroblast growth factor-1 (FGF-1) and fibroblast growth factor receptor 2 (FGFR2) contributed to the initiation of SBC in TA2 mice by regulating the cell cycle and promoting cell proliferation. FGF-1 expression in SBC tumor tissue was higher than that in their corresponding matched normal mammary gland tissues in TA2 mice by using an Affymetrix Mouse 430 2.0 array (6, 10). Recently, through high-throughput screening of MMTV insertion sites in mouse mammary tumors, Klijn et al. confirmed that a series of gene loci including *Wnt, fgf, fgfr,* R-spondin (*Rspo*), and platelet-derived growth factor receptor (*Pdgfr*) are directly involved in MMTV-induced mouse mammary tumors (14). Most of the induced breast tumors are mainly at *wnt1* and *fgf3* sites by MMTV infection in wild-type mice (15, 16). Furthermore, FGFR2 is also a common MMTV insertion site (14). High FGFR expression can activate the expression of STAT3 (17). This activation of STAT3 can regulate the expression of its downstream target genes in TNBC cells to promote cell proliferation, migration, and invasion (18). Phosphorylated STAT3 increases tumor cell proliferation, migration, and invasion by increasing the expression of genes such as b-cell lymphoma 2 (*Bcl2*), cyclin D1, and c-myc (19–21).

This study collected SBC and normal breast tissue, protein, and serum samples from TA2 mice with different numbers of pregnancies and measured the expression of FGF3, FGFR2, STAT3, and phosphorylated STAT3. Moreover, the MA-891 cell line derived from SBC of TA2 mice was used to investigate the molecular mechanisms of STAT3 in the initiation of TA2 SBC. Our results showed that the FGFR2/STAT3 signaling pathway may be related to SBC initiation in TA2 mice. Inhibition of STAT3 decreased proliferation, invasiveness, and migration in MA-891 and TA2 xenografts.

MATERIALS AND METHODS

TA2 Mice With SBC

The mice were purchased from Tianjin Medical University and divided into five groups containing five mice each group according to the number of pregnancies, including the non-pregnancy, two pregnancies, four pregnancies, six pregnancies and SBC, which were marked by 0, 2, 4, 6, and SBC, respectively. The mice were raised for more than 14 months and sacrificed according to the experimental requirements. When the number of pregnancies of the mice is more than 6, some mice developed into SBC. The breast cancer tissues, mammary glands, and whole blood from the different groups were collected. The Institutional Animal Care and Use Committee of Tianjin Union Medicine Center approved the animal experimentation protocols and all animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals established by the Chinese Council on Animal Care.

Human Breast Cancer Samples

Paraffin-embedded of human breast cancer samples ($n = 139$) were obtained from Tianjin Union Medical Center (Tianjin, China). The patients had been diagnosed with breast cancer and had not received medical treatment for breast cancer before surgical resection. These 139 cases of human breast cancer were divided into non-TNBC (117 cases, group I) and TNBC (22 cases, group II) groups according to clinicopathological results. The utilization of these tumor samples was permitted by the tissue bank of the Tianjin Union Medical Center and patient information was kept strictly confidential.

MA-891 Cell Line With Cryptotanshinone (CTS) and Stattic Treatment

The MA-891 cell line was obtained from KeyGEN BioTECH, Inc. (NanJing, China) and maintained in RPMI 1640 medium (Gibco,

Abbreviations: TA2, Tientsin Albino 2; STAT3, signal transducer and activator of transcription 3; MMTV, mouse mammary tumor virus; SBC, spontaneous breast cancer; FGF, Fibroblast growth factor; FGFR, fibroblast growth factor receptors; LTR, longer terminal repeats; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple-negative breast cancer; CTS, cryptotanshinone; PBS, phosphate-buffered solution.

USA) containing 10% heat-inactivated fetal bovine serum (FBS) (ExCell Biology, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 5% CO_2 incubator at 37°C. CTS (Solarbio, China) and Stattic (Selleck Chemicals, USA) were used to treat the cells. Stattic (Selleck Chemicals, USA) and CTS (Solarbio, China) were dissolved in DMSO for different concentration.

Transient siRNA Transfection

The siRNA sequences targeted to the mouse STAT3 were synthesized by Shanghai Gene-pharma including three siRNA interference sequences, one positive control sequence (GAPDH), one negative control (NC) sequence (sequences of siRNAs have been listed in **Supplementary Table 1**). Three STAT3 transfection sequences including 2315, 1415, 1107 were used to inhibit the expression of STAT3. When the cells were 60–70% confluent in six-well plates (2×10^5 cells/well), Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 1× Opti-MEM (Gibco, USA) were used to dilute the STAT3 negative control siRNA or STAT3 siRNA following the manufacturer's protocol, and the mixture was added to the cells. The cells were harvested for 48 h after transfection to examine the effect of targeted protein knockdown with western blots.

Wound-Healing Assay

Wound-healing assays were used to evaluate the migration abilities of control cells compared to the cells treated with CTS and Stattic. Detailed information was provided in the **Supplementary Materials and Methods**.

Cell Viability Cell Counting Kit-8 (CCK8) Assay

MA-891 cells were seeded in 96-well plates at 2,000 cells per well and incubated for 12 h. These cells were divided into groups and every group was repeated in triplicate. Detailed information was provided in the **Supplementary Materials and Methods**.

Cell Migration and Invasion Assay

Migration and invasion assays of the Control, CTS-treated (60 μM) and Stattic-treated (2 μM), and STAT3 knockdown cells (STAT3i-1415) were performed as described previously (22) and every group was repeated in triplicate. The detailed information was provided in the **Supplementary Materials and Methods**.

Plate Clone Formation Assay

Cell proliferation was assessed by plate clone formation assay and the detailed information was provided in the **Supplementary Materials and Methods**.

ELISA Measurement of Serum FGF3 Concentration in TA2 Mice With Different Number of Pregnancies

After standing for 30 min, whole blood samples from TA2 mice with different numbers of pregnancies were centrifuged for 15 min at 3,000 g to separate the serum. The serum levels of FGF3 were then determined using ELISA kits (Wuhan ColorfulGene Biological Technology, China).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from normal breast tissue in TA2 mice with different numbers of pregnancies and SBC was isolated using TRizol reagent (Invitrogen, USA) and reversely transcripted into cDNA according to the manufacturer's instructions (Novcare Biotech, China). The level of MMTV-LTR expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The MMTV-LTR primer sequences were 5′GACATGAAACA ACAGGTACATGA3′ and 5′GGACTGTTGCAAGTTTACTC 3′ (full length 339 bp). The GAPDH primer sequences were 5′ACCACAGTCCATGCCATCAC3′ and 5′TCCACCACCCTG TTGCTGTA3′ (full length 452 bp).

Western Blot Assay

Western blot analysis was performed as our previously described (23–25). Information about the primary antibodies and reagents is listed in the **Supplementary Materials and Methods**. Detailed information on the antibodies is listed in **Supplementary Table 2**. All these western blot assays were repeated three times.

Immunocytochemical (ICC) Staining and Immunohistochemical (IHC) Staining

ICC and IHC staining were performed as previously described (7, 23). Detailed information, including the primary antibodies used, is provided in the **Supplementary Materials and Methods** and **Supplementary Table 2**.

Animal Experiments

Twenty female TA2 mice (6 weeks of age, 20–25g) were randomly divided into the control (5 mice) vs. CTS (5 mice), and control (5 mice) vs. Stattic (5 mice) groups. The mice were injected subcutaneously on the right flank with MA-891 cells (1×10^6 cells/mouse) suspended in 100 μL serum-free medium. The inhibitor groups were subcutaneously administered once every 2 days from the fifth day after inoculation. The concentrations of CTS and Stattic were 60 μM and 2 μM, respectively. Starting on the eighth day after injection, the tumors could be palpated and measured every 2 days. The tumor size was determined according to the formula: Tumor volume (mm^3) = (length × $width^2$)/2 and the tumor growth curve was plotted. On the 18th day after inoculation, all mice were sacrificed and the tumor tissues were removed and photographed. Fresh tumor tissues from each mouse were frozen for western blot analysis.

Scoring of IHC Staining

For the scoring of immunostained tissue sections, both the intensity and percentage of positive cells were evaluated according to the methods described by Fei et al. (22, 24). The detailed information is shown in the **Supplementary Materials and Methods**.

Statistical Analysis

SPSS Statistics for Windows, version 17.0 (IBM Corp., USA) was used to evaluate the data. One-way ANOVA was used to compare the differences in MMTV mRNA and protein

expression. Kruskal–Wallis test was performed to compare the differences of the protein expression levels. Other comparisons were performed with two-tailed Student's *t*-test and Pearson's chi-square ($\chi 2$) test. In this study, $P < 0.05$ was considered statistically significant.

RESULTS

Morphological Features in Breast Tissue of TA2 With Different Number of Pregnancies and SBC

H&E staining of tissues including mammary glands of mice with different number of pregnancies and SBC showed gradually increased hyperplasia with increasing pregnancy. The structure of mammary duct and lobule is intact in the normal mammary glands of TA2 mice without a history of pregnancy (**Figure 1Aa**). Breast tissue from mice with two pregnancy times showed mild hyperplasia of the mammary gland epithelium, without obvious changes of the glandular tube (**Figure 1Ab**). Breast tissue of mice with four pregnancy times showed obvious hyperplasia of the mammary gland epithelium was obvious and slightly dilated glandular tube (**Figure 1Ac**). TA2 mice with six pregnancy times showed severe hyperplasia of the mammary gland epithelial cells and highly dilated duct. The shape of mammary glands was irregular and some were cystoid. The glands were also larger (**Figure 1Ad**). The tissue of SBC were composed of numerous solid tumor nests and glandular structures with few stroma (**Figure 1Ae**).

Expression of FGFR2, STAT3, p-STAT3^{Tyr705}, and p-STAT3^{Ser727} and MMTV-LTR Amplification in TA2 Mice With Different Number of Pregnancies and SBC

Our previous studies confirmed that the presence of MMTV was related to SBC initiation in TA2 mice (4, 5, 7). Moreover, there have been reports that MMTV indirectly promotes breast tumor formation by inserting into *FGF3* and *FGFR2* (14). To clarify the relationships among MMTV, FGFR2/STAT3, and SBC initiation, RT-PCR was used to detect MMTV-LTR expression in normal breast tissue from TA2 mice with different numbers of pregnancies and SBC. The results confirmed a significant increase in MMTV-LTR amplification in breast tissue with increasing number of pregnancies compared to the level in the breast tissue of TA2 mice without pregnancy; moreover, SBC had the highest expression of (**Figures 1B,Ea**). Furthermore, ELISA was used to detect the serum FGF3 level in the different groups and the results showed that the level of FGF3 in TA2 with SBC was the highest and the differences between TA2 with different number of pregnancies and SBC had significant significances (**Figure 1C**). No significant differences were observed in the serum levels of FGF3 among TA2 mice with and without pregnancy. Analysis of FGFR2, STAT3, p-STAT3^{Tyr705}, and p-STAT3^{Ser727} expression by western blot of normal breast tissue with different number of pregnancies and SBC (**Figure 1D**) showed gradually increasing expression FGFR2, p-STAT3^{Tyr705}, and p-STAT3^{Ser727} with increasing numbers of pregnancies. FGFR2, p-STAT3^{Tyr705} and p-STAT3^{Ser727} expression levels were highest in the SBC group. Compared to those in the breast tissue from TA2 mice without pregnancy, the expression of FGFR2 (**Figure 1Eb**), p-STAT3^{Tyr705} (**Figure 1Ec**), and p-STAT3^{Ser727} (**Figure 1Ed**) increased in TA2 mice with different numbers of pregnancies and SBC, with statistically significant differences (**Figures 1Eb–d**). Normal breast tissue and SBC had the lowest and highest STAT3 expression, respectively. There were no differences among breast tissue of TA2 mice with different numbers of pregnancies (**Figure 1Ee**). Furthermore, IHC showed that FGFR2 and p-STAT3^{Ser727} expression gradually increased in the breast tissue of TA2 with different number of pregnancies (**Figures 1Fb–d,g–i**) and SBC (**Figures 1Fe,j**) compared to that in normal breast tissue (**Figures 1Fa,f**), which was consistent with the western blot results.

CTS and Stattic Inhibited STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, Cyclin D1 and, c-myc Expression in MA-891 Cells

To study the mechanisms of STAT3 in the initiation of TA2 SBC, MA-891 cells derived from SBC of TA2 mice were treated with two kinds of STAT3 small molecule inhibitors (CTS and Stattic). Western blot showed that CTS and Stattic inhibited STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc expression (**Figure 2A**), with significant differences in expression between the treatment and control groups for STAT3 (**Figure 2Ba**), p-STAT3^{Ser727} (**Figure 2Bb**), p-STAT3^{Tyr705} (**Figure 2Bc**), Bcl2 (**Figure 2Bd**), cyclin D1 (**Figure 2Be**), and c-myc (**Figure 2Bf**). In addition, the expression and subcellular localization of these proteins was also observed in the MA-891 cells before and after CTS and Stattic treatments by ICC (**Figure 2C**). In control MA-891 cells, STAT3, p-STAT3^{Ser727}, and p-STAT3^{Tyr705} were mainly located in the nucleus. Cyclin D1 was located in the nucleus to play a role in cell proliferation. Bcl-2, which can inhibit apoptosis, was mainly located in the cytoplasm while c-myc was mainly located in the nucleus. After treatment with CTS and Stattic, no obvious changes in the localization and expression of total STAT3 were observed (**Figures 2Ca,g,m**). However, the expression of p-STAT3^{Ser727} (**Figures 2Cb,h,n**) and p-STAT3^{Tyr705} (**Figures 2Cc,i,o**) decreased compared to those the control cells. Additionally, compared to the control cells, the expression of Bcl2 (**Figures 2Cd,j,p**), cyclin D1 (**Figures 2Ce,k,q**), and c-myc (**Figures 2Cf,l,r**) also decreased in MA-891 cells after treatment.

Down-Regulation of STAT3 Expression Inhibited MA-891 Cell Proliferation

To determine the effect of small molecular inhibitors on MA-891 cell viability, we carried out CCK8 detection after CTS and Stattic treatment. After 24 h incubation, the absorbance of the control and treated cells was measured (**Figure 3A**), showing dose-dependent effects of the two inhibitors on cell viability. The inhibitory effect on the viability of MA-891 cells increased with the concentration of CTS and Stattic. Compared to the control cells, the differences for DMSO, 10, 20, 40, and 60 μM

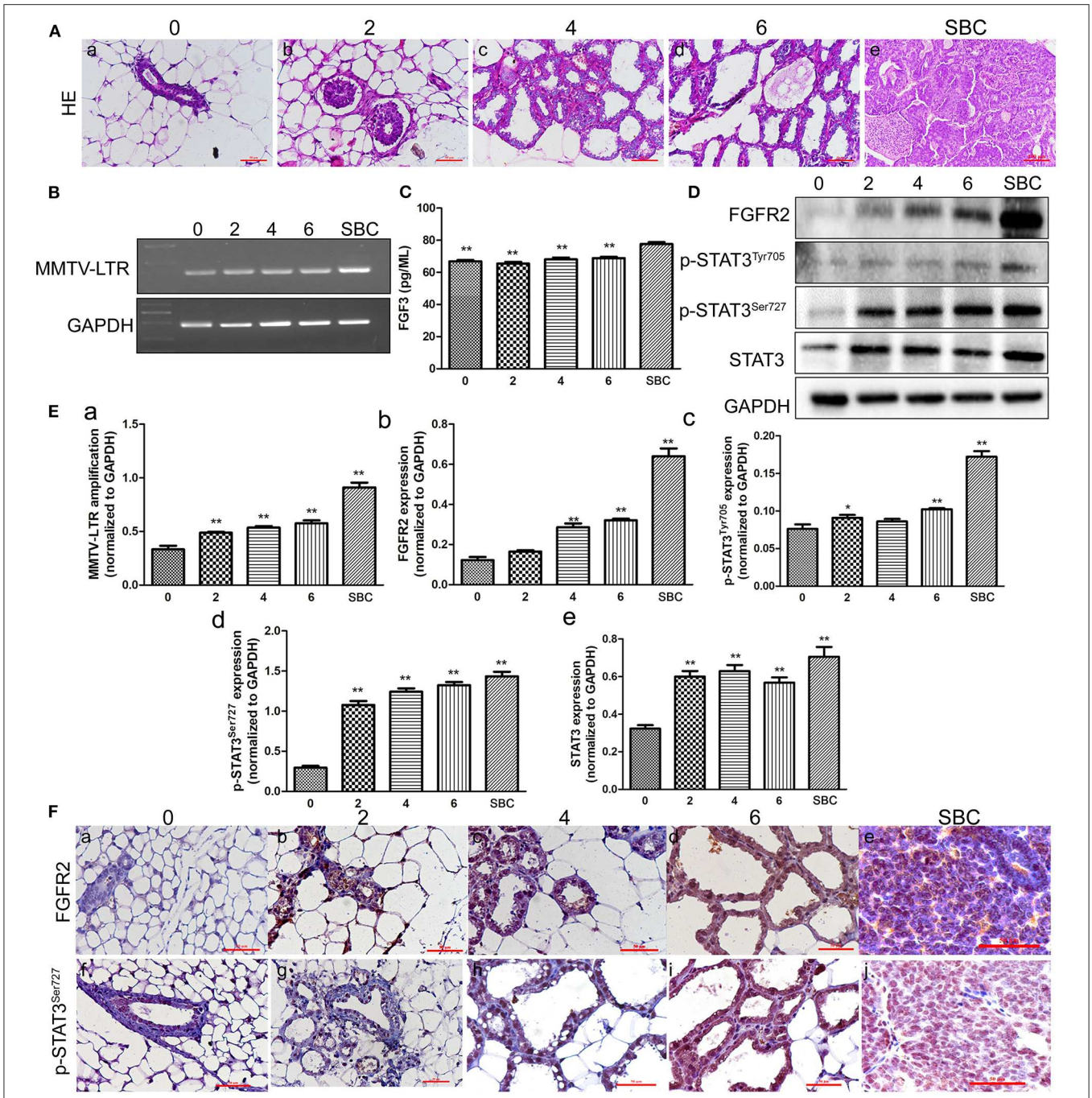

FIGURE 1 | Expression of mouse mammary tumor virus-long terminal repeat (MMTV-LTR) and related proteins in the serum and breast tissue of Tientsin Albino 2 (TA2) mice with different numbers of pregnancies and spontaneous breast cancer (SBC). **(A)** (a–e) Morphological changes in the breast tissues in TA2 mice with zero, two, four, and six pregnancies and SBC, respectively (H&E, ×40). **(B)** Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of MMTV-LTR from TA2 mice with no, two, four, and six pregnancies and SBC, respectively. **(C)** Serum FGF3 concentrations in TA2 mice with different numbers of pregnancies and SBC by enzyme-linked immunosorbent assay (ELISA). **(D)** Expression levels of fibroblast growth factor receptor 2 (FGFR2), phosphorylated signal transducer and activator of transcription 3 (p-STAT3)^{Tyr705}, p-STAT3^{Ser727}, and STAT3 by western blot analysis in TA2 mice with different numbers of pregnancies and SBC. **(E)** Histograms showing quantitative differences in MMTV-LTR and related protein expression in the serum and breast tissue of TA2 mice with different number of pregnancies and SBC. Each bar represents the mean ± standard deviation (SD) of three independent experiments. **(F)** Immunohistochemical staining of FGFR2 and p-STAT3^{Ser727} (IHC, ×40). (a–e). FGFR2 staining in breast tissues of TA2 mice with 0, 2, 4, and 6 pregnancies and with SBC, respectively. (f–j). p-STAT3^{Ser727} staining in breast tissues of TA2 mice with no, two, four, and six pregnancies and SBC, respectively. Statistically differences are indicated: $^{**}P < 0.001$; $^{*}P < 0.05$.

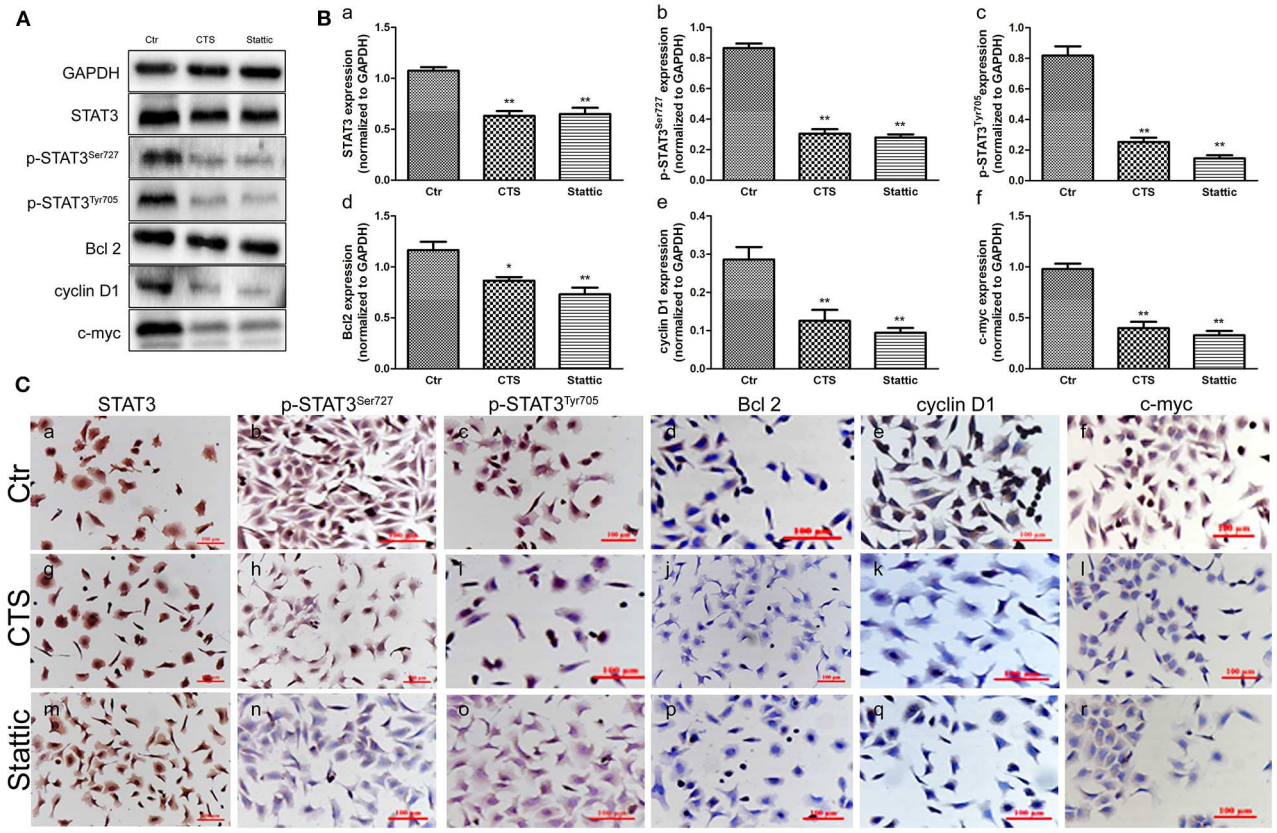

FIGURE 2 | Protein expression in control and cryptotanshinone (CTS) and Stattic-treated MA-891 cells. **(A)** Western blot analysis showing STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc expression in control, CTS-treated, and Stattic-treated cells. **(B)** Histograms of quantitative differences in protein expression among control, CTS and Stattic-treated MA-891 cells. Each bar represents the mean ± standard deviation (SD) of three independent experiments. **(C)** Immunocytochemical (ICC) staining of STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc. (a–f). STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1 and c-myc staining in control MA-891 cells, respectively. (g–l). STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc staining in the CTS-treated group, respectively. (m–r). STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1 and c-myc in staining in the Stattic-treated group, respectively. Statistically differences are indicated: $^{**}P < 0.001$; $^{*}P < 0.05$.

CTS and for DMSO, 0.5, 1, 1.5, and 2 μM Stattic are shown in **Figures 3Aa,c,** respectively. The inhibitory effect on MA-891 cells with 60 μM CTS and 2 μM Stattic increased with the treatment time. Compared to the control cells, the differences for DMSO, 1, 2, 4, and 6 h of CTS and for DMSO, 30 min, 1, 2, and 4 h Stattic are shown in **Figures 3Ab,d**. Moreover, plate cloning assay was used to assess cell proliferation ability, with 2,000 (**Figures 3Ba,d,g**), 1,000 (**Figures 3Bb,e,h**), and 500 (**Figures 3Bc,f,i**) control, CTS-treated, and Stattic-treated MA-891 cells seeded in six-well plates, respectively. The results showed that CTS and Stattic inhibited clone formation. The proliferation ability in the control group was significantly higher than those in the CTS and Stattic treatment groups (**Figure 3Ga**).

Down-Regulation of STAT3 Expression Inhibited MA-891 Cell Migration and Invasion

To determine whether CTS and Stattic treatment affected cell migration in MA-891 cells, wound healing and transwell migration assays were performed using control, CTS-treated, and Stattic-treated cells. **Figure 3C** shows the results of the wound-healing assays at 0, 12, and 24 h. The areas covering the scratched surface gradually decreased with the extension of incubation periods (**Figures 3Ca–i**). Cell migration in the CTS-treated and Stattic-treated cells were decreased at 12 and 24 h compared to that at 0 h (**Figure 3Gb**). Moreover, results of the transwell migration assay confirmed a higher number of migrating cells for control MA-891 cells than for CTS-treated and Stattic-treated cells. To examine whether CTS and Stattic treatment affected cell invasion in MA-891 cells, we performed cell invasion assays using transwell assay with matrigel-coated inserts. The numbers of invading cells were markedly lower in the CTS and Stattic treatment groups compared to those in the control cells (**Figures 3Fa–c,e–g**). In order to avoid the off-targets effects, MA-891 cells after STAT3 knockdown were used to measure the migration and invasion ability of cells. Results of western blot showed that siRNA STAT3-1415 had the strongest inhibitory efficiency and was used in this study (**Figure 3D**). In addition, the expression of cyclin D1 and c-myc

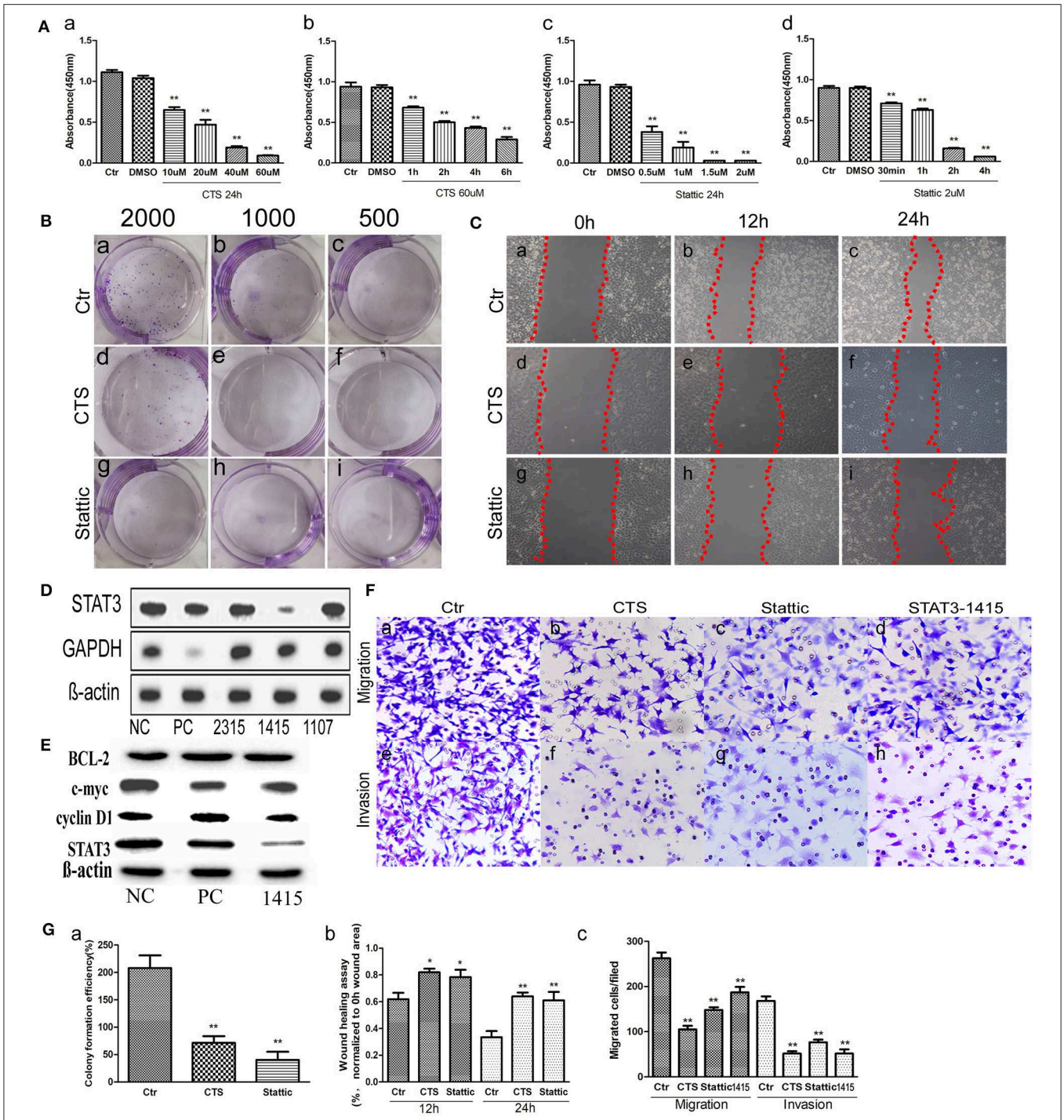

FIGURE 3 | Comparisons of proliferation, migration, and invasive ability in control, cryptotanshinone (CTS)-treated, and Stattic-treated MA-891 cells. **(A)** Cell counting kit-8 (CCK8) shows that CTS and Stattic inhibit viability in a time- and dose-dependent manner. (a) Comparisons of viability in control cells and cells treated with different concentrations of CTS for 24 h. (b) Comparisons of viability in control cells and cells treated with 60 μM of CTS for different times. (c) Comparisons of viability in control cells and cells treated with different Stattic concentrations for 24 h. (d) Comparisons of viability in control cells and cells treated with 2 μM Stattic for different time. **(B)** Cell proliferation ability based on clone formation. (a–c) Proliferation ability of 2,000, 1,000, and 500 control cells, respectively. (d–f) Proliferation ability of 2,000, 1,000, and 500 cells after CTS treatment, respectively. (g–i) Proliferation ability of 2,000, 1,000, and 500 cells after Stattic treatment, respectively. **(C)** Wound-healing assay in MA-891 cells at 0, 12, and 24 h, respectively, after different treatments. (a–c) Representative images in control cells. (d–f) Representative images after CTS treatment. (g–i) Representative images after Stattic treatment. **(D)** Western blot showed STAT3 and GAPDH expression in MA-891 cells with siRNA

(Continued)

FIGURE 3 | STAT3-2315, 1415, 1107, positive control, and negative control. **(E)** Western blot showed STAT3, cyclin D1, c-myc and Bcl2 expression in MA-891 cells with siRNA STAT3-1415, positive control, and negative control. **(F)** Transwell migration and invasion assay in MA-891 cells before and after treatment. (a–d) Migration ability in control, CTS-treated, Stattic-treated cells, and cells after STAT knockdown. (e–h) Invasion assay of control, CTS-treated, Stattic-treated cells and cells after STAT knockdown. **(G)** Histograms showing the quantitative results of the proliferation, migration, and invasive ability of MA-891 cells before and after treatment. Each bar represents the mean ± standard deviation (SD) of three independent experiments. Statistically differences are indicated: $^{**}P < 0.001$; $^{*}P < 0.05$. 1415: siRNA STAT3-1415. PC, positive control; NC, negative control.

decreased in MA-891 cells after siRNA STAT3-1415 treatment compared to the negative control and positive control. However, there were not obvious differences of Bcl2 expression among siRNA STAT3-1415, negative control and positive control MA-891 cells (**Figure 3E**). After STAT3 was inhibited, the numbers of migrating and invasive cells also decreased compared to those in the control cells (**Figures 3Fd,h**). Quantitative assessment of the transwell invasion assays showed significant differences of the invasive cell numbers for CTS, Stattic treatment and STAT3 knockdown (**Figure 3Gc**).

STAT3 Expression in TA2 SBC Tissues Promoted Tumor Growth

MA-891 cells derived from TA2 SBC were subcutaneously injected into the right flank of TA2 mice to investigate the effect of STAT3 on xenograft growth and proliferation. From the fifth day after injection, CTS and Stattic were administered to mice with xenografts once every 2 days (four times in total). Eight days after injection, xenografted tumors could be palpated on the flanks of the mice. All mice were sacrificed on the 18th day after injection and the tumor tissues were removed (**Figures 4Aa–d**). The tumor lengths and widths were measured and the volumes were determined. As shown in **Figure 4A**, the average xenograft volume in the CTS and Stattic treatment groups was significantly lower than it in the control group, with statistically significant differences between the two groups (**Figures 4Ae,f**).

Expression of FGFR2/STAT3 Signaling Pathway-Related Proteins in TA2 Xenografts Before and After CTS and Stattic Treatment

Proteins were extracted from tumor tissues in the four groups of animal xenografts for western blot analysis. The results showed that CTS and Stattic inhibited the expression of STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc (**Figure 4B**). Compared to the control, the protein expression levels of STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc were lower in the Stattic and CTS-treated groups (**Figures 4Ca–f**), indicating that STAT3 might be important for tumor development and progression in TA2 SBC.

STAT3 and FGFR2 Expression in Human Breast Cancer Tissues

To measure the STAT3 and FGFR2 expression levels and their clinicopathological significance, 139 cases of human breast cancer tissue including 117 non-TNBC and 22 TNBC were subjected to IHC staining for STAT3. The average STAT3 and FGFR2 staining index in the TNBC group (group II) were higher than them in the non-TNBC group (group I) and the difference had statistical significance (**Figure 4D**) (**Table 1**).

DISCUSSION

The TA2 mouse model, an ideal animal model for breast cancer research, has a high incidence of SBC. Our previous studies showed the tumorigenesis of TA2 SBC was estradiol and progesterone-dependent. High levels of estradiol and progesterone during pregnancy bind to the HRE of MMTV-LTR to promote the amplification of MMTV and induce the initiation of SBC in TA2 mice (7). MMTV usually integrates into *Wnt, fgf, fgfr, Rspo,* and *Pdgfr*-related locus sites, which contribute to carcinogenic protein amplification. Previous studies have shown that *Wnt* and *fgf3* insertion sites play important roles in TA2 SBC, consistent with our previous findings that Wnt/β-catenin is involved in SBC development and progression in TA2 mice (5).

FGFs and FGFRs are involved in different physiological processes and tumor development associated with proliferation, survival, differentiation, migration, and apoptosis by activating the STAT3, MAPK, and other pathways (26, 27). FGF3, a member of the FGF family, regulates several processes including brain developmental pattern, and limb development by binding, and activating FGFRs in cell surface (28). FGF3/FGFRs are also associated with cellular proliferation, infiltration, and invasiveness during the initiation and development of cancer (29, 30). FGFR1, FGFR2, FGFR3, and FGFR4 are the members of receptor tyrosine kinase subfamily (31). FGF/FGFR2 signaling axis plays an important role in the development of breast cancer. Single nucleotide polymorphisms in intron 2 of the FGFR2 gene are associated with the incidence of breast cancer (32–34).

TNBC is a subtype of invasive breast cancer with ER, PR, and HER-2 negativity that accounts for approximately 15–20% of all breast carcinomas (35, 36). TNBC is also more likely to occur in childbearing women and the prognosis of patients was poor (37). Recent studies have shown that abnormal STAT3 expression plays a vital role in TNBC (12). Similar to FGFR2, STAT3 can also promote tumor growth. In static-phase cells, STAT3 remains in the cytoplasm at its inactive form. Once phosphorylated and activated, STAT3 can translocate to the nucleus to provide transcriptional activity for specific target genes including Bcl2, cyclin D1, c-myc, etc. Upon its activation, this transcription factor regulates malignant tumor proliferation, survival, and metastasis (11, 38). The activity of STAT3 increases in TNBC and regulates proliferation, metastasis, and radiochemotherapy resistance, suggesting that targeting of STAT3 signaling might be an effective therapy in TNBC (38, 39).

This study measured the expression of FGFR2/STAT3 signaling pathway-related proteins and the amplification of

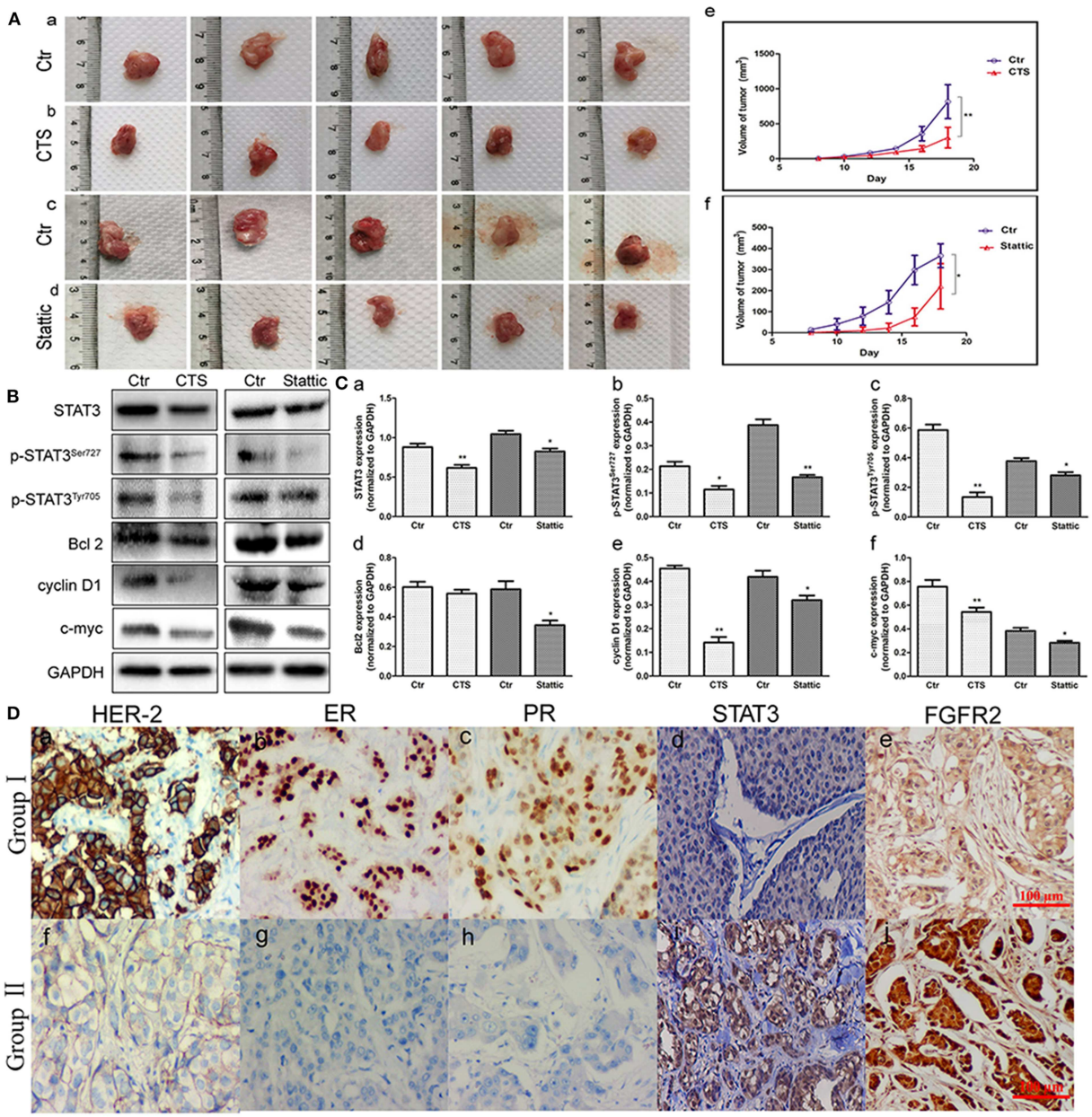

FIGURE 4 | Fibroblast growth factor receptor 2/signal transducer and activator of transcription 3 (FGFR2/STAT3) signaling pathway-related proteins associated with the development of animal xenografts from MA-891 and human breast cancer. **(A)** Gross pictures of tumor masses (each tumor was from one mouse) after the 18th day. (a–d) Tumor tissue of TA2 xenografts from control MA-891 cell injection (a), control MA-891 cell injection and cryptotanshinone (CTS) administration (b), control MA-891 cell injection (c), and control MA-891 cell injection and Stattic administration, respectively (d). (e–f) Growth curves of animal xenografts from control and CTS administration (e), control and Stattic administration (f), respectively. **(B)** Western blot analysis of the expression of proteins including STAT3, p-STAT3^{Ser727}, p-STAT3^{Tyr705}, Bcl2, cyclin D1, and c-myc in xenografts with control and CTS and Stattic treatments. **(C)** Histograms of quantitative differences in protein expression. Each bar represents the mean ± standard deviation (SD) of three independent experiments. **(D)** Immunohistochemical (IHC) staining of human epidermal growth factor receptor 2 (HER-2), estrogen receptor (ER), progesterone receptor (PR), and STAT3 in human breast cancer tissues (IHC ×100). (a–c) HER-2-, ER-, and PR-positive breast cancer. (d) STAT3 expression in non-TNBC. (e) FGFR2 expression in non-TNBC. (f) HER-2-negative breast cancer. (g) ER-negative breast cancer. (h) PR-negative breast cancer. (i) STAT3 expression in TNBC. (j) FGFR2 expression in TNBC. Statistically differences are indicated: $^{**}P < 0.001$; $^{*}P < 0.05$.

MMTV-LTR in TA2 mice with different number of pregnancies and SBC. The results showed gradually increasing breast tissue expression of FGFR2, p-STAT3^{Ser727}, and p-STAT3^{Tyr705} with increasing number of pregnancies and highest expression in SBC, a finding consistent with the amplification of MMTV-LTR. The expression of total STAT3 in mice with a history of pregnancy and SBC was higher than that in mice without a history of pregnancy. Furthermore, the serum FGF3 expression

TABLE 1 | Differences of the average STAT3 and FGFR2 staining index between non-TNBC and TNBC.

		Group	Number	Average staining index	Z	P
STAT3	Non-TNBC	I	117	3.299 ± 2.00	−4.265	0.001
	TNBC	II	22	5.50 ± 2.22		
FGFR2	Non-TNBC	I	117	3.94 ± 1.23	−4.230	0.001
	TNBC	II	22	5.23 ± 1.07		

in SBC was higher than those in TA2 mice with different numbers of pregnancies. Third, to determine the role of the FGFR2/STAT3 signaling pathway in the development of SBC in TA2 mice, two kinds of STAT3 small molecule inhibitors were used to inhibit STAT3 expression and phosphorylation in MA-891 cells and the mouse xenograft model. The results showed decreased proliferation, migration, and invasion in MA-891 cells after treatment. Meanwhile, the average volumes of xenografts in the CTS and Stattic treatment groups were significantly decreased compared to that in the control group. The expression of important downstream proteins of the FGFR2/STAT3 signaling pathway including Bcl2, cyclin D1, and c-myc decreased in MA-891 cells and animal xenografts after CTS and Stattic treatment. CST, a natural product isolated from Salvia miltiorrhiza Bunge, can significantly inhibit the $STAT3^{Tyr705}$ phosphorylation and the dimerization of STAT3 (40). Stattic can inhibit $STAT3^{Tyr705}$ and $STAT3^{Ser727}$ phosphorylation (41). When STAT is phosphorylated and forms dimerization, it can translocate into the nucleus and become a transcription factor. However, in the resting cells, STAT3 retains in the cytoplasm. In this study, results of western blot showed that CTS and Stattic inhibited STAT3, p-$STAT3^{Ser727}$, p-$STAT3^{Tyr705}$ expression. In addition, CTS and Stattic could also inhibit the expression of basal STAT3. We speculate that basal STAT3 accumulated into the cytoplasm after the phosphorylation inhibited by CTS and Stattic. The cytoplasmic STAT3 expression might be regulated by the upstream proteins and decreased via negative feedback. In order to avoid the off-targets effects, siRNA was used to inhibit the expression of STAT3 and the ability of migration and invasion of MA-891 decreased after STAT3 knockdown. Fourth, we also measured STAT3 and FGFR2 expression in human breast tissue, observing significantly higher expression in TNBC than them in non-TNBC. These results indicate that the FGFR2/STAT3 signaling pathway may promote SBC initiation in TA2 mice.

In summary, the results of the present study provide evidence that FGF3 and FGFR2 expression and STAT3 phosphorylation are associated with the number of pregnancies, which increased the occurrence of SBC in TA2 mice. Inhibition of STAT3 can decrease proliferation, invasiveness, and migration in MA-891 cells and the growth of TA2 xenografts. As a key transcription factor, STAT3 may be a potential therapeutic target for patients with TNBC. Based on our previous studies, the high incidence of TA2 SBC associated with the gravidity, the frequency of pregnancy, and presence of MMTV. The detail molecular mechanism of TA2 spontaneous breast cancer is very complex and more experiments are needed to confirm the relationship between FGFR2/STAT3 signaling pathway and the tumorigenesis in TA2 mice in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Review Board of Tianjin Union Medicine Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Tianjin Union Medicine Center.

AUTHOR CONTRIBUTIONS

SZ designed the study and interpreted data contributed to manuscript writing and approved the manuscript before submission. JD, KL, and QZ collected and analyzed data and approved the manuscript before submission. ZL, FF and KZ collected, analyzed, and interpreted the data, and approved the manuscript before submission. HZ, MZ, and YZ collected data, gave constructive comments on the manuscript, and approved the manuscript before submission.

FUNDING

This work was supported in part by grants from the National Natural Science Foundation of China (81672426) and the Foundation of Committee on Science and Technology of Tianjin (17ZXMFSY00120 and 17YFZCSY00700).

ACKNOWLEDGMENTS

We acknowledge editage service for the manuscript language edit.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00652/full#supplementary-material

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 07 August 2020
doi: 10.3389/fcell.2020.00762

Cryptorchidism and Testicular Tumor: Comprehensive Analysis of Common Clinical Features and Search of SNVs in the *KIT* and *AR* Genes

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Allelic variants in genes implicated in the development of testicular germ cell tumor (TGCT) could be present in patients with cryptorchidism (CO). Currently; the mechanisms explaining this relationship are still unknown. In this study the common clinical features in patients with CO and TGCT and 6 variants of *KIT* and *AR* genes associated to TGCT were analyzed. Population analyzed included 328 individuals: 91 patients with CO; 79 with TGCT, 13 of them with previous CO diagnosis, and 158 healthy males. Of the 13 patients with TGCT and history of CO, one patient (7.7%) presented the heterozygous form of the variant rs121913507 and two patients (15.4%) presented homozygote genotype for the variant rs121913506 in *KIT* gene. Interestingly, the heterozygous form for the variant rs121913506 of *KIT* gene was identifying in all of 13 patients. The rs201934623, rs774171864, and rs12014709 variants of the *AR* gene did not show any clinical association. Our results strongly support that genetic component in CO could be conditioning for the development of TGCT. Notably, *KIT* gene variants might be determinants in the pathological association between TGCT and CO.

Keywords: SNVs, *KIT*, *AR*, isolated cryptorchidism, testicular germ cell tumor

OPEN ACCESS

Edited by:
Isabel R. Schlaepfer, School of Medicine, University of Colorado, United States

Reviewed by:
Erika Di Zazzo, University of Molise, Italy
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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 April 2020*
Accepted: *21 July 2020*
Published: *07 August 2020*

Citation:
Landero-Huerta DA, Vigueras-Villaseñor RM, Yokoyama-Rebollar E, García-Andrade F, Rojas-Castañeda JC, Herrera-Montalvo LA, Díaz-Chávez J, Pérez-Añorve IX, Aréchaga-Ocampo E and Chávez-Saldaña MD (2020) Cryptorchidism and Testicular Tumor: Comprehensive Analysis of Common Clinical Features and Search of SNVs in the KIT and AR Genes. Front. Cell Dev. Biol. 8:762. doi: 10.3389/fcell.2020.00762

INTRODUCTION

Cryptorchidism (CO) or undescended testis (OMIM#219050), is the most common genitourinary malformation in newborn males. Many reports indicate that boys with CO have an overall relative risk (RR) of 4.8 (95% confidence interval 4.0–5.7) of develop testicular germ cell tumors (TGCT, OMIM#273300; Dieckmann and Pichlmeier, 2004; Kratz et al., 2010), which is the most frequent solid tumor in men between 15 and 44 years of age. TGCT is classified generally as seminoma

(sTGCT) and non-seminoma (nsTGCT; Kratz et al., 2010). Although the association of CO and TGCT has been clinically established, the mechanisms leading to carcinogenesis are still unknown (Ferguson and Agoulnik, 2013). Due to the presence of common genetic factors in the etiology of both pathologies, it is possible infer the existence of a molecular genetic relationship between CO and the development of TGCT (Vigueras-Villaseñor et al., 2015).

Studies that have been focused on searching diagnostic markers for TGCT proposed several single nucleotide variants (SNVs) in genes such as *POU5F1*, *DND1*, *KIT*, *KITLG*, *AR*, *DMTR1*, *SPRY4*, *BCL2*, *NANOG*, *TGFBR2*, *PTEN*, *AKT1*, *PDE11A*, *GATA4,* and *THOC1* (Dalgaard et al., 2011; Turnbull and Rahman, 2011; Landero-Huerta et al., 2017; Litchfield et al., 2017). Proto-oncogene tyrosine kinase receptor (*KIT*) and the androgen receptor (*AR*) are relevant in the development of TGCT. Although it has not been shown that genetic variants in these genes are responsible for causing CO or Germ cell neoplasia *in situ* (*GCNIS*) a precursor lesion to TGCT, both genes have a role in testicle development and testicular carcinogenic process, influencing directly as in the case of *AR*, through non-genomic pathways (Walker, 2003). The *KIT* gene codifies a class III homodimeric receptor with tyrosine kinase activity in humans. KIT is activates by its ligand KITLG codified by the *KITLG* gene (Agarwal et al., 2014). Both, receptor and ligand are essential for survival, migration and differentiation of the early germ cells (gonocytes; Sheikine et al., 2012). Currently, gonocytes have been proposed as responsible for the development of *GCNIS* (Vigueras-Villaseñor et al., 2015; Berney et al., 2016). On the other hand, the *AR* gene produces a homodimeric cytosolic nuclear receptor, which binds to androgens and induces gene transcription (Li and Al-Azzawi, 2009; Davis-Dao et al., 2011). AR is essential in the inguinal-scrotal phase of testicular descent during the male fetal stage by controlling the normal gonocyte proliferation in the testis (Merlet et al., 2007; Hutson et al., 2015). Interestingly, AR is overexpressed in *GCNIS* and it has been found in gonocytes unable to differentiate properly (Merlet et al., 2007).

Although several studies clearly show a high risk of develop testicular neoplasia in patients with CO, there are no studies that correlate SNVs in patients with TGCT and CO. Therefore, the aims of this study were to analyze the relevant common clinical features in Mexican patients with isolated CO and TGCT, and to identify SNVs in the *KIT* and *AR* genes by allelic discrimination in patients with TGCT and history of CO. This study reports clinical features and genetic variant that may support the early diagnosis of TGCT in pediatric patients with isolated CO.

MATERIALS AND METHODS

Subjects

The study included 328 individuals referred to the Urology Service of the National Institute of Pediatrics and National Institute of Cancerology in Mexico, from 2006 to 2017. The patients were divided in two groups as follows: the first group consisted in 91 patients with confirmed diagnosis of isolated or non-syndromic CO; the second group included 79 patients with confirmed diagnosis of TGCT, 66 of them without history of CO, and 13 patients with confirmed history of isolated CO. In all patients the diagnosis were validated by clinical history, physical examination and imaging studies in all cases. Subsequently, clinical data was obtained and patients were classified according to clinical features. In addition, 158 healthy men without history of CO or TGCT were included as a control group.

All patients and healthy men were Mexican descent, at least two generations, with 46, XY normal karyotype. This study is part of the project with registration number INP-01/2016, approved by the Research and Ethic Committee of INP and all patients included in the study had previously signed the informed consent.

Genotyping of Allelic Variants

DNA was obtained from peripheral blood sample from patients with CO, patients with TGCT without history of CO and healthy individuals according to standard protocols QIA-AMP DNA blood mini kit, [Qiagen, Vienna, Austria]. DNA from patients with both conditions (TGCT and history of CO) was obtained from the testicular tumor samples embedded in paraffin a according to the manufacturer's protocol FFPE RNA/DNA Purification Plus Kit, [Norgen Biotec Corp, Ontario, Canada]. Subsequently, DNA samples were used for genotyping analysis of rs121913507 (D816V), rs121913506 (D816H), and rs121913514 (N822K) SNVs in the *KIT* gene and rs201934623 (P392S), rs774171864 (A299T), and rs12014709 (g.67718624T > G) SNVs in the *AR* gene. The genotyping was performed through allelic discrimination according to established protocol of TaqMan [BMG chemistry Applied Biosystems, Foster City, CA, United States]. The genotyping rate was 99.9% and 30% of the randomized samples, which showed 100% reproducibility in duplicate trials for the 6 SNVs.

Statistical Analysis

The comparison between the genotypes obtained and the clinical data was performed using SPSS v21 statistical package. Chi-square test or Fisher's exact test were used for the SNVs analysis in different groups; in all cases, the P value < 0.05 was considered significant. The statistical power was calculated from the frequency of the minor allele of the variants with a significant P value due to the small number of individuals included in this study. Finally, the allelic frequencies of the SNVs in the population were compared with the frequencies reported in other populations in the HapMap and in the project of the 1000 genomes of European Ancestry (EUR), African Ancestry (AFR), East Asian Ancestry (EAS), South Asian Ancestry (SAS), Latino Ancestry (AMR), and Mexican Ancestry in Los Angeles (MXL).

Analysis of Linkage Disequilibrium and Haplotypes

Haplotype association analysis was performed with the variants located in the same gene. Those variants with a D' value equal or greater than 0.8 were considered in linkage disequilibrium (LD) and haplotypes formed by our cases and controls were

compared using Haploview 4.2 software (Barrett et al., 2004), and then correlating only the patients with bilateral and unilateral CO phenotypes. The epistasis between variants located in different loci were assayed by a Multifactor Dimensionality Reduction (MDR) method in the MDR 3.0.2 software (Moore et al., 2006), since all *P* values were greater than 0.05 and Cross Validation (CV) values equal to 10/10, were considered.

Analysis of Population Structure

The analysis of rs121913506 (D816H), rs121913514, rs774171864, and rs12014709 SNVs in 328 individuals were performed. The software STRUCTURE 2.3.4. (Pritchard Lab, Stanford University) was used to test the stratification within the samples. A mixing model consisting of a burning period of 1,000,000 and 1,000,000 repetitions with a $k = 2$ was used. It should be noted that, although the ancestry informative markers (AIMS) analysis was not performed, the SNVs allowed us to evidence the structural phenomenon of our population. The value of $\delta > 1$ indicated that majority of the analyzed individuals were mixed (Rosenberg, 2002).

RESULTS

Clinical Features Associated to CO and TGCT

At the time of diagnosis, the patients with CO ($n = 91$) had an average age of 3.5 ± 0.3 years, and those with TGCT had 26.5 ± 0.9 years [TGCT with CO ($n = 13$) = 24.3 ± 2.5 years, and TGCT without CO ($n = 66$) = 26.9 ± 1.0 years].

In patients with CO, the frequency of bilateral CO was 48.3% (44/91); all patients with CO underwent orchidopexy, but 73.6% (68/91) underwent orchidopexy after 18 months of age (2–16 years; data not shown), and only 16.5% (15/91) required orchiectomy due to the presence of testicular atrophy (**Table 1**). In addition, 13.2% (12/91) of the patients reported family history of CO and only 2.2% (2/91) reported family history of TGCT (**Table 1**).

In the group of TGCT patients, 16.5% (13/79) had CO, of them, 38.5% (5/13) underwent orchidopexy in an average of 8.8 years of age. All patients of the TGCT group (79/79) underwent orchiectomy at TGCT diagnosis. The most frequent histological type of TGCT patients was nsTGCT [61% (48/79)]. The subgroup of 13 patients with TGCT and CO did not report family history of CO or TGCT, while in the subgroup of TGCT without CO, only 4.5% (3/66) patients had family history of TGCT (**Table 1**).

Genotyping

In order to identify a genetic marker associated to CO condition or to genetic susceptibility to develop TGCT in patients with CO, 6 SNVs were analyzed in this study. Three SNVs in the *KIT* gene, including rs121913507 (D816V), rs121913506 (D816H), and rs121913514 (N822K) were evaluated. While the 3 SNVs analyzed in the *AR* gene were rs201934623 (P392S), rs774171864 (A299T), and rs12014709 (c.66718624 T/G). Initially the subgroups of patients with TGCT and history of CO ($n = 13$) were compared with those patients with TGCT without CO ($n = 66$). Posteriorly, we compared the subgroup of TGCT and CO patients with isolated CO patients ($n = 91$), and finally, with healthy controls ($n = 158$; **Table 2**). Results showed that the variant rs121913507 (D816V) of the *KIT* gene was found in 1/13 (7.7%) of patients with TGCT and CO in a heterozygous form, while the rest of patients [12/13 (92.3%)] presented a homozygote genotype (AA). The comparison among the rest of the patients and healthy controls did not show significant statistically differences [TGCT + CO *vs* TGCT ($P = 0.162$), TGCT + CO *vs* CO ($P = 0.125$), and TGCT + CO *vs* Controls ($P = 0.076$), respectively]. The variant rs121913506 (D816H) of the *KIT* gene was found in heterozygous form in 11/13 (84.6%) patients with TGCT and CO, while 2/13 (15.4%) patients with TGCT and CO had a homozygote genotype (CC). In contrast to the variant rs121913507 (D816V), the comparison between the variant rs121913506 (D816H) of patients with TGCT and CO with the rest of the patients and healthy controls showed statistically significant differences [TGCT + CO *vs* TGCT ($P = 0.025$), TGCT + CO *vs* CO ($P = 0.015$), and TGCT + CO *vs* Controls

TABLE 1 | Clinical features of the patients.

Clinical features		Patients with isolated CO $n = 91$ (%)	Patients with TGCT $n = 79$	
			TGCT with CO $n = 13$ (%)	TGCT without CO $n = 66$ (%)
Age at diagnosis		3.5 ± 0.3	24.3 ± 2.5	26.9 ± 1.0
CO	Bilateral	44 (48.3)	7 (53.8)	NA
	Left	25 (27.5)	3 (23.1)	
	Right	22 (24.2)	3 (23.1)	
Orchidopexy	Yes	91 (100)	5 (38.5)	NA
	No	0 (0)	8 (61.5)	
Orchiectomy	Yes	15 (16.5)	13 (100)	66 (100)
	No	76 (83.5)	0 (0)	0 (0)
Family history of CO	Positive	12 (13.2)	0 (0)	0 (0)
	Negative	79 (86.8)	13 (100)	66 (100)
Family history of TGCT	Positive	2 (2.2)	0 (0)	3 (4.5)
	Negative	89 (97.8)	13 (100)	63 (95.5)
TGCT	Bilateral	NA	1 (7.7)	0 (0)
	Left		4 (30.8)	36 (54.5)
	Right		8 (61.5)	30 (45.5)
Clinical stage at diagnosis	I	NA	4 (30.8)	33 (50)
	II		7 (53.8)	15 (21.7)
	III		1 (7.7)	12 (18.2)
	IV		1 (7.7)	6 (9.1)
Metastasis	Positive	NA	7 (53.8)	24 (36.4)
	Negative		6 (46.2)	42 (63.6)
Tumor histological type	nsTGCT	NA	10 (76.9)	38 (57.6)
	sTGCT		3 (23.1)	28 (42.4)

nsTGCT, Non-Seminomatous Testicular Germ Cells Tumor; sTGCT, Seminomatous Testicular Germ Cells Tumor; and NA, Not Apply.

(P = 0.005), respectively]. The analysis of the rs121913514 (N822K) variant of the *KIT* gene between all groups did not show any differences (**Table 2**).

The results of the analysis of the SNVs in the *AR* gene showed that the rs12014709 (g.67718624T > G) variant located in the intronic region of gene presented different genotypes, however, no statistically significant differences resulted between the groups (**Table 2**). The variants rs201934623 (P392S) and rs774171864 (A299T) of the *AR* gene did not show any differences.

TGCT With History of CO Patients and the *KIT* Gene

Considering that the group of patients with TGCT and CO showed significant differences in the genotypes of the *KIT* gene, we analyzed their association with significant clinical features (**Table 3**). In the first analysis, the patients 2 and 3 that presented the variant rs121913506 (D816H) in homozygous form, were young and suffered unilateral CO (although in opposite side), however, both presented different tumor histological type and only one of them developed metastasis. By the other hand, the patient 13 presented the variant rs121913507 (D816V) in heterozygous form, he had history of bilateral CO and TGCT was unilateral, the tumor type was nsTGCT and he did not develop metastasis.

Interestingly, higher frequency of metastasis was observed in patients with TGCT and previous CO (n = 13) compared to those who presented only TGCT without history of CO (n = 66) [53.8% (7/13) *vs* 36.4% (24/66)] and histological type of nsTGCT [76.9% (10/13) *vs* 57.6% (38/66)] (**Table 1**). Furthermore, only 38.5% (5/13) of patients with TGCT and previous CO underwent orchidopexy in an age average of 8.8 years, while the rest [8/13 (61.5%)] of these patients did not have orchidopexy. The nsTGCT histological type [87.5% (7/8) *vs* 60% (3/5)], metastasis [75% (6/8) *vs* 20% (1/5)], and more advanced tumor stage were most frequently presented in these 8 patients that did not underwent orchidopexy (**Table 3**).

Allelic Frequencies

The allelic frequencies for the 3 variants of the *KIT* gene and 3 variants of the *AR* gene analyzed in this study were search in the literature and in different worldwide databases for general population, to compare these information to the allelic frequencies observed in our results. Only the variants rs121913507, rs201934623, rs774171864, and rs12014709 coincided with the reported frequencies (**Table 4**).

Haplotype blocks conformed by variants located in the same gene were not identify. In the same way, MDR analysis did not show any significant statistical gene-gene interaction. However, the analysis of the population structure of the 4 of the 6 SNVs, a value of $\delta = 2.27$ was identified.

DISCUSSION

Despite the high rate of spontaneous descent during the first year of life, CO is still one of the most common congenital malformations among males worldwide, with a frequency of 1–3%; and is one of the risk factors for development TGCT (Banks et al., 2013). Besides, TGCT is the most common cancer in young men between 15 to 44 years (Trabert et al., 2014), with an age-adjusted rate of 11.1 per 100,000 men (world standard). Among the solid tumors with higher mortality, TGCT are found in the

TABLE 2 | Genotypic frequency of the allelic variants in *KIT* and *AR* genes in Mexican population.

SNV	Patients with TGCT and CO *n* (%)	Patients with TGCT *n* (%)	*P* value TGCT + CO *vs* TGCT	Patients with CO *n* (%)	*P* value TGCT + CO *vs* CO	Healthy Controls *n* (%)	*P* value TGCT + CO *vs* Control
KIT GENE							
D816V (A/T) rs121913507 c.54733155	AA = 12 (92.3) AT = 1 (7.7) TT = 0 (0)	AA = 66 (100) AT = 0 (0) TT = 0 (0)	0.162	AA = 91 (100) AT = 0 (0) TT = 0 (0)	0.125	AA = 158 (100) AT = 0 (0) TT = 0 (0)	0.076
D816H (G/C) rs121913506 c.54733154	GG = 0 (0) GC = 11 (84.6) CC = 2 (15.4)	GG = 0 (0) GC = 66 (100) CC = 0 (0)	0.025	GG = 0 (0) GC = 91 (100) CC = 0 (0)	0.015	GG = 0 (0) GC = 158 (100) CC = 0 (0)	0.005
N822K (A/T) rs121913514 c.54733174	AA = 0 (0) AT = 13 (100) TT = 0 (0)	AA = 0 (0) AT = 66 (100) TT = 0 (0)	NA	AA = 0 (0) AT = 89 (97.8) TT = 2 (2.2)	0.765	AA = 0 (0) AT = 154 (97.5) TT = 4 (2.5)	0.693
AR GENE							
Intronic (T/G) rs12014709 c.66718624	TT = 0 (0) TG = 13 (100) GG = 0 (0)	TT = 2 (3.0) TG = 63 (95.5) GG = 1 (1.5)	0.736	TT = 4 (2.5) TG = 80 (87.9) GG = 7 (7.7)	0.415	TT = 4 (2.5) TG = 143 (90.5) GG = 11 (7.0)	0.508
P392S (C/T) rs201934623 c.67546320	CC = 13 (100) CT = 0 (0) TT = 0 (0)	CC = 66 (100) CT = 0 (0) TT = 0 (0)	NA	CC = 91 (100) CT = 0 (0) TT = 0 (0)	NA	CC = 158 (100) CT = 0 (0) TT = 0 (0)	NA
A299T (C/T) rs774171864 c.67546042	CC = 0 (0) CT = 13 (100) TT = 0 (0)	CC = 0 (0) CT = 66 (100) TT = 0 (0)	NA	CC = 0 (0) CT = 91 (100) TT = 0 (0)	NA	CC = 0 (0) CT = 158 (100) TT = 0 (0)	NA

NA = Not Apply.

TABLE 3 | Clinical features of the patients with TGCT and isolated CO.

Patient	Age at diagnosis of TGCT	CO side	Age of orchidopexy	Tumor side	Tumor histological type	Metastasis	Clinical stage at diagnosis	Genotype
	Patients with orchidopexy							
1	16 years	Bilateral	8 years	Right	nsTGCT	No	I	
2	18 years	Right	8 years	Right	sTGCT	No	I	D816H/D816H
3	19 years	Left	12 years	Left	nsTGCT	Retroperitoneum	II	D816H/D816H
4	24 years	Right	10 years	Right	nsTGCT	No	I	
5	27 years	Bilateral	6 years	Right	sTGCT	No	I	
	Patients without orchidopexy							
6	16 years	Bilateral	NA	Right	nsTGCT	Retroperitoneum	II	
7	17 years	Bilateral	NA	Left	nsTGCT	Pelvis	II	
8	22 years	Left	NA	Left	sTGCT	Retroperitoneum	II	
9	22 years	Bilateral	NA	Bilateral	nsTGCT	No	II	
10	23 years	Right	NA	Right	nsTGCT	Lung	IV	
11	27 years	Left	NA	Left	nsTGCT	Retroperitoneum	II	
12	36 years	Bilateral	NA	Right	nsTGCT	Lung	III	
13	49 years	Bilateral	NA	Right	nsTGCT	No	II	Wild type/D816V

NA = Not Apply.

TABLE 4 | Allelic frequencies of the variables analyzed in the population studied.

Gene	SNV	Allele	ALL	EUR	AFR	EAS	SAS	AMR	MXL	Mexican
KIT	rs121913507 D816V	A	1	1	1	1	1	1	1	1
		T	0	0	0	0	0	0	0	0
	rs121913506 D816H	G	—	—	—	—	—	—	—	0.50
		C	—	—	—	—	—	—	—	0.50
	rs121913514 N822K	A	—	—	—	—	—	—	—	0.49
		T	—	—	—	—	—	—	—	0.51
AR	rs201934623 P392S	C	0.99	1	1	1	0.96	1	0.99	1
		T	0.01	0	0	0	0.04	0	0.01	0
	rs774171864 A299T	C	1	1	1	1	1	1	1	0.50
		T	0	0	0	0	0	0	0	0.50
	rs12014709 Intronic	T	0.85	0.91	0.58	1	0.96	0.91	0.96	0.49
		G	0.15	0.09	0.42	0	0.04	0.09	0.04	0.51

There are no data available on the frequencies of the variant (—), ALL, All gnomeAD, EUR, European Ancestry, AFR, African Ancestry, EAS, East Asian Ancestry, SAS, South Asian Ancestry, AMR, Latino Ancestry, and MXL, Mexican Ancestry in Los Angeles.

fifth place with a mortality rate of 1.3 per 100,000 men (Global Cancer Observatory, 2020). In spite of clinical relationship between CO and TGCT the molecular mechanisms underlying both diseases are still unknown. Significantly, although the incidence of CO and TGCT has increased worldwide, in Mexico there are few epidemiological data about both diseases.

In this study, we analyzed the clinical manifestations of patients with CO, patients with TGCT and patients with TCGT and history of CO. In the group of patients with CO we found that the incidence of bilateral disease was higher than those reported for Hispanic population [48.3% (44/91) *vs* 19.7% (25/127)] (Davis-Dao et al., 2012). However, other findings were similar to other studies. Surgical intervention in 70% of the patients with CO done after 2 years (Williams et al., 2018) compared with 73.6% in our study, and the family history of TGCT in our patients with CO and in Italian population [2.2% (2/91) *vs* 2.1% (15/721; Foresta et al., 2008)] (**Table 1**).

In addition, in the group of TGCT patients we did not find CO history, compared to other reports who found CO history in 5–10% (Kratz et al., 2010) and 14.6% (18/123) of TGCT patients (Garolla et al., 2005). On the other hand, we identify 4.5% patients with TGCT without CO (3/66) with at least one family member affected by the same neoplasm, and a global frequency of 3.8% (3/79 patients with TGCT), these were according to other authors for familiar TGCT (Mai et al., 2009; Rapley and Nathanson, 2010); we also observed higher frequency of nsTGCT in our patients with TGCT, similar to Hispanic population (Woldu et al., 2018; **Table 1**).

CO is an important risk factor for the development of TGCT, and this relationship has been clinically established, however, the molecular mechanism between failure in

testicular descent and the development of malignancy, is still unknown (Ferguson and Agoulnik, 2013). Several genes have been associated to the development of TGCT (Rapley and Nathanson, 2010; Landero-Huerta et al., 2017), but only a few of them, such as *KIT* gene expression (Vigueras-Villaseñor et al., 2015), or polyQ inserts in exon 1 of the *AR* gene (Ferlin et al., 2005; Davis-Dao et al., 2011; Hutson et al., 2015; Fukawa and Kanayama, 2018) have been analyzed in patients with CO, without establish a relationship between CO and TGCT.

Therefore, we focused on determine the relationship between 6 SNVs in both *KIT* and *AR* genes and TGCT and CO in Mexican population. The contrast of the patients with TGCT and history of CO with TGCT without CO, or those with CO as well as control group showed statistical differences in the presence of the homozygous form (CC) of the rs121913506 (D816H) variant of the *KIT* gene in two patients with TGCT and history of CO, these results suggested that CO patients with C allele in homozygous form might have a higher risk of development TGCT. The frequency of C allele in homozygous form in our group of TGCT Mexican patients was similar to the reported in Japanese population (Sakuma et al., 2003) [2.5% (2/79) *vs* 2.9% (1/34)]. Although the other SNVs did not show statistical differences, we consider pertinent not to discard them as important SNVs in Mexican population, therefore, these results must be explored in higher number of samples. In previous reports, the rs121913506 (D816H) variant has been considered as mutation related to TGCT (Looijenga et al., 2003), however, we identified the same variant in heterozygous form in the majority of patients and even in the control group, therefore our population could have a different genotype.

In the group of patients with TGCT, we considered the simultaneous presence of TGCT and CO as the most important factor, so we analyzed individually this subgroup of 13 patients, and we found that the variants rs121913507 (D816V) and rs121913506 (D816H) of the *KIT* gene were identified in 3 patients (2, 3, and 13 patients; **Table 3**); however, there were not association between these genetic variants and any particular phenotype or clinical feature. However, the results obtained from the clinical characteristics indicated that the patients with TGCT with previous CO could express a more aggressive phenotype of the TGCT, which itself is already severe (**Table 3**). These results suggest that the relationship between CO and severe TGCT phenotypes could be due to the participation of the function of multiple genes and not exclusively by *KIT* and *AR*. In particular, we observed that patients who did not undergo orchidopexy at right time had a more severe TGCT phenotype (**Table 3**). We could highlight the importance of performing orchidopexy in a timely manner, minimizing the risk and complications in the pediatric patient with CO (**Table 3**), and avoiding undescended testes to be subject to different abnormal stress conditions (Williams et al., 2018).

Additionally, the comparison of the allelic frequencies for the rs121913506 (D816H) and rs121913514 (N822K) variants in other populations was not possible due to lack of available information in the HapMap and in the project of the 1000 genomes. However, the frequencies for the allele A of the variant rs121913507 (D816V) in the *KIT* gene, and for the allele C of the variant rs201934623 (P392S) in the *AR* gene, were similar to that found in all populations. In the case of the both alleles of the variant rs774171864 (A299T), the frequencies were different to all databases, and the allelic frequencies of the variant rs12014709 were similar only with the AFR. Finally, we identified a mixed population in population structure analysis, according to previously reports for Mexican population with predominantly Amerindian and EUR (Salazar-Flores et al., 2015).

In conclusion, the results of this study support the fact that CO is a risk factor for the development of TGCT at molecular level. *KIT* gene variants rs121913507 and rs121913506 might be common among TGCT and CO. However, more studies must perform to clarify these results and to find predictive biomarkers for TGCT and CO patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the project with registration number INP-01/2016, approved by the Research and Ethics Committee of INP. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MC-S contributed to conception and design. DL-H, MC-S, FG-A, and JR-C contributed to the development of the methodology. MC-S, RV-V, EY-R, IP-A, and EA-O performed the analysis and interpreted the data. DL-H, MC-S, and RV-V contributed to writing, review, and revision of the manuscript. LH-M, JD-C, and JR-C provided administrative, technical, or material support. RV-V, MC-S, and DL-H contributed to study supervision. All authors contributed to the article and approved the submitted version.

FUNDING

The authors thank CONACyT for financial support CONACyT FOSISS (Project 272641/2017) and Instituto Nacional de Pediatría INP: 01/2016. CONACyT for the scholarship granted to DL-H (No. 429258), DL-H submitted this article to fulfill the graduate requirement for a Ph.D. degree at Posgrado en Ciencias Naturales e Ingeniería of Universidad Autónoma Metropolitana Unidad Cuajimalpa.

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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.

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frontiers in Oncology

MINI REVIEW
published: 08 October 2020
doi: 10.3389/fonc.2020.581515

Recent Discoveries in the Androgen Receptor Pathway in Castration-Resistant Prostate Cancer

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The androgen receptor (AR) is the main therapeutic target in advanced prostate cancer, because it regulates the growth and progression of prostate cancer cells. Patients may undergo multiple lines of AR-directed treatments, including androgen-deprivation therapy, AR signaling inhibitors (abiraterone acetate, enzalutamide, apalutamide, or darolutamide), or combinations of these therapies. Yet, tumors inevitably develop resistance to the successive lines of treatment. The diverse mechanisms of resistance include reactivation of the AR and dysregulation of AR cofactors and collaborative transcription factors (TFs). Further elucidating the nexus between the AR and collaborative TFs may reveal new strategies targeting the AR directly or indirectly, such as targeting BET proteins or OCT1. However, appropriate preclinical models will be required to test the efficacy of these approaches. Fortunately, an increasing variety of patient-derived models, such as xenografts and organoids, are being developed for discovery-based research and preclinical drug screening. Here we review the mechanisms of drug resistance in the AR signaling pathway, the intersection with collaborative TFs, and the use of patient-derived models for novel drug discovery.

Keywords: androgen receptor, castration-resistant prostate cancer, transcription factors, octamer transcription factor 1, preclinical models

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *09 July 2020*
Accepted: *27 August 2020*
Published: *08 October 2020*

Citation:
Obinata D, Lawrence MG, Takayama K, Choo N, Risbridger GP, Takahashi S and Inoue S (2020) Recent Discoveries in the Androgen Receptor Pathway in Castration-Resistant Prostate Cancer. Front. Oncol. 10:581515. doi: 10.3389/fonc.2020.581515

INTRODUCTION

Prostate cancer is one of the most common causes of cancer-related death among men in Western countries. At diagnosis, most prostate cancers rely on the androgen receptor (AR) signaling for growth and survival. In this pathway, the AR is bound by ligands, such as dihydrotestosterone (DHT), and regulates the expression of target genes (1–5). In addition, the AR collaborates with cofactors, including transcription factors (TFs), which bind to specific DNA elements in regulatory regions of AR-responsive genes. Since AR collaborative TFs fine-tune androgen-responsive gene expression, it is important to further elucidate their role in the progression of prostate cancer.

In normal prostate epithelium, the AR suppresses proliferation and promotes differentiation (6); however, during carcinogenesis prostate cancer cells develop "lineage-addiction," where the AR promotes tumor progression (7). Given the importance of the AR pathway in prostate cancer, it is the target of most treatments for advanced disease. Androgen deprivation therapy (ADT), through surgical or pharmacological castration, is initially effective at reducing tumor burden. ADT is administered alone or in combination with chemotherapy or AR-signaling inhibitors (8). A subset of cancer cells withstand treatment and eventually develop into castration-resistant prostate cancer (CRPC), which proliferates despite castrate concentrations of circulating androgens. Since AR signaling persists in most cases of CRPC, patients receive further treatment with AR signaling inhibitors (abiraterone acetate, enzalutamide, apalutamide, and darolutamide) based on whether they have metastatic or non-metastatic disease (9–12). Yet, tumors inevitably acquire further resistance, often by reactivating AR signaling. Once patients fail an AR signaling inhibitor, further treatments include another AR-directed therapy, chemotherapy, or if there are genomic defects in homologous recombination repair genes, a PARP inhibitor (13, 14). However, as CRPC is ultimately lethal, there is an ongoing need for new treatments.

An important step in developing novel therapies is testing their effectiveness in preclinical models. Although there is a longstanding paucity of preclinical models of CRPC, larger collections of patient-derived models are providing new tools to validate and prioritize candidate treatments for clinical trials. In this review, we examine mechanisms of castration-resistance involving the AR and collaborating TFs, new strategies for targeting tumors with these features, and the use of different patient-derived models for testing these novel treatments.

MECHANISMS OF CASTRATION-RESISTANCE THROUGH ALTERATIONS OF THE AR

The AR gene on Xq11-13 consists of 8 exons encoding the N-terminal domain (NTD; 555 amino acids; exon 1), DNA-binding domain (DBD; 68 amino acids; exons 2 and 3), hinge region (40 amino acids; exon 4), and ligand binding domain (LBD; 295 amino acids; exons 4–8) (15, 16). Binding of androgens to the LBD triggers an intramolecular interaction with the NTD, which in turn interacts with AR co-activators (17, 18).

Amplifications of the *AR* locus are one the most common mechanisms of castration-resistance, and they often encompass an enhancer located ∼700 kilobases upstream (19–21). In some tumors, the *AR* gene and enhancer are amplified independently of each other (21). The *AR* enhancer is bound by several transcriptional activators, including FOXA1, GATA2, NKX3.1, HOXB13, and the AR itself (20). Amplifications of the *AR* and its enhancer are associated with higher levels of AR expression, and over-expressing the AR in prostate cancer cell lines causes enzalutamide-resistance (20, 21). Accordingly, patients with amplifications of the *AR* locus and/or enhancer are often resistant to AR-directed therapies, including enzalutamide and abiraterone acetate (22, 23). In preclinical studies with VCaP cells, which have an *AR* amplification and express high levels of the *AR*, darolutamide had a lower IC50 than enzalutamide and apalutamide in suppressing proliferation (24), suggesting that potent inhibition of the AR may be required for tumors with this mechanism of resistance.

The conformation of the AR can be disrupted by point mutations, which commonly arise in CRPC and mediate resistance to AR-directed treatments (25, 26). Occasionally, two AR mutations can occur in the same tumor (27–30). Point mutations often occur in the LBD, causing gain-of-function in ligand binding, so the AR is activated by other steroids, and antagonists, like enzalutamide, are converted into agonists (31–33) (**Supplementary Table 1**). Since AR mutations confer resistance to particular antagonists, they are potential predictive biomarkers for AR-directed inhibitors. Enzalutamide may not be suitable for tumors with AR mutations that convert it into a partial agonist (F877L, H875Y/T878A, F877L/T878A) (27). Darolutamide might be more effective for these tumors, since it remains an antagonist despite these AR mutations (34). In addition, darolutamide has unique flexibility that allows it to bind the W742C/L mutated ligand-binding pocket, unlike enzalutamide (35). However, the utility of AR mutations as biomarkers needs confirmation in patients. For example, the F877L AR mutation converts apalutamide into a partial agonist *in vitro*, but neither this mutation nor T878A was a common cause of acquired resistance to apalutamide in a phase I/II trial (36).

In addition to AR mutations, constitutively active AR splice variants (ARVs) can mediate castration resistance (37). Increased expression of ARVs can arise through amplifications or structural rearrangements of the AR gene in CRPC (20, 30, 38, 39). Among numerous ARVs, AR-V7, and ARv567es have been studied in the most detail. AR-V7 includes exons 1/2/3, encoding the NTD, followed by a cryptic exon (**Figures 1A,B**) (37). ARv567es includes exons 1/2/3/4/8, but skips exons 5/6/7 (40). Since both variants lack the LBD, they are not bound by most AR signaling inhibitors, so can sustain AR-driven gene expression. The lack of the AR hinge region in AR-V7 may also promote therapy resistance. SPOP (speckle type POZ protein), an E3 ubiquitin ligase that is upregulated by enzalutamide treatment, usually binds to the hinge region of the AR and induces its degradation (41). By escaping this ubiquitin degradation pathway, AR-V7 may enable enzalutamide resistance. In addition, the hinge region usually mediates microtubule binding and translocation of the AR into the nucleus (42, 43). Since AR-V7 lacks the hinge region, its transport is independent of microtubules, enabling resistance to taxane chemotherapy, which targets microtubules, unlike ARv567es which still contains the hinge region (43, 44). Cell-cycle or cell-division associated genes such as ubiquitin-conjugating enzyme E2 C (UBE2C) are unique AR-V7 targets, contributing to cell proliferation under androgen-depleted conditions (45, 46). Recent functional analyses demonstrated the importance of various splicing factors, which are highly expressed in CRPC tissues (47–50). Enhanced expression of splicing factors would promote their recruitment to pre-mRNA, facilitating the mRNA splicing process. Thus, altered splicing

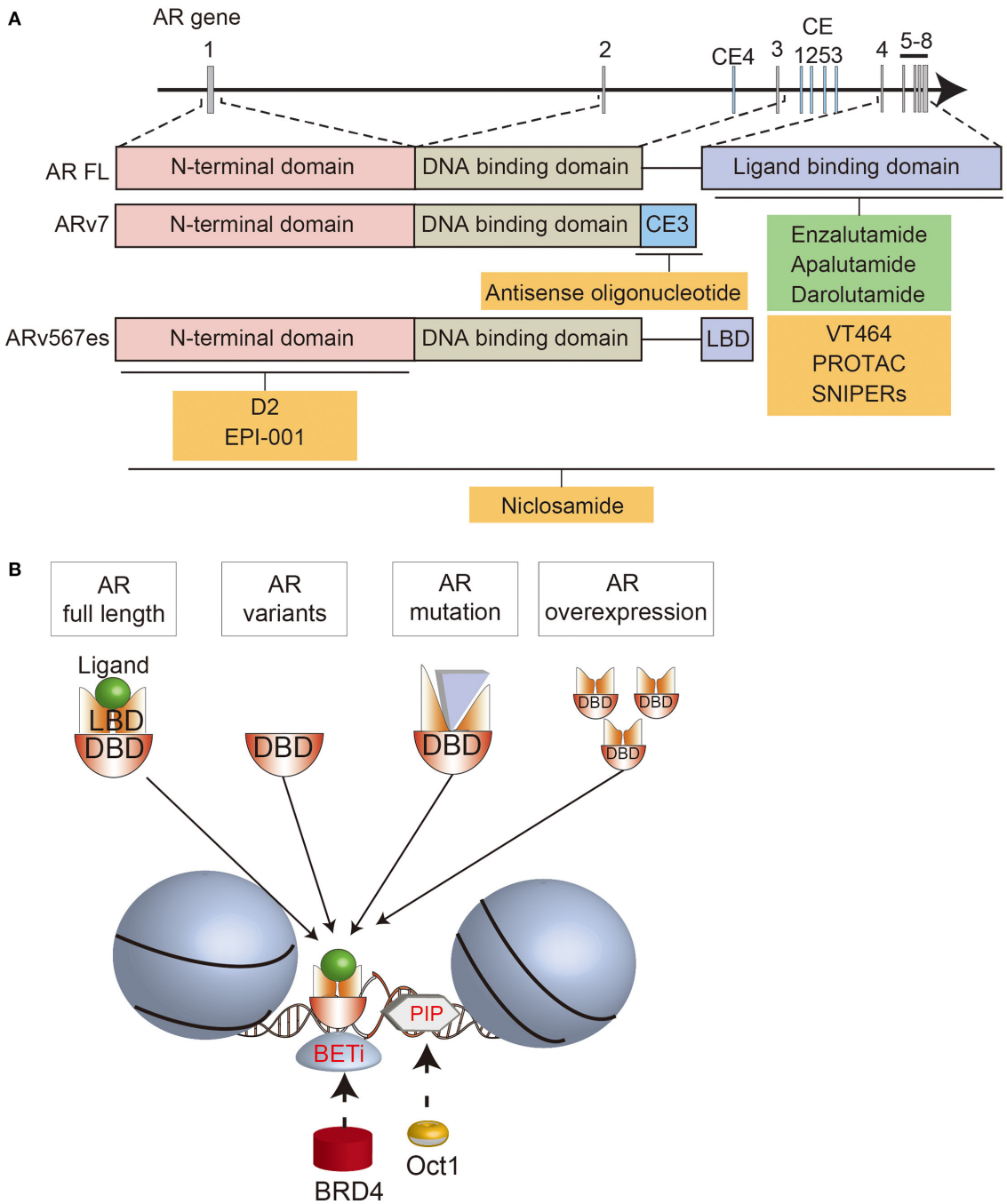


FIGURE 1 | Schematic summary of the AR structure, AR-directed treatments, and interactions with BRD4 and OCT1. **(A)** Overview of the AR locus, the structure of full length (FL) and variant (AR-V7, ARv567es) forms of the AR, and various AR-targeted treatments that are approved (green) or in development (yellow). **(B)** Summary of the interactions between different forms of the AR, BRD4, and OCT1 on chromatin.

machinery would result in a dysregulated AR splicing process. Splicing factor, proline- and glutamine-rich (PSF/SFPQ) is responsible for wide-ranging upregulation of spliceosome gene expression in CRPC to activate a broad range of oncogenic pathways, including AR (48). Thus, these studies provide an intriguing insight into prostate cancer progression through splicing machinery.

NEW STRATEGIES FOR DIRECTLY TARGETING THE AR IN CRPC

In an effort to overcome resistance to current treatments, new therapies are being developed to target the AR. Some compounds have a similar mechanisms-of-action to existing AR-directed treatments. Like abiraterone actetate, the new compound VT464

(seviteronel) is a CYP17A1 inhibitor that suppresses androgen biosynthesis (51). Unlike abiraterone acetate, VT464, selectively inhibits the 17,20-lyase rather than 17α-hydroxylase reactions, so it is proposed that the combination with prednisone is not necessary. However, phase 1 testing of VT464 suggested that there is minor inhibition of CYP17 hydroxylase (52), so low-dose dexamethasone is being administered with VT464 in ongoing trials with prostate cancer patients (53, 54). Abiraterone acetate and VT464 both also function as competitive AR antagonists, including of AR mutants, with VT464 more potent than abiraterone in cells with the T878A AR mutation (55–58).

An alternative strategy is to deplete the AR in prostate cancer cells. This may be done by blocking gene expression with antisense oligonucleotides targeting different regions of *AR* pre-mRNA transcripts (59–62). Antisense oligonucleotides against exon 1 reduce full-length AR and ARV expression, while antisense oligonucleotides against cryptic splicing signals specifically downregulate AR-V7 expression (60, 62). The AR can also be depleted using selective AR degraders (SARD), which bind to the AR and induce proteasome-mediated degradation (63). Some SARDs bind to both the N- and C-termini of the AR, so also promote degradation of ARVs (64). Preclinical studies suggested that niclosamide, an approved treatment for parasitic worms, could be repurposed as a SARD, since one of its effects is degradation of ARVs. Although the combination of niclosamide with enzalutamide or abiraterone significantly reduced the growth of castrate-resistant cells (65–67), a phase I trial showed that inhibitory concentrations of niclosamide could not be achieved in patients (68). Therefore, this approach must rely on newer generations of SARDs being developed (64).

Another way of inducing AR degradation is with proteolysis targeting chimeras (PROTAC) or SNIPERs (specific and non-genetic inhibitor of apoptosis protein [IAP]-dependent protein erasers). These heterobifunctional small molecules contain a ligand that binds to the target protein, such as an AR antagonist, linked to another ligand that engages the ubiquitin ligase complex (69–71). Since current AR-targeted PROTACs bind to the LBD, they induce degradation of full-length AR, but not ARVs. Nevertheless, they still inhibit the growth of enzalutamide-resistant cells, emphasizing the ongoing importance of full-length AR in many cases of CRPC (71).

A different strategy for directly targeting the AR is to disrupt its interactions with other molecules. D2 is a peptidomimetic that disrupts the interaction between the AR and a co-regulator, PELP1 (proline, glutamate and leucine rich protein 1), by mimicking the LXXLL motif in the AF2 domain of the AR C-terminus (72). By blocking this interaction, D2 inhibits nuclear translocation of the AR and reduces the growth of prostate cancer cells. EPI-001 also blocks the interactions between the AR and coactivators, and is notable because it binds the NTD (73). Thus, EPI-001 also inhibits ARVs (74). EPI-001 inhibits the growth of prostate cancer cell lines *in vitro* and *in vivo*, and had increased activity in combination with docetaxel (74, 75). However, off-target effects have been identified, highlighting the difficulty of targeting the AR NTD (76). EPI-506 was developed as a successor to EPI-001 (77), but was required at high doses in a phase I trial due to low potency and a short half-life. Therefore, the development of N-terminal AR inhibitors is ongoing (78).

Compounds are also being developed to block the interaction of the AR with DNA. This could target both full-length and variant forms of the AR, which contain the DBD. For example, AR binding to specific androgen response element sequences can be blocked using PI polyamides, N-methylimidazole (Im) and N-methylpyrrole (Py) amino acids that bind to the minor groove of DNA with high affinity and sequence specificity (79–81). PI polyamides that bind particular AREs can suppress androgen-responsive gene expression (82), and inhibit binding of RNA polymerase II to the transcription start site of AR-driving genes (83).

INDIRECTLY TARGETING AR SIGNALING VIA COFACTORS AND COLLABORATING TRANSCRIPTION FACTORS

Cofactors, including coregulators and TFs, are also necessary for AR-regulated gene expression. Whilst coregulators directly bind to activation function (AF) 1 or 2 domains of the AR, TFs bind to DNA elements near AR binding sites (84). Some TFs are also pioneer factors that facilitate AR recruitment to target regions through chromatin remodeling (85). Dysregulation of TFs can dramatically change the pattern of AR responsive gene expression. Indeed, there are differences in AR binding regions and coordinating TFs between treatment-naïve and castration-resistant prostate cancer (86). AR binding sites that are unique to CRPC were not AR-regulated in treatment-naïve prostate cancer cells or enriched in binding of common AR collaborative TFs, such as MYC (86). MYC is a oncogenic transcription factor that plays a critical role in prostate cancer progression by influencing diverse molecular mechanisms (87).

The importance of cofactors and collaborative TFs makes them potential therapeutic targets for indirectly targeting the AR. There are numerous strategies for targeting different AR interacting proteins, so here we focus on two notable examples, bromodomain and extra terminal domain (BET) proteins and OCT1 (POU2F1; POU class 2 homeobox 1) that collaborate with MYC.

BET PROTEINS

The BET family of epigenetic readers, including BRD2/3/4 (bromodomain containing 2, 3, and 4) and BRDT (bromodomain testis associated), bind to acetylated histones and regulate the expression of downstream genes such as MYC (88).

BET proteins are therapeutic targets in different tumor types, but are of particular interest in prostate cancer because they affect the expression and activity of the AR pathway (89). BET proteins directly interact with the NTD of the AR (90). Moreover, BRD4 has numerous shared DNA binding loci with full-length AR and AR-V7 (90, 91). With FOXA1, BRD4 and AR-V7 bind to canonical AR target genes, but with ZFX they bind to non-canonical genes related to cell cycle, autophagy, and WNT signaling (91). Accordingly, BET

inhibitors downregulate the expression of AR target genes, as well as MYC (90, 91). BET inhibitors also decrease AR-V7 levels by regulating alternative splicing (92, 93). This culminates in reduced growth of prostate cancer cell lines, organoids and xenografts treated with BET inhibitors, including enzalutamide-resistant models (90, 92, 94, 95).

The promising preclinical data for BET inhibitors suggests that they are potential new treatments for CRPC, functioning in part by indirectly targeting the AR. Numerous BET inhibitors are clinical development and some are in phase I/II clinical trials enrolling men with CRPC, such as ABBV-075 (mivebresib) and MK-8628/OTX015 (birabresib) (89). So far, prostate cancer patients in these trials have still had progressive or stable disease, although a partial response has been reported (89, 96, 97). Ongoing trials are also testing combination treatments of BET inhibitors with AR-directed treatments, PARP inhibitors, chemotherapy and immunotherapy (89, 98). For example, a phase 1b/2a trial of the BET inhibitor ZEN-3694 in combination with enzalutamide demonstrated that the treatment had acceptable tolerability in men with metastatic CRPC who had previously failed abiraterone or enzalutamide (99). Encouragingly, a subset of these patients had prolonged progression-free survival with the combination therapy, including those with tumors with low AR activity.

A challenge in the clinical development of BET inhibitors is overcoming toxicity and off-target effects, so new forms of BET inhibitors are being developed. Using the PROTAC approach, BET degraders target BET proteins for ubiquitination and proteasomal destruction (71, 100). In addition, compounds have been developed to selectively target one of the two bromodomains (BD1 and BD2) within BET proteins (101, 102). BET degraders and selective bromodomain inhibitors both inhibit the growth of prostate cancer cells *in vitro* and *in vivo* (71, 100, 101). Therefore, ongoing trials, combination treatments and new compounds, may provide opportunities to treat CRPC by targeting BET proteins.

OCT1

Another canonical AR collaborative TF is OCT1. Of the eight OCT proteins, OCT1 is most widely expressed, and is related to the pluripotency master regulator OCT4 (103, 104). OCT1 acts downstream of pioneer factors that make histone modifications to support AR binding to target regions. GATA2 (GATA binding protein 2) and OCT1 work in a hierarchical network where GATA2 is recruited with AR, followed by OCT1 binding to its motifs. Increased immunoreactivity of OCT1 is correlated with worse prognosis of localized prostate cancer (105). OCT1 is also highly expressed in other cancers, including gastric and colorectal cancer (106, 107). Interestingly, in MYC-driven lung adenocarcinoma, OCT1 binding sites were enriched in a set of genes regulated by MYC (108), suggesting that OCT1 and MYC may also co-regulate a subset of androgen responsive genes in prostate cancer. Furthermore, OCT1 interacts with PARP-1 and BRCA1 (109, 110). OCT1 enhances breast cancer aggressiveness, and BRCA1 catalyzes OCT1 degradation to inhibit tumorigenicity (110). PARP inhibitors are often effective for cancers with *BRCA1* mutations, however some tumors are resistant (111). These findings suggest that OCT1 may have a significant effect when used in combination with PARP inhibitors.

Of the genes that are jointly regulated by OCT1 and the AR in prostate cancer, acyl-CoA synthetase long-chain family member 3 (ACSL3) is the mostly highly differentially expressed (112). ACSL3 in turn increases AKR1C3 (aldo-keto reductase family 1 member C3) expression, enhancing the backdoor pathway of androgen synthesis that confers resistance to abiraterone (113, 114). Beyond ACSL3, the genome-wide network of OCT1 target genes in CRPC is enriched in factors such as anillin actin binding protein (ANLN) and DLG associated protein 5 (DLGAP5) that regulate proliferation and migration (115, 116).

Although there are few drugs targeting OCT1, PI polyamides have been developed that block the interaction between OCT1 and specific DNA binding sites. A PI polyamide targeting the OCT1 binding sites of ACSL3 suppresses ACSL3 expression and inhibits the growth of CRPC by repressing global OCT1 chromatin association and AR signaling (112). These preclinical data support further development of compounds targeting OCT1 in CRPC.

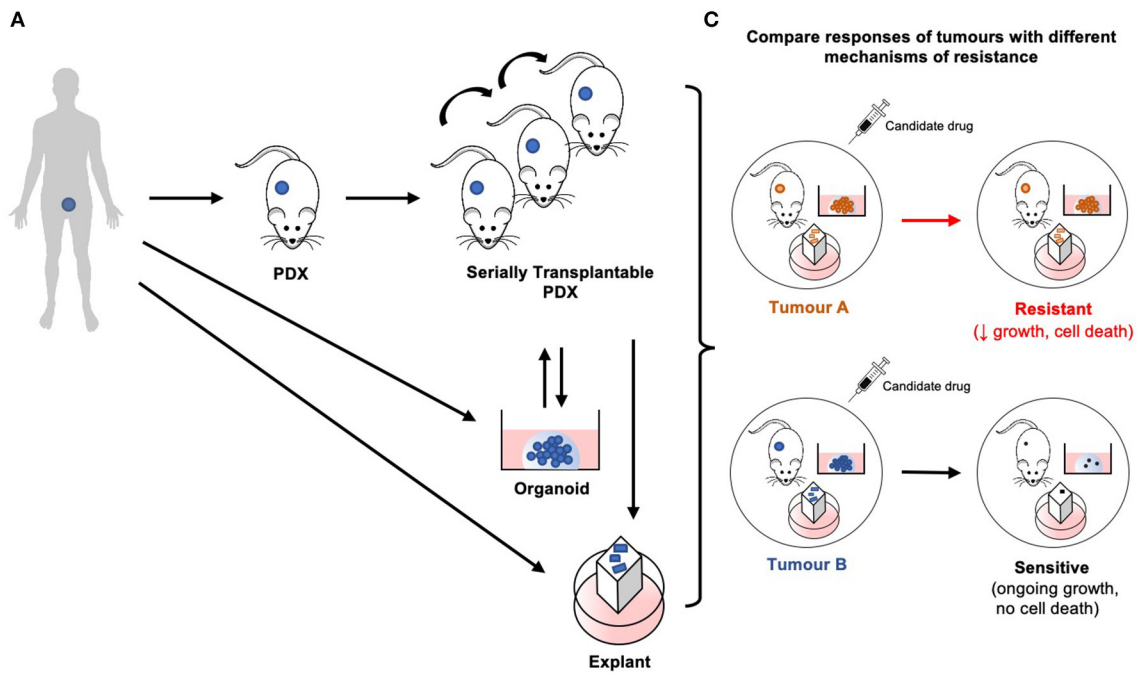
PATIENT-DERIVED MODELS FOR TESTING NEW TREATMENTS FOR CRPC

As novel compounds are developed to directly and indirectly target the AR, their efficacy must be tested with appropriate preclinical models. Unfortunately, the development of preclinical models of CRPC lags behind the evolving understanding of CRPC and changes in clinical practice. Most studies use a small collection of cell lines, including LNCaP, PC3, DU145, VCaP, 22RV1, and LAPC4 cells (117). These cells are very well-characterized and have been used for important discoveries. They have different mechanisms of castration-resistance, such AR amplification and expression of AR-V7 in VCaP cells, and an intragenic duplication of the *AR* gene and expression of several AR isoforms in 22Rv1 cells (40, 47, 118, 119). An important use of prostate cancer cell lines is high-throughput screening, including in the NCI-60 panel (120, 121). With this approach, cell lines can be used to identify drug targets with genome-wide genetic screens, such as with siRNA or CRISPR-Cas9, and treated with large compound libraries to identify candidate drugs for further evaluation (50, 122). Nevertheless, this small number of cell lines does not encompass the heterogeneity of CRPC. To address this challenge, there are ongoing efforts to develop new *in vivo*, *ex vivo*, and *in vitro* models from patient specimens.

The ability to establish patient-derived xenografts (PDXs) from patient tumors has advanced with the use of more highly immune-deficient strains of mice. Yet, PDXs are often more difficult to establish from prostate cancer compared to other malignancies, due to low take rates (10–40%) and long latency periods (up to 12 months) (123). Nevertheless, several groups have established collections of serially transplantable prostate

cancer PDXs (30, 124–128). At least 51 PDXs of CRPC have been established, primarily from patients who failed ADT, but fewer from men treated with second generation AR-directed therapies (125). To simulate androgen deprivation, PDXs of CRPC are often grafted in castrated mice, with circulating androgen levels equivalent to patients treated with abiraterone (129). By increasing the number of models of CRPC, PDXs provide new opportunities to study the mechanisms of castration resistance, including mutations and ARVs. PDXs are also useful for testing whether candidate therapeutics are effective at reducing the growth of tumors with diverse alterations in the AR pathway. The typical endpoint to determine whether drug treatment reduces the growth rate of PDXs is decreased tumor volume, or ideally regression.

Like all experimental models, PDXs have limitations, so they can be integrated with other patient-derived models to maximize the advantages and offset the limitations of each approach (**Figures 2A–C**). PDXs provide a rigorous way to evaluate *in vivo* drug responses, but the experiments are expensive, labor-intensive, low throughput and have long timeframes. Explants and organoids are complementary models that address these limitations. Explants are intact pieces of tissue maintained for several days *ex vivo* on filters or gelatin sponges, so they retain the native tissue architecture and microenvironment (130, 131).



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	PDX	Organoid	Explant
Rapid expansion	+	+++	-
Generation time	+++	+	-
Genetic manipulation	+	++	-
Maintenance of tumour architecture	+++	+	+++
Screening throughput	+	+++	++
Timescale of assay	+/++/+++	+	+

FIGURE 2 | Establishment and application of patient-derived models for preclinical testing of new treatments for CRPC. **(A)** PDXs are established from human patient tumor tissue, and are considered serially transplantable when repassaged into additional host mice and expanded. Explants and organoids can be established directly from fresh patient specimens or from PDXs. PDXs may also be established from organoids. **(B)** Each model is unique with its own advantages and limitations. **(C)** Therefore, by integrating these models established from tumors with different resistance mechanisms, preclinical therapeutic evaluation can be performed with greater rigor and efficiency.

Organoids are digested prostate tissue grown in extracellular matrix solutions, such as Matrigel. Explants and organoids can be established from fresh patient specimens or PDXs, which are renewable sources of tissue (30, 132, 133). These patient-derived models are higher throughput and can be used to rapidly test whether compounds affect proliferation and apoptosis. These *ex vivo* and *in vitro* cultures are also useful for testing tool compounds that have poor bioavailability or are not available in sufficient quantities for *in vivo* experiments. Explants and organoids can also be used for experiments that are challenging with PDXs, including large-scale dose responses of single or combination treatments, genetic manipulation, and short-term time points for mechanistic studies (132, 134, 135). This bridges the gap between high-throughput experiments with cell lines, and *in vivo* treatments with PDXs. Therefore, by combining different patient-derived models established from different cases of CRPC, it will be possible to test the next generation of therapies with greater rigor and efficiency to help prioritize them for further clinical trials.

CONCLUSION

Over the last decade, the introduction of new treatments for CRPC has extended patient survival, but tumors still eventually fail treatment. The increasingly detailed understanding of the underlying mechanisms of resistance has facilitated the development of novel compounds that use alternative approaches to target the AR pathway, directly or indirectly. Two examples of drug targets are BET proteins, with BET inhibitors in ongoing clinical trials for prostate cancer, and OCT1, with novel compounds in preclinical development. Whether these compounds are effective as monotherapies, or should be used in combination with other treatments is still under investigation. Nevertheless, growing collections of patient-derived models, spanning xenografts, organoids and explants, are providing ways to test the efficacy of these candidate drugs across a wider spectrum of tumors. Collectively, this ongoing effort will provide a rich pipeline of new treatments for further validation in clinical trials.

AUTHOR CONTRIBUTIONS

DO, ST, and SI: conceived the concepts. DO, ML, and NC: wrote the first draft of the manuscript. KT and GR: revise the first draft. All authors reviewed and approved of the final manuscript.

FUNDING

The authors are supported by funding from the National Health and Medical Research Council, Australia (fellowship to GR 1102752, project grants 1138242, 1156570), the Department of Health and Human Services acting through the Victorian Cancer Agency (fellowship to ML MCRF18017), the EJ Whitten Foundation, the Peter and Lyndy White Foundation, P-CREATE (Grant No. JP18ck0106194 from AMED, Japan), Takeda Science Foundation, and JSPS KAKENHI (Grant Nos. JP19H03793 and JP19K09740).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.581515/full#supplementary-material

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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.

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frontiers in Oncology

REVIEW
published: 09 October 2020
doi: 10.3389/fonc.2020.580617

Androgen Receptor Signaling and Metabolic and Cellular Plasticity During Progression to Castration Resistant Prostate Cancer

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Metabolic reprogramming is associated with re/activation and antagonism of androgen receptor (AR) signaling that drives prostate cancer (PCa) progression to castration resistance, respectively. In particular, AR signaling influences the fates of citrate that uniquely characterizes normal and malignant prostatic metabolism (i.e., mitochondrial export and extracellular secretion in normal prostate, mitochondrial retention and oxidation to support oxidative phenotype of primary PCa, and extra-mitochondrial interconversion into acetyl-CoA for fatty acid synthesis and epigenetics in the advanced PCa). The emergence of castration-resistant PCa (CRPC) involves reactivation of AR signaling, which is then further targeted by androgen synthesis inhibitors (abiraterone) and AR-ligand inhibitors (enzalutamide, apalutamide, and daroglutamide). However, based on AR dependency, two distinct metabolic and cellular adaptations contribute to development of resistance to these agents and progression to aggressive and lethal disease, with the tumor ultimately becoming highly glycolytic and with imaging by a tracer of tumor energetics, ^{18}F-fluorodoxyglucose (^{18}F-FDG). Another major resistance mechanism involves a lineage alteration into AR-indifferent carcinoma such a neuroendocrine which is diagnostically characterized by robust ^{18}F-FDG uptake and loss of AR signaling. PCa is also characterized by metabolic alterations such as fatty acid and polyamine metabolism depending on AR signaling. In some cases, AR targeting induces rather than suppresses these alterations in cellular metabolism and energetics, which can be explored as therapeutic targets in lethal CRPC.

Keywords: androgen receptor, castration-resistant prostate cancer, metabolic reprogramming ^{18}F-FDG, neuroendocrine, aerobic glycolysis, fatty acid metabolism, mitochondria

OPEN ACCESS

Edited by:
Isabel R. Schlaepfer, University of Colorado, United States

Reviewed by:
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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *06 July 2020*
Accepted: *25 September 2020*
Published: *09 October 2020*

Citation:
Uo T, Sprenger CC and Plymate SR (2020) Androgen Receptor Signaling and Metabolic and Cellular Plasticity During Progression to Castration Resistant Prostate Cancer. Front. Oncol. 10:580617. doi: 10.3389/fonc.2020.580617

INTRODUCTION

Normal cells gain distinctive capabilities to overcome the restrictions in the tissue of origin to initiate primary tumor formation (1–3). The phenotypic traits in the original environment often determine the molecular processes that drive the progression to advanced and metastatic tumors (4, 5). This is true for reprogramming of cellular and energy metabolism during cancer progression (6–8).

"The Warburg effect (aerobic glycolysis)" observed by Otto Warburg nearly a century ago is the phenomenon that cancer cells preferentially convert most glucose to lactate even in the presence of oxygen by which mitochondrial oxidative phosphorylation can proceed to generate ATP more efficiently (9, 10). His original hypothesis also emphasized that dysfunction of mitochondria is the initiating factor for cancer formation (11, 12). While not maximizing ATP production/glucose, aerobic glycolysis permits cancer cells to efficiently convert glucose into the biomass (e.g., nucleotides, amino acids, and lipids) for cell growth and proliferation (13, 14). As opposed to Warburg's notion, most-if not all-cancer cells rely on functional mitochondria for their survival (13, 15).

Prostate cancer (PCa) is unique from a metabolic perspective. Ironically, the normal prostatic epithelial cell is one of the best cell types that fit to the original Warburg's theory: mitochondria must be dysfunctional to get higher rate of glycolysis. Instead, primary PCa does not exhibit the Warburg effect. Contrary to other cancer cells, malignant transformation involves the conversion from energy-inefficient ("glycolytic") secretory epithelial cells to energy-efficient ("oxidative") PCa cells (16–20) (**Figure 1**).

Androgen receptor (AR) plays pivotal roles in both normal and malignant prostate cells. Indeed, AR transcriptional program supports PCa viability during the course from primary tumor formation to progression to metastasis. AR has the capabilities of regulating virtually all aspects of cellular metabolism (glucose, lipid, amino acid, nucleotides, etc.) (20–24). Conversely, pre-receptor control of "androgen" metabolism, which is dictated by tissue localization and abundance of steroidogenic enzymes and metabolism, ultimately determines activity of the holoreceptor for the transcriptional output (25, 26). Nevertheless, PCa exhibits specified metabolic and energetic phenotypes depending on the stage of disease progression (18, 19, 23). For example, while AR signaling persists, the transition from oxidative to glycolytic metabolism occurs during the progression to advanced PCa (27–29). Lipogenesis is continuously maintained by AR during the development of PCa (30–32). AR antagonism is highly effective in counteracting AR signaling thus altering associated metabolic programs, but tumors evolve by acquiring androgen-independent AR activation in adenocarcinoma or bypassing AR requirement through transdifferentiation to more aggressive and lethal AR-indifferent carcinomas (33). This cellular transformation results in drastic metabolic adaptation to promote aerobic glycolysis (29, 34, 35).

Understanding of the relationship between these distinctive metabolic features and AR signaling in PCa will lead to identification of metabolic vulnerabilities that offer the opportunity for diagnosis and therapy. In this review, we will characterize metabolic phenotypes of PCa in relation to AR signaling and review the current knowledge of metabolism-based imaging tools and therapeutic interventions to target cancer metabolism.

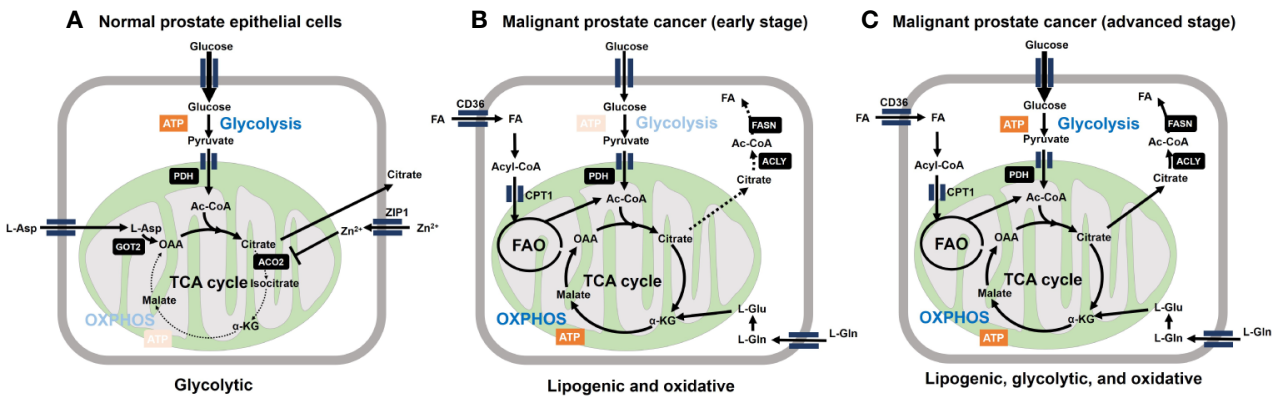

FIGURE 1 | Metabolic reprogramming is involved in malignant transformation of prostatic cells. **(A)** Normal prostate epithelial cells express zinc transporter ZIP1 facilitating intracellular accumulation of zinc ion, which contributes to inhibition of m-aconitase (ACO2) at mitochondria. This inhibition results in truncation of tricarboxylic acid cycle (TCA) cycle and release of citrate to the extracellular space. Citrate production is supported by increasing the substrate pools for citrate synthase, acetyl-CoA (Ac-CoA) and oxaloacetic acid (OAA) at the mitochondria. OAA is supplied as the result of action of mitochondrial aspartate aminotransferase (GOT2) on L-aspartate. The level of mitochondrial acetyl-CoA is associated with increased expression of pyruvate dehydrogenase E1 component subunit alpha (PDHE1α) of pyruvate dehydrogenase complex (PDH). From bioenergetic point of view, normal prostatic cell is supported by aerobic glycolysis. **(B)** Marked decrease in zinc levels due to depletion of ZIP1 represents an essential early event in the development of PCa malignancy, which relieves m-aconitase to establish a complete TCA cycle. These metabolic alterations are functionally related to low citrate level and the general low avidity of ^{18}F-FDG in primary PCa. Fatty acids (FA) are incorporated through CD36, followed by CPT1-mediated entry into mitochondria to serve as the substrate for fatty acid oxidation (FAO). L-Glutamine also serves as the precursor of TCA cycle intermediates after conversion into L-glutamate. ATP-citrate lyase (ACLY) cleaves citrate to produce acetyl-CoA to serve as the substrate for fatty acid synthase (FASN). **(C)** Further malignant transformation promotes glycolysis (through increased expression of glycolytic enzymes). While lipogenic trait is enhanced, multiple combinations of/all energy source pathways are theoretically available at this stage. Therefore, it is important to determine which metabolic pathway dominates for survival of given tumors for the future metabolism-based precision therapy.

ANDROGEN ACTION IN PROSTATE FUNCTION AND METABOLISM: ZINC, TRUNCATED TRICARBOXYLIC ACID CYCLE CYCLE, CITRATE METABOLISM

Androgens are hormones required for development and maintenance of the male reproductive system. The functions of prostatic cells in both normal and malignant condition have been characterized by the relationship to the status and availability of androgen and its cognate receptor AR. Upon binding to androgen, AR which is otherwise sequestered in the cytoplasm translocates to nucleus and acts as sequence-specific dimerized transcription factors (36).

The unique metabolic processes in the prostate are well adapted to fulfill the major function as a secretory tissue to generate prostatic fluid comprised of high concentration of citrate along with zinc, lipids, and kallikrein enzymes including prostate-specific antigen (PSA) (37, 38) (**Figure 1**). Typically, citrate is either retained and oxidized in the mitochondria to generate energy as an essential intermediate in the citric acid cycle, or is exported into the cytoplasm where it is cleaved by ATP citrate lyase (ACLY) to generate acetyl-CoA, which is used for fatty acid (FA) synthesis (39).

The normal human prostate retains the capability of accumulating the highest levels of zinc in any soft tissue of the human body through expression of specific zinc transporters (ZIP1–4 for uptake and ZnT1–10 for release) (40). High levels of mitochondrial zinc inhibit mitochondrial aconitase, resulting in truncation of tricarboxylic acid cycle (TCA) cycle at the first step of citrate oxidation (17, 41). Androgen signaling enhances citrate production by increasing the substrate pools for citrate synthase, acetyl-CoA and oxaloacetic acid (OAA) at the mitochondria. The level of mitochondrial acetyl-CoA is associated with increased expression of pyruvate dehydrogenase E1 component subunit alpha (PDHE1α) (42). Aspartate uptake is through the excitatory amino acid transporter SLC1A1/EAAC1 (43). Followed by transamination processes to generate OAA at the mitochondria (44). Mammalian cells typically produce ~38 ATP/glucose through the combined actions of glycolysis and TCA cycle oxidation on glucose. On the other hand, the normal prostatic epithelia can generate only ~14 ATP/glucose due to truncation in TCA cycle resulting in the loss of ~24 ATP/glucose (19).

Marked decrease in zinc levels due to depletion of ZIP1 represents an essential early event in the development of PCa malignancy (45), which relieves mitochondrial aconitase to establish a complete TCA cycle (18, 19). These metabolic alterations are functionally related to low citrate level and the general low avidity of ^{18}F-FDG in primary PCa (46, 47).

AR DRIVES PCA BY REGULATING CENTRAL METABOLISM

Multi-omics studies (transcriptome, proteomics, cistrome, and metabolome) define the AR as a master regulator that orchestrates cellular metabolism to fuel proliferation and growth of PCa cells (20–22, 48, 49). Specifically, AR transcriptionally regulates multiple pathways of energy and biomass supply, including glycolysis, mitochondrial respiration, metabolism of FA (synthesis, ß-oxidation, and uptake), nucleotides, amino acids, and polyamines. Thus, drastic metabolic alterations are expected to inevitably follow AR inhibition and re/activation during the progression to lethal CRPC along with AR antagonism therapy.

Glucose Metabolism

AR determines bioenergetic traits through regulation of components in glycolytic pathway (GLUT1, HK1, HK2, and PFK2/PFKFB) and pyruvate flux into mitochondria (PDH, MPC2) (21, 42, 50). AR signaling increases expression of glucose-6-phosphate dehydrogenase (G6PD) which directs glucose-6-phosphate from glycolysis to the pentose phosphate pathway (PPP) for generation of NADPH and nucleotide precursors (51). The conversion from pyruvate to lactate is catalyzed by LDH proteins including AR-target LDHA (52, 53). Hyperpolarized ^{13}C magnetic resonance spectroscopic imaging (MRSI) demonstrates that *in vivo* conversion [1-^{13}C] lactate into [1-^{13}C] pyruvate occurred more efficiently in PDX models of AR-driven CRPC than those of AR-negative PCa (54). Monocarboxylate transporter MCT4 is upregulated in CRPC and contributes to completion of successful aerobic glycolysis through secretion of lactate. Indeed, MCT4-targeting antisense oligonucleotides (ASO) provide significant tumor suppressive activity in cellular and xenograft models of CRPC (55). Overall, AR is capable of promoting both glycolysis and pyruvate oxidation, indicating AR's predominant roles in both glycolytic and oxidative PCa tumors.

FA Metabolism

AR regulates FA metabolism by controlling expression of more than 20 enzymes involved in many aspects of lipid metabolism, including uptake, trafficking, synthesis, and degradation (32, 49).

AR and the master regulator of lipid homeostasis sterol regulatory-element binding protein (SREBP) regulate each other in a positive feedback system (32, 49, 56, 57). SREBP's transcriptional targets include ELOV6 and SCD1 (58) while fatty acid synthase (FASN) and ACC (acetyl-CoA carboxylase) are co-targeted by both SREBP and AR (49, 59). Thus, AR activation accelerates FA synthesis, particularly as the form of mono-unsaturated and saturated FA (31, 32). Conversely, AR inhibition leads to marked reduction of *de novo* lipogenesis and permits incorporation of dietary FA enriched in polyunsaturated FA which are prone to lipid peroxidation when subjected to oxidizers such as arsenic trioxide (60, 61).

In addition to citrate oxidation, fatty acid oxidation (FAO) is the dominant energy producing pathway through decomposition of *de novo* or exogenous FA (62–65). Both FA synthesis and FAO have been recently shown to play key roles in cancer cell growth and proliferation (49, 63). This is an apparently contradictory situation where catabolism and anabolism of the same group of metabolites co-exist in the same cells. Also, FA synthesis and FAO have traditionally been considered incompatible due to the inhibitory effects of malonyl-CoA (the product of ACC1 which serves as the substrate for FASN) on carnitine palmitoyltransferase 1 (CPT1) in

the carnitine shuttle (the rate limiting step for the transport of FA into the mitochondria) (66). Nevertheless, pharmacological or genetic inhibition of FASN resulted in decreased FAO as well as oxygen consumption, suggesting the existence of simultaneous FA synthesis and oxidation in the cells (67). Moreover, combined inhibition of FASN and FAO produced additive therapeutic effects in PCa, demonstrating that two pathways coexisting and feeding each other in some situations (65, 68). More excitingly, CPT1A-mediated FAO is reportedly to linked to epigenetics by supplying acetyl-CoA for histone acetylation (69).

On the other hand, there is growing body of evidence that PCa utilizes exogenous FA derived from diet or adipocytes (70–72). Blockade of this incorporation by CD36 inhibition is antitumorigenic (71). These reports emphasize that pharmacological intervention in FA metabolism has therapeutic benefit.

Amino Acids

AR regulates amino acid catabolism through expression of amino acid transporters (LAT1, LAT3, ASCT1, ASCT2) (43, 73–76). LATs and ASCTs are for bulky and small neutral amino acids, respectively. In particular, ASCT2 prefers the conditionally essential amino acid glutamine as the substrate. Glutamine undergoes glutaminolysis to generate TCA cycle intermediates *via* glutamate production as an alternative energy source, providing pharmacological glutamine starvation as a therapeutic strategy (74, 77–80).

One Carbon Metabolism Network

AR regulates one-carbon metabolism network consisting of the two folate cycle pathways (DHFR, GNMT, SARDH), and methionine cycle (MAT, AHCY) which interact with trans-sulfuration pathway (CBS, CTH) and polyamine synthesis (ODC1, AMD1) (81–83). The methionine cycle contributes to the formation of *S*-adenosyl-methionine (SAM), the universal methyl donor for protein and DNA methyltransferase reactions(84). Thus, this metabolism may contribute to AR-driven malignant progression by promoting DNA synthesis and changing DNA and histone methylation status (81). As discussed below, availability of SAM determines neuroendocrine PCa (NEPC) status which is AR independent (85).

Addiction to Altered Metabolism

Dependence of AR on reprogrammed metabolic characteristics occurs in FA and ornithine metabolism. AR signaling is blunted when genetic or pharmacological inhibition of the rate-limiting enzymes in the pathways, such as ODC1, FASN, and CPT1 (67, 68, 82).

METABOLIC PLASTICITY IN RELATION TO ANTI-AR THERAPY AND THE RESISTANCE MECHANISMS

Since 1950s, inhibition of AR activity has remained a mainstay in the treatment of advanced PCa (86–89). Although most patients with PCa initially respond to AR inhibition, they eventually develop castration-resistant PCa (CRPC) (36, 90, 91). The emergence of CRPC usually involves reactivation of AR signaling(92–97), which is then further targeted by as androgen synthesis inhibitors (abiraterone) and AR-ligand inhibitors (enzalutamide, apalutamide, and daroglutamide) (98, 99). Nevertheless, resistance to these agents and progression to lethal disease are essentially universal by developing adaptive resistance to these target therapies through two distinct groups of mechanisms based on AR dependency (100) (**Figures 2**, **3**). Continued AR activation occurs by multiple mechanisms including increased AR expression in close association with enhanced intracrine or paracrine androgen synthesis (**Figure 2**, Group 1), AR gene mutations enabling promiscuous ligand interaction, and expression of constitutively active AR variants (AR-Vs)(Group 2) (100–103). AR antagonism can also promote lineage crisis and cellular plasticity to bypass AR blockade and generate neuroendocrine PCa (NEPC)(Group 1, 2→Group 3, Group 4→Group 3) (33, 104–106). Transformation into treatment-induced NEPC (t-NEPC) requires lineage plasticity in adeno-PCa to bypass AR blockade along with three major events: (i) The loss of AR expression. (ii) Alternative splicing of REST transcript by SRRM4 leading to the loss of REST activity that represses neuroendocrine gene expression. (iii) Activation of NE transcription factors e.g. ASCL1 and BRN2 that determine the commitment to a NE lineage (105–108). Among many other factors, EZH2 stands out to regulate NEPC-specific gene expression through epigenetic machinery (105). Importantly, N-MYC forms a transcription repressor complex with EZH2 to repress AR transcription program (109). It is noteworthy that AKT1-mediated phosphorylation drives a non-epigenetic mode of EZH2 action as AR coactivator to support androgen-independent AR activation during CRPC development and progression. Another emerging cell type is double negative PCa (DNPC)(Group 1, 2→Group 4), which is negative for both AR and neuroendocrine markers and may represent an intermediate phenotype between AR expressing adenocarcinoma and the neuroendocrine phenotype (110).

Alteration in Pre-Receptor Control of Dihydroxytestosterone Metabolism

5α-Reduction of testosterone (T) in prostate results in the formation of the more potent ligand dihydroxytestosterone (DHT) to activate AR. Thus, ADT is the frontline treatment and directed toward disruption of T-DHT-AR axis by suppression of gonadal T by medical or surgical castration (26). Resistance to gonadal T depletion namely CRPC is associated with AR activity which is achieved by a gain-of-function in AR itself and/or sufficient intratumoral amounts of T and DHT to activate AR (25, 26, 101). Metastatic prostate tumor cells synthesize their own androgens through *de novo* steroidogenesis, which involves upregulation of enzymes required for stepwise synthesis from cholesterol to T and DHT (93, 111). Another strategy requires adrenal synthesis and supply of dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) which are converted to Δ^4-androstenedione (AD) by 3β-hydroxysteroid dehydrogenase/isomerase (HSD3B1) in PCa (25, 26). AD is converted to DHT through canonical (AD→T→DHT) or alternative ("backdoor")

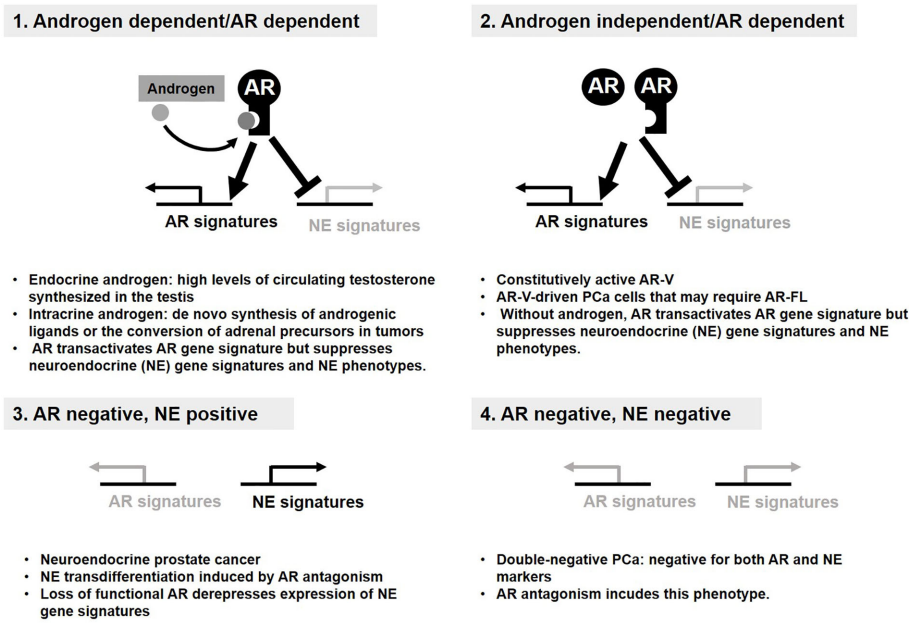

FIGURE 2 | Androgen receptor (AR) status defines four distinct groups of prostate cancer (PCa). Four distinct groups of PCa display the resistance mechanism to anti-AR therapy. AR signaling supports survival and growth of PCa and suppresses transdifferentiation into neuroendocrine. (1, 2). Loss of AR signaling derepresses expression of NE gene signatures required for NE phenotypes (3). Double-negative PCa bypasses AR requirement without NE phenotype (4).

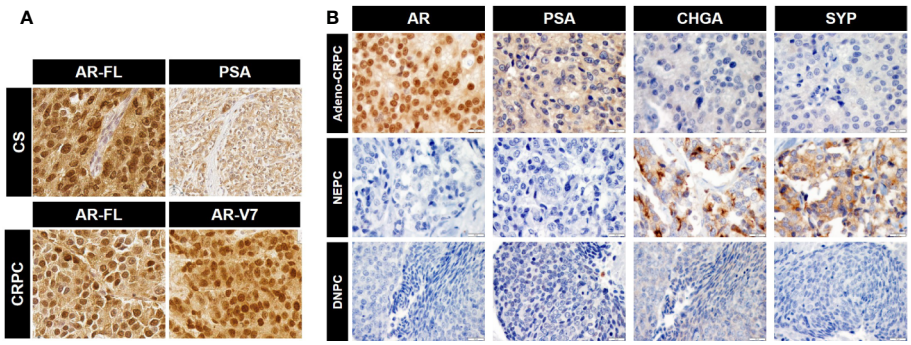

FIGURE 3 | Immunohistochemical images for the expression of androgen receptor (AR), prostate-specific antigen (PSA), and neuroendocrine PCa (NEPC) markers. **(A)** Expression of full-length AR (AR-FL) and PSA in castration-sensitive (CS) PCa and AR-FL and AR-V7 in CRPC. Note uniform nuclear staining of AR-V7. CS and CRPC correspond to group (1) and (2) in **Figure 2**, respectively. **(B)** Expression of specific markers for each CRPC type. CRPC adenocarcinoma are positive for AR and its transcriptional target PSA but negative for NEPC markers chromogranin A (CHGA) and synaptophysin (SYP). NEPC is positive for NEPC markers and negative for AR and PSA. DNPC is negative for AR, PSA, and NEPC markers (image courtesy of Dr. Colm Morrissey at University of Washington). Representative images for the data in **Figure 2** (2: Adeno-CRPC, 3: NEPC, 4: DNPC). Scale bar=20 μm.

pathway involving the intermediate androstenedione to form DHT. Importantly, a gain-of-function mutation in HSD3B1 (N367T) leads to stabilization of the enzyme which confers two distinct survival benefits to PCa (112, 113). This variant supports CRPC status to bypass depletion of gonadal testosterone by facilitating the synthesis of AD thus flux to DHT from adrenal DHEA and DHEA-S (112). The resistance to anti-AR therapy is acquired by this variant which more efficiently converts the androgen synthesis inhibitor abiraterone into the precursor of potent AR agonist (113).

AR Dependent Mechanisms: Full-Length AR (AR-FL) and AR-V7 Specific Signaling?

As discussed above, AR is the master regulator of cellular metabolism. The questions remain as to whether AR-Vs are simply a constitutively active substitute for liganded-AR-FL to

control cellular metabolism. Do AR-V7 and AR-FL differentially contribute to a selective adaptation during metabolic rewiring that occurs in CRPC progression? To answer this question, androgen-treated LNCaP and LNCaP engineered to co-express AR-V7 were used to extract AR-FL and AR-V7 signaling, respectively (114). AR-V7 specific metabolic signatures include reduced citrate level as a result of enhanced utilization rather than a failure to synthesize citrate. AR-V7 enhanced glycolytic flux more effectively than AR-FL with enhanced conversion of glutamine to citrate *via* reductive carboxylation (114). These findings suggest that AR-V alters flux of a subset of metabolites to provide growth advantage. As of yet, no such data has been generated to address the functional contribution of endogenous AR-Vs to bioenergetic phenotypes.

AR Indifferent CRPC: Drastic Metabolic Changes Are Associated With Cellular Lineage Alterations

MYC family proteins regulate virtually all genes involved in glycolysis not only by controlling their express levels but shifting alternative splicing toward glycolytic isoform PKM2 over PKM1 (115, 116). Moreover, MYC, increases mitochondrial export of acetyl groups as the form of citrate and the resulting acetyl-CoA contributes to histone acetylation by histone acetyltransferase GCN5 (117). Indeed, there exists the interplay between the epigenetic landscape and metabolism (118). For example, pyruvate generated from glycolysis is the main substrate for acetyl-CoA, a central metabolite coordinating the activity of the histone acetyltransferase (HAT) enzymes. Increased expression of the histone lysine demethylase KDM8 is observed in the context of treatment-induced NEPC and transactivated expression of EZH2 (119). Mechanistically, the KDM8-mediated PKM2 nuclear translocation results in the transcriptional activation of glycolytic program, including GLUT1, HK2, PKM2, LDHA, etc.) and downregulation of genes for pyruvate dehydrogenase complex (PDHA1 and PDHB1) to reduce the direction of pyruvate to mitochondria. As a proof of concept, inhibition of glycolysis lead to growth inhibition (119). Phosphoglycerate dehydrogenase (PHGDH) is the first enzyme branching from glycolysis in the serine biosynthesis which involved in one-carbon metabolism to supply S-adenosyl methionine (SAM) (120). SAM in turn serves as the substrate for DNA and protein methyltransferases. Cancer metabolism is linked to epigenetics in this scenario. Upregulation of PHGDH is common in NEPC thus facilitating methylation-related epigenetic modifiers such as EZH2 (105).

POSITRON EMISSION TOMOGRAPHY–BASED METABOLIC PHENOTYPING

In vivo metabolic phenotyping involves the steps for profiling and characterizing energetic phenotypes of tumors, which has a great diagnostic value for PCa patients. In this regard, ^{18}F-FDG, ^{18}F- or ^{11}C-labeled acetate, and ^{18}F- or ^{11}C-labeled choline represent the three most studied positron emission tomography (PET) radiotracers in the PCa field (121, 122). Biochemical characteristics of tumors correlate well with uptake of each radiotracer (**Figure 4**). Acetate uptake is increased concomitantly with elevated FASN activity (123, 124). Upregulation of choline kinase (CK), which is associated with malignancy, promotes phosphorylation of choline to be incorporated in cellular membrane as the form of phosphatidylcholine (125, 126). While both acetate and choline uptake serve as a basis of powerful PET imaging, it has been well accepted that PCa displays less avidity to ^{18}F-FDG (46, 47). However, largely depending on the disease phase, 84% of mCRPC patients have at least one ^{18}F-FDG positive metastasis. Moreover, 85% of ^{18}F-FDG positive metastasis displayed positivity for another tracer ^{18}F-fluorodihydrotestosterone (^{18}F-FDHT) used as indicator of AR(-FL) expression (29). On the other hand, prostate specific membrane antigen (PSMA) is "imageable" AR-target gene product (127). Thus, ^{68}Ga-PSMA-PET imaging reflects relative changes in treatment-dependent AR activity thus providing high diagnostic values (128). The expression levels of glucose uptake–associated genes, including GLUTs and hexokinases to provide a genomic rationalization for the previously reported ^{18}F-FDG avidity of PSMA-suppressed PC tumors such as NEPC and DNPC (35, 129). Non-invasive imaging tools have not been available for oxidative phosphorylation in tumors. Oxidative tumors can be monitored by the agent 4-[^{18}F]fluorobenzyl triphenylphosphonium (^{18}FBnTP) whose uptake is driven by mitochondrial membrane potential ($\Delta\Psi_m$) (130). Thus, combined use of these diagnostic tools will be powerful to characterize bioenergetic phenotypes of PCa tumors and determine treatment options.

TUMOR METABOLISM IN CRPC IS OBSERVED ACROSS VARIOUS CANCER TYPES?

As discussed above, PCa develops adaptive resistance to AR-targeting therapy through two distinct groups of mechanism based on AR dependency. In addition to alterations in AR structure of function, AR-dependent mechanism involves aberrant pre-receptor metabolism of steroids which is arguably unique to CRPC. AR-independent paths include transdifferentiation into NEPC and DNPC. Nevertheless, advanced CRPC, NEPC, and DNPC are ultimately addicted to aerobic glycolysis which is associated with high avidity of FDG in PET scan (29, 35). Ironically, Warburg effects occur in virtually all types of cancers and may represent the final form of tumor metabolism (13, 23, 131). Consistently, systems biology approach was used to analyze the expression of metabolic genes across 20 different cancer types and their impact on clinical outcome, which demonstrates that downregulation of mitochondrial genes is associated with the worst clinical outcome across all cancer types (132). Interdependence of AR and FASN drives AR-dependent CRPC progression (67), but overexpression of FASN is the rule rather than the exception in many types of cancers (133). Cancer cells appear to undergo a tissue-specific metabolic rewiring, which converges toward a common metabolic landscape. One may ask "What metabolic programs differentiate one cancer type from the others?". A recent report from the Vander Heiden group

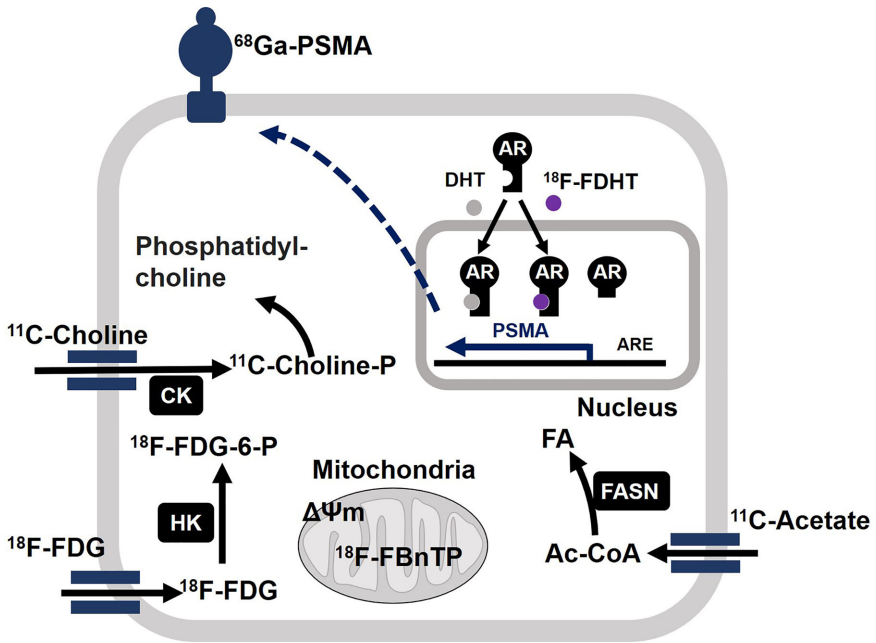

FIGURE 4 | Molecular basis of actions of positron emission tomography (PET) radiotracers in prostate cancer (PCa). Acetate is converted to acetyl-CoA (Ac-CoA) which serves as a substrate for FASN to produce fatty acids (FA). After enzymatic modification by choline kinase (CK), ^{11}C-choline is incorporated into cell membrane as the form of phosphatidylcholine. After incorporation into cell, ^{18}F-FDG undergoes phosphorylation by hexokinase (HK) and accumulates as the form of ^{18}F-FDG-6-P. Mitochondrial membrane potential ($\Delta\Psi_m$) drives accumulation of 4-[^{18}F]fluorobenzyl triphenylphosphonium (^{18}F-FBnTP) at mitochondria. ^{18}F signal is indicative of respiration-competent functional mitochondria. Binding of ligand dihydrotestosterone (DHT) activates full-length AR as a transcriptional factor to upregulate target genes such as PSMA. Accordingly, the presence of full-length AR can be monitored by ^{18}F-FDHT. It is noteworthy that constitutively active AR variant fails to bind to ^{18}F-FDHT. Accordingly, ^{18}F-FDHT negativity does not necessarily mean tumors are negative for any form of AR. ^{68}Ga-labeled antagonistic ligand for PSMA can be used to monitor tumors with active AR signaling.

specifically addressed this issue by testing whether tissue-of-origin dictates cancer dependence on specific metabolic pathways (134). Mouse models of pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung carcinoma (NSCLC) have the same genetic background with Kras mutation and TP53 deletion, While PDAC tumors have decreased branched-chain amino acids (BCAA) uptake, NSCLC tumors incorporate free BCAAs into tissue protein and use BCAAs as a nitrogen source while PDAC tumors have decreased BCAA uptake. Expression pattern of BCAA metabolizing enzymes in original tissues reflect these metabolic differences in tumors, arguing both tumor genetics and tissue context define cancer dependence on specific metabolic pathways (134). While TP53 and RB1 are commonly tumor suppressive in many cancer types (135), their combined inactivation promotes cell plasticity in PCa to undergo NEPC differentiation (136, 137). In this scenario, PCa-specific metabolic status might permit this lineage transition.

THERAPEUTIC INTERVENTIONS

Two biological events are emerging as hallmarks of cancer: reprogramming of energy metabolism and evading immune destruction (1). The latter is an active area of research as cancer immunotherapy. Metastatic PCa with CDK12 inactivating mutations (3-7% incidence) has durable responses to PD-1 blockade by checkpoint inhibitors (138, 139). As for targeted therapy in cancer metabolism, gain of function mutations in isocitrate dehydrogenases (IDH1 and IDH2) result in the production of the "oncometabolite" 2-hydroxyglutarate (140, 141). Targeting mutant IDH is attractive but limited in PCa: IDH mutations account for only 1-2% of PCa incidence, which is much lower than other tumors, e.g. glioma (~50%) (142, 143). For PCa, dysregulated FA metabolism, which is mechanistically linked to aberrant AR and/or SREBP signaling (49, 144), has multiple candidate factors for pharmacological inhibition, including SREBP (fatostatin) (145), acetyl-CoA carboxylase (ND-646, GS-0976) (146, 147), and SCD1 (Merck Frosst Cpd 3) (148). IPI-9119 (67) and TVB-2640 (80) are selective FASN inhibitors for potential clinical use. Treatment with IPI-9119 led to disruption of the interdependence between AR and FASN and extensive reduction in AR signaling (67). Energy disruptors aim to reduce intracellular ATP level by inhibiting glycolysis or disturbing mitochondrial mechanisms leading to oxidative phosphorylation (33, 149). Several options are available for pharmacological inhibition of glucose metabolism: glucose uptake (phloretin) (150) and glycolytic enzymes (3-bromopyruvate and Koningic acid for GAPDH) (151, 152). Complex I (NADH–quinone oxidoreductase) is the largest respiratory complex of the mitochondrial oxidative phosphorylation system (153). Complex I inhibition has been shown to be a potential

clinical repressor of prostate growth based on early correlative and retrospective studies in men with PCa who had received metformin for treatment of their associated diabetes mellitus (154, 155). Thus, mitochondrial energy metabolism emerges as cancer therapy target (156). In addition to direct inhibition on oxidative phosphorylation (BAY87-2243 and IACS-010759 for complex I) (157, 158), the strategies can be developed to prevent entry of the precursors of TCA cycle intermediates into mitochondria. Glutamine utilization can be prevented by inhibiting glutamine uptake and metabolism (CB839 for glutaminase and V9302 for ASCT2) (159–161). CPT1 inhibition prevents the entry of FA into mitochondria and thus downstream FAO (65). On the other hand, MSDC-0160 inhibits pyruvate entry into mitochondria by mitochondrial pyruvate carrier (144).

Therapeutic targets in cancer metabolism in many cases exist even in the normal cells, which adds potential toxicity and non-specificity to drugs targeting metabolic pathways (162). It is necessary to define their specific action in the context of tumor initiation and progression. The successful application of metabolic inhibitors will lie in accurate metabolic phenotyping and stratification of tumors to predict which respond to the given drugs.

DISCUSSION

We have described how PCa is unique from other cancers from the metabolic point of view. In addition, AR signaling persists in normal and malignant prostatic cells except for when AR antagonism triggers the transition to highly glycolytic AR-indifferent carcinoma. AR determines virtually all aspects of cellular metabolism while a selected phenotype is dominant depending on the stage of disease progression. Accordingly, the question remains as to what directs AR toward specified metabolic preference. The underlying mechanisms may include the presence of AR-Vs, differential actions of AR co-regulators, epigenetics, and tumor microenvironment. Understanding and targeting the selective AR-metabolome axis may provide the unique therapeutic opportunity for AR-driven CRPC which is resistant to current anti-AR therapy.

Except for targeting mutant IDHs, metabolic inhibitors are potentially active regardless of tumor genetic subtype and thus beneficial to the large majority of men with CRPC who are not currently candidates for precision medicine (e.g., DNA repair defects for PARP inhibitors or CDK12 loss for immunotherapy) (138, 163). Nevertheless, appropriate tumor imaging at spatial resolution (e.g., use of PET radiotracers) may facilitate select effective metabolic therapy by determining what bioenergetic phenotype dominates in tumors (glycolytic, lipogenic, or oxidative) (121, 122). For instance, FASN inhibition may be selected when ^{11}C acetate uptake suggests tumors are lipogenic. High avidity to ^{18}F-FDG is supported by expression of glycolytic gene signature in NEPC, providing a rationale to target glucose metabolism for therapy. Tumor plasticity adds another layer of complexity to PCa as it develops and spreads. Altered metabolic pathways may be dispensable or indispensable depending on the stage of tumor progression. This is true for *de novo* FA synthesis whose pharmacological inhibition is detrimental in some cases (antitumorigenic regardless of availability of exogenous lipids) but tolerable in others (e.g., rescued by lipids derived from diet and adipose tissues) (67, 70).

To develop effective metabolism-based target therapy (164), it is crucial to identify metabolic pathways that define the stage of tumor progression depending on AR and cellular lineage status. The success of future therapies may be enhanced by the combination of the prescribed metabolic inhibitors such as metformin and statins (155, 165).

AUTHOR CONTRIBUTIONS

TU and SRP conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

VA merit review award (I01BX003324) and DoD award (W81XWH-17-1-0323 W81XWH-20-1-0146, W81XWH-17-1-0484) to SRP. NCI P50 CA097186, and the Institute for Prostate Cancer Research at the University of Washington and Fred Hutchinson Cancer Research Center.

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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.

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ORIGINAL RESEARCH
published: 29 October 2020
doi: 10.3389/fcell.2020.571677

Epidermal Growth Factor Is Essential for the Maintenance of Novel Prostate Epithelial Cells Isolated From Patient-Derived Organoids

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Prostate cancer (PCa) is the second leading cause of cancer-related mortality and morbidity among males worldwide. Deciphering the biological mechanisms and molecular pathways involved in PCa pathogenesis and progression has been hindered by numerous technical limitations mainly attributed to the limited number of cell lines available, which do not recapitulate the diverse phenotypes of clinical disease. Indeed, PCa has proven problematic to establish as cell lines in culture due to its heterogeneity which remains a challenge, despite the various *in vitro* and *in vivo* model systems available. Growth factors have been shown to play a central role in the complex regulation of cell proliferation among hormone sensitive tumors, such as PCa. Here, we report the isolation and characterization of novel patient-derived prostate epithelial (which we named as AUB-PrC) cells from organoids culture system. We also assessed the role of epidermal growth factor (EGF) in culturing those cells. We profiled the AUB-PrC cells isolated from unaffected and tumor patient samples via depicting their molecular and epithelial lineage features through immunofluorescence staining and quantitative real-time PCR (qRT-PCR), as well as through functional assays and transcriptomic profiling through RNA sequencing. In addition, by optimizing a previously established prostate organoids culture system, we were able to grow human prostate epithelial cells using growth medium and EGF only. With these data collected, we were able to gain insight at the molecular architecture of novel human AUB-PrC cells, which might pave the way for deciphering the mechanisms that lead to PCa development and progression, and ultimately improving prognostic abilities and treatments.

Keywords: prostate epithelial cells, prostate cancer, organoids, lineage markers, RNA-seq, EGF

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

Received: 11 June 2020
Accepted: 12 October 2020
Published: 29 October 2020

Citation:
Cheaito K, Bahmad HF, Jalloul H, Hadadeh O, Msheik H, El-Hajj A, Mukherji D, Al-Sayegh M and Abou-Kheir W (2020) Epidermal Growth Factor Is Essential for the Maintenance of Novel Prostate Epithelial Cells Isolated From Patient-Derived Organoids. Front. Cell Dev. Biol. 8:571677. doi: 10.3389/fcell.2020.571677

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer and second leading cause of cancer-related deaths among males worldwide, with an estimated annual incidence of 191,930 in the United States in 2020, and estimated deaths of 33,330 per year (Siegel et al., 2020). PCa usually contains multifocal lesions (with varying genetic alterations) and is heterogenous at the molecular,

cellular and architectural levels (Zhang et al., 2016), which makes obtaining a homogenous material for molecular analysis difficult (Abate-Shen and Shen, 2000; Bahmad et al., 2020b; Daouk et al., 2020). The heterogeneity of this tumor also renders choosing the best therapy for each patient (castration therapy, surgery, radiotherapy, or chemotherapy) very challenging (Karantanos et al., 2013; Zhang et al., 2016).

Understanding the molecular pathways involved in PCa has been hindered by numerous technical limitations. These mainly relate to the limited number of PCa cell lines available, which do not recapitulate the diverse phenotypes of clinical disease (Ziaee et al., 2015). Nonetheless, the need for representative *in vitro* and *in vivo* models that recapitulate different stages of PCa (Daoud et al., 2016; Daouk et al., 2020; Bahmad et al., 2020b), especially castration-resistant prostate cancer (CRPC), has led to numerous attempts to establish cell lines from human prostate carcinomas (Van Bokhoven et al., 2003). Prostate carcinomas, however, have been the most challenging to establish continuous cell lines from Cunningham and You (2015) and Huang et al. (2016). Approximately 30 reported human prostate cell lines have been described and used for research purposes from 1970 to the present (Van Bokhoven et al., 2003). Due to contamination of putative prostate cell lines, those cells turned out to be derivatives of previously established prostate carcinoma cell lines such as DU145 and PC-3 (Chen, 1993; MacLeod et al., 1999; Pan et al., 2001; Van Bokhoven et al., 2001, 2003). It is thus important to select prostate cell lines that accurately depict its molecular features in order to address research questions appropriately, preferably generated from primary human tissue, bearing in mind that generating a "new primary" PCa cell line is very challenging (Sobel and Sadar, 2005).

A novel promising technology has been recently developed to study tissue homeostasis through a three-dimensional (3D) organoid culture system (Koo et al., 2011). These organoids that mimic the structures of tissues *in vivo*, can grow "indefinitely" in culture and remain phenotypically and genetically stable (Sato et al., 2009; Schwank et al., 2013a,b; Drost et al., 2016). It is believed that they stem from single multipotent stem cells or progenitors capable of differentiation and self-organization to form structures morphologically and functionally resembling the corresponding *in vivo* organ (Bartucci et al., 2016; Bahmad et al., 2020a). Currently, organoids are being established from a variety of organs, including the colon, stomach, and prostate among others (Barker et al., 2010; Eiraku et al., 2011; Jung et al., 2011; Sato et al., 2011; Antonica et al., 2012; Huch et al., 2013; Koehler et al., 2013; Lancaster et al., 2013; Stange et al., 2013; Sachs and Clevers, 2014; Taguchi et al., 2014; Takasato et al., 2014; Agarwal et al., 2015; Drost et al., 2016). Karthaus et al. adapted this culture method to PCa and described an R-spondin1-based 3D culture method through which normal human and murine prostate epithelial cells can be cultured indefinitely without genetic manipulation, in an *in vitro* 3D system that models prostate glandular structure (Karthaus et al., 2014).

Herein, we employed the 3D organoid culture system to generate patient-derived prostate epithelial (American University of Beirut-Prostate Cells; AUB-PrC) cells *in vitro* in an attempt to establish new cells without any genetic manipulation. Since EGFR ligands (such as EGF) and other growth factors have been shown to mediate epithelial cell repair of bronchial cells (Barrow et al., 1993; Burgel and Nadel, 2004), breast cancer (Fitzpatrick et al., 1984; Kim et al., 2012), and PCa cells (Peehl et al., 1996; Festuccia et al., 2005), we hypothesized that EGF might have a role in prostate epithelial cell growth in culture as well. This is supported by the notion that human recombinant EGF is known to be essential for the growth of PCa cells cultured in keratinocyte growth media (Bahmad et al., 2018). We characterized the novel generated primary AUB-PrC cells for molecular and epithelial lineage features through immunofluorescence (IF) staining and quantitative real-time PCR (qRT-PCR), as well as through functional assays and transcriptomic profiling through RNA sequencing.

MATERIALS AND METHODS

Patients Selection

Samples from different stages of human prostate adenocarcinomas were obtained from consented treatment-naïve patients undergoing radical prostatectomy at the American University of Beirut Medical Center (AUBMC). Appropriate Institutional Review Board (IRB) approval was obtained. After getting written informed consents from the patients, primary tissue samples collected were used only if this doesn't compromise the diagnosis or staging. A sample was taken from each patient from the area most likely to be involved with cancer (from the core of the cancerous lesion) and a sample from the unaffected area (far from the tumor site) according to the urologist's and pathologist's recommendation.

A total of seven treatment-naïve patients with PCa diagnosis were enrolled in our study and tested for PSA level at the time of operation. Prostate tissue specimens were collected, weighed, and characterized then assigned a Gleason score, International Society of Urological Pathology (ISUP) grade group, and TNM cancer staging by a pathologist at AUBMC. Among the seven patients included, there was no cancer invasion to nearby lymph nodes and the cancer had not metastasized to other parts of the body (**Supplementary Table S1**).

Collection, Dissection, and Processing of Patient Prostate Tissue Specimens

The collected fresh prostate tissues (ranging from 3 to 5 mm in size) were directly put in a 50 mL conical tube containing "human prostate growth medium" right after the surgery, sent to the research laboratory, and kept at 4°C until processing (within 6 h to maximize the reliability of organoids generation). Using sterile scalpel blades, prostate tissue fragments were minced into approximately 0.1–0.5 mm diameter pieces and washed with "human prostate growth medium" to get rid of cellular debris. Part of minced fragments were used for organoids culturing and the remaining fragments were used for RNA extraction and sequencing.

Prostate tissue fragments designated "unaffected" (U) and "tumor" (T) and minced using sterile scalpel blades were kept overnight in a humidified incubator containing 5% CO_2 at

37°C with 5 mL of 5 mg/mL collagenase type II (Gibco™; cat #17101-015) in Advanced DMEM-F12 medium (adDMEM/F12) (Gibco™; cat #12634-010) with ROCK inhibitor (Y-27632) (Santa Cruz; cat #sc-281642A) to digest the tissue. The next day, cells were washed with adDMEM/F12, then centrifuged at 200 g for 5 min at 4°C. The pellet was resuspended in 1 mL TrypLE™ (ThermoFisher; cat #12605-010) with Y-27632 and digested for approximately 15 min at 37°C. The pellet was then washed once with adDMEM/F12 and centrifuged at 200 g for 5 min at 4°C. Digested tissue was placed in ice-cold Matrigel™ (Corning Life Sciences; cat #354230) and pipetted up and down several times to mix. Around 20,000 cells in a 40 μL drop of 90% Matrigel™ were plated into the middle of one well of a 24-wells culture plate which was placed upside down in the 37°C incubator for 15 min to allow the Matrigel™ to solidify. Then, 500 μL of pre-warmed (37°C) human prostate growth medium plus Y-27632 was added gently into each well. Media was replenished every 3 days using human prostate growth medium plus Y-27632. ROCK inhibitor (Y-27632) was added fresh to the culture medium on the same day medium is changed for the first week after plating only.

Human Prostate Growth Medium Components

"Human prostate growth medium" was prepared using adDMEM/F12 supplemented with 1% Penicillin/Streptomycin (v/v) (Biowest; cat #L0022-100), 0.2% Gentamicin/Amphotericin B (v/v) (Thermo Fisher; cat #R01510), 0.2% plasmocin prophylactic (v/v) (Invivogen; cat #ant-mpp), 10 mM HEPES (Gibco™; cat #15630-056) and 2 mM GlutaMAX (Gibco™; cat #35050-061). For organoids culturing, organoid medium components specified in **Supplementary Table S2** were added (Cheaito et al., under review).

Culturing of Patient-Derived Prostate Epithelial (AUB-PrC) Cells

After passaging the organoids, leftover two-dimensional (2D) cells were detached using TrypLE™ and then transferred to T25 plates previously coated with 1% collagen-I. Cells were supplemented with "human prostate growth medium" plus ROCK inhibitor (Y-27632) and incubated at 37°C in a CO_2 incubator. These patient-derived 2D cells were split at a ratio of 1:2 every 3–4 days where 50% of cells were frozen down in FBS + 10% DMSO (v/v) and stored in liquid nitrogen, and 50% were maintained in culture using same conditions and medium.

American University of Beirut-Prostate Cells (AUB-PrC) cells from patients (unaffected and tumor parts) were named as follows:

- **AUB-PrC-U#:** Patient # unaffected prostate epithelial cells
- **AUB-PrC-T#:** Patient # tumor prostate epithelial cells

(# designates the patient number from 1 to 7)

Cells were frozen in fetal bovine serum (FBS) (Sigma-Aldrich; cat #F9665) + 10% dimethyl sulfoxide (DMSO) (Scharlau; cat #SU01571000) as P0 cells to serve as a stock of patient's derived cells for later use. All cells were kept as a stock in liquid nitrogen.

Immunofluorescence (IF) Analysis of Cells

Indirect immunofluorescence analysis was used to characterize prostate epithelial lineage markers, including CK8 and CK5. Cells were grown on collagen-I coated coverslips. Adherent cells were then fixed using 4% PFA (v/v) in PBS for 20 min, then permeabilized with 0.5% Triton X-100 (v/v) in PBS for 20 min. Non-specific sites were blocked by incubation in blocking buffer [0.1% BSA (v/v), 0.2% Triton X-100 (v/v), 0.05% Tween-20 (v/v) and 10% NGS (v/v] in PBS) for 1 h [bovine serum albumin (BSA) (v/v) (Amresco; cat #0332-100G), normal goat serum (NGS) (v/v) (ThermoFisher; cat #16210064), Tween-20 (Sigma-Aldrich; cat #P1379), and Triton X-100 (v/v) (Sigma-Aldrich; cat #T9284)]. Cells were then incubated overnight with specific primary antibodies at 4°C. After washing with PBS containing 0.1% Tween-20 (v/v), cells were incubated with the corresponding secondary antibodies, then washed gently and mounted with anti-fade reagent Fluoro-gel II with DAPI (Electron Microscopy Sciences; cat #17985-51). Immunofluorescence images were captured using the Carl Zeiss Axio Observer.Z1 with 40 × oil reflector and confocal microscopic analyses was performed using Zeiss LSM710 laser scanning confocal microscope, both utilizing the Carl Zeiss ZEN 2013 image software.

The following antibodies were used: mouse monoclonal anti-CK8 (1/200 dilution; BioLegend; cat #MMS-162P), rabbit polyclonal anti-CK5 (1/200 dilution; BioLegend; cat #PRB-160P), rabbit polyclonal anti-CK14 (1/200 dilution; BioLegend; cat #PRB-155P), rabbit polyclonal anti-Vim (1/50 dilution; Santa Cruz Biotechnology; cat #sc-5565) Alexa 568 goat anti-mouse IgG (ThermoFisher; cat #A-11004), and Alexa 488 goat anti-rabbit IgG (ThermoFisher; cat # A-11034). All secondary Alexa Fluor antibodies were used at 1/200 dilution. Fluoro-gel II with DAPI (Electron Microscopy Sciences, PA) was used for mounting.

Total RNA Extraction and Purification

Total RNA was extracted from corresponding samples using both TriZol (ThermoFisher; cat #15596026) and RNAeasy Mini Kit (Qiagen; cat #74104) according to manufacturer's protocols with modifications. Patient tissues, organoids, and organoid-derived AUB-PrC cells were washed once with 1 mL of PBS prior to the addition of 1 mL of TriZol reagent, which were used to isolated total RNA (upper aqueous phase) after the addition of 0.2 mL of Chloroform followed by centrifugation at 12,000 rpm for 15 min at 4°C. Isolated RNA phase were mixed with 70% ethanol with equal volumes followed by purification of RNA using RNAeasy Mini spin column (Qiagen) according to the manufacturer's protocol. Concentrations and integrity (RNA integrity number—RIN) of isolated RNA were determined using ThermoScientific™ NanoDrop 2000™ and Agilent BioAnalyzer 2100™, respectively.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of Cells

For cDNA preparation, the Quantitect Reverse Transcription Kit (Qiagen; cat #205311) was utilized. cDNA was diluted in

a 1:10 volume ratio of DEPC. mRNA expression of normal and tumor samples were analyzed by RT-PCR (Bio-rad CFX™ Manager Software; cat #1845000) using the ΔC_t method and the SYBR green system. All reactions were performed using 2X SYBR Green master mixes each containing 2 μL template cDNA, 0.5 μL of each primer mix (forward and reverse), 5 μL buffer containing SYBR Green (Applied Biosystems; cat #A46111) and 2.5 μL of RNase free water (Primers used are listed in **Supplementary Table S3**).

The PCR reaction consisted of a DNA denaturation step at 95°C for 5 min, followed by 40 cycles (denaturation at 95°C for 10 s), then annealing at the appropriate temperature of 60°C for each primer for 30 s, and finally an extension step at 72°C for 10 min. For each experiment, reactions were performed in duplicates and expression of individual genes was normalized to the house keeping gene *GAPDH*. Gene expression was calculated through the following equation: $\Delta C_t = C_{t\ (target)} - C_{t\ (GAPDH)}$. The amount of endogenous target gene relative to a calibrator (*GAPDH*) became $2^{-\Delta\ Ct}$.

RNA Sequencing of AUB-PrC Cells vs. Their Corresponding Tissue Counterparts

RNA-Seq Library Preparation and Sequencing

RNA samples from two patients (patients 4 and 5) with total concentrations of > 0.5 ng/μl and RIN > 8 were used for library preparation. RNA Sequencing (RNA-Seq) libraries were prepared using Illumina® TruSeq Stranded mRNA prep kit (Illumina; cat #RS-122-2101) accordingly with the manufacturers LS protocol. Samples were barcoded, multiplexed and sequenced (100 bp pair-end) using the Illumina® Hi-Seq 2500 platform at New York University Abu Dhabi (NYUAD) Genomic Core facility (Abu Dhabi, U.A.E.).

Transcriptome Data Computational Analysis (Subject to Change)

DESeq2 computational pipeline was used to estimate the raw count reads aligned to the reference genome (Love et al., 2014). Computing methods were run on a Linux based command system on NYUAD High Performance Computing (HPC) server platform Dalma. Correlation (i.e., Principle Component Analysis—PCA) analysis were generated by RNA-Seq START (Shiny Transcriptome Analysis Resource Tool) application, via the New York University Abu Dhabi Center of Genomic and Systems Biology (NYUAD-CGSB) Bioinformatics Online Analysis and Visualization Portal[1] (Nelson et al., 2017). The data discussed in this paper have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE148937[2].

Gene Array Data Analysis

Differentially expressed gene (DEG) features (3,383 and 4,250 significantly differentially expressed transcripts between the AUB-PrC cells and their corresponding tissues in the unaffected and tumor samples, respectively) were subjected to Gene Ontology (GO) term and gene set enrichment analyses using GSEA, Cytoscape, and EnrichmentMap bioinformatics tools (Reimand et al., 2019). The database of pathway gene sets used for pathway enrichment analysis was downloaded from http://baderlab.org/GeneSets and it includes eight data sources: MSigDB (C2 collection) (Subramanian et al., 2005), NCI (Schaefer et al., 2009), Institute of Bioinformatics (IOB), NetPath (Kandasamy et al., 2010), HumanCyc (Romero et al., 2005), Reactome (Croft et al., 2011), GO (Ashburner et al., 2000), MSigDB (C3 collection; Specialty GMTs mirs, transcription factors) (Subramanian et al., 2005), and Panther (Mi et al., 2005; **Supplementary Table S4**).

MTT Cell Growth Assay

MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) (Sigma-Aldrich; cat #M5655-1G) cell growth assay was used, according to the manufacturer's instructions (Mosmann, 1983; Riss et al., 2004; Van Meerloo et al., 2011), to measure the *in vitro* cell proliferation and growth of the unaffected and tumor patient-derived AUB-PrC cells under the three different culturing conditions:

- Condition control "All Factors" was prepared as described in **Supplementary Table S2**
- Condition "All Factors – EGF" included all other components except EGF
- Condition "EGF alone" included adDMEM/F12 medium + EGF only (10 ng/mL)

AUB-PrC cells were derived from tissue samples of 3 different patients (Patients 5, 6, and 7), including the unaffected and the tumor sample for each. Cells were seeded at a density of 4×10^3 cells/well in 100 μL in triplicates in 96-well culture plates and incubated overnight at 37°C in a humidified incubator containing 5% CO_2, before being exposed to the different culturing conditions for 72 h. Media was changed at 24 and 48 h. The reduced MTT optical density (OD) was measured by the microplate ELISA reader (Multiscan EX) at a wavelength of 595 nm. The percentage of cell viability was presented as percentage growth using the OD ratio of cells relative to condition "All Factors." The average percentage cell viability in each condition was derived from the mean of triplicate wells of three independent experiments.

Cell Viability (Trypan Blue Exclusion Method)

Unaffected and tumor AUB-PrC cells from three patients were seeded, in triplicates, in 12-well plates at a density of 5×10^4 cells per well. Cells were then cultured under the three different culturing conditions used in the MTT assay for up to 72 h. Viable cells were collected and counted using trypan blue dye (Sigma-Aldrich; cat #T8154-100ML) exclusion method after 72 h (Strober, 2001). Cell viability was expressed as percentage growth relative to condition "All Factors." The data are derived from the mean of triplicates wells.

[1] http://tsar.abudhabi.nyu.edu/

[2] https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148937

Statistical Analyses

Statistical analysis was performed using GraphPad Prism 7 software. Student's *t*-test was used to analyze gene expression. To determine statistical significance of differences in *in vitro* cell proliferation and viability of the unaffected and tumor patient-derived AUB-PrC cells between the three culturing conditions related to EGF, two-way ANOVA test was performed followed by multiple comparisons using Bonferroni *post hoc* analysis. All $P < 0.05$ were considered significant.

RESULTS

Isolation of Patient-Derived Prostate Epithelial (AUB-PrC) Cells From 3D Organoids

Starting from the prostate organoids protocol and using the same culture medium (Cheaito et al., under review), AUB-PrC two-dimensional (2D) cell lines (unaffected and tumor) were successfully generated. After the 1st week of organoids culture (**Figure 1A**), cells started invading the three-dimensional (3D) Matrigel™ droplet and proliferating in 2D cultures on the bottom of the plates (**Figure 1B**). Collagen-I allowed the spreading of cells and maintained their healthy morphology when propagated for continuous passages reaching more than 28 passages with successful repeated freeze-thaw cycles (**Figure 1C**).

Immunofluorescence Characterization of AUB-PrC Cells for Prostate Epithelial Lineage Markers

Using immunofluorescence, we characterized AUB-PrC cells derived from three treatment-naïve patients for prostate epithelial lineage markers. AUB-PrC cells displayed key characteristics of epithelial cells, showing that when such cells are further apart from each other, they form extensions that fill the gaps *in vitro*. We also demonstrated that tumor AUB-PrC cells display elongated epithelial cell features compared to their unaffected counterparts (**Figure 2A** and **Supplementary Figure S1**). Those key characteristics of epithelial cells show that when such cells are further apart from each other, they tend to form extensions to fill the gaps *in vitro*. Morphological differences were further depicted in immunofluorescent staining of AUB-PrC cells using lineage epithelial cell markers, including CK8 (luminal epithelial cell marker) and CK5 (basal epithelial cell marker). Both unaffected and tumor AUB-PrC cells showed evidence of CK8 + and CK5 + expression (**Figure 2B** and **Supplementary Figure S2A**) with no difference in expression noticed between unaffected and tumor cells. Since PCa cells are more prone to lose their epithelial phenotype in favor of a more mesenchymal phenotype, which is a trigger for aggressiveness and metastasis (Cheaito et al., 2019), we employed immunofluorescent staining of tumor AUB-PrC cells using CK8 (luminal epithelial cell marker) and vimentin (VIM; mesenchymal cell marker), showing evidence of VIM + expression (**Supplementary Figure S2B**).

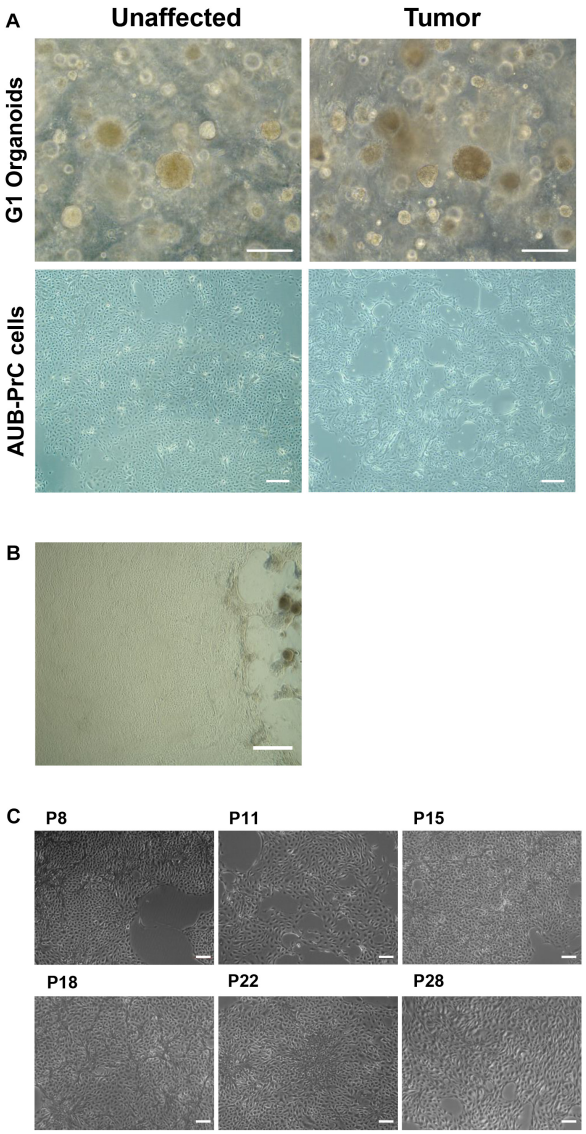

FIGURE 1 | Isolation of patient-derived AUB-PrC cells using organoids culture conditions. Representative bright-field images showing established prostate organoids (generation 1, G1) from unaffected and tumor prostate patient samples {patient 2 with Grade Group 3 [Gleason Score 7(4 + 3)]; patient characteristics in **Supplementary Table S1**} grown in culture (Scale bar = 200 μm) **(A)**, and AUB-PrC cells established and grown on 1% collagen type-I coated plates (Scale bar = 200 μm) **(B)** maintaining their healthy morphology when propagated for continuous passages (passages P8, P11, P15, P18, P22, and P28 are shown) reaching more than 28 passages with successful repeated freeze-thaw cycles (Scale bar = 100 μm) **(C)**.

Expression of Prostate Epithelial Lineage Genes in AUB-PrC Cells

Next, we sought to characterize the novel patient-derived cell lines with respect to specific primers relative to *GAPDH*, for experimental value $n = 1$, done in technical duplicates, using quantitative reverse transcription-PCR (qRT-PCR) analysis. We assessed mRNA expression levels of several genes including

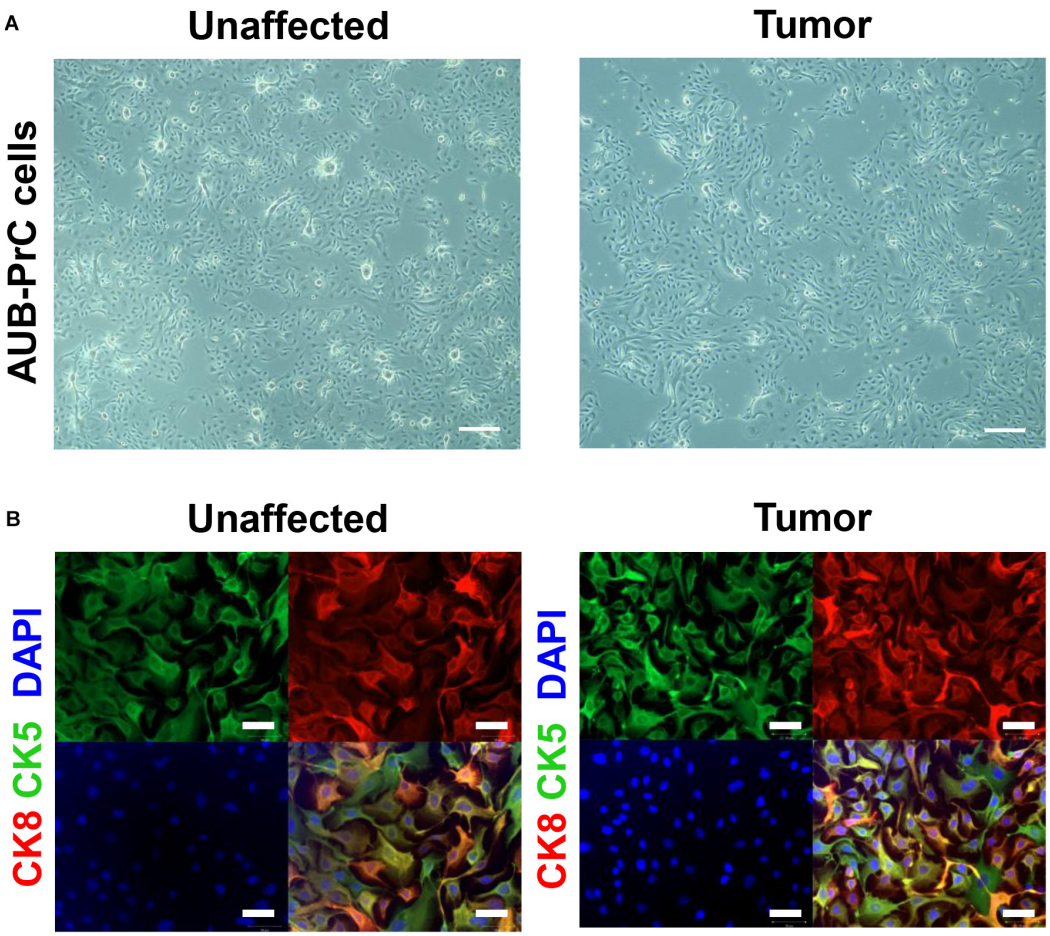

FIGURE 2 | Morphologic and immunofluorescent epithelial lineage characterization of AUB-PrC cells. **(A)** Representative bright-field images of AUB-PrC cells from unaffected and tumor prostate patient samples {patient 1 with Grade Group 5 [Gleason Score 9(5 + 4)]; patient characteristics in **Supplementary Table S1**}, displaying key characteristics of epithelial cells and showing that when cells are far apart from each other, they form extensions that fill the gaps *in vitro*. Scale bar = 200 μm. **(B)** Representative immunofluorescence images of AUB-PrC cells from unaffected and tumor prostate patient samples {patient 3 with Grade Group 2 [Gleason Score 7(3 + 4)]; patient characteristics in **Supplementary Table S1**} stained for the lineage epithelial cell markers, CK8 (luminal epithelial cell marker) and CK5 (basal epithelial cell marker), and the nuclear counterstain DAPI illustrating CK8 + /CK5 + intermediate character. Scale bars = 50 μm.

epithelial cell markers (*CDH1* and *CDH2*), prostate luminal epithelial markers (*CK8* and *CK18*), basal epithelial markers (*NKX3.1* and *P63*), and other markers known to be aberrated in the prostate or maintain stemness (*AR* and *CD44*, respectively).

In our study, patient 1 showed significantly increased expression level of E-cadherin (*CDH1*) and decreased levels of N-cadherin (*CDH2*) (**Figures 3A,B**). Although patient 1 has high ISUP group 5, this does not exclude the possibility that the cancer cells still retain cell adhesion epithelial phenotype. This is consistent with the epithelial behavior of those cells which when grown apart from each other in culture tend to form extensions and fill the gaps *in vitro*, as mentioned previously. Also, we found significantly increased expression of the luminal epithelial cell markers (*CK8* and *CK18*) in patient 2 AUB-PrC cells (**Figures 3C,D**). Besides, a pathway known to be central to prostate cells proliferation and survival (Song et al., 2009) was found to be dysregulated in the AUB-PrC cells from all three patients, depicting upregulation of *NKX3.1* among those patients and down-regulation of *AR* in patient 2 AUB-PrC cells (**Figures 3E,F**). Stem cell markers, such as *P63* (basal stem cell marker) and *CD44* were found to be upregulated in AUB-PrC cells from patients 1 and 2 (**Figures 3G,H**). Notably, stem cell-expressing population of AUB-PrC cells may be responsible for the regenerative potential that allows these patient cells to be maintained in culture for many passages, especially cells derived from tumor samples. It is noteworthy mentioning that since patients might have different genetic backgrounds, it is expected to have them convey different gene expression profiles.

Whole-Transcriptome Sequence Analysis of AUB-PrC Cells vs. Their Corresponding Tissues

We then sought to study transcriptomic features that signify AUB-PrC cells vs. their corresponding tissues in unaffected and tumor samples. We performed paired-end (100 base pair)

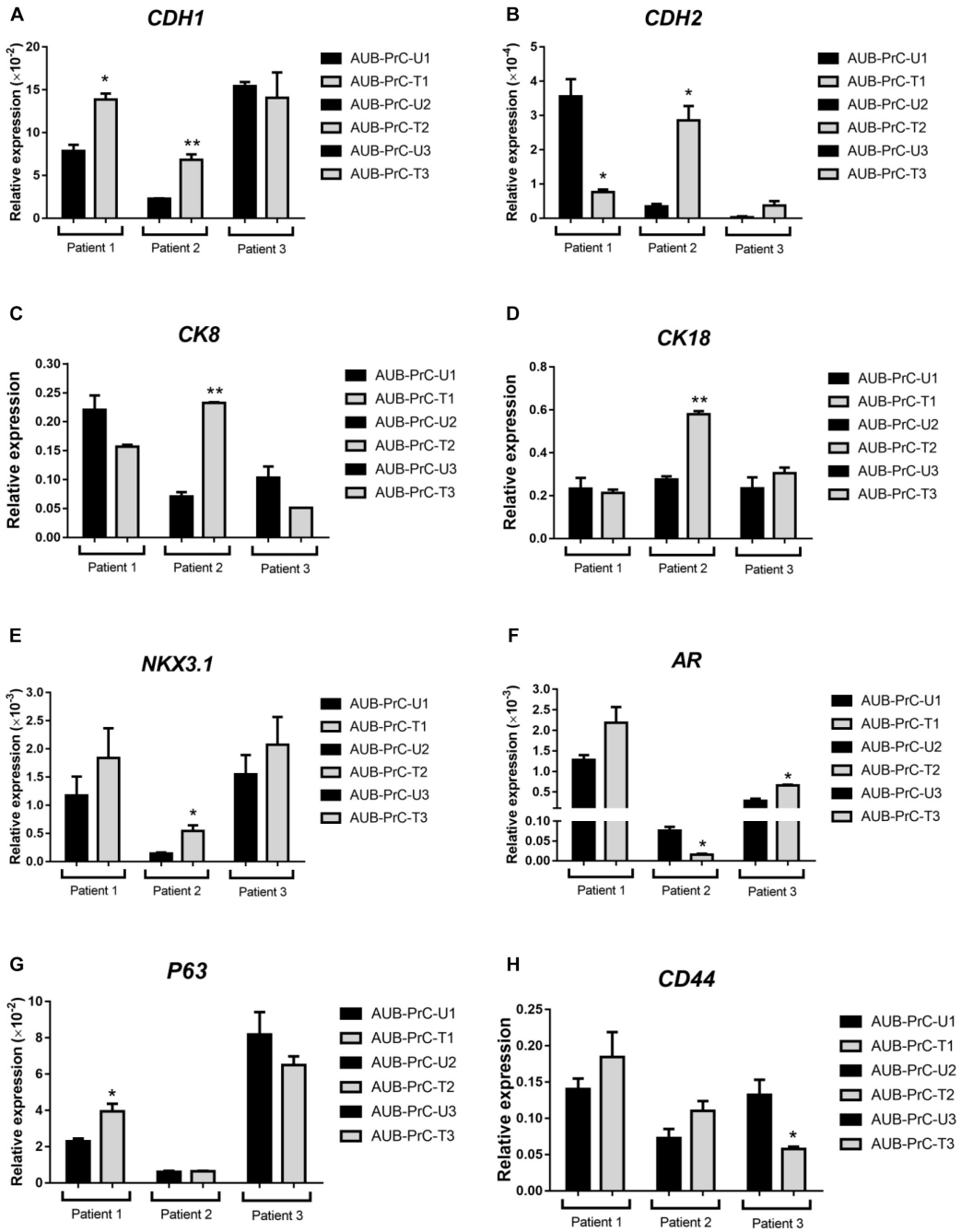

FIGURE 3 | Expression of different prostate epithelial lineage and stem cell markers determined using qRT-PCR analysis. qRT-PCR expression analysis depicted aberrations in epithelial cell markers (**A:** *CDH1* and **B:** *CDH2*), prostate luminal epithelial markers (**C:** *CK8* and **D:** *CK18*), basal epithelial cell marker (**E:** *NKX3.1*), stem cell markers (**G:** *P63* basal stem cell marker and **H:** *CD44*), and other markers know to aberrated in PCa (**F:** *AR*). For each patient {patient 1 with Grade Group 5 [Gleason Score 9(5 + 4)]; patient 2 with Grade Group 3 [Gleason Score 7(4 + 3)]; patient 3 with Grade Group 2 [Gleason Score 7(3 + 4)]; patients characteristics in **Supplementary Table S1**}, reactions were performed in biological duplicates and expressions of individual genes was normalized to the house keeping gene *GAPDH*. Data were plotted relative to the unaffected cells (AUB-PrC-U) for each patient. Relative expression value are presented as means + SD (two technical replicates) (*P <0.05; **P <0.01; by Student's t-test).

RNA-sequencing using the Hi-Seq 2500 Illumina platform to delineate DEG features between patient-derived AUB-PrC cells and their corresponding tissue counterparts (two biological replicates – with technical duplicate for each – in each group).

Based on statistical significance using p-adj < 0.05 cut-off, we identified 3,383 and 4,250 transcripts that were significantly differentially expressed between the AUB-PrC cells vs. their corresponding tissue counterparts in each of the unaffected

and tumor samples, respectively (722 up-regulated and 2,661 down-regulated in unaffected samples and 1,092 up-regulated and 3,158 down-regulated in tumor samples; **Supplementary Tables S5, S6**). The volcano plots in **Figures 4A,B** represent an overview of DEGs with a threshold set at p-adj < 0.05. The DEG expression heatmaps for unaffected and tumor samples are presented in **Figures 4C,D**, and interestingly the venn diagram identified DEG that are uniquely expressed in the Unaffected

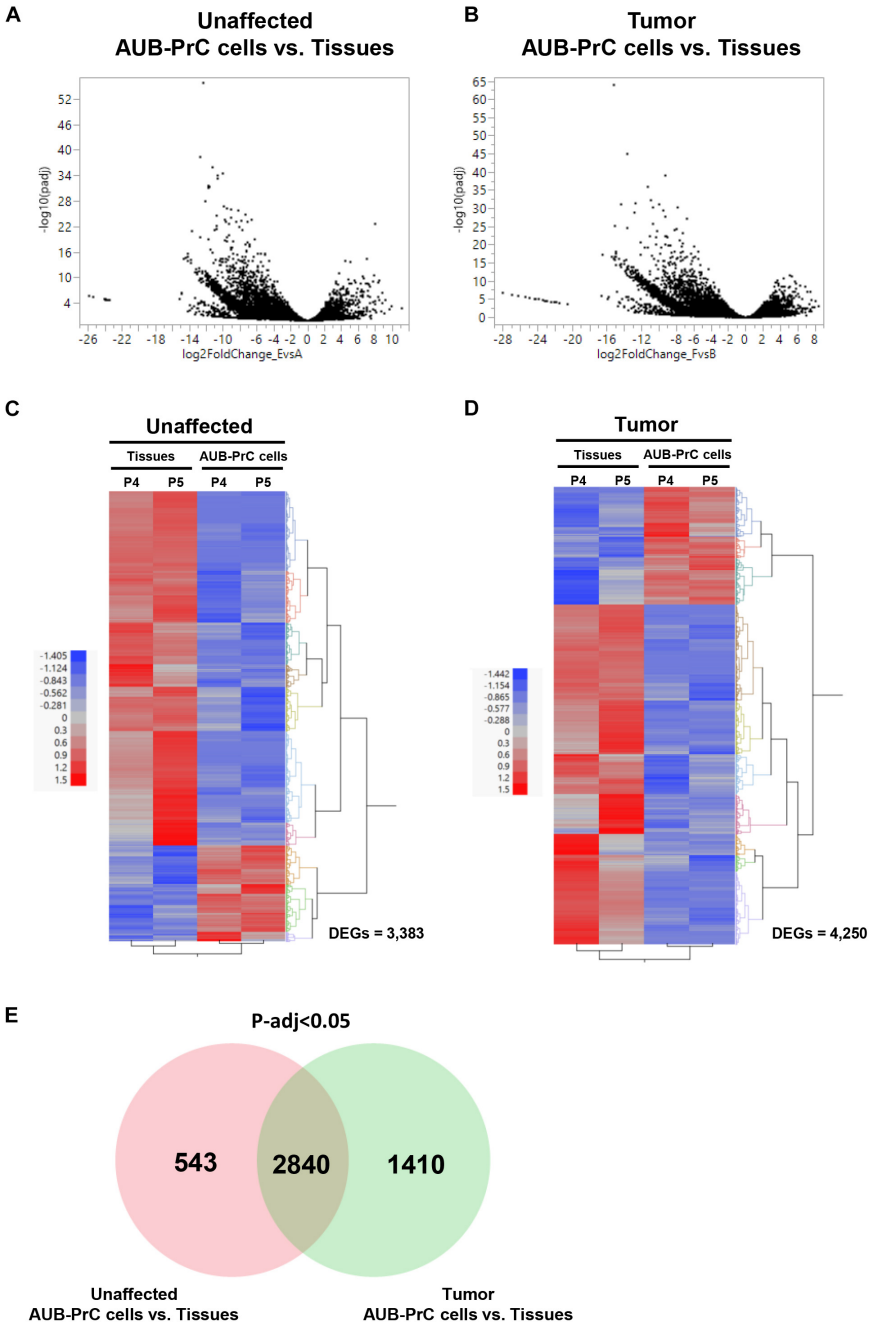

FIGURE 4 | RNA-sequencing of patient-derived AUB-PrC cells relative to their corresponding tissues in unaffected and tumor samples. RNA-Seq was performed using the Hi-Seq 2500 Illumina platform to delineate the differentially expressed genes (DEG) as described in "Materials and Methods" section. Volcano plots **(A,B)** and Venn diagram **(E)** demonstrating an overview of the DEGs. The threshold was set at p-adjusted < 0.05. Differentially expressed transcripts (n = 3,383 and 4,250 in unaffected and tumor samples) between AUB-PrC cells and tissue counterparts {two biological replicates and two technical duplicates in each group; patient 4 with Grade Group 1 [Gleason Score 6(3 + 3)] and patient 5 with Grade Group 3 [Gleason Score 7(4 + 3)]; patients characteristics in **Supplementary Table S1**} were identified using statistical criteria detailed in "Materials and Methods" section. **(C,D)** Heatmaps and the hierarchical cluster analyses of the differentially expressed genes for unaffected **(C)** and tumor **(D)** samples. Red represents the upregulated genes and blue represents the downregulated genes.

samples (543) vs. the tumor samples (1410) (**Figure 4E** and **Supplementary Table S7**).

GO Term Analysis Venn Diagram

Gene Ontology (GO) analysis of the DEGs lists [unique genes in unaffected AUB-PrC cells vs. tissue [543 DEGs], unique genes in tumor AUB-PrC cells vs. tissue (1410 DEGs), and common genes between unaffected and tumor (2840 DEGs)] isolated based on the venn diagram were further analyzed via DAVID platform (Huang Da et al., 2009). Focusing solely on biological processes with a cutoff of $p < 0.05$, several terms were identified. In the unaffected AUB-PrC cells vs. tissue, there were 41 terms (**Supplementary Table S8**) showing top five significant enrichments of GO:0043065∼positive regulation of apoptotic process (16 genes), GO:0001755∼neural crest cell migration (6 genes), GO:0045746∼negative regulation of Notch signaling pathway (5 genes), GO:2000379∼positive regulation of reactive oxygen species metabolic process (5 genes), and GO:0090074∼negative regulation of protein homodimerization activity (3 genes) (**Figure 5**—top panel). In the tumor AUB-PrC cells vs. tissue, there were 58 term (**Supplementary Table S9**) showing top 5 significant enrichments GO:0006887∼exocytosis (14 genes), GO:0045909∼positive regulation of vasodilation (8 genes), GO:0051090∼regulation of sequence-specific DNA binding transcription factor activity (7 genes), GO:0001525∼angiogenesis (25 genes), and GO:0019233∼sensory perception of pain (10 genes) (**Figure 5**—middle panel). Whereas common genes shared between both DEGs list consisted of 414 terms (**Supplementary Table S10**) that included top 5 significant enrichments of GO:0007155∼cell adhesion (158 genes), GO:0030198∼extracellular matrix organization (83 genes), GO:0007165∼signal transduction (249 genes), GO:0006954∼inflammatory response (109 genes), and GO:0006955∼immune response (109 genes) (**Figure 5—lower** panel).

AUB-PrC Cells Demonstrate Upregulation of Prostate Epithelial Lineage mRNA Expression

DEGs genes that characterize prostate basal, luminal, and intermediate epithelia (Wang et al., 2001) along with other genes known to be aberrated in prostate tissue and cancer and growth factors genes were found to be dysregulated in AUB-PrC cells *vs.* tissues in unaffected and tumor samples (**Table 1**).

Next, we pursued to confirm some of the gene features that were identified by the RNA-Seq analysis to be differentially expressed in AUB-PrC cells relative to their corresponding tissue counterparts. RNA-Seq analysis had revealed the upregulation of the prostate luminal epithelial lineage marker *CK8* and basal stem cell marker *P63* in AUB-PrC cells compared to their tissue counterparts (**Table 1**). It also showed the downregulation of other genes, such as *AR*, *VIM*, and *TWIST1* in those cells. Consistent with the RNA-Seq results, quantitative real-time PCR analysis of AUB-PrC cells from patient 5 and its tissue counterparts (three technical replicates each) showed upregulation of *CK8* and *P63* genes in AUB-PrC cells compared to their tissue counterparts (**Supplementary Figure S3A**, upper panels) and downregulation of *AR*, *VIM*, and *TWIST1* (**Supplementary Figure S3A**, lower panels). Molecular characterization was also performed on AUB-PrC cells and tissue sections from patients 4 and 5 on which RNA-Seq analysis was done. Immunofluorescent staining showed evidence of high CK8 + and CK5 + expression among cells and their counterpart tissues, with low expression of VIM+ (**Supplementary Figure S3B**). Results are consistent with

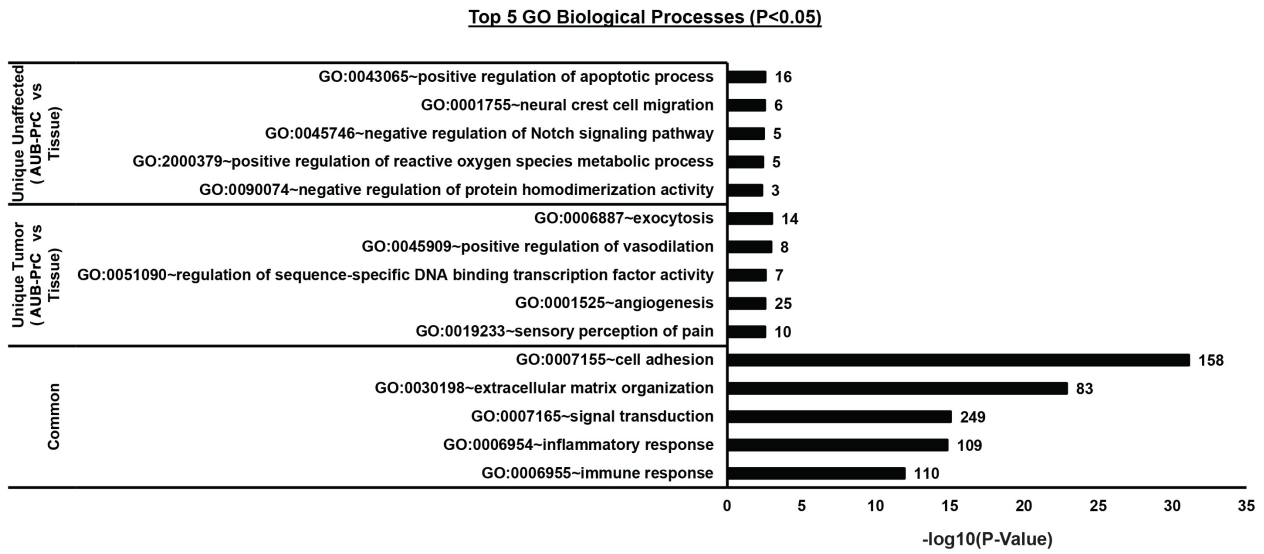

FIGURE 5 | Biological processes Gene Ontology (GO) analysis of the DEGs lists between AUB-PrC cells vs. tissue in unaffected and tumor samples. [Unique genes in unaffected AUB-PrC cells vs. tissue (543 DEGs), unique genes in tumor AUB-PrC cells vs. tissue (1410 DEGs), and common genes between unaffected and tumor (2840 DEGs)] were isolated based on the Venn diagram and further analyzed via DAVID platform (Huang Da et al., 2009) to identify the top 5 GO biological processes.

TABLE 1 | List of DEGs genes commonly representing prostate lineage markers and other markers related to prostate diseases along with their expression levels in AUB-PrC cells relative to their corresponding tissue counterparts in unaffected and tumor samples.

Markers	DEGs gene symbols	DEGs gene names	AUB-PrC cells vs. Tissues			
			Unaffected samples		Tumor samples	
			FC (log2)	*p*-adj	FC (log2)	*p*-adj
Basal epithelial	*KRT5*	Keratin 5	4.81	0.00034	4.77	0.00034
	KRT14	Keratin 14	5.7	1.51E-09	4.37	6.16E-06
	TP63	Tumor protein p63	2.32	0.07827	2.96	0.01257
	NKX3.1	NK3 Homeobox 1	−3.3	0.00118	−2.9	0.00498
Luminal epithelial	*KRT8*	Keratin 8	1.48	0.16463	2.99	0.00043
	KRT13	Keratin 13	2.78	0.40733	5.12	0.05105
	KRT18	Keratin 18	1.04	0.40985	2.35	0.01291
Intermediate epithelial	*KRT19*	Keratin 19	3.57	0.01804	2.99	0.04917
Cadherins	*CDH1*	E-cadherin	1.55	0.27936	2.05	0.09614
	CDH2	N-cadherin	−5.08	0.0475	−4.34	0.00446
Prostate cancer related	*AR*	Androgen receptor	−3.12	0.01436	−2.81	0.02722
	VIM	Vimentin	−1.99	0.25782	−1.05	0.58212
	CD44	CD44 Molecule	2.27	0.00253	3.22	4.37E-06
	FOXA1	Forkhead Box A1	0.92	0.58522	1.19	0.39047
	TWIST1	Twist Family BHLH Transcription Factor 1	−2.06	0.37524	−4.25	0.01980
	IL6	Interleukin 6	−7.78	0.00111	−10.92	9.39E-07
	TMPRSS2	Transmembrane Serine Protease 2	−2.90	0.08178	−4.22	0.00371
	ERG	ETS Transcription Factor ERG	−3.40	2.49E-05	−3.27	4.73E-05
Growth factors	*FGF10*	Fibroblast Growth Factor 10	−9.38	9.95E-05	−9.55	6.35E-05
	FGFR1	Fibroblast growth factor receptor 1	−2.61	0.00081	−3.33	7.76E-06
	FGF2	Basic fibroblast growth factor (β-FGF)	−3.44	0.00076	−3.05	0.00242
	EGFR	Epidermal growth factor receptor	1.40	0.29082	2.18	0.04582
	EGF	Epidermal growth factor	0.84	0.77265	−1.77	0.41791
	NTF3	Neurotrophin-3	−6.68	0.00198	−8.20	0.00072

DEG, differentially expressed gene; FC, fold change.

the RNA-Seq results showing upregulation of *CK8* and *CK5* genes and downregulation of *VIM* (**Table 1**).

GSEA Identifies Enrichment of Growth Factor and Epithelial Lineage-Related Signaling Pathways in AUB-PrC Cells Relative to Their Tissue Counterparts

We sought to build enrichment maps to evaluate DEGs and their related pathways in our datasets (**Figure 6** and **Supplementary Tables S11, S12**) using Cytoscape 3.7.2 software (EnrichmentMap tool). Using gene set enrichment analysis (GSEA), we identified significantly altered pathways in AUB-PrC cells relative to their corresponding tissue counterparts (**Supplementary Tables S13, S14**). Results indicated significant differences (FDR < 0.01, NOM $p < 0.05$) in the enrichment of the gene sets database (Human_GOBP_AllPathways_no_GO_iea_April_01_2020_symbol.gmt; **Supplementary Table S4**). We selected the 20 most significantly enriched signaling pathways, based on normalized enrichment score (NES) (**Supplementary Figures S4, S5**). Results indicated the unaffected data set was enriched for cell cycle pathways, E2F signaling, TP53 transcriptional regulation, Rb signaling, mitosis, and epithelial differentiation pathways while the treated data set was enriched for cell cycle pathways, PLK1 signaling, DNA irradiation damage and cellular response via ATR, and epithelial differentiation pathways. Other pathways that are found to be enriched in AUB-PrC cells and are of specific interest in prostate diseases include cell adhesion molecules (CAMs), cholesterol biosynthesis and metabolism pathways, ErbB-2 signaling, c-Myc pathway, and other cancer pathways which can be further explored in future work to look for novel potential therapeutic targets for PCa.

EGF Is Essential to Grow Patient-Derived AUB-PrC Cells in Culture

Based on an observation made during the organoids' optimization experiment (Cheaito et al., under review), we noticed that EGF withdrawal from the medium affected the ability to derive AUB-PrC cells negatively (data not shown). So, we further investigated the importance of EGF for the growth of AUB-PrC cells by growing them under 3 conditions; condition 1 includes prostate organoids growth medium (as described in **Supplementary Table S2**), condition 2 includes prostate organoids growth medium without EGF, and condition 3 includes adDMEM/F12 with EGF only (10 ng/mL) (**Figure 7A**).

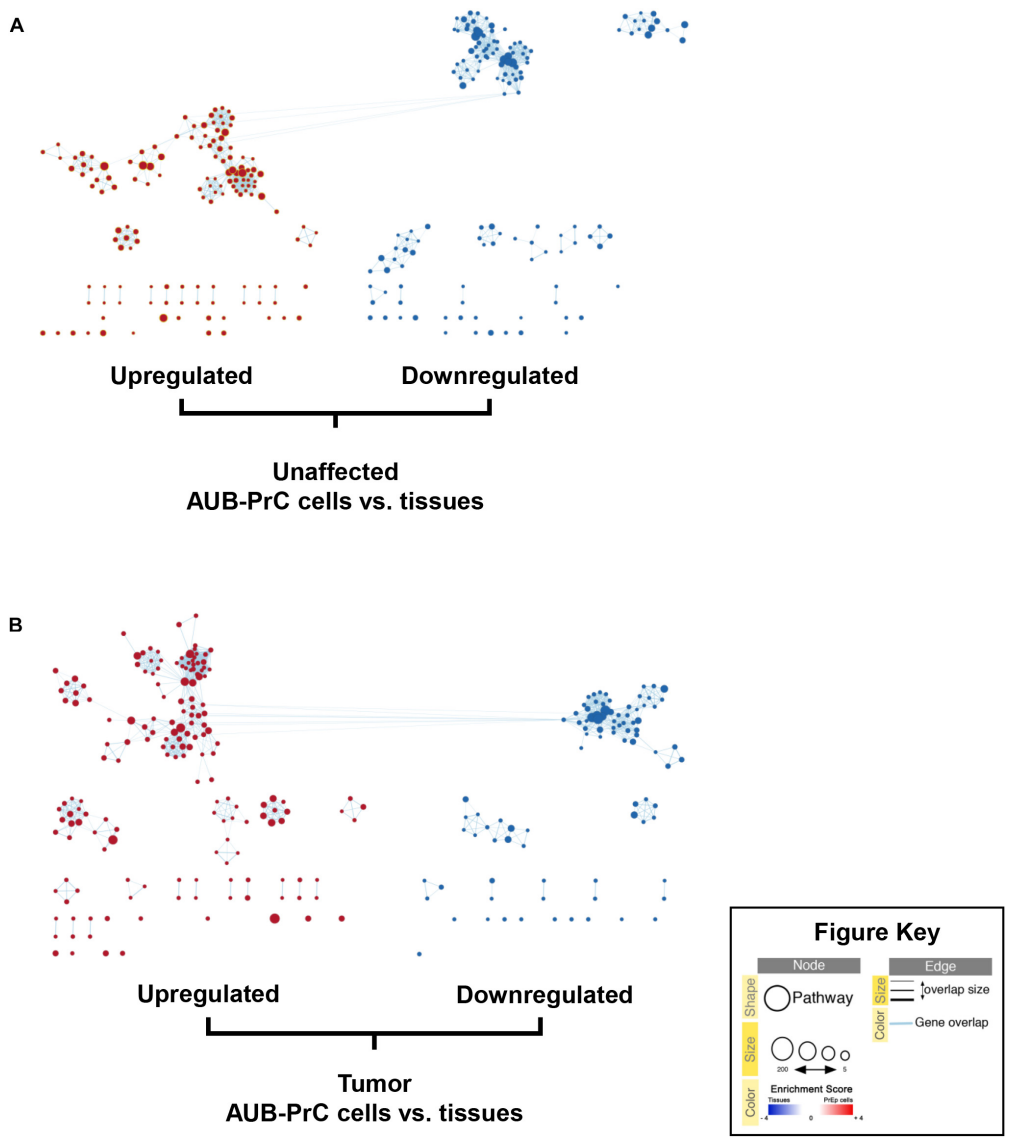

FIGURE 6 | Enrichment maps of pathways enriched in upregulated genes (red) and downregulated genes (blue) in AUB-PrC cells vs. tissues. Enrichment maps of pathways among prostate unaffected **(A)** and tumor **(B)** samples were built using EnrichmentMap analysis on Cytoscape 3.7.2 software. Each node (circle) represents a distinct pathway (red representing upregulated pathways and blue representing downregulated pathways), and edges (lines) represent the number of genes overlapping between two pathways, determined using the similarity coefficient.

AUB-PrC cells derived from tissue samples from 3 different patients (Patients 5, 6, and 7), including the unaffected and the tumor sample, were seeded under the three different conditions. MTT and Trypan Blue assays were performed showing, a significant reduction in cell viability and cell proliferation when EGF was removed from the medium, while EGF alone demonstrated the ability to maintain the growth of AUB-PrC cells. Indeed, there was no significant difference in both cell proliferation and cell viability between condition 1 and condition 3 for all three patients' derived AUB-PrC cells (**Figures 7B,C**). To further confirm that condition 3 "EGF alone" can support the growth of both luminal and epithelial cells, AUB-PrC cells growing under 3 conditions described above were immunostained with luminal marker CK8 and basal marker CK14. The results obtained showed similar morphologies and expression patterns of luminal and basal markers in both condition 1 and condition 3, which confirms that EGF alone can substitute the cocktail of 12 components included in condition 1.

DISCUSSION

Epithelial organ remodeling (such as PCa, breast cancer, and colon cancer) is a major contributing factor to worldwide morbidity and mortality. It is difficult to translate basic epithelial research into clinical therapy due to the lack of relevant

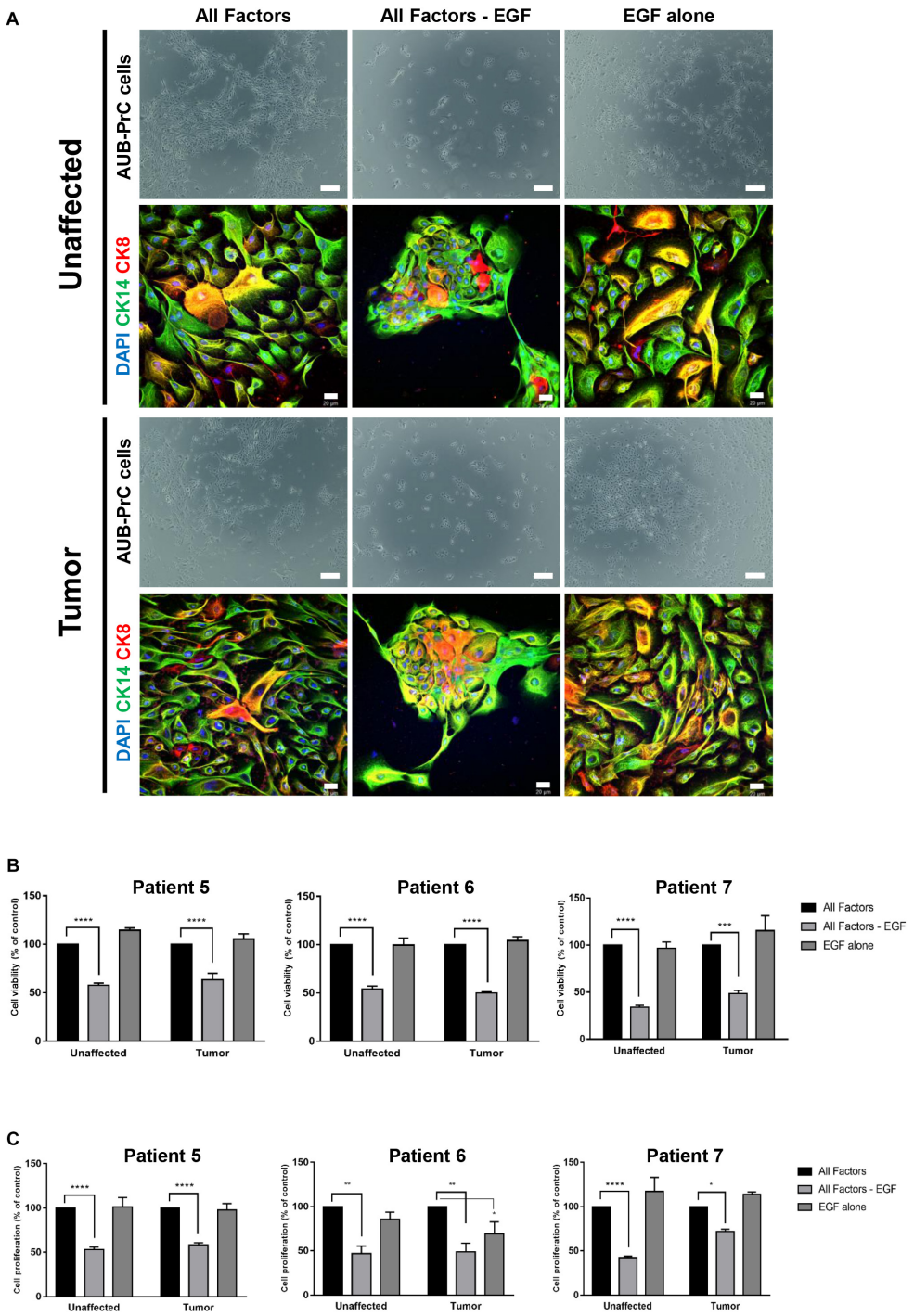

FIGURE 7 | EGF is essential to grow patient-derived AUB-PrC cells in culture. **(A)** Representative bright-field images of AUB-PrC cells established from unaffected and tumor organoids {patient 7 with Grade Group 3 [Gleason Score 7(4 + 3)]; patient characteristics in **Supplementary Table S1**} and grown under different conditions; condition 1 "All factors" with prostate organoids growth medium, condition 2 "All factors -EGF" with prostate organoids growth medium without EGF, and condition 3 "EGF alone" with adDMEM/F12 with EGF only (10 ng/mL). Scale bar = 200 μm. Representative Immunofluorescent images of AUB-PrC cells {patient 7 with Grade Group 3 [Gleason Score 7(4 + 3)]; patient characteristics in **Supplementary Table S1**} grown under different conditions as described previously are stained with the prostate lineage epithelial markers CK8 and CK14. The nuclei were stained with anti-fade reagent Fluorogel II with DAPI. The images were acquired using the Zeiss LSM 710 laser scanning confocal microscope (Zeiss), and images were processed using the Carl Zeiss ZEN 2013 image software. Scale bar = 20 μm. **(B)** Cell viability was determined using the trypan blue exclusion assay. **(C)** Cell proliferation was determined in triplicates using the MTT cell proliferation assay {patient 5 with Grade Group 3 [Gleason Score 7(4 + 3)]; patient 6 with Grade Group 2 [Gleason Score 7(3 + 4)]; patient 7 with Grade Group 3 [Gleason Score 7(4 + 3)]; patients characteristics in **Supplementary Table S1**}. Data represent an average of triplicate measurements and are reported as mean ± SEM. (Two-way ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; different conditions compared to condition 1 "All Factors," Bonferroni's multiple comparisons test).

preclinical models (Hynds and Giangreco, 2013). The challenges of PCa research include inter- and intra-patient heterogeneity and the scarcity of appropriate *in vitro* and *in vivo* models that depict the vast molecular aberrations that occur in PCa (Van Bokhoven et al., 2003; Tsai et al., 2018). In this context, many genetic aberrations in PCa are poorly studied, and their effects on therapeutic response are not known (Vela and Chen, 2015). Despite its prevalence, PCa has proven very difficult to propagate *in vitro* and is highly underrepresented with very few cell lines available among the thousands of cancer cell lines in public repositories (Gao et al., 2014).

The heterogeneous nature of PCa has made it difficult to understand the factors involved in the onset and progression of the disease (Shen and Abate-Shen, 2010). In the last few years several efforts have been made to delineate the complex genomic landscape of PCa (Baca and Garraway, 2012). Moreover, considering that PCa is fairly indolent, the development of treatment approaches that delay its onset or progression is likely to have a significant impact on outcome. Indeed, the scarcity of human PCa cell lines has always hindered our understanding of the disease etiology and progression, and therefore the need for novel cell lines representing the heterogeneity of the disease is of eminent importance. Along those lines and starting from organoids, we aimed at generating novel patient-derived cell lines representing unaffected and tumor prostate tissues.

Starting from the organoids protocol and using the same culture medium (Cheaito et al., under review), human prostate two-dimensional (2D) cell lines (unaffected and tumor) – which we named as AUB-PrC cells – were successfully generated. After the first week of organoids culture, 2D cells started invading the three-dimensional (3D) Matrigel™ droplet and proliferating on the bottom of the culture plates. These cells were successfully derived whenever organoids were established; nonetheless, their maintenance in culture was very challenging. Consequently, to maintain them in culture, we attempted to optimize the culture conditions by using different matrices. Interestingly, collagen-I allowed the spreading of cells and maintained their healthy morphology when propagated for continuous passages reaching more than 28 passages. The favored adhesion of PCa cells to collagen-I represents a possible explanation for these results. Indeed, the most frequent site of human PCa metastasis is the bone and collagen-I represents the most abundant protein within the skeleton (Buckwalter et al., 1996). In addition, it has been previously demonstrated that collagen-I induces the attachment and proliferation of PCa cells (Kiefer and Farach-Carson, 2001).

We sought to characterize the novel patient-derived AUB-PrC cells using immunofluorescence (IF), qRT-PCR, and RNA-Seq analyses (**Table 2**). AUB-PrC cells depicted a distinctive epithelial cell morphology expressing CK8 and CK5 prostate epithelial lineage markers. Yet, PCa cells are more prone to lose their epithelial phenotype in favor of a more mesenchymal phenotype, which is a trigger for aggressiveness and metastasization. Indeed, our results showed that tumor AUB-PrC cells demonstrate some vimentin (mesenchymal cell marker) expression as well which further validate our point (**Supplementary Figure S2**). qRT-PCR results indicated a trend in mRNA expression levels of several genes involved in prostate lineage differentiation and other genes known to aberrated in PCa.

We also studied the transcriptomic features and delineated the DEGs that signify AUB-PrC cells *vs.* their corresponding tissues in unaffected and tumor samples, followed by gene set enrichment analysis (GSEA), demonstrating upregulation of epithelial differentiation pathways and gene features. Herein, we showed that the AUB-PrC cells that have been isolated from patient-derived organoids cultures are of prostate epithelial lineage based on expression of different markers including CK5, CK8, AR, and Nkx3.1, and hence they represent the tissue of origin. Nevertheless, we expected to see variations in the transcriptomic analysis between the cells and their corresponding tissues because we are comparing cells grown *in vitro* in 2D *vs.* primary cells or tissues. In other words, we are comparing epithelial cells that are growing under selective pressure *in vitro* to those that are nascent non-manipulated tissues that contain intact microenvironment with all its components.

Among the DEGs identified by RNA-sequencing were upregulated ones that include keratins (*KRT5*, *KRT8*, *KRT13*, *KRT14*, *KRT18*, and *KRT19*), *TP63*, *CDH1*, *EGFR*, *CD44*, and *FOXA1*, and other downregulated genes such as *NKX3.1*, *TWIST1*, *IL6*, *TMPRSS2*, *ERG*, *AR*, *CDH2*, and growth factor genes (*FGF10*, *FGF2*, *FGFR1*, *EGF*, and *NTF3*). We sought

TABLE 2 | Table summarizing major characteristics of patients and AUB-PrC cell lines generated.

Patient #	Cell lines	Gleason score	ISUP grade group	Immunofluorescent staining relative to unaffected cells			mRNA expression of different prostate epithelial lineage and stem cell markers relative to unaffected cells							
				CK8	CK5	VIM	*CDH1*	*CDH2*	*CK8*	*CK18*	*NKX3.1*	*AR*	*p63*	*CD34*
Patient 1	AUB-PrC-U1 and AUB-PrC-T1	9(5 + 4)	Grade group 5	↑	↑	↓	↑	↓	↓	↓	↑	↑	↑	↑
Patient 2	AUB-PrC-U2 and AUB-PrC-T2	7(4 + 3)	Grade group 3	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑
Patient 3	AUB-PrC-U3 and AUB-PrC-T3	7(3 + 4)	Grade group 2	↑	↑	↓	↓	↑	↓	↑	↑	↑	↓	↓
Patient 4	AUB-PrC-U4 and AUB-PrC-T4	6(3 + 3)	Grade group 1	↑	↑	↑	–	–	–	–	–	–	–	–
Patient 5	AUB–PrC-U5 and AUB-PrC-T5	7(4 + 3)	Grade group 3	↑	↑	↑	–	–	–	–	–	–	–	–
Patient 6	AUB-PrC-U6 and AUB-PrC-T6	7(3 + 4)	Grade group 2	↑	↑	↓	–	–	–	–	–	–	–	–
Patient 7	AUB-PrC-U7 and AUB-PrC-T7	7(4 + 3)	Grade group 3	↑	↑	↑	–	–	–	–	–	–	–	–

AUB-PrC, American University of Beirut-Prostate Cells; ISUP, International Society of Urological Pathology; ↑, increased; ↓, decreased.

to validate some of those genes using qRT-PCR and IF analyses and results were indeed consistent with the RNA-Seq data. Interestingly, the observed mRNA expression patterns recapitulate the architecture of prostate tissues where luminal secretory cell layers express prominent levels of *CK8* and *CK18*, underlying basal cell layers express *CK5*, *CK14* and *TP63*, and intermediate epithelial cells express *KRT19* (Wang et al., 2001; Van Leenders and Schalken, 2003; Peehl, 2005; Cheaito et al., 2019). *KRT13*, which was upregulated in AUB-PrC cells, has been also proposed to be a marker of stem/progenitor-like cell state. In PCa, this gene has been shown to be enriched in benign stem-like cells displaying androgen-resistance and was identified in tumors that have the potential to metastasize to the bone (Liu et al., 2016). Likewise, *TWIST1* which plays a role in PCa bone metastasis, was downregulated in AUB-PrC cells in our study (Gajula et al., 2013; Chang et al., 2015).

Intercellular adhesion is a key factor in epithelial tissue morphogenesis and maintenance, and disruption of this adhesion is an important factor in cancer (Balzer and Konstantopoulos, 2012). Cadherins are a family of calcium-dependent cell– CAMs with well-established roles in cell–cell recognition, intercellular junction organization and cell differentiation. The role of cadherins, particularly the epithelial (E)-cadherin, has been studied in detail in relation to metastatic potential and prognosis in carcinoma. In our study, RNA-seq revealed upregulation of epithelial *CDH1* and downregulation of mesenchymal *CDH2*, verifying the epithelial nature of AUB-PrC cells (Tomita et al., 2000).

One of the initiating events in prostate tumorigenesis is downregulation of the homeobox gene *NKX3.1*. It is described as the "gatekeeper" for PCa initiation (Barbieri et al., 2013), and was found to be downregulated in AUB-PrC cells in our study. Chromosomal rearrangements involving the *ETS* family of transcription factors, such as *TMPRSS2-ERG* fusions, are mostly detected after initiation and not as an initial event, thus they are commonly associated with PCa progression (Tomlins et al., 2005; Shen and Abate-Shen, 2010). In our human AUB-PrC cells, those genes were found to be downregulated. Along the line, AUB-PrC cells demonstrated downregulation of growth factor genes including *FGF10*, *FGF2*, *FGFR1*, *EGF,* and *NTF3*, all of which are essential for development and progression of PCa (Polnaszek et al., 2003; Memarzadeh et al., 2007; Corn et al., 2013; Yang et al., 2013; Mandel et al., 2018).

In this same context, and although EGF was designated as an essential component for establishing and maintaining prostate organoids in culture (Karthaus et al., 2014), we were interested in studying its effect on the *in vitro* culturing and growth of AUB-PrC cells. In brief, we investigated the importance of EGF by growing AUB-PrC cells in three different conditions: prostate organoids growth medium (will all 12 factors), prostate organoids growth medium without EGF, and adDMEM/F12 media with EGF only. Remarkably, our results demonstrated enhanced growth and maintenance of those cells in the presence of EGF alone, while a significant reduction in cell viability and proliferation was noticed when EGF was removed from the medium. These data are consistent with the substantial role of EGF in stimulating cell motility and migration of epithelial cells from various tumors, including PCa (Lu and Kang, 2010; Montanari et al., 2017). Further, we stained the cells grown under the three conditions with prostate luminal epithelial marker CK8 and basal epithelial marker CK14, and found similar cell morphologies and expression patterns in conditions 1 (complete organoids media) and 3 (EGF alone), confirming that EGF by itself is sufficient to substitute the cocktail of 12 components included in condition 1.

Lastly, it is important to emphasize that it is very crucial to establish new cell line models of cancers especially when some of those are scarce as in the case of PCa. Cancer cell lines are considered powerful tools for studying the mechanisms of tumorigenesis especially if the cancer harbors heterogeneity features such as in PCa. Those cancer cell lines are considered fundamental pre-clinical models to assess the efficacy of anti-cancer therapeutics. The available cell lines in PCa do not really recapitulate the huge heterogeneity of the disease and data inferred from small number of cell lines cannot be really generalized as a representative of the pathophysiology of that disease. The major PCa cell lines used are of Caucasian origin (LnCap, DU145, PC3, and VcaP) and hence might not genetically represent the different world populations. Our novel cell lines represent a novel cohort of Middle Eastern patients. Importantly, those novel cell lines are derived from treatment-naïve patients and therefore the cancer cells are considered primitive in terms of treatment response. This can shed more light on the etiology of the disease as it will not be masked by different therapeutic modalities.

Limitations

Our work has several limitations. First, we acknowledge that the sample size might be small, but since we are dealing with patient tissues, it is indeed difficult to obtain large number of prostate tissues to work on just after the surgery. Second, some experiments were not performed on all the seven patients included, and this is due to the fact that obtaining tissue samples from patients is challenging including the small size of the certain samples that we receive and the small number of cells we get. Third, although samples were taken from each patient from the area most likely to be involved with cancer (from the core of the cancerous lesion) and from the unaffected area (far from the tumor site) based on an assessment made by the urologist and pathologist, no definite conclusion can be made to whether the unaffected sample is not genetically modified or might contain niche of cancerous cells. Forth, since PCa starts as an adenocarcinoma (epithelial origin), we tend to refer to the cell lines as epithelial PCa cell lines. However, PCa cells are more prone to lose their epithelial phenotype in favor of a more mesenchymal phenotype, which is a trigger for aggressiveness and metastasization. Herein, our results showed that tumor AUB-PrC cells demonstrate some vimentin (mesenchymal cell marker) expression as well. Fifth, we acknowledge that it is crucial to assess the AUB-PrC cell lines' ability to engraft in animal models to provide information also about its potential employment *in vivo*, which can be employed in future studies. In addition, 3D culture experiments using Matrigel or Collagen Type I can be performed also to try and distinguish between malignant and non-malignant

cells. Also, validating some of the significantly DEGs at a protein level using western blotting is interesting to be addressed in future studies assessing molecular aberration and signaling underlying our newly developed AUB-PrC cell lines. Sixth, our RNA seq results revealed that growth factors are among the DEGs identified in AUB-PrC cells vs. tissues, including *FGF10*, *FGF2*, *FGFR1*, and *NTF3*, all of which are essential for development and progression of PCa. For the scope of this paper, we have only worked on EGF. Nevertheless, it would be very interesting to assess the roles of the other growth factors. Lastly, and as all those newly derived cells are considered biological replicates from unique patients and therefore represent different cell models, it becomes crucial to subject them to targeted sequencing or whole genome sequencing to fully characterize the genomic landscape of each cell line/patient.

CONCLUSION

The derivation of novel models to express the diverse array of aberrations seen in PCa is essential in detecting specific stages of the disease, classifying PCa based on specific molecular alterations, and selecting the most appropriate therapy for each patient. In this manuscript, we were able to generate and characterize different cell models representing different PCa patients from Middle-Eastern background and having a common feature of being treatment-naïve. We successfully demonstrated the importance of growth factors in modeling of prostate diseases by showing that the newly isolated prostate cells are capable of growing in culture in the presence of EGF alone. Yet, it is of utmost importance to further analyze the differential transcriptomic features between tumor and unaffected samples to better understand PCa at a subcellular level. Our findings provide a prospect to better understand prostate diseases, especially PCa, and pave the way for deciphering the mechanisms that lead to PCa development and progression, and ultimately improving prognostic abilities and treatments.

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**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of the American University of Beirut. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KC: investigation, methodology, writing- original draft preparation, writing- reviewing and editing, visualization, validation. HB: resources, software, formal analysis, investigation, methodology, data curation, writing- original draft preparation, writing- reviewing and editing, visualization, validation. HJ, OH, and HM: investigation, methodology, writing- reviewing and editing, validation. AE-H and DM: investigation, writing- reviewing and editing, visualization, validation. MA-S: project administration, supervision, software, formal analysis, methodology, data curation, writing- original draft preparation, writing- reviewing and editing, visualization, validation. WA-K: conceptualization, project administration, supervision, writing- original draft preparation, writing- reviewing and editing, validation, visualization, funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by funding from the Medical Practice Plan (MPP) at the American University of Beirut Faculty of Medicine (AUB-FM) (to WA-K) and funding from New York University Abu Dhabi (NYUAD) Center for Genomics and Systems Biology (to MA-S). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We want to thank all members of the Abou-Kheir's and Al-Sayegh's Laboratories for their support. In addition, we would like to thank all members of the core facilities in the DTS Building for their help and support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.571677/full#supplementary-material

Supplementary Figure 1 | Morphologic analysis of AUB-PrC cells from patients 2 and 3. Representative bright-field images of AUB-PrC-U2 and AUB-PrC-T2 cells **(A)** and AUB-PrC-U3 and AUB-PrC-T3 cells **(B)**, displaying key characteristics of epithelial cells {patient 2 with Grade Group 3 [Gleason Score 7(4 +3)]; patient 3 with Grade Group 1 [Gleason Score 7(3 +4)]; patients characteristics in **Supplementary Table S1**}. Scale bar 200 μ m.

Supplementary Figure 2 | Immunofluorescent epithelial lineage characterization of AUB-PrC cells for CK8/CK5 and CK8/VIM **(B)**. Representative immunofluorescence images of AUB-PrC cells from unaffected and tumor prostate patient samples {patient 1 with Grade Group 5 [Gleason Score 9(5 +4)]; patient 2 with Grade Group 3 [Gleason Score 7(4 +3)]; patient 3 with Grade Group 2 [Gleason Score 7(3 +4)]; patient 4 with Grade Group 1 [Gleason Score 6(3 +3)]; patient 6 with Grade Group 2 [Gleason Score 7(3 +4)]; patient characteristics in **Supplementary Table S1**} stained for the lineage epithelial cell markers, CK8 (luminal epithelial cell marker), CK5 (basal epithelial cell marker), and VIM (mesenchymal cell marker), and the nuclear counterstain DAPI illustrating CK8 +/CK5 **(A)** and CK8+/VIM **(B)** characters. Scale bars 20 μ m.

Supplementary Figure 3 | Validation of dysregulated gene expression in AUB-PrC cells relative to their tissue counterparts. **(A)** Upregulation of *CK8* and

P63 and downregulation of *AR*, *VIM*, and *TWIST1* in AUB-PrC cells compared to tissues [patient 5 with Grade Group 3 [Gleason Score 7(4 +3)]; patient characteristics in **Supplementary Table S1**} was validated by qRT-PCR and analyzed using the $2^{-\Delta Ct}$ method by normalization to *GAPDH*. Reactions were performed in technical triplicates and expression of individual genes was normalized to the house keeping gene *GAPDH*. Data were plotted relative to the tissue counterparts. Relative expression values are presented as means + SD (three technical replicates) (*P <0.05; **P <0.01; by Student's t-test). **(B)** Representative Immunofluorescent images of AUB-PrC cells {patient 4 with Grade Group 1 [Gleason Score 6(3 +3)] and patient 5 with Grade Group 3 [Gleason Score 7(4 +3)]; patients characteristics in **Supplementary Table S1**} stained with the prostate lineage epithelial markers CK8, CK5, and VIM. The nuclei were stained with anti-fade reagent Fluorogel II with DAPI. The images were acquired using the Zeiss LSM 710 laser scanning confocal microscope (Zeiss), and images were processed using the Carl Zeiss ZEN 2013 image software. Scale bar 200μ m.

Supplementary Figure 4 | Gene set enrichment analysis (GSEA) of the top 20 signaling pathways activated in AUB-PrC cells relative to their tissue counterparts among the unaffected samples. Comparison of data sets indicated unaffected AUB-PrC cells had enrichment of cell cycle pathways, E2F signaling, TP53 transcriptional regulation, Rb signaling, mitosis, and epithelial differentiation pathways.

Supplementary Figure 5 | Gene set enrichment analysis (GSEA) of the top 20 signaling pathways activated in AUB-PrC cells relative to their tissue counterparts among the tumor samples. Comparison of data sets indicated tumor AUB-PrC cells had enrichment of cell cycle pathways, PLK1 signaling, DNA irradiation damage and cellular response via ATR, and epithelial differentiation pathways.

Supplementary Table 1 | Patients' clinical characteristics.

Supplementary Table 2 | Overview of specific components and their respective concentrations added to prepare human prostate organoids culture medium. Adopted and modified from Drost et al. (2016).

Supplementary Table 3 | Primer sequences and annealing temperatures for select human genes.

Supplementary Table 4 | Gene set database used for pathway enrichment analysis (Human_GOBP_AllPathways_no_GO_iea_April_01_2020_symbol.gmt; downloaded from http://baderlab.org/GeneSets).

Supplementary Table 5 | List of all differentially expressed genes (DEGs) between AUB-PrC cells and their tissue counterparts in unaffected samples.

Supplementary Table 6 | List of all differentially expressed genes (DEGs) between AUB-PrC cells and their tissue counterparts in tumor samples.

Supplementary Table 7 | List of all differentially expressed genes (DEGs) between AUB-PrC cells and their tissue counterparts that are unique to unaffected samples (543), unique to tumor samples (1410), and common between the two (2840).

Supplementary Table 8 | List of biological processes identified that are unique to the unaffected AUB-PrC cells vs. tissue. There were 41 terms showing top five significant enrichments of GO:0043065~positive regulation of apoptotic process (16 genes), GO:0001755~neural crest cell migration (6 genes), GO:0045746~negative regulation of Notch signaling pathway (5 genes), GO:2000379~positive regulation of reactive oxygen species metabolic process (5 genes), and GO:0090074~negative regulation of protein homodimerization activity (3 genes).

Supplementary Table 9 | List of biological processes identified that are unique to the tumor AUB-PrC cells vs. tissue. There were 58 terms showing top five significant enrichments of GO:0006887~exocytosis (14 genes), GO:0045909~positive regulation of vasodilation (8 genes), GO:0051090~regulation of sequence-specific DNA binding transcription factor activity (7 genes), GO:0001525~angiogenesis (25 genes), and GO:0019233~sensory perception of pain (10 genes).

Supplementary Table 10 | List of biological processes identified that are common between unaffected and tumor AUB-PrC cells vs. tissue. There were 414 terms showing top five significant enrichments of GO:0007155~cell adhesion (158 genes), GO:0030198~extracellular matrix organization (83 genes), GO:0007165~signal transduction (249 genes), GO:0006954~inflammatory response (109 genes), and GO:0006955~immune response (109 genes).

Supplementary Table 11 | Gene set annotations of the enrichment map presented in **Figure 6A** corresponding to the unaffected AUB-PrC cells vs. tissues identified using Cytoscape 3.7.2 software (EnrichmentMap tool).

Supplementary Table 12 | Gene set annotations of the enrichment map presented in **Figure 6A** corresponding to the tumor AUB-PrC cells vs. tissues identified using Cytoscape 3.7.2 software (EnrichmentMap tool).

Supplementary Table 13 | Signaling pathways that are activated in unaffected AUB-PrC cells vs. tissues identified using gene set enrichment analysis (GSEA).

Supplementary Table 14 | Signaling pathways that are activated in tumor AUB-PrC cells vs. tissues identified using gene set enrichment analysis (GSEA).

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 05 November 2020
doi: 10.3389/fcell.2020.590192

Dietary Energy Modulation and Autophagy: Exploiting Metabolic Vulnerabilities to Starve Cancer

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

Received: 31 July 2020
Accepted: 14 October 2020
Published: 05 November 2020

Citation:
Cozzo AJ, Coleman MF, Pearce JB, Pfeil AJ, Etigunta SK and Hursting SD (2020) Dietary Energy Modulation and Autophagy: Exploiting Metabolic Vulnerabilities to Starve Cancer. Front. Cell Dev. Biol. 8:590192. doi: 10.3389/fcell.2020.590192

Cancer cells experience unique and dynamic shifts in their metabolic function in order to survive, proliferate, and evade growth inhibition in the resource-scarce tumor microenvironment. Therefore, identification of pharmacological agents with potential to reprogram cancer cell metabolism may improve clinical outcomes in cancer therapy. Cancer cells also often exhibit an increased dependence on the process known as autophagy, both for baseline survival and as a response to stressors such as chemotherapy or a decline in nutrient availability. There is evidence to suggest that this increased dependence on autophagy in cancer cells may be exploitable clinically by combining autophagy modulators with existing chemotherapies. In light of the increased metabolic rate in cancer cells, interest is growing in approaches aimed at "starving" cancer through dietary and pharmacologic interventions that reduce availability of nutrients and pro-growth hormonal signals known to promote cancer progression. Several dietary approaches, including chronic calorie restriction and multiple forms of fasting, have been investigated for their potential anti-cancer benefits, yielding promising results in animal models. Induction of autophagy in response to dietary energy restriction may underlie some of the observed benefit. However, while interventions based on dietary energy restriction have demonstrated safety in clinical trials, uncertainty remains regarding translation to humans as well as feasibility of achieving compliance due to the potential discomfort and weight loss that accompanies dietary restriction. Further induction of autophagy through dietary or pharmacologic metabolic reprogramming interventions may enhance the efficacy of autophagy inhibition in the context of adjuvant or neo-adjuvant chemotherapy. Nonetheless, it remains unclear whether therapeutic agents aimed at autophagy induction, autophagy inhibition, or both are a viable therapeutic strategy for improving cancer outcomes. This review discusses the literature available for the therapeutic potential of these approaches.

Keywords: cancer, autophagy, fasting, metabolism, caloric restriction, cancer therapy

INTRODUCTION

The overarching term *autophagy* is generally recognized to encompass three distinct processes: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy utilizes an isolation membrane called an *autophagosome* to sequester and transport protein aggregates or organelles to lysosomes for degradation (Mizushima et al., 2004; Kuma and Mizushima, 2010). In contrast, microautophagy involves direct engulfment of cytoplasmic components through invagination of the lysosomal membrane, while chaperone-mediated autophagy targets select cytosolic proteins and translocates them to the lysosome in a chaperone-dependent manner (Mizushima and Komatsu, 2011; Shaid et al., 2013). In this review, we will focus on pharmacologic and dietary approaches that have been examined for their potential to modulate dependence of cancer cells macroautophagy, referred to hereafter simply as autophagy.

In a growing tumor, cancer cells are faced with increased metabolic demands in a microenvironment characterized by dysfunctional vascularization, hypoxia, and fierce competition for a limited supply of nutrients (Dewhirst et al., 2008; Zhang et al., 2008). Under the harsh conditions of the tumor microenvironment, the highly conserved catabolic process of autophagy can support cancer cell metabolism through supply of critical metabolites via degradation and recycling of precise cargo such as misfolded proteins, dysfunctional mitochondria, and pathogens, as well as non-selective engulfment of bulk cytoplasmic components (Nakatogawa et al., 2009; Kuma and Mizushima, 2010; Boya et al., 2013; Shaid et al., 2013). Early studies investigating the effects of autophagy inhibition have utilized genetic silencing of key autophagy genes, effectively disrupting the autophagy cascade and providing more insight into the roles of autophagy in cancer initiation and aggressive features in cancer cells (Cufi et al., 2012).

Dietary interventions that restrict caloric intake may induce autophagy in normal and/or cancerous cells, and there is increasing interest in using these interventions clinically with the ultimate goal of manipulating systemic fuel availability to "starve" a developing tumor. Herein we discuss the roles of autophagy in cancer initiation, tumor progression, and therapeutic response. In addition, we provide: (i) an overview of the underlying molecular biology following restriction of dietary energy intake through approaches such as caloric restriction and various forms of fasting; (ii) we summarize the limited evidence from associated clinical trials that have utilized these interventions as an approach to improving treatment outcomes or reducing the toxic side effects of chemotherapy (Raffaghello et al., 2008; Lee et al., 2010); (iii) we address some of the currently available pharmacological approaches for both induction and inhibition of autophagy; and (iv) we briefly discuss the potential for synergy between dietary or pharmacologic energy manipulation and autophagy inhibition.

METABOLIC AND HORMONAL REGULATION OF AUTOPHAGY

Basal autophagy occurs constitutively through the signaling of hormones and growth factors (Rabinowitz and White, 2010), facilitating the maintenance of cellular homeostasis by removing redundant or damaged organelles and generating metabolites used to provide energy to the cell or create new macromolecules (Boya et al., 2013). In contrast, autophagy is induced above basal levels under conditions associated with cellular stress or low energy status, including a high AMP/ATP ratio, nutrient deprivation, and/or reduced growth factor signaling (Saha et al., 2018). The principal cellular regulators of autophagic flux are AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR), both of which function to integrate nutrient and energy signaling with cellular metabolism and various forms of fasting (Meijer and Codogno, 2011; Mihaylova and Shaw, 2011; Kim and Guan, 2015).

AMPK is an evolutionarily conserved serine/threonine protein kinase that acts as a key sensor of cellular energy status. Upon activation, AMPK works to restore energy homeostasis by activating an array of catabolic pathways including autophagy, as well as phosphorylating and inactivating mTOR (Hardie et al., 2016). High AMP/ATP ratios and glucose deprivation are the primary signals for AMPK activation (Gowans et al., 2013; Zhang et al., 2017).

mTOR, also a serine/threonine kinase, is a master regulator of cellular growth and proliferation in response to nutrient and hormone signaling; namely, amino acid concentrations and insulin-like growth factor 1 (IGF1) and/or insulin levels (Avruch et al., 2006). In order to activate downstream anabolic pathways, mTOR complex 1 (mTORC1) must be recruited to the lysosome (Lawrence and Zoncu, 2019). The protein complex GATOR1 functions to inhibit mTOR activation via GTP hydrolysis of the heterodimeric Rag GTPases responsible for recruiting mTOR to the lysosomal surface (see **Figure 1**; Bar-Peled et al., 2013; Panchaud et al., 2013). The activity of GATOR1 is regulated by amino acid concentrations—specifically levels of leucine, arginine, and methionine. Leucine and arginine, functioning through SESTRIN and CASTOR respectively, interact with GATOR2 to inhibit mTOR upon amino acid deprivation (Kim J. S. et al., 2015; Saxton et al., 2016; Wolfson et al., 2016). Leucyl-tRNA synthetase (LRS) functions as another leucine sensor and positive regulator for mTORC1 as a GTPase-activating protein (GAP) for RagD (Lee et al., 2018). SAMTOR, an inhibitor of mTOR and sensor for S-adenosylmethionine, is responsible for mTOR inactivation in the context of methionine deprivation (**Figure 1**), which improves insulin sensitivity and extend lifespan in rodents (Orentreich et al., 1993; Gu et al., 2017).

Growth factor signaling involving insulin and IGF1 is another well-established upstream regulator of mTOR that further integrates host nutrient status with cellular metabolism. Both insulin and IGF1 activate the PI3K/AKT signaling axis upon binding to their tyrosine kinase receptor, resulting in increased activation of mTORC1 at the lysosomal surface (Saxton and Sabatini, 2017). Insulin, a peptide hormone

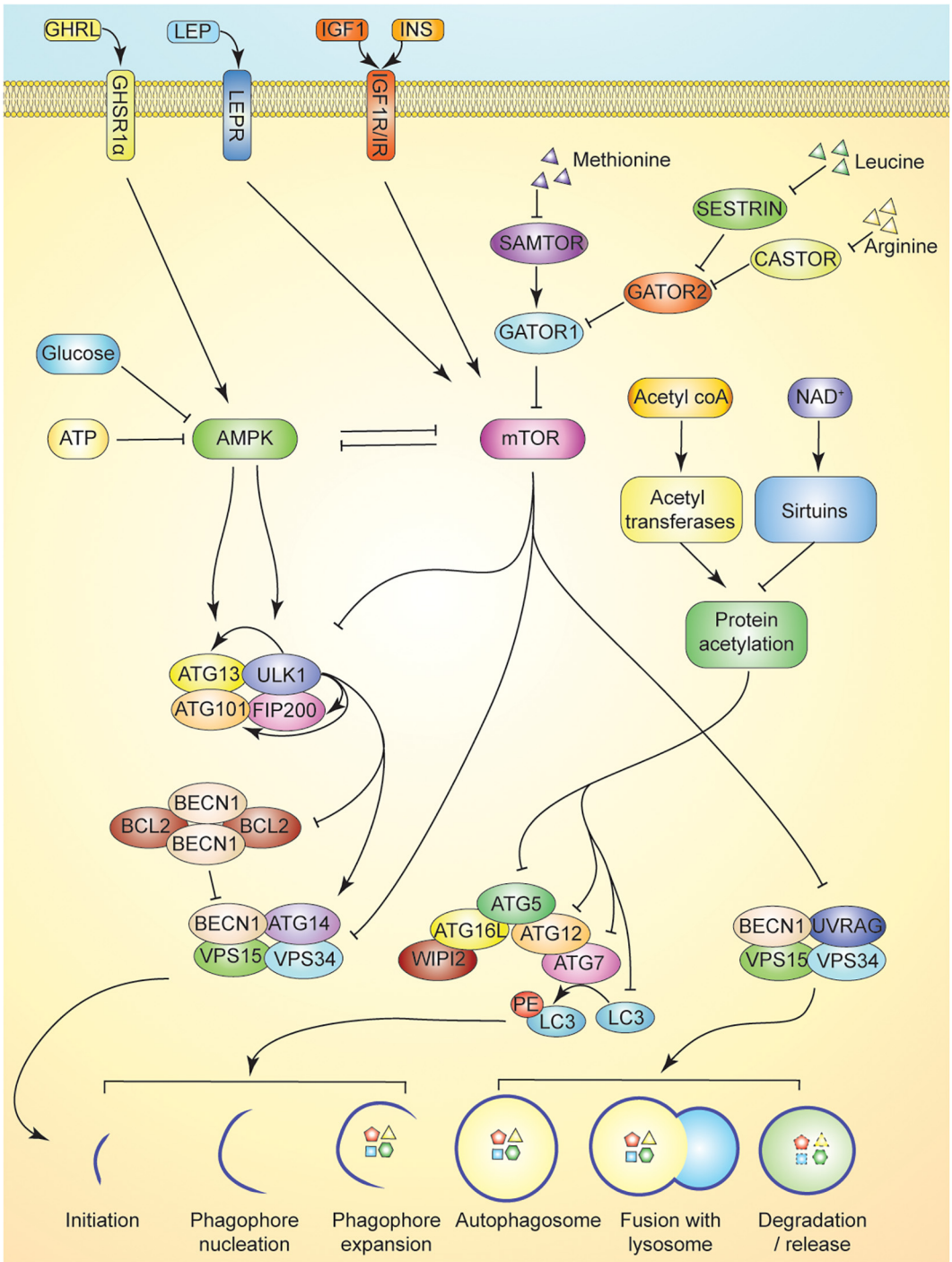

FIGURE 1 | Overview of autophagy and its regulation. Nutrient sensing in autophagy induction is multifaceted. Activation of the ULK serine threonine kinase complex induces autophagy by promoting release of BECN1 from BCL2 inhibitory heterotetramers, and promoting the association of BECN1 with ATG14, VSP15, and VSP34 in Class III PI3K complex I. This complex is responsible for initiating isolation membrane formation. AMPK activation in response to cellular energy status activates ULK complex by phosphorylation of ULK1 and ATG13. Activation of autophagy is antagonized by mTOR inhibition of AMPK, ULK complex, and Class III PI3K complex I and II. mTOR activity is maintained by intracellular leucine, arginine, and methionine levels. Leucine and arginine inhibit SESTRIN and CASTOR, respectively, to promote GATOR2 inhibition of GATOR1, a key negative regulator of mTOR activity. Methionine, through production of SAM inhibits SAMTOR to suppress GATOR1 activity. Activation of growth factor signaling via hormones upstream of mTOR (e.g., leptin, insulin, and IGF1) further suppresses autophagy. GHRL signaling in contrast can activate AMPK to promote autophagy. Maintenance of protein acetylation by acetyltransferases is enabled by ready supply of acetyl-coA and suppresses the activity of ATG5-ATG12 complex further limiting autophagy induction. Activation of sirtuins by elevation of NAD^+ levels promotes autophagy by reducing such inhibitory acetylation and enabling ATG5-ATG12 complex to lipidate LC3.

produced by pancreatic β-cells, is released in response to elevated blood glucose (Braun et al., 2011). Hyperglycemia is a hallmark of metabolic syndrome and is associated with insulin resistance, aberrant glucose metabolism, chronic inflammation, and the production of other metabolic hormones such as IGF1, leptin, and adiponectin (Braun et al., 2011). IGF1, a peptide growth factor produced primarily by the liver, is typically bound to IGF binding proteins (IGFBPs), which regulate the amount of free IGF1 bioavailable to bind to the IGF1 receptor (IGF1R) to induce growth or survival signaling (Pollak, 2012). In metabolic syndrome, the amount of bioavailable IGF1 increases via hyperglycemia-induced suppression of IGFBP synthesis and/or hyperinsulinemia-induced promotion of hepatic GH receptor expression and IGF1 synthesis (Braun et al., 2011). Elevated circulating IGF1 is an established risk factor for many cancer types (Pollak, 2012).

Autophagy is initiated by the activation of Unc-51 like autophagy activating kinase 1 (ULK1). Upon activation, ULK1 phosphorylates autophagy-related protein 13 (ATG13) and focal adhesion kinase family interacting protein of 200 kD (FIP200), promoting the association of a protein complex involving ULK1 and the non-catalytic subunits ATG13, FIP200, and ATG101 (Hurley and Young, 2017). This ULK1 signaling complex links cellular energy status with autophagy induction, as AMPK activates the complex by binding and phosphorylating ULK1 on S317 and S777, while mTOR phosphorylates S757, blocking ULK1 association with AMPK (Kim et al., 2011). Thus, ULK1 signaling is responsive to both ATP levels (through AMPK) and amino acid levels (through mTOR) (Mihaylova and Shaw, 2011; Meijer et al., 2015).

Activation of the ULK1 complex initiates the formation of the phagophore, which requires translocation of the complex to an endoplasmic reticulum domain enriched for the lipid phosphatidylinositol 3-phosphate [PI(3)P] (Axe et al., 2008; Itakura and Mizushima, 2010). ULK1 also promotes the activation of Beclin 1 (BECN1)-containing PI3K class III complexes by disrupting the formation of inhibitory BECN1/BCL2 heterotetramers (Pattingre et al., 2005). Two distinct PI3K complexes, consisting of BECN1, vacuolar protein sorting protein 15 (VPS15), VPS34 and either ATG14 (complex I) or UV radiation resistance-associated gene protein (UVRAG) (complex II), are critical to phagophore initiation and autophagosome maturation, respectively (Backer, 2016). BECN1 and ATG14 on the PI3K class III complex I are phosphorylated by ULK1, activating the complex. Activation and recruitment of PI3K complex 1 to the site of autophagosome formation drives nucleation of the phagophore membrane and generation of PI(3)P, which is essential for recruiting additional ATG proteins and PI(3)P effectors, such as WIPI (Lamb et al., 2013). PI3K class III complex II promotes downstream fusion of the autophagosome with an endosome-lysosome, resulting in the breakdown of sequestered cellular components (Liang et al., 2008). mTOR directly inhibits the lipid kinase activity of both PI3K class III complexes through phosphorylation of ATG14 and UVRAG (Yuan et al., 2013; Kim Y.M. et al., 2015).

Following nucleation of the phagophore via the PI3K class III complex I, two conjugation systems involving ubiquitin-like proteins associate with the membrane to aid in phagophore expansion and autophagosome formation. ATG12 is first activated by ATG7 before binding irreversibly to ATG5, which interacts further with a small coiled-coil protein, ATG16, to form the larger ATG12-ATG5-ATG16L complex (Mizushima et al., 2011). The complex is recruited to the phagophore membrane and functions as an E3-like ligase to mediate the lipidation of microtubule-associated protein light chain 3 (LC3) with phosphatidylethanolamine (PE) (Fujita et al., 2008). LC3-PE can be localized to both the inner and outer membranes of the autophagosome, and upon autophagosome maturation, the lipidated LC3 on the outer membrane gets deconjugated by Atg4 (Chen and Klionsky, 2011). The ATG proteins then dissociate from the membrane before its closure into an autophagosome, while lipidated LC3 remains attached to the inner surface of the autophagosome. LC3 is believed to aid in expansion and closure of the isolation membrane, and is a widely used marker for identifying autophagosomes and monitoring autophagic flux (Tanida et al., 2004). LC3 also serves as a binding motif for multiple mitophagy-associated receptors such as BNIP3 and FUNDC1, allowing for delivery of the autophagosome membrane to the mitochondria for receptor-mediated mitophagy (Liu et al., 2012; Wu et al., 2014). LC3 also plays a critical role in ubiquitin mediated autophagy/mitophagy, where it binds LC3 interacting region (LIR) motifs of proteins such as p62 (SQSTM1, Sequestosome 1), OPTN (Optineurin), and NBR1(NBR1 Autophagy Cargo Receptor) which serve as a bridge between ubiquitinated cargo and autophagy machinery (Chen et al., 2019).

AUTOPHAGY AND CANCER

Autophagy in Cancer Initiation

Basal autophagy exerts a protective role in suppressing malignant transformation and early tumorigenesis by regulating cellular homeostasis and metabolism through the degradation of intracellular components (Yun and Lee, 2018). Autophagy was initially thought to be a tumor suppressive mechanism because BECN1, key in phagophore formation, is a haploinsufficient tumor suppressor with monoallelic loss in several human breast, prostate, and ovarian cancers (Liang et al., 1999; Qu et al., 2003). However, this finding is confounded by the location of *BECN1* adjacent to the well established tumor suppressor breast cancer 1, early onset (*BRCA1*) on chromosome 17q21. Nonetheless, the cellular "quality control" resulting from the unfolded protein response, preservation of genomic stability, and prevention of reactive oxygen species (ROS) accumulation point to autophagy as a mechanism suppressing cancer initiation (Bhutia et al., 2013; Yun and Lee, 2018). Cancer cells exhibit reduced proteolysis or autophagic activity when compared with non-transformed cells (Gunn et al., 1977; Kisen et al., 1993; Bhutia et al., 2013).

Although human cancers largely lack evidence of genetic inactivation of core autophagy machinery, various murine models have revealed that knockouts of key autophagic genes promote tumorigenesis (Amaravadi et al., 2016). In addition

to its role in initiating autophagy (Vega-Rubín-de-Celis, 2019), BECN1 is essential for early embryonic development and regulates growth factor receptor signaling (Yue et al., 2003; Rohatgi and Shaw, 2016). As a result, biallelic deletions of BECN1 cannot be studied because of lethality in animal models. In a model of immortalized mouse mammary epithelial cells (IMMECs) in nude mice, monoallelic BECN1 loss increased sensitization to metabolic stress, induced DNA damage response, and stimulated gene amplification in support of mammary tumorigenesis (Karantza-Wadsworth et al., 2007). Furthermore, *BECN1* overexpression in MCF7 breast carcinoma cells reduced tumorigenesis in nude mice (Liang et al., 1999). Moreover, *BECN1* heterozygosity in MMTV-*Wnt1* mice revealed increased WNT-1 driven mammary tumorigenesis compared with wildtype controls (Cicchini et al., 2014). Similarly, mice with either a monoallelic deletion for autophagy and Beclin1 regulator 1 (*Ambra1*) or a biallelic deletion for SH3 Domain Containing GRB2 Like, Endophilin B1 (*SH3GLB1*, aka *Bif-1*) revealed higher rates of spontaneous tumor incidence (Cianfanelli et al., 2015). UVRAG, another critical autophagy protein, is a component of Class III PI3K complex II, and activates BECN1 to enable phagophore formation (Liang et al., 2006). Mutated UVRAG has been reported to suppress autophagy and promote tumor growth in colorectal cancers (He et al., 2015).

The role of autophagy in early breast tumorigenesis remains unresolved. Murine models of hereditary breast cancer showed that monoallelic loss of *BECN1* reduces tumorigenesis and facilitates p53 induction (Huo et al., 2013). Similarly, *Palb2*$^{f/f}$; *Wap-cre* mice with monoallelic loss of *BECN1 (BECN1*$^{\pm}$) experienced a significant delay in mammary tumor formation compared with mice homozygous in *BECN1* expression (Huo et al., 2013). Additionally, Gong et al. (2013) showed that BECN1 is essential for the tumorigenicity of breast cancer stem-like cells. BECN1 competes with myeloid cell leukemia sequence 1 (MCL1), an antiapoptotic BCL2 family member, for stabilization by a common deubiquitinase, USP9X (Elgendy et al., 2014). Accordingly, loss of BECN1 may result in MCL1 accumulation (Elgendy et al., 2014). Hence, BECN1 may regulate breast cancer initiation through an autophagy-independent pathway.

Genetic disruption of other autophagy-related genes has also revealed autophagy-associated regulation of cancer initiation in other cancer types. ATG7 is essential for hematopoietic stem cell (HSC) maintenance, and deleting ATG7 in LSK (Lin^{-}Sca-1^{+}c-Kit^{+}) cells resulted in HSC dysfunction, increased DNA damage, elevated reactive oxygen species (ROS), and myeloproliferation. Histologically, the infiltrating myeloid cells in these ATG7-deficient mice reportedly bear semblance to acute myeloid leukemia (Mortensen et al., 2011). Additionally, mice with systemic mosaic deletion of *Atg5* and liver specific *Atg7* deletion develop benign liver tumors more frequently than wildtype control mice (Takamura et al., 2011). Impairing autophagy through *in vivo* tissue-specific deletion of *Atg7* in pancreatic epithelial cells revealed increased inflammation, ROS accumulation, and mitochondrial damage, markers of oxidative stress that are well known risk factors for promoting cancer initiation (Antonucci et al., 2015).

Autophagy may also protect against cancer via suppression of oxidative stress via modulation of nuclear factor erythroid 2-related factor 2 (Nfe2l2/Nrf2)/kelch-like ECH-associated protein 1 (Keap1) and SQSTM1/p62 pathway (Komatsu et al., 2010; Jiang et al., 2015). p62 is a selective substrate of autophagy and cargo adapter that can disturb the Nfe2l2-Keap1 association, leading to the selective degradation of Keap1 and translocation of Nfe2l2 to activate antioxidant stress response genes (Taguchi et al., 2012). Under normal conditions, p62 is degraded by autophagy via its LC3 interaction region (LIR), but impaired autophagy leads to the accumulation of oncogenic p62 aggregates (Pankiv et al., 2007). Thus, the connection of oxidative stress to cancer promotion and the abnormal accumulation of p62 in several breast (Thompson et al., 2003; Li S. S. et al., 2017) and other cancers (Kitamura et al., 2006; Inoue et al., 2012; Saito et al., 2016) may in part explain the tumor suppressive effects of autophagy.

Autophagy Dependence in Cancer Malignancy and Response to Therapy

In contrast to the protective role of autophagy in maintaining function and integrity in normal cells, following transformation, autophagy promotes progression and metastasis in several cancer types, thus revealing the "double edged" role of autophagy in cancer (Huo et al., 2013). In established tumors, autophagy may also act as an essential adaptive response to promote growth and overcome cellular stressors (White, 2015). For example, a variety of human cancers with mutations in the oncogene *Ras*—including pancreatic ductal adenocarcinoma (PDAC), bladder, large cell lung, colon, and prostate cancers—have high levels of basal autophagy *in vivo* even under nutrient-replete conditions, and are subsequently more sensitive to pharmacological autophagy inhibition (Guo et al., 2011). Constitutive activation of the GTPase KRAS promotes mitogen-activated protein kinase (MAPK) signaling as well as increased dependence on autophagy (Guo et al., 2011). Some types of Ras-driven cancers, such as those with *H-Ras*^{V12} or *K-Ras*^{V12} mutations, display up-regulated levels of basal autophagy despite active mTORC1 (Grotemeier et al., 2010; Guo et al., 2011). Signaling through the Ras/Extracellular Signal-Regulated Kinase (ERK) pathway also induces autophagy through BECN1 (Grant, 2008; Mendoza et al., 2011; Wong et al., 2015; Butler et al., 2017). These cancers are considered "autophagy addicted," as they not only require autophagy in the absence of nutrients but also depend on autophagy for tumor growth (Guo et al., 2011). In these tumors, mTOR can be bypassed as a regulator of autophagy (Perera et al., 2015; Wong et al., 2015).

An illustrative example of *Ras*-driven autophagy addiction is PDAC, a highly aggressive cancer with a near 100% KRAS mutation frequency and a 5-year survival rate of less than 5 percent (Siegel et al., 2018; Waters and Der, 2018). To investigate the interplay between autophagy and *Ras*-mediated tumorigenesis, Guo et al. (2011) transduced non-tumorigenic immortal baby mouse kidney cells (iBMK) with *H-ras*^{V12} or *K-ras*^{V12} and evaluated tumor growth in the presence or absence of

the key autophagy genes *Atg5* and *Atg7*. The chronic impairment of autophagy significantly reduced tumor formation in nude mice (Guo et al., 2011). Interestingly, in $KRAS^{G12D}$-driven humanized mouse models of pancreatic ductal adenocarcinoma (PDAC), deletion of *Atg5* or *Atg7* leads to development of premalignant pancreatic lesions, while preventing further progression to malignancy (Rosenfeldt et al., 2013; Yang et al., 2014).

In HER2-positive breast cancer, the precise role of autophagy in tumorigenesis and tumor progression is currently being investigated. Recent work has demonstrated that HER2-positive breast cancer cells utilize lower levels of basal autophagy compared to HER2-negative breast cancers under normal conditions, but under stressed conditions, induce autophagy to a greater extent (Bortnik et al., 2016). This differential induction of autophagy was mediated in part through activation of ATG4B, a protease that cleaves pro-LC3B to form LC3-I during autophagosome formation (Bortnik et al., 2016). Interestingly, a recent study by Vega-Rubin-de-Celis et al. (2018) demonstrated a novel mechanism of autophagy suppression via interaction of HER2 with BECN1. HER2-positive breast cancer patients with allelic loss of *BECN1* have worse clinical prognosis, suggesting that suppression of autophagy through this interaction may have pro-tumorigenic effects. Indeed, disruption of this interaction using a small molecule, Tat-Beclin 1, in mice bearing BT-474-VH2 xenografts resulted in increased autophagy induction and reduced tumor progression as effectively as treatment with the tyrosine kinase inhibitor lapatinib (Vega-Rubin-de-Celis et al., 2018).

Autophagy plays a role in nearly every phase of the metastatic cascade, including modulation of tumor cell motility and invasion, cancer stem cell viability and differentiation, resistance to anoikis, epithelial-to-mesenchymal transition, tumor cell dormancy and escape from immune surveillance, and establishment of the pre-metastatic niche (for a recent review on this topic the reader is referred to Mowers et al., 2017). Importantly, autophagy is also upregulated in response to stressful stimuli such as DNA damage induced by cytotoxic agents, contributing to treatment resistance (Kroemer et al., 2010). For example, increased autophagy induction in response to treatment with the HER2-directed therapies trastuzumab and lapatinib has been implicated as a mechanism of drug resistance. Compared to trastuzumab-sensitive SKBR3 breast cancer cells, trastuzumab-resistant JIMT-1 cells constitutively utilize higher levels of autophagy in order to sustain proliferative activities (Cufi et al., 2013). Similarly, treatment of HER2-positive cells with lapatinib has been shown to increase autophagy induction, which, if sustained, allows cells to survive and develop drug-resistance (Tang et al., 2012; Lozy et al., 2014; Chen et al., 2016).

PROMOTION OF AUTOPHAGY THROUGH NUTRIENT OR ENERGY RESTRICTION

Collectively, the studies described above highlight the context-dependent role of autophagy in cancer incidence and progression. Thus, it may be unsurprising that both autophagy induction and autophagy inhibition have shown promise as viable therapeutic strategies for improving cancer outcomes. Evidence for autophagy induction achieved through nutrient or energy restriction is described below.

Approaches for and Cellular Impact of Dietary Energy Restriction

Approaches to restricting dietary energy intake include caloric restriction (CR) and fasting. CR is a dietary manipulation which decreases typical (*ad libitum*) caloric intake by 20–40% without incurring malnutrition (Mitchell et al., 2015) and has potent anticancer effects in both developing and established cancer (O'Flanagan et al., 2017). On the other hand, fasting involves short term reduction of caloric intake to 0–500 calories for defined intervals of time, typically while consuming water alone, or, in the case of partial fasting regimens, consuming vegetable broths and/or fruit juices (Wilhelmi, de Toledo et al., 2013). Intermittent fasting regimens involve cycles of short-term reduction of caloric intake in intervals ranging from 1 to 3 days per week. This method encompasses protocols for whole-day fasting, time-restricted feeding, and alternate-day fasting (Tinsley and La Bounty, 2015). Whole day fasting indicates total deprivation from caloric intake for periods typically ranging from 24 to 48 h per week, either consecutively or non-consecutively, with *ad libitum* feeding on remaining days (Tinsley and La Bounty, 2015). Time-restricted feeding regimens define consecutive periods of *ad libitum* feeding that range from 3 to 12 h per day with complete fasting during the remaining hours. Intermittent fasting can also be achieved through alternate day fasting or by following the 5:2 diet. In clinical and preclinical protocols for the 5:2 diet, caloric consumption is restricted to approximately 25% of energetic needs on fasting days, with *ad libitum* feeding on the remaining days of the feeding cycle (Patterson et al., 2015).

Under conditions of low nutrient availability, such as those that occur during fasting, autophagic flux is increased in normal and malignant cells to liberate metabolic substrates via degradation of intracellular structures such as damaged proteins and mitochondria. For example, during fasting periods of 12–24 h, mice experience an induction of autophagy in several tissues, including the liver, kidney, and neurons (Komatsu et al., 2005; Alirezaei et al., 2010; Takagi et al., 2016). Specifically, fasting potently activates AMPK in multiple tissues, including skeletal muscle, adipocytes, and the hypothalamus (**Figure 2**; Kajita et al., 2008; Lim et al., 2010; Bujak et al., 2015). Interestingly, endocrine signaling involving ghrelin, a gut-brain peptide upregulated during periods of fasting, has tissue-specific effects on AMPK activation, as ghrelin activates AMPK in neurons and the hypothalamus yet inhibits AMPK in cardiomyocytes (**Figure 2**; Toshinai et al., 2001; Andersson et al., 2004; Wang et al., 2014; Bayliss et al., 2016).

Both the ATG12- ATG5- ATG16L and LC3 conjugation systems are regulated by protein acetylation status—further tying autophagic flux to cellular energy and nutrient status (Bánréti et al., 2013). High levels of acetyl CoA, characteristic of a fed, high-energy state, repress autophagy through acetylation of

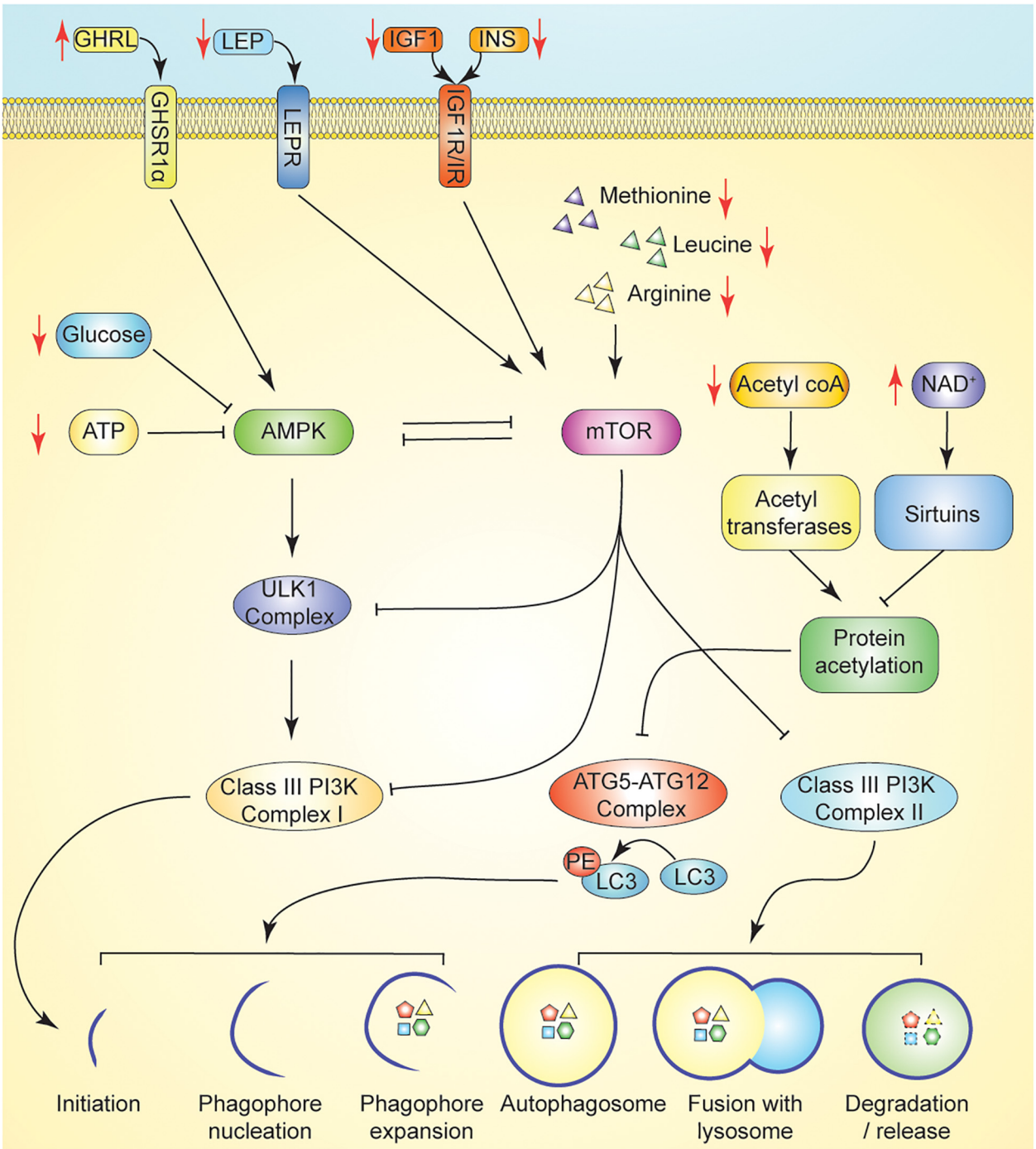

FIGURE 2 | Promotion of autophagy through nutrient or energy restriction. Caloric restriction (CR) suppresses insulin, IGF1, and leptin, each of which suppress autophagy via activation of mTOR following binding to their cognate receptor. Induction of ghrelin by CR promotes activation of AMPK to promote autophagy. Dietary energy modulation by CR limits availability of key nutrient regulators of autophagy including amino acids and glucose. In CR, AMPK signaling is induced in response to reduced ATP and/or glucose concentrations. ATG5-ATG12 complex activity is regulated by the availability of acetyl-coA and the activity of deacetylases. In CR, reduced acetyl-coA limits protein acetylation by reducing substrate availability for acetyltransferases. CR induced increase in NAD + levels promote the activity of sirtuins. Key: Black lines reflect regulation at basal states; Black arrows reflect activation, while black T bars reflect inhibition. Red arrows reflect changes in hormone/metabolite availability induced by fasting which may modulate autophagy.

ATG5, ATG7, ATG12, and LC3 by the p300 acetyltransferase (Lee and Finkel, 2009; Mariño et al., 2014). Conversely, increased expression and activity of the NAD^+-dependent sirtuin 1 (sirt1), inducible by caloric restriction, stimulates autophagy via direct deacetylation of the Atg and LC3 machinery (**Figure 2**; Cohen et al., 2004; Lee et al., 2008). Fasting and caloric restriction result in an increase in the cellular NAD^+/NADH ratio, resulting in high concentrations of the NAD^+ substrate necessary for sirtuin activity (Hayashida et al., 2010). Other sirtuins regulate autophagy indirectly. Sirt2 has been implicated in autophagy modulation through its role in inactivating cytosolic FoxO1, which, under starvation conditions, disassociates from Sirt2 and promotes autophagy via acetylation of lysine residues on Atg7 (Zhao et al., 2010). Sirt3, the primary mitochondrial histone deacetylase, plays a key role in oxidative stress homeostasis through its role in deacetylation of superoxide dismutase 2 (SOD2), a major mitochondrial antioxidant enzyme. Caloric restriction and oxidative stress increase the expression of Sirt3, which is now recognized as a critical component of multiple autophagy inducing pathways (Qiu et al., 2010; Pi et al., 2015; Shi et al., 2015; Liu et al., 2018).

Systemic Impact of Dietary Energy Restriction

CR and fasting promote longevity in model organisms via reprogramming of endocrine signaling and systemic metabolism, reducing exposure to oxidative stress, and improved mitochondrial function (Michalsen and Li, 2013). Autophagy has been implicated in CR-mediated effects on longevity, and animal models have also demonstrated that this induction of autophagy is necessary for survival during fasting, as it is required to prevent fatal hypoglycemia and cachexia (Karsli-Uzunbas et al., 2014). In the context of cancer, chronic CR has demonstrated tumor suppressive effects in breast, colon, and pancreatic cancers in animal models (Brandhorst et al., 2015; Di Biase et al., 2016; Rossi et al., 2017).

In rodents, fasting and CR modulate similar metabolic targets, but elicit distinct physiological responses (Lee and Longo, 2011). During periods of fasting, serum glucose levels decrease and hepatic glycogen stores diminish within 24 h (Longo and Mattson, 2014). Alternative metabolic pathways are upregulated to provide substrates for energy utilization; for example, gluconeogenesis is activated to provide glucose to specific tissues, primarily the brain. Additionally, β-oxidation of free fatty acids released from adipose tissue is upregulated, while the ketone bodies β-hydroxybutyrate and acetoacetate, released as a byproduct of β-oxidation and from the conversion of ketogenic amino acids, are utilized in the process of ketolysis (Longo and Mattson, 2014). Fasting also results in pronounced endocrine changes, as discussed below. In mice, intermittent fasting regimens are modeled by completely removing food for approximately 24–48 h every 5–7 days (Longo and Mattson, 2014). This intervention decreases fasting insulin, glucose concentrations, total plasma cholesterol, and triglycerides as effectively as continuous CR (Varady et al., 2007). Within a 48 h fasting period, blood glucose decreases by roughly 50% (Jensen et al., 2013).

Metabolic benefits from CR and/or fasting have also been demonstrated in humans. Adherence to these dietary restriction protocols promotes modest weight loss and reductions in total plasma cholesterol and triglyceride concentrations, glucose, and low-density lipoprotein cholesterol (Johnson et al., 2007; Klempel et al., 2013; Rothschild et al., 2014). For example, a 48 h fast in rodents results in weight loss of approximately 20 percent of total body weight, compared to a 4 day fast in humans which results in less than 2 percent weight loss (Pietrocola et al., 2017b). While blood glucose levels in humans decrease after 2 days in the fasted state, clinically acceptable glucose levels are maintained within this period (Lieberman et al., 2008). Additionally, in humans and mice, IGF1 levels decrease by approximately 30 and 70 percent, respectively, during periods of fasting ranging from 24 to 72 h (Dorff et al., 2016). Alternatively, IGF1 decreases by 25 percent with continuous CR in mice, but in humans does not decline unless CR is also accompanied by restriction of protein intake (Lee and Longo, 2011). An intermittent fasting regimen that restricted calories by 85% on alternate fasting days in mice resulted in decreases in IGF1, leptin, and visceral fat, and increased levels of adiponectin (Varady et al., 2009). Thus, a variety of dietary energy restriction approaches are available to reduce circulating IGF1.

A reduction in circulating IGF1 and insulin levels in humans may result in increased autophagic flux through downregulation of the PI3K/AKT/mTOR pathway (**Figure 2**; Thissen et al., 1994). Leptin, an adipokine, is another known regulator of energy expenditure and neuroendocrine signaling, and is associated with cancer progression (Garofalo and Surmacz, 2006). Leptin has tissue-specific effects on autophagy; however, it is predominantly associated with autophagy inhibition via the PI3K/Akt/mTOR signaling pathway (Maya-Monteiro and Bozza, 2008; Wang et al., 2012; Cassano et al., 2014). In both obese and normal weight humans, fasting and CR also decrease serum concentrations of leptin, consistent with its classical role as a satiety hormone (Boden et al., 1996; Weigle et al., 1997; Rogozina et al., 2011).

Intermittent fasting regimens have not consistently been demonstrated to improve insulin and glucose control (Patterson et al., 2015). One study that compared the metabolic impact of intermittent CR (2 days per week) vs. continuous CR (7 days per week) in overweight, premenopausal women demonstrated that intermittent CR resulted in a greater reduction in fasting insulin levels and insulin resistance (Harvie et al., 2011). In both interventions, similar decreases in leptin, C-reactive protein, LDL cholesterol, and triglycerides were achieved, but the differences in glycemic control that followed adherence to each respective regimen indicate that different mechanisms may be driving the metabolic alterations (Harvie et al., 2011).

Dietary Energy Restriction During Cancer Therapy

In response to fasting and fasting-mimicking diets, normal cells enter a state characterized by decreased cellular division, reduced metabolic activity, and increased utilization of repair pathways,

resulting in chemo-protective effects (Raffaghello et al., 2008; Lee et al., 2010). Decreased levels of bioavailable serum IGF1 and reduced activation of the PI3K/Akt/mTOR axis are implicated in both the longevity effects of CR as well as this fasting-induced stress resistance in normal cells (Raffaghello et al., 2008; Lee et al., 2010). Conversely, as malignant cells are unable to downregulate their oncogene-driven metabolic programs, their sensitivity to chemotherapeutics is retained or even increased following bouts of short-term fasting, resulting in destruction of cancer cells by chemotherapy in a phenomenon termed *differential stress-sensitization* (Raffaghello et al., 2008; Lee et al., 2010).

Though the impact of dietary energy restriction on cancer progression in humans has not yet been fully characterized, interventions which reduce caloric intake during cytotoxic chemotherapy may improve therapeutic efficacy while reducing undesirable side effects in untransformed cells (Buono and Longo, 2018). In humans, side effects from cytotoxic chemotherapies include nausea, vomiting, gastrointestinal inflammation, central and peripheral neurotoxicity and neuropathy, bone marrow toxicities such as myelosuppression and febrile neutropenia, and long-term sequelae including cardiovascular disease and increased risk of secondary malignancies (Nurgali et al., 2018). These side effects are non-trivial and may result in physical and emotional stress that poses an obstacle to treatment, negatively influencing patient outcomes.

Numerous short-term fasting protocols, including intermittent fasting, periodic fasting, and fasting-mimicking diets, have been tested for their ability to improve efficacy and tolerability of chemotherapy cycles (Brandhorst and Longo, 2016). Unlike intermittent fasting, periodic fasting regimens last for 3 days or longer and are repeated every 2 or more weeks, while fasting-mimicking regimens use a plant-based low carbohydrate and low-protein diet that is indicated for use every 3 to 4 weeks (Longo and Mattson, 2014; Brandhorst et al., 2015). There are numerous clinical trials registered on ClinicalTrials.gov investigating the impact of fasting or other dietary energy restriction approaches on response to chemotherapy across a wide variety of cancer types. Most of these trials to date have focused on tolerability of the fasting or fasting-mimicking regimen in combination with chemotherapy as well as measurable side effects in human subjects. We will discuss below representative trials for which final or interim results have been peer-reviewed for publication or submitted as abstracts for presentation at major conferences.

Bauersfeld et al. (2018) conducted a randomized, individually controlled cross-over trial wherein subjects with breast and ovarian cancers underwent a modified fasting protocol for multiple 60 h periods over the course of three out of six cycles of chemotherapy (36 h before to 24 h after the chemotherapy; subjects were fasted during either the first three cycles or the second three cycles). Subjects were allowed a maximum daily intake of intake of 350 kcal during fasting periods and reported improved quality-of-life and reduced self-reported fatigue following therapy when therapy was administered during a fasting period (Bauersfeld et al., 2018). Greater benefit was perceived when subjects were fasted during the first three chemotherapy cycles as opposed to the second three cycles (Bauersfeld et al., 2018).

Cheng et al. (2014) reported a protective effect of prolonged fasting cycles during chemotherapy against chemotherapy-induced myelosuppression in mice, as well as preliminary findings suggesting myeloprotective effects of fasting in humans. Similarly, de Groot et al. (2015) investigated whether fasting for 24 h before receiving (neo) adjuvant TAC-chemotherapy therapy and for a subsequent 24 h after completing therapy could reduce hematological toxicity in subjects with stage II and III HER2-negative BC, using γ-H2AX in peripheral blood mononuclear cells (PBMCs) as a proxy marker for chemotherapy toxicity in normal somatic cells. No significant differences were observed in the frequency of grade I, II, III, or IV side effects due to fasting; however, fasted subjects experienced attenuated bone marrow toxicity as well as a smaller and less consistent increase in markers of chemotherapy-induced DNA damage in PBMCs compared to non-fasted subjects (de Groot et al., 2015). Of note, while fasting significantly reduced IGF1 as compared with baseline values, final IGF1 serum values did not differ across the two treatment arms (de Groot et al., 2015). Similar findings suggesting protection against bone marrow toxicity and DNA damage in circulating PBMCs were also reported following prolonged fasting (48–72 h) in subjects receiving platinum-based combination chemotherapy without concurrent radiation across a variety of cancer types (Dorff et al., 2016). Limitations of this study include a small sample size and the lack of a non-fasted control group (Dorff et al., 2016). Importantly, the safety of completely abstaining from food for periods of 2 or more days has been demonstrated in a medically-supervised setting with the majority of cancer patients experiencing minimal adverse reactions (de Groot et al., 2015; Bauersfeld et al., 2018; Finnell et al., 2018).

Taken together, the quantitative biomarker-based data available to support fasting-induced differential stress resistance in humans during chemotherapy is limited but compelling. Considering the importance of autophagy in protection against genotoxic insult and cellular transformation, future studies should address whether autophagy induction in normal cells underlies the reduced severity of chemotherapy-induced side effects and/or increased rate of cellular repair in normally functioning cells in response to dietary restriction.

Dietary Energy Restriction in Cancer Cachexia

Cancer cachexia is a catabolic wasting syndrome characterized by anorexia and progressive loss of muscle and adipose tissue mass (Aoyagi et al., 2015). The combination of hypermetabolism and the anorectic effect of elevated IL-6 contribute to a chronic caloric deficit of approximately 200-450 kcals per day in weight-losing patients with cachexia (Kumar et al., 2010; White, 2017). Elevated IL-6 also leads to the release of glucocorticoids, which contribute to muscle wasting in cancer cachexia (White, 2017). While parenteral nutritional supplementation provides some benefit, as does enteral tube feeding (Amano et al., 2020), there is little effect on mortality in response to oral dietary supplementation (Baldwin et al., 2012).

Given the strong relationship between caloric intake and mortality in patients with cachexia, CR/fasting interventions in patients with advanced cancers may not be advised. Some of the metaboendocrine effects of CR—including improved insulin sensitivity, reduced leptin, and increased ghrelin—have been independently considered as approaches for intervention in cancer cachexia. For example, treatment with ghrelin has arisen as a promising treatment option in cancer cachexia, improving appetite, food consumption, and body composition (Khatib et al., 2018). While CR is associated with elevation of ghrelin, by definition of CR this elevation cannot translate into improved caloric intake. Additionally, low leptin predicts poor survival in cancer cachexia (Mondello et al., 2014), while induction of autophagy in response to CR contributes to muscle wasting in mouse models of cachexia (Penna et al., 2013, 2019).

Other approaches used to combat cachexia have included several immunomodulatory agents, which dampen proinflammatory signaling (Aoyagi et al., 2015). While inflammatory signaling pathways are reduced by CR, growth factor signaling is also reduced (Hursting et al., 2013); thus it is unclear whether the anti-inflammatory aspects of CR promote or impair retention of skeletal muscle mass, or perhaps even further exacerbate wasting. In an experimental model of cachexia, CR preserved grip strength but did not otherwise alter the course of cachexia (Levolger et al., 2018). It should also be noted that in this study CR was not compared against other protective interventions (Levolger et al., 2018). In summary, while CR may appear to promote a protective metaboendocrine state, limited evidence support a protective role for CR and much of the existing literature implicate CR as a potentially deleterious intervention in the context of cachexia. Thus, any consideration of CR or fasting in cancer therapy should include assessment of the patient's risk of cachexia.

PHARMACOLOGICAL AUTOPHAGY MODULATION AS AN APPROACH TO CANCER TREATMENT

Perturbing Growth Factor Signaling as a Mimetic of Dietary Energy Restriction

Reductions in circulating IGF1 may be an important driver of the potent anticancer effects of dietary restriction, as fasting and CR result in enhanced cancer cell apoptosis, reduced angiogenesis, and alterations in key metabolites and systemic signaling pathways downstream of IGF1/IGF1R (O'Flanagan et al., 2017). As a reduction in bioavailable IGF1 is a common theme in response to dietary energy restriction interventions, it is tempting to speculate that inhibitors of IGF1 signaling could be used as a metabolic reprogramming intervention and mimetic of energy restriction (**Figure 3**), yielding some of the protective effects of fasting on chemotherapy toxicity. IGF1 is a nutrient-sensitive endocrine hormone that is primarily secreted by the liver. Upon binding of IGF1 to its cognate receptor, insulin-like growth factor receptor 1 (IGF1R), autophosphorylation events lead to the activation of two signaling axes—MAPK and PI3K (class I)/AKT/mTOR—which promote increased cell proliferation, inhibition of autophagy, and evasion of cell death (Meynet and Ricci, 2014; O'Flanagan et al., 2017). Human studies have demonstrated that modest protein restriction in a chronic CR regimen modulates anti-cancer effects associated with decreased IGF1 levels (Fontana et al., 2008). However, monoclonal antibodies directed at IGF1R have resulted in unexpected toxicity in human subjects when combined with chemotherapy (Langer et al., 2014; Di Cosimo et al., 2015; Baselga et al., 2017), while several small molecule inhibitors of IGF1R have not yielded clinical benefit when used as single agents in clinical trials (Fassnacht et al., 2015; Chiappori et al., 2016; Gradishar et al., 2016; Bergqvist et al., 2017). Yet, small molecule IGF1R inhibitors—as well as inhibitors of other components of the IGF1R pathway—may still hold clinical potential when used in combination therapies. For example, combination of AXL1717 (picropodophyllin), an IGF1R pathway inhibitor, with gemcitabine HCl and carboplatin yielded an acceptable toxicity profile in previously untreated, locally advanced, or metastatic NSCLC (Holgersson et al., 2015). Similarly, BMS-754807 is a reversible small molecule inhibitor of IGF1R and insulin receptor (IR) (Carboni et al., 2009) that has demonstrated effectiveness *in vitro* in combination with anti-cancer therapies for the treatments of breast, pancreatic, colon, lung, and gastric cancers (Carboni et al., 2009). IGF1R inhibition may also be an approach to tackling drug resistance in HER2-overexpressing breast cancers, as one of the potential mechanisms of resistance to trastuzumab occurs through upregulation of IGF1R and subsequent cross-phosphorylation and activation of HER2 (Chakraborty et al., 2017).

Direct inhibition of mTOR has also been investigated as an approach to manipulation of the PI3K/Akt/mTOR axis (**Figure 3**). Combination of the PI3K/mTOR inhibitor buparlisib with fulvestrant resulted in a significant increase in median progression-free survival yet an unacceptable toxicity profile in postmenopausal women with hormone-receptor-positive, HER2-negative, advanced breast cancer (Di Leo et al., 2018). However, mTOR inhibition via temsirolimus or everolimus, a derivative of rapamycin that inhibits activation of mTORC1 by binding to FKBP12 (also known as RAD001 or Affinitor), in combination with liposomal doxorubicin and bevacizumab was well tolerated and yielded an increase in objective response rate in patients with metaplastic TNBCs bearing PI3K/AKT/mTOR pathway aberrations (Basho et al., 2017).

Treatment with everolimus downregulates the nutrient-sensing effects of mTOR and results in reduced protein synthesis, cellular proliferation, and glucose uptake, as well as increased autophagic flux (Jobard et al., 2017). In HER2-positive breast cancer patients treated with trastuzumab plus everolimus, serum metabolomic analysis revealed that this combination modulated a physiological state similar to that which occurs during fasting where lipolysis and autophagy are upregulated and gluconeogenesis and glycogenolysis are decreased (Jobard et al., 2017). The combination of everolimus with HER2-directed therapies is also a promising approach to combat drug resistance. In drug resistant HER2-positive breast tumors, trastuzumab treatment increases phosphorylation of PDK1 and mTOR, which

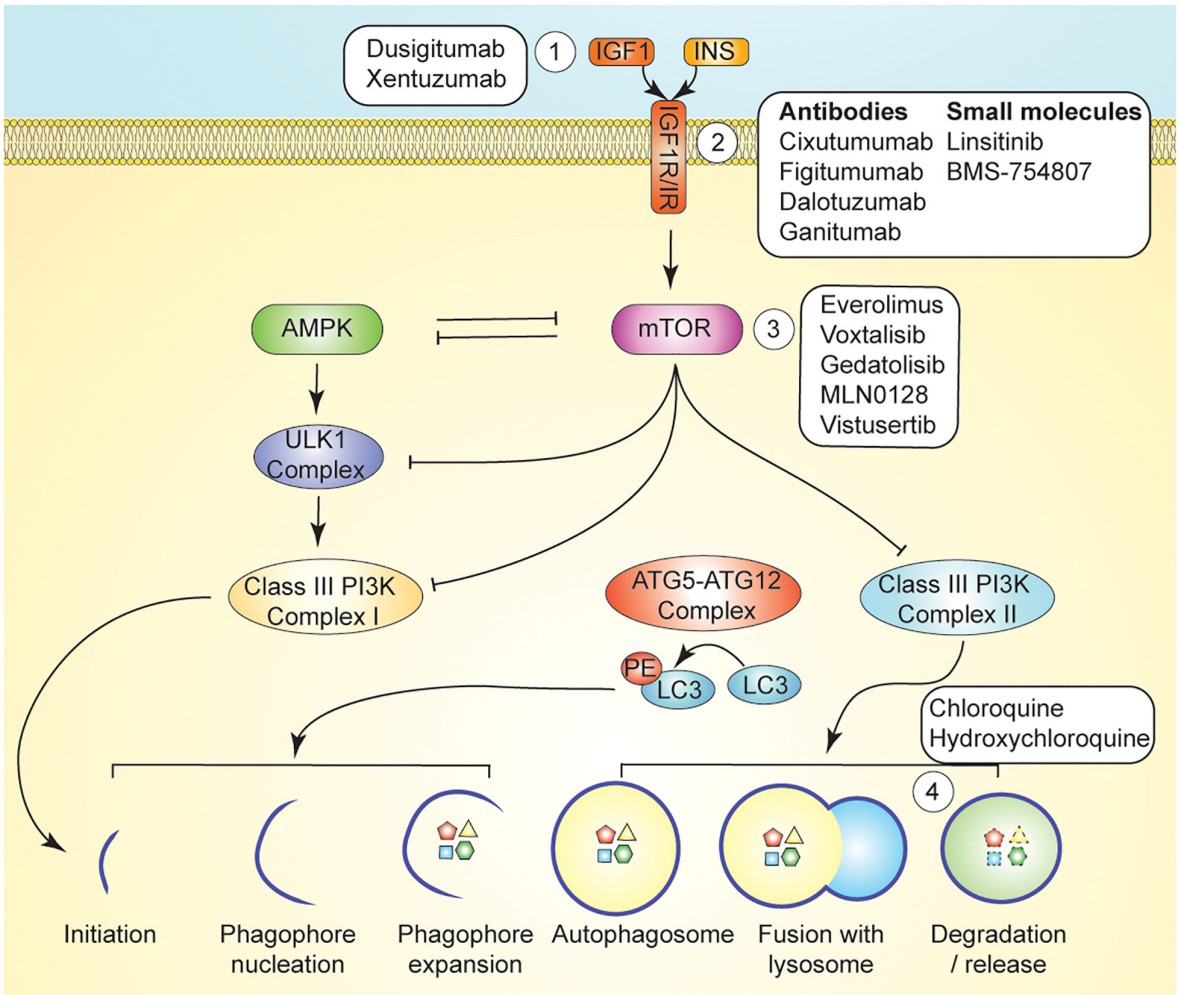

FIGURE 3 | Pharmacological Autophagy Modulation. mTOR signaling regulates induction of autophagy via inhibition of AMPK, ULK1, and Class III PI3K signaling. Autophagy can be induced by inhibition of growth factor signaling upstream of mTOR by ligand-targeting monoclonal antibodies (Kuma and Mizushima, 2010), receptor-targeting monoclonal antibodies, or small molecule inhibitors (e.g., AXL1717 and BMS-754807) (Mizushima et al., 2004). mTOR signaling is directly inhibited with compounds such as temsirolimus, and everolimus (Shaid et al., 2013). Autophagy can be inhibited with lysosomotropic agents such as chloroquine and hydroxychloroquine, which inhibit maturation of the autophagolysosome (Mizushima and Komatsu, 2011).

activates ribosomal protein S6 kinase beta-1 (S6K1) and promotes anabolic activities (Huynh et al., 2017). Blocking this escape pathway with everolimus is one approach to improve the efficacy of trastuzumab.

Metabolic Reprogramming Interventions (MRIs)

In addition to direct inhibition of growth factor signaling, a pharmacological strategy currently being investigated to treat cancer involves the combination of metabolic reprogramming interventions (MRIs) with traditional cytotoxic chemotherapies. These approaches are based on the identification of compounds that mimic the beneficial effects of caloric restriction without the need for challenging dietary changes. A subclass of MRIs is termed caloric restriction mimetics (CRMs), which induce a metabolic reprogramming in cancer cells intended to recapitulate the biochemical effects of dietary energy restriction.

CRMs exert their anticancer effects by increasing autophagic flux in response to a reduction in cellular protein acetylation (Madeo et al., 2014). This increase in autophagic flux results in an increase in extracellular ATP, a potent chemoattractant for professional phagocytes (Corriden and Insel, 2012), and therefore promotes immunogenic cell death. Pietrocola et al. (2016) provide compelling evidence that robust immunosurveillance and increased chemotherapeutic efficacy following nutrient deprivation are dependent upon an increase in cancer cell autophagy; the increased chemosensitivity and enhanced immunity were reversible upon intraperitoneal injection of recombinant IGF1). Importantly, success of antineoplastic therapies in the long-term is largely determined by their ability to reinstate robust and prolonged anticancer immunosurveillance (Kepp et al., 2014).

Pharmacological Inhibition of Autophagy

Despite gaps in our understanding of autophagy's complete role in cancer, pharmacological inhibition of autophagy is currently being investigated for potential use as adjuvant therapy, as inhibition of autophagy causes metabolic instability that can be exacerbated in combination with therapy (Pascolo, 2016). Chloroquine (CQ) is a pharmacological agent that indirectly inhibits autophagy by preventing endosomal acidification, resulting in inhibition of lysosomal enzymes that require an acidic pH and disrupting the maturation of the autophagolysosome (**Figure 3**; Solomon and Lee, 2009; Amaravadi et al., 2011). In preclinical models of Ras-driven pancreatic cancers, CQ has been shown to effectively reduce cell growth, tumorigenicity, and oxidative phosphorylation (Yang et al., 2011). Yang et al. (2011) demonstrated that CQ potently retards *in vitro* proliferation and anchorage-independent growth of several different human pancreatic cell lines. Moreover, CQ treatment significantly increased survival in a transgenic, Kras-driven murine model of PDAC and diminished *in vivo* growth of a human PDAC cell line in immunocompromised mice (Yang et al., 2011).

However promising, the translational relevance of these findings is limited. Subcutaneous injection of pancreatic cell lines precludes investigation into factors within the pancreatic tumor microenvironment that may hinder or promote tumor cell survival in the face of autophagy ablation. Similarly, the use of immunocompromised mice prevents identification of potentially important effects of autophagy inhibition on tumor immunosurveillance (Li Y. Y. et al., 2017; Pietrocola et al., 2017a). Of note, a phase II pharmacodynamic study that used the chloroquine analog hydroxychloroquine (HCQ, which has been shown to have decreased toxicity in humans compared to CQ) to inhibit autophagy in patients with metastatic PDAC showed no significance in progression-free survival (Wolpin et al., 2014).

Many chemotherapies—such as gemcitabine, which is commonly used to treat PDAC, or platinum-based compounds used in the treatment of primary and metastatic breast cancers—induce autophagic flux, and the putative cytoprotective roles of autophagy may limit the efficacy of chemotherapy (Donohue et al., 2013; Hashimoto et al., 2014; Jiang et al., 2017). Therefore, CQ in combination with chemotherapy may present an attractive therapeutic strategy to increase the cytotoxicity of treatment regimens (Hashimoto et al., 2014). Indeed, combination treatment with chloroquine and gemcitabine showed increased efficacy in delaying tumor growth of patient-derived PDAC xenografts relative to the use of either as a single agent (Balic et al., 2014). A phase I clinical trial in patients with metastatic or unresectable PDAC reported no dose-limiting toxicities following a combination of CQ and gemcitabine; furthermore, of the nine patients enrolled in the trial, 3 patients showed a partial response while two patients exhibited stable disease (Samaras et al., 2017). At the time of this review, the Abramson Cancer Center of the University of Pennsylvania is actively recruiting patients with advanced primary or metastatic PDAC to explore the efficacy of hydroxychloroquine in combination with gemcitabine or another chemotherapeutic agent (ClinicalTrials.gov: NCT01506973). Collectively these findings suggest that CQ and its analogs may have the potential to improve clinical outcomes in PDAC treatment when used in combination with current standard-of-care chemotherapy approaches.

Consistent with findings in PDAC, CQ-associated increases in therapeutic potency of chemotherapeutic agents have also been reported in preclinical TNBC studies. Gemcitabine induced mTOR-independent autophagy in MDA-MB-231 cells *in vitro*; accordingly, combination treatment with CQ and gemcitabine resulted in increased apoptotic cell counts compared to treatment with only gemcitabine (Chen et al., 2014). Similarly, a model of human TNBC using subcutaneous patient derived xenografts (PDX) in nude mice reported that the addition of CQ potentiated the effects of adriamycin and cyclophosphamide treatment by significantly reducing primary tumor size and multiplicity of lung metastases (Lefort et al., 2015).

Chloroquine and its analogs have also shown promise in situations of acquired therapeutic resistance, a frequent challenge faced in TNBC treatment (Kim et al., 2018). Compared with the parental MDA-MB-231 cell line, anthracycline-resistant MDA-MB-231 cells showed heightened levels of basal autophagy (Chittaranjan et al., 2014), prompting Chittaranjan et al. (2014) to test whether the use of autophagy inhibition could improve outcomes in cases of therapy resistance. Indeed, combination treatment with epirubicin and HCQ increased therapeutic efficacy by significantly reducing PDX tumor growth compared with saline controls and epirubicin alone (Chittaranjan et al., 2014). CQ in combination with carboplatin also reduced tumor growth in carboplatin-resistant TNBC orthotopic xenografts, potentially through depletion of cancer stem cells (Liang et al., 2016).

Additional Considerations of Autophagy Modulation

Several autophagy inhibitors are available, and their mechanisms, and potential for modulation of pathways other than autophagy, are distinct. In addition to its ability to prevent completion of the autophagic process, CQ has been implicated in tumor vessel normalization (Maes et al., 2014), suppression of macrophage endocytosis to improve nanoparticle delivery (Wolfram et al., 2017), and increased sensitivity to cisplatin in breast cancer cells (Maycotte et al., 2012), each of which were shown to occur via mechanisms independent of autophagy suppression. With this in mind, based on promising preclinical data, there are a growing number of clinical trials investigating the potential for use of CQ as adjuvant therapy, including a Phase II trial testing the efficacy and safety of CQ in combination with taxane or taxane-like chemotherapeutic agents in the treatment of advanced or metastatic BC who were non-responders to AC therapy (ClinicalTrials.gov; NCT01446016).

Perhaps in the context of chemotherapeutic resistance, an approach combining cancer therapies with interventions that increase dependence on autophagy (e.g., through manipulation of dietary energy intake or pharmacologic interventions such as MRIs or growth factor inhibition) will expose a metabolic

weakness that could be exploited with autophagy inhibitors. Indeed, the results of Lashinger et al. (2016) showed that conditions of CR in combination with genetic autophagy ablation in a *Ras*-driven model of pancreatic cancer had greater effects on decreasing tumor volume and progression than either condition in isolation. The effect of chemotherapy under these conditions was not explored. Combining autophagy induction and inhibition increased radiosensitivity of colorectal cancer cells in culture (Shiratori et al., 2019). However, a phase 1 trial combining the Akt inhibitor MK-2206 with hydroxychloroquine in patients with advanced solid tumors resulted in a substantial number of drug-related adverse events and minimal evidence of antitumor activity (Mehnert et al., 2019). Interestingly, use of these drugs in combination altered the pharmacokinetics of both drugs (Mehnert et al., 2019), which may have impacted toxicity. Perhaps in combination the dosages of these drugs should be reduced, or hydroxychloroquine should be tested in combination with other autophagy inducers.

Notably, some have reported that cancer cell autophagy is required for immunogenic cell death yet dispensable for chemotherapy-induced cell death (Michaud et al., 2011). Antunes et al. (2017) demonstrated increased chemotherapeutic efficacy following nutrient deprivation in an autophagy-independent manner. These findings argue for caution regarding the use of autophagy inhibitors in the absence of chemotherapeutic resistance, as an inhibition of primary tumor growth may be concomitant with impairment of anti-tumor immunosurveillance and an elevated risk of recurrence. Longitudinal resection studies in mice addressing the potential for recurrence following autophagy inhibition during treatment may be helpful in untangling this research question.

CONCLUSION

In sum, cancer cells often exhibit an increased dependence on autophagy, both for baseline survival and as a response to stressors such as chemotherapy or a decline in nutrient availability. Numerous hormonal and metabolic cues direct autophagic induction in cancer. There is evidence to suggest that the increased dependence on autophagy in cancer cells may be exploitable clinically by combining autophagy modulators with existing chemotherapies. Fasting appears to hold promise for reducing dose-limiting side effects of chemotherapy in humans. However, it remains unclear whether therapeutic agents aimed at autophagy induction, autophagy inhibition, or both are a viable therapeutic strategy for improving cancer outcomes. In light of the burgeoning interest in precision medicine, identification of oncogenic drivers associated with increased susceptibility to fasting, autophagy induction or inhibition may hold clinical promise.

AUTHOR CONTRIBUTIONS

AC contributed to concept development, writing, preparation, figure design, and submission of this article. MC contributed to concept development, writing, preparation, and figure design. JP, AP, and SE contributed to concept development and writing. SH contributed to concept development, writing, preparation, figure design, and submission of this article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the National Cancer Institute (R35CA197627) and funding through the Breast Cancer Research Foundation (BCRF-19-073) to SH. SE received funding through the UNC Office for Undergraduate Research as a Summer Undergraduate Fellowship.

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 12 November 2020
doi: 10.3389/fcell.2020.579160

miR-9-5p as a Regulator of the Androgen Receptor Pathway in Breast Cancer Cell Lines

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Breast cancer (BC) is the most diagnosed carcinoma and the leading cause of cancer death in female over 100 countries. Thanks to the advance in therapeutic strategies, patients' survival has improved. However, the lack of response to treatments and drug resistance are still a main concern, demanding for new therapeutic approaches, in particular for the advanced stages of the disease. Androgen receptor (AR) is gaining increasing interest as a fourth targetable receptor in BC, however, its regulation in BC cells is still poorly understood. MicroRNAs (miRNAs) regulate gene expression post-transcriptionally. Here, we identified miR-9-5p as an inhibitor of AR expression, we validated the inverse correlation between miR-9-5p and AR in primary BC samples and we further identified a feedback loop in which androgen agonists of AR up-regulate miR-9-5p. We also provided evidence that miR-9-5p elicits anti-proliferative effects in BC cell lines regardless of their estrogen receptor status. Finally, we showed that miR-9-5p can revert AR-downstream signaling even in presence of AR-agonists, highlighting the role of this miR in the hormonal response of BC. In conclusion, this study supports the role of miR-9-5p as an anti-proliferative miR in BC and as a central modulator of AR-signaling response to circulating androgens in BC.

Keywords: microRNAs, miR-9-5p, androgen receptor, breast, cancer

OPEN ACCESS

Edited by:
Marzia Di Donato, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Pia Giovannelli, University of Campania Luigi Vanvitelli, Italy
Luciano Cascione, Istituto Oncologico della Svizzera Italiana, Switzerland

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

Received: *01 July 2020*
Accepted: *20 October 2020*
Published: *12 November 2020*

Citation:
Bandini E, Fanini F, Vannini I, Rossi T, Plousiou M, Tumedei MM, Limarzi F, Maltoni R, Fabbri F, Hrelia S, Cho WCS and Fabbri M (2020) miR-9-5p as a Regulator of the Androgen Receptor Pathway in Breast Cancer Cell Lines. Front. Cell Dev. Biol. 8:579160. doi: 10.3389/fcell.2020.579160

INTRODUCTION

Despite progresses in its global management approaches, breast cancer (BC) still remains the most diagnosed carcinoma and the leading cause of cancer death in women over 100 countries (Bray et al., 2018). In 2020 it was estimated an incidence of 276,480 cases and 42,170 deaths in women due to BC in the United States (Siegel et al., 2020). Although several therapeutic approaches have provided remarkable improvements in survival, drug resistance and the lack of response to treatments are still a main issue, suggesting a great need to exploit novel therapeutic strategies in

Abbreviations: AR, Androgen receptor; BC, Breast cancer; EMT, Epithelial-mesenchymal transition; FFPE, Formalin-fixed paraffin-embedded; miRNAsmicroRNAs; DHT, Dihydrotestosterone; DHEA, Dehydroepiandrosterone.

particular in the advanced stages (Zhao et al., 2019). Androgen receptor (AR) is a member of the steroid nuclear receptors family that mediates the biological effects of androgens. It is considered an emerging target in the treatment of patients characterized by the lack of expression of hormone receptors (the so called "Triple-Negative Breast Cancer" or TNBC), and in other BC subtypes, despite its role is still controversial (Takagi et al., 2018; Vidula et al., 2019). In recent years, several molecules have been investigated as crucial regulators of transcription, and translation of genes involved in carcinogenesis, and microRNAs (miRNAs) are among the most studied. They belong to a large family of small non-coding RNAs and deserve great attention since they can modulate the expression of tumor suppressor genes and oncogenes, affecting the signal pathways in cancer cells. However, little is known about miRNA regulation of hormonal signaling, especially androgen signaling, in BC, and how this affects hormone-therapy. Several studies have been performed in order to identify a miRNA signature that could predict their potential role as prognostic or predictive biomarkers for patient management. In particular, miR-9-5p dysregulation has been reported in many cancer types, but its role as a tumor suppressor gene or as an oncomiR remains unclear, especially in BC. Some studies reported miR-9-5p as a negative prognostic marker related to more aggressive clinical pathological features and as involved in epithelial-mesenchymal transition (EMT) (Almeida et al., 2010; Gwak et al., 2014; van Schooneveld et al., 2015; Bastos et al., 2016; Jang et al., 2017; Cheng et al., 2018). AR has emerged as a fourth receptor in BC and as a promising target, especially for those patients who do not respond to current hormone receptor-targeted therapies. In this study, we investigated the role of miR-9-5p in the AR pathway. We showed that AR is a target of miR-9-5p and we identified a novel miR-9-5p/AR feedback loop with important implications for the response of BC to anti-androgen therapy.

MATERIALS AND METHODS

Cell Cultures

The human breast carcinoma cell lines MDA-MB-453 and MCF-7 were purchased from the American Type Culture Collection (ATCC; Manassas, Virginia, United States), and the cell line T-47D was purchased from Zooprophylactic Institute of Genova (Italy). MDA-MB-453 were maintained in Leibovitz's L-15 medium (ATCC 30-2008, United States) supplemented with fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, United States) to a final concentration of 10%, according to the manufacturer's information sheet. MCF-7 was maintained in Eagle's Minimum Essential Medium (EMEM) medium (ATCC 30-2003, United States) supplemented with FBS to a final concentration of 10%. T-47D were maintained in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (Euroclone, Italy), and FBS to a final concentration of 10%. One percent of Penicillin-streptomycin (PAA, Carlo Erba Reagents, Italy), and 0.002% of MycoZap Prophylactic (Lonza Group Ltd., Switzerland) were added to all mediums. The cultures were maintained in a Heraeus incubator, in atmosphere composed of 95% air, and 5% CO_2, except for MDA-MB-453 that required a free gas exchange with atmospheric air. Every 4 days we proceeded to sub-cultivation of cell lines by using Trypsin-EDTA (Life Technologies, United States). Cell lines were tested every 2 months with MycoAlert^{TM} Mycoplasma Detection Kit (Lonza Group Ltd., Switzerland) to check a possible contamination by mycoplasma. In experiments where hormonal treatments were performed, medium phenol red free added of FBS charcoal stripped was used (Gibco, Thermo Fisher Scientific, United States).

Patient Sample

Paired FFPE tumor and adjacent non-tumor tissue from 11 BC patients (5 Triple Negative Breast Cancers TNBCs, and 6 Luminal A) were obtained at the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRST, S.r.l, IRCCS, in Meldola (FC), Italy. Histological and clinical characteristics are listed in **Supplementary Table S1**. Written informed consent was obtained from all patients before sample analyses. The study was approved by the Ethical Committee of our institute and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before the first sampling.

Drug Treatments

Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (Merck, Germany) in powder formulation and a 10 μM working solution was obtained by resuspending it in dimethyl sulfoxide (DMSO); DHT was then used at 10 nM and DMSO never exceeded 0.1%. Dehydroepiandrosterone (DHEA) was provided by the Pharmacy of our Institute at a concentration of 7.6 mM dissolved in sterile water, and 50 μM solution was used to perform experiments.

Pre-miRNAs Transfection

Pre-miRNAs (pre-miRNA miR precursors, Ambion, Life Technologies, United States), and the corresponding negative control, SCR (Pre-miR miRNA precursor scrambled negative control #1, Ambion, Life Technologies, United States) were used to transfect BC cell lines at a final concentration of 100 nM through TransIT-X2® Dynamic Delivery System (Mirus Bio LLC, United States), in accordance with the instructions provided by the manufacturer. Opti-Mem Medium (Gibco, Thermo Fisher Scientific, United States) was used for transfection complex. Depending on the type of final analysis, transfections were stopped and pellets collected at 24–48–72 h. MiRNA transfection efficiencies were evaluated by Real-time quantitative PCR (qRT-PCR).

AR Silencing

SMARTpool siGENOME AR siRNA (Dharmacon), and the corresponding negative control, siGENOME Non-Targeting siRNA #1 (Dharmacon), were transfected into BC cell lines at a final concentration of 50 nM, through TransIT-X2® Dynamic Delivery System (Mirus Bio LLC, United States), in accordance with the instructions provided by the manufacturer. Opti-Mem

Medium (Gibco, Thermo Fisher Scientific, United States) was used for transfection complex. Transfections were stopped and pellets collected at 48 h. AR transfection efficiencies and miR-9-5p expression were evaluated by qRT-PCR.

Extraction of RNA and Proteins

RNA was isolated from BC cells with mirVana™ miRNA Isolation Kit (Invitrogen, Thermo Fisher Scientific, United States), following the manufacturer's instruction. RNA isolation from 2-to-4 10 μm thick slices FFPE was performed by using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Invitrogen, Thermo Fisher Scientific, United States) following the protocol provided by the manufacturer. The quantification of extracted RNA was carried out using NanoDrop ND-1,000 (Thermo Fisher Scientific, United States). Total proteins were extracted, keeping samples on ice, with 1X RIPA lysis buffer (Santa Cruz Biotechnology, United States) with the addition of 10 μl of PMSF, 10 μl of sodium orthovanadate and 15 μl of protease inhibitors per ml of 1X RIPA lysis buffer, as recommended by the datasheet. The lysates were centrifuged at 4°C for 30 min. Then, supernatant was transferred to another tube. Proteins were quantified through BCA Protein Assay (Pierce, Thermo Fisher Scientific, United States), and using a Multiscan EX microplate reader (Thermo Fisher Scientific, United States), at a wavelength of 490 nm.

Protein Expression Analysis

Western blotting was used to evaluate the expression of AR. Proteins (50 μg) were denatured and separated by electrophoresis using a gel Criterion TGX Stain Free Gel Precast 4–20% (Bio-Rad Laboratories, CA, United States), and Laemmli Sample Buffer (Bio-Rad Laboratories, CA, United States) with 5% of β-mercaptoethanol (Carlo Erba Reagents, Italy), in 1:1 ratio with the sample. Electrophoretic run was performed at a constant voltage of 180V in a TRIS/Glycine/SDS 1X buffer (Bio-Rad Laboratories, CA, United States). Trans-Blot Transfer Turbo midi-format 0.2 μm PVDF membrane (Bio-Rad Laboratories, CA, United States) using the Trans Blot Turbo System (Bio-Rad Laboratories, CA, United States). The membrane was subsequently incubated for at least 2 h at room temperature in a solution of Tween 20 (Bio-Rad) at 0.1% and 1X Dulbecco's phosphate buffered saline (DPBS) (Invitrogen, Thermo Fisher Scientific, United States) supplemented with 5% nonfat dry milk (Bio-Rad Laboratories, CA, United states) in order to facilitate the saturation of non-specific binding sites. Primary antibodies and dilutions used are the following: anti-Androgen Receptor (1:1,000; ab133273, Abcam, United States), anti-Vinculin clone FB11 (1:1,000; Biohit, United Kingdom), anti-β-actin Antibody HRP (1:50,000; ab49900, Abcam, United States). Secondary antibodies HRP-conjugated goat anti-mouse and anti-rabbit (1:5,000; Santa Cruz, United States) and Precision Plus Protein Western C Strep Tactin-HRP Conjugate (1:10,000; Bio-Rad Laboratories, CA, United States) were used. The secondary HRP-conjugated antibodies were used for 1 h at room temperature. The membranes, after washes with T-PBS, were developed using Clarity Western ECL reagent (Bio-Rad Laboratories), and images acquired through Chemidoc (Bio-Rad Laboratories), and analyzed using ImageJ Software (Wayne Rasband, NIH, United States).

miRNA and mRNA Expression Analysis

MiRNAs expression analysis was performed using the TaqMan miRNA Assays (Applied Biosystems, Thermo Fisher Scientific, United States). Briefly, the molecule of complementary DNA (cDNA) was synthesized using 10 ng of RNA as template, specific primer and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, United States). mRNAs expression was evaluated with the use of the TaqMan Gene Expression Assays (Applied Biosystems, Lifetechnologies, United States). The cDNA was synthesized using 80 ng of RNA as a template and the TaqMan RT PCR Kit (Applied Biosystems, Thermo Fisher Scientific, United States). qRT-PCR was performed with Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, United States) using cDNA, TaqMan probes and TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, United States). To determine the basal expression of miR-9-5p in BC cell lines, a reference RNA composed of total RNA from nine human tissues or cell lines was used (Total RNA Breast Human, #750500, Agilent Technologies, United States). Experiments were conducted in triplicate and normalized to RNU48, RNU44 and GAPDH, used as endogenous controls. Relative expression levels were calculated using the method of comparative Ct ($2^{-\Delta\ \Delta\ Ct}$ method).

Immunohistochemistry

During surgery some tumor samples were obtained and fixed in neutral buffered formalin, then embedded in paraffin. For each patient, 5 μm thickness sections were mounted on positive-charged slides (Bio Optica, Milan, Italy). AR expression was performed using the Ventana Benchmark ULTRA staining system (Ventana Medical Systems, Tucson, AZ, United States) with the Optiview DAB Detection Kit (Ventana Medical Systems). AR (SP107 Cell Marque, Ventana Medical Systems) antibody pre-diluted by the supplier was used. The slides were incubated for 16 min and automatically counterstained with hematoxylin II (Ventana Medical Systems). AR positivity was detected and semiquantitatively quantified as the percentage ratio between immunopositive tumor cells and the total number of tumor cells. All samples were evaluated by two independent observers. Any disagreement (>10% of positive cells for the different markers) was resolved by consensus after joint review using a multihead microscope. Biomarker determination was performed according to European Quality Assurance guidelines

CellTiter-Glo Luminescent Cell Viability Assay

CellTiter-Glo Luminescent Cell Viability Assay (Promega, United States) was used to determine the number of viable cells after hormone treatments and transfections. It is based on the quantification of present ATP that indicates the existence of metabolically active cells. The procedure, according to the

instructions provided by the manufacturer, requires the adding of a single reagent (CellTiter-Glo Reagent) directly to cultured cells. This system produces a cell lysis and the generation of a luminescent signal, captured through a GloMax luminometer (Promega, United States), proportional to the ATP content, which, in turn, is directly proportional to the number of viable cells.

Flow Cytometry

All samples were analyzed by using a FACSCanto^{TM} cytofluorimeter (BD Biosciences). Data acquisition (10,000 events were collected for each sample) was performed by using the FACS Diva^{TM} software (BD Biosciences), as recommended by the manufacturer. After cells were seeded in 6-well plates and treated with DHT 10 nM or DHEA 50 μM, or after miR-9-5p transfection they underwent different protocols.

Bromodeoxyuridine assay was used to determine the percentage of S-phase cells. Cells were incubated with a 60 μM Bromodeoxyuridine (BrdU) solution in 1 ml of medium and then centrifuged and fixed with 70% cold ethanol. The day after cells were washed in PBS 1X and incubated with the following reagents in this order: HCL 2M (Carlo Erba Reagents, Italy), sodium tetraborate (Sigma-Aldrich, Merck, Germany), and Tween 20 0.5% (Bio-Rad Laboratories, CA, United States) + BSA 1% (Thermo Fisher Scientific, United States). Finally, cells were incubated with a 1:50 dilution of anti-BrdU antibody (Thermo

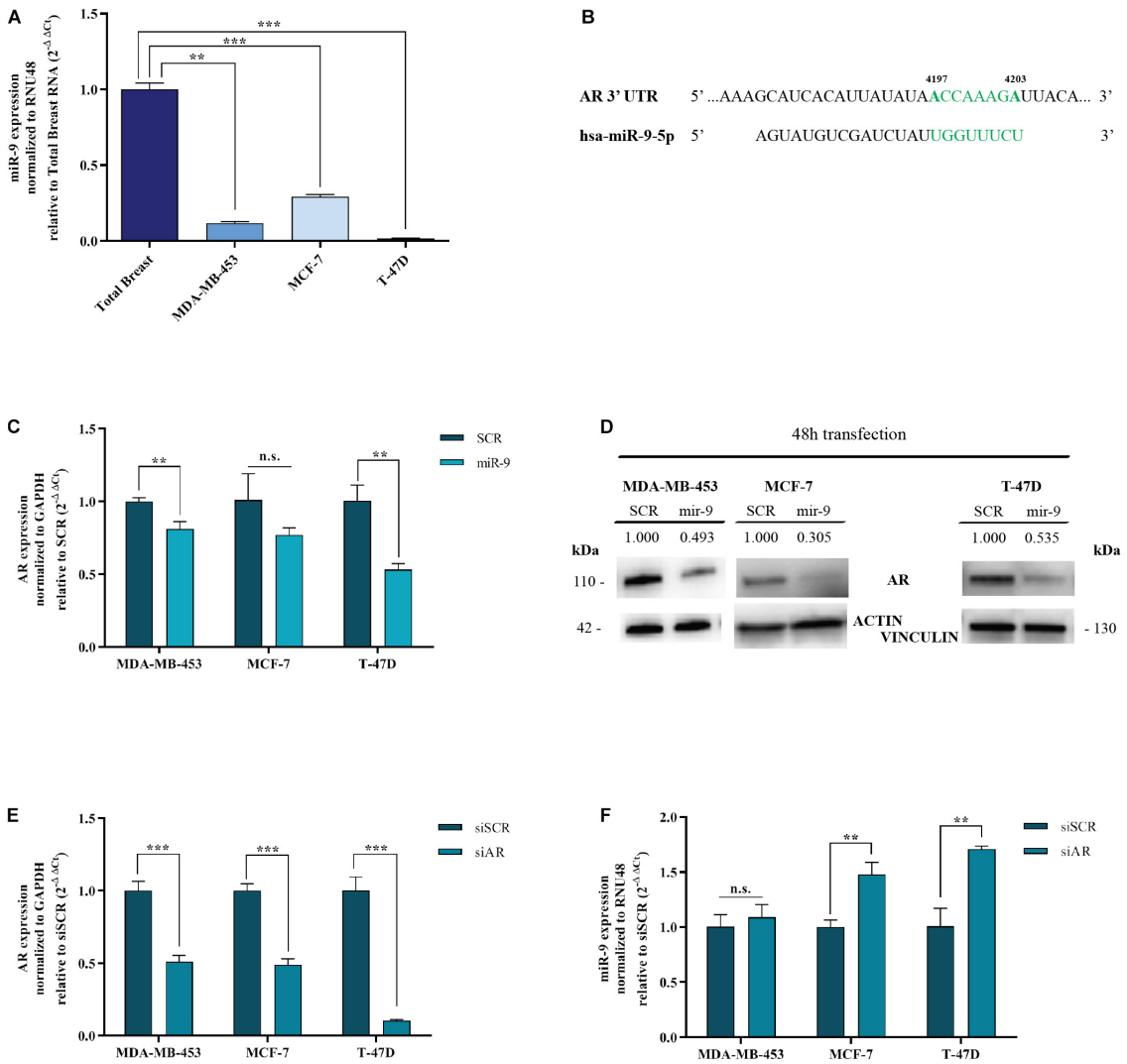

FIGURE 1 | MiR-9-5p is downregulated in BC cell lines and down-regulates AR. **(A)** MiR-9-5p (mir-9) expression was measured by qRT-PCR in three BC cell lines and compared to Total Breast RNA. **(B)** Predicted miR-9-5p target sites in the AR 3′UTR mRNA, as shown by the Software TargetScan Human Release v6.2. in green, the seed region of miR-9::AR mRNA predicted interaction. **(C)** mRNA expression level of AR in MDA-MB-453, MCF-7, and T-47D cell lines, 48 h after transfection with pre-miR-9-5p (miR-9), or scrambled (SCR). **(D)** Protein expression level of AR in MDA-MB-453, MCF-7 and T-47D cell lines, 48 h after transfection with pre-miR-9-5p (miR-9), or scrambled (SCR). **(E)** AR levels after transfection of MDA-MB-453, MCF-7, and T-47D cell lines with AR siRNA (siAR) compared to scrambled (siSCR). **(F)** MiR-9-5p (miR-9) expression in MDA-MB-453, MCF-7, and T-47D cell lines after 48 h transfection with AR siRNA (siAR) or scrambled (siSCR). $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Multiple *t*-test, corrected for multiple comparison using the Holm-Sidak method in **(A–D)**.

Fisher Scientific, United States) for 1 h, followed by 1 h of a FITC-conjugated secondary antibody (Dako, Agilent Technologies, United States) diluted 1:250. After antibody incubation, cells were washed with PBS 1X, stained with propidium iodide solution and incubated overnight at 4°C. Samples were processed and analyzed the day after.

For cell cycle evaluation, cells were harvested after each treatment timepoint, fixed in 70% ethanol, and stained in a solution containing 10μg ml/1 of propidium iodide (Sigma-Aldrich), 10,000 U ml/1 of RNase (Sigma-Aldrich), and 0.01% of NP40 (Sigma-Aldrich). After 30–60 min, the samples were analyzed. Data were elaborated using the ModFit LT^{TM} software v.4.1.7 (Verity Software House), and expressed as fractions of cells in the different cell-cycle phases.

Cignal Reporter Assay

Cignal Reporter Assay Kit (Qiagen, United States) was used to assess the activation of AR transduction pathway, by measuring the activities of downstream AR transcription factors (both increases and decreases). One microgram of AR-responsive reporter (a mixture of inducible transcription factor responsive construct and constitutively expressing Renilla luciferase construct) and negative control (a mixture of non-inducible reporter construct and constitutively expressing Renilla luciferase construct), along with pre-miR-9-5p and the corresponding negative control SCR (100 nM), were diluted in Opti-Mem Medium (Gibco, Thermo Fisher Scientific, United States). The diluted nucleic acids were mixed with TransIT-X2 Mirus (Temaricerca, Italy). Following 24 h transfection, cells were treated with DHT 10 nM for additional 24 h, so that 48 h post-transfection cells were harvested into cell lysis buffer (Promega, United States). Luciferase activity was measured using the Dual-Luciferase Assay System (Promega, United States). The change in the activity of AR signaling pathway was determined by comparing the normalized luciferase activities of the reporter in treated *versus* untreated transfectants (SCR vs. miR-9-5p and SCR + DHT vs. miR-9-5p + DHT). The activity ratio Firefly:Renilla was calculated from experimental and control transfections. Then, ratios from AR responsive reporter transfections were divided by ratios from negative controls to obtain relative luciferase units (RLU).

Data Analysis

Data obtained from qRT-PCR were analyzed by Life Technologies TM 7500 Software v2.0.6 for 7500 Fast Real Time PCR System (Life Technologies, Thermo Fisher Scientific, United States). Relative expression levels were calculated using the method of comparative Ct ($2^{-\Delta\ \Delta\ Ct}$). The images of the Western Blot, acquired through Chemidoc, were analyzed using ImageJ Software (Wayne Rasband, NIH, United States). Viability experiments were conducted in 96 wells plate, using 8 wells for every sample. Statistical analyses were performed using GraphPad Prism 8 (version 8.4.2) statistical software (GraphPad

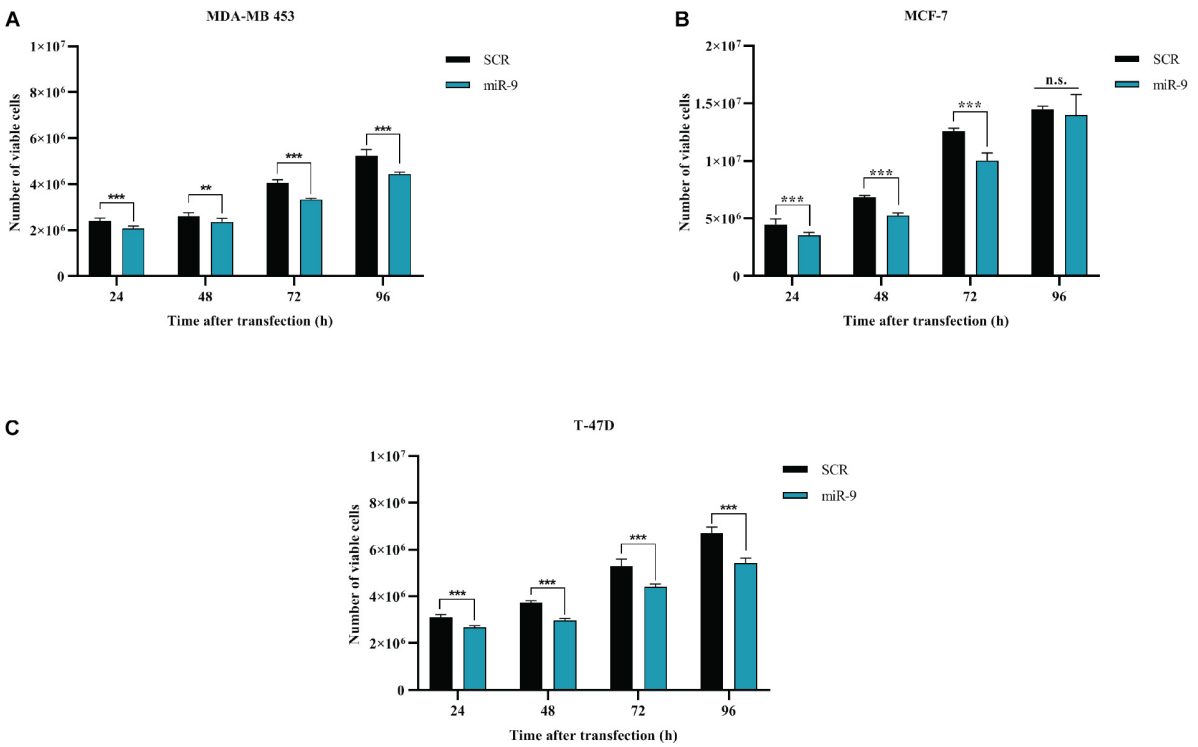

FIGURE 2 | Effects of miR-9-5p (mir-9) on proliferation of BC cell lines **(A)** MDA-MB-453 **(B)** MCF-7 (**C**) T-47D. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Multiple *t*-test, corrected for multiple comparison using the Holm-Sidak method in **(A–C)**.

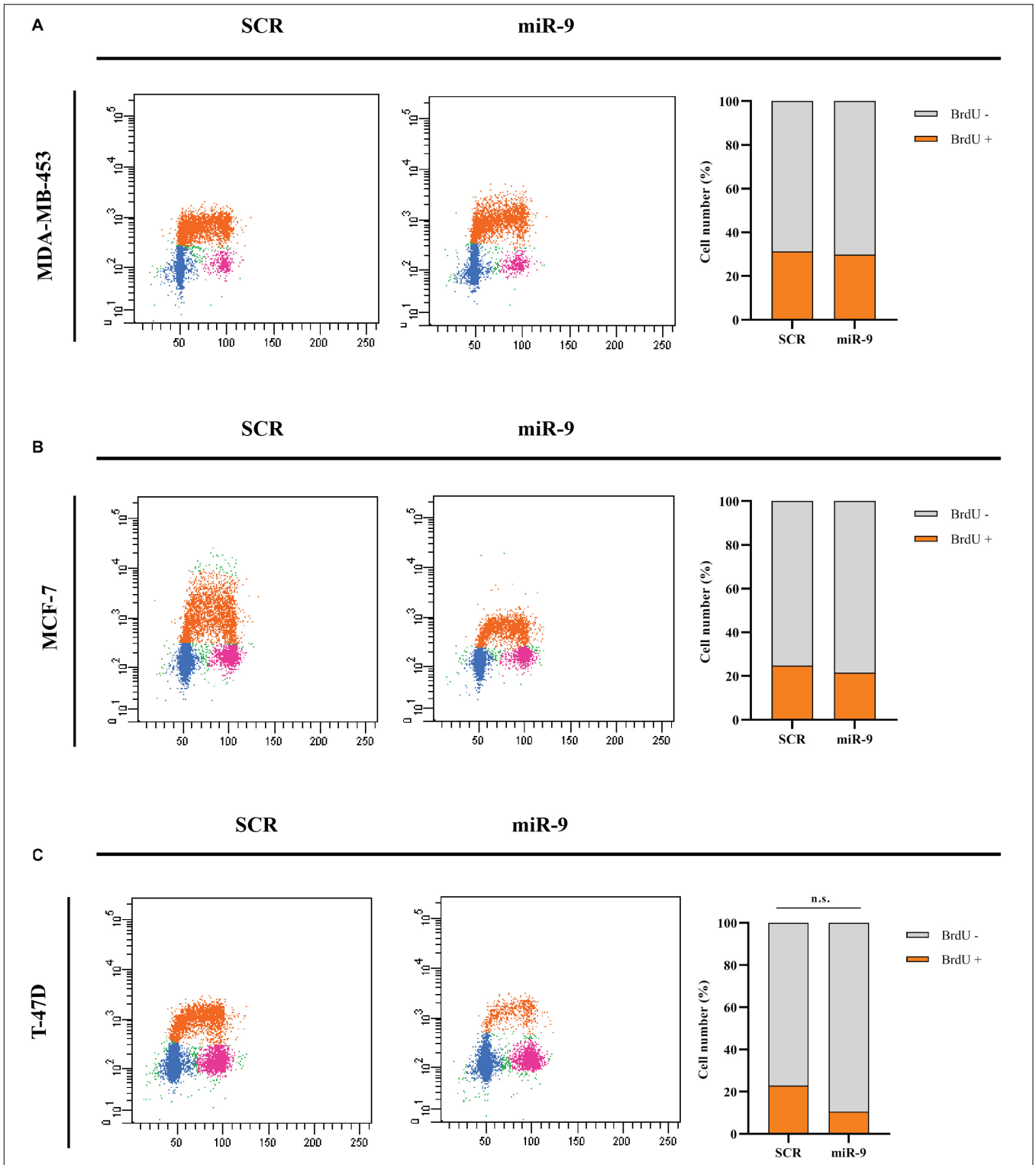

FIGURE 3 | Cytofluorimetric evaluation of the effects of miR-9-5p (miR-9) on DNA replication at 72 h post-transfection. Cytofluorimetric dot plots of proliferating MDA-MB-453 **(A)**, MCF-7 **(B)**, and T-47D **(C)** BC cell lines, following BrdU incorporation, and anti-BrdU antibody incubation. The percentage of cells in S phase, which results in BrdU positive cells (BrdU+) was presented in the histograms on the right.

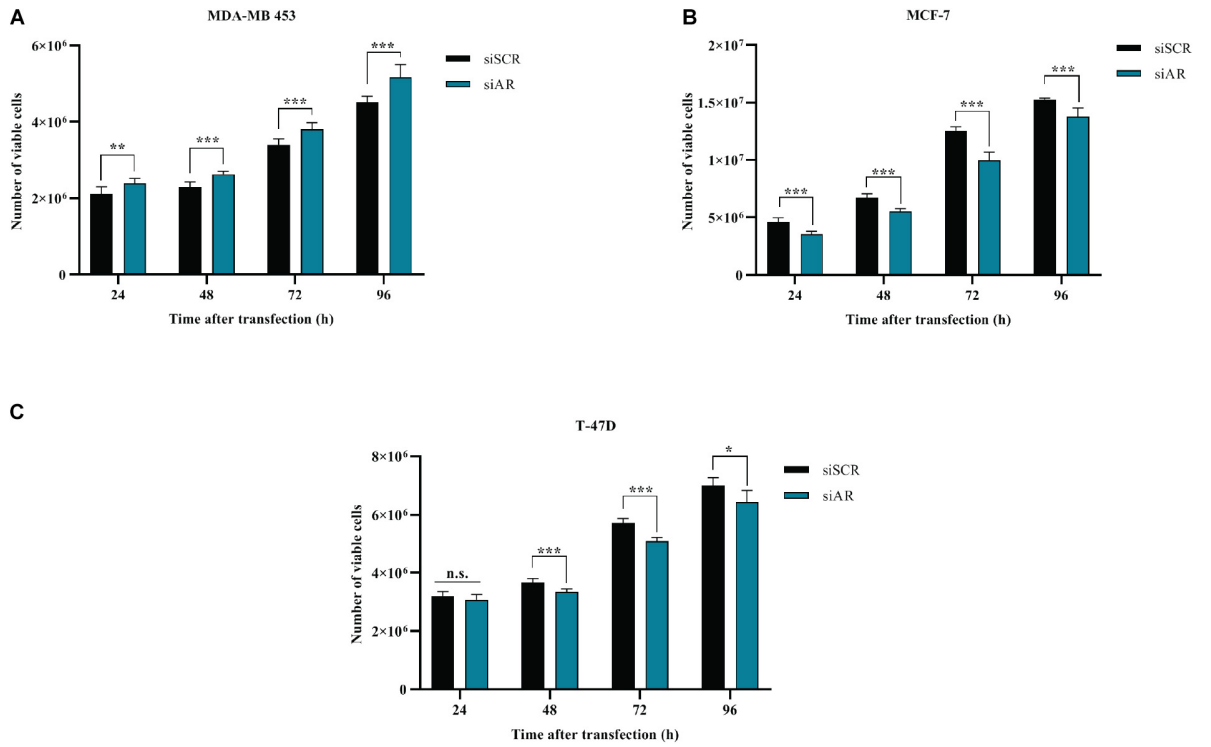

FIGURE 4 | Effects of AR silencing (siAR) on proliferation of BC cell lines **(A)** MDA-MB-453 **(B)** MCF-7 **(C)** T-47D. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Multiple *t*-test, corrected for multiple comparison using the Holm-Sidak method in **(A–C)**.

Software, United States). Statistical significance was indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

MicroRNA-9-5p Is Downregulated in BC Cell Lines

MDA-MB-453 are molecular apocrine BC cell lines (AR^{+}, ER^{-}) (Robinson et al., 2011). MCF-7 and T-47D are luminal A BC cell lines (AR^{+}, ER^{+}) (Dai et al., 2017). We performed qRT-PCR for miR-9-5p to assess the endogenous expression of miR-9-5p in these 3 different BC cells lines, compared to human Total Normal Breast RNA (Agilent Technologies). All cell lines show a low endogenous expression of miR-9-5p when compared to Total Normal Breast RNA (**Figure 1A**).

MicroRNA-9-5p Silences AR

AR is an emerging target for hormone therapy in BC. However, the factors regulating its expression are not understood. Intriguingly, miR-9-5p is predicted to target AR by TargetScan Software v7.2 (Agarwal et al., 2015) and miRWalk database (Medical Faculty Mannheim of the University of Heidelberg) (**Figure 1B**). To assess whether miR-9-5p silences AR, MDA-MB-453, MCF-7 and T-47D cells were transfected with miR-9-5p or a scrambled oligonucleotide as a control (SCR) and the expression of AR was measured both at the mRNA and at the protein level by qRT-PCR and immunoblotting, respectively. We observed a significant down-regulation of AR expression in all three tested BC cell lines both at the mRNA (**Figure 1C**) and at the protein level (**Figure 1D**).

AR Silencing Upregulates miR-9-5p in ER+ Cell Lines

Intriguingly, when we silenced AR endogenous expression with a pool of siRNAs (a mixture of 4 siRNA provided as a single reagent) (**Figure 1E**), upregulation of miR-9-5p was observed (compared to a siRNA negative control) but only in ER+ BC cell lines MCF-7 and T-47D, with no effects on the ER- cell line MDA-MB-453 (**Figure 1F**). Overall, these data indicate that while miR-9-5p downregulates AR expression regardless of the ER status of BC cell lines, AR silencing up-regulates miR-9 only in ER^{+} cell lines.

MicroRNA-9-5p Reduces BC Cell Growth

BC cells were transfected with the pre-miR-9-5p mimic, or a scrambled miRNA as a control (SCR), and *in vitro* cell growth at 24–48–72–96 h post-transfection was evaluated. MiR-9-5p increased expression significantly reduced the proliferation of all three BC cells at all tested times (**Figures 2A–C**). Overexpression of miR-9-5p was verified by qRT-PCR (**Supplementary Figures S1A–C**). In order to study the effects of mir-9-5p on cell proliferation, we further quantified replicating

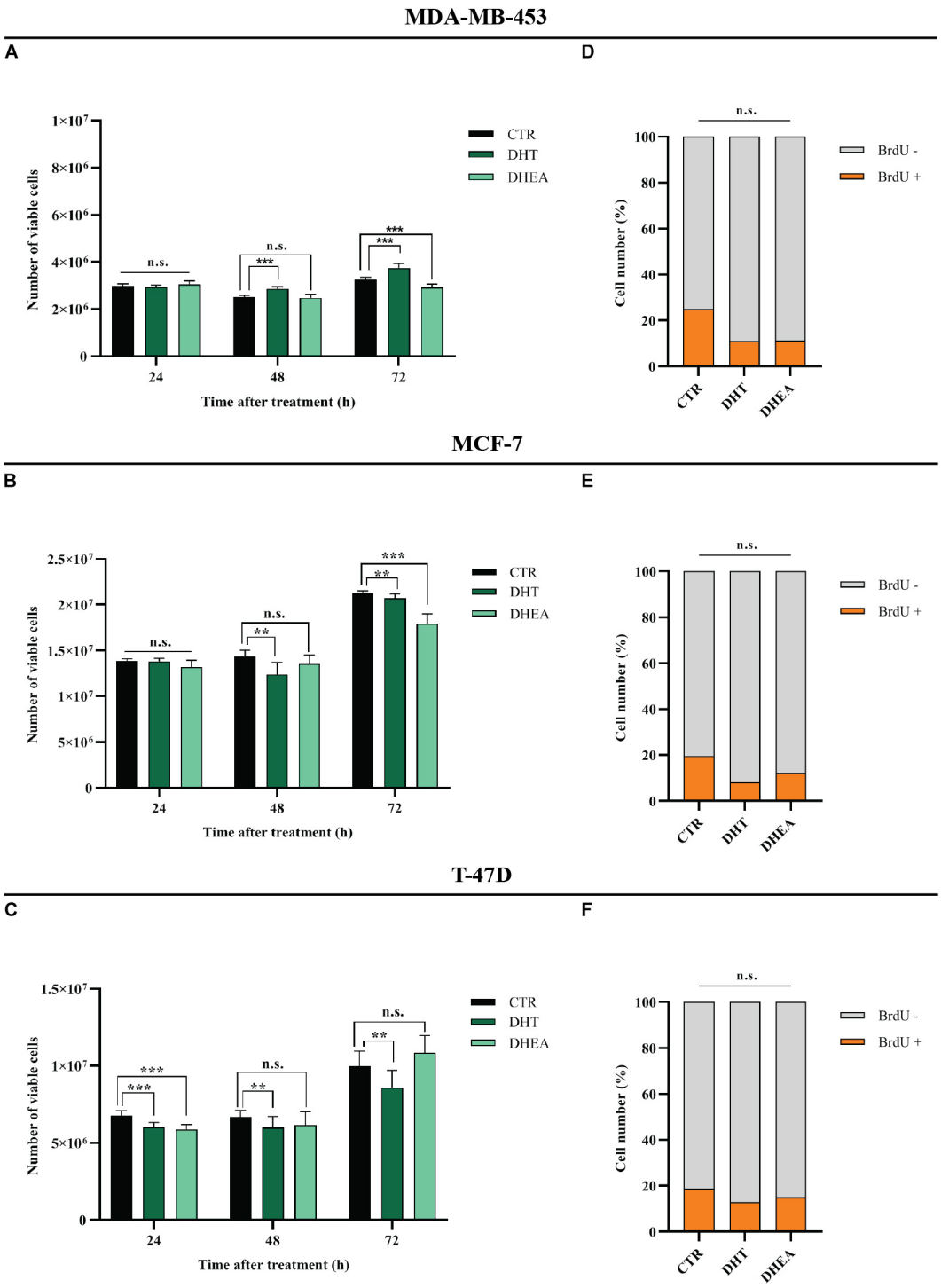

FIGURE 5 | Effect of DHT and DHEA on proliferation of BC cell lines at the indicated time points **(A–C)** and on DNA replication at 48 h post-transfection **(D–F)**. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Multiple *t*-test, corrected for multiple comparison using the Holm-Sidak method in **(A–F)**.

DNA by performing a BrdU assay. While only little difference was observed in MDA-MB-453 and MCF-7 cell lines at 72 h (**Figures 3A,B**), we observed a notable reduction of DNA replication in T-47D cell line transfected with pre-miR-9-5p compared to scrambled (25 vs. 6.2%) at 72 h (**Figure 3C**) which persisted even at 96 h (**Supplementary Figure S2**), suggesting that cells did not enter S phase. These results were confirmed also by cell cycle analysis using propidium iodide, which showed a

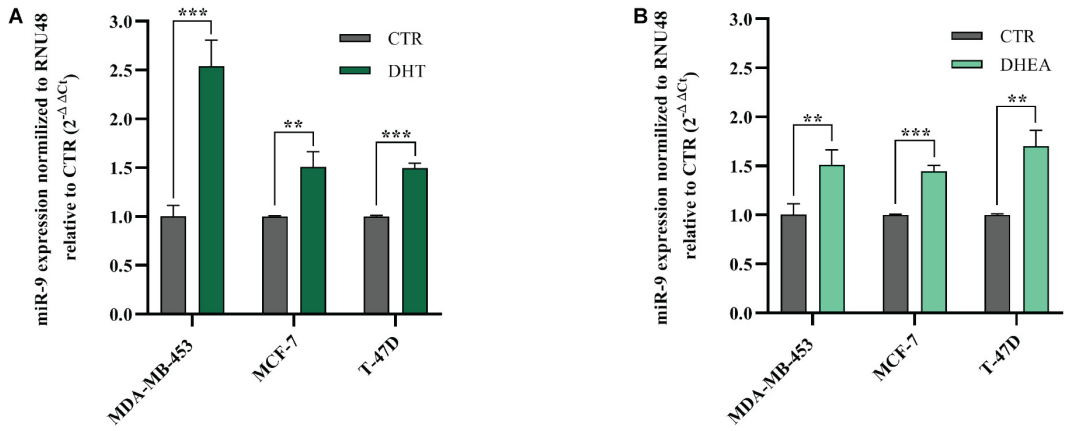

FIGURE 6 | Effects of DHT and DHEA on miR-9-5p (miR-9) expression at 24 h post-transfection **(A,B)**. $^{**}p < 0.01$, $^{***}p < 0.001$. Multiple *t*-test, corrected for multiple comparison using the Holm-Sidak method in **(A,B)**.

reduction of T-47D cells in S-phase and a block in G1-phase when miR-9-5p was overexpressed (**Supplementary Figures S3–S5**).

AR Silencing Reduces Proliferation of ER+ Cells Lines

BC cells were transfected with a pool of AR siRNAs (a mixture of 4 siRNA provided as a single reagent) (**Figure 1E**) and *in vitro* cell growth at 24–48–72–96 h post-transfection was assessed. AR silencing did not reduce the proliferation of ER- MDA-MB-453 (**Figure 4A**), but significantly reduced the proliferation of the two ER+ cell lines MCF-7 and T-47D (**Figures 4B,C**). These data are consistent with the inhibitory effect of miR-9-5p on BC proliferation and with the fact that silencing of AR expression up-regulates miR-9-5p only in ER+ cells and not in the ER- MDA-MB-453 BC cells.

DHT and DHEA Reduce Growth of BC Cell Lines

MDA-MB-453, MCF-7, and T-47D cell lines express AR (**Figure 1D**). In order to assess the effects of circulating AR-agonists on BC cell proliferation, we treated BC cells with DHT and DHEA, respectively. DHT showed a cell growth promoting effect only in MDA-MB-453 at 48 and 72 h (**Figure 5A**), while it displayed a significant inhibitory effect in MCF-7 at 48 and 72 h (**Figure 5B**) and in T-47D at all tested time-points (**Figure 5C**). Nevertheless, BrdU assay indicated a reduction trend in percentage of cells in S phase for all three cell lines tested, although the statistical analysis did not yield any significant differences between treated and untreated group (**Figures 5D–F**). DHEA showed inhibition of cell growth in MDA-MB-453 and MCF-7 cell lines at 72 h (**Figures 5A,B**) and in T-47D only at 24 h after treatment (**Figure 5C**). Similarly to DHT-treated cells, also for DHEA-treated cells the BrdU assay showed a decrease of cell number in S phase for all three tested cell lines although the statistical analysis did not yield any significant differences between the two groups (**Figures 5D–F**).

DHT and DHEA Up-Regulate miR-9-5p

Next, we decided to assess whether AR agonists DHT and DHEA were able to modulate miR-9-5p expression, which is endogenously low in the three BC cell lines included in this study, compared to normal breast cells (**Figure 1A**). We treated MDA-MB-453, MCF-7, and T-47D cells with DHT or DHEA, and we observed that miR-9-5p was significantly upregulated in all cell lines both by DHT (**Figure 6A**) and DHEA (**Figure 6B**). Since miR-9-5p down-regulates AR protein expression (**Figure 1D**), we conclude that a feedback loop occurs between miR-9-5p and AR, in which AR agonists can induce miR-9-5p expression which, in turn, inhibits AR protein levels in all three BC cell lines and regardless of their ER status.

MicroRNA-9 Inhibits AR Transcriptional Activity

It has been shown that miR-9-5p silences AR in BC cell lines. In order to assess whether this reflects in an effect of miR-9-5p on AR-mediated downstream signaling, we performed the AR Cignal Reporter Assay. We observed that while miR-9-5p over-expression had minimal effects on the AR signaling (as measured by the luciferase activity of the Cignal reporter plasmid) and in absence of AR agonists (DHT and DHEA), miR-9-5p over-expression was able to reverse the activation of AR signaling even in presence of AR agonists (**Figures 7A–C**). These findings suggest that miR-9-5p has a leading effect in shaping AR-mediated downstream pathway and that the effects of miR-9-5p on the AR downstream signaling mostly occur in presence of the AR agonists (**Figures 7A–C**).

MicroRNA-9 and AR Expression Show an Inverse Correlation in BC Patients

In order to evaluate if miR-9-5p was downregulated in BC patients, we performed a qRT-PCR analysis in 11 primary BC samples. Results showed that miR-9-5p was significantly downregulated in tumor tissues (T) compared to healthy adjacent ones (N) in all patients (**Figure 8A**), and an inverse correlation

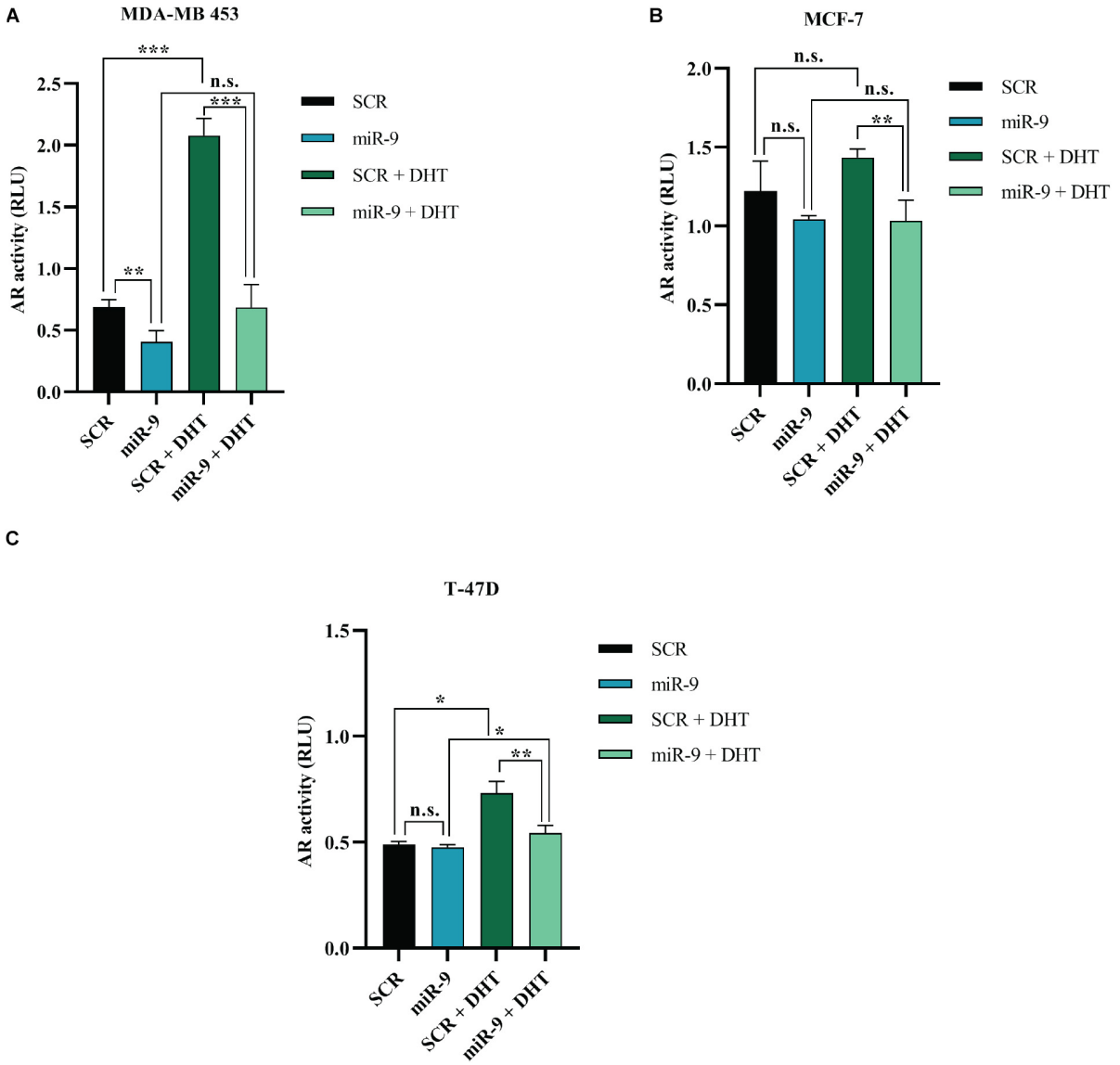

FIGURE 7 | Effects of miR-9-5p (mir-9) on AR signaling. AR transcriptional activity as measured with the AR Cignal reporter assay at 48 h in MDA-MB-453 **(A)**, MCF7 **(B)**, and T47D **(C)** cells. The modulation of AR transcription factor was determined by comparing the normalized luciferase activities of the reporter in treated vs. untreated transfectants (SCR vs. miR-9, SCR vs. SCR + DHT, miR-9 vs. miR-9+DHT, and SCR+DHT vs. miR-9+DHT), in all the BC cell lines MDA-MB-453 **(A)**, MCF-7 **(B)**, and T-47D **(C)**. Firefly: Renilla activity ratios were calculated from experimental and control transfections. Then, ratios from AR responsive reporter transfections were divided by ratios from negative controls to obtain relative luciferase units (RLU). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Multiple *t*-test, corrected for multiple comparison using the Holm-Sidak method in **(A–C)**.

between miR-9-5p and AR expression was observed in tumor tissues (**Figure 8B**) validating in patient samples our findings that miR-9-5p is a regulator of AR expression in BC. Expression of AR in FFPE samples was confirmed by immunohistochemistry (IHC) (**Supplementary Figures S6A,B**).

DISCUSSION

In the last few years, AR has emerged as a new potential target for the treatment of BC patients. Indeed, circulating androgens are present at physiological conditions in women, and their levels vary depending on hormonal needs and in relation to pre- or post-menopausal state (Giovannelli et al., 2018). Approximately 50–80% of BCs are positive for AR, but the prognostic and predictive value of its expression in BC is still controversial (Yang et al., 2020). MiRNAs are increasingly implicated in regulating tumorigenesis of BC, and among them miR-9-5p is attracting great attention. Its role is currently still debated since it has shown functions both as an oncomir and as a tumor suppressor gene (Gravgaard et al., 2012; Selcuklu et al., 2012; Orangi and Motovali-Bashi, 2019; Sporn et al., 2019).

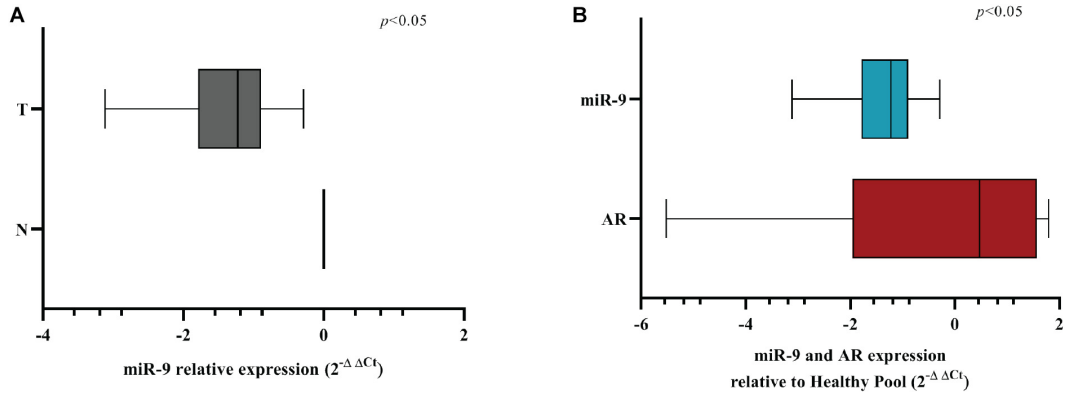

FIGURE 8 | MiR-9-5p is downregulated in BC FFPE samples. **(A)** Box-plot representation of the miR-9-5p (miR-9) qRT-PCR expression in adjacent normal (N) vs. tumoral (T) samples for all FFPE samples examined (n = 11). **(B)** Box-plot representation of the miR-9-5p (miR-9) and AR qRT-PCR expression in all FFPE samples examined (n = 11). The expression of AR and miR-9 have been normalized to GAPDH and RNU44, respectively. A non-parametric unpaired t-test (Kolmogorov-Smirnov test) method in **(A**,**B)**. Box-plots have been presented on a ln-scale.

The current evidence points toward a BC stage-dependent role of miR-9-5p: as a tumor suppressor gene in the early stage of BC, and as a promoter of the metastatic progression, essentially playing as an oncogene, at later stages (Li et al., 2020). For instance, Gravgaard et al. (2012) observed that miR-9-5p may be involved in the metastatic process, with a higher expression in distant metastases compared to the corresponding primary tumor. Low miR-9-5p expression was also found to be associated with a better prognosis, smaller tumors, earlier stage and ER+ cancers (Sporn et al., 2019). Controversial studies attributed downregulated miR-9-5p to anti-proliferative and pro-apoptotic functions in BC cells compared to healthy ones (Selcuklu et al., 2012; Orangi and Motovali-Bashi, 2019). Others described an unclear role of miR-9-5p in tumor tissues and BC cells, suggesting that its function may depend on different subtypes of BC or progression level (Krell et al., 2012; Hasanzadeh et al., 2016; Shi et al., 2017). Curiously, a group reported different expression levels of miR-9-5p in tissues compared to serum of BC patients (Tavakolian et al., 2019). Previous findings revealed that miR-9-5p expression can be influenced by steroid hormone receptors, and that it is implicated in a regulatory mechanism signaling in BC, besides being involved in affecting the biology of the tumor microenvironment (Zhuang et al., 2012; Pillai et al., 2014; Baroni et al., 2016; Barbano et al., 2017). Interestingly, Baldassari et al. (2018) demonstrated that a combined treatment with miRNAs, including miR-9-5p, enhances the activity of specific anti-BC drugs *in vitro*, even on the most aggressive HER2+ and TNBC subtype. However, the functional implications of miR-9-5p in AR signaling have not yet been deeply explored in BC. Today, among the main therapies for advanced BC in Tamoxifen-resistant BCs and TNBCs, are Bicalutamide and Enzalutamide, first and second generation AR-directed antagonists (Salvi et al., 2019). One of the first phase II trials involving Bicalutamide revealed evidence of androgen blockade and good tolerance in patients with AR+, ER– metastatic BC (Gucalp et al., 2013). Later, a phase II study enrolling patients with locally advanced or metastatic AR+ TNBC, confirmed the antitumor activity and safety of Enzalutamide (Traina et al., 2018). Other studies, including the one by Lu et al. (2019), described the combination of Bicalutamide and Aromatase inhibitor in patients with ER+/AR+ advanced BC. However, in this cohort, it was not reported a synergistic activity, suggesting that more large-sample clinical trials should be managed in this population to better understand how to overcome endocrine resistance (Lu et al., 2019). At present, there are no standard targeted therapies for the treatment of AR+ patients, given that AR is recognized as a protein whose regulation is still under discussion. Targeting the AR signaling could represent one of the challenging approaches to overcome the lack of response to current available therapies, and miRNAs belong to the most appealing candidates to be used for such therapeutic purposes. Here we show that miR-9-5p, which is downregulated in BC cell lines and in primary BC FFPE samples compared to healthy counterparts, downregulates AR, both at the mRNA and at the protein level, and that it is upregulated after androgens stimulation, regardless of the ER status. These findings suggest a possible feedback loop in which AR stimulation induces miR-9-5p in BC cells, which in turn silences AR expression and prevents AR downstream signaling even in presence of AR agonists. To our knowledge, this is the first evidence that demonstrates such an intertwined loop between androgens, miR-9-5p and AR in BC (**Supplementary Figure S7**). While the pro- or anti-proliferative effect of miR-9-5p in BC is still debated, in our cell line models miR-9-5p elicits an anti-proliferative effect (3 different cell lines and 3 different time points). Interestingly, the anti-tumoral effect of miR-9-5p is independent of the ER status, providing the rationale for the study of this miRNA as a possible therapeutics especially in subtypes of BC such as TNBC, currently with a dismal prognosis. Cell Titer-Glo assay was used to assess the number of viable cells based on the number of metabolically active cells, whereas BrdU incorporation assay was used to obtain quantitative information on cells that are actively replicating their DNA. The different results obtained with the two methods should be interpreted in light of the intrinsic differences between the two assays. We observed a significant decrease of cell proliferation in

ER+ cells MCF-7 and T-47D, after DHT exposure, while in ER- cell line MDA-MB-453 although it induced a slight increase of cell metabolic activity, it seemed to decrease the percentage of cells in S phase, overall supporting an antiproliferative effect. It is believed that the androgen/estrogen imbalance can promote tumor progression depending on the predominant hormone. In addition, aromatizable androgens such as androstenedione and testosterone may have both anti-proliferative or pro-proliferative effect depending on several variables, i.e., the activity of estrogen- or androgen- synthesizing enzymes (aromatase and 5α-reductase, respectively), the intracellular ER/AR expression ratio, and the concentration of circulating androgens (Secreto et al., 2019). About the latter, it has been suggested that they could have a role as independent molecules but also as a substrate for estrogen synthesis albeit limited to AR+/ER+ BC (Giovannelli et al., 2018). Interestingly, in the absence of ER-α more than a half of AR binding events had a pattern analogous to that of ER-α in ER+ cells, promoting ER target genes expression and cell growth, thus suggesting a role of AR as a ER-α mimic (Robinson et al., 2011). All together, these findings highlight the need to better understand the androgen/estrogen network, in order to clarify the different behaviors observed in BC subtypes and patients, especially in relation to the presence or absence of ER, as we showed in our data. Since miR-9-5p over-expression decreases BC cell proliferation, our findings rise the very provocative question whether AR antagonists might actually weaken the anti-proliferative effects of miR-9-5p, by preventing androgen-induced up-regulation of miR-9-5p in BC cells. Certainly further studies should be conducted to better understand this point and clarify how miR-9-5p levels and modulation could affect anti-AR treatments in AR+ BC. In support to a possible correlation between miR-9-5p and AR, we further reported an inverse correlation between miR-9-5p and AR expression in a set of paired FFPE tumor and adjacent non-tumor BC patients. Obviously, the small size of our patient cohort represents a limitation of this study, together with the fact that we did not interrogate the effects of miR-9-5p in AR regulation in an *in vivo* model. Future studies will cover these limitations but the fact that an inverse correlation between miR-9-5p and AR expression in primary samples was achieved already in such a small number of patients, should be interpreted as encouraging and superior to any animal model data. In summary, we identified miR-9-5p as a tumor suppressor gene in BC, regardless of ER status, capable of down-regulating AR in BC cells and to inhibit AR downstream signaling even in presence of androgen agonists. We finally report that miR-9-5p is induced by AR agonists, supporting the existence of an androgen/miR-9-5p/AR feedback circuitry that should be accounted for when exploiting AR antagonists as therapeutics for BC patients.

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 Romagna Ethics Committee (C.E.R.O.M.) and IRST IRCCS Medical Scientific Committee (CMS). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF: conceptualization, supervision. EB and FF: data curation, writing–original draft. FF: formal analysis. EB, IV, TR, MP, and FFb: investigation. RM: patients enrollement. MT and FL: immunoistochemistry and slide evaluation. EB: methodology. EB and MF: project administration. FF, IV, FFb, SH, WC, and MF: writing–review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was partially supported by Alma Mater Studiorum – University of Bologna for the Erika Bandini's Ph.D. study and research (Bandini, 2015). MF was the recipient of a Pablove Accelerator award. MF was supported by the National Institutes of Health (NIH/NCI) grant 5P30CA071789-20. Work in MF's lab was supported by the NIH/NCI grants R01CA215753 and R01CA219024.

ACKNOWLEDGMENTS

We thank Michela Tebaldi for the bioinformatic assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.579160/full#supplementary-material

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 08 December 2020
doi: 10.3389/fcell.2020.608412

Microenvironmental Determinants of Breast Cancer Metastasis: Focus on the Crucial Interplay Between Estrogen and Insulin/Insulin-Like Growth Factor Signaling

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OPEN ACCESS

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

Received: 20 September 2020
Accepted: 09 November 2020
Published: 08 December 2020

Citation:
Vella V, De Francesco EM, Lappano R, Muoio MG, Manzella L, Maggiolini M and Belfiore A (2020) Microenvironmental Determinants of Breast Cancer Metastasis: Focus on the Crucial Interplay Between Estrogen and Insulin/Insulin-Like Growth Factor Signaling. Front. Cell Dev. Biol. 8:608412. doi: 10.3389/fcell.2020.608412

The development and progression of the great majority of breast cancers (BCs) are mainly dependent on the biological action elicited by estrogens through the classical estrogen receptor (ER), as well as the alternate receptor named G-protein–coupled estrogen receptor (GPER). In addition to estrogens, other hormones and growth factors, including the insulin and insulin-like growth factor system (IIGFs), play a role in BC. IIGFs cooperates with estrogen signaling to generate a multilevel cross-communication that ultimately facilitates the transition toward aggressive and life-threatening BC phenotypes. In this regard, the majority of BC deaths are correlated with the formation of metastatic lesions at distant sites. A thorough scrutiny of the biological and biochemical events orchestrating metastasis formation and dissemination has shown that virtually all cell types within the tumor microenvironment work closely with BC cells to seed cancerous units at distant sites. By establishing an intricate scheme of paracrine interactions that lead to the expression of genes involved in metastasis initiation, progression, and virulence, the cross-talk between BC cells and the surrounding microenvironmental components does dictate tumor fate and patients' prognosis. Following (i) a description of the main microenvironmental events prompting BC metastases and (ii) a concise overview of estrogen and the IIGFs signaling and their major regulatory functions in BC, here we provide a comprehensive analysis of the most recent findings on the role of these transduction pathways toward metastatic dissemination. In particular, we focused our attention on the main microenvironmental targets of the estrogen-IIGFs interplay, and we recapitulated relevant molecular nodes that orientate shared biological responses fostering the metastatic program. On the basis of available studies, we propose that a functional cross-talk between estrogens and IIGFs, by affecting the BC microenvironment, may contribute to the metastatic process and may be regarded as a novel target for combination therapies aimed at preventing the metastatic evolution.

Keywords: breast cancer, metastasis, estrogen receptor, GPER, insulin/IGF signaling, tumor microenvionment, targeted therapies

INTRODUCTION

Breast cancer (BC) is the most common tumor in women and the second cause of cancer-related death worldwide (DeSantis et al., 2019). The metastatic evolution, which occurs in nearly 50% of BC patients, seriously thwarts the clinical management of the disease, thereby representing one of the main determinants of BC mortality (DeSantis et al., 2019). Consequently, enormous research effort is currently focused on a better understanding of the multiple molecular and biological factors facilitating the formation and spread of metastases. In this vein, gene signatures specifically discriminating between metastatic and non-metastatic tumors have been identified (Ramaswamy et al., 2003), allowing to postulate that the metastatic propensity is established in the early stages of oncogenesis by three major classes of genes: (i) genes controlling the metastasis initiation, (ii) genes controlling the metastasis progression, and (iii) genes controlling the metastasis virulence (Nguyen and Massagué, 2007). In addition, it is now recognized that most of these genes activated in cancer cells coopt microenvironmental signals to prompt the metastatic process in diverse tumor types, including BC. Indeed, the acquisition of metastatic features requires a complex and coordinated interaction between the epithelial BC cells and the surrounding tumor microenvironment, which is characterized by cellular (stromal fibroblasts, adipocytes, cancer stem cells (CSCs), and endothelial and immune cells) and non-cellular [growth factors and hormones, extracellular matrix (ECM) molecules, cytokines, and low oxygen] components that actively cooperate toward the metastatic landscape (Hanahan and Coussens, 2012). In this context, it should be mentioned that certain metabolic conditions associated with dysfunctional hormonal status, such as obesity and type 2 diabetes, may contribute to metastasis formation in BC, as suggested by epidemiological evidence indicating an elevated risk of metastasis in diabetic and obese patients (Park et al., 2017; Harding et al., 2020). Likewise, worse prognostic parameters have been detected in this subpopulation of BC patients (Schrauder et al., 2011; Zhao and Ren, 2016). Notwithstanding the aforementioned epidemiological correlations, the molecular mechanisms underlying the high risk and poor outcome of obese and diabetic BC patients are complex and multifactorial. First, adipose tissue does contribute to the local production of estrogens, which exert a potent stimulatory action on cancer cells binding to the classical estrogen receptor (ER), as well as the alternate G-protein–coupled estrogen receptor (GPER) (Barton et al., 2018). In addition, obesity facilitates the establishment of hyperinsulinemia and insulin resistance, thereby determining an unopposed activation of the insulin receptor (IR) and the insulin-like growth factor receptor (IGF-1R) (Lewitt et al., 2014), which are part of the complex insulin/IGF system (IIGFs). IIGFs comprises insulin, IGF-1, IGF-2 (IGFs) and cognate receptors (IR, IGF-1R, IR/IGF-1R hybrids, and IGF-2R also known as the mannose 6-phosphate receptor) and six IGF-binding proteins (IGF-BP1-6) (Frasca et al., 2008). IIGFs is deeply deregulated in diverse type of tumors, including BC, and it has been implicated in the acquisition of the metastatic potential (Frasca et al., 2008; Vigneri et al., 2015; Manzella et al., 2019). Noteworthy, both IIGFs and estrogen signaling promote paracrine responses that endow cross-communications within the diverse components of the breast tumor microenvironment toward metastatic progression. In addition, complex networks of molecular and functional connections between these signaling systems appear to elicit a relevant role in BC metastasis. Herein, we first provide a comprehensive analysis of the most significant components of the tumor microenvironment involved in the activation of metastatic programs in BC. Next, we emphasize the molecular and functional interplay between estrogen and IIGFs signaling in activating BC microenvironment toward the acquisition of a metastatic phenotype. Finally, we propose that targeting the dysfunctional interactions between the IIGFs and the estrogen pathways may represent a promising tool in comprehensive therapeutic approaches aimed at halting the aberrant microenvironment in the metastatic BC.

MICROENVIRONMENTAL PLAYERS INVOLVED IN BC METASTASIS

Despite being considered a biologically inefficient process, as only few of the cancer cells released in the bloodstream actually develop secondary tumors, metastases remain one of the most intriguing and investigated aspects of tumor biology for their huge impact on prognosis. Likewise, the vast majority of BC-related deaths are due to metastases, which target mainly the bone (50–75%), lung (17%), brain (16%), and liver (6%) (Wei and Siegal, 2017). BC cells can escape the primary tumor, sneaking into the circulatory system and reaching distant sites where the neoplastic cells can either form a novel tumor mass straight after or enter a dormant state that can end up in disease relapse. Accordingly, the formation of overt secondary tumors can occur even many years after the diagnosis of the primary disease, as tumor cells disseminated at secondary sites may remain indolent for protracted period of times, until systemic and local factors cooperate toward the waken-up of dormant tumors. The macroenvironmental and microenvironmental mechanisms regulating cancer cell detachment from primary site and colonization at secondary target tissues, as well as entry and exit from dormancy, are likely to determine the fate of incipient tumors and therefore the prognosis of patients. In this paragraph, we provide an overview of the main microenvironmental players involved in BC metastasis, in order to provide a propaedeutic outline for depicting the cooperation of estrogen and IIGF signaling in triggering metastasis dissemination. For descriptive purposes, the aforementioned players will be categorized according to their role in (i) metastasis initiation, (ii) metastasis progression, and (iii) metastasis virulence.

Metastasis Initiation

Metastasis initiation refers to the complex coordination of the biological processes determining tumor outgrowth and angiogenesis, thereby prompting cancer cell entry into the bloodstream. A better understanding of the microenvironmental mechanisms regulating the expression of genes involved in

metastasis initiation in BC is pivotal to deciphering the role of estrogenic and IIGFs signaling in the early stages of metastatic switch. In BC, the initiation of metastasis appears to be abundantly regulated by microenvironmental events that promote epithelial-to-mesenchymal transition (EMT), the formation of CSCs, the activation of neoangiogenesis, and the instigation of local invasion.

EMT occurs when epithelial cells are reprogrammed to acquire mesenchymal traits, endowing BC cells with increased detachment propensity, enhanced motility, and invasive capability, as well as augmented intravasation capacity (Polyak and Weinberg, 2009). Clearly, EMT entails a profound change in cytoskeleton organization and a marked inclination to loosen cell–cell junctions that disrupt the contiguity of the epithelium and facilitate the breaching of basement membrane. A number of environmental clues originating from diverse cell types within the tumor milieu may activate EMT programs in BC. The most important regulatory factors in EMT are hormones, growth factors [IGFs, hepatocyte growth factor (HGF), epidermal growth factor [EGF], platelet-derived growth factor [PDGF], transforming growth factor β (TGF-β)], and cytokines/chemokines (Polyak and Weinberg, 2009). In addition, developmental signaling pathways (Wnt, Notch, and Sonic hedgehog), ECM components (collagen, hyaluronic acid, integrins), and local hypoxia may contribute to the modulation of EMT (Polyak and Weinberg, 2009).

These stimuli converge on several EMT-inducing transcription factors such as Snail, Slug, Zeb1, Zeb2, Twist, FoxC2, and Goosecoid (Polyak and Weinberg, 2009), with the ultimate aim to repress CDH1 (E-cadherin) transcription, thereby reducing epithelial differentiation and promoting a mesenchymal phenotype. It is worth recalling the enormous heterogeneity of microenvironmental cell types involved in the production of breast EMT-inducing molecules. For instance, stromal cancer-associated fibroblasts (CAFs) expressing Snail1 are associated with a high degree of desmoplastic areas with anisotropic fibers, together with lymph node involvement and worse prognosis in infiltrating BC (Stanisavljevic et al., 2015). Likewise, Snail-1 depletion in CAFs hampered their paracrine activity toward metastatic invasion, as supported by animal models of BC co-xenografted with BC cells and Snail1-deficient CAFs (Alba-Castellón et al., 2016). Together with fibrous stroma, also adipose stroma is involved in BC EMT toward the acquisition of metastatic potential. In this regard, it has been shown that when cocultured with adipocytes, BC cells may acquire EMT-like phenotypic changes associated with Twist-1 activation and higher migratory and invasive capability (Lee et al., 2015). Extending these findings, transcription factors classically associated with EMT programs have been shown to impact also other aspects of BC progression, including inflammation and antitumor immunity. This is the case for the transcription factor ZEB (zinc finger E-box–binding protein 1), whose global transcriptional regulation profile has been investigated by chromatin immunoprecipitation and RNA sequencing, followed by gene set enrichment analysis of ZEB1-bound genes in BC cells. Using this approach, the authors identified a ZEB1-regulated inflammatory phenotype associated with the production of cytokines classically related with poor prognosis and metastasis, including interleukin 6 (IL-6) and IL-8 (Katsura et al., 2017). Of note, in EMT-activated BC cells, the immune checkpoint ligand PD-L1 was shown to be up-regulated in a Zeb-1–dependent manner (Noman et al., 2017), reinforcing the evidence that EMT-associated gene signatures correlate with increased inflammatory immune cell infiltration toward BC aggressiveness (Mak et al., 2016). It should be mentioned that EMT also serves as a reprogramming tool through which cancer cells acquire stemness features correlated with enhanced metastatic capability (Mani et al., 2008). According to the CSC hypothesis, a rare subpopulation of stem-like cells with tumorigenic, self-renewal and differentiation properties may generate all cell types within the tumor bulk (De Francesco et al., 2018b). Furthermore, metastatic proficiency is strictly linked to the abundance of cancer cells with stem features (Charafe-Jauffret et al., 2009). In cells undergoing EMT, mammosphere formation, used as readout for CSCs activity, is 10-fold more efficient, thereby corroborating the idea that the EMT process may serve as a source of CSCs (Mani et al., 2008). In this context, the adaptive response gene ATF3 has been proposed to integrate stromal signals coming from the tumor microenvironment with the acquisition of combined EMT/CSC features. More specifically ATF3, which is regulated by a number of extracellular signals including TGF-β, tumor necrosis factor α (TNF-α), and IL-1β, may promote morphological and molecular changes consistent with the activation of EMT, the increase of the CD24low–CD44high cells, the formation of mammospheres, the activation of motility programs, and breast tumorigenesis *in vivo* (Yin et al., 2010).

Transendothelial migration (TEM) precedes the dissemination of cancer cells in the circulation, thereby permitting intravasation. As a pivotal step in metastasis initiation, TEM entails a number of microenvironmental cellular and non-cellular actors. Indeed, endothelial cells, vessel-associated macrophages (VAMs) and tumor-associated macrophages (TAMs) play a key role in BC cell intravasation. For instance, VAMs secrete chemoattractant molecules to recruit cancer cells at the vessel interface, whereas BC cells themselves secrete colony-stimulating factor to attract macrophages in an auto-amplifying paracrine loop (Goswami et al., 2005). Moreover, macrophages-derived TNF-α induces the retraction of endothelial cells and their apoptosis, thus rendering vessels more loose and permeable for cancer cells invasion (Zervantonakis et al., 2012). Interesting evidences indicate that diverse signals from stromal CAFs led by TGF-β, PDGF, CXCL12/CXCR4, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs) can directly drive the process of intravasation through multiple mechanisms as ECM remodeling, enhanced vessel permeability, and aberrant angiogenesis (Guo and Deng, 2018).

Metastasis Progression

The reciprocal interaction between estrogen and IIGFs signaling in BC microenvironment facilitates metastasis progression, which refers to the multiple events occurring both in the primary tumors and at metastatic sites, immediately after intravasated cancer cells enter the circulation and reach target organs. Having

gained access to lymphatic vessels or capillaries, circulating BC cells disperse in the bloodstream in various directions before their extravasation at secondary site, an event that seems to be organ-specific and facilitated by numerous players like components of the TME [mesenchymal stromal cells (MSCs), CAFs, TAMs], circulating cancer cells, and extravasation factors. By using a murine BC model of lung metastasis, Yu and collaborators found that MSCs maintain an inhibitory tone on lung metastasis formation through the release of the inflammatory chemokine CXCL12 and the up-regulation of the cognate receptor CXCR7 in BC cells (Yu et al., 2017). However, this effect is reversed in the presence of TGF-β, thus indicating that the prometastatic effect of MSCs depends on the simultaneous activation of inflammatory pathways like TGF-β, which is known to be activated in CAFs (Yu et al., 2017). The rapid outgrowth and expansion of the neoplastic mass generates intratumor hypoxia, which activates compensatory biological responses mediated by the transcription factors HIF (hypoxia-inducible factors) 1 and 2 (Semenza, 2012). HIF-mediated gene transcription occurs at the primary tumor, at the premetastatic niche, and ideally in all the cellular components of the TME, with the ending result of boosting the formation of metastasis (Semenza, 2012). In BC, HIF triggers the production of angiogenic factors such as VEGF to support intravasation and extravasation (Semenza, 2012). Loss of HIF-1 in triple-negative BCs (TNBCs) was associated with decreased lung metastasis through the inhibition of L1 cell adhesion molecule, which mediates BC cells' physical interactions with endothelial cells at the pulmonary district (Zhang et al., 2012). Of note, HIF mediates the activation of signaling systems required for BC invasion like the HGF/MET pathway and the RhoA/Rock signaling (Semenza, 2012). Ideally contributing to all the steps necessary for metastasis formation and dissemination, gene transcription programs dependent on HIF activation pave the way for extravasation and invasion also by triggering deep transformations of ECM. These responses require the up-regulation of lysyl oxidase enzymes (LOX, LOXL2, and LOXL4), which are produced by hypoxic BC cells released in the bloodstream and accumulated at premetastatic niche, where they enable the remodeling of collagen and other ECM molecules toward the intravasation of circulating BC cells (Schito and Semenza, 2016). Interestingly, certain ECM molecules such as hyaluronan not only enable tumor stroma with mechanical properties facilitating BC cell motility, but also provide CAFs with enhanced migratory capability leading to the metastasis progression (McCarthy et al., 2018). Indeed, CAFs can be found at the primary and the metastatic stroma, as well as in the circulatory system. Circulating CAFs (cCAFs) can be detected individually or in CAFs clusters, as well as in heterotypic clusters with circulating tumor cells (CTCs). It has been suggested that cCAFs generate a suitable microenvironmental niche for metastasis seeding and growth together with the escape from immune surveillance (Duda et al., 2010). In support of this hypothesis, CAFs have been detected in premetastatic niches prior to the appearance of cancer cells. Extending these findings, Ao and collaborators detected cCAFs in almost 90% of patients with metastatic BC, whereas these cell types were detected in nearly the 20% of patients with localized disease and were absent in samples from healthy donors. These observations indicate that cCAFs may serve as a tool to track and perhaps anticipate the detection of CTCs (Ao et al., 2015). CTCs, which are found as single cells or as clusters (tumor emboli), are considered as precursors of metastatic colonies. Their biology and behavior strictly depend on the tumor of origin, as well as on microenvironmental factors. For instance, a metastasis-competent subset of clustered CTCs from BC patients oligoclonally derive from primary tumor cells and are held together by plakoglobin-mediated intercellular adhesion (Aceto et al., 2014). Interestingly, elevated expression of plakoglobin in BC samples correlates with worse prognostic index, including worse distant metastasis–free survival, thereby reinforcing the role of CTCs and related factors in metastasis formation (Goto et al., 2017). It has become increasingly recognized that TAMs contribute to the acquisition of malignant features in BC, through multiple mechanisms, including the formation and dissemination of metastasis. Indeed, TAMs contribute to BC cell migration and invasion, boost lymphangiogenesis and angiogenesis, participate in the formation of the metastatic niche and maintain a cross-communication with BC cells to support disease progression (Williams et al., 2016). Chemoattractant factors released by TAMs trigger tumor cells intravasation and their travel at distant sites such as lung and bone (Williams et al., 2016). Furthermore, TAMs secrete a number of proangiogenic mediators including EGF, PDGF, MIF, TNF-α, TGF-β, IL-8 and IL-1β, CCL2, and CXCL8 (Williams et al., 2016). Interestingly, intravasation of BC cells facilitated by TAMs can occur also in absence of local angiogenesis, as evidenced by multiphoton microscopy in animal models of BC (Williams et al., 2016). It is been largely demonstrated that paracrine signals between TAMs and BC cells establish positive feedback loops conducive to disease progression. In particular, EGF secreted specifically by TAMs but not by BC cells derived from primary tumors was shown to promote cell invasion (O'Sullivan et al., 1993) and the expression of CFS-1 in BC cells. Then, CSF-1 secreted by BC cells induced the production of EGF by TAMs (Goswami et al., 2005). The pharmacological manipulation of this paracrine cycle by inhibition of either EGFR or CSF-1R was sufficient to dampen BC cell migration and invasion (Goswami et al., 2005). On the basis of the above considerations, it is evident that the BC microenvironment at the metastatic site is profoundly different from that surrounding the primary tumor. Understanding these molecular and biological differences may represent a useful tool to manipulate the tumor microenvironment in order to control the metastatic progression.

Metastasis Virulence

A number of estrogen and IIGF-regulated genes control the so-called metastasis virulence, which refers to the events that contribute to the metastatic colonization. These multifaceted responses bestow biological advantages to the secondary rather than the primary tumor, facilitating the establishment of macrometastases once locally aggressive micrometastasis have been formed. Clearly, the mechanisms regulating these responses are particularly influenced by the organospecific tropism of metastatic BC cells; however, general dynamic mechanisms governing metastasis virulence can be described. First, BC

cells that successfully reach secondary sites are subjected to a mesenchymal–epithelial transition, which restores the epithelial phenotype. Afterward, neoplastic cells within the metastatic niche activate paracrine signaling that allow cell survival, resistance to apoptosis, evasion from immune surveillance, and colonization. Bone represents the main site for BC metastasis, particularly in the luminal subtypes of BC (Wei and Siegal, 2017). Metastatic BC cells hamper bone remodeling, promote bone degradation, and activate osteomimicry processes that facilitate the formation of macrometastasis (Awolaran et al., 2016). The initial trigger is represented by factors released by BC cells in the bone, including osteopontin (OPN), parathyroid hormone-related peptide (PTHrP), heparanase, IL-1, IL-6, and prostaglandin E2. These mediators contribute to the instigation of osteolytic processes by RANKL–RANK signaling. As a consequence of osteoclasts activation, bone is degraded through the involvement of cathepsin-K, MMP-9, and MMP-13. Growth factors stored in the bone matrix (TGF-β, IGF-1) are immediately released and in turn stimulate BC cells to secrete additional PTHrP in a vicious cycle (Waning and Guise, 2014). As it concerns brain metastasis from BC, their ability to adapt to the specific brain microenvironment is highlighted by the evidence that novel neurovascular units constituted by metastatic cells, together with microvascular cells, astrocytes, and neurons are immediately organized in the metastatic niche, where neoplastic cells may acquire a metabolic phenotype similar to the ones of resident cells (Neman et al., 2014). Very likely, this strict multicellular cooperation guarantees a better control on the brain blood barrier, thereby facilitating the access of additional CTCs, as well as an easy entry gate for nutrients. Interestingly, brain metastatic cells can activate adjacent astrocytic and glial cells that in turn secrete a number of tumor-stimulating cytokines, including IL-6, interferon γ (IFN-γ), tumor necrosis factor-α (TNF-α), TGF-β, IGF-1, and PDGF-1 (Wang et al., 2013), thereby supporting the role of the metastatic microenvironment in the evolvement of the secondary disease. In order to survive and colonize the hostile lung environment, BC metastases enact a deep remodeling of the premetastatic niche through the establishment of paracrine responses at the interface between host cells and cancer cells. For instance, BC cells exhibiting a preferential tropism for the lung fuse with lung fibroblasts and release their exosomes toward the production of proinflammatory S100 proteins that facilitate the survival of metastatic cells (Hoshino et al., 2015). Additionally, the mobilization of bone marrow–derived cells initiated by the HIF/LOX pathway in hypoxic BC cells triggers ECM remodeling in the lung and facilitates the systemic instigation of indolent cancer cells through the secretion of OPN. Interestingly, Ye et al. (2015) have unveiled the ability of an inflammatory microenvironment to impact on metastasis formation at the lung. More specifically, using a mouse model of BC, the authors found that a TGF-β–driven inflammatory signature drives the secretion of cytokines involved in the formation of the premetastatic niche such as S100A8, S100A9, Angpt2, and VEGF. Last, a metastasis-favorable microenvironment has been hypothesized for liver, where larger BC metastasis can be found compared to the lung. Along with fibroblasts and TAMs, liver-specific cellular components such as Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells cooperate toward the establishment of metastasis (Ma R. et al., 2015). Of note, liver metastases from BC cells show a peculiar metabolic profile compared to bone and lung metastases. The reduction of mitochondrial metabolism and the increased rate of conversion of pyruvate into lactate by PDK1 may suggest a specific metabolic adaptation to lack of nutrients and hypoxia. Likewise, PDK1 is recognized as one of the most important regulators of liver metastasis in BC (Dupuy et al., 2015).

ESTROGEN AND IIGFS SIGNALING IN BC

Having described the main biological events and molecular mediators orchestrating the microenvironmental responses involved in BC metastasis, in this paragraph we provide a brief but sound overview of the basic signaling mechanisms mediated by estrogen and IIGFs in BC (**Figure 1**). Despite the description of estrogen and IIGFs pathway in epithelial BC cells goes beyond the purpose of this review, a concise sketch of the mode of action of these transduction pathways is required to understand how estrogen and IIGFs signaling work together in landscaping BC microenvironment toward metastasis propagation.

Estrogen Signaling

Estrogenic signaling facilitates the establishment of BC metastasis by activating stimulatory responses that impact the initiation, progression, and virulence of metastatic genes. Most of these genes are transcriptionally regulated by the ERα, which is expressed in approximately 70% of breast tumors identifying estrogens as master regulators of breast malignant development (Katzenellenbogen and Frasor, 2004; Yager and Davidson, 2006; Kumar et al., 2011; Rondón-Lagos et al., 2016). Consequently, ERα is a main target of the current endocrine approaches in ERα-positive BCs (Howell et al., 2007). Estrogen-mediated gene transcription occurs through multiple independent and sometimes cooperating mechanisms that may lead to relevant biological responses. Unliganded ERα is principally located in the cytoplasm; however, upon ligand exposure, it dissociates from the heat shock proteins, dimerizes, and shuttles to the nuclear compartment (Stenoien et al., 2001). Then, ERα acts as a transcription factor binding to the estrogen-responsive elements (EREs) located on the promoter regions of target genes (Stenoien et al., 2001). Ligand-activated ERα may also regulate the transcription of genes in an ERE-independent manner through the interaction with other factors (McDonnell and Norris, 2002; Björnström and Sjöberg, 2005). For instance, interacting with c-fos and c-jun proteins at the AP-1–binding sites, ERα may regulate the transcription of genes as IGF-1 (Umayahara et al., 1994), collagenase (Webb et al., 1995), and cyclin D1 (Sabbah et al., 1999). In addition, ERα may contribute to rapid responses to estrogens by interacting with scaffold proteins such as caveolin-1 or signaling molecules, namely, G proteins, Src kinase, and Shc (Migliaccio et al., 1996; Razandi et al., 1999, 2002; Wyckoff et al., 2001; Song et al., 2002;

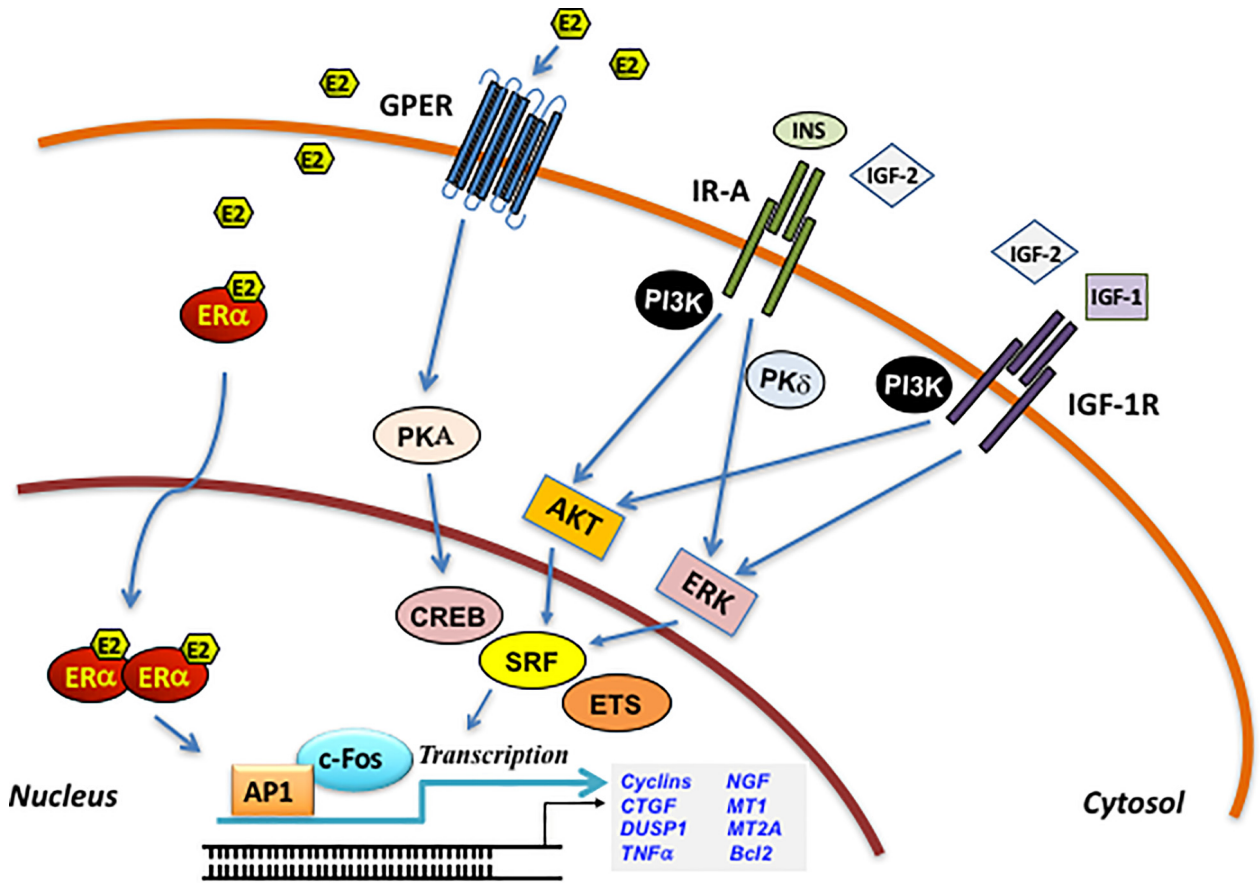

FIGURE 1 | Schematic illustration of the ERα/GPER and IIGFs cross-talk. Insulin, IGF-2, and IGF-1 bind to their specific receptors and stimulate rapid signals converging to the activation of PI3K, MAPK, and PKδ networks. These pathways, in turn, trigger the activation of transcription factors including CREB, SRF, and ETS, which favor c-fos induction and its recruitment to the AP-1 site. ERα/GPER activation by E2, through the activation of various intermediates, cross-talks with the IIGFs leading to enhanced mitogenic signals. PKA, protein kinase A; PKCδ, protein kinase C, δ isoform; MAPK, mitogen-activated protein kinases; PI3K, phosphatidyl-inositol-3-kinases; ERK, extracellular signal-regulated kinases; AKT, protein kinase B; CREB, cAMP-response element-binding protein; ETS, E26 transformation specific; SRF, serum response factor; c-fos, FBJ murine osteosarcoma virus; AP-1, activator protein-1; CTGF, connective tissue growth factor; DUSP1, dual specificity protein phosphatase 1; TNF-α, tumor necrosis factor α; NGF, nerve growth factor; MT1, metallothionein 1; MT2A, metallothionein 2A; Bcl2, B-cell lymphoma 2.

Auricchio et al., 2008; Levin and Pietras, 2008; Levin, 2009), and activate diverse extranuclear signaling cascades, such as Src, adenylyl cyclase, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and protein kinase C (PKC) (Migliaccio et al., 1996; Castoria et al., 2001). Likewise, upon estrogenic stimulation, ERα engages tyrosine kinase receptors as IGF1R, the EGF receptor, and ErbB2 (HER-2/neu), triggering relevant biological effects in diverse cell contexts, including BC cells (Kahlert et al., 2000; Chung et al., 2002; Razandi et al., 2003). For instance, the ERα-mediated activation of growth factor receptors may lead to the stimulation of the Ras/Raf/MAPK and Akt transduction cascades and then to growth responses (Kahlert et al., 2000; Chung et al., 2002; Razandi et al., 2003). Overall, the aforementioned nuclear and extranuclear-initiated pathways driven by ERα may control a variety of biological outcomes in mammary tumor cells, ranging from cell cycle, proliferation, chromatin remodeling to survival, and motility (Ballaré et al., 2003; Levin, 2003; Qiu et al., 2003; Castoria et al., 2004; Vicent et al., 2006; Giretti et al., 2008; Levin and Pietras, 2008; Giovannelli et al., 2012).

Along with ERα, additional mediators have been shown to convey estrogen signaling toward metastatic features. In this regard, the GPER, originally termed GPR30, is a seven-transmembrane protein belonging to the G-protein–coupled receptors superfamily, which mediates the action of estrogens in numerous normal and malignant cell contexts. For instance, several studies have reported a tumor promoting effects of GPER in BC. In this regard, estrogens were shown to trigger through GPER the SRC-mediated extracellular release of heparan-bound EGF and then the activation of EGFR in ER-negative BC cells (Filardo et al., 2000). Triggering rapid kinase-associated transduction pathways (i.e., ERK1/2, PI3K/Akt, Hippo/YAP/TAZ pathway), ion channels (i.e., calcium) and second messengers (i.e., cAMP), GPER may regulate the transcription of diverse genes such as c-fos, connective tissue growth factor (CTGF), EGR1, ATF3, metalloproteases, and cyclins (Pandey et al., 2009;

Zhou et al., 2015; Barton et al., 2018). The genomic responses to GPER activation may in turn influence BC cell growth, motility, and invasion (Lappano et al., 2014). Not only estrogens and estrogen-mimetic compounds, but also antiestrogens such as 4-hydroxytamoxifen, raloxifene, and ICI182,780, may act as GPER agonists and stimulate cell survival and proliferative transduction pathways (Filardo et al., 2000; Revankar et al., 2005; Pandey et al., 2009; Prossnitz and Arterburn, 2015). A functional role for GPER in breast tumorigenesis and particularly in metastasis has also been confirmed in transgenic mouse models of mammary tumorigenesis. At later stages of tumorigenesis, GPER knockout mice showed smaller tumors respect to wild-type mice along with a reduced growth rate, histologic features typical of low aggressive tumors, and decreased lung metastases (Marjon et al., 2014). Retrospective BC analysis further supported the contribution of GPER in BC progression. In this vein, immunohistochemical studies showed that GPER levels are positively associated with tumor size, distant metastases, and recurrence in BC specimens and inversely correlated with disease-free survival in tamoxifen-treated patients (Filardo et al., 2006; Liu et al., 2009; Ignatov et al., 2011). A recent bioinformatics analysis in ER-negative BCs has endorsed the aforementioned findings, proving that high GPER levels are both linked with promigratory and metastatic genes and positively correlated with a shorter disease-free interval (Talia et al., 2020). Nevertheless, some studies have reported a tumor suppressor function of GPER (Weißenborn et al., 2014; Martin et al., 2018), warranting further investigations in order to better appreciate the role of GPER in different cancer cell contexts.

Insulin/IGF Signaling

As stated above, IIGFs, an important growth regulatory pathway often overactivated in BC, is crucially implicated in the acquisition of metastatic features.

IIGFs consists of circulating ligands (insulin, IGF-1, and IGF-2), multiple receptors, and six IGF-binding proteins (Belfiore et al., 2009, 2017). The human IR exists in two isoforms (IR-A and IR-B) generated by alternative splicing of the IR gene with the exclusion (IR-A) or inclusion (IR-B) of 12 amino acids encoded by exon 11. The IR and the IGF-1R have highly homologous structures, but different functions. Given the high degree of homology, IR and IGF-1R can heterodimerize leading to the formation of insulin/IGF-1 hybrid receptors (HRs) (Belfiore et al., 1999, 2009). The IGF-2R lacks an intracellular tyrosine kinase domain and therefore does not transduce intracellular mitogenic signals, acting mainly as a buffer for modulating IGF-2 bioactivity through IR-A and IGF-1R (El-Shewy and Luttrell, 2009). The IIGFs has a significant role not only for normal mammary gland development but also in the onset and maintenance of the malignant phenotype of BC cells. As insulin and IGFs stimulate cell growth via mitogenic, antiapoptotic and chemotactic activity, many of the steps of the normal development of the mammary gland are recapitulated during the process of metastasis (Gallagher and LeRoith, 2011). Indeed, IIGFs is implicated in tumor progression and metastasis of both ER-positive and ER-negative BC cells (Bartella et al., 2012; De Marco et al., 2015) and frequently shows features of deregulation such as (i) overexpression and activation of IGF-1R, IR, and IR/IGF-1R hybrids in malignant cells, (ii) dysregulated expression and/or bioavailability of IGF-1 and IGF-2 in both malignant and stromal cells, and (iii) increased IR-A:IR-B ratio and establishment of IR-A/IGF2 autocrine/paracrine loops (Malaguarnera et al., 2012a). IR-A is also termed the "oncofetal" IR isoform as it exerts a pivotal role in promoting fetal growth by acting as a promiscuous receptor that binds not only insulin but also IGF-2, proinsulin, and IGF-1 (Belfiore and Malaguarnera, 2011; Malaguarnera and Belfiore, 2014; Belfiore et al., 2017). In fact, proinsulin, the insulin prohormone, which is increased in fetal life and insulin resistance conditions, is a high-affinity IR-A ligand (Malaguarnera et al., 2012b) and stimulates proliferation and migration in BC cells.

The increased IR-A:IR-B ratio in BC cells is likely due to multiple mechanisms leading to dysregulated expression of splicing factors involved in exon 11 skipping of the IR gene (Echeverria and Cooper, 2014) including mutations of the gene encoding for the SF3B1 splicing factor. In BC cells IR-A is considered to act as a hub for integrating signals coming from the circulation and connected with the nutritional status (insulin and proinsulin) and signals coming from the microenvironment (IGF-1 and IGF-2) (Belfiore et al., 2017). Insulin has a major orchestrating role in this context by increasing tissue IGFs' bioavailability through the dual action of enhancing IGF-1 production by the liver and concomitantly inhibiting IGF-BPs synthesis (Belfiore et al., 2017).

IR-A downstream signals show important differences when stimulated by either insulin or IGF-2, the latter being more mitogenic (Frasca et al., 1999). However, IR-A is intimately linked to the mitogenic MAPK/mammalian target of rapamycin (mTOR) cascade rather than to the PI3K/Akt metabolic cascade also in response to insulin (Frasca et al., 1999). As a consequence, BC cells do not share the insulin resistance of peripheral tissues of obese patients (Yee et al., 2020). Therefore, IR-A overexpression can be seen as a way BC cells exploit to overcome insulin resistance of obese patients and allow full response to the estrogen/IIGFs cross-talk (Belfiore et al., 2017). Additionally, IR-A overexpression increases the assembly of IR-A/IGF-1R hybrids that function as high-affinity binding sites for IGFs, thus amplifying signals from the microenvironment (Belfiore et al., 2009). In turn, IR-A–mediated biological responses are regulated by tumor stroma components, such as the proteoglycan decorin, which negatively modulates IGF-2 actions while leaving unaffected insulin/proinsulin effects. Thus, reduced levels of decorin associated with aggressive BCs enhance the activity of the IGF-2/IR-A loop (Morcavallo et al., 2014).

The relevance of this loop is underscored by studies showing that endocrine-resistant ER^+ BCs may have reduced expression of IGF-1R while expressing much higher levels of IR (Fagan et al., 2012; Yee, 2018). Similarly, data obtained in thyroid cancer indicate that loss of differentiation (Vella et al., 2002) and stem-like phenotype (Malaguarnera et al., 2011) are associated with high relative abundance of IR-A and IGF-2 secretion, while IGF-1R expression is generally reduced.

Although overexpression of IR and IGF-1R in cancer cells recognizes multiple mechanisms, which are reviewed elsewhere (Belfiore et al., 2017), a recently emerged non-mutational

mechanism involves the collagen receptor DDR1, which is up-regulated by IIGFs activation and by collagen (Vella et al., 2019a). In turn DDR1 up-regulates both IR and IGF-1R in a feed-forward loop (Matà et al., 2016; Vella et al., 2017) that may enhance BC metastasis potential (see below). Interestingly, DDR1 also regulates adipose cell aromatase and estrogen output by activating a mechanotransduction pathway (Ghosh et al., 2013) representing a relevant node in the estrogen/IIGFs cross-talk.

Not surprisingly, obesity and T2DM, both characterized by insulin resistance, are associated with an increased risk of postmenopausal BC and higher rates of tumor progression and recurrence; hyperinsulinemia has been found to be a major determinant of this risk (Schrauder et al., 2011; Lewitt et al., 2014; Park et al., 2017). In this line, several studies show that women with increased circulating levels of IGF-1 and low amount of IGFBP3 may have a high risk of BC and that high levels of IGF-1 are associated with BC progression and recurrence (Belfiore et al., 2017).

To further corroborate the importance of IR-A activation in BC patients, IR phosphorylation in BC cells was a significant marker of poor patient survival (Law et al., 2008). Moreover, a high IR-A:IR-B ratio was particularly associated with the luminal B subtype of ER^+/progesterone receptor–positive (PR^+)/$HER2^-$ BCs that are clinically characterized by a higher grade, positive lymph node involvement, and poorer relapse-free survival (Huang et al., 2011).

Notably, the IIGFs is widely implicated in the process of angiogenesis, which is essential for the metastatic dissemination of tumor cells. To metastasize, cancer cells must be able to form new vessels often in hypoxic environments. VEGF-A is an important mediator of angiogenesis and is under the transcriptional control of HIF-1 and HIF-2, transcription factors induced by hypoxia and growth factors (Bielenberg and Zetter, 2015).

Consistently, IGF-1Rs are expressed in isolated hemovascular endothelial cells, newly formed blood microvessels, and in lymphatic endothelium (Bar and Boes, 1984), and IGF-1 is able to up-regulate VEGF through HIF-1α in BC cells. Interestingly, GPER cooperates with HIF-1α for the transcriptional activation of VEGF induced by IGF-1 in vascular endothelial cells (De Francesco et al., 2017). IR-A is also markedly overexpressed in angiogenic vasculature in human tumors and stimulates endothelial cell proliferation and *in vivo* angiogenesis (Belfiore et al., 2009, 2017).

Similarly, lymphangiogenesis is an important mechanism by which tumor cells are disseminated via the lymphatic system and induce lymph node metastases, which occur in the early stages of BC development and may promote further spread of BC cells at distant sites (Fidler, 2003). Both IGF-1 and IGF-2 show the ability to induce angiogenesis and lymphangiogenesis in several *in vitro* and *in vivo* model systems (Bjorndahl et al., 2005). In particular, IGF-1 induces and promotes lymphangiogenesis through the induction of VEGF-C.

Along with angiogenesis and lymphangiogenesis, IIGFs have been implicated in the mechanisms of BC cell homing, which is necessary for colonization at secondary sites. In this regard, several evidences suggest that, upon exposure to cytokines and growth factors of bone microenvironment, BC cells undergo genetic alterations that may enhance their ability to survive and colonize the bone. IGF-1 and IGF-2 are among those molecules found in bone environment together with TGF-β, PDGF, and fibroblast growth factors (FGFs) (Wissmann and Detmar, 2006). Adding to this, oncogene mutations and other molecular abnormalities leading to STAT3 activation induce IGF-2 secretion and IR-A activation toward invasive features and resistance to antitumor treatments (Lee et al., 2006). For instance, IGF-2 secreted by epithelial mammary cells expressing c-Myc oncogene activates fibroblasts that acquire the ability to remodel the ECM, thus promoting epithelial cell invasion (De Vincenzo et al., 2019). Consistently, metastatic BC CAFs have protumorigenic properties induced by increased IGF-2 expression (Gui et al., 2019).

Metabolic reprogramming is a hallmark of cancer. It is worth mentioning that we recently showed that IR-A activation by insulin and IGF-2 plays a role in BC cells metabolic reprogramming by increasing both glycolysis and oxidative phosphorylation. IGF-2–activated IR-A especially enhanced BC cell metabolic flexibility, leading to the acquisition of malignant features consistent with cellular adaptation to a challenging microenvironment characterized by high energy demand (Vella et al., 2019b).

Finally, IGF-2/IR-A loop has also been implicated in EMT (Zelenko et al., 2016) and other stem-like features (Malaguarnera et al., 2011), which play a key role in cancer development and recurrence.

Overall, these studies clearly support a pivotal role for IIGFs in aggressive traits of BC supportive of metastatic phenotypes.

MICROENVIRONMENTAL INTERACTIONS BETWEEN ESTROGENIC SIGNALS AND IIGF CONDUCIVE TO BC METASTASIS

As previously mentioned, mounting evidence indicates that, in BC, signals mediated by estrogens and IIGFs shape the tumor microenvironment and drive metastatic evolution. Despite these signaling systems elicit profound direct actions on BC cells themselves, understanding the role of estrogens and IIGFs and their cooperation in landscaping the tumor microenvironment toward metastatic features (**Figure 2**) may unveil further layers of complexity toward novel therapeutic perspectives. Estrogen/ER-mediated BC progression does involve a bidirectional cooperation between BC cells and components of the surrounding stroma as blood vessels, immune cells, CAFs, and other types of cells (Lappano and Maggiolini, 2018; Rothenberger et al., 2018). Stromal cells may contribute to the progression of BCs acting as a main source of soluble and non-soluble secreted factors such as hormones, growth factors, cytokines, and ECM molecules, which regulate matrix remodeling, neoangiogenesis, migration, and invasion (Lappano et al., 2020a; **Tables 1**, **2**).

In CAFs, the estrogen-induced production of SDF-1α, occurring in an ERα-independent manner, may contribute to BC progression through the accumulation of cancer-infiltrating

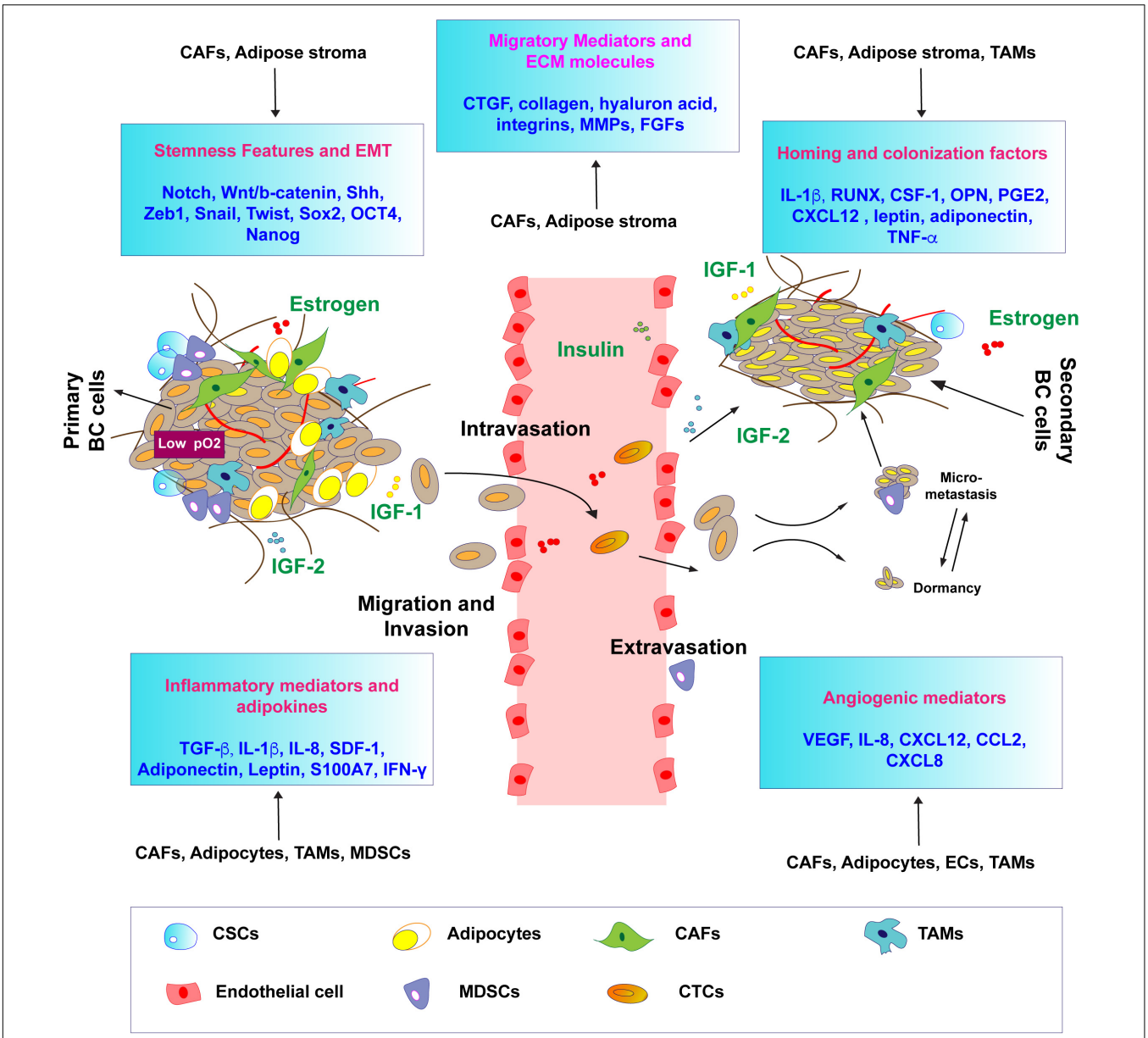

FIGURE 2 | Estrogen and IIGF-prompted microenvironmental responses conducive to BC metastasis. Schematic representation of the main biological responses and shared mediators (in boxes) regulated by both estrogen signaling and by IIGFs, shaping the tumor microenvironment toward metastatic progression. Both estrogen and IIGFs signaling regulate the expression of inflammatory, migratory, and angiogenic mediators by modulating paracrine responses in the tumor microenvironment. The activation of developmental pathways and EMT programs, under the control of estrogen and IIGFs-regulated genes, is responsible for the acquisition of stemness features associated with metastatic progression. Homing and colonization factors under the influence of estrogen and IIGFs trigger BC cells priming to the metastatic sites. CTCs, circulating tumor cell; CSCs, cancer stem cells; CAFs; cancer-associated fibroblasts; TAMs, tumor-associated fibroblasts; MDSCs, myeloid-derived suppressor cells.

myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment (Ouyang et al., 2016). In this context, it should be mentioned that growth factors released within the tumor microenvironment may modulate the function of ERα toward the development of breast malignant features (Bartella et al., 2012). Yet, CAFs may be targets of the stimulatory paracrine actions elicited by diverse molecules released by BC and/or other stromal cells (Kalluri, 2016). Among these molecules, IGF-1 and IGF-2 have been shown to be released by epithelial BC cells and drive the acquisition of the activated status in adjacent fibroblasts, toward increased migratory and invasive behavior (De Vincenzo et al., 2019). Conversely, IGF-1 released by CAFs triggered migratory effects in MDA-MB-231 BC cells and the formation of lung metastasis in an animal model of BC (Daubriac et al., 2018). Similarly, the paracrine release of IGF-1 by CAFs primed TNBC to metastasize the bone (Zhang et al., 2013). In parallel, the

TABLE 1 | Schematic representation of the EMT factors modulated by estrogen and IIGF signaling in BC.

EMT factor	Mediator	Model system	Mechanism involved	References
Snail	IGF-1R	Human mammary epithelial cells	Constitutively activated IGF-IR induces EMT through Snail1	Kim et al., 2007
NF-κB	IGF-1R	Human mammary epithelial cells	Constitutively activated IGF-IR induces EMT through Snail1	Kim et al., 2007
GDF15	IGF-1R	BC cells	GDF-15 activates IGF-1R-FoxM1 signaling to trigger EMT	Peake et al., 2017
TGF-β	IGF-1R	BC cells	IGF-1 and latent TGF-β promote MMPs activity and EMT	Walsh and Damjanovski, 2011
Twist, Zeb, Slug	IR	Immunodeficient hyperinsulinemic mouse models of T2DM and BC cells	Hyperinsulinemia induces IR-mediated EMT	Zelenko et al., 2016
Fibronectin and β-1 integrin	GPER	Tamoxifen-resistant BC cells	GPER/EGFR/ERK signaling upregulates β1-integrin expression and drives EMT	Yuan et al., 2015
IL-1β	E2/GPER	BC cells and CAFs	IL-1β/IL1R1 loop induces EMT	De Marco et al., 2016
Notch, HIF-1α	GPER	BC cells and CAFs	A cross-talk between Notch, HIF-1α, and GPER mediates EMT	De Francesco et al., 2018a
Notch	E2/GPER	BC cells and CAFs	Estrogenic GPER signaling triggers Notch-dependent EMT genes	Pupo et al., 2014
ECM molecules	ERα	BC cells	Loss of ERα triggers EMT	Bouris et al., 2015

TABLE 2 | Schematic representation of the main stromal mediators involved in metastatic progression by estrogen and IIGF signaling.

Mediator	Regulator	Stromal cell of origin	Target cell/tissue	Metastasis-promoting function	References
Aromatase	Leptin, IL-6	CAFs, adipocytes, ASCs	BC cells and microenvironment	E2 production, cell proliferation, migration, angiogenesis	Luo et al., 2014; Kamat et al., 2015; Sabol et al., 2019
IGF-1 and IGF-2	Oncogenic mutations	CAFs, adipocytes, ASCs	BC cells and microenvironment	Homing, colonization, angiogenesis, EMT, stemness features, CAF activation	Lee et al., 2006, 2016; De Vincenzo et al., 2019
CTGF	E2, IIGFs	CAFs	CAFs, BC cells	Migration, invasion	Madeo and Maggiolini, 2010; De Marco et al., 2013, 2014
Notch	E2	CSCs	CSCs, BC cells	Stemness features, migration, EMT, homing	Pupo et al., 2014
Collagen/DDR1	IIGFs	CAFs	BC cells and microenvironment	Migration, ECM remodeling	Matà et al., 2016; Vella et al., 2017
HIF-1α/VEGF	E2, IGF-1	CAFs	ECs	Angiogenesis	De Francesco et al., 2014, 2017
IL-1β	E2	CAFs	BC cells and microenvironment	Migration, invasion	De Marco et al., 2016
FGF-2	E2	CAFs	BC cells and microenvironment		Santolla et al., 2019
OSM	Adipose stroma	Adipose stroma	CSCs	EMT, stemness features	Lapeire et al., 2014; Sanchez-Infantes et al., 2014; West et al., 2014
PDGF	IGF-1	CAFs	BC cells and microenvironment	EMT, ECM remodeling, intravasation	Pasanisi et al., 2008; Guo and Deng, 2018

increased expression of IGF-2 detected in breast CAFs isolated from metastasis, compared to CAFs isolated from primary breast tumors (Gui et al., 2019), suggests that also this growth factor may play a relevant role in the paracrine actions mediated by tumor stroma and leading to the metastatic switch. Indeed, IGFs have been implicated in key stages of bone metastasis such as homing, dormancy, colonization, and expansion (Weilbaecher et al., 2011). In TNBCs, stromal CAFs were identified as the source of IGF-1 and CXCL12, which were shown to prime cells to home the CXCL12- and the IGF1-rich bone microenvironment, in a process dependent on CXCR4 and IGF-1R expression by cancer cells (Zhang et al., 2013). Both IGF-1 and IGF-2 appear to play important roles in bone colonization and expansion by metastasizing tumor cells. In a study, bone-derived IGFs stimulated metastasis of BC to bone by increasing cancer cell proliferation and survival, via AKT activation and recruitment of nuclear factor κB (NF-κB) (Hiraga et al., 2012). Further, culture medium from cells stimulated to undergo bone resorption was found to contain high concentrations of IGF-1; notably, the anchorage-independent growth of human BC cells cultured in this medium was inhibited by the IGF-1R–neutralizing antibody (Ab) αIR3, but not by Abs against TGF-β, FGF-1 or FGF-2, or PDGF-BB (Hiraga et al., 2012). Additionally, growth of human BC cells in a human adult bone model was facilitated by active

osteoclasts induced by RANKL, and IGFs released following bone resorption (Sangai et al., 2008). More specifically, CAF-derived IGF-2 triggered migratory effects in BC cells; this effect was elicited through the involvement of the collagen receptor DDR1 (Matà et al., 2016), which has emerged as a pivotal signaling mediator of the IIGFs. In fact, DDR1 not only serves as a receptor for collagen, but it also appears to work as an adaptor signaling molecule necessary for the transduction of IGF-mediated actions (Matà et al., 2016). Interestingly, non-canonical DDR1 signaling was shown to enable collagen action and multiorgan site metastatic reactivation of breast tumors mainly through the activation of the JAK2/STAT3 pathway and the manifestation of CSC traits (Gao et al., 2016). Therefore, collagen-DDR1 signaling may serve as one of the signaling pathways exploited for BC cells' exit from dormancy, formation of metastasis, and disease relapse. In this context, collagen-enriched ECM integrates hormonal responses toward the establishment of lung metastatic lesions (Jallow et al., 2019). *In vivo*, E2 was able to remodel ECM architecture in the peritumoral area and in the pulmonary premetastatic niche, thus suggesting that both collagen- and estrogen-mediated action may boost lung lesions in ER-positive tumors (Jallow et al., 2019). It should be recalled that the tumor microenvironment at metastatic sites is functionally and molecularly different from the microenvironment surrounding the primary tumor. In particular, a shift from ER-positive to ER-negative context has been detected during metastasis formation. Indeed, Forsare and collaborators interrogated primary and metastatic breast biopsies, as well as CTCs from blood samples serially collected at different timepoints, and demonstrated that the ER status evolves toward the loss of the receptor in CTCs, which reflect real-time tumor progression, as well as at distant metastasis, whereas ER is detectable at the primary tumor site (Forsare et al., 2020). Accordingly, CAFs isolated from primary and metastatic breast tumors were characterized by a differential miRNOma response to estrogens (Vivacqua et al., 2019). These observations suggest that in the microenvironment of breast tumors with aggressive phenotypes, additional mediators may be involved in the stromal response to estrogens. Among these, early studies showed that breast tumor–derived CAFs are stimulated by estrogens through a GPER-mediated nuclear function (Madeo and Maggiolini, 2010; Pupo et al., 2013; Lappano and Maggiolini, 2018). In this regard, GPER, along with the phosphorylated EGFR, was surprisingly recruited by estrogens to the promoter sequences of target genes in CAFs (Madeo and Maggiolini, 2010; Pupo et al., 2013, 2017). Hence, estrogenic GPER signaling fosters CAFs to produce a variety of secreted factors that fuel proliferation, migration, invasion, spreading, and EMT of nearby BC cells, as well as tubulogenesis in endothelial cells (De Francesco et al., 2013; Yuan et al., 2015; De Marco et al., 2016; Pisano et al., 2017; Cirillo et al., 2019; Santolla et al., 2019). In particular, the functional interaction of GPER with the EGFR, IGF1R, FGFR1, HIF-1α, and Notch transduction pathways may trigger the release of growth factors, such as CTGF, VEGF, and FGF2, and cytokines such as IL-1β that account for important paracrine actions mediated by CAFs toward BC growth and dissemination (Pandey et al., 2009; De Francesco et al., 2013, 2014, 2017, 2018a; Pupo et al., 2014; Ren et al., 2015; De Marco et al., 2016; Santolla et al., 2019; Lappano et al., 2020b). Interestingly, diverse studies have shown that GPER bridges together estrogenic signaling with IGF1R and IR-mediated action in the breast tumor microenvironment, independent of the ER status. For instance, the IGF-1/IGF-1R pathway triggers the up-regulation of GPER through the PKCδ/ERK/c-fos/AP1 transduction cascade in an ERα-dependent manner, leading to migratory effects in MCF7 BC cells (De Marco et al., 2013). The cross-talk between IGF-1R and GPER appears to represent a general stimulatory mechanism shared among diverse types of cancer, including mesothelioma and lung cancer (Avino et al., 2016). In addition, IGF-1 stimulation prompted a cross-talk between GPER and DDR-1 leading to cell migration and chemotaxis (Avino et al., 2016). In ER-negative breast CAFs, GPER was shown to be necessary for the stimulatory actions triggered by the metal zinc through the IGF-1R pathway toward CAFs and BC cell migration (Pisano et al., 2017). Furthermore, a functional interaction between GPER and HIF-1α triggered the IGF-1–mediated release of VEGF by CAFs, which prompted vessel-like assembly in endothelial cells. Altogether, these findings suggest that a complex network between ER, GPER, and IGF-1R stimulates the tumor microenvironment and especially CAFs to facilitate metastatic spread. Extending these findings, GPER was shown to be up-regulated not only by IGF-1 but also by insulin in both BC cells and CAFs, thus indicating that GPER may be included among the transduction mediators engaged by the IIGFs pathway in BC (De Marco et al., 2014). The positive correlation between GPER expression in CAFs and serum levels of insulin in BC patients further corroborates the role of insulin in promoting a dysfunctional microenvironment toward disease progression. It should be mentioned that both GPER and the IIGFs have been implicated in the aberrant activation of EMT programs (**Table 1**), which are known to promote metastasis initiation through multiple mechanisms, such as the gain of stemness properties. In this context, GPER was shown to trigger β1-integrin expression, leading to CAF-induced cell migration and EMT (Yuan et al., 2015). Likewise, estrogenic GPER signaling promoted EMT through the activation of the Notch pathway (Pupo et al., 2014), a signaling system involved in CSC maintenance and survival (De Francesco et al., 2018a). Moreover, in patient-derived xenografts from ER-negative BCs, GPER expression was shown to be higher in breast CSCs compared to the non-CSC counterpart (Chan et al., 2020); phosphoproteomic analysis identified the PKA and BAD-Ser118 as the main transduction mediators involved in GPER signaling in breast CSCs (Chan et al., 2020). Interestingly, GPER silencing reduced CSCs activity *in vitro* and tumor growth *in vivo* (Chan et al., 2020), thus reinforcing the involvement of this receptor in CSC functionality.

Despite the role of estrogenic GPER signaling in regulating breast CSC biology has been recently acknowledged, the contribution of ERs in both normal and CSC remains controversial (Sleeman et al., 2007). Indeed, estrogens appear to rely on receptors others than the classic ERα for the expansion of populations with stem-like features (Fillmore et al., 2010; Alferez et al., 2018). In this context, it should be mentioned that the ER target gene PR plays a key role in the regulation of stemness as evidenced in normal mammary gland

development, as well as in the context of breast neoplasia (Daniel and Lange, 2009; Axlund and Sartorius, 2012; Hilton et al., 2012; Finlay-Schultz and Sartorius, 2015; Knutson et al., 2017; Truong et al., 2019).

Likewise, the early dissemination of PR^+ BC cells has been demonstrated using animal models of BC (Hosseini et al., 2016). Extending these findings, PR signaling has been shown to synergize with ER pathway to regulate a number of effectors involved in stemness, metastatic proficiency, and resistance to therapy (Hilton et al., 2012; Finlay-Schultz and Sartorius, 2015; Mohammed et al., 2015; Diep et al., 2016). Among these mediators, the PR target gene insulin receptor substrate 1 (IRS-1), which is a relevant member of the IIGFs, may represent a novel node, bridging together ER-signaling and IIGF signaling by means of PR (Daniel and Lange, 2009).

As it concerns the IGF system, IGF-1R represents a very well-known driver of EMT and stem-related functions in normal and cancerous tissues. Stem-promoting signaling pathways such as Notch, Wnt/β-catenin and Shh may function upstream of IGF-1R to increase its expression (reviewed in Farabaugh et al., 2015): in addition, signaling cascades downstream of IGF-1R activate transcription factors involved in the control of EMT and stemness, such as Zeb1, NF-κB, Snail, Twist, Sox2, Oct4, Nanog (reviewed in Farabaugh et al., 2015). It has been reported that IGF-1 signaling has a critical role in BC progression by controlling both the maintenance of BCSCs and their EMT behavior (Chang et al., 2013). However, IGF-1 can enable EMT also through the activation of non-classical EMT factors; this is the case for transmembrane glycoprotein MUC1, which is frequently overexpressed in BC metastasis, and is up-regulated through the IGF-1R/PI3K/AKT pathway (Cordone et al., 2017). Intriguingly, targeting MUC1 may reverse BC stem cell phenotype, thereby supporting the role of MUC1 in metastatic dissemination. The mammary tissue is rich in adipocytes that produce multiple endocrine, inflammatory, and angiogenic factors involved in the growth and the acquisition of malignant and stem cell traits by adjacent breast tumor cells (Lee et al., 2015). Accordingly, a number of experimental evidences have supported the role of adipocytes in the establishment of metastasis in BC (Kamat et al., 2015). As mentioned above, estrogen production in adipocytes could be one of the mechanisms involved in the higher incidence and aggressiveness of BC observed in obese postmenopausal women. It is been demonstrated that aromatase activity in differentiated adipocytes, as well as in adipose stem cells, is fostered by the hormone leptin, as well as by other adipokines such as IL-6, with the result to increase local estrogen production and ERα signaling (Liu et al., 2013; Strong et al., 2013; Sabol et al., 2019). Beyond estrogen production, other obesity-related factors can contribute to the acquisition of metastatic phenotypes in BC patients. For instance, obesity is associated with a low-grade chronic inflammatory state, characterized by increased production of inflammatory mediators, together with enhanced IGF-1 and insulin signaling (Iyengar et al., 2013). In this context, it should be mentioned that inflammatory factors produced by adipose cells subjected to fat overload contribute not only to insulin resistance, but also to increased metastatic propensity. In conditions of obesity, the adipose tissue is highly inflammogenic as the stressed adipocytes undergo hypoxia and eventually death, thereby liberating several signaling molecules from dying cells. These damage-associated molecular patterns in turn attract immune cells such as macrophages, which enwrap dying adipocytes to form crown-like structures and foam cells. Inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-6, and HMGB1 are released either by adipocytes or activated macrophages to recall additional immune cells and perpetuate the inflammatory damage. Certain inflammatory mediators secreted from the adipose tissue of breast tumors have been shown to trigger direct stimulatory effects on BC cells. For instance, the migration rate of BC cells was increased after coculture with carcinoma adipose stromal cells; this effect was shown to be dependent on the up-regulation of the small calcium binding protein and inflammatory mediator named S100A7, which is correlated with adverse pathological parameters and poor relapse-free survival (Sakurai et al., 2017). Likewise, oncostatin M (OSM) and other adipokines released from tumor-associated adipose tissue prompted the activation of STAT3, and its target genes S100A7, S100A8, and S100A9 triggering increased cellular scattering and peritumoral neovascularization of orthotopic xenografts (West and Watson, 2010; Lapeire et al., 2014). Adding to this, cytokines such as IL-6, IL-1β, and TNF-α, as well as adipokines such as leptin and adiponectin released by bone marrow adipocytes, send homing signals for BC cells to colonize the bone tissue (Choi et al., 2018a). Thereafter, the process of metastasis priming at distant site can be facilitated by a number of adipocyte-derived paracrine factors whose expression is often regulated by both estrogens and IIGFs. This is the case for IL-1β, which is a transcriptional target of signals mediated by GPER (De Marco et al., 2016), ER (Ruh et al., 1998), and IGF-1R (Ho et al., 2017) toward increased invasiveness and metastatic aggressiveness (De Marco et al., 2016; Eyre et al., 2019). In addition, IL-1β is involved in the activation of obesity-induced insulin resistance and inflammation. In fact, reduced gene expression, protein abundance of insulin signaling molecules, and increased release of inflammatory mediators were observed in adipocytes stimulated with IL-1β (Gao et al., 2014). Furthermore, IL1-β was shown to promote stem-cell–like phenotypes and invasiveness in MCF7 BC cell through the up-regulation of IL-6 (Oh et al., 2016), which has been shown to be released not only by cancer cells but also by adipocytes, CAFs, and TAMs (Gyamfi et al., 2018; Xu et al., 2019). Beyond the ability to promote the release of proinflammatory molecules such as IL-1β and IL-6 in the tumor microenvironment, estrogens and IIGFs-mediated signals have been shown to cross-communicate with certain adipokines such as leptin. As mentioned previously, leptin increases the availability of estrogens and promotes migration, invasion, EMT, and CSC enrichment in BC (Strong et al., 2015). A well-documented cross-talk between leptin and IGF-1R signaling pathways has been shown to promote the migration and invasion of BC cells (Saxena et al., 2008). Furthermore, leptin pathway cooperates with ER-mediated signaling to trigger stimulatory actions in BC (Fusco et al., 2010).

TAMs may comprise up to 50% of the BC microenvironment (Obeid et al., 2013). TAMs regulate the secretion of growth factors, proinflammatory cytokines and chemokines leading

to the resistance to endocrine therapy, tissue remodeling, angiogenesis, suppression of immune responses, and tumor growth (Obeid et al., 2013). Consequently, TAMs are associated with an increased aggressiveness and worse outcomes in breast malignancy (Williams et al., 2016; Choi et al., 2018b). For instance, TAMs may induce tamoxifen resistance through the activation of the PI3K/Akt/mTOR transduction pathway in BC cells (Li et al., 2020). Likewise, macrophage differentiation in TAMs mediated by the Notch signaling may promote BC resistance to the aromatase inhibitors (Liu et al., 2017). Interestingly, BC cells exposed to conditioned medium from TAMs have been shown to exhibit loss of ERα expression, increase of the proliferative marker Ki67, and the activation of c-Src, PKC, and MAPK transduction pathways, further supporting a role for TAMs in the endocrine resistance and BC patients' prognosis (Stossi et al., 2012). Together with CAFs, TAMs are the main source of IGFs within both primary and metastatic tumors. High macrophage infiltration has been associated with a poor prognosis and increased rates of metastasis in several cancer types, as TAMs can facilitate blood vessel formation to support expanding tumor growth and aid tumor cell intravasation into vasculature (Chittezhath et al., 2014). Soluble factors present in the TME, such as IGFs, may recruit and influence macrophage behavior (Hao et al., 2012). For instance, macrophages have been shown to play a role in matrix organization through the secretion of MMPs that are capable to degrade and reorganize the matrix, as well as aid in tumor cell migration (Kessenbrock et al., 2010). Moreover, TAMs have been shown to facilitate the deposition of aligned collagen fibers during tumor development (Varol, 2019). The binding of these ECM proteins to adhesion receptors on the surface of macrophages promotes inflammatory and tumor-promoting macrophage activation (Hsieh et al., 2017). Alterations in ECM organization and composition in the tumor microenvironment result in increased matrix stiffness, primarily localized at the invasive front of breast tumors. These stiff regions are enriched in aligned collagen fibers and TAMs. Studies have demonstrated that substrate stiffness, which is associated with enhanced breast tumor progression, is another mechanical aspect of the ECM that can influence macrophage behavior. Matrix stiffness, increasing CCL2 levels, may recruit specific macrophage populations, which interact with collagen fibers and facilitate tumor cell dissemination. Thus, it is becoming clear that macrophages are sensitive to changes in the ECM and their mechanical environment. In agreement, activation of IIGFs in BC patients has been correlated with increased macrophage infiltration, advanced tumor stage, resistance to therapies, and poor prognosis (Campbell et al., 2011). Stroma-derived IGFs have been further investigated in BC progression and metastasis, and the therapeutic opportunity of blocking IIGFs in combination with chemotherapy has been also evaluated. For instance, the efficacy of paclitaxel, a chemotherapeutic agent commonly used for the treatment of invasive BC, has been shown to be increased by the concomitant block of IGFs (Ireland et al., 2018). Altogether, these findings indicate that estrogens and IIGFs may cooperate to elicit a multifaceted breast tumor–supporting action through CAFs, tumor-associated adipocytes and macrophages, and other important components of the tumor stroma. By shaping relevant paracrine interactions within the tumor microenvironment, estrogen and IIGFs signaling systems may play a key role in the development and progression of BC metastasis.

TABLE 3 | Main combination therapies targeting the IIGFs and estrogen signaling.

Combination therapies targeting		References or ClinicalTrials.gov identifiers
IIGFs	**Estrogens**	
Figitumumab	Exemestane	Ryan et al., 2011
Ganitumab	Exemestane or fulvestrant	Robertson et al., 2013
Linsitinib	Letrozole	NCT01205685
MEDI-573	Letrozole, anastrozole, or exemestane	NCT01446159

MANIPULATING THE CROSS-TALK BETWEEN ESTROGENIC SIGNALS AND IIGF TO HALT METASTATIC PROGRESSION

Hormone therapy targeting the ER-mediated pathway is largely used for ER-positive breast tumors, which account for approximately 75% of all BCs (Senkus et al., 2013). Despite the good outcome, certain ER-positive tumors may become resistant to treatments and relapse, leading to a poor prognosis (Osborne and Schiff, 2011; Ma C.X. et al., 2015). Multiple mechanisms responsible for the endocrine resistance have been proposed including the activation of escape pathways toward alternate proliferative and survival stimuli (Osborne and Schiff, 2011; Ma C.X. et al., 2015). In this vein, diverse BC subtypes commonly express high levels of main players of IIGFs (Bhargava et al., 2011; Bahhnassy et al., 2015). Therefore, targeting IIGFs has been suggested as a promising therapeutic approach in BCs (Christopoulos et al., 2018). Accordingly, many components of the IGFs have been indicated as suitable targets on the basis of the results obtained in preclinical studies (Motallebnezhad et al., 2016). Unfortunately, clinical trials, particularly phase III studies, performed in BC patients, provided rather disappointing data for the rise of adverse effects together with minimal clinical benefit (Philippou et al., 2017; Qu et al., 2017). Hence, strategies cotargeting the bidirectional network between the estrogen and IIGFs could be exploited toward successful treatments (**Table 3**). In this regard, in a clinical trial for advanced ER-positive BCs, the use of the IGF-1R Ab figitumumab combined with the aromatase inhibitor exemestane has provided encouraging results in patients without preexisting metabolic syndrome at the time of the enrollment (Ryan et al., 2011). On the contrary, the addition of the IGF-1R therapeutic monoclonal Ab ganitumab to exemestane or fulvestrant did not improve the outcomes (Robertson et al., 2013). Moreover, experimental findings indicating an increased ratio of IR-A:IR-B in ER-positive BCs (luminal B) have suggested that targeting both IR-A and IGF-1R, along with the estrogen signaling, may be

beneficial in these patients, therefore avoiding the compensatory cross-talk between IGF-1R and IR (Huang et al., 2011; Yee, 2012). In this regard, a phase II study (NCT01205685) investigated in ER-positive BC patients the potential antitumor activity of a dual IGF-1R/IR tyrosine kinase inhibitor, namely, linsitinib (OSI-906), used in combination with hormone therapy. Unfortunately, this study was ended because of the appearance of severe toxicities associated with the treatments. To date, much focus has been turned into the design of novel molecules showing an enhanced efficacy without adverse effects and the identification of natural compounds able to trigger the desired action. Picropodophyllotoxin (PPT) is an epimer of podophyllotoxin isolated from the roots of *Podophyllum hexandrum*, which has been used as an antitumor drug and insecticidal/antifungal agent (Liu et al., 2015; Zhi et al., 2017). Launched as an anticancer drug targeting specifically the IGF-1R autophosphorylation (Girnita et al., 2004), PPT was shown to prevent the paracrine recruitment of fibroblasts and their activation as CAFs by breast tumor cells expressing c-Myc (De Vincenzo et al., 2019). PPT was also evidenced to suppress the capacity of $CD24^{-}CD44^{+}$ BC stem cells to undergo the EMT process (Chang et al., 2013). Promising experimental data have been provided using a dual IGF-1R/IR tyrosine kinase inhibitor, named BMS-536924, which showed the capability to prevent proliferative and migratory features of BC cells (Law et al., 2008; Litzenburger et al., 2009), without adverse effects associated with the insulin deficiency (Dool et al., 2011). Furthermore, a tyrosine kinase inhibitor targeting both IGF-1R and IR, named BMS-754807, triggered an inhibitory response in TNBC cells characterized by an IGF signature (Litzenburger et al., 2011). Likewise, TNBC cells derived from mice inoculated with both cancer cells and mesenchymal stem cells exhibited a reduced formation of bone metastasis using the BMS-754807 (Zhang et al., 2013). Unfortunately, clinical evidence regarding the action of both BMS-536924 and BMS-754807 in breast tumors, either using each inhibitor alone or in combination with hormone therapeutics, is still lacking. The interaction of tumor cells with the surrounding stroma profoundly influences the etiology and progression of BC through multiple mediators including hormones, growth factors, and cytokines. For instance, tumor–stroma communications may provide within the breast microenvironment growth factors such as IGFs, which in turn activate the ER-mediated signaling (Bartella et al., 2012). Similarly, the alternate ER GPER interacts with the IGF-1R transduction pathways acting as a mediator of the multifaceted estrogen action on breast CAFs (De Marco et al., 2013, 2014, 2016; Lappano et al., 2013; De Francesco et al., 2017; Pisano et al., 2017). Together, novel therapeutic approaches targeting the tumor–stroma network are required in order to inhibit the various molecules secreted within the tumor microenvironment and the downstream pathways prompting the proliferation, invasion, and resistance to chemotherapy of the tumor cells. In this context, size-switchable nanoparticles that deliver chemotherapeutics and simultaneously halt the stimulatory action of important regulators of the cancer microenvironment have been proposed in order to improve the treatment outcomes (Cun et al., 2019). As a therapeutic option in BC, an approach targeting downstream effectors of the cross-talk occurring between estrogen and IIGFs has been also suggested. Among others, valuable candidates are the inhibitors of the PI3K pathway (Jia et al., 2008), which is mainly involved in the IGF-1R–mediated action (Ciruelos Gil, 2014; Kasprzak et al., 2017). Moreover, a cross-talk between the PI3K/AKT/mTOR and the ER transduction cascades may occur either directly or through the IGF-1R effector, namely, IRS-1 (Guvakova and Surmacz, 1997; Ciruelos Gil, 2014). Hence, this latter mediator could be considered as a further potential target of the estrogen and IGFs network in BC. Indeed, IRSs are adapter proteins that interact with both IR and IGF-1R toward the stimulation of cell growth, motility, and metastasis (Pirola et al., 2003; Becker et al., 2016). Serving as scaffolds in BC cells, IRSs activate other intermediate proteins including the PI3K/AKT/mTOR signaling (Law et al., 2008; Mirdamadi et al., 2015). Of note, estrogens trigger the up-regulation of IRS-1 activating the PI3K transduction pathway (Guvakova and Surmacz, 1997; Sisci et al., 2007). Accordingly, the silencing of IRS-1 enhanced the tamoxifen-induced cell death in BC cells (Cesarone et al., 2006) and abrogated the transcriptional activity of ER dependent by IGF-1 (Sisci et al., 2007).

Because of the multilevel paracrine actions elicited by both IGF-1 and IGF-2 in BC metastasis, it is plausible to hypothesize that the direct targeting of IGF-1 and/or IGF-2 would provide an interesting strategy in therapeutic setting. The ligand-neutralizing approach has been tested in preclinical and clinical studies in diverse types of solid tumors, including BC. For instance, the neutralizing human Ab MEDI-573 serves as a double inhibitor for IGF-1 and IGF-2. In animal models, MEDI-573 blocks tumor growth by halting the IGF-1R and IR-A signaling cascade (Iguchi et al., 2015). Because of the encouraging results, MEDI-573 is currently under investigation in a phase 1b/2 clinical trial in patients with metastatic $HR^{+}/HER2^{-}$ BC, in combination with aromatase inhibitors (NCT01446159). Preliminary data have shown that MEDI-573 suppresses IGF-1 and IGF-2 without generating dose-limiting toxicity including metabolic disorders (Iguchi et al., 2015). The monoclonal Ab neutralizing IGF-1 and IGF-2 named BI836845 is also being tested in a cohort of $HR^{+}/HER2^{-}$ metastatic BC patients, in combination with mTOR and aromatase inhibitors, in a phase 2 clinical trial (NCT02123823). Furthermore, Vaniotis et al. (2018) generated a soluble fusion protein consisting of the extracellular domain of human IGF-1R and the Fc domain of human IgG. This product, named IGF-TRAP, showed IGF-1 and IGF-2–binding activity with elevated affinity, which was threefold higher than that of insulin (Vaniotis et al., 2018). The IGF-TRAP exhibited potent anti-antimetastatic bioactivity in BC, thus representing a novel tool for better manipulation of metastatic disease (Vaniotis et al., 2018).

Strategies cotargeting both estrogen and the IGF signaling as well as the cross-communication with protumorigenic molecules such as the adipokine leptin or the proinflammatory cytokine IL1-β would appear to offer major beneficial effects with respect to the inhibition of a single signaling pathway. In this vein, it should be mentioned that leptin inhibition reversed the breast CSC phenotype (Giordano et al., 2016), as well as lessened the effects exerted by adipose-derived stem cells (ASCs) derived from obese BC patients on cancer cell growth (Strong et al.,

2013). Approved by the US Food and Drug Administration for the treatment of rheumatoid arthritis, the IL-1 antagonist anakinra showed in BC a remarkable safety record together with a suppressive action on the IL-1–related inflammatory effects (Wu et al., 2018). To date, a single pilot trial aimed at determining the safety of anakinra used along with chemotherapy in patients with metastatic BCs is currently undergoing (NCT01802970). Overall, these findings may suggest that investigating the potential of combination strategies might provide further cues and clinical advantages in BC patients.

DISCUSSION

Metastatic BCs continue to be a foremost challenge as they are almost always incurable, ultimately leading to death (DeSantis et al., 2019). The poor clinical prognosis is further exacerbated by the lack of effective targeted treatments and by acquired resistance to therapies. Notwithstanding the advances made with targeted therapies, the absence of defined molecular targets and the high tumor heterogeneity of metastatic BC have resulted in lack of benefit in several subgroups of these patients (Mutebi et al., 2020). The discovery of new molecular targeting agents for metastatic BC is therefore an unmet need. Metastatic disease and therapy resistance are highly correlated with intracellular activated pathways. While previous studies have been mainly focused on genetic and biological differences between primary and metastatic epithelial BC cells, more recently, attention has gradually shifted to the most important cellular components of tumor stroma ascribing an increasing importance to cells of tumor microenvironment (Hanahan and Coussens, 2012; Guo and Deng, 2018). During cancer progression, both malignant epithelial and stromal cells produce various components and/or remodelers of ECM that promote metastatic progression, establishing the concept that tumor microenvironment has an essential role in BC biology and therapeutic response (Hanahan and Coussens, 2012; Guo and Deng, 2018). Extensive differences in tumor stroma compared with normal stroma have been widely observed, and several studies have shown that tumor microenvironment may affect biology and progression of cancer cells influencing therapeutic response and clinical outcome (Cacho-Díaz et al., 2020). Differences in tumor microenvironment of primary tumor and metastatic lesions have been reported. For instance, tumor cells are more protected in metastatic lesions than in primary tumor by tumor microenvironment (Cacho-Díaz et al., 2020). Soluble factors secreted by tumor or stromal cells, as well as ligand–receptor interactions and downstream pathways activation, play a pivotal role. Thus, we can expect that the full comprehension of underneath defects could be precious in future therapeutic perspectives.

The importance of IIGFs and estrogenic signaling in BC is well-established, as is the cross-talk between these pathways. However, relatively little is known regarding the impact of this cross-talk in modulating BC cells/microenvironment interactions, especially regarding BC metastatic evolution. We have focused on evidence showing that, indeed, estrogen/IIGFs impacts on stroma at different levels and that, conversely, tumor stroma itself is a main source of soluble and non-soluble secreted molecules, which regulate ECM remodeling, neoangiogenesis, migration, and invasion. In particular, dysregulated expression and bioavailability of IGFs have been implicated in key stages of metastasis, while estrogenic signaling toward the development of breast malignant features (Bartella et al., 2012; De Marco et al., 2015). Noteworthy, estrogen production by adipocytes has been linked to the higher incidence and aggressiveness of BC in obese postmenopausal women (Park et al., 2017).

Hopefully, a better knowledge of the impact of the estrogen/IIGFs cross-talk in modulating BC metastasis by affecting tumor microenvironment could have translational implications. Interestingly, IIGFs is regulated by ER but becomes the reliant signaling pathway when the expression and activation of ER are lowered by long-term blockade of ER signaling. In parallel, GPER signaling, which contributes to tamoxifen resistance, is crucially involved in a bidirectional cross-talk with IIGFs (Bartella et al., 2012; De Marco et al., 2015).

As already mentioned, IIGFs and estrogen signaling pathways are molecularly interconnected and result in redundancies and compensations that contribute to BC aggressiveness. Consistently, IIGFs inhibition have been exploited to overcome BC resistance and improve clinical outcome; however, an ideal way to inhibit IGF-1R, IR-A, and hybrid IR-A in cancer is still lacking. To date, several potential strategies against IIGFs and estrogen system activation have been attempted, but targeting a single system has failed to improve clinical outcome. Definitely, we propose that a combined approach strategy is mandatory.

In summary, we believe that targeting the tumor–environment interaction by focusing on the estrogen–IIGFs cross-talk may represent an effective therapeutic option, especially in patients with hyperinsulinemia due to insulin resistance. However, further studies are still needed to explore this challenging therapeutic option.

AUTHOR CONTRIBUTIONS

VV, ED, and RL collected and discussed the literature and wrote the manuscript, in collaboration with MGM and LM. AB and MM coordinated the work, and substantially integrated the manuscript, which was revised and approved, in its final version, by all authors. All authors contributed to the conceptualization and design of the review.

FUNDING

Fondazione AIRC supported AB (IG n. 23369), MM (IG n. 21322), and ED (Start-Up Grant 21651). RL and MM acknowledge (i) the special award namely "Department of Excellence 2018-2022" (Italian Law 232/2016) to the Department of Pharmacy, Health and Nutritional Sciences of the University of Calabria (Italy), (ii) the "Sistema Integrato di Laboratori per L'Ambiente – (SILA) PONa3_00341."

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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.

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frontiers in Cell and Developmental Biology

SYSTEMATIC REVIEW
published: 15 December 2020
doi: 10.3389/fcell.2020.592616

Functional Gene Expression Differentiation of the Notch Signaling Pathway in Female Reproductive Tract Tissues—A Comprehensive Review With Analysis

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OPEN ACCESS

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

Received: *07 August 2020*
Accepted: *11 November 2020*
Published: *15 December 2020*

Citation:
Orzechowska M, Anusewicz D and Bednarek AK (2020) Functional Gene Expression Differentiation of the Notch Signaling Pathway in Female Reproductive Tract Tissues—A Comprehensive Review With Analysis. Front. Cell Dev. Biol. 8:592616. doi: 10.3389/fcell.2020.592616

The Notch pathway involves evolutionarily conserved signaling regulating the development of the female tract organs such as breast, ovary, cervix, and uterine endometrium. A great number of studies revealed Notch aberrancies in association with their carcinogenesis and disease progression, the management of which is still challenging. The present study is a comprehensive review of the available literature on Notch signaling during the normal development and carcinogenesis of the female tract organs. The review has been enriched with our analyses of the TCGA data including breast, cervical, ovarian, and endometrial carcinomas concerning the effects of Notch signaling at two levels: the core components and downstream effectors, hence filling the lack of global overview of Notch-driven carcinogenesis and disease progression. Phenotype heterogeneity regarding Notch signaling was projected in two uniform manifold approximation and projection algorithm dimensions, preceded by the principal component analysis step reducing the data burden. Additionally, overall and disease-free survival analyses were performed with the optimal cutpoint determination by Evaluate Cutpoints software to establish the character of particular Notch components in tumorigenesis. In addition to the review, we demonstrated separate models of the examined cancers of the Notch pathway and its targets, although expression profiles of all normal tissues were much more similar to each other than to its cancerous compartments. Such Notch-driven cancerous differentiation resulted in a case of opposite association with DFS and OS. As a consequence, target genes also show very distinct profiles including genes associated with cell proliferation and differentiation, energy metabolism, or the EMT. In conclusion, the observed Notch associations with the female tract malignancies resulted from differential expression of target genes. This may influence a future analysis to search for new therapeutic targets based on specific Notch pathway profiles.

Keywords: female tract, notch signaling, carcinogenesis, breast cancer, ovarian cancer, endometrial cancer, cervical cancer

INTRODUCTION

With a growing global burden, the prevention and management of female cancers remain challenging. Breast cancer (BC) accounted for a quarter of newly diagnosed cases followed by cervix uteri (CC), uterus corpus endometrial (EC), and ovarian carcinomas (OV) contributing 6.9, 5.3, and 3.6% of the total number of new cases diagnosed in 2018, respectively (Bray et al., 2018).

The female tract comprises internal and external organs that together form a system working in complexity to carry out several functions, basically related to reproduction. Regarding the importance of their mission, maintaining the homeostasis of these tissues seems challenging as well as extremely significant. Any unbidden deregulation of the homeostasis may result in poor outcomes, e.g., gynecological (including endometrial, cervical, and ovarian cancers) as well as breast malignancies (Bates and Bowling, 2013).

The Notch pathway is one of the key regulators in the development of breast, cervix, ovary, and uterine endometrium epithelial tissues and is commonly affected during carcinogenesis and cancer progression (Mitsuhashi et al., 2012; Groeneweg et al., 2014; Kontomanolis et al., 2018; Rodrigues et al., 2019). In the present essay, we comprehensively review Notch-driven gene expression differentiation of specific tissues that are simultaneously dependent on signaling by steroid hormones. Further, we discuss the alterations of Notch signaling at two levels of action: the canonical core signaling and downstream effects of signal transduction in the context of female tract tumorigenesis and cancer progression. We additionally enriched the current review with our new analyses involving The Cancer Genome Atlas (TCGA) expression data to present the complex view of Notch-driven carcinogenesis in hormone-dependent female tract tissues. We address the question of how does the Notch signaling orchestrate cellular differentiation and proliferation within the normal breast, ovarian, uterus endometrial, and cervical tissues in comparison with cancerous tissues, especially in the context of steroid hormone dependency.

Notch signaling is a highly conserved pathway that originated from genetic studies in Drosophila melanogaster, specifically from observations of mutant flies with notched wings (Dexter, 1914). This signaling mechanism stands out as a crucial player in the transmission of internal information, thus governing many processes at different stages of development from cell fate determination during embryogenesis to differentiation, growth, and apoptosis control in postnatal life (Artavanis-Tsakonas et al., 1999).

Regarding Notch's importance in multicellular organisms, it is surprisingly simple in molecular design, containing a relatively small number of canonical core members. In humans, there are four Notch receptors (Notch1–4) and five canonical ligands belonging to the Delta–Serrate–Lag (DSL) family (Jag1, Jag2, Dll1, Dll3, and Dll4) (D'Souza et al., 2010). In canonical signaling, transmembrane Notch receptor interacts by its extracellular domain with one DSL ligand on a neighboring cell and initiates a sequence of two proteolytic cleavage events: first, catalyzed by tumor necrosis factor α-converting enzyme (TACE), viz., disintegrin-metalloproteinase of ADAM family (Adam10, Adam17), and second, by intracellular γ-secretase complex (comprising Psen1, Psen2, Pen2, Aph1, and nicastrin) resulting in the release of the Notch intracellular domain (NICD). Processing of Notch receptors involves posttranslational fucosylation by O-fucosyltransferase 1 (Pofut1) in endoplasmic reticulum followed by further modifications carried out by lunatic (Lfng), manic (Mfng), or radical fringe (Rfng) that occurs in the Golgi and regulates interactions with ligands (Logeat et al., 1998). Moreover, interactions of an activation nature between extracellular domains of Notch receptors and ligands appear in the form of trans-activation between juxtaposed cells, whereas cis-inhibition blocks interactions between proteins co-expressed along the membrane of the same cell (Saxena et al., 2001). Afterward, NICD translocates to the nucleus, interacts with CBF-1/Suppressor of Hairless/Lag-1 DNA-binding protein (CSL), also known as recombination signal binding protein RBP-J, and, with the addition of mastermind-like 1 (Maml1), forms a trimeric coactivator complex leading to expression of Notch direct executives of the HES/HEY family (Hes1, Hes5, Hey1, Hey2, and HeyL) forwarding the signal downwards to the final effectors (Andersson et al., 2011). Both HES and HEY are to date the best-known mammalian representatives of primary CSL-related Notch signaling targets belonging to the basic helix-loop-helix (bHLH) family of transcription factors (TFs), acting mainly as gene transcription repressors (Fischer and Gessler, 2007). A wide variety of cellular processes and events that Notch signaling governs through HES/HEY may therefore be explained by a great abundance of targeted genes. Beside HES and HEY, additional direct targets of Notch have also been reported; some of them depend on Notch signals in multiple tissues, while others are limited to specific types, including, e.g., pivotal cell cycle regulators (e.g., p21, p27, and cyclin D1), growth factors (e.g., ErbB2), regulators of apoptosis, and other TFs (e.g., c-Myc and NF-κB) (Miele and Osborne, 1999; Miele, 2006; Miele et al., 2006). Notch signaling can also be initiated in a non-canonical, i.e., ligand-independent manner as has been identified mostly in undifferentiated cell populations (Deftos et al., 2000). To date, three types of non-canonical Notch signaling have been distinguished: Type I—CSL-independent, Type II—S3 cleavage-independent, and Type III—Notch cleavage- and NICD release-independent (Sanalkumar et al., 2010). Worth noting is the fact that thus Notch signaling may be activated independently of NICD formation, which suggests cross-talk of the Notch pathway upstream of NICD processing.

Remarkable Notch pleiotropy of its transcriptional output is a corollary to a regulation pattern that Notch undergoes through pre-existing states of chromatin set by upstream "pioneer" TFs. For instance, Ditadi et al. demonstrated that differentiation of adult-type hematopoietic cells in the dorsal aorto-gonadal-mesonephros (AGM) region is indeed dependent on Notch1 and, more importantly, placed Notch upstream of pioneer TFs such as Runx1, Myb, and Gata2 during this process. It suggests that at point of lineage decision branch, Notch may act in combination with particular pioneer TFs that activate secondary downstream TFs, which subsequently modify chromatin landscape to allow Notch initiation of different

transcriptional output preceding downstream cell fate decisions (Ditadi et al., 2015). It was shown that among pre-existing cellular potentials, Notch specifies cell fate commitment through two classical modes: first, lateral inhibition, and second, lateral induction. By the former mechanism, cells adopt a particular fate and simultaneously inhibit adjacent cells from developing in a similar manner. Conversely, the latter implies sustaining a particular state of cell or group of cells that leads surrounding cells to differentiate (Flores et al., 2000; Haines and Irvine, 2003). Besides, cell fate determination was linked to the asymmetry occurring between Jagged and Delta ligands that gives rise to cells in a specific possible state: Sender, Receiver, or Sender/Receiver hybrid phenotype. The Notch-Delta signaling represents the mechanism of lateral inhibition leading to the acquisition of the opposite fates by the two cells, where the first cell shows high ligand (Delta) and low receptor (Notch) expression on its surface, whereas the second cell shows low ligand (Delta) and high receptor (Notch) expression. Hereby, the first cell serves as a Sender and the other cell serves as a Receiver. In contrast, the Notch-Jagged signaling is an example of the lateral induction resulting in the acquisition of a similar fate by the two cells, where both cells have an intermediate expression level of both the receptors (Notch) and ligands (Jagged); therefore, both may act as Sender and Receiver (Sender/Receiver hybrid phenotype). Despite lateral inhibition, as well as lateral induction, occurring in physiology (e.g., neurogenesis control in vertebrates; Beatus and Lendahl, 1998 in the former and mammalian inner-ear development; Hartman et al., 2010 in the latter), it has also been associated with pathology such as tumor–stroma cross-talk frequently involving Notch-Jagged signaling (Boareto et al., 2015b). Furthermore, in contrast to other pathways, Notch does not involve secondary messengers to amplify the signals and is solely dependent on the nuclear concentration of NICD (Kovall, 2007). Each activated receptor molecule is being consumed, which yields one NICD, indicating a strict association of signaling input and output, making signal strength essential for eliciting a specific cellular response but, on the other hand, sensitizing Notch to even small deviations from baseline expression (Fanto and Mlodzik, 1999). Hence, relatively weak and short Notch signals may activate only some subset of targets genes, while stronger signals of longer duration (as, e.g., in tumor cells with the constitutively active Notch pathway) may activate larger extent of target genes and even govern genes that primarily remain out of Notch regulation at physiological doses (Aster et al., 2017). Hereby, Notch signaling becomes even more significant; its sensitivity to alterations in expression together with a diverse repertoire of supervised biological processes draws a clear conclusion that any deregulation may lead to severe disruption of a particular mechanism and a further perspective to carcinogenesis.

Notch in Tumorigenesis

Regarding paradoxical roles that Notch plays during development, either block or promotion of differentiation in a cell type/fate-dependent manner, both hyper- and hypoactivation of the pathway can lead to tumor formation and progression. Remarkably, effects of Notch deregulation, same as cellular outcomes, are tissue- and, therefore, cancer-specific and reflect the diverse roles of Notch in a different context in cancers. An emerging body of evidence revealed Notch implications in all fundamental hallmarks of cancer demonstrated by Hanahan and Weinberg (2000, 2011), which range from oncogenic to tumor suppressive dependent on cancer type and tissue of origin as well as a set of downstream effectors that are turned on or off (Radtke and Raj, 2003; Nowell and Radtke, 2017b) (**Figure 1**). Moreover, the Notch pathway belongs to the group of cell fate arbiters, which regulates the balance between differentiation and division. Vogelstein et al. in the review of cancer genome landscapes pointed selective growth advantage of cancerous cells due to favoring the latter process through Notch abrogation (Vogelstein et al., 2013).

Notch was for the first time linked to tumorigenesis in human T-cell acute lymphoblastic leukemia (T-ALL) through the identification of chromosomal translocation [t(7:9)(q34;q34.3)], resulting in the juxtaposition of Notch1 and T-cell receptor β promoter (TCF-β) truncating Notch1 (Ellisen et al., 1991, p. 1). Following this finding, Notch alterations have been reported in numerous tumors including solid and hematological malignancies. **Table 1** illustrates the Notch roles in exampling malignancies.

To date, the best-known oncogenic activity of Notch in human malignancies is heightening the expression of pro-growth and proliferative genes. Research supporting this phenomenon was in major part conducted in human and murine T-ALL *in vitro* and *in vivo* models and focused on Notch ability to increase the expression of one of the global regulators of growth metabolism—MYC (Sharma et al., 2006; Weng et al., 2006; Palomero et al., 2007; Dang et al., 2009). Besides, Swamy et al. demonstrated that Notch promotes the O-GlcNAcylation of proteins, which is dependent on a constant influx of glucose and glutamine. One of the proteins that is modified through this process is Myc, which suggests its potential role as a sensor of nutrient sufficiency downstream of Notch signaling promoting the further progression of the cell cycle (Swamy et al., 2016). Another study showed cross-talk between the Notch and the PI3K-Akt pathway that may enhance the Warburg effect through increasing expression of glucose transporters by Akt (Palomero et al., 2007). Additionally, Notch was also shown to interact with the hypoxia pathway through hydroxylate hypoxia-inducible factor (HIF). It was proposed to integrate hypoxia with epithelial-to-mesenchymal transition (EMT) in tumor cells since the Notch/hypoxia axis was presented in the control of stem cell (SC) differentiation (Gustafsson et al., 2005; Sahlgren et al., 2008). Notch itself was shown to be a key initiator of EMT (Timmerman et al., 2004; Zavadil et al., 2004; Niessen et al., 2008; Sahlgren et al., 2008). Specifically, it was suggested that Jag1 mediates activation of Notch signaling during triggering EMT in epithelial cells (Noseda et al., 2004). Another study revealed a direct interaction between Notch1 and Snail-1, yielding the downregulation of VE-cadherin and loss of contact inhibition *in vitro* (Timmerman et al., 2004). Notch has also been correlated with activation of NF-κB, another prosurvival TF; however, to date, the mechanism remains elusive. Espinosa et al. demonstrated that HES1 suppresses the expression of Cyld,

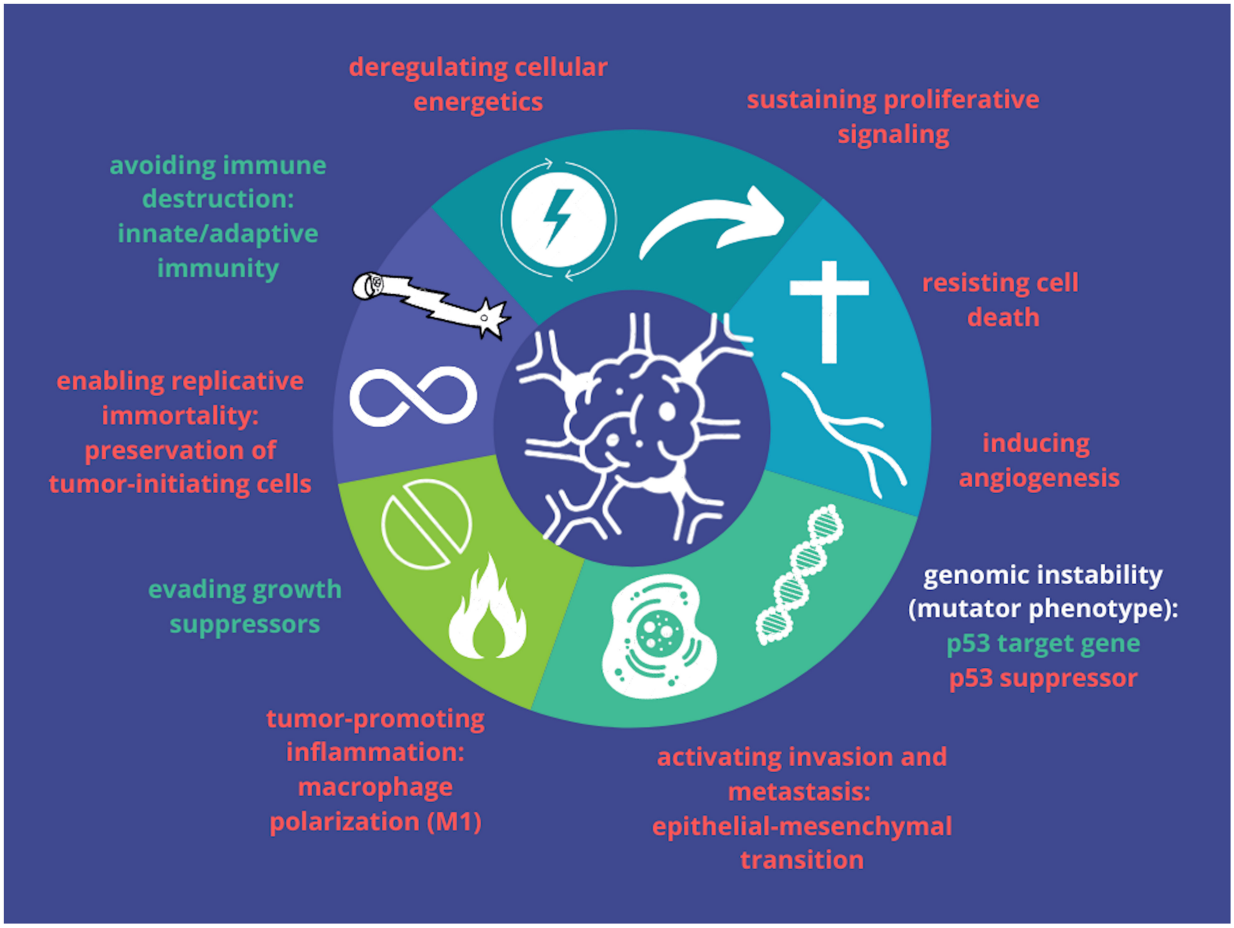


FIGURE 1 | Symbolic representation of cancer hallmarks that are proposed to be affected by Notch signaling. Oncogenic effects are shown in red and tumor-suppressive effects are in green (based on Aster et al., 2017).

a known inhibitor of NF-κB, thus leading to hyperactivation of NF-κB signaling and enhanced survival of T-ALL cells *in vitro* (Espinosa et al., 2010, p. 1). Finally, other oncogenic mechanisms that are employed by Notch include inhibition of apoptosis through downregulation of proapoptotic TF, Nur77, upregulation of Bcl2, IAP, and FLIP as well as inhibition of JNK activation.

On the other hand, Notch presents a tumor-suppressive character that was reported in several malignancies of squamous cell types such as head and neck, cutaneous, lung, bladder, and esophageal carcinomas and manifested itself through mutations predominantly found in Notch receptors (NOTCH1–4). Additionally, other alterations that reduced Notch activity were reported, e.g., loss-of-function mutations detected in Notch members like MAML1 and JAG2, and importantly, all these findings were confirmed in numerous *in vivo* studies employing murine models (Nowell and Radtke, 2017a). Leong et al. proposed another suppressive mechanism that Notch may be involved in inhibition of proliferation and induction of cell cycle arrest through increased expression of $p21^{Cip1}$ and $p27^{Kip1}$ as well as decreased β-catenin-mediated Wnt signaling (Leong and Karsan, 2006). Not only truncation or downregulation of Notch may have tumor-suppressive effects. Surprisingly, it was reported that the constitutive activation of Notch1 may suppress cellular growth in HPV-positive cervical cancer cells. The proposed mechanism comprises repression of viral E6/E7 expression by Notch through AP-1 downregulation that leads to increased p53 expression and prevents hyperphosphorylation of pRb. Alternatively, activation of Notch1 suppresses expression of E47, a helix-loop-helix TF, through ERK1/2 activation, hence inhibiting the progression of the cell cycle (Talora et al., 2002, 2005). A corollary to the above is a conclusion that a decrease in Notch1 activity appears to be more important during late tumorigenesis, rather than early tumor formation stage. Also, different Notch receptors may even have opposing effects within the same malignancy. In particular, Notch1 and Notch2 were shown to have antagonistic effects in embryonal brain tumor cell lines, where it went out that Notch2 promoted while Notch1 inhibited cell proliferation, soft agar colony formation, as well as xenograft growth (Fan et al., 2004, p. 2). In summary, it

TABLE 1 | Several examples reflecting diverse roles of Notch pathway in a cell- and cancer-specific manner.

Function of notch in	References
Oncogenic	
Blood	Koch and Radtke, 2011a,b
Brain	Fan et al., 2004, p. 2; Purow et al., 2005; Gao et al., 2007; Kanamori et al., 2007; Zhang X.-P. et al., 2008; Gaetani et al., 2010; Zhao et al., 2010
Lungs	Zheng et al., 2013
Breast	Pece et al., 2004; Reedijk et al., 2005; Ayyanan et al., 2006; Hu et al., 2006; Sansone et al., 2007; Shipitsin et al., 2007
Pancreas	Miyamoto et al., 2003; De La O et al., 2008; Plentz et al., 2009; Mazur et al., 2010; Maniati et al., 2011; Cook et al., 2012
Tumor suppressive	
Skin	Nicolas et al., 2003; Wang et al., 2011; Pickering et al., 2014
Esophagus	Alcolea et al., 2014; Gao et al., 2014; Song et al., 2014
Lungs	George et al., 2015
Liver	Viatour et al., 2011
Pancreas	Hanlon et al., 2010

becomes apparent that Notch has dualistic character during carcinogenesis, from oncogenic to tumor suppressive, which seems to be dependent on the cellular context and Notch cross-talk with other signaling pathways, although the protective character of Notch remains less well-understood than oncogenic and remains to be further investigated.

Notch in the Normal Development

Breast

Murine models have been found to be very useful in studies on mammary gland development and its tumoric transformation. The physiological development of murine mammary glands involves sequential steps of proliferation, morphogenesis, and differentiation events that ultimately lead to the formation of the epithelial duct system (Daniel and Smith, 1999). A large part of growth-associated and developmental processes occurs after birth and progresses among defined stages of puberty and pregnancy, ultimately leading to initiation of lactation (the above processes have been described in greater detail elsewhere; Smith and Boulanger, 2003). The remarkable essence in the context of mammary gland development is the interaction of multiple kinds, mesenchymal–epithelial, between epithelial, and involving the extracellular matrix (ECM), which are accompanied by apoptosis during involution of mammary gland after the lactation period. As expected, regarding the decisive role of Notch in determining cell fate, canonical signaling has been shown in several studies as an essential regulator of mammary cell communication during embryogenesis, SC self-renewal, cell lineage commitment, proliferation, and differentiation, as well as apoptosis in both murine and human mammary glands (Harrison et al., 2010; Takebe et al., 2011). Raafat et al. demonstrated temporal and spatial regulation of Notch in epithelial cells of mammary glands during development *in vivo*. In the adult tissues, NOTCH1–3 expression was increased from 5 weeks of age through early pregnancy onset followed by decrease observable with more advanced pregnancy stages and mammary gland involution after lactation (both apoptotic and quiescent mammary glands). Regarding receptors, NOTCH3 was the most abundant among all developmental stages in contrast to NOTCH4, whose expression was undetectable. Among other members of the Notch core, JAG1, DLL3, and HEY2 showed the highest expression among ligands and family of Hes/Hey genes analyzed during different stages of postnatal mammary gland development, respectively (Raafat et al., 2011). In turn, constitutively active NOTCH4 (Int3) controlled by mouse mammary tumor virus (MMTV) promoter in transgenic mice affected cell fate selection in the mammary epithelial cells, particularly arresting mammary gland development with a reduction in ductal growth and secretory lobule development that eventually led to the loss of lactation followed by transformation into poorly differentiated adenocarcinomas (Jhappan et al., 1992). Results referring to NOTCH4 have been confirmed in previously conducted studies involving the same transgenic model. In 2000, Soriano et al. proposed Notch4 as an oncoprotein presenting its constitutive activity in mammary glands, failing in the development of secretory lobules during gestation with further transformation in mammary tumors, hence making both findings consistent (Soriano et al., 2000).

In humans, breast tissue varies with the cyclic period throughout a woman's life. Puberty is characterized by the onset of the very rapid growth of breast accompanied by the expansion of blunt-ended primary and secondary ducts that ultimately branch into a complex tree with terminal ductal/lobular-alveolar units (TDLUs). The subsequent period between menarche and menopause exposes breasts to significant fluctuations in growth according to the clock of menstrual cycles. In turn, during pregnancy, the mammary gland is being extensively prepared for lactation through side branching and alveolar development. Subsequent cessation of milk production and involution represses the previous state, thereby resembling similarity to the virgin mammary gland. Such constant changes suggested the potential existence of mammary SCs (Williams and Daniel, 1983), which to date have been broadly studied and described (e.g., Kordon and Smith, 1998; Dontu et al., 2003; Shackleton et al., 2006; Stingl et al., 2006).

Recently, the epithelium of normal mammary gland has emerged in a form of a mixture of differentiated cell populations arranged in a hierarchical pattern with their stem and progenitor cells that are controlled by evolutionary pathways determining cell fate such as unsurprisingly, Notch signaling. Dontu et al. implicated Notch in self-renewal of the normal mammary SCs as well as progenitor compartments *in vitro* by applying a multicellular spheroids system for culturing putative mammary SCs (so-called "mammospheres"). This study indicated that the mammospheres' ability of self-renewal (equated with SC self-renewal capacity) was significantly increased in cultures enriched with a synthetic peptide derived from the Delta–Serrate–Lag2 (DSL) domain capable to activate the Notch pathway (Dontu et al., 2003, 2004). Another study investigated the role of Notch

in mammary SCs by applying different *in vitro* culturing systems consisting of genetically manipulated epithelial subpopulations followed by testing their repopulating abilities in the cleared mammary fat pad of mice. Worth noting, it was concluded that Notch is required to repopulate precursor populations at the early stages of establishing the hierarchy in the mammary epithelium (Bouras et al., 2008). Moreover, as reviewed by Melchor and Smalley, among different human mammary cell populations, genes involved in core Notch signaling exhibited differential expression patterns between two specific populations characterized by different colony-forming capacities: bipotent colony-forming cells (CFCs) and luminal-restricted CFCs. In particular, upregulation and downregulation of NOTCH4 was identified in the former and latter cells, respectively, in opposition to remaining Notch receptors (NOTCH1–3) as well as HES6. Further studies on NOTCH3 led to the conclusion that it may be considered as a key gene for the luminal cell commitment; although it was not explicitly stated, bipotent CFCs could correspond to stem progenitor cells, whereas the luminal CFCs may be considered as a linage-restricted progenitor population (Melchor and Smalley, 2008). In addition, distinct profiles of Notch1 expression were identified among different subtypes with remarkably high expression in the luminal-type cells (Bouras et al., 2008; Rodilla et al., 2015). Ultimate downregulation of Cbf-1/RBP-jk affected absolute SC number since it increased proliferation rate in SCs, although such an increase in proliferation had false bottom manifesting in disorganized side branching with a shifted contribution toward basal-type cells in the end buds and thus regulating the formation of more basal cell phenotypes. Similar effects were observed with overexpression of Numb, which is an endocytic negative regulator of Notch. In contrast, Notch1 upregulation was associated with commitment to the luminal cell lineage (more precisely: high keratin 8/18, Stat5, and p63 downregulation) (Bouras et al., 2008). Recently, *in vivo* imaging revealed basal SCs in the mammary gland of bipotent character that could yield in both myoepithelial and luminal cells (Rios et al., 2014) and Notch was found in charge during this process (Tiede and Kang, 2011; Junankar et al., 2015; Rodilla et al., 2015; Pamarthy et al., 2016).

Discovery of SCs entails the theory of tumor-initiating cells [TICs, also known as cancer stem cells (CSCs); both terms are used interchangeably] of large tumorigenic potential that drives carcinogenesis (Al-Hajj et al., 2003; Fu et al., 2014). By analogy to somatic SCs in organogenesis, tumors are composed of multiple cell types framed in a hierarchical pattern beginning with TICs that possess self-renewal capacity to repopulate the tumor. In breast carcinomas, TICs were initially characterized as lineage-negative (lin-) CD44+/CD24-/low cells (Al-Hajj et al., 2003). By applying the previously conceived methodology of culturing SCs in mammospheres, Ponti et al. cultured putative breast TICs *in vitro* in multicellular tumorspheres. As was demonstrated, tumorspheres, similarly to mammospheres, consist of undifferentiated cells able to self-renew and create another generation of tumorspheres involving cells differentiating into ductal and myoepithelial mammary lineages (Ponti et al., 2005). Investigations focusing on Notch signaling in tumorspheres derived from ductal carcinoma *in situ* (DCIS) showed a significant reduction of mammosphere production when the signaling was inhibited by either γ-secretase inhibitors (GSI), an anti-Notch4 monoclonal antibody (mAb), or gefitinib, the anti-EGFR compound, thereby suggesting indispensability of Notch for expansion of TICs in DCIS (Farnie et al., 2007). Moreover, these findings shed light on EGFR and Notch cooperation in TICs biology, which complies with regulatory feedback loop involving Notch and Her2 possibly maintaining TICs in HER2-enriched BCs (Korkaya and Wicha, 2009).

As described later in the section devoted to BC characteristics, it comprises a heterogeneous collection of molecular subtypes that differ in prognosis and available treatment options. To date, several studies suggested Notch activation in association with particular BC subtypes, especially triple-negative BC (TNBC). Although the relevance of Notch and how it influences the development of particular BC subtype are still elusive, the main explanation refers to its well-established role in SC lineage specification that was for the first time proposed in 2006 by Buono et al. Based on the Cre-mediated deletion model, the authors identified Notch maintaining luminal cell fate to the detriment of uncontrolled basal cell proliferation during alveolar development (Buono et al., 2006). To support the above hypothesis, another research revealed the indispensability of Notch3 during the commitment of bipotent progenitors to the luminal lineage (Raouf et al., 2008). Together with the aforementioned investigations of Bouras et al., the role of Notch in the expansion of the luminal progenitor population in the mammary glands became apparent. Furthermore, to explain the specific association of Notch in origins of TNBC, another model was suggested whereby aberrant Notch signaling contributes to the expansion of abnormal luminal progenitor population that ultimately initiate basal-like carcinoma; however, the model was only shown in carriers of BRCA1 mutation (Lim et al., 2009).

Ovaries, Endometrial Epithelium of the Uterus, Cervix, and Endocervix

Notch signaling is one of the most conserved developmental pathways in multicellular organisms such as mammals. Establishing its role in the development of the female reproductive system, i.e., ovaries, uterine endometrium, and cervix, is currently a major focus of multiple research. Even though the role of Notch in the development of these organs was very well-determined in model organisms including *D. melanogaster* and *C. elegans* (Andersson et al., 2011; Greenwald and Kovall, 2013), the insight into its function in normal gonads is very narrow, conversely to tumorigenesis.

Ovarian morphogenesis in mammals is a process that requires very precise spatial and temporal coordination of functions involving multiple types of cells, which is achieved by the mechanisms of endo-, para-, auto-, and juxtacrine signaling. The last type of signaling is remarkably executed by Notch as a contact-dependent pathway.

To date, Notch was revealed in both the embryonic and postnatal ovarian development, especially in essential events including follicle assembly and growth, meiotic maturation, vasculogenesis of ovaries, and production of steroid hormones.

Importantly, NOTCH2, JAG1, JAG2, HES1, and HEY2 were the most abundantly expressed among all Notch core members within embryonic ovaries (reviewed in Vanorny and Mayo, 2017).

To date, multiple evidence indicated the role of Notch in the development of ovaries in mammals. For instance, Vanorny et al. presented a model in which overexpression of JAG1 and JAG2 in the oocyte signals through NOTCH2 that is present among pregranulosa cells to take a part in the formation of germ cell syncytia and assembly of primordial follicles (Vanorny et al., 2014). On the other hand, studies inhibiting Notch signaling with either GSI or RNAi revealed disruption of multiple developmental processes ongoing in ovaries, hence indicating Notch involvement in meiotic progression and follicle assembly. Feng et al. demonstrated consequences of knockdown of NOTCH1 including delayed meiotic progression, defective oocyte growth, and aberrant primordial follicle assembly followed by the formation of multi-oocyte follicles within renal grafts of embryonic ovarian tissues (Feng et al., 2014, 2016). By employing an *ex vivo* ovarian culture system, it was shown that Notch inhibition delays syncytial breakdown, decreases granulosa cell proliferation, and grows the pool of faulty oocytes due to formation of the abrogated follicular niche (Trombly et al., 2009; Chen et al., 2014; Terauchi et al., 2016). Additional research revealed the emerging role of Notch in the development of mammalian ovaries involving the promotion of the growth and maturation of ovarian follicles through interactions between juxtaposed follicular cells as well as other interactions between cells of different types that require Notch signaling for proper luteinization and vasculature of the ovaries. It was shown that productive Notch signaling is an essential element for the local microenvironment, where the female germ cell develops distinct roles throughout developing ovarian follicles to ensure basic female reproductive functions (Vanorny and Mayo, 2017). Worth noting, multiple observations indicated that proper maintenance of Notch signaling requires an appropriate steroid hormone environment, thus confirming Notch cross-talk with steroid hormone signaling in both *in vitro* and *in vivo* conditions (Guo et al., 2012, 2).

In contrast to mammalian models, the number of studies correlating the Notch pathway with the development of the human ovaries is very low. Kristensen et al. presented transcriptional profiles of human preantral follicles and expression of Notch components that are dynamically regulated during follicle growth. Expression of Notch core members in human ovaries is slightly different in comparison with mammalian, although some common patterns may be recognized. In particular, JAG1, HES1, and HEY2 were upregulated in preantral follicles in contrast to NOTCH2, NOTCH3, NOTCH4, JAG2, HES4, HES5, and HES6, whose expression was lowered. Besides, HEY1 expression was dependent on the size of the preantral follicle (Kristensen et al., 2015). In turn, all Notch receptors (NOTCH1–4) and Jagged ligands (JAG1/2) were abundantly expressed in human cumulus granulosa cells (Tanriverdi et al., 2013).

The human endometrium is the tissue constantly being remodeled along with the menstrual cycle. Changes preceding ovulation involve the proliferative phase followed by a secretory phase of differentiation accompanied by morphological and functional alterations to become responsive in a limited time frame. Finally, the cycle continues to the late secretory phase and menstruation (Paiva et al., 2009). The Notch pathway is thought to regulate numerous biological processes including cell invasion, survival, apoptosis, and differentiation that are the essence of endometrial remodeling (Leong and Karsan, 2006). To date, very little is known about Notch signaling in the development of normal endometrium including endometrial stromal cell decidualization (Afshar et al., 2012). Some of the Notch members have been already identified in the endometrium throughout the menstrual cycle, although these findings were mainly dedicated to endometrial carcinoma and were inconclusive (Cobellis et al., 2008; Mitsuhashi et al., 2012). More recent investigations of Sinderen et al. localized Notch1 in both the endometrial glandular and luminal epithelium with the highest expression in the secretory phase, whereas Notch3 was detected in the endometrial luminal epithelium in the proliferative phase. Among ligands, Jag1 and Dll4 were found in the glandular and luminal epithelium with elevated levels in the secretory phase of the cycle, similarly to Dll1; however, the expression of the latter protein was restricted to the glandular epithelium only. Hes was moderately expressed in the glandular and luminal epithelium with elevated levels in the secretory phase; nevertheless, it was not clearly stated which particular Hes protein is mentioned (Van Sinderen et al., 2014).

Apart from the insufficiency of data directly involving Notch signaling in the development of normal endometrial tissue in humans, this pathway may be indirectly associated with its well-known functionality. More recent studies revealed that Notch participates in angiogenesis during uterine decidualization through *in vivo* studies in murine models, suggesting that the Notch pathway likely functions in mammalian decidual angiogenesis via coordinating VEGFR signaling in endothelial cells (Garcia-Pascual et al., 2014, p. 4; Shawber et al., 2015).

The female reproductive system is primarily formed from Müllerian ducts, which in turn give rise to, i.a., the oviducts, uterus, as well as cervix/endocervix and vagina, and is accomplished through the mesenchymal-to-epithelial transition (MET) and EMT. During embryogenesis, the Müllerian ducts are derived from the coelomic epithelium, initially originating from the intermediate mesoderm. Cells localized within the latter tissue undergo partial MET to form mesoepithelial cells lining the coelomic epithelium followed by either typical MET to form the epithelium of the Müllerian ducts or EMT to form the Müllerian ducts mesenchyme (Kobayashi and Behringer, 2003). Furthermore, the outer parts of Müllerian ducts fuse and form the urogenital canal, finally giving rise to the vagina, cervix, and uterus. The cervical lining undergoes a subsequent transition into the squamous type of epithelium, although despite the common origin of epithelium that is shared with a vagina, the phenotypic differences are thought to arise from other causes, i.e., mesenchymal signals driving the fate of epithelial cells during the squamous transformation of Müllerian vaginal epithelium that involves expression of p63, a transformation-related protein encoded by TP63 gene (Ince et al., 2002). Notably, canonical Notch signaling was recognized in the specification of

mesodermal cells during early embryogenesis through regulation of key TFs such as GATA family, Snail, and Twist, which are commonly activated in mesoderm formation. Moreover, a significant contribution of Notch signaling was also reported in EMT through the upregulation of Snail that is in turn required for mesoderm formation (Grego-Bessa et al., 2004; Timmerman et al., 2004). Also, Ferguson et al., by employing Amhr2-cre transgenic murine model of conditionally active NOTCH1 in the mesenchyme of the developing Müllerian duct, oviduct, uterine stromal cells, and granulosa cells in the ovary, demonstrated multiple developmental abnormalities, thus emphasizing the great importance of proper Notch signaling in the development of female reproductive tract (Ferguson et al., 2012, 2016). Additionally, the Notch-p63 regulatory loop has been established during embryogenesis by Tadeu and Horsley (2013), presuming that the formation of the cervix is also driven by the Notch pathway.

The endometrium of primates is characterized by a high and unique capacity to self-regenerate that occurs through a coordinated sequence of events involving strict regulation of differentiation of uterine progenitors accompanied by the promotion of an immune environment favoring the process of wound healing (Gellersen and Brosens, 2014). As aforementioned, Notch signaling is involved in the maintenance of progenitor cells, and its unique signature was found within human endometrial progenitors (Gargett et al., 2012). Moreover, few recent studies reported abrogation of endometrial regeneration and re-epithelialization with further consequences through deregulation in RBPJ expression, hence exposing the role of the Notch pathway in the functioning of the normal endometrium (Zhang et al., 2014a; Strug et al., 2018).

Notch in Cancer Development and Progression

To elucidate and broaden current insight into Notch roles as well as its contribution in the carcinogenesis of female tract organs such as breast, cervix, ovary, and uterine endometrium, we performed global analysis profiling expression of the Notch pathway at two levels of signaling: first, involving the core members, and second, involving downstream effectors targeted by *HES/HEY* genes that complement the literature review.

Methodology

Population structure and phenotype heterogeneity between major subtypes of BC, CC, OV, and EC accompanied by normal tissues were studied by applying the uniform manifold approximation and projection (UMAP) method, recently emerging as a novel machine learning approach for dimension reduction in large transcriptomic data, preceded by principal component analysis (PCA). To date, PCA was mainly applied as a first-line tool for the reduction of data dimensionality, especially in genomics. However, principal components (PCs) of the highest variance exhibit included information along with an increase in sample size at a very slow pace; thereby, multiple two-dimensional projections of lower variance are typically investigated to explore the data. In proceeding so, features of more subtle character may be tangled within projections. To bring such features to daylight in a two-dimensional system, non-linear transformation methods could be a more appropriate approach that emphasizes the local structure of the data. One of the commonly used non-linear methods is t-distributed stochastic neighbor embedding (t-SNE), although it struggles with datasets of large size, conversely to UMAP. By UMAP, a common practice is to initially reduce burden within data through applying PCA followed by reduction of dimensions projected to leading PCs and therefore extracting the only meaningful structure of given population while filtering out confounding noise (for those interested, principles of UMAP approach in the context of genomic data are very well described in Diaz-Papkovich et al., 2019). The spatial analysis was additionally enriched with mutations and CNV data as well as the clinical outcome of the core Notch members [i.e., disease-free survival (DFS) and overall survival (OS) analyses]. Arbitrarily made classification of patients into subgroups based on median gene expression, which is a common approach to include variables of continuous character (such as gene expression) in survival analyses, may result in misleading or insignificant conclusions due to improper stratification of patients. Therefore, the algorithm of cutpoint optimization accompanying DFS/OS analysis was employed. In brief, DFS/OS analysis is preceded by optimal cutpoint determination, which is defined as a cutpoint of the most significant split enabling patients to be categorized according to favorable or unfavorable prognosis based on the expression of a particular gene.

RESULTS AND DISCUSSION

The analysis of resultant total expression of 56 Notch core components among BC, CC, OV, and EC patients revealed clear spatial partitioning of each cancer type within UMAP spaces. Normal tissues of all organs have been clustered together, independently of tissue of origin, which suggests a nearly common profile of Notch signaling in normal sex hormone-dependent female tissues. If so, we addressed the question of how the Notch signaling alters in cancerous tissue such as BC, CC, OV, and EC vs. normal tissues. BC and CC tended to be the most distinct tumors regarding Notch core, as they formed separate clusters of samples, well-differentiated from each other and simultaneously from OV and EC in UMAP1 and UMAP2, respectively. OV and EC in turn seemed to be more similar to each other regarding UMAP1, albeit still different from BC and CC in UMAP2. Moreover, profiles of Notch core reflected internal partitioning of BC samples referring to PAM50-based classification, with basal-like subtype manifesting characteristics of a separate cluster of samples (**Figure 2**). The profiles of the Notch core components expression are shown in **Figure 3**.

Signaling by the Core—Ligands, Receptors, and Modulators

To date, the role of Notch and its core members has been of great research interest in various tumors. Starting with BC, the investigations conducted by Stylianou et al. became iconic in the field presenting the aberrant expression of Notch ligands,

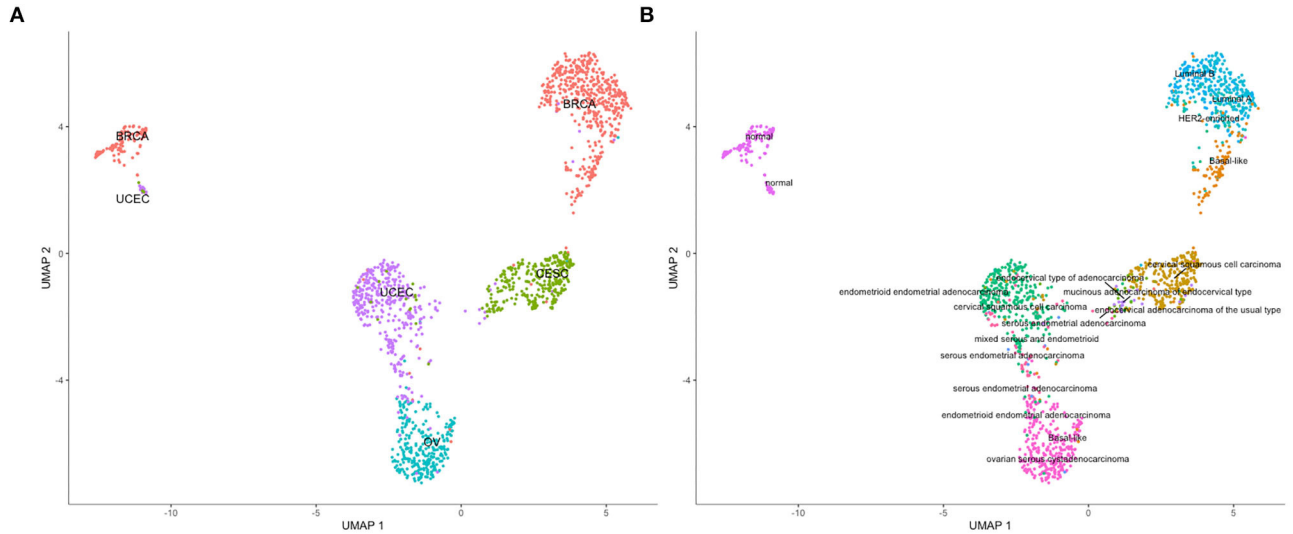


FIGURE 2 | Spatial profiling of BC, CC, OV, and EC accompanied by normal tissues regarding the expression of the Notch core components. **(A)** shows the distribution of the cancer types accompanied by the normal tissues and the **(B)** specifies subtypes of the tumors with a separate cluster of basal-like BC and normal tissues, independently of origin.

receptors as well as target genes among different BC cell lines. It was shown that attenuation of Notch signaling could revert the transformed phenotype of human BC *in vitro* (Stylianou et al., 2006). In particular, the available literature presents Notch1 as an oncogene. Its overexpression has been repeatedly correlated with BC progression as well as worse OS and DFS (Ercan et al., 2011; Yuan et al., 2015) and contributed to development and transition from DCIS to the invasive form of cancer (Farnie et al., 2007; Yuan et al., 2015). Additionally, Notch1 is involved in metastasis as high expression of NICD1 was attributed with sentinel lymph-node positive patients (Wieland et al., 2017). These findings were confirmed in a large bioinformatic meta-analysis involving 4,000 cases of human BCs correlating Notch signaling with increased risk of disease recurrence (Abravanel et al., 2015). However, alterations of Notch1 were reported predominantly in ER+/PR+/HER2+/− BCs (Dai et al., 2015), whereas mutations were more prevalent in HER2-negative tumors (Yi et al., 2017). As shown by numerous research, the role of Notch signaling in metastasis is even more eminent due to contribution in the process of EMT. Leong et al. showed the dependency of Jag1-Notch1-SLUG related to E-cadherin signaling. In particular, activation of Notch1 led to SLUG-facilitated repression of E-cadherin (Leong et al., 2007). Jag1-mediated signaling by Notch increased expression of mesenchymal markers such as N-cadherin, vimentin, Slug, Snail, Zeb1, as well as β-catenin to the detriment of E-cadherin repression (Chen et al., 2010; Brabletz et al., 2011; Bolos et al., 2013; Jian et al., 2013; Liu et al., 2014). Notch activity was also reported in hypoxia-induced EMT. In-depth studies revealed the involvement of Notch1 in hypoxia and CSC-related metastasis (Xing et al., 2011) and, hand in hand with high HIF, predicted worse patient outcomes and thus contributed to more aggressive BC phenotype (Ercan et al., 2012). Finally, Notch1 and Jag1 were related to tumor dormancy in the bone marrow environment able to induce metastasis through the Notch1/STAT3/LIFR signaling axis (Johnson et al., 2016), though overexpression of JAG1 was sufficient to induce bone metastasis (Sethi et al., 2011). Other studies reported Jag1 promoting angiogenesis in neighboring endothelial cells (Reedijk et al., 2005). Additionally, the significance of Jag1 was reported mainly in TNBC exhibiting high levels of NF-kB signaling. The induction of Jag1 in a NF-kB-dependent manner led to the expansion of CSC populations; however, it was observable only among basal-like subtypes (Yamamoto et al., 2013). Moreover, Boareto et al. in a series of their articles depicted the asymmetry between Notch signaling through Delta and that through Jagged affecting the phenotype acquired by the cell implicating worse clinical outcome of the disease. As a brief recap, Notch-Delta signaling allows only two states: Sender or Receiver; however, due to the Delta-Jagged asymmetry, the third possible state of a hybrid Sender/Receiver has arisen, whose relevance was revealed in angiogenesis and EMT (Boareto et al., 2015b). During angiogenesis, the endothelial cells adopt one of the phenotypes: a tip, leading to the formation of branching vessels, and a stalk, proliferating to develop the vessel. Hereby, Boareto et al. demonstrated the diversified effects of Delta-Jagged asymmetry in selecting the tip cell in response to VEGF, an angiogenic growth factor. Specifically, the domination of Notch-Jagged over Notch-Delta signaling destabilizes the tip and stalk cell fates toward the hybrid tip/stalk phenotype, leading to the chaotic, poorly perfused angiogenesis due to the formation of a new sprout that can migrate and develop filopodia. Thus, a hybrid tip/stalk phenotype gives the leading cell an advantage to rapidly exchange its position with a neighbor stalk to induce fast vessel branching that ensures an efficient supply of oxygen to rapidly growing tumors (Boareto et al., 2015a) and might be an explanation why Jag1 overexpression is

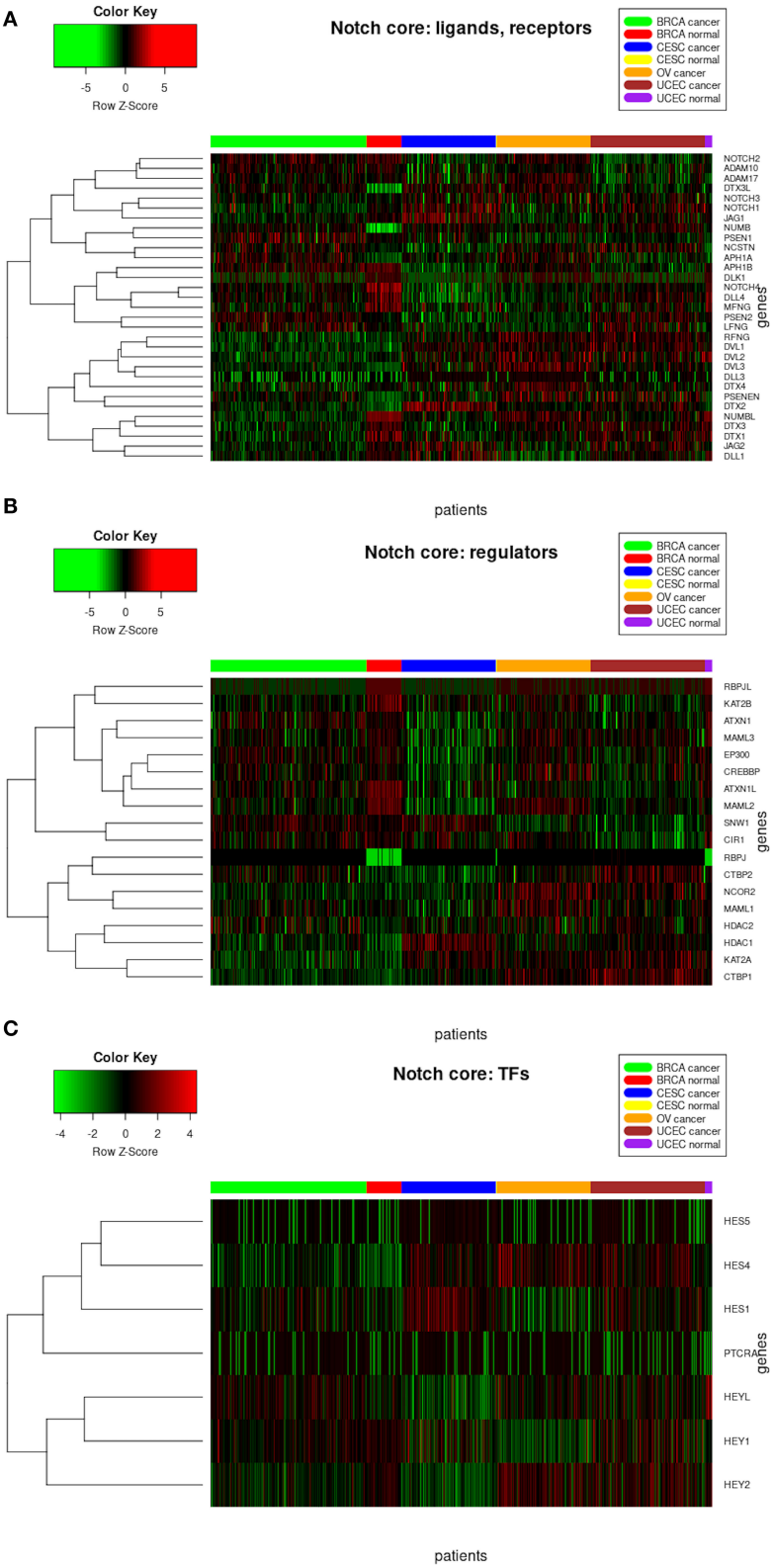


FIGURE 3 | Heatmap reflecting differential gene expression of Notch core members in cancerous and normal tissues of breast, cervix, ovary, and uterine endometrium divided into functional groups of **(A)** receptors, ligands, and associated regulators; **(B)** modulators of signal; and **(C)** Notch-specific transcription factors.

favored in the tumor environment, especially in tumor–stroma cross-talk (Li, 2014), while Dll4 acts as a brake on sprouting angiogenesis and supports physiological angiogenesis (Suchting et al., 2007). On the other hand, it is also believed that the Sender/Receiver hybrid state occurs in cells that underwent partial EMT and are progressing, hence enabling such cells to maintain the meta-stable hybrid epithelial/mesenchymal (E/M) phenotype. The Notch-Jagged signaling has been therefore a hallmark of more aggressive tumor characteristics linked with metastasis and tumor relapse through promoting the E/M hybrid and CSC-like characteristics. Bocci et al. showed that Notch-Jagged signaling might facilitate the formation of hybrid E/M cell clusters potentiating to dislodge from the primary tumor as clusters of CTCs aggravating tumor progression via tumor–stroma interactions (Bocci et al., 2019). These findings confirmed earlier research demonstrating Jag1 among metastasis effectors promoting the remodeling of metastasis niche (Cheung et al., 2016). Finally, *JAG1* knockdown significantly reduced tumor emboli formation in SUM149 BC cells (Bocci et al., 2019).

In the present study, we aimed to include the effects of Notch core alterations evaluated at two different levels: (1) comparison between cancerous vs. normal tissue, and (2) determination of expression cutpoint splitting patients into two subgroups of more/less favorable clinical outcome referring to the relative level of expression (above/below the estimated cutpoint) within cancer only marking the oncogenic or suppressive character of a particular gene. In the TCGA data, we observed opposed trends in NOTCH1 expression of ∼2-fold decrease among BC patients in comparison with normal breast tissue (**Table 2**). Nonetheless, OS and DFS analyses revealed decreased NOTCH1 expression associated with a more favorable prognosis (HR = 1.66, p = 0.047; cutp: HR = 3.14, p = 0.006; maxstat: HR = 3.13, p = 0.006, respectively). Since the lowered range of expression within BC cases was more favorable in terms of BC prognosis, this finding reaffirmed the oncogenic character of *NOTCH1* during breast carcinogenesis (**Tables 4**, **5**). Similarly, JAG1 was doubly decreased in BC vs. normal tissue (**Table 2**), although the lowered expression within BC only was more favorable regarding DFS, it confirmed the involvement of *JAG1* in the mechanism of the recurrence (cutp: HR > 100, p = 0.043; **Table 5**).

Similar findings were reported in OV, where Notch1 was associated with cellular growth through increased proliferation rate and colony formation capacity by NICD1 (Hopfer et al., 2005), similarly as observed among various OV cell lines (OVCAR3, SKOV3, CaOV3) (Rose et al., 2010). Immunohistochemistry (IHC) revealed the presence of Notch1 in 95% of serous OV; however, it was additionally observed in the marginal percentage of matched benign and normal ovarian controls (8 and 6%, respectively) (Wang et al., 2010), as opposed to NICD1, which was undetectable (Kluk et al., 2013). Few other studies associated Notch with OV recurrence, a phenomenon currently attributed to a tumorigenic and therapy-resistant subpopulation of TICs/CSCs (O'Connor et al., 2014). Consistent with the role of Notch in the maintenance of SCs, increased expression of NOTCH1 was found among spheroid ovarian CSCs (Zhang S. et al., 2008). Additionally, NOTCH1 significantly differentiated progression-free survival (PFS) according to TP53 mutation status, and its overexpression correlated with worsened prognosis, although no association with OS was observed (Zhou et al., 2016). In the case of EC, Notch1 showed higher expression compared to normal endometrium, independently of layer or phase of the menstrual cycle, as shown by IHC. The expression increased with the advanced International Federation of Gynecologists and Obstetricians (FIGO) stage. It was also associated with deep myometrial invasion, vessel involvement, and ovarian metastasis and translated into the poorer OS, especially in combination with JAG1 (Mitsuhashi et al., 2012). Cobellis et al. identified Notch1 upregulation in hyperplasia and carcinomas compared to polyps, concluding the oncogenic role in EC tumorigenesis (Cobellis et al., 2008). Importantly, the evaluation of NOTCH1 expression at the RNA level showed an inverse suppressive character of the receptor, in contradiction to the previous studies (Jonusiene et al., 2013). Our analysis showed that no significant alterations of NOTCH1 were found among EC patients, although survival analysis revealed the dualistic effects. Lowered NOTCH1 correlated with improved survival (cutp: HR = 2.22, p = 0.026; maxstat: HR = 3.56, p = 0.002; **Table 4**), whereas its elevation was associated with favorable DFS prognosis (cutp: HR = 0.376, p = 0.007; maxstat: HR = 0.36, p = 0.022; **Table 5**).

Regarding CC, Notch has been revealed as a key mechanism in transformation and cancer progression. The primary oncogenic mechanism involved activation of NICD1 that was shown to phenocopy activation of Ras (considered as second hit accompanying HPV-related E6/E7 oncogenic activity in transforming immortalized HaCaT keratinocytes) (Rangarajan et al., 2001, p. 1). Tumorigenic properties of CC cells are modulated by Notch1 and RhoC. Co-expression of both molecules was observed in primary CC biospecimens and Notch1 KO resulted in the downregulation of RhoC followed by a decrease in cell migration and invasion *in vitro* (Srivastava et al., 2010). Nevertheless, some studies also showed the tumor-suppressive character of Notch signaling in CC. For instance, high expression of Notch1 resulted in growth arrest of cervical tumor-derived cells (Talora et al., 2002, 2005; Wang et al., 2007). Greater levels of Notch components were observed in CC samples compared to normal tissues or high-grade lesions (Daniel et al., 1997; Campos-Parra et al., 2016; Rong et al., 2017). Besides, most invasive CCs exhibited Notch1 expression, prevalently observed within the cytoplasm, conversely to cervical intraepithelial neoplasia (CIN) samples, where nuclear localization of Notch1 was identified. The former finding was also correlated with CC progression, although the latter indicated poorer clinical outcomes (Vazquez-Ulloa et al., 2018). Tripathi et al. reported in turn that both nuclear and cytoplasmic Notch1 expression was decreased through the progression of cervical lesions, from non-neoplastic to precancerous lesions to a tumor, and this finding was additionally confirmed (Tripathi et al., 2014). We found doubly higher expression of NOTCH1 in cancerous tissue vs. normal tissue (**Table 2**) and lowered expression that predicted better survival of CC patients (cutp: HR = 1.73, p = 0.058; maxstat: HR = 1.67, p = 0.049; **Table 4**).

To date, very little is known about Notch2, especially in the context of the remaining receptors that have been widely

TABLE 2 | Summary statistics on Notch core components including logFC accompanied by frequency of mutations and CNVs.

	BRCA			CESC			OV†		UCEC		
	logFC*	**mut [%]**	**CNV [%]**	**logFC***	**mut [%]**	**CNV [%]**	**mut [%]**	**CNV [%]**	**logFC***	**mut [%]**	**CNV [%]**
ADAM10	−0.17	–	–	0.74	–	–	–	–	−0.02	–	–
ADAM17	−0.21	–	–	1.65	–	–	–	–	0.52	–	–
APH1A	0.79	–	12.3	0.24	1	3.1	0.3	10.2	0.52	–	7.1
APH1B	−0.63	–	–	−1.79	–	–	–	–	−1.4	–	–
ATXN1	−0.047	–	–	−1.05	–	–	–	–	−1.21	–	–
ATXN1L	−0.93	–	–	−0.36	–	–	–	–	−0.61	–	–
CIR1	−0.14	–	–	−0.67	–	–	–	–	−1.06	–	–
CREBBP	−0.1	1.8	4.9	−0.35	7.2	1.7	2.2	4.2	−0.47	8.9	0.9
CTBP1	0.19	–	–	−0.15	–	–	–	–	0.32	–	–
CTBP2	0.4	–	–	−0.3	–	–	–	–	0.27	–	–
DLK1	−7.38	–	–	–	–	–	–	–	–	–	–
DLL1	−1.37	–	–	−0.7	–	–	–	–	−1.76	–	–
DLL3	1.13	–	–	–	–	–	–	–	1.21	–	–
DLL4	−0.82	–	–	−2.5	–	–	–	–	−1.24	–	–
DTX1	−2.31	0.6	0.2	−0.09	0.5	0.3	0.3	2.2	−0.25	2.8	1.1
DTX2	0.56	–	–	1.76	–	–	–	–	1.01	–	–
DTX3L	2.12	–	–	1.03	–	–	–	–	0.01	–	–
DTX3	−1.44	–	–	−0.94	–	–	–	–	−0.22	–	–
DTX4	−0.89	–	–	0.83	–	–	–	–	0.04	–	–
DVL1	0.23	–	–	0.07	–	–	–	–	0.18	–	–
DVL2	−0.24	–	–	−0.74	–	–	–	–	−0.53	–	–
DVL3	0.38	–	–	0.74	–	–	–	–	0.4	–	–
EP300	−0.23	1.6	0.2	−0.16	10.8	2.4	0.3	2.5	0.02	8.9	1.7
HDAC1	0.36	0.8	0.8	1.07	–	0.7	0.3	6.5	0.23	1.6	2.6
HDAC2	0.38	–	–	0.54	–	–	–	–	0.14	–	–
HES1	−0.17	–	–	1.39	–	–	–	–	0.67	–	–
HES4	1.04	–	–	1.2	–	–	–	–	1.54	–	–
HES5	−0.28	–	–	1.26	–	–	–	–	0.11	–	–
HEY1	−0.98	0.2	9.8	−0.3	0.5	1	–	8.6	0.6	–	2.8
HEY2	−2	–	–	−1.51	–	–	–	–	−0.02	–	–
HEYL	0.85	–	–	−3.38	–	–	–	–	−2.15	–	–
JAG1	−0.74	–	–	0.38	–	–	–	–	−0.07	–	–
JAG2	−0.95	–	–	0.66	–	–	–	–	0.67	–	–
KAT2A	0.04	–	–	0.72	–	–	–	–	0.25	–	–
KAT2B	−1.4	–	–	−1.1	–	–	–	–	−1.23	–	–
LFNG	0.38	–	–	−0.04	–	–	–	–	0.43	–	–
MAML1	−0.01	–	–	−0.09	–	–	–	–	−0.19	–	–
MAML2	−2.54	1.2	2	−0.5	2.6	4.4	0.9	8.1	−0.11	3.2	1.5
MAML3	−0.55	–	–	−1.13	–	–	–	–	−1.14	–	–
MFNG	−1.48	–	–	−2.38	–	–	–	–	−1.31	–	–
NCOR2	0.03	1	1.8	−0.2	4.1	–	0.3	3.5	0.2	6	2
NCSTN	0.43	0.2	10.7	0.55	1	3.1	–	5	0.94	3.2	4.6
NOTCH1	−0.81	0.6	1.2	0.83	5.7	1	1.3	4.5	0.03	3.2	2.4
NOTCH2	−0.46	2	12.1	−0.18	3.6	2.7	1.3	11	−0.38	5.6	6.1
NOTCH3	−0.03	1	2	0.82	4.1	2.1	0.9	16.6	0.55	6.5	7.2
NOTCH4	−1.67	1	1	−2.46	6	2.4	1.6	6.4	−1.22	4.8	1.9
NUMB	−2.37	–	–	−1.74	–	–	–	–	−1.42	–	–
NUMBL	1.84	–	–	0.03	–	–	–	–	0.35	–	–
PSEN1	0.22	–	–	0.37	–	–	–	–	0.21	–	–

(Continued)

TABLE 2 | Continued

	BRCA			CESC			OV†		UCEC		
	logFC*	mut [%]	CNV [%]	logFC*	mut [%]	CNV [%]	mut [%]	CNV [%]	logFC*	mut [%]	CNV [%]
PSEN2	0.71	–	–	−0.85	–	–	–	–	0.09	–	–
PSENEN	1.02	–	–	0.68	–	–	–	–	1	–	–
PTCRA	1.42	–	–	0.61	–	–	–	–	2.09	–	–
RBPJL	–	–	–	–	–	–	–	–	−9.63	–	–
RBPJ	–	–	–	–	–	–	–	–	–	–	–
RFNG	0.01	–	–	−0.65	–	–	–	–	0.19	–	–
SNW1	0.06	–	–	0.05	–	–	–	–	−0.66	–	–

	BRCA		CESC		OV		UCEC		
	mut [%]	CNV [%]	mut [%]	CNV [%]	mut [%]		CNV [%]	mut [%]	CNV [%]
TP53	32.9	1.8	4.6	0.3	87.7	1.7	27.8		1.1
DNMT1	0.6	1.6	1.5	2.7	0.6	11.2	3.6		5.8
HDAC1	0.8	0.8	–	0.7	0.3	6.5	1.6		2.6
HDAC4	0.6	1	1	5	0.6	3.4	4.8		1.3
HDAC7	–	0.6	0.5	0.7	0.3	2.6	2.8		3.2

† No comparison of expression due to lack of normal tissue samples in case of OV.
**In cancerous vs. normal tissue.*

described; for instance, its relevance in tumorigenesis of EC or CC has not been established and remains elusive. What has been established is a regulatory role of a transcriptional and functional character that Notch2 plays in governing signals from Notch1 and Notch3 in BC (Shimizu et al., 2002). Also, its alterations affected the luminal cellular hierarchy during the specification of mammary epithelial lineages (Sale et al., 2013). Among BC cases, Notch2 mutations were the most prevalent (Lee et al., 2016) and correlated with better prognosis, especially visible in low-grade tumors (Kim et al., 2016). Our study confirmed the increased CNV frequency of 12.1% in NOTCH2 with no relevance to the level of expression or survival in BC (**Tables 2**, **4**, **5**). In OV, higher expression of NOTCH2 correlated with worse PFS, independently of TP53 mutations, especially in grade II (Parr et al., 2004). We observed similar associations of NOTCH2 with OS and DFS as Zhou et al. with PFS. In particular, lower expression was correlated with better prognosis, regardless of the status of TP53 mutations (**Tables 4**, **5**). Our study also revealed an interesting finding that NOTCH2 significantly modulates the survival of EC patients. The initial evaluation of cutpoints stratifying EC patients into subgroups of differential survival turned our attention to the diverse results computed by the algorithms (cutp: cutpoint = 3285, HR = 2.42, $p = 0.015$; maxstat: cutpoint = 1299, HR = 7.31, $p = 0.02$; **Tables 4**, **5**). We thereby assumed that there is a potential third distinguishable group of patients of moderate survival prognosis regarding NOTCH2 and confirmed that with a proper algorithm, finally showing the improving survival prognosis with decreasing NOTCH2 expression (**Table 6**).

Notch3 tends to exert dualistic, i.e., oncogenic and suppressive roles. On one side, Notch3 has been widely shown in mammary carcinogenesis (Dievart et al., 1999; Yamaguchi et al., 2008; Pradeep et al., 2012; Yi et al., 2017), specifically increasing proliferation of luminal cells through cyclin D1, Myc, and Akt (Pradeep et al., 2012). This finding refers to the earlier established regulatory role of Notch3 in the commitment of luminal cells lineage from bipotent progenitors (Raouf et al., 2008). Long-term hormonal therapies were frequently shown as eventually leading to therapy resistance, independently of ER status. Indeed, Notch3 has been indicated as a factor that may contribute to the acquisition of the resistance through the IL6/STAT3/Notch3 axis that causes a departure from metabolic dormancy. Inhibition of IL6 in BC *in vitro* resulted in the downregulation of Notch3 followed by resensitization to hormonal therapies, e.g., tamoxifen (Sansone et al., 2016). It was also demonstrated that in response to TGF-β produced by bone marrow osteoblasts, increase in Notch3 and Jag1 promoted osteoblast differentiation and bone metastasis formation (Zhang et al., 2010; Sethi et al., 2011). In contrast, overexpression of Notch3 could also be tumor suppressive, as shown by Chen et al., through the upregulation of Cdh1 leading to the accumulation of $p27^{Kip1}$ and cell cycle arrest at the G0 to G1 phase transition *in vitro* (Chen et al., 2016, 1). On the other side, Notch3 may inhibit EMT in BC through a novel mechanism comprising the upregulation of GATA3 (Lin et al., 2018, 3). Notch3 was also negatively correlated with chemoresistance (Gu et al., 2016, p. 3). Concerning Notch4, its oncogenic role has been to date mostly described in murine models (reviewed in detail elsewhere; Politi et al., 2004). However, Notch4 was also related to the formation and maintenance of CSCs in BC that surpassed Notch1 in efficacy in that context (Azzam et al., 2013). On the other hand, Notch4 was currently presented to sensitize BC cells *in vitro* to TRAIL-induced apoptosis (Naik et al., 2015). It was also correlated with poor prognosis followed by anti-estrogen

treatment, although short-term therapies resulted in increased activity of CTCs through Jag1/N and otch4 activation (Simoes et al., 2015). Our analysis indicated the tumorigenic character of NOTCH4, especially associated with disease recurrence (cutp: HR = 5.37, $p = 0.002$; maxstat: HR = 5.24, $p = 0.002$; **Table 5**) and survival (HR = 1.96, $p = 0.053$; **Table 4**) in BC; nonetheless, the expression dropped as compared to the normal tissue (logFC = −1.67; **Table 2**).

The Notch signature in OV was primarily recognized in studies aiming to identify diagnostic markers of epithelial OV in human samples and *in vitro* cultures. Interestingly, Notch3 overexpression has been accompanied by amplification localized within NOTCH3 locus that has been identified among serous high-grade OV [confirmed by multiple techniques: SNP genotyping, fluorescent *in situ* hybridization (FISH), IHC] and studies including TCGA Network (Park et al., 2006; Nakayama et al., 2007). Our study confirmed the former of the increased prevalence of CNVs within NOTCH3 (16.6%) in OV (**Table 2**). Among ligands, Jag1 was mainly identified within OV cells and surrounding peritoneal mesothelial cells. Additionally, it was shown that Jag1 activation of signaling through Notch3 promoted cell proliferation and adhesion (Choi et al., 2008). Jung and collaborators conducted expression profiling of Notch in serous OV vs. benign tissues and reported elevation of NOTCH3, JAG1, and JAG2 as well as corresponding higher levels of Notch3 and Jag1 proteins. Furthermore, NOTCH3 was correlated with poor OS and resistance to chemotherapy, although at the protein level, Notch3 was correlated with the advanced stage of the disease, lymph node, and distant metastasis (Jung et al., 2010). High NOTCH3 was, in contrast, more favorable regarding PFS (Zhou et al., 2016). Our analysis did not confirm the relevance of NOTCH3 in either OS or DFS, although we identified the beneficial effects of relatively higher JAG1 (HR = 0.612, $p = 0.034$) and JAG2 (HR = 0.675, $p = 0.042$) in the latter (**Tables 4**, **5**) during ovarian carcinogenesis.

Another study involving NICD3 *in vitro* cultures with lowered endogenous Notch concentration demonstrated elevated expression of SC-associated genes such as NANOG and OCT4, thereby highlighting the relevance of Notch signaling in CSC biology (Park et al., 2010). Notch was also implicated in promoting tumor invasiveness and metastasis through participation in the process of EMT, which in OV has been associated with chemoresistance and SC-like characteristics (Marchini et al., 2013). It was shown that the upregulation of NICD3 in the serous OV cell line (OVCA429) triggers EMT. This finding was confirmed by noticeable alterations in cellular morphology conformed to remind fibroblasts and differential levels of mesenchymal markers and epithelial markers (high Slug, Snail, α-actin vs. low E-cadherin). Moreover, the cells were resistant to carboplatin-induced apoptosis in comparison with control OVCA429 cells (without NICD3 overexpression) (Gupta et al., 2013). Moreover, several studies described the role of Notch signaling in angiogenesis, specifically in serous OV. Microarray-based differential gene expression (DGE) analysis comparing profiles between endothelial cells from high-grade serous OV and endothelial cells from benign ovaries revealed upregulation of JAG1, whose subsequent RNAi silencing reduced tube formation and migration of endothelial cells (Lu et al., 2007). The IHC-based analysis reflected in turn overexpression of Dll4 in tumor and endothelium in over 70% of OV samples that were ultimately correlated with worse OS in contrast to Dll4-low samples (Hu et al., 2011). Conversely, we found beneficial effects of DLL4 upregulation in the context of patients' survival (cutp: HR = 0.71, $p = 0.02$; maxstat: HR = 0.725, $p = 0.04$; **Table 4**) and OV recurrence (HR = 0.661, $p = 0.04$; **Table 5**); admittedly, the evaluation of the trend was based on the level of mRNA.

The case of resistance to platinum-based therapies that is frequently observed in OV and results in recurrence of the disease is still being widely discussed. It also remains a major obstacle, whose overcoming is of the greatest urgency concerning patients' prognosis. Regarding well-established Notch association with CSCs and further relevance of CSCs in a mechanism of acquiring drug resistance, the Notch pathway has recently become a major focus in attempts to understand failures of OV management. Generally, drug-resistant and self-renewing CSCs have been considered as a potential cause of disease recurrence among advanced stage OV patients post platinum-based therapy that is co-observed with multidrug-resistant (MDR) phenotype. Currently applied therapies target the bulk of tumor cells, which rapidly divide and do not exhibit CSC-related characteristics; therefore, the reduction in primary tumor mass is observed, although it simultaneously extricates the niche of drug-resistant CSCs (Gupta et al., 2009). Recent studies reported the specific involvement of Notch3 and Jag1 (Munoz-Galvan et al., 2019) in the mechanism of OV resistance to treatment and its recurrence. In the former, upregulation of NOTCH3 was observed in tumor high-grade serous OV vs. normal ovarian samples and correlated with significantly shorter survival. Moreover, the cluster of Notch signaling was identified in the network-based analysis and related to the prediction of OV response to platinum treatment. These findings were confirmed *in vitro* involving tumorspheres enriched in CSCs, showing elevated Notch signaling, especially NOTCH3; similar observation was made among particular OV patients resistant to platinum therapy. Finally, inhibition of Notch signaling via GSI *in vitro* implicated in a significant reduction in the formation of tumorspheres treated with either cis- or carboplatinum (Munoz-Galvan et al., 2019). The latter investigations emphasized that, as was previously mentioned, the formation of CSCs is essentially regulated by EMT. In turn, the Notch pathway has been widely demonstrated as a critical regulatory mechanism of the EMT process as was also confirmed therein. Specifically, Jag1 seemed to have a more prevailing role in mediating EMT in cisplatin-resistant cells than Jag2, which agrees with earlier studies (Choi et al., 2008; Steg et al., 2011) defining Jag1 as the main ligand of the Notch pathway in OV. Furthermore, these investigations confirmed (in fact, already established; Androutsellis-Theotokis et al., 2006) the existence of cross-talk between Jag1 and STAT3 (major player of JAK/STAT signaling that determines cell polarity, especially in the progression of EMT in cancer), their physical interactions, and the effects of their deadly cross-talk leading to the promotion of the EMT and thus reinforced the invasion and migration capacity of cisplatin-resistant ovarian cancer cells both *in vitro* and *in vivo* (Yang et al., 2019, 3). Liu et al. as well-referred to

Jag1-induced stemness and chemoresistance to platinum-based therapies in OV and surprisingly revealed GATA1 as upstream TF that targets Jag1, thereby activating the Notch pathway and ultimately resulting in OV progression and development of chemoresistance (Liu et al., 2020, p. 1). Therefore, Jag1 may be considered as a linking molecule between other signaling pathways that exert progression of OV in cooperation with Notch signaling. In addition to the aforementioned findings, it was shown that in the absence of the Wnt signaling, the Jag1-activated Notch pathway sustains the proliferation and migration of OV cells *in vitro* and mouse xenograft models (Bocchicchio et al., 2019).

The Notch pathway was also attributed to the dissemination of OV cells through evading cell death in a very specific manner. Generally, high-grade serous OV originates from fallopian tube ECM-exfoliated cells that underwent a tumorigenic transformation; thereby, it may be concluded that escape from anoikis and survival in an anchorage-independent manner is the essence of OV spread. Importantly, Notch has already been implicated in anchorage-independent survival, e.g., NOTCH3 drives resistance to anoikis (Brown et al., 2015, 2). A very recent study cross-referencing functional CRISPR/Cas9 genome-wide knockout screen as well as metabolomics and transcriptomics to identify pathways promoting a state of anchorage independency in high-grade serous OV demonstrated Notch enrichment (as the whole pathway) as well as specific alterations of NOTCH1 and NOTCH3. Moreover, since the Notch pathway contributes to fatty acid (FA) transport (Jabs et al., 2018) and angiogenesis, the conclusion was drawn that it promotes the spread of OV cells in part through FA metabolism, which makes the peritoneal omentum the site of first choice of OV cells to disseminate (Wheeler et al., 2019).

Finally, Notch receptors were correlated with the prognosis of OV patients. High NOTCH3 was more favorable regarding PFS in all OV cases. Elevated expression of NOTCH4 was in turn significantly correlated with more favorable OS in all OV cases; however, the difference in prognosis was not so evident; nonetheless, we also observed such correlation in our research (HR = 0.602, $p = 0.04$; **Table 4**). Further correlations with clinicopathological parameters were additionally established such as better OS prognosis regarding upregulated NOTCH4 among grade III OV patients (Zhou et al., 2016).

Significantly higher expression of Notch3, Jag1, and Dll4 was also reported among EC compared to normal endometrium, regardless of the layer of the endometrium (Mitsuhashi et al., 2012). Cobellis et al. examined in a similar way the expression of Notch4 and Jag1 in normal endometrial samples of pre- and postmenopausal women and compared it with unmatched pathologic samples including, i.a., EC. Conversely, Notch4, and Jag1 decreased with increasing histological grade. Concerning the above, the authors concluded that Notch4 exhibited a more suppressive character (Cobellis et al., 2008). DidŽiapetriene et al. reported alterations in Notch signaling evaluated at the RNA level (qPCR). The study included quantification of Notch receptors (NOTCH1–4), ligands (JAG1, JAG2, and DLL1), and HES1, and in turn revealed significant decrease in expression of all analyzed genes in EC compared to matched, adjacent non-tumor endometrium (Jonusiene et al., 2013; Lachej et al., 2019). Moreover, NOTCH4 and DLL1 were downregulated more likely in stage IB than IA tumors (Sasnauskiene et al., 2014). Our analysis revealed diverse alterations in the expression of Notch ligands and receptors. Conversely to NOTCH3, JAG2, and DLL3, NOTCH4, DLL1, and DLL4 showed lowered expression in EC in comparison with normal endometrium (**Table 2**). Consistent with the above, we also identified oncogenic effects of DLL3 on survival (cutp: HR = 2.29, $p = 0.02$; maxstat: HR = 2.6, $p = 0.007$; **Table 4**) and disease recurrence (cutp: HR = 3.15, $p = 0.005$; maxstat: HR = 2.95, $p = 0.002$; **Table 5**). Among ligands, JAG2 demonstrated similar effects on OS (cutp: HR = 2.17, $p = 0.05$; maxstat: HR = 2.16, $p = 0.05$; **Table 4**), whereas lowered expression strongly correlated with more favorable DFS (cutp: HR = 0.193, $p < 0.001$; maxstat: HR = 0.215, $p < 0.001$) the same as DLL1 (cutp: HR = 0.396, $p = 0.009$; maxstat: HR = 0.388, $p = 0.012$; **Table 5**). Regarding receptors, lowered expression of NOTCH3 was associated with better survival (HR = 2.6, $p = 0.005$; **Table 4**) and DFS prognosis (HR = 2.71, $p = 0.006$; **Table 5**), whereas NOTCH4 was insignificant.

To date, no reports on the significance of either Notch ligands or receptors (excluding Notch1) were found in the area of CC. Our study revealed a decrease in DLL1 and DLL4 expression in CC vs. normal tissue, conversely to JAG1 and JAG2, which were elevated (**Table 2**). Regarding the receptors, there is only one study from 2016 of Sun et al. that demonstrated overexpression of intracellular domains of Notch receptors (NICD1–4) significantly reducing cell proliferation in HeLa cells (Sun et al., 2016, p. 2). As we described earlier, the initiation of Notch cascade may occur in a non-canonical way, independently of receptor activation, and this fact affects the possibility of referring these findings to the classical evaluation of the receptor relevance. In our study, NOTCH3 showed ∼2-fold lowered expression than in normal tissue, in contrast to NOTCH4, which was significantly elevated (**Table 2**). In the context of survival, ligands including DLK1, DLL1, DLL3, DLL4, and JAG1 as well as the NOTCH4 receptor were oncogenic, as a lowered expression of these molecules correlated with improved OS in CC, although with diverse effects on DFS. In particular, DLL3, JAG1, and NOTCH4 retained their relevance as in OS, whereas lowered DLL4 correlated with better prognosis (**Tables 4**, **5**).

Additionally, it is worth recalling the specific BC subtype, i.e., TNBC (esp. basal-like BC) characterized by very poor overall outcome increasingly regarded as a separate clinical entity. Recently, Notch signaling also emerged in pathogenesis and disease progression of TNBC. Its receptors have been related to multiple mechanisms reflecting the aggressive character of TNBC that were already described in previous sections, but deserves additional emphasis. Notch importance in TNBC starts from regulatory effects of Notch receptors on TICs behavior through the association of Notch signaling in maintenance and expansion of mammary CSCs and finally ending with a correlation between expression of Notch receptors and aggressive clinical course of the disease, including invasiveness and chemoresistance. Pathological activation of Notch1 has been considered as a key event in the etiology of TNBCs. Moreover, it contributed to a more aggressive phenotype of TNBC as well as the

TABLE 3 | Summary statistics on Notch core components including logFC according to the PAM50 subtypes of BC.

	logFC			
	Basal-like	**HER2-enriched**	**Luminal A**	**Luminal B**
ADAM10	−0.45	−0.12	−0.1	−0.19
ADAM17	0.28	−0.06	−0.27	−0.27
APH1A	1.04	0.7	0.7	0.77
APH1B	−1.73	−1.31	−0.12	−0.58
ATXN1	−0.63	−1.06	−0.89	−1.12
ATXN1L	−0.55	0.07	0.08	−0.16
DLK1	−6.99	−5.63	−7.16	–
DLL1	−1.56	−1.35	−0.95	−1.99
DLL3	4.04	1.32	0.55	1.03
DLL4	−1.07	−0.54	−0.69	−1.01
DTX1	−1.95	−2.39	−2.08	−3.1
DTX2	0.8	0.91	0.52	0.33
DTX3	2.01	1.95	2.12	2.23
DTX3L	−2.11	−1.79	−1.2	−1.35
DTX4	−0.13	0.07	−1.1	−1.12
DVL1	0.54	0.11	0.25	0.19
DVL2	−0.01	−0.47	−0.28	−0.22
DVL3	0.74	0.36	0.3	0.4
EP300	−0.33	−0.24	−0.21	−0.23
HDAC1	0.51	0.19	0.31	0.49
HDAC2	1.11	0.85	0.17	0.29
HES1	−0.42	0.04	−0.14	−0.23
HES4	1.16	1.34	0.99	0.85
HES5	−0.75	−0.24	0.03	−0.5
HEY1	−1.32	−1.07	−0.77	−1.14
HEY2	−1.84	−2.33	−1.85	−2.5
HEYL	0.17	0.81	1.14	0.66
JAG1	−0.68	−1.03	−0.52	−1.06
JAG2	−0.56	−1.23	−0.97	−1.13
KAT2A	0.17	−0.57	−0.06	0.32
KAT2B	−1.53	−1.44	−1.35	−1.38
LFNG	−1.42	1.03	0.62	0.64
MAML1	−0.08	−0.17	0.03	0.03
MAML2	−1.13	−2.73	−2.46	−3.23
MAML3	−1.45	−0.81	−0.33	−0.6
MFNG	−1.54	−1.7	−1.34	−1.81
NCOR1	−0.57	−0.6	−0.3	−0.23
NCOR2	−0.16	−0.21	0.11	0.08
NCSTN	0.57	0.24	0.44	0.36
NOTCH1	0.16	−0.81	−0.9	−1.03
NOTCH2	−0.34	−0.54	−0.32	−0.7
NOTCH3	0.1	0.11	0.02	−0.24
NOTCH4	−1.96	−1.7	−1.43	−1.87
NUMB	−0.55	−0.28	−0.29	−0.4
NUMBL	0.3	−0.4	−0.23	−0.23
PSEN1	−0.19	0.31	0.33	0.23
PSEN2	0.49	0.54	0.78	0.84
PSENEN	0.9	1.09	1.1	1
PTCRA	1.05	2.1	1.47	1.31
RBPJ	–	–	–	–
RBPJL	–	–	–	–
RFNG	0.11	−0.25	0.05	0.06
SNW1	0.06	0.03	0.06	0.09

(Continued)

further progression of the disease, especially through Notch–EMT interactions (Giuli et al., 2019). The role of Notch2 has not been clearly explained in the context of TNBC, although *in vitro* studies suggested its ambiguous role in the pathogenesis. Signaling through Notch2 initiated by Jag1 and/or Dll4 together with FYN/STAT5 was reported to maintain the mesenchymal phenotype of cells in basal-like BC. It was demonstrated that Notch2 silencing via siRNA reduced expression of mesenchymal markers such as vimentin, Snai1/2, Twist, and Zeb1 (Lee et al., 2018). In turn, Notch3 was defined as an essential factor for TNBC to acquire more aggressive features. Lastly, elevated Notch4 promotes the mesenchymal phenotype and maintains stemness during the progression of TNBC (Giuli et al., 2019). Interestingly, by specifying the PAM-50 subtype, we agree with the literature trend of increasing NOTCH1 expression. The level of the expression was slightly higher than in normal tissue, although the observation was made only in basal-like BC (**Table 3**). Additionally, among ligands, DLL3 demonstrated significantly higher expression in comparison with other subtypes (logFC = 4.04 vs. 1.32, 0.55, and 1.03 in basal-like, HER2-enriched, luminal A and B BC, respectively; **Table 3**).

To date, the significance of the essential modulators of Notch receptors and ligands is often being omitted in specific cancer types, thus offering limited insight into Notch signaling during carcinogenesis. As a brief recap, the intramembrane activation of the Notch signaling occurs in a cascade of successive cleavage events: SI proteolysis performed by Fringe (Lfng, Mfng, and Rfng), SII proteolysis performed by TACE (Adam17), and SIII proteolysis performed by γ-secretase complex (Psenen, Psen1, Psen2, Ncstn, Aph1a, and Aph1b).

Of the Fringe family, Lfng was recognized as a tumor suppressor. Zhang et al. revealed that mammary-specific deletion of *LFNG* induced the origins of basal-like and claudin-low breast tumors accompanied by the accumulation of NICD followed by an increase in the expression of Notch targets and amplification of the *Met/Caveolin* locus, hence facilitating Jag/Notch signaling to promote basal-like BC (Xu et al., 2012). Similarly, Lfng was also shown as suppressive in prostate cancer (Zhang et al., 2014b) and pancreatic cancer (Zhang J. et al., 2016; Zhang S. et al., 2016). In turn, *MFNG* was highly expressed in claudin-low BC and its silencing reduced migratory potential and tumorsphere formation as well as decreased the stem-like population of cells *in vivo* (Zhang et al., 2015). Our study showed the overexpression of *LFNG* in BC and EC in comparison with corresponding normal tissues. *MFNG* was strongly decreased with the lowest expression

in CC vs. normal tissue. *RFNG* was similarly lower in CC than in its normal compartment with no significant alterations in BC and EC (**Table 2**). Consistent with Zhang's research, we observed downregulation of *LFNG* in basal-like BC, despite overexpression in the HER-2 enriched and luminal BC subtypes in comparison with normal breast tissue (**Table 3**). Importantly, we did not observe any association of either *LFNG* or *MFNG* with OS (**Table 4**). Regarding DFS, lowered *LFNG* was associated with better prognosis in BC (cutp: HR = 1.92, $p = 0.039$; maxstat: HR = 2.03, $p = 0.022$), as opposed to OV (cutp: HR = 0.681, $p = 0.0352$; maxstat: HR = 0.674, $p = 0.0341$) and EC (cutp: HR = 0.325, $p = 0.00254$; maxstat: HR = 0.364, $p = 0.00884$; **Table 5**). Heightened *MFNG* predicted better outcomes in BC (cutp: HR = 0.353, $p = 0.0143$; maxstat: HR = 0.337, $p = 0.0101$), CC (cutp: HR = 0.215, $p = 0.00556$; maxstat: HR = 0.296, $p = 0.0165$), and EC (cutp: HR = 0.347, $p = 0.0432$; maxstat: HR = 0.345, $p = 0.0419$) in contrast to OV (HR = 1.63, $p = 0.00173$; **Table 5**). Surprisingly, our analysis revealed suppressive character of *RFNG*, whose higher expression was associated with more favorable OS in BC (cutp: HR = 0.221, $p = 0.00146$; maxstat: HR = 0.242, $p = 0.00299$), CC (maxstat: HR = 0.595, $p = 0.0457$), and OV (cutp: HR = 0.633, $p = 0.0366$; maxstat: HR = 0.624, $p = 0.0341$; **Table 4**), and more favorable DFS in BC (HR = 0.341, $p = 0.011$) and EC (HR = 0.412, $p = 0.0204$; **Table 5**).

Multiple studies reported oncogenic activity of *ADAM17* and its overexpression promoting tumorigenesis and disease progression in various cancers including, among others, BC (Shen et al., 2016) and TNBC (Caiazza et al., 2015). *ADAM17* has also been proposed as a therapeutic target, especially in OV, to enhance the efficiency of platinum-based therapies and diminish the acquisition of secondary chemoresistance (Hedemann et al., 2018). In our study, *ADAM17* was decreased in BC compared to the breast normal compartment; however, specifically in basal-like BC, the expression was higher than in normal tissue (**Table 3**). Similarly, the expression was higher in CC vs. normal tissue (**Table 2**). The survival analysis confirmed the oncogenic character of *ADAM17* as the lowered expression predicted better OS in CC (cutp: HR = 2.22, $p = 0.00373$; maxstat: HR = 2.46, $p = 0.001$; **Table 4**). Regarding disease recurrence, higher *ADAM17* was associated with better outcomes in BC (HR = 0.198, $p = 0.00275$), whereas lowered expression was more favorable in CC (maxstat: HR = 2.5, $p = 0.0383$) and EC (cutp: HR = 3.3, $p = 0.000521$; maxstat: HR = 3.04, $p = 0.00326$; **Table 5**). In addition, among CC patients, we were able to distinguish third survival group differing in prognosis regarding the drop in *ADAM17* expression, indicating an improving prognosis (**Table 6**).

Among the remaining Notch regulators such as Deltex (*DTX1*, *DTX2*, *DTX3*, *DTX3L*, and *DTX4*), Numb (*NUMB* and *NUMBL*), and Dvl (*DVL1*, *DVL2*, and *DVL3*), we observed diversified effects in tumors of different sites in the female tract. However, to date, the literature devoted to their involvement in the carcinogenesis of the female tract organs is very limited, and for that reason, the results of our analysis have been presented in **Tables 2–5** and have not been hereby discussed.

Signaling by the Core—Signal Transduction and HES/HEY TFs

Activation of the Notch signaling leads to the formation of an effector complex (CSL) consisting of RBP-J, specific co-activators [MAML family and histone acetyltransferases (HATs)], and co-repressors (CtBP, histone deacetylases HDAC, CIR, and ATXN1/L) to consequently derepress or activate promoters of *HES/HEY* genes. RBP-J is thus the most essential primary effector of the Notch signaling prompting to analyze its alterations, especially in tumorigenesis. The model systems of human BC revealed depletion of *RBPJ* resulting in increased cell survival and enhanced tumorigenicity due to the signal relegation to MYC and NF-κB (Kulic et al., 2015); however, it was reported as generally enhancing tumor growth and metastases in *Drosophila* (Liefke et al., 2010). In the present study, we observed that higher expression of *RBPJ* correlated with improved survival or more favorable disease-free prognosis in CC, OV, and EC, although surprisingly lowered levels were associated with better DFS in BC (**Tables 4**, **5**). Among RBP-J regulators, *MAML1*, the main Notch co-activator, has been linked with the EMT and BC progression. In the knockdown studies involving MCF7 and MDA-MB-231 BC cell lines, it was concluded that *MAML1* may be considered as a negative regulator of EMT, thus limiting the rate of metastasis and BC relapse. Nevertheless, the relevance of the other regulators has not been elucidated. Our analyses indicated the downregulation of MAML family in BC, CC, and EC in comparison with their corresponding normal compartments (**Tables 2**, **3**); nonetheless, the effects of expression of specific MAML on either OS or DFS varied (**Tables 4**, **5**). Among histone acetyltransferases, *CREBBP* and *EP300*, we observed an increased frequency of mutations and CNVs, especially in CC and EC (*CREBBP*: 7.2% mutated cases and 1.7% CNV in CC, 8.9% mutated cases and 0.9% CNV in CC; *EP300*: 10.8% mutated cases and 2.4% CNV in CC, 8.9% mutated cases and 1.7% CNV in EC; **Table 2**). Moreover, as shown in **Tables 4**, **5**, *ATXN1*, *CREBBP*, *CTBP1/2*, *KAT2A/B*, *HDAC1/2*, *CIR1*, or *SNW1* significantly differentiated patient outcomes reflecting the oncogenic or suppressive character of specific genes, which, to our best knowledge, is the first study describing their relevance in the female tract malignancies.

The Notch signaling ultimately leads to activation of Notch-specific TFs of the HES/HEY family triggering the cellular response through their downstream target effectors associated with processes such as apoptosis, proliferation, EMT, etc. Recently, Hes1 was shown in the maintenance of breast CSCs, metastasis, and halting the drug-induced apoptosis (Liu et al., 2015). Besides, the overactivation of Hes1 and Hes5 was observed among CC cases compared to CIN or normal cervical epithelia and furthermore correlated with poor prognosis of early-stage CC patients (Liu et al., 2007) that likely affected cell differentiation and promoted survival of CSCs through Notch–Hash interactions (Liu et al., 2010). We observed downregulation of *HES1* and *HES5* in BC, with the lowest values in basal-like BC, in comparison with normal breast tissue, whereas both were overexpressed in CC or EC vs. corresponding normal compartments. *HEY1*, apart from alterations of expression, was

TABLE 4 | Summary table of OS analysis.

	Cutp				Maxstat			
	BRCA	CESC	OV	UCEC	BRCA	CESC	OV	UCEC
ADAM10	2.02*	2.05*	1.47*		2.16**	1.98*	1.55*	
ADAM17		2.22**				2.46***		
APH1A	0.359***		0.654**	2.53*	0.349***		0.65**	2.53*
APH1B		2.44***	1.43*			2.45***	1.48*	
ATXN1			0.607*	0.365**				0.411*
ATXN1L	1.91*		1.9**		2.42**			
CIR1		0.161***		0.423*	0.331*	0.512*		0.423*
CREBBP		0.59*	1.42*			0.59*	1.42*	
CTBP1	0.415**				0.357**			
CTBP2	0.482**			0.41**	0.451**			0.399**
DLK1		2.36*				2.52**		
DLL1		2.25*	1.69**			1.67*	1.68**	
DLL3	0.593*	2.62*		2.29*				2.6**
DLL4		2.79***	0.71*			3.02***	0.725*	
DTX1	0.386*	0.441**			0.386*	0.437**		
DTX2		0.317***	1.45*			0.321***		
DTX3	0.21***	0.408*			0.44*		1.64*	<0.001*
DTX3L		0.494*				0.518*		
DTX4	0.461*				0.461*			
DVL1		2.83**	1.49*			2.83**		
DVL2	0.522*				0.522*			2.55**
DVL3				2.14*				2.14*
EP300								
HDAC1								
HDAC2	2.11**	1.74*	1.43*		2.08**	1.75*	1.43*	2.7**
HES1								1.98$^{(0.059)}$
HES4	0.402***	1.99*	0.702*	0.325**	0.402***	2.13**	0.702*	0.374**
HES5	1.86*	0.525*			2.35**	0.323*		
HEY1								2.03$^{(0.0539)}$
HEY2	0.56*		0.723*		0.56*	2.54*	0.691*	
HEYL		2.27*				2.49**		
JAG1		1.73*				1.9*		
JAG2				2.17$^{(0.0522)}$				2.16$^{(0.053)}$
KAT2A	0.267*							
KAT2B	2.27**	0.524*			2.27**	0.542*	0.734*	0.44*
LFNG		0.569$^{(0.0556)}$				0.569$^{(0.0556)}$		
MAML1						1.94*		
MAML2	0.546*				0.546*			
MAML3	0.61$^{(0.0534)}$	1.69*		2.94**	0.61$^{(0.0534)}$			3.52***
MFNG		0.552*		0.32***	2.42$^{(0.0514)}$	0.529*	1.35$^{(0.059)}$	0.35**
NCOR1	2.52*				2.49*			
NCOR2	0.52*	2.34**		0.426*	0.459*	2.51***		0.419*
NCSTN		3.29**		0.175$^{(0.0522)}$		4.92**		0.169*
NOTCH1	1.66*	1.73$^{(0.0577)}$		2.22*	1.66*	1.67*		3.56**
NOTCH2			1.43*	2.42*			1.44*	7.31*
NOTCH3				2.6**				2.6**
NOTCH4	1.96$^{(0.0528)}$	1.92*				1.96*	0.602*	
NUMB			1.55**	0.269***			1.54**	
NUMBL	0.59*	0.253*	1.35$^{(0.0544)}$		0.548*	0.253*		

(Continued)

TABLE 4 | Continued

	Cutp				Maxstat			
	BRCA	CESC	OV	UCEC	BRCA	CESC	OV	UCEC
PSEN1		1.88*		0.264*		2.14**		0.343**
PSEN2	0.576*	1.89*	0.407**			1.99**	0.453***	0.35*
PSENEN	0.311**			0.411*	0.311**			0.287***
PTCRA		0.506*				0.542*		
RBPJ		0.489**		0.483*		0.478*		0.46*
RBPJL				3.13**				2.43**
RFNG	0.221**		0.633*		0.242**	0.595*	0.624*	
SNW1			0.485*				0.532*	

Value represents HR with statistical significance, and the color indicates expression level correlating with favorable prognosis: red—higher expression favorable, blue—lower expression favorable (level of the expression is considered relative to the determined cutpoint).
$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$.

more frequently mutated in ∼10% of BC and OV cases. On the other hand, *HEY2* was decreased in all of the tumors compared to the normal compartments, whereas *HEYL* levels dropped only in CC and EC (**Tables 2**, **3**). We also observed associations of *HES* and *HEY* genes with patients' survival and tumor recurrence (**Tables 4**, **5**), which may originate from differential activation patterns of the downstream effectors associated with the most essential biological processes frequently deregulated during carcinogenesis.

Signaling by Notch—The Downstream Effects

Yet, the Notch roles have been well-established in embryogenesis and adult life. Numerous research demonstrated how Notch orchestrates two principal processes such as cell fate determination and maintenance of SCs (e.g., Fiuza and Arias, 2007; Andersson et al., 2011; Hori et al., 2013; Siebel and Lendahl, 2017). Both great cellular machinery entail and initiate an effect of downstream dissemination of Notch signals through *HES* and *HEY* TFs. An excellent illustration of the above is a number of Notch downstream targets that we have identified and employed in this study through the GTRD database of ChIP-seq-identified TF binding sites. Analysis of HES1, HES5, HEY1, HEY2, and HEYL targets resulted in a total of 3,054 different genes. To provide a wider understanding of the mechanisms regulated downstream to Notch signaling, independently of tumorigenesis, we performed over-representation analysis (ORA) of biological terms among identified HES/HEY targets. The most essential processes have been shown in **Figure 4**. Beside broadly considered development, the most pivotal mechanisms are attributed to cellular death (apoptosis), DNA repair, proliferation, differentiation, cell cycle, and tissue architecture/remodeling-associated processes (adhesion, motility, ECM interactions, and EMT). However, the significance of these effects in the context of carcinogenesis and disease progression driven by Notch signaling is bypassed, and to date, only limited evidence of very specific context can be found in the literature, thus indicating lack of the comprehensive view of that area. Thereby, beyond the relevance of the Notch core components on the carcinogenesis of the female tract, we additionally included the second dimension of our considerations, which is the analysis and review of the major biological processes associated with tumorigenesis and/or progression that are targeted by HES/HEY downstream to Notch signaling among BC, CC, OV, and EC.

The abundance of biological processes that occur distinctly dependently of the tissue type and genetic alterations, especially during carcinogenesis, and are driven by Notch as a distant effect of the core signaling, has found its reflection in the grouping of BC, CC, OV, and EC within UMAP spaces regarding the resultant expression of the 3,054 downstream targets of Notch. These results indicated a common biological response posterior to Notch activation among the normal tissues, regardless of the diverse influence of hormonal regulation. If so, the question is how are the downstream effects of Notch signaling differentiated by the pathway in the tumors of the female tract. These malignancies vary regarding the clinical course of the disease as well as their biology, and these differences tend to originate from differential Notch signaling as a superior regulator. The findings were similar to the previous UMAP clustering concerning the expression of the core components, although of greater contrasts between the groups. In particular, BC and CC were the most distinct clusters of samples, as they were separated along with UMAP1 and UMAP2 spaces. OV and EC formed more similar clusters regarding UMAP1, of more different characteristics than to the core signaling along UMAP2. Moreover, basal-like BC formed a very distinct entity of samples, the same as the normal tissues independently of the primary origin (**Figure 5**).

According to the biological processes recognized among the Notch downstream targets, we further focused on sets of genes associated with the major mechanisms that are abrogated during carcinogenesis and progression, especially concerning the cancer hallmarks, such as apoptosis, adhesion and EMT, proliferation, and Warburg effect, and revealed the expression

TABLE 5 | Summary table of DFS analysis.

	Cutp				Maxstat			
	BRCA	**CESC**	**OV**	**UCEC**	**BRCA**	**CESC**	**OV**	**UCEC**
ADAM10			1.67**	2.84**			1.67**	3.91***
ADAM17	0.514*			3.3***	0.511*	2.5*		3.04**
APH1A		2.86**		2.44*		2.86**		2.44*
APH1B	0.198**	0.398*		3.05**	0.198**		0.559*	3.01**
ATXN1	1.94*				3.36*			
ATXN1L								
CIR1			1.54*				1.61*	
CREBBP			1.62**	2.34*		2.33*	1.62**	2.66**
CTBP1	2.34*				2.96*		1.58*	
CTBP2	0.488*	<0.001*		3.16**	0.485*	<0.001*		3.73***
DLK1			0.653*					
DLL1			1.46(0.0518)	0.396**				0.388*
DLL3	1.78(0.0573)	2.18*	2.18(0.0546)	3.15**		2.18*		2.95**
DLL4	2.86*	0.46(0.056)			2.86*		0.661*	
DTX1	0.483*		0.686*				0.652*	
DTX2	2.13*	0.17***		0.382*	2.29*	0.17***		0.268***
DTX3	0.207***				0.35**			
DTX3L		0.409*				0.391**		
DTX4								
DVL1	0.515*	2.36*	1.46*			2.52*		
DVL2	0.501*							
DVL3	2.06*			2.43*	2.05*			2.74**
EP300								
HDAC1		0.473*				0.473*		
HDAC2	2.35**				2.31**			
HES1		2.2*		0.434*		2.63**	0.6*	0.434*
HES4			0.643**	0.491*			0.615**	0.329**
HES5	1.82*		0.688(0.0508)	0.471(0.0522)	2.1*			
HEY1	2.85*		0.665*		3.66**		0.568*	
HEY2			0.64**	0.458*		2.54*	0.64**	0.458*
HEYL	3.51*	3.11*			4.91*	4.31**		
JAG1	100>*	3.23**				3.23**	0.612*	
JAG2			0.675*	0.193***				0.215***
KAT2A	<0.001*					2.24*		
KAT2B		0.426*						
LFNG	1.92*		0.681*	0.325**	2.03*	2.21(0.0513)	0.674*	0.364**
MAML1				2.11*				2.4*
MAML2			1.35(0.0562)			<0.001*		
MAML3		100>*			2.26*	100>*		
MFNG	0.353*	0.215**	1.63**	0.347*	0.337*	0.296*	1.63**	0.345*
NCOR1								
NCOR2		0.323**		2.23*		0.368**	2.11(0.0533)	
NCSTN	5.59(0.0553)	2.25*				2.41*		0.434*
NOTCH1	3.14**			0.376**	3.13**		0.732(0.05)	0.36*
NOTCH2			1.37*				1.6**	
NOTCH3				2.71**				2.71**
NOTCH4	5.37**	2.6*			5.24**	2.75*		
NUMB	0.46*				0.503*		1.7*	
NUMBL	0.433*	3.88***		2.91*	0.476**	3.76***		3.52**

(Continued)

TABLE 5 | Continued

	Cutp				Maxstat			
	BRCA	CESC	OV	UCEC	BRCA	CESC	OV	UCEC
PSEN1	0.531*		1.4*	0.368**	0.56(0.0569)	0.448*	1.41*	0.336**
PSEN2	0.525*		0.632**				0.449**	
PSENEN						0.523*		2.16*
PTCRA	2.7**	0.337*	0.71*		2.7**	<0.001*	0.675*	
RBPJ	2.97***		0.71*		3.22***		0.675*	
RBPJL				2.72**		2.68*		3.17**
RFNG	0.341*			0.412*	0.341*			0.412*
SNW1								2.16*

Value represents HR with statistical significance, and the color indicates expression level correlating with favorable prognosis: red—higher expression favorable, blue—lower expression favorable (level of the expression is considered relative to the determined cutpoint).
$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$.

profiles reflecting distinct spatial partitioning of BC, CC, OV, EC, and corresponding normal tissues. **Figure 6** presents heatmaps of predefined sets of specified ontology, which confirm the former findings and emphasize how pleiotropic are distant effects of Notch signaling and the significance of the pathway during the essential events of carcinogenesis followed by a progression of the disease, especially in the female tract organs.

Normal development is generally controlled by a balance between cell proliferation and apoptosis, although the tumorigenesis occurs not only due to uncontrolled proliferation, but simultaneously reduced apoptosis. This balance essentially determines the overall growth or regression of cancer in response to various factors such as chemo- and radiotherapy or hormonal treatments, which all act by inducing apoptosis. Thus, expression profiles of apoptosis and proliferation-associated genes allow delineating of the biology of the individual tumors that may be further exploited to clinical advantage. Observable deregulation affecting the efficacy of the apoptotic mechanism may also be considered as a potential cause of treatment failure. Recapping, Notch as an arbiter of cell fate is a superior regulator of both processes, as, depending on the context, it orchestrates rate of proliferation and apoptotic cell death (Miele and Osborne, 1999). To date, it has been established that increased rates of apoptosis are related to the tumors of advanced grades and the ER-negative cells of BC. Moreover, the tumors of more aggressive character showed increased apoptosis and proliferation as well as correlated with a worse prognosis. Besides, the mitotic activity index (MAI) was shown as a very strong prognostic factor associated with the tumor size and lymph node status (van Diest et al., 2004). The findings on CC, OV, and EC linked the resistance to chemo- and hormone therapies with the impaired apoptosis and shifted the balance toward uncontrolled proliferation. Nonetheless, it is worth emphasizing that high rates of proliferation accompanied by relatively high rates of apoptosis are a manifestation of at least partial persistence of the physiological control mechanisms over the tumorigenesis.

The EMT is a complex process of acquiring by the epithelial cell a mesenchymal phenotype through a cascade of biological events. During carcinogenesis, these changes involve loss of adhesion, remodeling of the cytoskeleton architecture, as well as altered cell polarization, detachment from the ECM, migration, and intra- and extravasation, ultimately leading to the formation of the metastasis. From a morphological point of view, the EMT is characterized by the epithelial dedifferentiation to the mesenchymal phenotype usually accompanied by a loss of E-cadherin followed by increased expression of N-cadherin, vimentin, and cellular proteases. Thereby, the EMT represents the transitory state in the disease progression from the organ confined to a metastatic spread. To date, the Notch pathway has been shown as a key factor in the promotion and regulation of the EMT. The major regulatory mechanism involves direct transcriptional activation of Snail expression, a crucial TF promoting the repression of E-cadherin (Kar et al., 2019). These alterations have been associated with progression, metastasis, and more aggressive clinical course of BC (De Francesco et al., 2018), CC (Rodrigues et al., 2019), OV (Huang et al., 2019), and EC (Makker and Goel, 2016), although the accompanying Notch overexpression was observed especially among the basal-like BC (Fedele et al., 2017). Notably, these findings focused on the core signaling omitting the distant effects of the Notch pathway regarding the adhesion and EMT-associated processes. **Figure 6B** demonstrates how the profiles of expression of the Notch target genes involved in adhesion and EMT are differentiated across the female tract malignancies, reflecting the diverse biology of each specific type of tumor irrespective of the simultaneous signaling by steroid hormones.

The aberrancies of the Notch pathway were also shown to contravene the cell energetics. The signals forcing cells to proliferate at an enormous rate affect the utilization of the nutrients, especially the glucose uptake. Of note, the cancer cells tend to alter their metabolism to satisfy the high demands for various compounds, thus ensuring further growth and invasion. This involves glycolytic shift resulting in increased glycolysis

TABLE 6 | Summary table of OS analysis regarding the three groups.

Gene	Tumor	Comparison	HR*
HES5	BRCA	Low vs. high	0.397**
		Low vs. medium	0.744
		Medium vs. high	0.507(0.0529)
ADAM17	CESC	Low vs. high	0.146***
		Low vs. medium	0.377*
		Medium vs. high	0.492*
DLL1		Low vs. high	0.293**
		Low vs. medium	0.73
		Medium vs. high	0.456*
HES4		Low vs. high	0.141***
		Low vs. medium	0.428*
		Medium vs. high	0.479*
HES5		Low vs. high	8.17**
		Low vs. medium	2.21(0.0627)
		Medium vs. high	2.93(0.0581)
JAG1		Low vs. high	0.39*
		Low vs. medium	0.661
		Medium vs. high	0.392*
NOTCH1		Low vs. high	0.51*
		Low vs. medium	0.679
		Medium vs. high	0.728
NOTCH1	UCEC	Low vs. high	0.237**
		Low vs. medium	0.523
		Medium vs. high	0.348(0.0686)
NOTCH2		Low vs. high	0.105*
		Low vs. medium	0.152*
		Medium vs. high	0.422*

Value represents HR with statistical significance, and the color indicates expression level correlating with unfavorable prognosis: red—higher expression unfavorable, blue—lower expression unfavorable (level of the expression is considered relative to the determined cutpoint).
$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$.

that occurs irrespective of the mitochondrial respiration, known as the Warburg effect (Warburg, 1925). Recently, it has been shown that even a weak impulse of Notch activity may elicit continuing metabolic changes resembling the Warburg effect (Slaninova et al., 2016). In the studies on BC, Martinez-Outschoorn et al. concluded that the acidic microenvironment resulting from the ongoing Warburg effect provides a favorable niche for generating the CSCs (Martinez-Outschoorn et al., 2011), the hypothesis that has been further extended in the study of Goodman and collaborators revealing company of the high Notch activity (Goodman, 2012). Besides promoting tumorigenesis, the metabolic changes associated with the Warburg effect were also shown to increase the drug resistance (Bhattacharya et al., 2016). On the other hand, many studies are more and more often emphasizing the importance of the tricarboxylic acid (TCA) cycle (commonly known as the Krebs cycle), the major route for oxidative phosphorylation, during the carcinogenesis (Anderson et al., 2018). We observed that the Notch downstream effectors associated with the energetics of the cell reflected various profiles of the Warburg effect among the gynecological malignancies. Remarkably, the expression of FH encoding fumarase, an enzyme that catalyzes the reversible hydration/dehydration of fumarate to malate during the TCA cycle, demonstrated opposite expression patterns in the tumors compared to the normal tissue (**Figure 6D**) and its deregulation complies with the reports (Eng et al., 2003).

Finally, we performed WGCNA to elucidate the "otherness" factor of the cluster representing the basal-like BC in UMAP. The analysis revealed the module of 1,336 genes belonging to the Notch downstream targets that shared co-expression patterns in association with BC subtype. Beyond the major differences visible between cancer and normal breast tissue, basal-like was the most distinct, although similar to HER2-enriched. The latter also exhibited partial similarity to luminal subtypes, which were roughly homogeneous (**Figure 7A**). Regarding biological processes that these genes were involved in, we identified 190 terms that met the significance threshold. The most interesting were related to cell cycle, EMT, mesenchymal cell differentiation, DNA repair, G1/S and G2/M transition of the mitotic cell cycle, histone modification, SC differentiation, steroid hormone-mediated signaling pathway, and cellular response to steroid hormone signaling as well as establishment or maintenance of cell polarity, which very well represent differential biology and various clinical course of distinct BC subtypes (**Figure 7B**).

CONCLUDING REMARKS

Notch pathway is one of the few major regulatory mechanisms during tissue development. Its deregulation affects normal proliferation and differentiation leading to aberrancies in tissue architecture and was also reported as an essential player in carcinogenesis and cancer progression including the female reproductive tract (breast, cervix, ovary, and uterine endometrium). Our analysis showed distinct gene expression profiles of Notch pathway members as well as their target genes.

Interestingly, though examined cancers show separated models of the Notch pathway and its targets, gene expression of all normal tissues is much more similar to each other than to its cancerous compartments, despite the different influence of hormone signaling, i.e., through estrogen. Such Notch-driven cancerous differentiation resulted in a case of opposite associations mainly with DFS and to less extent with OS that consequently reflects very distinct profiles of the target genes, including genes associated with cell proliferation and differentiation, energy metabolism, or the EMT. Expression of apoptotic genes differed among all cancers, but despite that, the most visible were differences between normal and cancerous tissues of the same type. Our analysis revealed that the Notch signaling pathway not only has a distinct influence on different female reproductive tract tissues but also demonstrated various roads of carcinogenesis. The differentiation of BC, CC, OV, and EC regarding the expression of the Notch core components visible in **Figure 2** arose from the alterations in specific parts

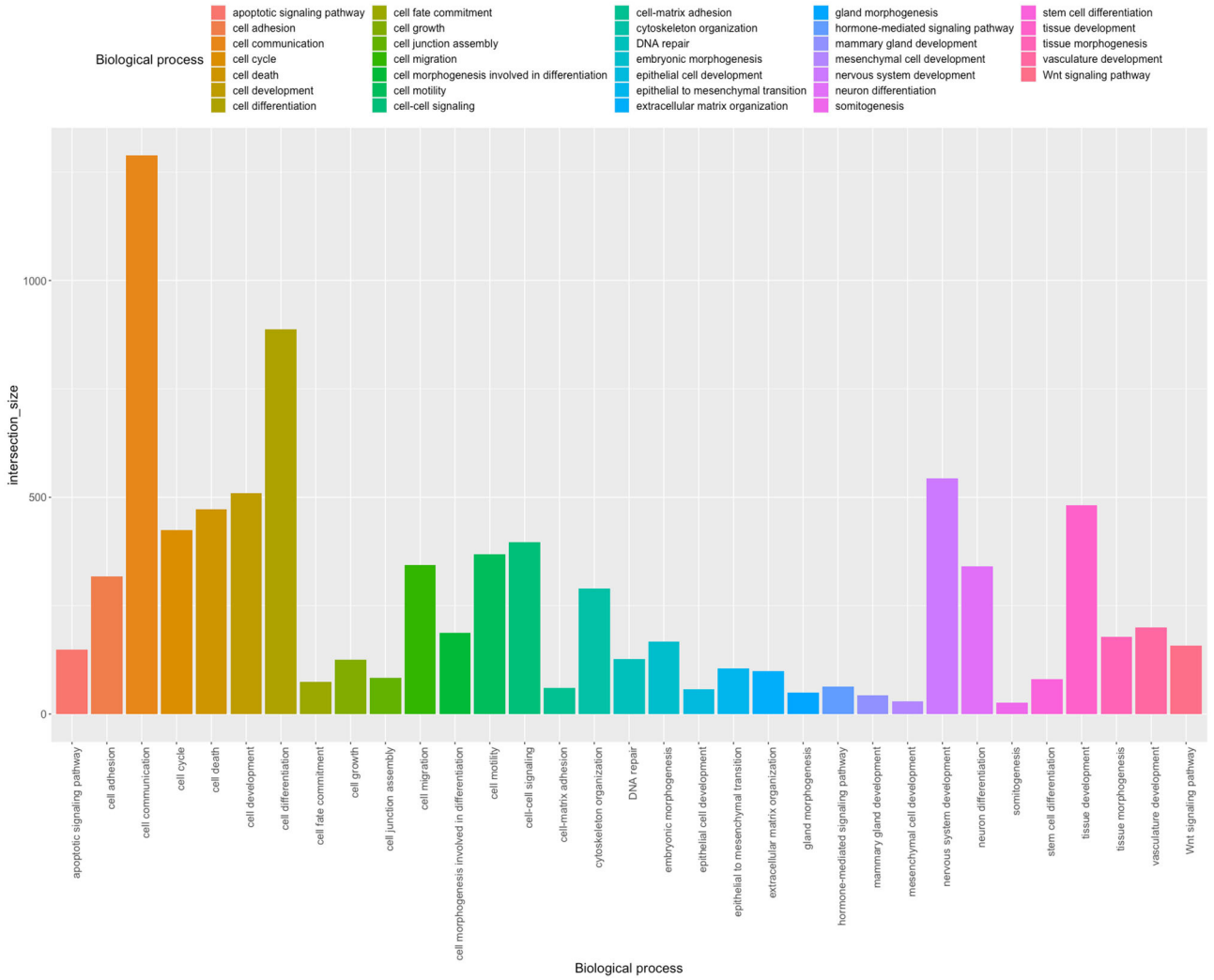


FIGURE 4 | Functional annotation of HES and HEY downstream targets.

of the Notch pathway. BC and CC were closely related in the UMAP dimensions and, simultaneously, different from OV and EC forming another cluster of similar traits. By analogy, we observed the same trends in Notch-driven survival, which have been summarized in **Table 7**. It seems that the major differences in the Notch signaling originate from the different patterns of Notch activation through ligands of Delta and Serrate families. Despite all tumors showing the common profile of Notch receptors favoring lowered expression in terms of DFS, Delta and Serrate were similarly correlated with better prognosis in BC and CC, although different from OV and EC. However, the executors of Notch processing such as Fringe (SI cleavage), TACE (ADAM17 and SII cleavage), and γ-secretase complex (SIII cleavage) as well as the modulators (Dvl, Numb, and Deltex) seem to process the Notch signal similarly across the female tract tissues. The lowered activity of the *CSL* (RBP-J) effector complex was more favorable in BC, CC, and EC as opposed to OV with various profiles of co-activators and co-repressors, which could likely affect the signal transduction. Finally, the last members of the core signaling, *HES* and *HEY* TFs, reflected in trends the activation pattern of Delta and Serrate ligands and decreased expression in BC and CC, but the increased expression in OV and EC was associated with improved disease-free outcomes. The signaling map differing BC, CC, OV, and EC drawn by alterations in single genes may therefore serve as marker profiles resulting in specific clinical outcomes, which in turn originate from alterations of the downstream targets and associated biological processes. We based this comparative summary on the DFS as it seems to be less biased with the general condition of patients, coexisting diseases, and other clinical factors affecting OS; however, the trends in both analyses were largely consistent (**Tables 4**, **5**). Quite simple signaling connections are functionally very differentiated; therefore, several mechanistic experiments are required to find

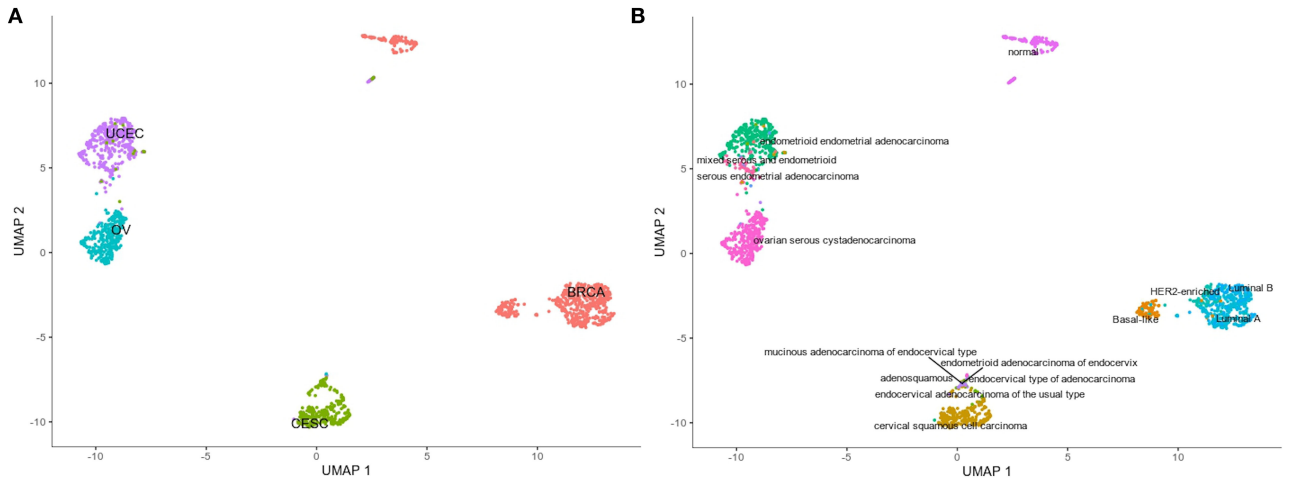


FIGURE 5 | Spatial profiling of BC, CC, OV, and EC accompanied by normal tissues regarding the expression of downstream targets of Notch signaling. **(A)** shows the distribution of the cancer types accompanied by the normal tissues and the **(B)** specifies subtypes of the tumors with differentiated basal-like BC and normal tissues, independently of origin.

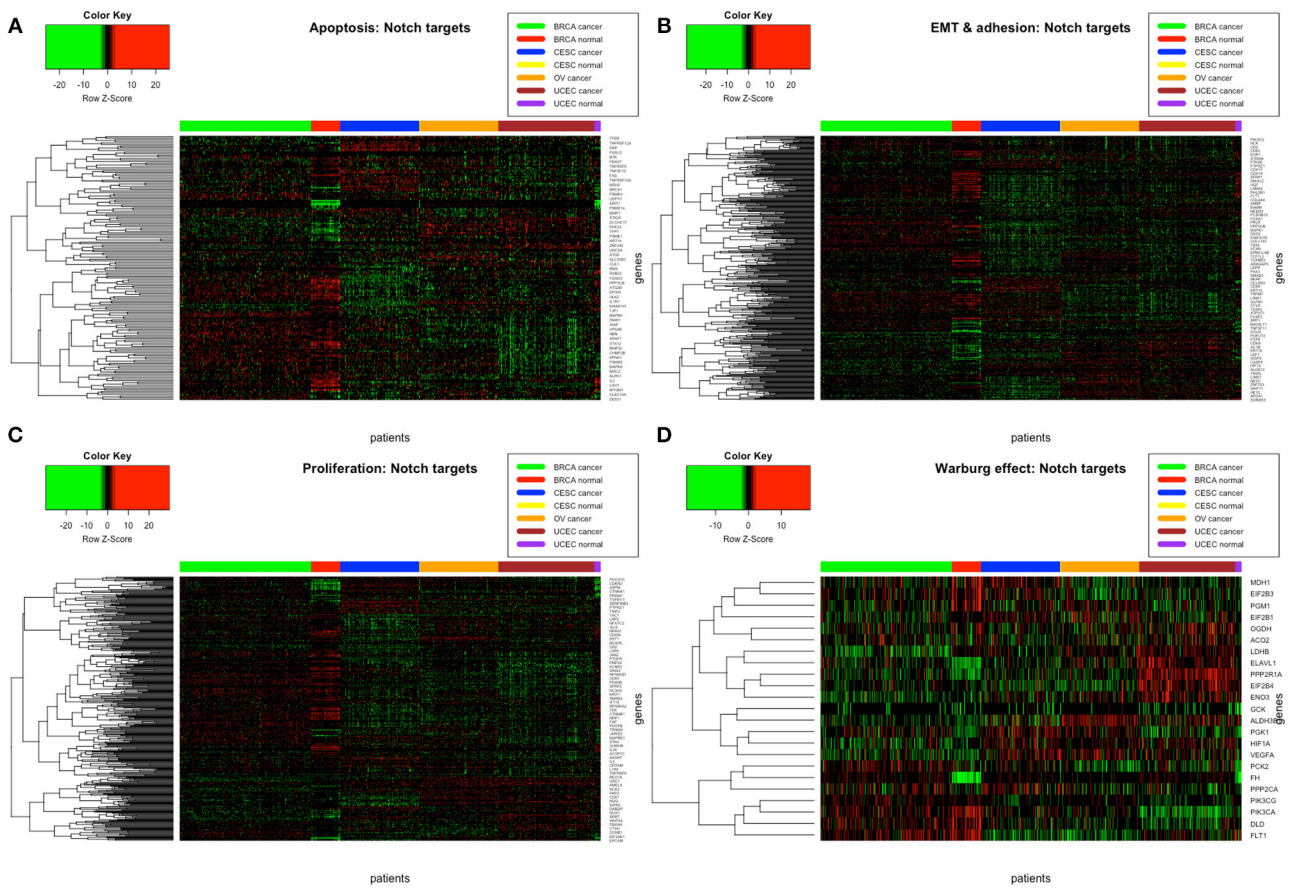


FIGURE 6 | The profiles of expression of the Notch downstream effectors associated with **(A)** apoptosis, **(B)** adhesion and EMT, **(C)** proliferation, and **(D)** cell energetics, i.e., Warburg effect.

and explain every specific change in expression of particular Notch pathway members. On the other hand, we may conclude that observed OS and DFS Notch pathway associations resulted from differential expression of target genes. This may direct a future analysis to search for new therapeutic targets based on specific Notch pathway profiles.

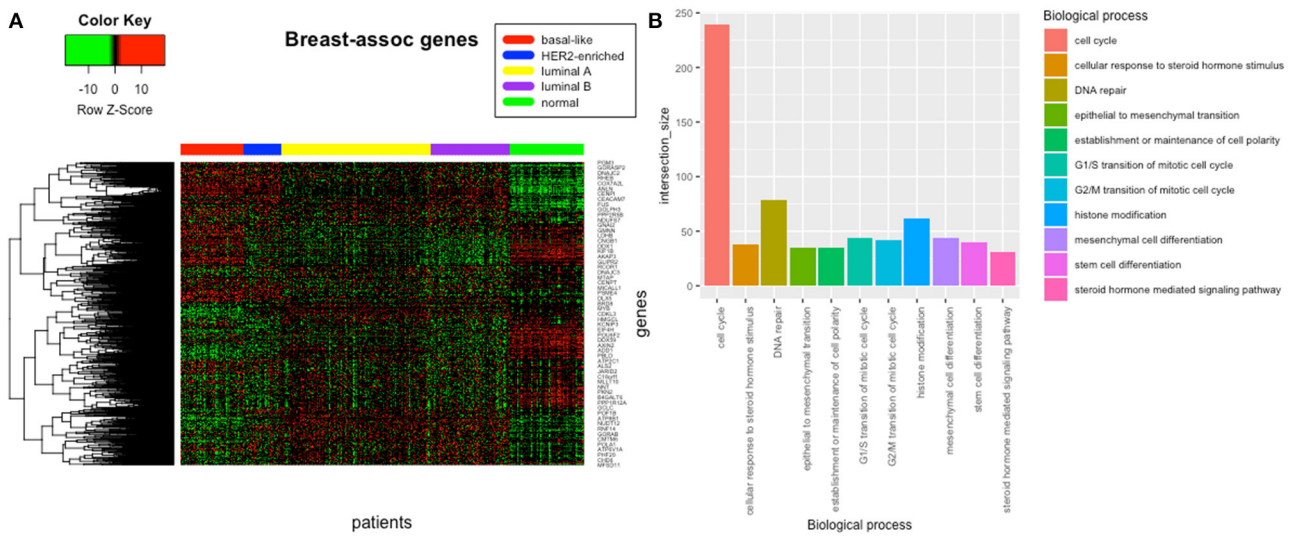


FIGURE 7 | The Notch-derived profiles of expression characterizing subtypes of BC **(A)** with functional annotation of the biological processes **(B)**.

MATERIALS AND METHODS

Data Acquisition

The four TCGA cohorts including breast invasive carcinoma (BRCA; BC), cervical and endocervical cancers (CESC; CC), ovarian serous cystadenocarcinoma (OV), and uterine corpus endometrial carcinoma (UCEC; EC) were obtained from The Cancer Genome Atlas repositories through GDAC Firehose in the form of expression (RNAseqV2, level 3, RSEM normalized, data status of 28th Jan 2018) with corresponding clinical data. Patients that missed corresponding expression/clinical data were excluded from further analyses. Moreover, among BC, only female patients with available PAM50 classifier were analyzed. The summary and sizes of cohorts used in the study are shown in **Table 8**. Normal, paired solid tissues were additionally retrieved through R-dedicated package TCGA-Assembler (Wei et al., 2018).

Pathway-Associated Data

The scheme of a core signaling through Notch was accessed through the KEGG database (hsa04330) (Kanehisa and Goto, 2000; Kanehisa et al., 2019). The list of core members participating in the pathway was downloaded from MSigDB (Liberzon et al., 2015) according to the corresponding KEGG gene set. Detailed lists of genes involved in the Notch pathway are available in the GitHub repository (https://github.com/orzechmag/notchfemaletract).

Genes classified as downstream targets of the Notch pathway were identified through literature-based, well-known pathway-specific TFs. Subsequently, targets of the aforementioned executive TFs were identified through the GTRD database, which comprises a collection of ChiP-seq documented TF binding sites for human (Yevshin et al., 2017, 2019). Finally, Ensembl Gene ids were converted into Gene Symbols using the db2db tool from bioDBnet (Mudunuri et al., 2009). Detailed lists of target genes retrieved from GTRD are available in the GitHub repository (https://github.com/orzechmag/notchfemaletract).

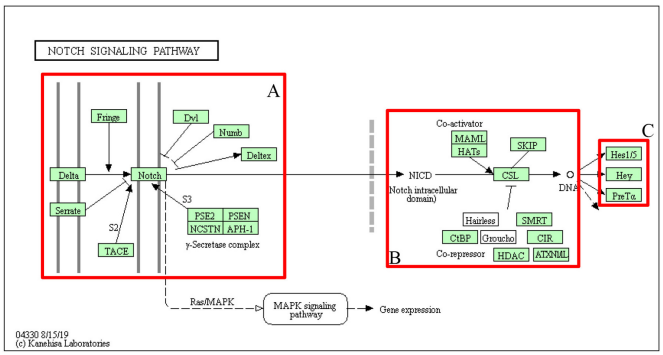
Pathway-Associated Global Profiling of Tumors

Population structure of BC, CC, OV, and EC accompanied by normal tissues was studied by applying the UMAP method, preceded by a PCA pre-processing step regarding the expression of core members of Notch as well as expression of its downstream effectors as two separate models through employing Monocle3 R package. Monocle3 is primarily dedicated to analyzing single-cell RNA-seq (scRNA-seq); however, except for trajectory-based analysis of tissue/lineage-specific differentiation, available tools (e.g., PCA pre-processing, UMAP, visualization tools, and suite for DGE analysis) are of general usage and therefore were applicable.

Alterations of Pathway Core Members—DGE and Mutations

Basic alterations between cancerous and normal tissue were identified through the calculation of logarithmized fold change (logFC, i.e., $\log_2$FC) applied on members of the core of the Notch pathway. logFC was calculated between tumor and its matched normal tissue except OV as its corresponding normal tissue was not available in TCGA. Profiles of expression were shown by employing heatmaps generated with heatmap.2() function in R with the complete agglomeration method and Spearman distance metric. Moreover, mutations and copy number alterations (CNAs) occurring in pathway core genes accompanied by TP53 and DNA processing-associated enzymes such as DNMT1 (DNA methyltransferase 1) and HDAC1, HDAC2, HDAC4, and HDAC7 (histone deacetylases 1, 2, 4, and 7) were identified via cBioPortal (Cerami et al., 2012; Gao et al., 2013) among respective cohorts of BC, CC, OV, and EC.

TABLE 7 | The summary of Notch signaling differentiating BC, CC, OV, and EC.

Group	Gene	BC	CC	OV	EC
					
A: LIGANDS, RECEPTORS, AND MODULATORS					
Delta	*DLL1*				↑
	DLL3		↓		↓
	DLL4	↑		↑	
Serrate	*JAG1*	↓	↓	↑	
	JAG2			↑	↑
Fringe	*LFNG*	↓		↑	↑
	MFNG	↑	↑	↓	↑
	RFNG	↑			↑
TACE	*ADAM17*	↑	↓		↓
Notch	*NOTCH1*	↓			↑
	NOTCH2			↓	
	NOTCH3				↓
	NOTCH4	↓	↓		
Dvl	*DVL1*	↑	↓	↓	
	DVL2	↑			
	DVL3	↓			↓
Numb	*NUMB*	↑		↓	
	NUMBL	↑	↓		↓
Deltex	*DTX1*	↑		↑	
	DTX2	↓	↑		↑
	DTX3	↑		↓	
	DTX3L		↑		
	DTX4				
γ-secretase complex	*PSENEN*		↑		↓
	PSEN1	↑		↓	↑
	PSEN2	↑		↑	
	NCSTN		↓		↑
	APH1A		↓		↓
	APH1B	↑	↑	↑	↓
B: SIGNAL TRANSDUCTION					
MAML	*MAML1*				↓
	MAML2		↑		
	MAML3	↓	↓		
HATs	*CREBBP*		↓	↓	↓
	EP300				
	KAT2A	↑		↓	
	KAT2B		↑		

(Continued)

TABLE 7 | Continued

Group	Gene	BC	CC	OV	EC
SKIP	*SNW1*				↓
CSL	*RBPJL*		↓		↓
	RBPJ	↓		↑	
CtBP	*CTBP1*	↓		↓	
	CTBP2	↑	↑		↓
HDAC	*HDAC1*		↑		
	HDAC2	↓			
ATXN1/L	*ATXN1*	↓			
	ATXN1L				
CIR	*CIR1*			↓	
SMRT	*NCOR2*		↑		↓
C: TRANSCRIPTION FACTORS					
Hes1/5	*HES1*		↓	↑	↑
	HES5	↓			
Hey	*HEY1*	↓		↑	
	HEY2		↓	↑	↑
	HEYL	↓	↓		
PreTα	*PTCRA*	↓	↑	↑	

The up arrow indicates the higher expression of a particular Notch member associated with favorable DFS prognosis and the down arrow indicates the lowered expression of a particular Notch member correlating with better DFS.

TABLE 8 | Sizes and classification of the cohorts used in the study.

	No. of tumor samples	No. of matched normal samples
BC:	505	
- Basal-like	97	113
- Luminal A	228	
- Luminal B	122	
- HER2-enriched	58	
CC:	304	
- Cervical squamous cell carcinoma	252	3
- Endocervical adenocarcinoma of the usual type	6	
- Adenosquamous	5	
- Endometrioid adenocarcinoma of endocervix	3	
- Endocervical type of adenocarcinoma	21	
- Mucinous adenocarcinoma of endocervical type	17	
OV	301	Not available
EC:	370	24
- Serous endometrial adenocarcinoma	57	
- Mixed serous and endometrioid endometrial adenocarcinoma	10	
- Endometrioid endometrial adenocarcinoma	303	

Notch-Specific Survival Analysis

Significance of core pathway members has been investigated in terms of clinical outcome; therefore, DFS and OS analyses were conducted. The analysis was performed with Evaluate Cutpoints

system (Ogluszka et al., 2019) involving cutp, maxstat, and rolr algorithms of cutpoint determination in correlation with survival time and clinical outcome according to the following clinical parameters: "patient.person_neoplasm_cancer_status" and "patient.vital_status" as event indicator and "patient.days_to_last_followup" and "patient.days_to_death" as a time of observation for DFS and OS, respectively.

Variability of Genes Associated With Specific Biological Processes Governed by the Notch Signaling

Among downstream effectors of Notch signaling, we identified sets of genes involved in major biological processes of indisputable relevance and contribution in carcinogenesis and disease progression such as apoptosis, adhesion (including EMT-related markers), proliferation, and Warburg effect. The sets of genes were created based on MSigDB collections of ontological terms (C5, BP: GO biological processes) and involved all terms that were widely associated with apoptosis, adhesion, EMT, proliferation, and cancer energetics (i.e., Warburg effect). Subsequently, each ontology was defined among downstream targets of the Notch signaling pathway resulting in the final sets of genes. Profiles of expression were presented in the form of heatmaps, analogously to the previous section. Gene sets of all ontological terms are available in the GitHub repository (https://github.com/orzechmag/notchfemaletract).

Identification of the Basal-Like "Otherness" Factor Among the Subtypes of BC

Regarding the fact that basal-like BC formed a separate cluster in UMAP dimensions from the remaining BC subtypes, it may be considered as a distinct molecular characteristic with an inclination to become a separate disease entity (as in fact remains in line with literature reports). Thereby, we aimed to define the set of genes contributing to distinct characteristics of the basal-like subtype followed by functional annotation to define abrogated biological processes among downstream targets of the Notch pathway. Modules of genes sharing a common profile of expression with BC subtype were determined by applying weighted gene co-expression network analysis (WGCNA) with a soft-thresholding approach ($\beta = 4$) within the R environment (Langfelder and Horvath, 2008). The further analysis comprised functional annotation of genes concerning biological processes through g:Profiler (Raudvere et al., 2019) and visualization expression profiles in heatmaps, analogously to the previously described. The WGCNA R code is available in the GitHub repository (https://github.com/orzechmag/notchfemaletract).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: GDAC Firehose (https://gdac.broadinstitute.org/) and GitHub (https://github.com/orzechmag/notchfemaletract).

AUTHOR CONTRIBUTIONS

AB was responsible for study design, data interpretation, and revision of the manuscript. DA and MO were responsible for data preparation, data analysis, study results, data interpretation, and writing of the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the National Science Center, Poland 2016/23/N/NZ2/02372.

ACKNOWLEDGMENTS

The results shown here are in whole based on data generated by the TCGA Research Network: https://www.cancer.gov/tcga.

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 14 January 2021
doi: 10.3389/fcell.2020.601006

Role of Fibroblast Growth Factor 23 (FGF23) and αKlotho in Cancer

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Together with fibroblast growth factors (FGFs) 19 and 21, FGF23 is an endocrine member of the family of FGFs. Mainly secreted by bone cells, FGF23 acts as a hormone on the kidney, stimulating phosphate excretion and suppressing formation of 1,25$(OH)_2D_3$, active vitamin D. These effects are dependent on transmembrane protein αKlotho, which enhances the binding affinity of FGF23 for FGF receptors (FGFR). Locally produced FGF23 in other tissues including liver or heart exerts further paracrine effects without involvement of αKlotho. Soluble Klotho (sKL) is an endocrine factor that is cleaved off of transmembrane Klotho or generated by alternative splicing and regulates membrane channels, transporters, and intracellular signaling including insulin growth factor 1 (IGF-1) and Wnt pathways, signaling cascades highly relevant for tumor progression. In mice, lack of FGF23 or αKlotho results in derangement of phosphate metabolism and a syndrome of rapid aging with abnormalities affecting most organs and a very short life span. Conversely, overexpression of anti-aging factor αKlotho results in a profound elongation of life span. Accumulating evidence suggests a major role of αKlotho as a tumor suppressor, at least in part by inhibiting IGF-1 and Wnt/β-catenin signaling. Hence, in many malignancies, higher αKlotho expression or activity is associated with a more favorable outcome. Moreover, also FGF23 and phosphate have been revealed to be factors relevant in cancer. FGF23 is particularly significant for those forms of cancer primarily affecting bone (e.g., multiple myeloma) or characterized by bone metastasis. This review summarizes the current knowledge of the significance of FGF23 and αKlotho for tumor cell signaling, biology, and clinically relevant parameters in different forms of cancer.

Keywords: Ca^{2+}, calcitriol, inflammation, malignancies, phosphate

OPEN ACCESS

Edited by:
Marzia Di Donato, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Gabriella Castoria, Second University of Naples, Italy
Mohammed S. Razzaque, Lake Erie College of Osteopathic Medicine, United States

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

Received: *31 August 2020*
Accepted: *15 October 2020*
Published: *14 January 2021*

Citation:
Ewendt F, Feger M and Föller M (2021) Role of Fibroblast Growth Factor 23 (FGF23) and αKlotho in Cancer. Front. Cell Dev. Biol. 8:601006. doi: 10.3389/fcell.2020.601006

FIBROBLAST GROWTH FACTOR 23 (FGF23)

The human *fibroblast growth factor 23* (*FGF23*) gene localized on chromosome 12p13 was discovered in 2000 (*Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23*, 2000, ADHR Consortium, 2000). FGF23 is a member of the family of fibroblast growth factors (FGFs) and a proteohormone of 32 kDa (Yamashita et al., 2000; Yamazaki et al., 2002). It is characterized by endocrine and paracrine effects in contrast to most other FGFs, which do not act as classical hormones (Angelin et al., 2012). Endocrine FGF23 is primarily produced by bone cells and released into the bloodstream (Riminucci et al., 2003; Yoshiko et al., 2007). Low *Fgf23* expression was detected in other tissues, such as spleen, thymus, small intestine, liver, kidney,

heart, and brain (Yamashita et al., 2000; Yoshiko et al., 2007). The secretion of the biologically active hormone into the blood is controlled by proteolytic cleavage of the full-length, intact FGF23 molecule by a furin/furin-like proprotein convertase between ^{179}Arg and ^{180}Ser (Shimada et al., 2001). The susceptibility of FGF23 to proteolytic degradation is regulated by UDP-N-acetyl-alpha-D galactosamine: polypeptide N-acetylgalactosaminyltransferase 3 (GalNT3)-mediated *O*-glycosylation at threonine 178 and phosphorylation at serine 180 by the enzyme family with sequence similarity 20 member C (FAM20C) (Tagliabracci et al., 2014). Target organs of FGF23 include kidney and parathyroid glands (Ben-Dov et al., 2007; Gattineni et al., 2009). In the former, FGF23 inhibits the reabsorption of phosphate by down-regulating the membrane abundance of NaPiIIa, the major Na^+-coupled phosphate transporter of the proximal tubule (Gattineni et al., 2009). Moreover, FGF23 suppresses the synthesis of $1,25(OH)_2D_3$, active vitamin D, by inhibiting key enzyme 1-α-hydroxylase (encoded by *Cyp27b1*) in the kidney (Chanakul et al., 2013). In the parathyroid glands, FGF23 down-regulates the production and secretion of parathyroid hormone (PTH) (Ben-Dov et al., 2007). This way, FGF23 is part of a hormone circuit additionally involving PTH and $1,25(OH)_2D_3$ and regulating phosphate and vitamin D metabolism, as well as impacting on Ca^{2+} (Blau and Collins, 2015). These endocrine effects of FGF23 are mediated by FGF receptors (FGFRs) including FGFR1c, FGFR3c, and FGFR4 with αKlotho (KL) serving as a scaffolding protein, which is needed to enhance the binding affinity of FGF23 (Gattineni et al., 2009, 2011; Chen G. et al., 2018). Other effects of locally produced FGF23 are, at least in part, paracrine and include the regulation of inflammation in hepatocytes (Singh et al., 2016), the induction of cardiac hypertrophy (Faul et al., 2011), or inhibition of neutrophils (Rossaint et al., 2016). At least some of these effects are independent of KL (Quarles, 2019). The plasma concentration of FGF23 goes up in many acute and chronic diseases (Gutierrez et al., 2005). In chronic kidney disease (CKD), high FGF23 plasma levels are observed prior to hyperparathyroidism or hyperphosphatemia (Isakova et al., 2011). FGF23 predicts progression and outcome in CKD (Hasegawa et al., 2010). Independently of kidney disease, FGF23 is associated with carotid atherosclerosis (Rodríguez-Ortiz et al., 2020), fibrosis, and poorer prognosis in heart failure (Roy et al., 2020) and prognosis in heart failure with preserved ejection fraction (Kanagala et al., 2020). In another cohort, however, the role of FGF23 for patients with heart failure was less clear (Stöhr et al., 2020). Dyslipidemia is associated with higher FGF23 levels (Mirza et al., 2011). Inflammatory conditions also up-regulate FGF23 (Czaya and Faul, 2019). Hence, FGF23 is discussed as a biomarker correlating with progression and outcome in some significant diseases of high burden (Schnedl et al., 2015).

Abbreviations: $1,25(OH)_2D_3$, active vitamin D; ADAM10, A disintegrin and metalloproteinase domain-containing proteins 10; ADAM17, A disintegrin and metalloproteinase domain-containing proteins 17; ALPL, alkaline phosphatase; BACE1, β-APP cleaving enzyme 1; CKD, chronic kidney disease; CRC, colorectal cancer; CYP, cytochrome P 450; DDLPS, dedifferentiated liposarcoma; DLBLC, diffuse large B-cell lymphoma; EGR-1, early gene response transcription factor 1; EMT, epithelial to mesenchymal transition; EOC, epithelial ovarian cancer; ER, endoplasmic reticulum; ERK1/2, extracellular receptor signal-related kinase ½; FAM20C, family with sequence similarity 20 member C; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FOXO, forkhead box O; GalNT3, UDP-*N*-acetyl-alpha-D galactosamine:polypeptide *N* acetylgalactosaminyltransferase 3; GFR, glomerular filtration rate; GBM, glioblastoma multiforme; GSK-3β, glycogen synthase kinase-3β; HCC, hepatocellular carcinoma; HIF-1α, hypoxia-inducible factor 1α; HMGB1, high-mobility group protein B 1; HPSE, heparanase; HR, hazard ratio; IGF-1, insulin-like growth factor 1; IL, interleukin; KL, αKlotho; KLB, βKlotho; KLG, γKlotho; Klph, Klotho lactase-phlorizin hydrolase-related protein; MAPK, mitogen-activated protein kinase; MDS, myelodysplastic syndromes; miR, micro ribonucleic acid; MM, multiple myeloma; mTOR, mammalian target of rapamycin; NaPiIIa, sodium phosphate cotransporter 2a; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; PDAC, pancreatic ductal adenocarcinoma; PPAR, peroxisome proliferator–activated receptor; PI3K, phosphoinositide 3-kinase; PTH, parathyroid hormone; RCC, renal cell carcinoma; ROMK1, renal outer medullary potassium channel 1; sKL, soluble Klotho; SCC, squamous cell carcinoma; SNP, single-nucleotide polymorphism; SOX17, sex determining region Y (SRY) – related high-mobility group (HMG) box protein family member 17; TNM, tumor, nodes, metastasis; TIO, tumor-induced osteomalacia; TRPV5, transient receptor potential ion channel 5; VDR, vitamin D receptor; Wnt, wingless-related integration site.

αKlotho

The *αKlotho* (referred to as KL) gene was identified in 1997. In mice, a mutation of the *Kl* gene causes a syndrome of rapid aging including a drastically shortened life span and further age-associated diseases and symptoms affecting most organs and tissues such as atherosclerosis, osteoporosis, skin atrophy, infertility, or emphysema (Kuro-O et al., 1997). KL is mainly expressed in the kidney but also in the central nervous system (cerebellum, cerebral cortex, spinal cord) and in other tissues with detectable but lower expression such as thyroid gland, aorta, urinary bladder, ovary, skeletal muscle, pancreas, prostate gland, testis, or the adrenal gland (Kuro-O et al., 1997; Lim et al., 2015). However, it has not been clear for a long time how KL develops its function until it was discovered that the phenotype of the *Kl* knockout mouse is similar to the *Fgf23* knockout mouse. The mice exhibit high serum phosphate levels, soft tissue and vascular calcification, increased expression of renal sodium phosphate cotransporter NaPiIIa, and 1-α-hydroxylase, accompanied by high serum levels of $1,25(OH)_2D_3$ (Tsujikawa et al., 2003; Nakatani et al., 2009; Razzaque, 2009a). Moreover, it could be shown that the ablation of $1,25(OH)_2D_3$ signaling in mice lacking a functional vitamin D receptor prevents the premature aging phenotype in $Kl^{-/-}$ mice (Anour et al., 2012; Andrukhova et al., 2017). Deficiency of both *1-α-hydroxylase* and *Kl* prevents soft tissue and vascular calcification and normalizes the high Fgf23 and low PTH levels paralleled by Kl deficiency in mice (Ohnishi et al., 2009). These findings assign KL an important physiological role in the regulation and maintenance of phosphate homeostasis (Razzaque, 2009b). The human *KL* gene is located on chromosome 13q12 and ranges over 50 kb with 5 exons and 4 introns (Matsumura et al., 1998). It encodes the KL protein, which shows homology with family I β-glycosidases and is a 135-kDa single-pass transmembrane protein (Kuro-O et al., 1997; Chen et al., 2007; Xu and Sun, 2015; Dalton et al., 2017). The protein comprises a N-terminal short signal sequence, the large ectodomain

containing two internal repeats termed KL1 and KL2 mediating KL activity and function, the transmembrane domain, and a short intracellular domain (Kuro-O et al., 1997; Kuro-O, 2008; Xu and Sun, 2015) (**Figure 1**). Three different isoforms can be distinguished: full-length transmembrane KL, the 130-kDa shed soluble form (sKL), and the shorter truncated secreted variant of KL (65 kDa) (Kuro-O et al., 1997; Shiraki-Iida et al., 1998; Dalton et al., 2017). sKL consists of the KL1 and KL2 domain but lacks the transmembrane and intracellular domain. It arises because of proteolytic cleavage, termed α-cut, of full-length transmembrane KL on the cell surface by α-secretases A disintegrin and metalloproteinase domain-containing proteins 10 and 17 (ADAM10 and ADAM 17) and the β-APP cleaving enzyme 1 (BACE1) (Chen et al., 2007; Bloch et al., 2009; Xu and Sun, 2015). The residual transmembrane fragment undergoes an intramembrane proteolytic degradation by γ-secretases (Bloch et al., 2009). Moreover, another cleavage mechanism of KL by ADAM10 and ADAM17, termed β-cut, generates the two 65-kDa fragments KL1 and KL2 (Chen et al., 2007). Therefore, after shedding, sKL protein enters blood, urine, or cerebrospinal fluid as KL1 or KL2 only or both KL1 and KL2 and exerts its functions in other tissues and organs (Imura et al., 2004; Akimoto et al., 2012; Xu and Sun, 2015; Dalton et al., 2017). sKL inhibits insulin growth factor 1 receptor (IGF-1R)/phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) signaling and activates forkhead box O (FOXO) (Kurosu et al., 2005; Yamamoto et al., 2005). It increases glucose uptake and glycogen storage and reduces lipid accumulation and insulin resistance through PPARα expression (Gu et al., 2020) corroborating the role of KL and underlying signaling in glucose metabolism and adipocyte maturation as discussed recently (Razzaque, 2012). Other KL downstream effects are the activation of extracellular signal–related kinase 1/2 (ERK1/2) (Maekawa et al., 2011), inhibition of Wnt signaling (Liu et al., 2007), or reduction of inflammation (Maekawa et al., 2009). Moreover, sKL is involved in the stimulation of ion channels and transporters including transient receptor potential ion channel TRPV5 (Chang et al., 2005; Cha et al., 2008) or renal outer medullary potassium channel 1 (ROMK1) (Cha et al., 2009). The secreted isoform of KL is processed by alternative RNA splicing in the internal splice donor site of exon 3, containing a N-terminal signal sequence and KL1 only (Matsumura et al., 1998). In contrast to KL and sKL, the secreted isoform has not been detected *in vivo* yet (Kuro-O, 2019).

Progressing CKD is associated with decreased renal KL expression and loss of renal function (Koh et al., 2001; Komaba et al., 2010; Hu et al., 2011). Lower KL expression correlates with more cardiovascular events in patients on hemodialysis

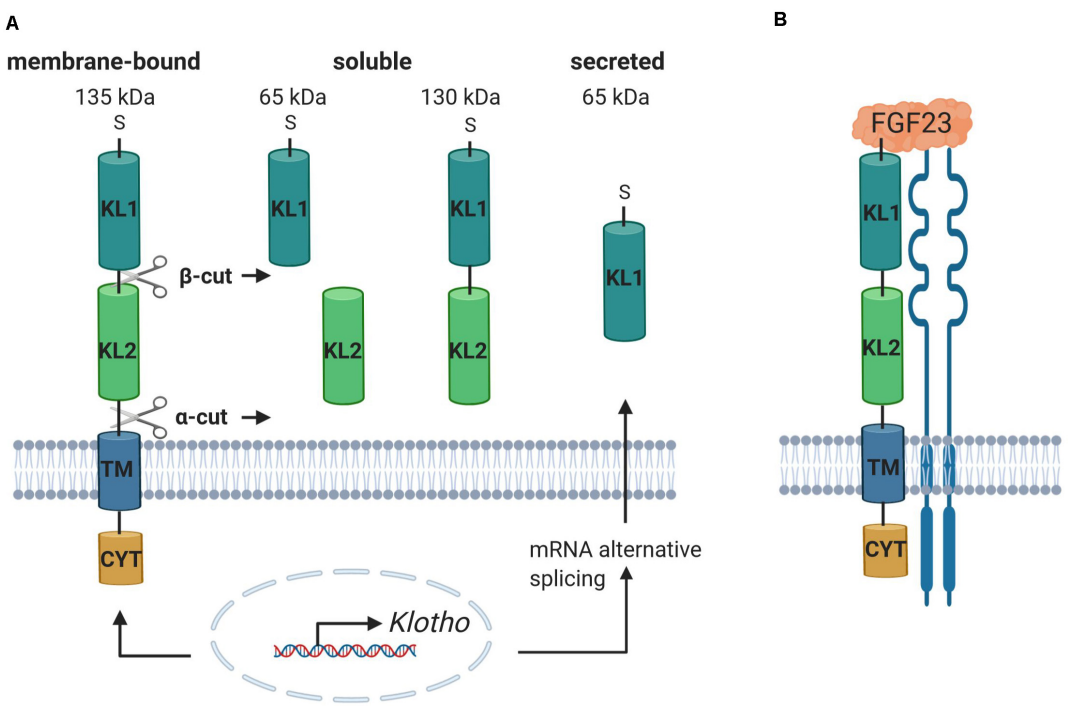

FIGURE 1 | Klotho includes different isoforms and binds to FGFR, facilitating the binding and subsequent signal transduction of FGF23. **(A)** The *KL* gene encodes a single-pass transmembrane protein, comprising an N-terminal short signal sequence (S), the ectodomain, containing the internal repeats KL1 and KL2, the transmembrane domain (TM), and a short cytoplasmic domain (CYT). It exists as full-length 135-kDa membrane-bound KL, the 130-kDa shed soluble KL (sKL) isoform, and the truncated secreted variant of KL. Proteolytic cleavage of full-length KL due to α-cut and/or β-cut produces sKL, containing either KL1, KL2, or both. Alternative RNA splicing of *KL* mRNA generates the secreted isoform, containing an N-terminal signal sequence and KL1 only. **(B)** The complexation of KL with FGFR enables the binding of FGF23, resulting in the formation of a trimeric complex, which activates the downstream signaling pathway. Created with BioRender.com.

(Memmos et al., 2019). In addition, KL inhibits inflammation (Maekawa et al., 2009; Zhao et al., 2011) and oxidative stress (Kimura et al., 2018; Qian et al., 2018), conditions enhanced in CKD (Mihai et al., 2018) and cardiovascular diseases (Dhiman et al., 2015).

In addition, the KL family includes two other members, termed βKlotho and γKlotho (referred to as KLB and KLG hereinafter). The *Klb* gene, identified in 2000, shows sequence similarity to *Kl* and encodes a single-pass transmembrane protein (Ito et al., 2000). KLB is localized in the cell membrane and mainly expressed in the liver and adipose tissue, where it forms a complex with FGFR1 and FGFR4, and mediates metabolic functions of FGF19 and FGF21 (Kurosu et al., 2007; Ogawa et al., 2007; Xu and Sun, 2015). FGF19 controls bile acid synthesis through suppression of *Cyp7a1* (Kurosu et al., 2007). Thus, $Klb^{-/-}$, $Fgf15^{-/-}$, and also $Fgfr4^{-/-}$ mice lack Cyp7a1 suppression, resulting in increased bile acid production and excretion (Inagaki et al., 2005; Ito et al., 2005). Moreover, KLB is necessary for FGF21 signaling, which is expressed mainly in the liver, where it is involved as downstream target of peroxisome proliferator–activated receptor α (PPARα) in metabolic adaptation to fasting but also in adipose tissue, where it modulates lipolysis and glucose uptake (Kurosu et al., 2007; Arner et al., 2008; Suzuki et al., 2008; Dolegowska et al., 2019). The *Klph* gene was found in mice, encoding the Klotho lactase-phlorizin hydrolase-related protein, which is mainly expressed in the eyes but also in the kidney, adipose tissue, and skin (Ito et al., 2002; Fon Tacer et al., 2010). This novel member of the KL family is also termed KLG. KLG interacts with FGFR1b, 1c, 2c, and 4 and promotes activation of FGF signaling by FGF19 in HEK293 cells (Fon Tacer et al., 2010).

FGF23 AND CANCER

As detailed below and summarized in **Table 1**, the implications of FGF23 in cancer biology are thus far sparser than the known role of its coreceptor KL in tumor diseases. This may, in large part, be due to the fact that KL acts as a tumor suppressor in various types of cancer, whereas such a function is not established for FGF23. A role of FGF23 in malignancies is most clearly proven in the case of tumor-induced osteomalacia (TIO) or oncogenic hypophosphatemic osteomalacia (Larsson et al., 2003). This is a rare paraneoplastic syndrome due to a tumor excessively producing FGF23, which, in line with its main endocrine effects, induces renal phosphate excretion, as well as reduction of $1,25(OH)_2D_3$. As a consequence of both, the patients suffer from osteomalacia, demineralized bone (Yamazaki et al., 2002; Larsson et al., 2003). Benign soft tissue (mesenchymal) tumors are most frequently responsible for TIO (Boland et al., 2018), but also malignancies including colon adenocarcinoma (Leaf et al., 2013), ovarian cancer (Lin et al., 2014), small cell carcinoma of the lung (Sauder et al., 2016), anaplastic thyroid carcinoma (Abate et al., 2016), B-cell non-Hodgkin lymphoma (Elderman et al., 2016), breast cancer (Savva et al., 2019), and intracranial tumors (Colazo et al., 2020) can produce FGF23. If the causative tumor cannot be identified, the anti-FGF23 monoclonal antibody KRN23 may be therapeutically useful in TIO (Minisola et al., 2017).

Hematologic Malignancies

Because bone is the main site of FGF23 production, malignancies typically affecting or arising from bone may have a link to FGF23. In patients with bone metastasis due to different solid

TABLE 1 | Associations of FGF23 with cancer.

Cancer	FGF23 level	FGF23 effect	References
Tumor-induced osteomalacia	↑ Cancer tissues ↑ Serum	↑ Renal phosphate wasting ↓ $1,25(OH)_2D_3$ → Osteomalacia	Yamazaki et al., 2002; Larsson et al., 2003; Leaf et al., 2013; Lin et al., 2014; Abate et al., 2016; Elderman et al., 2016; Sauder et al., 2016; Boland et al., 2018; Savva et al., 2019; Colazo et al., 2020
Bone metastasis	↑ Serum		Mansinho et al., 2019
Myelodysplastic syndromes	↑ Serum ↑ Erythroid precursors	↓ Bone mineralization; microarchitecture ↑ *Alpl*; *Runx2* ↑ Anemia	Weidner et al., 2020
Multiple myeloma	↑ Serum ↓ Cells	↑ *EGR-1* and *HPSE* → Impacts tumor growth	Suvannasankha et al., 2015
Prostate cancer	Expression in cells ↔ Serum	↑ Cell proliferation and tumor invasion ↑ MAPK and AKT → Impacts tumor growth	Lee et al., 2014; Feng et al., 2015; Vlot et al., 2018
Endometrial cancer	↔ Serum		Cymbaluk-Płoska et al., 2020
Ovarian cancer	↑ Serum ↑ Cells		Tebben et al., 2005
Colorectal cancer	Serum level may rise ↑ Stool		Jacobs et al., 2011; Wang H.-P. et al., 2014
Breast cancer	↑ Cell mRNA		Aukes et al., 2017
Urothelial carcinoma	↑ Serum		Li et al., 2019
Prolactinoma	↔ Serum		Arslan et al., 2017

tumors, a higher FGF23 plasma concentration is associated with shorter survival and shorter time to skeletal-related events (Mansinho et al., 2019). Patients with myelodysplastic syndrome (MDS) characterized by impaired hematopoiesis in the bone marrow have a higher FGF23 plasma concentration that is associated with anemia and lower bone mineralization (Weidner et al., 2020). In mice, MDS is paralleled by *Fgf23* expression in erythroid precursor cells (Weidner et al., 2020). Multiple myeloma (MM) is characterized by painful bone lesions. MM cells exhibit KL-dependent FGF23 signaling, and intact FGF23 plasma levels are elevated in MM patients (Suvannasankha et al., 2015).

Prostate Cancer

FGF23 single-nucleotide polymorphisms (SNPs) are associated with increased risk of prostate cancer (Kim et al., 2014a). *FGF23* expression is enhanced in patients with castration-resistant prostate cancer, as well as FGF23/FGFR1/KL in different prostate cancer cell lines (Lee et al., 2014). FGF23 acts as an autocrine factor in prostate cancer cells stimulating tumor invasion and cell proliferation (Feng et al., 2015). According to another study, *KL* expression is reduced due to promoter hypermethylation (Seo et al., 2017). FGF23 down-regulation suppresses tumor growth *in vivo* (Feng et al., 2015). FGF23 production may be subject to autocrine stimulation through FGFR in prostate cancer (Feng et al., 2012; Wu et al., 2013; Lee et al., 2014). According to one study, the FGF23 plasma level is unchanged in prostate cancer (Vlot et al., 2018), although prostate cancer cells may stimulate FGF23 expression in osteocytes (Choudhary et al., 2018). Bone metastasis may account for the high FGF23 levels and symptoms of TIO observed in patients with prostate cancer according to other studies (Nakahama et al., 1995; Cotant and Rao, 2007; Chiam et al., 2013).

Gynecologic Tumors

In endometrial cancer, no change in the FGF23 plasma concentration is observed (Cymbaluk-Płoska et al., 2020), whereas the FGF23 plasma concentration goes up in advanced-stage epithelial ovarian cancer (EOC) (Tebben et al., 2005), and a defined FGF23 SNP is associated with better prognosis in this tumor entity (Meng et al., 2014). Breast cancer may be associated with oncogenic osteomalacia and raised FGF23 levels (Savva et al., 2019). *FGF23* mRNA expression is high in breast cancer cells, and FGF produced by tumor cells contributes to metastatic lesions (Aukes et al., 2017). Furthermore, FGFR signaling may be highly relevant for breast cancer oncogenesis (Navid et al., 2020). According to a phase 0/1 clinical trial, combined aromatase and FGFR1 inhibition in breast cancer results in a surge in the FGF23 plasma concentration (Quintela-Fandino et al., 2019).

FGF23 IN OTHER FORMS OF CANCER

The plasma FGF23 concentration may rise in colorectal adenoma (Jacobs et al., 2011), and FGF23 excretion is enhanced in the stool from patients with colorectal carcinoma (Wang H.-P. et al., 2014). In urothelial carcinoma, an increase in the FGF23 plasma concentration is reported (Li et al., 2019). In patients with prolactinoma, the FGF23 plasma concentration is unaltered, and there is only a minor effect of FGF23 on bone loss in these patients, if any (Arslan et al., 2017). Progression of hepatocellular carcinoma (HCC) is not linked to altered FGF23 expression (Zou et al., 2018).

It is important to keep in mind that most of the aforementioned studies on FGF23 and different types of cancer report associations, not necessarily causative relationships.

αKlotho SIGNALING PATHWAYS RELEVANT FOR CANCER

The development of cancer, its progression, and metastasis are a complex process. Initially, cells are exposed to harmful genetic or epigenetic alterations resulting in dysregulated signaling pathways. Subsequently, the modified cells escape homeostatic checks and elimination (Sever and Brugge, 2015). Typical dysregulated pathways in cancer include IGF-1R, PI3K/AKT1/mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK)/ERK, glycogen synthase kinase-3β (GSK-3β), or Wnt/β-catenin signaling. Many of them are controlled by KL (Sopjani et al., 2015; Badve and Kumar, 2019). Moreover, aging is a major driver of cancer (Aunan et al., 2017). Also in view of rapid aging of Kl-deficient mice (Kuro-O et al., 1997), it is intriguing to speculate that KL signaling in many tissues is implicated in cancer development and may be a possible target in cancer prevention or therapy. The role of KL in different forms of cancer is summarized in **Table 2**.

THE ROLE OF αKlotho IN CANCER

Breast Cancer

In 2008, KL was revealed as a tumor suppressor in breast cancer (Wolf et al., 2008). According to this study, normal breast tissue exhibits higher KL expression than ductal carcinoma *in situ* or invasive ductal carcinoma. Also, in less-differentiated breast cancer cell lines, *KL* expression is lower than in the non-tumor breast cell line MCF-12A or in well-differentiated MCF-7 breast cancer cells. KL overexpression reduces, whereas RNAi-mediated KL down-regulation enhances breast cancer cell proliferation. *KL* overexpression activates the FGF pathway, whereas *KL* overexpression and sKL attenuate IGF-1R activation and its downstream targets AKT1, GSK-3β, and ERK1/2 (Wolf et al., 2008). *In vitro* and *ex vivo*, methylation of the *KL* promoter in breast cancer cells is negatively correlated with *KL* mRNA abundance, suggesting a role of epigenetic silencing of KL in breast cancer (Rubinek et al., 2012; Dallol et al., 2015). Also dietary methyltransferase inhibition with green tea polyphenols and histone deacetylase inhibition with sulforaphane up-regulate epigenetically silenced KL in breast cancer cells (Sinha et al., 2015). sKL may exert further antitumor effects in breast cancer by regulating endoplasmic reticulum (ER) Ca^{2+} storage, as well as inner mitochondrial membrane potential and Ca^{2+} transport (Shmulevich et al., 2020). Heterozygosity for a certain *KL* gene

TABLE 2 | Associations of KL with cancer.

Cancer	KL level	Mechanism of KL change	KL effect	References
Breast cancer	↓ Cancer tissues ↓ Cell lines	Epigenetic silencing; *KL* variant	↓ Cell proliferation ⊣ IGF-1R/AKT/GSK-3β and ERK1/2 ↑ FGF pathway → Regulation of endoplasmic reticulum Ca^{2+} storage	Wolf et al., 2008, 2010; Rubinek et al., 2012; Dallol et al., 2015; Sinha et al., 2015; Shmulevich et al., 2020
Colorectal cancer	↓ Cancer tissues ↓ Cell lines	Epigenetic silencing; *miR-15b*; NF-κB and IGF-1R activity; *KL* variant	↓ Cell survival; proliferation ↓ Tumor growth; weight; volume ↑ Cell cycle arrest ↑ Apoptosis ⊣ IGF-1R/PI3K/AKT ⊣ ERK and HIF-1α ⊣ NF-κB ⊣ Wnt3a/β-catenin signaling	Gan et al., 2011; Pan et al., 2011; Li et al., 2014, 2016, 2018; Yang et al., 2014; Bordonaro and Lazarova, 2015; Perveez et al., 2015; Arbel Rubinstein et al., 2019; Liu et al., 2019; Xie et al., 2019, 2020; Kamal et al., 2020; Son et al., 2020
Lung cancer	↓ Cancer tissues ↓ Cell lines	*miR-10b*; Ras8 activity	↓ Cell proliferation; growth; invasiveness; migration ↑ Apoptosis ⊣ IGF-1R/AKT ⊣ Wnt3a/β-catenin signaling ↓ IL-6 and IL-8 → Sensitizes for cisplatin via PI3K/AKT or autophagy	Chen et al., 2010, 2012, 2016, 2019; Wang X. et al., 2011, Wang et al., 2013; Huang et al., 2015; Chen B. et al., 2018
Hepatocellular cancer	↓ Cancer tissues ↓ Cell lines	Epigenetic silencing	↓ Colony formation; proliferation; migration; invasion ↑ Apoptosis; autophagy ⊣ Wnt/β-catenin signaling ⊣ IGF-1R/AKT/ERK ↑ VEGFR2/PAK1 → ↑ migration; invasion	Chen et al., 2013; Shu et al., 2013; Xie et al., 2013b; Sun et al., 2015; Tang et al., 2016b
Squamous cell carcinoma	↓ Cancer tissues	Epigenetic silencing	⊣ *N*-cadherin → Regulation of EMT	Adhikari et al., 2017; Ibi et al., 2017
Pancreatic cancer	↓ Cancer tissues ↓ Cell lines	Epigenetic silencing; *miR-199a*	↓ Colony size and number; tumor growth ↑ Chemotherapeutic effects ⊣ IGF-1R/AKT/ERK1/2 ⊣ mTOR ⊣ FGF2	Abramovitz et al., 2011; Jiang et al., 2014; Zhang et al., 2020
Gastric carcinoma	↓ Cancer tissues ↓ Cell lines	Epigenetic silencing; *miR-199a*	↓ Growth ⊣ IGF-1R/PI3K/mTOR ⊣ ERK1/2 ↑ Apoptosis	Wang L. et al., 2011; Xie et al., 2013a; He et al., 2014
Prostate cancer	↓ Cell lines	Epigenetic silencing; *KL* SNP		Kim et al., 2014b; Seo et al., 2017
Renal cell carcinoma	↓ Cancer tissues ↓ Cell lines		↓ Cell proliferation; migration; invasion; motility; EMT ⊣ IGF-1R ⊣ PI3K/AKT/GSK-3β/Snail ⊣ EGF-1 dependent p38MAPK activation	Zhu et al., 2013; Gigante et al., 2015; Kim et al., 2016; Dehghani et al., 2018
Ovarian cancer	↓ Cancer tissues ↓ Cell lines		↓ Cell proliferation ↓ Tumor growth and tumor-associated inflammation ⊣ IGF-1/ERK1/2	Lojkin et al., 2015; Yan et al., 2017
Melanoma	↓ Aged cells	PPARγ; HMGB1 and NF-κB activity	↓ Cell motility ⊣ Wnt5a-mediated filamin A cleavage	Camilli et al., 2011; Xie et al., 2016; Behera et al., 2017
Thyroid cancer	↓ Cancer tissues		↓ Cell proliferation ↑ Apoptosis ⊣ Stanniocalcin-1	Dai et al., 2016; Pawlikowski et al., 2019
Urothelial carcinoma of the bladder	↓ High- grade cancer tissues			Hori et al., 2016

(Continued)

TABLE 2 | Continued

Cancer	KL level	Mechanism of KL change	KL effect	References
Glioblastoma multiforme		Epigenetic silencing	↓ Cell viability	Peshes-Yeloz et al., 2019
Cervical carcinoma	↓ Cancer tissues ↓ Cell lines	Epigenetic silencing	↓ EMT ⊣ Wnt/β-catenin signaling	Lee et al., 2010; Chang et al., 2012
Dedifferentiated liposarcoma	↓ Cancer tissues		↓ Cell proliferation ↑ Apoptosis → Sensitizes to ER stress ⊣ IGF-1–induced Ca^{2+} and ERK1/2 signaling	Delcroix et al., 2018
T-cell lymphoma and diffuse large B-cell lymphoma	↓ Cancer tissues ↓ Cell lines		↓ Cell proliferation ↑ Apoptosis ⊣ IGF-1R/AKT/ERK1/2	Zhou et al., 2017a,b

variant (KL-VS) is associated with an even higher breast cancer risk of patients with *BRCA1* mutation prone to developing breast cancer (Wolf et al., 2010).

Colorectal Cancer

Epigenetic silencing through *KL* promoter hypermethylation is observed in different colon cancer cell lines (Pan et al., 2011). Also, in human colorectal cancer (CRC) specimens, *KL* promoter methylation with reduced *KL* mRNA is frequent (Gan et al., 2011; Pan et al., 2011; Li et al., 2014; Yang et al., 2014; Perveez et al., 2015; Arbel Rubinstein et al., 2019; Liu et al., 2019; Son et al., 2020). According to some studies, methylation status and reduced *KL* expression are independent of age, gender, TNM stage, histological grade, or tumor differentiation (Pan et al., 2011; Yang et al., 2014; Perveez et al., 2015). Others found an association of KL expression with decreased survival of CRC patients (Liu et al., 2019) or TNM stage, invasiveness, and lymph node metastasis (Li et al., 2016; Arbel Rubinstein et al., 2019). Moreover, a recent study observed an association between *KL* variants and an increased risk of CRC (Kamal et al., 2020). Overexpression of *KL* or *KL1* fragment or treatment with sKL decreases surviving colonies and cell proliferation and induces cell cycle arrest and apoptosis of colon cancer cells (Pan et al., 2011; Arbel Rubinstein et al., 2019). Mice colon cancer cells transfected with KL exhibit lower tumor growth, weight, and volume (Li et al., 2014). The same holds true after treatment with sKL1 (Arbel Rubinstein et al., 2019). Similar to breast cancer, KL might be tumor-suppressing by inhibiting IGF-1R–dependent PI3K/AKT signaling (Li et al., 2014) or aerobic glycolysis via ERK/hypoxia-inducible factor 1α (HIF-1α) (Li et al., 2018) in CRC. Also, down-regulation of Wnt3a/β-catenin signaling and apoptosis are induced by KL in CRC cells (Bordonaro and Lazarova, 2015; Arbel Rubinstein et al., 2019; Xie et al., 2020). *miR-15b* may contribute to reduced KL expression in CRC because higher *miR-15b* levels in CRC patients compared to healthy subjects, those with metastasis than without, and those with cancer recurrence than without are described (Li et al., 2016). In CRC cells, inflammation-inherent nuclear factor κB (NF-κB) and IGF-1R activity further lowers KL expression, increasing cell proliferation and invasion (Xie et al., 2019). Conversely, KL blocks NF-κB activation (Liu et al., 2019).

Lung Cancer

KL is down-regulated in lung cancer cells and tissues and even more so in chemotherapy-resistant lung cancer (Chen et al., 2012, 2016; Chen B. et al., 2018). KL inhibits lung cancer cell proliferation, growth, invasiveness, and migration and fosters apoptosis (Chen et al., 2010, 2012, 2016, 2019; Wang X. et al., 2011; Wang et al., 2013), effects, at least in part, dependent on IGF-1R/AKT (Chen et al., 2010; Wang et al., 2013) and Wnt3a/β-catenin signaling (Chen et al., 2012, 2019) and on reduced interleukin 6 (IL-6) and IL-8 production (Chen B. et al., 2018). *MiR-10b* lowers, Ras-related GTPase Ras8 up-regulates KL expression in non–small-cell lung cancer cells (Huang et al., 2015). Patients with large-cell neuroendocrine lung carcinoma or small-cell lung cancer with KL expression have better outcome than those without KL expression pointing to KL being a potential biomarker (Usuda et al., 2011a; Vanoirbeek et al., 2011; Brominska et al., 2019). This could not be confirmed for sKL in lung cancer (Pako et al., 2020). KL may sensitize lung cancer cells to apoptosis induction by cisplatin via PI3K/AKT signaling (Wang et al., 2013) or due to decreased autophagy (Chen et al., 2016).

Hepatocellular Cancer

HCC cells and HCC tissue exhibit reduced KL expression (Shu et al., 2013; Xie et al., 2013b; Sun et al., 2015; Tang et al., 2016b), a phenomenon again explained by epigenetic silencing of the *KL* promoter through hypermethylation and acetylation (Xie et al., 2013b). *KL* promoter methylation is associated with a poorer prognosis (Xie et al., 2013b), whereas KL expression is inversely related to histological grade and clinical stage in HCC (Tang et al., 2016b). *KL* overexpression or treatment with recombinant KL or sKL decreases colony formation, cell proliferation, migration, and tumor invasion while inducing apoptosis and autophagy through inhibition of Wnt/β-catenin (Sun et al., 2015; Tang et al., 2016b) and IGF-1R/AKT/ERK signaling (Shu et al., 2013). According to another study, however, KL activates vascular endothelial growth factor receptor 2/p21-activated kinase 1, resulting in cell death resistance and favoring tumor migration and invasion (Chen et al., 2013). Thus, higher KL expression is associated with cirrhosis, venous invasion, tumor multiplicity, and a lower

overall survival in HCC patients according to this study (Chen et al., 2013).

Squamous Cell Carcinoma

Lower KL and higher DNA methyltransferase 3a (enzyme required for epigenetic alteration of *KL* promoter activity) are typical of the transition from normal tissue to oral dysplastic lesions to oral squamous cell carcinoma (SCC) (Adhikari et al., 2017). *KL* promoter methylation may predict survival prognosis in head and neck SCC with conflicting results (Alsofyani et al., 2017; Zhu et al., 2019). Higher *KL* gene expression is again associated with better survival, and *KL* methylation with gender, tumor grade, and site (Zhu et al., 2019). Survival of patients with esophageal SCC is better if the tumor expresses KL (Tang et al., 2016a). Moreover, KL expression is inversely correlated with invasion depth, histological grade, clinical stage, and lymph node metastasis in esophageal SCC (Tang et al., 2016a). In lung SCC, KL expression is associated with invasiveness (Ibi et al., 2017). KL inhibits *N*-cadherin and regulates epithelial–mesenchymal transition (EMT) (Ibi et al., 2017). Also, in cervix SCC, KL is reduced (Aviel-Ronen et al., 2016).

Pancreatic Cancer

Pancreatic adenocarcinoma tissue or human pancreatic adenocarcinoma cell lines Panc1, MiaPaCa2, and Colo357 are characterized by reduced KL expression compared to normal pancreatic tissue (Abramovitz et al., 2011). Epigenetic silencing due to a hemimethylated *KL* promoter may account for this (Abramovitz et al., 2011). Overexpression of KL or recombinant sKL reduce survival and size of the cancer cell colonies and potentiates chemotherapeutic effects (Abramovitz et al., 2011). They inhibit IGF-1R and its downstream signaling effectors IRS-1, AKT1, and ERK1/2 as well as FGF2 pathway activation (Abramovitz et al., 2011). sKL injection also reduces tumor growth in mice (Abramovitz et al., 2011). KL expression is positively, p-IGF-1R abundance negatively, correlated with lower TNM stage and pathological grade (Jiang et al., 2014). Higher methylation of the *KL* promoter in pancreatic ductal adenocarcinoma compared to normal pancreatic tissue worsens outcome (Jiang et al., 2014). *miR-199a* lowers KL expression in pancreatic adenocarcinoma Panc1 cells (Zhang et al., 2020). KL inhibits mTOR as downstream target of AKT1 and MEK/ERK signaling in Panc1 cells (Zhang et al., 2020).

Gastric Carcinoma

KL promoter hypermethylation with decreased gene expression is typical of gastric carcinomas and gastric carcinoma cell lines (Wang L. et al., 2011). KL overexpression inhibits growth and ERK1/2 activity, resulting in apoptosis of AGS and MKN28 gastric carcinoma cells (Wang L. et al., 2011). Promoter hypermethylation correlates with poorer survival of patients with gastric cancer, making it an independent prognosis factor (Wang L. et al., 2011). Restoration of KL expression reduces p-IGF-1R, p-PI3K, and p-mTOR in GC-7901 cells (Xie et al., 2013a). Similar to pancreatic cancer (Zhang et al., 2020), *miR-199a* influences KL expression in gastric cancer (He et al., 2014). The human sex determining region Y (SRY)–related high-mobility-group (HMG) box protein family member 17 (SOX17) protein also binds to the *KL* promoter in gastric cancer cells, thereby inducing KL expression (Yang et al., 2020).

Prostate Cancer

A KL single-nucleotide polymorphism (*rs3752472*) is associated with the risk of prostate cancer (odds ratio = 1.85) (Kim et al., 2014b). Methylation in the *KL* CpG island region KL-M3, including −593 to −406 bp, accounts for the down-regulation of *KL* mRNA in prostate cancer cell lines DU145 and PC-3 (Seo et al., 2017). The same region is unmethylated in 22Rv1 prostate cancer cells exhibiting *KL* mRNA expression (Seo et al., 2017). The *KL* promoter in 22Rv1 cells is hypomethylated, and in DU145 and PC-3 cells hypermethylated (Seo et al., 2017).

Renal Cell Carcinoma

In renal cell carcinoma (RCC) tissue and cell lines, KL protein and mRNA expression are reduced (Zhu et al., 2013; Gigante et al., 2015; Kim et al., 2016; Dehghani et al., 2018). KL expression is negatively associated with TNM stage, tumor size, shorter overall and progression-free survival (Zhu et al., 2013; Gigante et al., 2015). *KL* overexpression in RCC cells down-regulates PI3K/AKT/GSK3-β/Snail signaling, thereby inhibiting cell migration, invasion, and EMT (Zhu et al., 2013). Moreover, KL inhibits epidermal growth factor 1–dependent p38MAPK activation and IGF-1R signaling in Caki-1 cells compromising cell motility and proliferation (Zhu et al., 2013; Kim et al., 2016; Dehghani et al., 2018).

Ovarian Cancer

Results regarding the role of KL in ovarian cancer are controversial. According to a clinical study of 189 EOC patients, 73.5% of patients exhibit detectable KL expression. sKL is associated with high tumor grade, suboptimal tumor debulking results, disease progression [hazard ratio (HR) = 1.97], and death (HR = 2.09), possibly due to KL supporting the tumor with energy and angiogenesis (Lu et al., 2008). Others found reduced KL expression in different human EOC cell lines and specimens, as well as inhibition of proliferation of different EOC cell lines upon sKL treatment or KL overexpression (Lojkin et al., 2015; Yan et al., 2017). KL suppresses IGF-1–induced ERK 1/2 phosphorylation in OVCA-432 and SKOV-3 cells (Lojkin et al., 2015). KL expression is lower in ovarian cancer and is associated with decreased survival (Yan et al., 2017). In mice, KL-expressing A2780 tumor cells grow more slowly than KL-negative tumor cells (Yan et al., 2017). KL suppresses a tumor-associated inflammatory response in mice with ovarian cancer, thereby contributing to a more favorable outcome (Yan et al., 2017).

Melanoma

In different melanoma cell lines, a mutually inhibitory effect of Wnt5a and KL expression is established impacting on metastasis (Camilli et al., 2011). The effect of KL on Wnt5a internalization and signaling is dependent on heparan sulfate proteoglycans (Camilli et al., 2011). Moreover, KL inhibits Wnt5a-mediated

filamin A cleavage through calpain, an effect contributing to reduced motility of melanoma cell lines (Camilli et al., 2011). Melanoma cells exhibit *KL* expression, depending on the age of surrounding fibroblasts (Behera et al., 2017). Older patients' melanoma cells show lower KL expression (Behera et al., 2017). Treatment of melanoma cells with media of aged fibroblasts results in increased Wnt5a expression and less *KL* mRNA expression, compared to incubation with media of young fibroblasts (Behera et al., 2017). KL expression in melanoma cells is enhanced by PPARγ, and KL or PPARγ agonist rosiglitazone treatment reduce melanoma growth in mice (Behera et al., 2017). HMG protein B1 (HMGB1) activates NF-κB and inhibits KL expression melanoma cell lines (Xie et al., 2016).

Thyroid Cancer

KL overexpression and sKL induce apoptosis and compromise proliferation of thyroid cancer cell lines FTC133 and FTC238, an effect presumably dependent on stanniocalcin-1 (Dai et al., 2016). Low differentiation is paralleled by reduced KL expression in human thyroid cancer (Pawlikowski et al., 2019).

OTHER FORMS OF CANCER

KL is a possible tumor suppressor in urothelial carcinoma of the bladder (Hori et al., 2016, 2018). KL expression in glioblastoma multiforme (GBM) correlates with outcome (Trošt et al., 2016; Peshes-Yeloz et al., 2019). sKL decreases viability of GBM cell lines, and reduced KL expression is due to epigenetic *KL* promoter methylation in these cells (Peshes-Yeloz et al., 2019). Similar epigenetic mechanisms of down-regulation of *KL* expression are effective in human specimens of invasive cervical carcinoma and cell lines (Lee et al., 2010). Secreted KL acts as a tumor suppressor in CaSki cervical carcinoma cells by inhibiting canonical Wnt signaling and *c-MYC* and *Cyclin D1* expression (Lee et al., 2010). *KL* overexpression in SiHa cells down-regulates

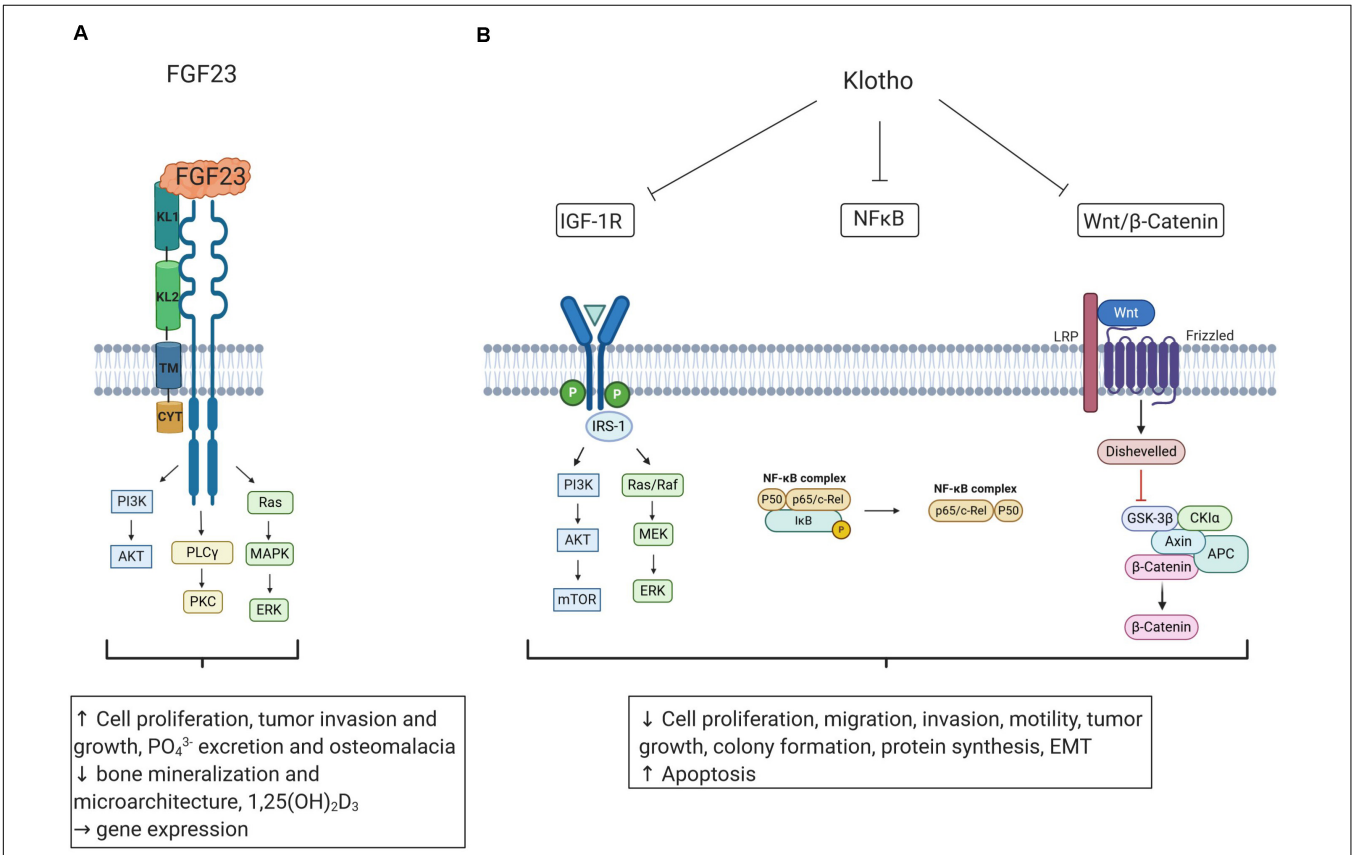

FIGURE 2 | The influence of FGF23 and Klotho on oncogenic and tumor-suppressing pathways. **(A)** FGF23 binds to FGFRs and coreceptor KL and may impact cell proliferation, tumor growth, and bone gene transcription. Tumor-induced elevation of FGF23 production may cause phosphate wasting, $1,25(OH)_2D_3$ reduction, and osteomalacia. **(B)** Klotho (KL) is a tumor-suppressor inhibiting pathways relevant for tumorigenesis including IGF-1R, Wnt/β-catenin, and NF-κB signaling, resulting in decreased cell proliferation, invasion, migration, tumor growth, protein synthesis, and EMT and inducing apoptosis. Figure according to Sachdeva et al. (2020). Created with BioRender.com. Insulin-like growth factor 1 receptor (IGF-1R); phosphoinositide 3-kinase (PI3K); mammalian target of rapamycin (mTOR); mitogen-activated protein kinase (MAPK); mitogen-activated protein kinase kinase (MEK); extracellular receptor signal-related kinase (ERK); insulin receptor substrate 1 (IRS-1); nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB); iκappaB kinase (IκB); low density lipoprotein receptor-related protein (LRP); glycogen synthase kinase-3β (GSK-3β); adenomatous polyposis coli (APC); phospholipase Cγ (PLCγ), protein kinase C (PKC); epithelial to mesenchymal transition (EMT); phosphate (PO_4^{3-}), $1,25(OH)_2D_3$ (active vitamin D).

β-catenin, *c-MYC*, and *cyclin D1* signaling, as well as EMT (Lee et al., 2010; Chang et al., 2012).

KL expression correlates with overall survival and is lower in dedifferentiated liposarcoma (DDLPS) than in adipose tissue (Delcroix et al., 2018). *KL*-overexpressing DDLPS blunts IGF-1–induced Ca^{2+} and ERK1/2 signaling, reducing proliferation, inducing apoptosis, and sensitizing cells to ER stress (Delcroix et al., 2018).

Also in T-cell lymphoma and diffuse large B-cell lymphoma (DLBLC), KL overexpression attenuates IGF-1R, ERK1/2 and AKT signaling (Zhou et al., 2017a,b). Moreover, in biopsies and cell lines of T-cell lymphoma and DLBLC, KL expression is reduced correlating with shorter survival. *KL* overexpression in T-cell lymphoma and DLBLC cell lines lowers proliferation and enhances apoptosis (Zhou et al., 2017a,b).

FGF23/KL AND THE CANCER MICROENVIRONMENT

As summarized in **Figure 2**, KL is a potent regulator of IGF-1R and Wnt/β-catenin signaling, and these pathways are highly relevant for the cancer microenvironment (Huang and Du, 2008; Sanchez-Lopez et al., 2016). Local hypoxia is typical of advanced cancers activating HIF-1 (Petrova et al., 2018). KL inhibits HIF-1α in CRC (Li et al., 2018). Conversely, HIF-1α increases ectopic FGF23 expression in patients with TIO (Zhang et al., 2016). Hypoxia fosters accumulation of tumor-associated macrophages in the tumor microenvironment and mediates inflammation (Lewis and Murdoch, 2005). Interestingly, cultured macrophages express FGF23, which up-regulates cell number and their tumor necrosis factor α expression (Masuda et al., 2015; Han et al., 2016). Thus, FGF23 production and local inflammation may be interdependent in the microenvironment of the tumor depending on hypoxia, HIF-1α activation, and tumor-associated macrophages. Furthermore, FGF23 possibly contributes to a bone-like microenvironment in phosphaturic mesenchymal tumor, mixed connective tissue variant (PMTMCT), through FGFR1c/KL, inducing enhanced FGF23 production by the tumor cells and worsening TIO (Kinoshita et al., 2019). Clearly, further studies are warranted to address this important issue.

FGF23/KL, PHOSPHATE HOMEOSTASIS, AND CANCER

FGF23/FGFR/KL regulate renal phosphate handling (Gattineni et al., 2009). Moreover, FGF23 indirectly impacts on phosphate by inhibiting $1,25(OH)_2D_3$ formation (Chanakul et al., 2013) and by affecting PTH (Krajisnik et al., 2007; Kawakami et al., 2017). Hence, FGF23/KL have a central role in the interaction of bone, kidney, small intestine, and parathyroid gland, maintaining phosphate homeostasis (Razzaque, 2009b). Serum phosphate levels are higher in patients with cancer than in healthy individuals (Papaloucas et al., 2014). Higher phosphate concentrations in men are related to a higher overall cancer risk (Wulaningsih et al., 2013), and higher phosphate intake accelerates tumorigenesis in mice (Lee et al., 2015), uncovering phosphate as a possible factor in cancer (Brown and Razzaque, 2018). Accordingly, CKD patients, often exhibiting hyperphosphatemia and $1,25(OH)_2D_3$ deficiency, have an increased risk of cancer (Wong et al., 2009, 2016; Park et al., 2019). $1,25(OH)_2D_3$ may have anti-cancer activity (Vanoirbeek et al., 2011). According to Brown's hypothesis, hyperphosphatemia is an important factor in tumorigenesis and at the same time causes an endocrine reduction of $1,25(OH)_2D_3$, which in turn is associated with an increased risk of cancer (Brown, 2019). For this hypothesis, FGF23/KL plays an important role due to its pivotal function in phosphate handling. Definitely, further research on pathological derangements of phosphate homeostasis is warranted to uncover the relationship between FGF23/KL dysregulation, disturbed phosphate homeostasis, and cancer development.

CONCLUSION

KL seems to be an universal tumor suppressor in many different tumor entities owing to its inhibitory effect on pro-survival intracellular pathways including IGF-1R/PI3K/AKT or Wnt signaling. Often, cell culture studies revealed similar actions of sKL and overexpression of transmembrane KL in different types of cancer. Whether targeting KL can be therapeutically exploited in cancer must be investigated in future trials. In most studies and types of cancer, higher abundance of sKL is associated with a more favorable prognosis, presumably due to its down-regulatory effect on major prosurvival signaling cascades required for cancer progression. The investigations into the role of FGF23 in cancer have so far revealed two important aspects in general: In those forms of cancer affecting bone or originating from it such as MM or prostate cancer, FGF23 signaling may directly contribute to cancer biology/progression. In many other tumor entities, the biological role of an elevation of the plasma FGF23 concentration is still enigmatic, but FGF23 may serve as a (tumor) biomarker. In TIO, treatment with anti-FGF23 monoclonal antibody offers a beneficial therapeutic intervention. In other malignancies affecting bone including prostate cancer or MM, an anti-FGF23 approach may also be useful as enhanced FGF23 or FGF23 signaling is typical of these tumor entities. Clearly, this and the role of FGF23-dependent phosphate metabolism in cancer require further studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

MFö's work in the field of FGF23 was supported by the Deutsche Forschungsgemeinschaft (Fo 695/2-2 and Fo 695/6-1).

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 22 January 2021
doi: 10.3389/fcell.2020.621070

Determinants and Functions of CAFs Secretome During Cancer Progression and Therapy

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Multiple lines of evidence are indicating that cancer development and malignant progression are not exclusively epithelial cancer cell-autonomous processes but may also depend on crosstalk with the surrounding tumor microenvironment (TME). Cancer-associated fibroblasts (CAFs) are abundantly represented in the TME and are continuously interacting with cancer cells. CAFs are regulating key mechanisms during progression to metastasis and response to treatment by enhancing cancer cells survival and aggressiveness. The latest advances in CAFs biology are pointing to CAFs-secreted factors as druggable targets and companion tools for cancer diagnosis and prognosis. Especially, extensive research conducted in the recent years has underscored the potential of several cytokines as actionable biomarkers that are currently evaluated in the clinical setting. In this review, we explore the current understanding of CAFs secretome determinants and functions to discuss their clinical implication in oncology.

Keywords: cancer-associated fibroblast (CAF), secretome, secreted factors, growth factors, cancer, tumor microenvironment (TME), metastasis, therapy

OPEN ACCESS

Edited by:
Marzia Di Donato, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Ferdinando Auricchio, Second University of Naples, Italy
Paolo Paoli, University of Florence, Italy
Fernando Calvo, Consejo Superior de Investigaciones Científicas (CSIC), Spain

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

Received: *24 October 2020*
Accepted: *07 December 2020*
Published: *22 January 2021*

Citation:
Linares J, Marín-Jiménez JA, Badia-Ramentol J and Calon A (2021) Determinants and Functions of CAFs Secretome During Cancer Progression and Therapy. Front. Cell Dev. Biol. 8:621070. doi: 10.3389/fcell.2020.621070

INTRODUCTION

Cancer-associated fibroblasts (CAFs) are contributing to the production of a wide variety of secreted factors impacting tumor progression by directly regulating malignant cancer cells aggressiveness or by indirectly reprogramming tumor immunity and angiogenesis (Sahai et al., 2020). Hence, molecular and functional inter- and intra-tumoral heterogeneity of CAFs has been a recent focus in oncology research. It is widely accepted that the functional phenotype of CAFs is in part determined by the cell of origin, including but not restricted to local resident fibroblasts (D'Arcangelo et al., 2020; Sahai et al., 2020). An alternative hypothesis advocating for CAF being a cell state depending on autocrine and paracrine signaling rather than a cell type has also been proposed (Kalluri, 2016). Whether CAFs functional heterogeneity is maintained among different solid tumor types or is a constant evolutionary state is still a debated question. However, recent studies have been investigating the determinants of CAFs secretome and their therapeutic interest across different tumor types. Molecular biomarkers predicting the risk of relapse and the potential benefit from treatments are currently needed for clinical decision-making. In an attempt to reach a more comprehensive evaluation of tumors, many CAFs-secreted factors have been included in gene expression signatures that are considered suitable prognostic tools for clinical diagnosis and prognostication (Berdiel-Acer et al., 2014; Karlan et al., 2014; Yue et al., 2019). As a matter of fact, recent advances in the understanding of CAFs secretome determinants and functions have brought to light the multiple benefits of using CAFs-secreted factors as actionable biomarkers for cancer diagnosis, treatment, and prognosis.

DETERMINANTS OF CAFS SECRETOME

Crosstalk With Tumor Cells and TME Components

Autocrine- and Paracrine-Secreted Factors

Multiple autocrine loops impacting CAFs secretome have been discovered in the recent years (**Figure 1**). Among them, members of the transforming growth factor beta (TGF-beta) superfamily are known to be the main inducers of CAFs activation. Of note, CAFs secrete large amounts of TGF-beta isoforms 1, 2, and 3 (reviewed in Kalluri, 2016). In turn, secreted TGF-beta maintains a self-sustained active state -typically regarded as myofibroblasts- characterized by alpha smooth muscle actin (alpha-SMA) expression (Orimo et al., 2005; Kojima et al., 2010). Other members of the TGF-beta superfamily, such as Nodal, induce pro-tumorigenic phenotypes in fibroblasts from melanoma and colorectal cancer (CRC) (Li et al., 2019). Likewise, activin A has showed the ability to induce a secretory phenotype in CAFs via the SMAD-2-mediated transcriptional regulation of genes encoding extracellular matrix (ECM) components, ECM regulators, and soluble factors (Cangkrama et al., 2020). Alternatively, CAFs secretome may be maintained during tumor progression through enhanced stromal cell-derived factor (SDF)-1 autocrine signaling loops and increased co-expression of receptors, such as TGF-beta-RI and CXC receptor (CXCR)-4 (Kojima et al., 2010). In this sense, Scherz-Shouval et al. reported an increased production of TGF-beta-2 and SDF-1 factors by CAFs upon heat shock factor (HSF)-1 cytoplasmic translocation to the nucleus (Scherz-Shouval et al., 2014). HSF-1 is a transcription factor that mediates the cellular response to different types of stress, such as hypoxia or proteotoxic stress (Dayalan Naidu and Dinkova-Kostova, 2017).

Complex paracrine signaling through cancer cell-secreted factors also regulates CAFs secretome during tumor progression (**Figure 1**) and may depend on genetic alterations occurring in cancer cells. Indeed, KRAS mutant but not KRAS wild-type pancreatic ductal adenocarcinoma (PDAC) cancer cells induce CAFs activation through CXCR-2, leading to a nuclear factor-kappa B (NF-κB)-mediated secretion of pro-tumoral cytokines interleukin (IL)-4, IL-10, and IL-13 (Awaji et al., 2020). Several additional studies have reported a pro-tumorigenic crosstalk between cancer cells and CAFs. For instance, kallikrein (KLK)-4 produced by malignant and premalignant prostate lesions can act on normal fibroblasts through the activation of protease-activated receptor (PAR)-1, which leads to a CAF pro-angiogenic secretory phenotype characterized by the increased expression of Dickkopf-related protein 1 (DKK-1), growth differentiation factor 15 (GDF-15), hepatocyte growth factor (HGF), IL-8, and vascular endothelial growth factor (VEGF) and the decreased expression of insulin-like growth factor-binding protein (IGFBP)-3, monocyte chemoattractant protein 1 (MCP-1), and platelet-derived growth factor (PDGF)-AA (Kryza et al., 2017). In PDAC, S100 calcium-binding protein A11 (S100A11) secreted by cancer cells activates the surrounding fibroblasts through the S100A11–receptor for advanced glycation end products (RAGE)–tumor progression locus 2 (TPL2)–cyclooxygenase 2 (COX2) pathway to promote metastatic progression (Mitsui et al., 2019).

CAFs activation may also occur through paracrine signaling with non-cancer cells. For example, cytokines produced by immune cells during the inflammatory process may instruct CAFs function. Among them, IL-1β produced by immune cells in early hyperplastic lesions activates normal fibroblasts to become CAFs *via* the NF-κB pathway (Erez et al., 2010). Alternatively, granulin secretion by macrophages activates resident fibroblasts into tumor-promoting myofibroblasts sustaining metastatic growth in PDAC (Nielsen et al., 2016).

In addition to local intra-tumoral autocrine and paracrine signaling, systemic signaling involving steroid hormones, such as estrogens and androgens, is also able to modulate CAFs secretome, through binding to their receptors expressed in CAFs (Clocchiatti et al., 2018; Rothenberger et al., 2018). For instance, estrogens (E2) regulate the expression of several microRNAs (miRNAs) in breast cancer (BC)-derived CAFs (Vivacqua et al., 2019). In gastric cancer, estrogens stimulate CAFs secretion of IL-6, thereby promoting signal transducer and activator of transcription (STAT)-3 pathway-dependent cancer cells proliferation and invasion (Zhang Y. et al., 2020). Similarly, in prostate cancer (PCa), the activation of CAFs–androgen receptor (AR) with dihydrotestosterone modulates the secretion of pro-tumorigenic factors impacting cancer cell growth (Tanner et al., 2011). Conversely, AR blockade in CAFs decreases the expression of pro-tumorigenic factors, such as insulin-like growth factor (IGF)-1, fibroblast growth factor (FGF)-7, FGF-10, SDF-1, HGF, and TGF-beta 2 (Yu et al., 2013).

As illustrated by the opposite gene expression programs regulated upon FGF and TGF-beta pathway activation

Abbreviations: AKT, protein kinase B; ATRA, all-trans retinoic acid; CCL, C–C motif chemokine; COX2, cyclooxygenase 2; CXC, C–X–C motif chemokine; CXCL, CXC ligand; CXCR, CXC receptor; DKK-1, Dickkopf-related protein 1; FGF, fibroblast growth factor; GDF-15, growth differentiation factor 15; GM-CSF, granulocyte-macrophage colony-stimulating factor; HER3, epidermal growth factor receptor 3; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; HspA1A, heat shock 70 kDa protein 1A; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; IGF, insulin-like growth factor; IGF1R, IGF 1 receptor; IGFBP, insulin-like growth factor-binding protein; JAK, Janus kinase; JNK, Jun N-terminal kinase; KLK, kallikrein; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor; MMP, matrix metallopeptidase; mTOR, mammalian target of rapamycin; MYH-11, myosin 11; NF-κB, nuclear factor-kappa B; NRG, neuregulin; OXPHOS, oxidative phosphorylation system; P38 MAPK, p38 mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PDGFRB, platelet-derived growth factor receptor beta; PDPN, podoplanin; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; POSTN, periostin; PAR, protease-activated receptor; RAGE, receptor for advanced glycation end products; miRNA, microRNA; mRNA, messenger RNA; lncRNA, long non-coding RNA; S100A11, S100 calcium-binding protein A11; SATB-1, special AT-rich sequence-binding protein-1; SDF, stromal cell-derived factor; SFRP4, secreted frizzled-related protein 4;Shp, Src homology 2-containing protein tyrosine phosphatase; SMAD, acronym for a signal transducer family; SOCS, suppressor of cytokine signaling 1; STAT, signal transducer and activator of transcription; TAZ, transcriptional coactivator with PDZ-binding motif, also known as Wwtr1; TET, ten-eleven translocation enzyme; THBS, thrombospondin; TIMP, tissue inhibitors of MMP;TNC, tenascin C; TNF, tumor necrosis factor; TPL2, tumor progression locus 2; VCAM, vascular cell adhesion protein; VEGF, vascular endothelial growth factor; VEGFA, VEGF type A; YAP, yes-associated protein.

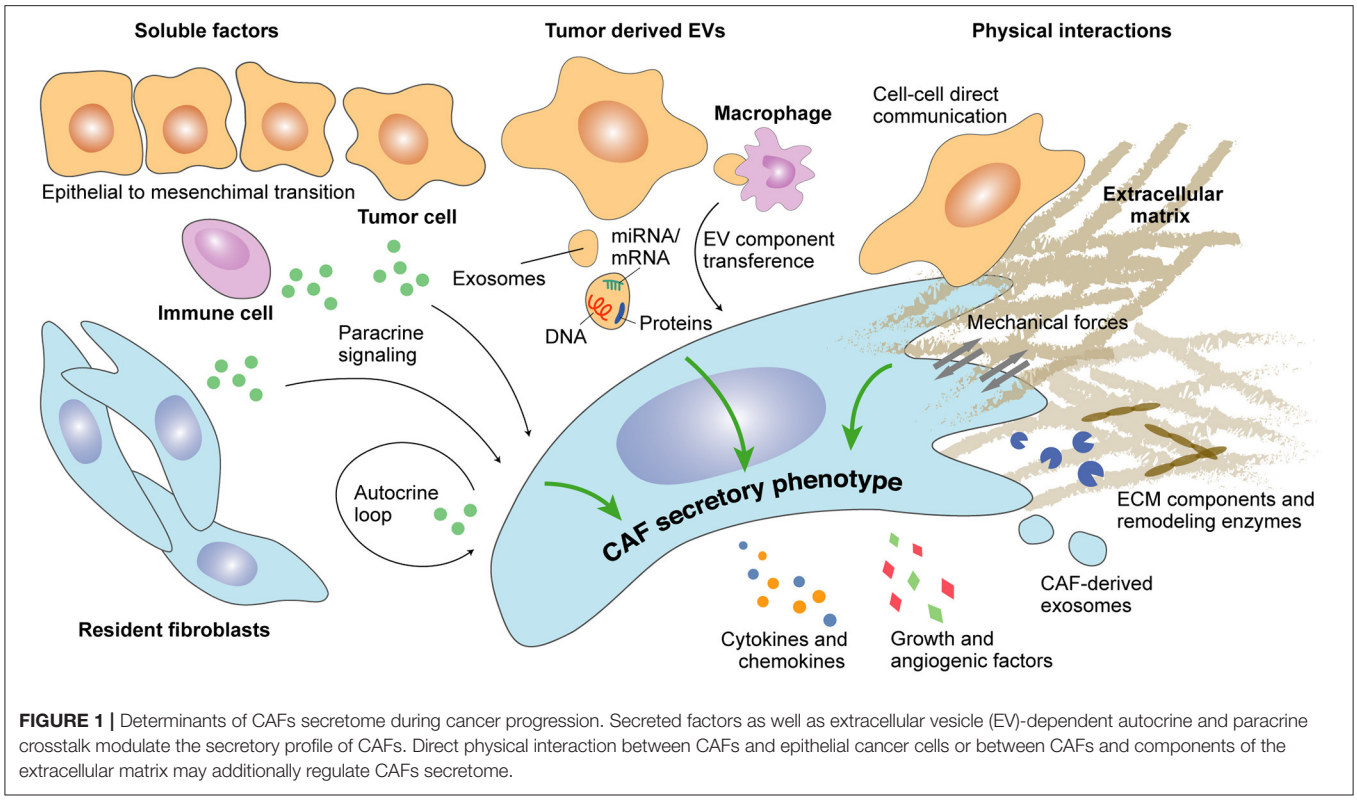

FIGURE 1 | Determinants of CAFs secretome during cancer progression. Secreted factors as well as extracellular vesicle (EV)-dependent autocrine and paracrine crosstalk modulate the secretory profile of CAFs. Direct physical interaction between CAFs and epithelial cancer cells or between CAFs and components of the extracellular matrix may additionally regulate CAFs secretome.

(Bordignon et al., 2019), CAFs secretome heterogeneity may be the result of a delicate balance between autocrine and paracrine signaling stimulated simultaneously and activating CAFs in different ways. It is worth noting that autocrine and paracrine triggering cues educating CAFs secretory functions may be tumor-dependent or even cancer subtype-dependent. Thus, the identification of multiple CAFs activation and secretion programs could greatly improve current molecular classification of cancer.

Extracellular Vesicles

Extracellular vesicles (EVs) are spherical membrane formations comprising exosomes and microvesicles, which can carry different molecules, such as proteins, DNAs, non-coding RNAs, and miRNAs/mRNAs. While both are playing a key role in distant intercellular communication, exosomes are derived from the endosomal system, and microvesicles are produced by the plasma membrane (reviewed in van Niel et al., 2018). EV-based intercellular communication between cancer cells and the tumor microenvironment (TME)–including CAFs (**Figure 1**)–promotes cancer progression in multiple ways (reviewed in Han et al., 2019); however, EVs' ability to induce a secretory phenotype in CAFs still remains an open field of research (Webber et al., 2015). In some cases, tumor cell-derived EVs contain typical mitogenic factors, such as TGF-beta, which in ovarian cancer induces a pro-tumoral secretome leading to increased proliferation, motility, and invasiveness of ovarian cancer cells (Giusti et al., 2018). Furthermore, an increasing body of evidence points to the importance of EV-derived miRNA. For instance, in gastric cancer, EVs containing miR155, miR193b, and miR210 prime CAFs to secrete inflammatory chemokines, such as CXC ligand (CXCL)-1 and CXCL-8, through the activation of the Janus kinase (JAK)/STAT and NF-κB signaling pathways (Naito et al., 2019). Similarly, NF-κB activation in CAFs by EV-derived miR-1247-3p in metastatic hepatocellular carcinoma (HCC) leads to a tumor-promoting secretion of IL-6 and IL-8 (Fang et al., 2018). Alternatively, EV-derived miR-210 and miR-155-5p induce a pro-angiogenic switch in CAFs through the activation of the JAK-2/STAT-3 pathway in lung cancer and melanoma (Zhou et al., 2018; Fan et al., 2020). Additional signaling pathways have been associated with miRNA-driven secretory stimulation. For instance, EVs containing miR-10b contribute to the enhanced TGF-beta expression in CAFs through the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway in CRC (Dai et al., 2018). Finally, EVs containing coding mRNA have also been reported to trigger a secretory phenotype in CAFs. In this sense, cancer cell-derived EVs containing mRNA coding for CXCR-4 and IGF-1R promote CAFs secretion of growth factors, such as VEGF in acute myeloid leukemia (Huan et al., 2013).

Interestingly, EV-induced CAFs may in turn shed additional EVs that will further support tumor growth by conferring enhanced proliferative and survival capabilities to cancer cells, thus establishing an EV-mediated bidirectional intercommunication (Savardashtaki et al., 2019; Yang et al., 2019). For instance, CAFs exposed to cancer cell-derived EVs in Hodgkin lymphoma are primed to secrete EVs together with pro-inflammatory cytokines and angiogenic factors (Dörsam

et al., 2018). Similar to cancer cell-derived EVs, CAF-derived EVs contain commonly miRNA, which has been described to promote migration and resistance to treatment in several tumors (Dourado et al., 2019; Hu et al., 2019; Qin et al., 2019; Sun et al., 2019; Wang J.-W. et al., 2019). Besides miRNA, CAF-derived EVs containing mitochondrial mRNA can educate cancer cells to increase oxidative phosphorylation system (OXPHOS) metabolism, which consequently induces escape from dormancy (Sansone et al., 2017).

EV-mediated activation of CAFs can also occur indirectly through stromal cell mediators present in the TME. Indeed, a recent study showed that tumor-associated macrophages incorporate and transfer cancer cell-derived EVs to CAFs, which allows the formation of a pro-tumorigenic microenvironment (Umakoshi et al., 2019).

Moreover, EVs content may be changing during tumor progression, which could result in a temporal modulation of CAFs secretory phenotypes. Supporting this notion, EVs derived from primary or metastatic CRC promote different CAF functional profiles, switching from a pro-angiogenic to a more ECM remodeling phenotype (Rai et al., 2019). Therefore, EV-mediated communication between tumor cells and CAFs may depend on alterations in the composition of secreted EVs during tumor progression. However, additional research is needed to confirm this hypothesis.

Cell–Cell Contact and Mechanical Interactions

CAFs/cancer cells physical interactions can modulate CAFs functions (Yamaguchi and Sakai, 2015), and the high complexity of this interplay has been recently illustrated (Arwert et al., 2020). For instance, CAFs may sense cancer cell genomic stress through cytoplasmic transcytosis and respond by expressing interferon (IFN)-β1, leading to an increased production of chemokines, cytokines, and other inflammatory factors [CXCL-1 and 10, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage migration inhibitory factor (MIF), IL-6, and IL-8, among others] (Arwert et al., 2020). Hetero-cellular gap junction communications have also been described between CAFs and cancer cells. This mechanism promoting epithelial-to-mesenchymal transition (EMT), migration, and invasiveness appears to be rather unidirectional—from CAFs toward cancer cell (Luo et al., 2018); however, a better understanding of the underlying molecular mechanisms is still required.

Mechano-sensing between CAFs and the surrounding ECM may equally modulate CAFs secretome. For example, matrix stiffness and contractile forces have been shown to determine CAFs behavior through different mechano-sensitive pathways. Among them, non-canonical YAP pathway activation is promoting CAFs ECM remodeling and angiogenic functions (Calvo et al., 2013). Indeed, secretion of pro-angiogenic VEGF-A by CAFs depends on factors involved in YAP pathway activation (Calvo et al., 2015).

These findings suggest a significant role of physical interactions in determining CAFs secretory phenotype. Remarkably, spatial (invasive front or tumor core) as well as temporal (early or late stage cancer) characteristics may influence mechano-sensing and should be considered when studying forces instructing CAFs secretory functions (Acerbi et al., 2015; Bauer et al., 2020).

Spatial and Temporal Plasticity

Intra-tumor heterogeneity of CAFs functional subtypes and their differential spatial pattern are already a widespread knowledge (Lambrechts et al., 2018; Awaji and Singh, 2019; Neuzillet et al., 2019). Thus, an effect of CAFs intra-tumoral location upon their secretory function may be expected. Remarkably, fibroblasts located next to the invasive front of BC show higher capacity to induce cancer cells migration and EMT in comparison with those located in the epicenter of the tumor (Gao et al., 2010). In pancreatic cancer, two spatially separated subtypes were identified (**Figure 2**). An inflammatory CAFs subtype (iCAFs), distant from cancer cells, showed a secretory phenotype with high interleukin and chemokine production [leukemia inhibitory factor (LIF), IL-6, IL-11, IL-1, CXCL-1] in comparison with periglandular myofibroblastic CAFs (myoCAFs), specialized in stromal remodeling functions (Öhlund et al., 2017). However, these two subtypes may be the borders of a functional spectrum depending on IL-1-R1 expression, conditioned by the balance between TGF-beta (pro-myoCAFs) and IL-1/JAK/STAT (pro-iCAFs) signaling activation (Biffi et al., 2019). An additional antigen-presenting CAFs (apCAFs) population activating CD4+ T cells was recently identified in PDAC (**Figure 2**), suggesting CAFs subtype-specific immunomodulatory capacity (Elyada et al., 2019). Along this line, Neuzillet and colleagues highlighted the complexity of CAFs heterogeneity in PDAC, describing at least four different functional and spatially distributed subtypes depending on periostin (POSTN), myosin 11 (MYH-11), and podoplanin (PDPN) expression (Neuzillet et al., 2019). Similarly, in BC (**Figure 2**), a subset of myofibroblastic CAFs (CAF-S1: CD-29^{Med} FAP^{Hi} FSP-1^{Low-Hi} αSMA^{Hi} PDGFRb^{Med-Hi} CAV-1^{Low}) secreting differentially higher amount of CCL-11, CXCL-12, CXCL-13, and CXCL-14 was predominantly detected close to epithelial tumor cells (Costa et al., 2018). The other myofibroblastic (αSMA^{Hi}) CAF-S4 subtype was preferentially located within the tumor tissue, whereas CAF-S3 (FSP-1^{Med} and PDGFRb^{Med-Hi}) was detected in the juxta tumoral healthy tissue. CAF-S2 subtype (low or negative expression of stromal markers) appeared to be equally distributed in both areas (Costa et al., 2018).

For greater complexity, temporal evolution of CAFs during tumor progression makes it difficult to assess the exclusive effect of spatial distribution (Kalluri, 2016; D'Arcangelo et al., 2020). Indeed, Nidogen-2, a protein secreted by "vascular CAFs" in murine BC model, was firstly detected among perivascular cells but relocated within the tumor stroma at later stages of tumor progression (Bartoschek et al., 2018). In PDAC patients, different phenotypes were detected by CAFs secretome analysis of primary tumors and matched metastatic tissue (Öhlund et al., 2017). In addition, the above-mentioned S1 and S4 CAFs subsets have been recently found to be enriched in metastatic BC lymph nodes in comparison with matched primary tumor tissue (Costa et al., 2018; Pelon et al., 2020). In this setting, CXCL-12 secretion was found to be responsible of CAF-S1

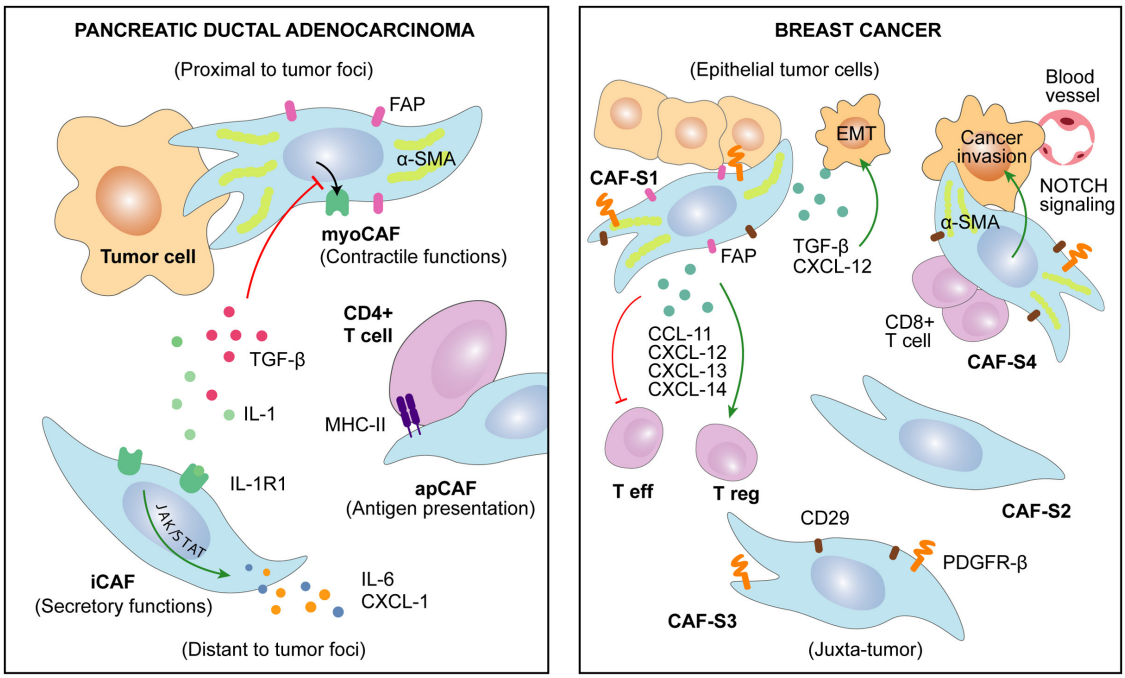

FIGURE 2 | Several CAFs subtypes have been recently described in cancers of distinct origin. CAFs heterogeneity is associated with specific markers and discrete functions. Remarkably, CAFs characteristics may be related to their spatial localization in the tumor.

tumor-promoting phenotype, whereas CAF-S4 seemed to be specialized in NOTCH-dependent contractile and remodeling functions (Pelon et al., 2020). Similarly, CAFs isolated from prostate tumors at different stages revealed that CAFs secretome evolves during PCa development. While localized PCa-derived CAFs are characterized by FGF-7 secretion, CAFs from metastatic PCa showed increased levels of matrix metallopeptidase (MMP)-11 and heat shock 70 kDa protein 1A (HspA1A) (Eiro et al., 2017).

As previously mentioned, increasing evidence suggests that PCa-derived CAFs secretome is modulated by AR signaling (Yu et al., 2013; Cioni et al., 2018). Interestingly, AR expression by CAFs evolves during PCa progression (Olapade-Olaopa et al., 1999; Gevaert et al., 2018). Indeed, metastatic PCa and castration resistance PCa display significantly lower stromal AR expression than localize tumor- and androgen-dependent PCa (Li et al., 2008; Singh et al., 2014). These observations suggest that a temporal regulation of CAFs secretome may occur through the modulation of hormone receptors expression during cancer progression.

Overall, a complex relationship between temporal and spatial influence may instruct CAFs secretome. However, it is still unknown whether tumor progression orchestrates CAFs function and plasticity according to spatial factors or, conversely, distinct CAFs spatial subtypes are modeling tumor dynamics.

Anti-cancer Therapy as a Determinant of CAFs Secretome

Chemotherapy

Several studies have addressed the modulatory effect of standard chemotherapy (CT) on the TME. CT induces shrinkage of cancer cells compartment resulting in an increased representation of stromal cells in residual tumors (Goto et al., 2017). In this context, factors secreted by CAFs after CT may significantly enhance tumor regrowth from residual cancer cells (Hisamitsu et al., 2019). Current evidence points to CT-induced DNA damage being a key mechanism influencing the repertoire of CAF-secreted factors after treatment (**Figure 3**). Indeed, CAFs support tumor regrowth after DNA damage-mediated NF-κB signaling activation through secretion of WNT-16B (Sun et al., 2012). Alternatively, treatment of lung adenocarcinoma with cisplatin enhances IL-11 secretion by CAFs, which in turn promotes resistance of cancer cells to CT through the STAT-3 signaling pathway (Tao et al., 2016). Of note, CAF-induced IL-11/STAT-3 cell–cell survival signaling has been reported in other tumor types (Calon et al., 2012). Thus, CAF-dependent IL-11 resistance mechanism may be of potential relevance in cancers of distinct origin. Similarly, a direct effect of gemcitabine and 5-FU regimen toward a tumor-supportive secretory phenotype of CAFs has been described in PDAC. In that case, higher expression of secreted factors [intercellular adhesion molecule 1 (ICAM-1), IL-6, GM-CSF, IL-8, CXCL-1] upon treatment has

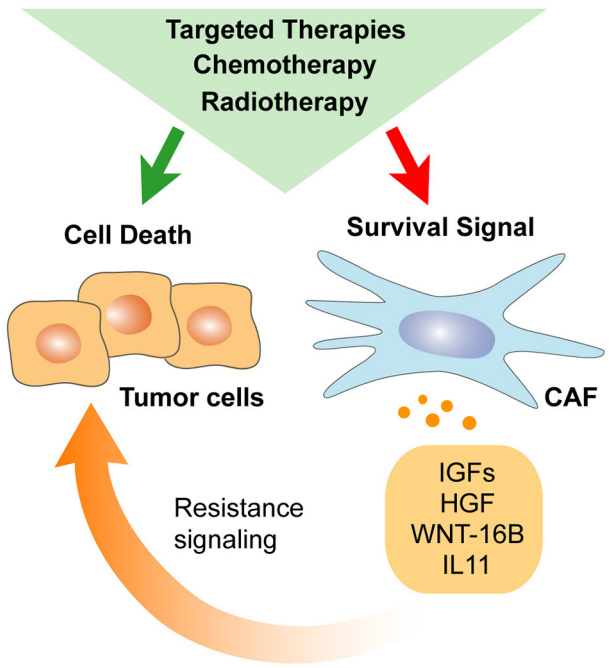

FIGURE 3 | Anti-cancer therapy impacts CAFs secretome. Stromal elements develop their own response to systemic chemotherapy, radiotherapy, and targeted therapy. As a result, CAFs response to treatment may diminish direct anti-cancer drug efficiency by promoting cancer cells self-renewal and survival.

been associated with the activation of stress-associated mitogen-activated protein kinase (MAPK) signaling pathway [Jun N-terminal kinase (JNK) and P38 MAPK] (Toste et al., 2016). Finally, the indirect effect of CT on CAFs secretome may also occur due to the dysregulated crosstalk between cancer cells and CAFs. For instance, Nab-paclitaxel treatment increases CXCL-10 expression in pancreatic cancer cells, leading to lower IL-6 secretion by CAFs subsequently impairing migration and invasive capabilities of cancer cells (Feng et al., 2018).

Radiotherapy

Similarly to CT, radiotherapy (RT) has been reported to impact the secretome of CAFs (**Figure 3**). Rødningen and colleagues showed that ionizing radiation altered the fibroblasts expression of genes involved in ECM remodeling as well as Wnt and IGF signaling to support cancer stemness and resistance to therapy (Rødningen et al., 2005; Wang Z. et al., 2019). Persistent DNA damage, induction of senescence, and TGF-beta pathway activation rather than cellular death are some of the proposed mechanisms modulating CAFs secretome upon RT (Ansems and Span, 2020). Importantly, a vast body of evidence suggests that RT enhances paracrine signaling between fibroblasts and cancer cells through the IGF and TGF-beta pathways (Barker et al., 2015; Tommelein et al., 2018). Promotion of EMT, enhanced cancer stemness properties, and increased resistance to therapy are among the various effects exerted by RT-activated CAFs (Arshad et al., 2015; Barker et al., 2015). For instance, IGF-1 activates the IGF-1R/AKT/mTOR survival pathway in CRC, and CXCL-12 promotes EMT and invasion in pancreatic cancer (Li D. et al., 2016; Tommelein et al., 2018). Alternatively, RT-activated CAFs may also support intra-tumoral angiogenesis (Hellevik et al., 2013). Interestingly, several studies have reported that the immunoregulation exerted by CAFs remains unaltered after ionizing RT (Hellevik et al., 2013; Gorchs et al., 2015; Berzaghi et al., 2019). Thus, combination with immune-targeted therapies may be an attractive clinical approach for RT-treated patients.

Targeted Therapy

Originally designed to be specific to cancer cells, targeted therapies may also produce a range of undesirable off-target effects on stromal cells and particularly on CAFs secretome. For instance, specific secretion of HGF and neuregulin (NRG)-1 by fibroblasts upon exposure to vemurafenib -a BRAF inhibitor- was reported in melanoma (Fedorenko et al., 2015). Of note, the activation of MET by HGF has been suggested as a potential mechanism of acquired tyrosine kinase inhibitor (TKI) resistance to gefitinib in epidermal growth factor receptor (EGFR)-mutant non-small cell lung cancer (NSCLC) (Yano et al., 2008) and to vemurafenib itself in BRAF-mutant melanoma (Wilson et al., 2012). In PCa, HGF and NRG-1 have been identified as non-cell-autonomous drivers of antiandrogen resistance. In this setting, NRG-1/epidermal growth factor receptor 3 (HER-3) signaling axis blockade demonstrated promising tumor growth suppression (Zhang Z. et al., 2020). More recently, Hirata and colleagues reported alternative CAF-induced resistance to BRAF inhibition. In this setting, BRAF inhibitor reprograms CAFs by enhancing platelet-derived growth factor receptor (PDGFR) activity, thus increasing ECM production –thrombospondin (THBS)-1/2, tenascin C (TNC), or POSTN, among other matrix components– and stiffness to potentiate cancer cells tolerance to treatment (Hirata et al., 2015). These data illustrate how CAFs functions can be affected even by targeted therapies to enable the emergence of resistance to treatment.

Epigenetic Regulation of CAFs Secretome

CAF phenotype may be driven by epigenetic deregulation rather than by somatic mutations (Qiu et al., 2008; Bianchi-Frias et al., 2016; Pidsley et al., 2018). DNA methylation, histone modifications, chromatin remodeling, and non-coding RNA–micro RNA (miRNA) and long non-coding RNA (lncRNA)–are the well-described molecular mechanisms behind the epigenetic CAF reprogramming and have been thoroughly reviewed elsewhere (Marks et al., 2016; Melissari et al., 2020; Pan and Zheng, 2020). Interestingly, several studies support the hypothesis that an *epigenetic switch* would initiate the activation process leading to a stable CAF cell state with tumor-supportive secretory phenotype (Albrengues et al., 2015; Kalluri, 2016).

For instance, multiple genes coding for secreted factors, such as IL-1a, CCL-5, and CCL-26; show differential hypomethylation patterns in CAFs and are consequently overexpressed in pancreatic cancer. In this case, paracrine lactate secreted by PDAC cells leads to ten-eleven translocation (TET) enzyme activation triggering the epigenomic reprogramming (Bhagat et al., 2019). Remarkably, different epigenetic mechanisms can merge into the same signaling cascade as happening

for the STAT-3 pathway. Indeed, SOCS-1 methylation and downregulation in PDAC-associated CAFs enhance STAT-3-induced IGF-1 expression (Xiao et al., 2016) to promote the survival and proliferation of pancreatic cancer cells (Kopantzev et al., 2017). Alternatively, LIF-induced methylation of Shp-1 promoter abrogates its expression leading to a STAT-3-mediated CAF pro-invasive phenotype (Albrengues et al., 2015).

In conclusion, in the absence of genomic mutations, epigenetic alterations may be seen as key determinants of CAF phenotype and could become promising targets for cancer treatment. Importantly, several clinical trials are currently exploring the benefits of DNA methylation therapies in solid tumors including colorectal (NCT01896856), pancreatic (NCT03264404), or prostate cancer (NCT03572387). Even though cancer cells are the intended target, the above-mentioned data suggest a potential effect of DNA methylation therapies over CAFs that will need to be carefully assessed in treated patients.

CAFS SECRETOME AS DETERMINANT OF TUMOR DEVELOPMENT

Cancer Cells Self-Renewal

A vast body of evidence indicates that CAFs secretome may contribute to tumor progression by enhancing cancer stemness (**Figure 4**). Indeed, CAF-secreted IGF-2 as well as IL-6 and IL-8 produced by CD-10+ GPR-77+ CAFs are promoting cancer stemness and tumor formation in lung cancer (Chen et al., 2014; Su et al., 2018). Similarly, PCa-derived CAFs with decreased expression of AR promote stemness in cancer cells through IFN-γ and M-CSF secretion (Liao et al., 2017). Importantly, cancer stemness induced by CAF-secreted factors may involve different pathways. For instance, PI3K/AKT pathway activation drives the progression of anal squamous cell carcinoma through IGF-2 secretion by the PDGFRB+ CAFs population (Cacheux et al., 2019). Similarly, IGF-2-secreting CAFs promote tumor regrowth and decreased latency after primary resection in CRC (Unger et al., 2017). Alternatively, NOTCH signaling may be triggered by CAF-derived CCL-2 to induce stem cell features as observed in BC cells (Tsuyada et al., 2012). Finally, WNT signaling activation by CAF-secreted HGF in colorectal adenocarcinomas or by CAF-secreted POSTN in head and neck squamous cell carcinoma and BC cells may also promote cancer stemness and further metastatic initiation (Vermeulen et al., 2010; Malanchi et al., 2011; Yu et al., 2018).

In addition to cytokine-driven signaling, cancer cells self-renewal and aggressiveness may also depend on metabolic cues originating from the TME (reviewed in Reina-Campos et al., 2017; Li et al., 2020). Specifically, CAFs originating lactate and glutamine production as well as autophagic alanine secretion are sustaining cancer cells metabolism, stemness, and progression to metastasis (Fiaschi et al., 2012; Curry et al., 2013; Sousa et al., 2016; Yang L. et al., 2016). The specific mechanisms enabling CAFs to deliver distinct nutrients to cancer cells are being explored. However, CAFs response to the surrounding constraints, such as the epigenetic reprogramming induced by chronic hypoxia, is likely to play a relevant role in metabolic reprogramming of CAFs (Becker et al., 2020).

Metastatic Cancer Cells Spreading

As mentioned above, CAF-secreted factors may also drive cancer progression by enhancing the metastatic potential of cancer cells (**Figure 4**). For instance, while TGF-beta activates different genetic programs in a wide range of stroma cells, the main contributor to its pro-tumorigenic influence seems to be an autocrine loop maintaining the secretion of pro-metastatic factors by CAFs (Calon et al., 2012). In addition, CAF-secreted TGF-beta promotes paracrine reprogramming of epithelial cancer cells by inducing EMT in tumors of distinct origin (Zhuang et al., 2015). In CRC, TGF-beta induces IL-13 expression in fibroblasts, and subsequent TGF-beta/IL-13 synergy activates an EMT program in epithelial cells (Scharl et al., 2013). Alternatively, Calon and colleagues described TGF-beta-activated CAFs secreting IL-6 and IL-11 leading to enhanced STAT-3-dependent survival and spreading of metastatic CRC cells (Calon et al., 2012). Similar observations were made in gastric cancer where CAF-secreted IL-6 induced EMT and metastasis through STAT-3 pathway activation (Wu et al., 2017). Alternatively, CAF-secreted vascular cell adhesion protein (VCAM)-1 has been shown to stimulate lung cancer cells migration and invasion (Zhou et al., 2020).

Besides their autocrine and paracrine activities regulating progression to metastasis, CAFs-secreted factors may also support cancer spreading by influencing ECM deposition at both the primary and metastatic sites. For example, Chakravarthy and colleagues described that TGF-beta-activated CAFs could induce the expression of large ECM components related to cancer progression (Chakravarthy et al., 2018). In this regard, TGF-beta-activated CAFs may secrete POSTN in order to increase proliferation, motility, and invasive properties in head and neck cancer (HNC) cells (Qin et al., 2016). Similarly, CAF-secreted POSTN may also promote tumor progression in CRC through YAP/TAZ activation in cancer cells (Ma et al., 2020). Of note, Deng and colleagues showed that the YAP-activated Wnt/β-catenin pathway promotes colon tumorigenesis (Deng et al., 2018). Hence, the overall data suggest a complex paracrine regulation of the Wnt pathway in cancer cells by TGF-beta-activated CAFs through POSTN secretion and YAP activation in order to enhance tumor progression and metastasis formation.

Immunomodulation

Somewhat contradictory evidences of CAFs immunoregulatory functions have been described during cancer progression (**Figure 4**). While increased abundance of CAFs clearly predicts immune exclusion and immunotherapy failure, a complete depletion of CAFs was associated with decreased intra-tumoral immune infiltration (Özdemir et al., 2014; Chakravarthy et al., 2018). One reason explaining opposite functions of CAFs in modulating immune infiltration may reside in the fact that different CAFs subpopulations with distinct properties are coexisting inside the tumor. For example, among the four CAF subsets identified in BC, only CAF-S1 subtype characterized by high FAP expression promoted immunosuppression by secreting

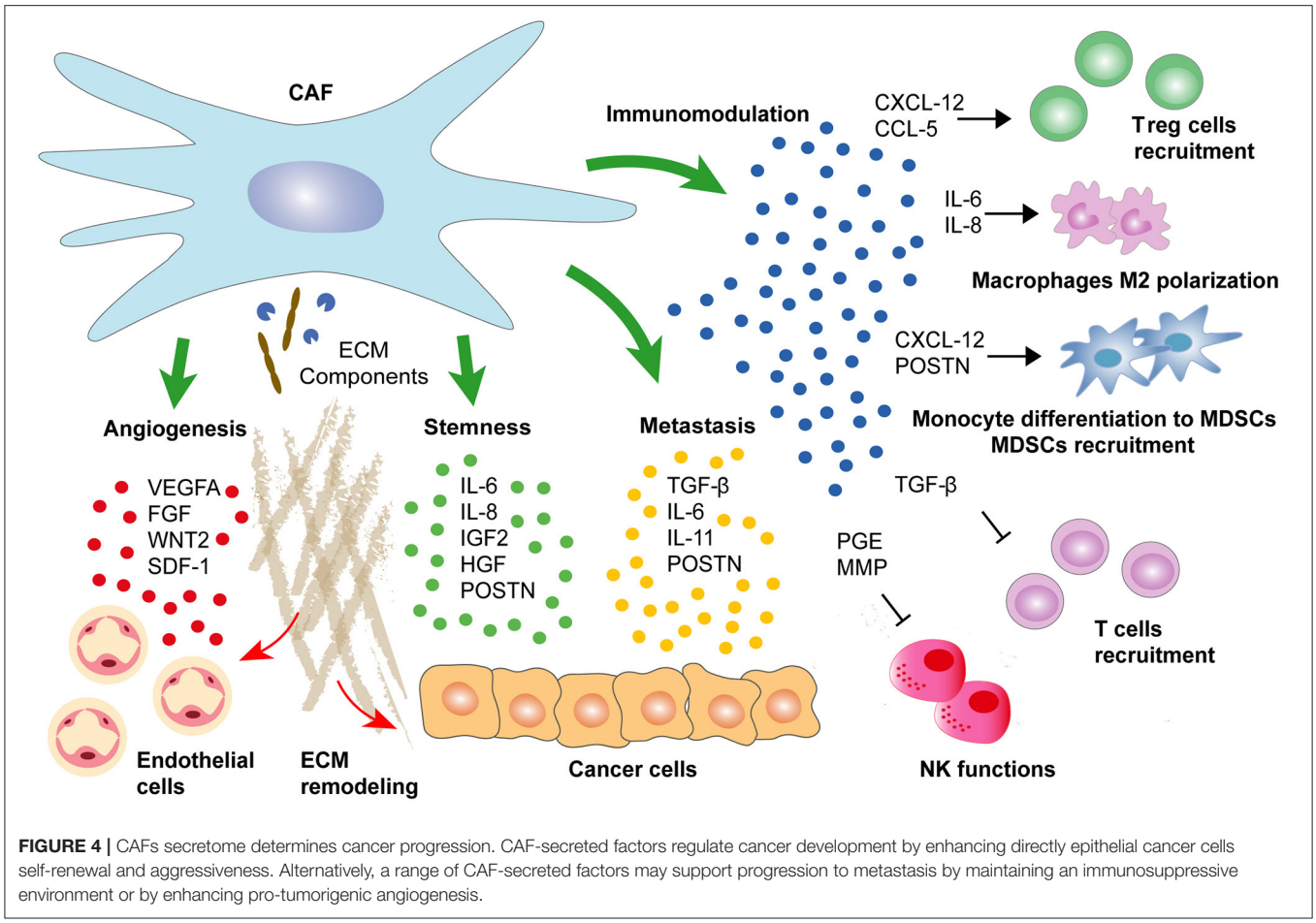

FIGURE 4 | CAFs secretome determines cancer progression. CAF-secreted factors regulate cancer development by enhancing directly epithelial cancer cells self-renewal and aggressiveness. Alternatively, a range of CAF-secreted factors may support progression to metastasis by maintaining an immunosuppressive environment or by enhancing pro-tumorigenic angiogenesis.

CXCL-12 and enhancing immunosuppressive T regulatory cells (T_{reg}) capacity to block T effector cells proliferation (Costa et al., 2018). Importantly, FAP+ CAFs are also the main producers of CXCL-12 in PDAC, and their specific depletion increases immunological control over tumor growth (Kraman et al., 2010; Feig et al., 2013). In this setting, the decreased expression of CXCL-12 leads to intra-tumoral T cell accumulation, which in turn synergizes with immuno- or chemotherapy to reduce cancer cells abundance (Feig et al., 2013; Sherman et al., 2014).

In addition to CXCL-12, a wide range of CAF-produced chemokines has been associated with tumor immunosuppression. For example, CAF-secreted CCL-5 induces CD4+CD25+ T_{reg} cells in BC (Tan et al., 2011; Costa et al., 2018). Notably, CCL-5 dependent T_{reg} cells recruitment was associated with lung metastasis in BC mouse model (Tan et al., 2011). CAFs also participate in immunosuppressive myeloid cell recruitment and differentiation by secreting SDF-1 to attract monocytes and to induce their differentiation into myeloid-derived suppressor cells (MDSCs) contributing to cancer immune evasion (Gabrilovich and Nagaraj, 2009; Deng et al., 2017). Alternatively, Wang and colleagues showed that CAF-secreted POSTN promoted MDSCs infiltration to establish a pre-metastatic niche during breast tumor metastasis through AKT and STAT-3 pathway activation (Wang Z. et al., 2016). Similarly, FAP+ CAF-secreted CCL-2 mediates STAT-3 pathway activation in MDSC to promote liver tumor growth and to suppress T cells proliferation during colorectal carcinogenesis (Chun et al., 2015; Yang et al., 2016). Alternatively, CAF-secreted IL-6 and IL-8 may promote monocytes differentiation into M2 macrophages and further recruitment in the tumor to diminish natural killer (NK) cells cytotoxicity against CRC cells (Cho et al., 2018; Zhang et al., 2019). Finally, CAFs may directly interfere with NK anti-tumoral functions through prostaglandin E2 (PGE2) secretion or MMPs production decreasing NK receptor ligands expression in tumor cells, thus reducing NK receptor-dependent cytotoxic activity (Balsamo et al., 2009; Ziani et al., 2017).

As mentioned earlier, CAFs are major producers of TGF-beta, a key contributor to tumor immunosuppression (Calon et al., 2015; Batlle and Massagué, 2019). For instance, TGF-beta produced by CAFs was correlated with CD8+ T cells exclusion, which in turn has been associated with resistance to anti-PD-L1 antibody-based immune checkpoint inhibitor therapy (Mariathasan et al., 2018). In this context, TGF-beta pathway blockade through TGF-beta receptor (TGFBR)-1 inhibition or TGF-beta blocking antibody enabled T cells infiltration into the tumor, restoring the anti-tumor immunity induced by the anti-PD-L1 antibody in BC and CRC pre-clinical models

(Mariathasan et al., 2018; Tauriello et al., 2018). In contrast, TGFBR-2 deletion in BC induces MDSCs recruitment to the TME, reducing T cell proliferation and activation (Yang et al., 2008). Interestingly, TGFBR-2 deletion has been associated with the increased expression of stromal TGF-beta leading to MDSCs recruitment of indifferent cancers including HNC or PCa (Lu et al., 2006; Franco et al., 2011). These data suggest that the genetic ablation of TGFBR-2 enhances the secondary activation of TGF-beta that may indirectly promote intra-tumoral immunosuppression.

Angiogenesis

Neovascularization is an important process during tumorigenesis. Interestingly, CAFs are the main source of VEGF-A, the most potent pro-angiogenic factor, which binds to its cognate VEGF receptor (VEGFR)-2 expressed on endothelial cells (**Figure 4**) (Fukumura et al., 1998; Apte et al., 2019). Noma and colleagues showed that TGF-beta pathway activation in CAFs was essential to induce VEGF secretion (Noma et al., 2008). In addition, Sánchez-Elsner and colleagues demonstrated a synergistic cooperation between SMADs and hypoxia-inducible factor (HIF)-1 alpha proteins to drive VEGF expression, suggesting a potential link between TGF-beta pathway and hypoxia to promote tumor angiogenesis (Sánchez-Elsner et al., 2001). Nevertheless, there is controversial evidence about the role of TGF-beta in tumor angiogenesis suggesting a differential expression of TGF-beta ligands during different phases of angiogenesis (Pardali et al., 2010). For instance, Liu and colleagues showed that TGFBR-1 inhibitor and VEGF synergistically induced tumor angiogenesis through α5-integrin upregulation (Liu et al., 2009). In contrast, TGF-beta-TGFBR-1 signaling has been involved in the promotion of BC angiogenesis through MMP-9 upregulation and through CAF-secreted TGF-beta and SDF-1 in HCCs (Safina et al., 2007; Yang J. et al., 2016).

In addition to promoting VEGF-dependent angiogenesis, CAFs may enhance tumor angiogenesis through additional paracrine signaling between stromal and cancer cells (Orimo et al., 2005; Unterleuthner et al., 2020). For instance, CAF-derived CXCL-12 leads to CXCL-8 secretion by PDAC cells inducing new vessel formation by endothelial cells (Matsuo et al., 2009). Alternatively, CAF-secreted galectin-1 induces VEGF-A production by BC cells to promote tumor angiogenesis (Tang et al., 2016). FGF has also been recognized as a potent pro-angiogenic factor involved in tumor angiogenesis (Compagni et al., 2000). Correspondingly, Pietras and colleagues demonstrated that cervical carcinoma-derived CAFs are secreting FGF-7 and FGF-2, inducing both epithelial proliferation and tumor angiogenesis (Pietras et al., 2008).

Finally, CAFs not only are directly promoting tumor angiogenesis by secreting pro-angiogenic factors but also act indirectly by participating in ECM remodeling (**Figure 4**) (De Palma et al., 2017). For example, CAFs may support vascularization through biomechanical activity and ECM stiffness promoted by MMP activity (Bordeleau et al., 2017; Sewell-Loftin et al., 2017). Importantly, CAFs are the most important suppliers of ECM-associated proteins essential to vascular formation including TNC production resulting in pro-angiogenic paracrine signaling (Newman et al., 2011; Rupp et al., 2016).

Resistance to Anti-cancer Therapy

In addition to modulating tumor progression, CAFs secretome may also promote resistance to systemic and targeted therapies. For instance, CAF-secreted SDF-1 upregulates the expression of special AT-rich sequence-binding protein-1 (SATB-1) in pancreatic cancer cells, which not only sustains pancreatic tumor growth but also mediates gemcitabine resistance (Wei et al., 2018). Furthermore, CAF-derived IGF-1 and IGF-2 induce CT resistance in pancreatic cancer (Ireland et al., 2016). IGF-2 also increases insulin receptor/IGF 1 receptor (IGF1R) axis activation in cancer cells to enhance resistance to anti-EGFR-targeted therapy in cholangiocarcinoma (Vaquero et al., 2018). In the same line, fibroblast-secreted HGF activates both the MAPK and PI3K/AKT pathways contributing to BRAF-targeted therapies' primary resistance in melanoma and in a subset of colorectal and glioblastoma cancer cells (Straussman et al., 2012).

Regarding hormonotherapy, CAFs-secreted factors contribute to estrogen receptor alpha (ER-α) phosphorylation in BC cells, thereby promoting tamoxifen resistance (Pontiggia et al., 2012). In addition, Li and colleagues reported that letrozole, an aromatase inhibitor lowering estrogen production, had opposite functional effects on CAFs secretome (Li K. et al., 2016). Letrozole reduces CCL-2, CCL-5, and CXCL-1 expression in CAFs, possibly contributing to its efficacy against BC cells. Conversely, letrozole also increases CAFs secretion of POSTN, a factor involved in BC progression and metastasis (Kyutoku et al., 2011; Li K. et al., 2016). In PCa, Zhang and colleagues, described that CAF-secreted NRG-1 activates the HER-3 signaling pathway leading to resistance to antiandrogen therapy (Zhang Z. et al., 2020). Notably, NRG-1 expression in PCa-derived CAFs is increased after antiandrogen therapy that may contribute to potentiate resistance to treatment (Zhang Z. et al., 2020).

Consequently, the presence in the tumor of a range of secreted factors involved in resistance to therapy may be the result of CAFs' own response to anti-cancer treatment. Indeed, CT induces senescence-associated secretory phenotype in fibroblasts, typically characterized by the increased secretion of cytokines, chemokines, and growth factors previously associated with tumor progression and treatment resistance (Demaria et al., 2017). More specifically, Sun and colleagues demonstrated that primary prostate fibroblasts increased WNT-16B expression in response to CT. In this setting, WNT-16B enhanced prostate tumor growth and diminished CT cytotoxicity against cancer cells (Sun et al., 2012). Finally, previously mentioned IL-11 secreted by CAFs upon cisplatin-based CT induced STAT-3 pathway activation and chemoresistance in lung cancer cells (Tao et al., 2016).

PROGNOSTIC AND PREDICTIVE VALUE OF CAFS SECRETOME

The identification of tumor features predicting prognosis, risk of relapse, and benefit from treatment is absolutely essential

for clinical decision-making in oncology. In this sense, a better understanding of the TME has provided multiple prognostic and diagnostic biomarkers in cancers of distinct origin (Bremnes et al., 2011). For instance, CAFs biomarkers (Ha et al., 2014; Dourado et al., 2018), tumor:stroma ratio quantification (Kemi et al., 2018; Vangangelt et al., 2018), and stromal gene expression profiling (Finak et al., 2008; Frings et al., 2013; Calon et al., 2015) are powerful tools predicting clinical outcome. However, addressing the exclusive impact of CAFs secretome on cancer prognosis remains a major challenge, and to our knowledge, there is no example of CAF-secreted biomarkers currently applied to the clinical setting.

Cytokines and chemokines have been proposed as prognostic factors in different types of cancer. Yet, their pleiotropic nature and multiple cellular origin as well as their distinct releasing patterns make their use as biomarkers especially challenging. Circulating IL-6 has been associated with cancer progression and poor prognosis in melanoma, BC, and gastrointestinal tumors, among others (Salgado et al., 2003; Hoejberg et al., 2012; Vainer et al., 2018), but only few studies have assessed IL-6 cellular origin. In bladder cancer patients for instance, CAF-derived IL-6 and ACTA-2 (αSMA coding gene) co-upregulation correlated with poor survival (Goulet et al., 2019). In esophageal adenocarcinoma, although IL-6 serum levels did not correlate with patients' outcome, the expression of ADAM-12, a surrogate marker for IL-6-producing CAFs, predicted poor prognosis after neoadjuvant chemoradiation (Ebbing et al., 2019). CXCL chemokines are additional examples of promising prognosis biomarkers associated with CAFs functions. Indeed, CAF-derived CXCL-14 expression was correlated with shorter recurrence-free survival in estrogen receptor negative, triple negative, and basal-like BC (Sjöberg et al., 2016), as well as in additional tumor types (reviewed in Westrich et al., 2020). Similarly, CXCL-1 and CXCL-8 positivity in CAFs was significantly associated with poor prognosis in gastric cancer patients (Naito et al., 2019), whereas CXCL-8,−10, and−11 CAFs expression correlated with resistance to neoadjuvant CT and poor prognosis in BC (Xu et al., 2020).

As previously mentioned, CAFs-secreted growth factors, such as HGF or IGFs, are involved in resistance to targeted therapy and may be of use to predict response to treatment. Indeed, HGF expression was correlated with innate resistance to BRAF inhibition, and increased HGF plasma levels predicted worse overall survival (OS) and progression-free survival (PFS) in BRAF-mutant melanoma patients (Straussman et al., 2012; Wilson et al., 2012). Alternatively, CAF-secreted IGF-binding proteins have been proposed as a potential therapeutic target and prognostic biomarker in pancreatic cancer and other tumor types (Thomas and Radhakrishnan, 2020).

Additional CAFs-secreted proteins, such as VCAM-1, THBS-2, and POSTN, involved in cancer progression were recently evaluated as potential actionable biomarkers. In lung cancer, the soluble fraction of VCAM-1 predicted relapse and lower OS, whereas high POSTN expression was associated with shorter PFS in ovarian cancer patients (Ryner et al., 2015; Zhou et al., 2020). Of note, a recent meta-analysis confirmed the value of POSTN as a biomarker predicting poor outcome in different solid tumors (Yang et al., 2020). Finally, increased THBS-2 predicted decreased OS in CRC patients (Wang X. et al., 2016).

CAF-derived exosomes transferring miRNA (Au Yeung et al., 2016; Fang et al., 2019; Qin et al., 2019) and lncRNA (Qu et al., 2016) to tumor cells have been reported to promote treatment resistance in different tumor types and were also evaluated as potential predictive biomarkers. In this context, serum detection of plasma exosomal miR-196a levels correlated with tumor size, lymph node metastasis, advanced tumor stage, and poor OS while accurately discriminating chemoresistant and sensitive patients in HNC (Qin et al., 2019). Of note, suppressing or interfering with CAF-derived exosomes transference to cancer cells has been proposed as a novel therapeutic approach (Li et al., 2017).

Similarly to soluble factors, many ECM-related products are contributing to the predictive power of stromal-originating gene signatures (Andriani et al., 2018; Yuzhalin et al., 2018; Jiang et al., 2019). ECM remodeling enzymes, such as MMPs and tissue inhibitors of MMP (TIMP), which are not exclusively but mainly released by CAFs, have also been suggested to predict cancer progression and response to treatment (Liu et al., 2019). Among them, Eiró et al. assessed the potential influence over patients' prognosis of TIMP-2 expression by CAFs at the tumor center and the invasive front of early stage BC. TIMP-2 resulted to be a potent poor outcome predictor at both locations (Eiró et al., 2015). Many members of the MMP family promoting tumor invasion through ECM degradation are also impacting cancer prognosis. For instance, increased MMP-1 expression by CAFs was associated with high risk of relapse in stage II CRC, and CAFs expression of MMP-9,−11, and−13 correlated with shorter relapse-free survival in BC (González et al., 2009; Eiro et al., 2019). Remarkably, the relevance of MMPs for prognosis might be cancer specific, as illustrated by MMP-2, which predicts poor OS and PFS in NSCLC but associates with better survival in patients with high grade BC (Leinonen et al., 2008; Niemiec et al., 2013).

Along this line, other CAF-secreted ECM components, such as collagens, have been recently recognized as important contributors to cancer progression and as potential liquid biopsy biomarkers in different tumor types (Giussani et al., 2019). Serum level of PRO-C3 –a biomarker of collagen III production– is a promising example predicting poor OS in PDAC patients and may be a non-invasive actionable biomarker for desmoplasia-targeting therapies (Willumsen et al., 2019). Interestingly and probably due to TGF-beta capacity to stimulate collagens production by CAFs, serum levels of collagen fragments have been associated with response to TGF-beta-targeted therapy (Nissen et al., 2019).

Overall, these exciting findings are calling for further validation of CAF-derived biomarkers in order to improve the standard of care and decision-making in oncology. However, it is worth noting that methodological variability will be especially relevant when transferring biomarkers from bench to bedside. Indeed, evaluating either a single or a panel of factors through distinct techniques may complicate the validation of CAFs secretome-based prognostic and predictive tools in the clinical setting (Paulsson and Micke, 2014). Spatial and temporal heterogeneity (see the Determinants of CAFs Secretome section)

may be an additional issue that could be overcome by liquid biopsy, a non-invasive method allowing real-time evaluation of CAF-secreted biomarkers circulating in the bloodstream (Herrera et al., 2019).

CAF-SECRETED FACTORS AS A THERAPEUTIC TARGET

Targeting the CAFs Secretome Regulating Cancer Cells Proliferation

Since CAFs complete depletion or blockade of fibroblast-rich tumor stroma formation resulted in decreased anti-tumor immune infiltration and more aggressive tumors, recent strategies have rather focused on the regulation of CAFs originating paracrine and autocrine signaling (Özdemir et al., 2014; Rhim et al., 2014). For instance, CAFs reprogramming by vitamin A and vitamin D was shown to inhibit tumor-supportive secretome associated with cancer progression (Froeling et al., 2011; Sherman et al., 2014). Remarkably, gemcitabine and either vitamin A or vitamin D regimen resulted in significant tumor burden reduction in PDAC pre-clinical models (Sherman et al., 2014; Carapuça et al., 2016). Hence, a phase II trial is currently evaluating the combination of CT and vitamin D in PDAC patients (NCT03415854). Yet, a previous phase II trial did not show any survival benefit with high doses of vitamin D3 compared with standard doses in combination with CT in metastatic CRC patients (Ng et al., 2019).

Importantly, vitamin A and vitamin D reprograming strategies are both associated with TGF-beta –a key autocrine and paracrine mediator of CAFs signaling– pathway inhibition. Indeed, vitamin D receptor ligands decrease fibroblast activation by TGF-beta (Ding et al., 2013), whereas all-trans retinoic acid (ATRA) –the active metabolite of vitamin A– inhibits the fibroblasts capacity to release active TGF-beta, thus impeding autocrine TGF-beta activation (Sarper et al., 2016). Of note, ATRA reprogramed fibroblasts are in addition reducing Wnt–β-catenin signaling in the surrounding cancer cells through SFRP-4 secretion (Froeling et al., 2011).

TGF-beta has emerged as a potential therapeutic target in oncology. Notably, TGF-beta inhibitor in combination with gemcitabine improved the OS in locally advanced and metastatic PDAC patients (Melisi et al., 2018). A phase I/II trial is currently testing the ability of TGF-beta inhibitor to restore the sensitivity to CT in patients with TGF-beta-activated program in metastatic CRC resistant to CT (NCT03470350). The analysis of the TGF-beta-activated program in CAFs provided several additional therapeutic targets. Among them, IL-6 and IL-11 are secreted interleukins activating STAT-3-dependent survival and spreading of metastatic CRC cancer cells (Calon et al., 2012). IL-11 or IL-6 inhibitors displayed potent anti-cancer activity in pre-clinical models of cancer of distinct origins (Coward et al., 2011; Putoczki et al., 2013; Song et al., 2014). Siltuximab, an IL-6 inhibitor, did not show benefit as monotherapy in CT-pretreated castration-resistance PCa patients (Dorff et al., 2010). However, additional IL-6 pathway inhibitors are currently tested in combination with CT in patients with breast (NCT03135171), pancreatic (NCT04258150, NCT02767557), or liver cancer (NCT04338685). Among CAF-secreted molecules, ECM components have also raised special interest as therapeutic targets. Yet, clinical trials failed to demonstrate the clinical benefit from COL-3, MMP-2, and MMP-9 inhibitors in patients (Chu et al., 2007). Similarly, PEGPH20, a PEGylated recombinant human hyaluronidase, did not add benefit to standard CT in PDAC patients (Tempero et al., 2020).

Off-target effects and indirect resistance to treatment involving CAFs functions may explain the lack of benefit of current stroma-targeting therapies. For example, Casanovas and colleagues described that VEGFR blockade induces tumor hypoxia and paradoxically triggers pro-angiogenic factors production, such as FGF family members (Casanovas et al., 2005). Likewise, CAFs derived from anti-VEGF-resistant tumors are secreting PDGF-C in order to reactivate tumor angiogenesis (Crawford et al., 2009). A potential solution may come from multi-targeted therapies, such as nintedanib, a triple angiokinase inhibitor blocking VEGFR, FGFR, and PDGFR. Nintedanib reduces the CAFs expression of IL-6, IL-8, VEGF, and VCAM-1 as well as OPN and showed important clinical benefit in combination with CT in NSCLC patients (Hilberg et al., 2008; Reck et al., 2014; Yamanaka et al., 2020).

Overcoming CAFs-Induced Immunosuppression

Several preclinical studies have recently underscored the relevance of TGFBR-1 targeting in order to activate an anti-tumor immune response. TGFBR-1 inhibitor (galunisertib) and anti-PD-L1 combination treatment increased intra-tumoral T cell infiltration and activation, thereby promoting a potent anti-tumor response in CRC and BC pre-clinical models. Characteristically, this effect was associated with a reduction of fibroblasts activation and an increased anti-tumor immune genes expression (Holmgaard et al., 2018; Mariathasan et al., 2018; Tauriello et al., 2018). Curiously, anti-PD-L1 treatment alone enhanced the expression of TGF-beta activated CAFs biomarkers, suggesting that TGF-beta pathway inhibition is essential to enhance anti-PD-L1 therapy (Holmgaard et al., 2018). In this context, two phase I trials are being conducted to evaluate the benefit of galunisertib in combination with immune checkpoint inhibitors (anti-PD-1, anti-PD-L1) in refractory metastatic cancer patients (NCT02423343, NCT02734160). In contrast, Zhao and colleagues showed that another TGFBR-1 inhibitor (TEW-7197) failed to increase anti-PD-L1 treatment efficacy in melanoma mouse model. TEW-7197 promoted fibroblasts proliferation and diminished PD-L1 expression in cancer cells through MMP-9 secretion by CAFs. Interestingly, the authors reported increased TGFBR-2 expression upon treatment (Zhao et al., 2018). In this context, previously mentioned TGFBR-2-induced secondary activation of TGF-beta could potentially contribute to CAFs activation and to combination treatment failure (Lu et al., 2006; Franco et al., 2011).

Bintrafusp alfa, a bi-functional fusion protein composed of an anti-PD-L1 antibody and a TGF-beta "trap," was recently designed to potentiate immune checkpoint and

TGF-beta inhibitors regimen. Bintrafusp alfa induces tumor regression and decreased spontaneous metastasis in a CRC mouse model by activating CD8+ T cells and NK cells while abrogating CAFs activation in the tumor (Lan et al., 2018). Clinical benefit of bintrafusp alfa is currently under evaluation in several cancer patients' trials including NSCLC (NCT03840902), BC (NCT03579472), HNSCC (NCT04247282), and CRC (NCT03436563).

Remarkably, previously mentioned ATRA-mediated CAFs reprograming increases T cells infiltration in PDAC while blocking CAFs autocrine TGF-beta activation (Ene-Obong et al., 2013; Sarper et al., 2016). In view of the above-mentioned data on TGF-beta inhibition, pancreatic cancer patients who will be treated with ATRA (NCT04241276) may as well benefit from combination regimen with immune checkpoint inhibitors.

Finally, CAF-secreted factors, such as FN and TNC, have been targeted for antibody-based delivery of immune enhancers specifically to the tumor site in order to concentrate their effect on cancer tissue and decrease treatment-associated toxicity on healthy tissue (Pasche and Neri, 2012). For example, L19-TNF and L19-IL-2 immunocytokines conjugate an antibody-recognizing FN with cytokines (TNF-α and IL-2, respectively) (Neri, 2019). Preclinical data already suggested the robust anti-cancer potential of L19-based immunocytokines (Lieverse et al., 2020), and phase II and III trials are currently testing the clinical benefit of L19-TNF-a and L19-IL-2 in patients with soft tissue sarcoma (NCT03420014) or melanoma (NCT02938299, NCT03567889).

CONCLUSIONS

From a general perspective, CAFs should be considered as a heterogeneous and dynamic stromal component which evolution to distinct functional subpopulations is paralleling the tumorigenic process. Spatio-temporally regulated factors and crosstalk with other TME components drive this co-evolution from local tissue resident fibroblasts and other cell types to distinct CAFs subtypes. Indeed, the intra-tumoral location effect over CAFs function is determined by spatial distribution of tumor cell-derived factors (**Figure 1**). Among them, autocrine or paracrine communications and EVs interchange have been described to define CAF phenotypes. However, the influence of other cell populations–infiltrating immune cells, endothelial cells–, and biomechanical stress (ECM stiffness) also differentially distributed within the TME may play an important role in modulating CAFs behavior. Among other potential functions associated with CAFs, there is solid evidence of several well-defined cell sub-specializations with either contractile and ECM remodeling functions or secretory and immunomodulating functions (**Figure 2**).

The potential role of CAFs secretome for predicting patient's outcome and response to treatment has also been intensively investigated. Overall findings illustrate the potential benefit of using CAFs secretome biomarkers to improve patient's selection and treatment follow-up. Notwithstanding their potential as actionable biomarkers, current knowledge is still mostly providing descriptive information of individual secreted factors but does not advocate for their translation into the clinical setting. Comprehensive -omics analyses are currently being used for an extensive characterization of CAFs secretome in order to discover and validate robust biomarkers and novel targets within the stroma (Principe et al., 2018). In this sense, liquid biopsy appears to be a promising method for real-time evaluation of the different components of CAFs secretome, allowing the detection of CAF-derived soluble factors, CAF-derived exosomes, and even circulating CAFs as potential biomarkers (Herrera et al., 2019).

Recent findings suggest a clear impact of both systemic and targeted anti-cancer therapies upon CAFs secretome that will need to be carefully assessed in view of patients' response to treatment (**Figure 3**). Understanding the impact of CAFs secretome on treatment resistance after therapy exposure will provide original tools to monitor patient's response. The implementation of clinical criteria evaluating components of the CAFs secretome will help in refining patients' selection for suitable therapies and improve oncological outcomes.

While CAFs contribution over tumor development is still a matter of research, CAFs secretome has already demonstrated its potential as a target for original therapeutic strategies in a wide range of cancers (**Figure 4**). However, a better understanding of CAFs secretome has also underscored the importance of defining the cell-type specific response to secreted factors. For instance, TGF-beta is a pro-metastatic cytokine currently targeted in multiple clinical trials (NCT04031872, NCT02452008, NCT03834662, NCT04574583). Yet, TGF-beta is also considered as a tumor suppressor due to its cytostatic effect on cancer cells (Akhurst and Hata, 2012). Another example is IL-11, a pro-metastatic factor that can be successfully targeted by IL-11 signaling antagonist to reduce cancer progression (Putoczki et al., 2013). Until recently, IL-11 was better known for its capacity to stimulate platelet production, and for decades, cancer patients have been treated with rhIL-11 to overcome CT-induced thrombocytopenia (Isaacs et al., 1997; Cairo et al., 2005; Wu et al., 2012). These findings underscore the importance of carefully assessing every cell-type specific response to secreted factors before therapeutic translation into the clinical setting.

CAFs may not be understood and targeted as a unique family anymore, and a detailed definition of CAFs secretome is still an unmet need. For instance, CAFs and senescent fibroblasts coexisting within the TME are able to secrete equivalent factors. Hence, CAFs secretome and senescence-associated secretory phenotype (SASP) may have overlapping tumor-promoting effects (Sahai et al., 2020).

Different treatment strategies, such as targeting a specific functional subtype of CAFs, reprogramming CAFs backwards to a tumor-suppressor phenotype, or even switching between distinct functional subtypes, are currently being addressed. Along this line, a major challenge in precisely defining CAFs heterogeneity and therapeutically targeting CAFs secretome will reside in a better comprehension of CAFs spatiotemporal evolution during tumor progression.

Overall, it is reasonable to believe that increasing our understanding of CAFs inter- and intra-tumoral heterogeneity will be key to fully grasp CAFs secretome diverseness and potentiate anti-cancer therapy.

AUTHOR CONTRIBUTIONS

JL, JM-J, and AC participated to the manuscript preparation and revision (writing, reviewing, discussing, and editing). JB-R made substantial contribution to the manuscript revision. All listed authors approved this manuscript for publication.

ACKNOWLEDGMENTS

This work has been supported by grants to AC from Instituto de Salud Carlos III with FEDER/FSE co-financing (CP16/00151; PI17/00211), to JL from the Spanish Association against Cancer (AECC; Junior Clinician fellowship).

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 05 February 2021
doi: 10.3389/fcell.2020.630147

Molecular Basis of LH Action on Breast Cancer Cell Migration and Invasion via Kinase and Scaffold Proteins

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OPEN ACCESS

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

Received: 16 November 2020
Accepted: 24 December 2020
Published: 05 February 2021

Citation:
Mondaca JM, Uzair ID, Castro Guijarro AC, Flamini MI and Sanchez AM (2021) Molecular Basis of LH Action on Breast Cancer Cell Migration and Invasion via Kinase and Scaffold Proteins. Front. Cell Dev. Biol. 8:630147. doi: 10.3389/fcell.2020.630147

Breast cancer (BC) is a major public health problem affecting women worldwide. Approximately 80% of diagnosed cases are hormone-dependent breast cancers. These hormones are known to stimulate tumor development and progression. In this setting, tentative evidence suggests that luteinizing hormone (LH) may also play a role in tumors. In BC cells that express functional LH receptors (LHR), this hormone regulates cell migration and invasion by controlling several kinases that activate actin cytoskeletal proteins. In this article, we show that LH induces phosphorylation of paxillin and its translocation toward the plasmatic membrane, where focal adhesion complexes are assembled. This process is triggered via a rapid extra-gonadal LHR signaling to Src/FAK/paxillin, which results in the phosphorylation/activation of the nucleation promoter factors cortactin and N-WASP. As a consequence, Arp2/3 complexes induce actin polymerization, essential to promote cell adhesion, migration, and invasion, thus enhancing metastatic spread of tumoral cells. Our findings provide relevant information about how gonadotrophins exert their action in BC. This information helps us understand the extragonadal effects of LH on BC metastasis. It may provide new perspectives for therapeutic treatment, especially for women with high serum levels of gonadotrophins.

Keywords: LH, cortactin, N-WASP, migration and invasion, breast cancer

INTRODUCTION

Women produce a range of reproductive hormones. Their secretion patterns vary throughout their lifetime. In premenopausal women, hormone levels fluctuate due to physiological variations associated with the menstrual cycle. Postmenopausal women have lower estrogen and higher gonadotrophin concentrations, but in general, their hormone levels are fairly constant (Hall et al., 2000; Hernandez et al., 2005). Gonadotrophins, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are glycoproteins that act on the gonads to regulate development and reproduction. These hormones are produced by the anterior pituitary gland. Their secretion

is induced by the gonadotrophin-releasing hormone (GnRH), which is released in a pulsatile manner in response to circulating levels of estrogens and progesterone via a negative feedback (Tsutsumi and Webster, 2009).

The role of LH in the female gonads is well established. It exerts its action by binding to specific LH receptors (LHR) that are part of the heptahelical G-protein-coupled receptor family (Ascoli et al., 2002). Thus, LH influences the production of androgens, which are aromatized to estrogens. After ovulation, gonadotrophins act to maintain progesterone levels, controlling steroidogenesis and gametogenesis (Gharib et al., 1990).

Although ovaries are the classic and single questionless target for gonadotrophins, recent research has demonstrated that receptors for this hormone are also present in normal and tumoral extragonadal tissue, including in the breast (Meduri et al., 1997, 2003; Jiang et al., 2002; Rao and Lei, 2007; Sanchez et al., 2016). In fact, LHR is widely expressed in breast tumors (Meduri et al., 1997, 2003) and BC cell lines (Bodek et al., 2003; Sanchez et al., 2016, 2018). Significantly higher LH levels and LHR expression have been found in invasive tumors, suggesting that LH upregulation could play a key role in breast carcinogenesis (Silva et al., 2002; Hudelist et al., 2009). We recently reported that LH modulates the expression of a set of genes implicated in tumorigenesis in BC cells and that the circulating levels of gonadotrophins are directly correlated with tumor growth in an *in vivo* model of BC (Sanchez et al., 2018). For all these reasons, and in accordance with findings of other groups, we suggest that an LH-triggered mechanism of action exists that could affect the development and/or progression of BC (Tanaka et al., 2000; Meduri et al., 2003; Hudelist et al., 2009; Sanchez et al., 2016, 2018).

The worst prognoses of BC are closely linked with the ability of tumors to generate metastasis at distant sites. Around 90% of deaths in BC patients are due to the development of metastasis (Redig and McAllister, 2013). This process occurs through specific steps that result in alterations in the adhesion, migration, and invasion properties of tumor cells, ultimately triggering metastatic spread (Jiang et al., 2015).

A dynamic reorganization of the actin cytoskeleton is key to the metastatic process. It is modulated by the action of several fundamental kinases and scaffold proteins, such as Src and focal adhesion kinase (FAK) (McLean et al., 2005). In our previous work, we identified that LH/LHR stimulates BC cell migration and invasion via a rapid signal to Gαi/Gβ in an Src and FAK-dependent pathway (Sanchez et al., 2016). Phosphorylated FAK recruits and activates paxillin, a scaffold protein that acts as a docking site for many actin cytoskeletal regulators (Shortrede et al., 2016).

Cortactin is a scaffold protein involved in branching of actin filaments (Uzair et al., 2019). When cortactin is activated, it relays signals from Src/FAK-paxillin to the Arp2/3 complex, leading to actin nucleation (Kruchten et al., 2008). N-WASP belongs to the family of the Wiskott-Aldrich syndrome proteins (WASPs). It acts as a scaffolding protein, recruiting signals from cdc42 GTPases for their regulation. Cortactin and N-WASP synergistically control the Arp2/3 complex (Uruno et al., 2001), enhancing the formation of actin-based protrusive structures involved in cell migration and invasion (Frugtniet et al., 2015).

Although elevated LH levels have been associated with a worse prognosis (Pujol et al., 2001), knowledge about the molecular mechanism by which LH exerts its action in BC remains poor. The aim of the present article was therefore to further our understanding of the molecular signaling induced by LH on BC cell morphology and motility. In particular, we were interested in investigating the influence of LH on the migratory, invasive, and metastatic potential of BC cells.

MATERIALS AND METHODS

Cell Culture and Treatments

The T-47D human breast carcinoma cell line was obtained from the American Type Culture Collection (ATCC, United States). T-47D cells were grown in RPMI 1640 supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS), penicillin, and streptomycin under 5% CO_2 atmosphere at 37°C. Prior to the experiments investigating non-transcriptional effects, BCs were kept in a medium containing no FBS for 8 h. LH (Luveris 75 IU) was obtained from the Merck Serono Laboratory. The concentration was chosen to mimic follicular phase levels (5 mIU/ml), since it induces a greater phosphorylation/activation on key regulatory proteins of cell motility (Sanchez et al., 2016). Different chemical inhibitors were used: 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo-(3,4-d) pyrimidine (PP2, 10 μM) was from Calbiochem, and FAK inhibitor (FAKi, 1 μM), Wiskostatin (10 μM), and CK-666 (4 μM) were from Santa Cruz Biotechnology. Whenever an inhibitor was used, the compound was added 45–60 min before starting the active treatments. PP2, FAKi, Wiskostatin, and CK-666 were dissolved in DMSO.

Immunoblottings

Cell lysates were separated by SDS-PAGE in 8–10% gels and transferred into polyvinylidene difluoride (PVDF) membranes. Antibodies used were p-FAK^{Y397} (611807), FAK (610088), and Arp3 (612135) (BD Transduction Laboratories); actin (sc-1615), LHR (sc-25828), c-Src (sc-5266), paxillin (sc-31010), p-paxillin^{Y118} (sc-365020), cortactin (sc-11408), p-cortactin^{Y466} (sc-101611), N-WASP (sc-13139), Arp2 (sc-15389), and p-Tyr (sc-7020) (Santa Cruz Biotechnology); p-N-WASP$^{S484/485}$ (ab1964) (Chemicon International); p-Src^{Y418} (ab4816) (Abcam); and p-Arp2^{T237} (orb155730) (Biorbyt). Primary and secondary antibodies were incubated with the membranes using standard techniques. Immunodetection was accomplished using enhanced chemiluminescence and recorded with a quantitative digital imaging system (ChemiDoc XRS with Image Lab, Bio-Rad).

Cell Immunofluorescence

T-47D cells were grown on coverslips and exposed to different treatments. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) for 30 min at

room temperature. Cells were incubated with antibodies against p-N-WASP$^{S484/485}$ (Chemicon International) and p-paxillin^{Y118} and p-cortactin^{Y466} (Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with DyLight 594 and/or fluorescein-conjugated secondary antibody (FITC 1:150; Vector Laboratories). Cells were then incubated with Texas Red–phalloidin (Sigma-Aldrich) for 30 min. After washing, the nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and mounted with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was visualized using a Nikon Eclipse E200 microscope and recorded with a high-resolution DP70 Olympus digital camera.

Co-immunoprecipitation Assay

T-47D cells were harvested with ice-cold lysis buffer containing 20 mM Tris–HCl pH 7.4, 10 mM EDTA, 100 mM NaCl, IGEPAL 0.5%, 3 μl/ml protein inhibitor cocktail (Sigma Aldrich, P8340), 3.3 μl/ml phosphatase inhibitor cocktail (Sigma Aldrich, P0044), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 3 mM sodium orthovanadate. In lysis buffer, 500 μg/μl of protein were mixed with 2 μg of FAK, cortactin, or Arp2 primary antibody and incubated for 1 h at 4°C with gentle rocking. Then, 40 μl 1:1 of protein-A agarose (sc-2001, Santa Cruz Biotechnology) was added. The mixture was gently rocked for 2 h at 4°C and centrifuged at 12,000×*g* for 5 min at 4°C. The supernatant was removed, and the immunoprecipitates washed with 500 ml of 20 mM Tris–HCl pH 7.4, 10 mM EDTA, 150 mM NaCl, 1% IGEPAL, 1 mM Na_3VO_4, 50 mM NaF, 0.1 mg/l PMSF, 0.3 mg/l aprotinin, and 0.01% protease inhibitor mixture (Sigma-Aldrich). Immunoprecipitated proteins were separated under reducing and denaturing conditions by 10% SDS-PAGE and transferred to a PVDF membrane. Non-specific binding was blocked with 3% BSA in PBS–Tween 20. Membranes were incubated with anti-FAK, anti-cortactin, anti-Arp2, anti-Arp3, and p-Tyr antibodies.

Gene Silencing With RNA Interference

Synthetic small interfering RNAs targeting paxillin (siRNA paxillin), Cdc42 (siRNA Cdc42), and control siRNAs were purchased from Santa Cruz Biotechnology. SureSilencing shRNA Plasmid Human LHCGR (Cat KH01310G) and control shRNA were purchased from SuperArray Bioscience Corporation. The siRNAs were used at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). T-47D BC cells were treated 48 h after siRNA transfection. The efficacy of gene silencing was checked with western blot analysis and found to be optimal at 48 h. Control experiments demonstrating selectivity and efficacy of silencing of the different targets can be found in **Figures 1D**, **2A**.

Transfection Experiments

A dominant negative construct for cortactin (cortactin^{3YF}, a non-phosphorylated mutant of cortactin) was generously donated by Dr. John Cooper (Washington University School of Medicine, United States). The inserts were cloned in pcDNA 2AB Flag-cortactin 3YF (Tehrani et al., 2007). A mutant construct for N-WASP (N-WASP$^{\Delta\ VCA}$, N-WASP truncation mutant ΔVCA domain) that lacks the carboxy-terminal domain necessary to activate Arp2/3 complex (Kovacs et al., 2011) was provided by Dr. Alpha Yap (University of Queensland, Australia). The plasmids (10 μg) were transfected into T-47D cells using Lipofectamine 2000 (Invitrogen, United States). BC cells were treated 24–48 h after transfection. The efficacy of transfection was checked with western blot analysis and found to be optimal at 36 h.

Adhesion Assay

Five hundred thousand cells per well were seeded into six-well plates on coverslips previously coated with 1% sterile gelatin and exposed to different treatments. The cells were incubated at 37°C for 2 h. Non-adherent T-47D cells were then removed by gentle washing with PBS. The attached cells were fixed with 4% paraformaldehyde and stained with 10% ethanol/crystal violet for 20 min. Cells of attached images were captured and counted in 10 randomly chosen fields per well using a Nikon Eclipse E200 microscope coupled to a high-resolution CCD digital camera, as previously described (Flamini et al., 2014).

Cell Migration Assay (Wound-Healing Assay)

Cell migration was assayed with razor scrape assays. Briefly, a razor blade was pressed through the confluent T-47D BC cell monolayer into the plastic plate to mark the starting line. T-47D cells were swept away on one side of that line. Cells were washed, and 2.0 ml of RPMI 1640 containing steroid-deprived FBS and gelatin (1 mg/ml) was added. To prevent cell proliferation, cytosine β-D-arabinofuranoside hydrochloride (Sigma) (10 μM), a selective inhibitor of DNA synthesis that does not inhibit RNA synthesis, was used 1 h before the test substance was added. Migration was monitored for 48 h. Cells were digitally imaged, and the migration distance was measured using a Nikon Eclipse E200 microscope and recorded with a high-resolution DP70 Olympus digital camera. Percentage of migration was calculated and reported as a percentage of the control.

Cell Invasion Assay

Cell invasion was assayed using the BD BioCoat™ Growth Factor Reduced (GFR) Matrigel™ Invasion Chamber (BD Bioscience, United States). In brief, after rehydration of the GFR Matrigel inserts, the test substance was added to the wells. An equal number of control inserts (no GFR Matrigel coating) were prepared as control. Added into the inserts was 0.5 ml of T-47D cell suspension (2.5×10^4 cells/ml). Cytosine β-D-arabinofuranoside hydrochloride (Sigma) (10 μM), a selective inhibitor of DNA synthesis that does not inhibit RNA synthesis, was used 1 h before the test substance was added to prevent cell proliferation. The chambers were incubated for 48 h at 37°C, 5% CO_2 atmosphere. After incubation, the non-invading cells were removed from the upper surface of the membrane using cotton-tipped swabs. The cells on the lower surface of the membrane were then stained with Diff-Quick stain. The invading cells were observed and photographed under the microscope at 100 × magnification. Cells were counted in the central field of triplicate membranes.

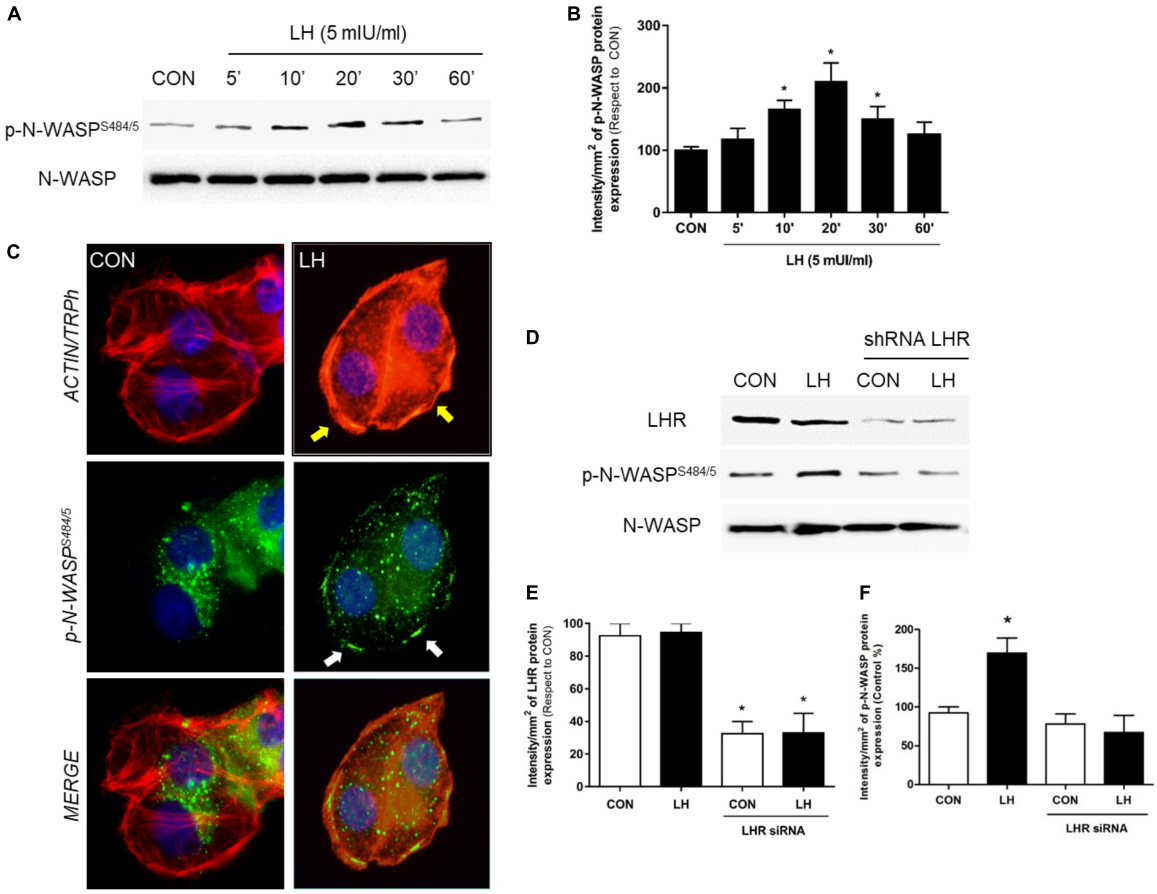

FIGURE 1 | LH signals to LHR, enhancing N-WASP phosphorylation. **(A)** T-47D cells were treated for different times (0–60 min) with LH (5 mIU/ml). The total cell amounts of wild-type N-WASP and phosphorylated N-WASP (p-N-WASP$^{S484/5}$) are shown by western blot analysis. **(B)** Phospho-N-WASP densitometry values were adjusted to N-WASP intensity and then normalized to the control sample. *$P < 0.05$ vs. control. **(C)** T-47D BC cells were treated with LH (5 mIU/ml) for 20 min. Cells were stained with phospho-N-WASP$^{S484/5}$ linked to fluorescein isothiocyanate (FITC, green), actin fibers with Texas Red phalloidin (red), and nuclei were counterstained with DAPI. Yellow arrows indicate actinic cytoskeleton reorganization to the periphery of the cell membrane. White arrows indicate membrane-localized p-N-WASP. **(D)** T-47D BC cells were transfected with shRNA vs. LHR or with vehicle, and protein analyses for LHR, N-WASP, and phospho-N-WASP$^{S484/855}$ were performed on cell lysates with or without treatment for 20 min with LH. The total cell amounts of wild-type LHR, N-WASP, and phospho-N-WASP$^{S484/485}$ are shown by western blot. **(E,F)** LHR and phospho-N-WASP densitometry values were adjusted to N-WASP intensity and then normalized to the control sample. The results are expressed as the mean ± *SD* of the measurements. *$P < 0.05$ vs. CON, control. All experiments were performed in triplicate; representative images are shown.

Statistical Analysis

Statistical analysis of the data was performed with one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple-comparisons test using GraphPad Prism 5.03 software. $P < 0.05$ was considered statistically significant. All values are expressed as mean ± *SD* of three independent experiments.

RESULTS

LH Triggers N-WASP Phosphorylation Through LH Receptor

N-WASP is essential in the regulation of actin nucleation, leading to changes in cell morphology and consequently stimulating cell migration, invasion, and metastasis. We have previously reported that N-WASP is phosphorylated after 17β-estradiol and triiodothyronine treatment (Sanchez et al., 2010; Shortrede et al., 2016; Uzair et al., 2019), increasing cell membrane structure formation implicated in cellular movement. Therefore, as a first approach to establish the extragonadal actions of LH on BC cell motility, T-47D cells were treated with follicular-phase levels of LH (5 mIU/ml) for different times (0–60 min) to analyze the expression and phosphorylation of N-WASP, a key regulator of actin cytoskeleton reorganization (Frugtniet et al., 2015). We found that LH promoted a rapid increase of N-WASP phosphorylation on Ser$^{484/485}$ in a time-dependent and transient manner. This effect was highest after 20 min and returned to basal levels after 60 min (**Figures 1A,B**).

In parallel, we performed an immunofluorescence assay to evaluate the cellular location of N-WASP after LH treatment

(5 mIU/ml, 20 min). Phosphorylated N-WASP$^{S484/485}$ was homogeneously distributed throughout the cytoplasm in the control cells. In cells exposed to LH, a rapid actinic cytoskeleton reorganization from the cytoplasm to the periphery of the cell membrane occurred (**Figure 1C**, yellow arrows). Also, p-N-WASP$^{S484/485}$ translocated to the edge of the membrane where it co-localized with actin fibers (**Figure 1C**, white arrows), promoting a thickening of the membrane (**Figure 1C**, black arrows) and inducing actinic nucleation.

In order to determine whether LHR is involved in the control of N-WASP, we silenced LHR with a specific shRNA. This resulted in a significant reduction of LHR expression along with a dramatic decrease in p-N-WASP$^{S484/485}$ after LH treatment (**Figures 1D–F**), thus confirming the role of LHR in N-WASP phosphorylation.

LH Induces a Dynamic Actin Cytoskeletal Reorganization via Src/FAK/Paxillin in BC Cells

Focal adhesion complexes (FA) undergo changes that ultimately lead to metastatic spread (Sanchez et al., 2010). We therefore analyzed proteins involved in FA, such as Src, FAK, and paxillin, in BC cells. We observed that a rapid pulse of LH for 20 min increases FAK^{Y397} and paxillin^{Y118} phosphorylation (**Figures 2A–C**). The presence of a specific FAKi reduced both FAK^{Y397} and paxillin^{Y118} phosphorylation, while the use of siRNA paxillin decreased paxillin^{Y118} phosphorylation (**Figures 2A–C**). These results suggest the existence of a signaling pathway involving FAK and paxillin in the regulatory mechanism of LH on BC cells.

We used immunofluorescence to evaluate the subcellular localization of p-paxillin^{Y118} in T-47D cells. We observed that LH increased paxillin^{Y118} phosphorylation and translocation from the cytoplasm to the membrane, generating FA (**Figures 2D–E**). Treatment with Src inhibitor (PP2), FAKi, and siRNA paxillin impaired these LH-induced events.

LH Signals to Cortactin Through a Paxillin-Dependent Signaling Pathway

Since cortactin is another key regulator of cell motility, migration, and invasion, we determined its phosphorylation in T-47D cells exposed to 5 mIU/ml of LH for different times (0–60 min) (**Figures 3A,B**). Maximal cortactin^{Y466} phosphorylation was found at 20 min; it receded to basal levels after 60 min. A similar pattern of phosphorylation was found with N-WASP (**Figures 1A,B**).

To test whether LH induces actin cytoskeleton reorganization via cortactin, we performed an immunofluorescence assay. Cells treated with LH for 20 min triggered cortactin^{Y466} phosphorylation and translocation to FA, where it co-localized with the thickening membrane (**Figures 3C,D**, yellow arrows). Treatment with siRNA paxillin and a dominant negative construct for cortactin (cortactin^{3YF}) impaired phosphorylation and the consequent translocation of p-cortactin^{Y466} to the plasma membrane (**Figures 3C,D**). These results suggest that LH signals to cortactin via paxillin.

LH Controls the Arp2/3 Complex via the FAK/Paxillin/Cortactin/N-WASP Cascade

The Arp2/3 complex is central to the rapid actin network formation toward the periphery of the cell membrane, which is required to build cellular structures for cell motility, such as filopodia and lamellipodia. This complex is activated by two regulator proteins, cortactin and N-WASP, which act alone or synergistically (Helgeson et al., 2014) to promote actin branching and enhance BC cell migration and invasion (Uzair et al., 2019). To continue identifying the signaling pathway triggered by LH on BC cells, we assessed the role of FAK, cortactin, N-WASP, and the Arp2/3 complex, which are the main regulators of FA and actin nucleation (Uzair et al., 2019). We performed two co-immunoprecipitation assays (IP) in BC cells treated with LH (5 mIU/ml, 20 min) in the presence or absence of PP2. We found that the basal interaction between FAK/cortactin and FAK/Arp3 was significantly reduced when cells were treated with LH. This effect was impaired by the use of the specific inhibitor PP2 (**Figure 4A**). In addition, we observed that LH reduced the interaction of cortactin/FAK and cortactin/Arp3 compared to control, but PP2 treatment blocked this reduction (**Figure 4B**). Our results suggest that, in basal condition, the FAK/cortactin/Arp3 subunit interacts in BC cells. After LH treatment, this interaction was partially dissociated, leading to FAK phosphorylation in Tyr^{397} via the Src kinase (**Figures 4A,B**). FAK phosphorylation in Tyr^{397} is fundamental for a conformational change that allows FAK protein to expose the Tyr$^{397/407/576/577/861/925}$ residues for autophosphorylation (McLean et al., 2005; Sanchez et al., 2010).

In parallel, we tested the role of several kinases and scaffold proteins involved in the signaling to the Arp2/3 complex. T-47D cells treated with LH increased Src^{Y419}, FAK^{Y397}, paxillin^{Y118}, cortactin^{Y466}, and N-WASP$^{S484/485}$ phosphorylation, and these increments were prevented by the use of PP2. Blockade with FAKi resulted in a visible inhibition of FAK^{Y397}, paxillin^{Y118}, cortactin^{Y466}, and N-WASP$^{S484/485}$ phosphorylation, whereas transfection with cortactin^{3YF} prevented phosphorylation of cortactin^{Y466} and N-WASP$^{S484/5}$ (**Figure 4C**).

We also assessed whether phosphorylation of cortactin and N-WASP may regulate the Arp2/3 complex after LH stimulation. We found that LH significantly increased N-WASP$^{S484/5}$ and Arp2^{T237} phosphorylation. Transfection with cortactin^{3YF} and the specific inhibitor for N-WASP (Wiskostatin) resulted in a reduction of N-WASP$^{S484/5}$ and Arp2^{T237} phosphorylation. The use of the Arp2/3 complex specific inhibitor (CK-666) also prevented Arp2^{T237} phosphorylation (**Figure 4D**). Furthermore, we demonstrated that Arp2 is activated after LH treatment, as shown by the increase of phospho-tyrosine in Arp2 immunoprecipitates (**Figures 4E,F**). This effect was abolished by CK-666. All these findings suggest a signaling cascade involving LHR, Src, FAK, paxillin, cortactin, and N-WASP

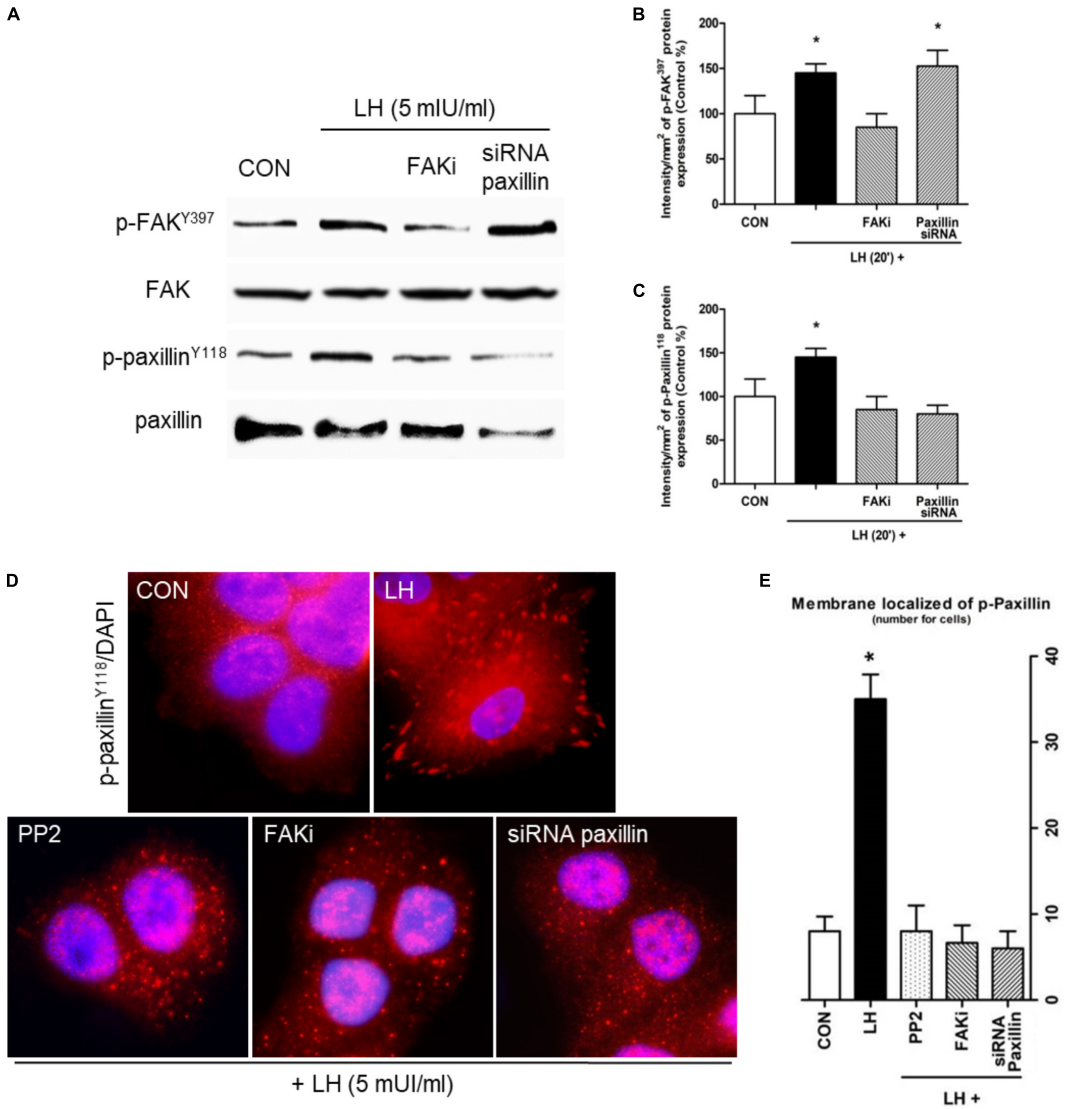

FIGURE 2 | LH promotes FAK and paxillin phosphorylation through LHR. **(A)** Cells were treated with LH (5 mIU/ml) for 20 min in the presence or absence of FAKi (1 μM) and siRNA paxillin. The total cell amounts of wild-type FAK and paxillin, phospho-FAK^{Y397}, and p-paxillin^{Y118} are shown by western blot. **(B,C)** Phospho-FAK^{Y397} and phospho-paxillin^{Y118} densitometry values were adjusted to FAK and paxillin intensity, respectively, and then normalized to the control sample. Results are expressed as the mean ± *SD*. $^{*}P < 0.05$ vs. control. **(D)** BC cells were stained vs. phospho-paxillin^{Y118} linked to DyLight 594 and nuclei counterstained with DAPI. **(E)** Quantification of the membrane-localized p-paxillin^{Y118} in the different conditions. Results are expressed as dots/cells (mean ± SD). $^{*}P < 0.05$ vs. CON, control. Membrane-localized p-paxillin^{Y118} was counted in 40 different cells. All experiments were performed in triplicate, and representative images are shown.

that mediates the regulatory effects of LH on the Arp2/3 complex in BC cells.

LH Effect on BC Cell Adhesion, Migration, and Invasion

Metastasis occurs through specific steps that result in alterations in the adhesion, migration, and invasion properties of tumor cells (Jiang et al., 2015). Thus, to relate the molecular action of LH on BC cell motility, we performed cell adhesion, migration, and invasion assays. Treatment with LH significantly enhanced the ability of T-47D cells to adhere to a gelatin matrix in comparison with the control cells (**Figure 5A**). The adhesion capacity of the cells was diminished when they were exposed to specific inhibitors or silencers, such as PP2, siRNA paxillin, cortactin^{3YF} and Wiskostatin compared to LH treatment alone (**Figure 5A**).

We then performed a wound-healing assay to evaluate cell migration in T-47D cells exposed to LH (5 mIU/ml). Treatment with LH significantly increased BC cell migration after 48 h of exposition compared to control cells (**Figure 5B**). Blockade of Src, FAK, paxillin, cortactin, N-WASP, and the Arp2/3 complex reduced the migration process, demonstrating the involvement of these proteins in LH-induced cell migration.

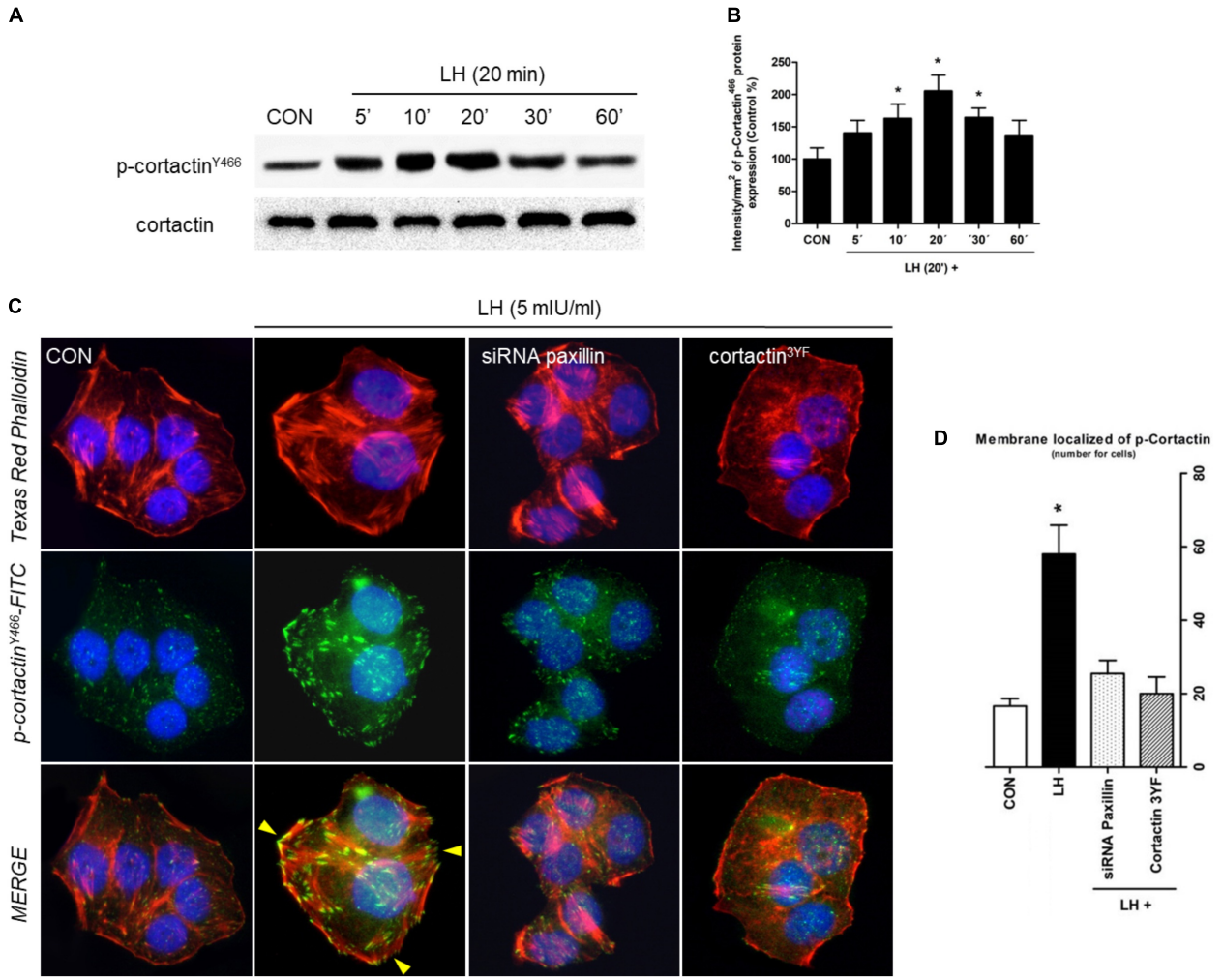

FIGURE 3 | LH induces dynamic actin remodeling in BC cells. **(A)** BC cells were treated for different times (0–60 min) with LH (5 mIU/ml). The total cell amounts of wild-type cortactin and phosphorylated cortactin (cortactin^{Y466}) are shown by western blot analysis. **(B)** Phospho-cortactin^{Y466} densitometry values were adjusted to cortactin intensity and then normalized to the control sample. $^*P < 0.05$ vs. control. **(C)** T-47D cells were treated with LH for 20 min in the presence or absence of a siRNA paxillin or cortactin^{3YF} construct. Cells were stained with phospho-cortactin^{Y466} linked to FITC, actin was stained with phalloidin linked to Texas Red, and nuclei were counterstained with DAPI. Yellow arrows indicate membrane-localized p-cortactin^{Y466}. **(D)** Quantification of the membrane-localized p-cortactin in the different conditions. Results are expressed as dots/cells (mean ± *SD*). $^*P < 0.05$ vs. CON, control. Membrane-localized p-cortactin^{Y466} was counted in 40 different cells. All experiments were performed in triplicate, and representative images are shown.

To determine the capacity of BC cells to invade the surrounding environment, we performed three-dimensional invasion assays using Matrigel. LH-treated cells showed an enhanced invasion after 24 h compared with control cells (**Figures 5C,D**). Inactivation of Src, paxillin, cortactin, Cdc42, N-WASP, and the Arp2/3 complex suppressed LH-induced BC cell invasion (**Figures 5 C,D**).

DISCUSSION

Breast cancer develops in a hormonal environment that determines tumor behavior and influences clinical response to therapy (Redig and McAllister, 2013). Most BCs express estrogen receptors, indicating estrogen dependence of the tumor (Tokunaga et al., 2014), and are treated with therapies that reduce estrogen levels or inhibit their receptors. Since gonadotrophins and their receptors are involved in estradiol synthesis, alterations in their signaling could modify estrogen levels and, consequently, influence BC progression. Some studies have reported that LH and LHR genes may suffer mutations that alter estrogen levels. Hence, exposure to LH may increase BC risk (Haavisto et al., 1995; Powell et al., 2003). Gonadotrophins have been proposed as modulators in BC development, but the available evidence is limited and inconsistent (Mann et al., 2003; Ziecik et al., 2007; Huhtaniemi, 2010).

The key findings of this work are that LH regulates the cell adhesion, migration, and invasion processes via the phosphorylation of fundamental actin cytoskeletal proteins in T-47D BC cells. As we recently reported, this process is

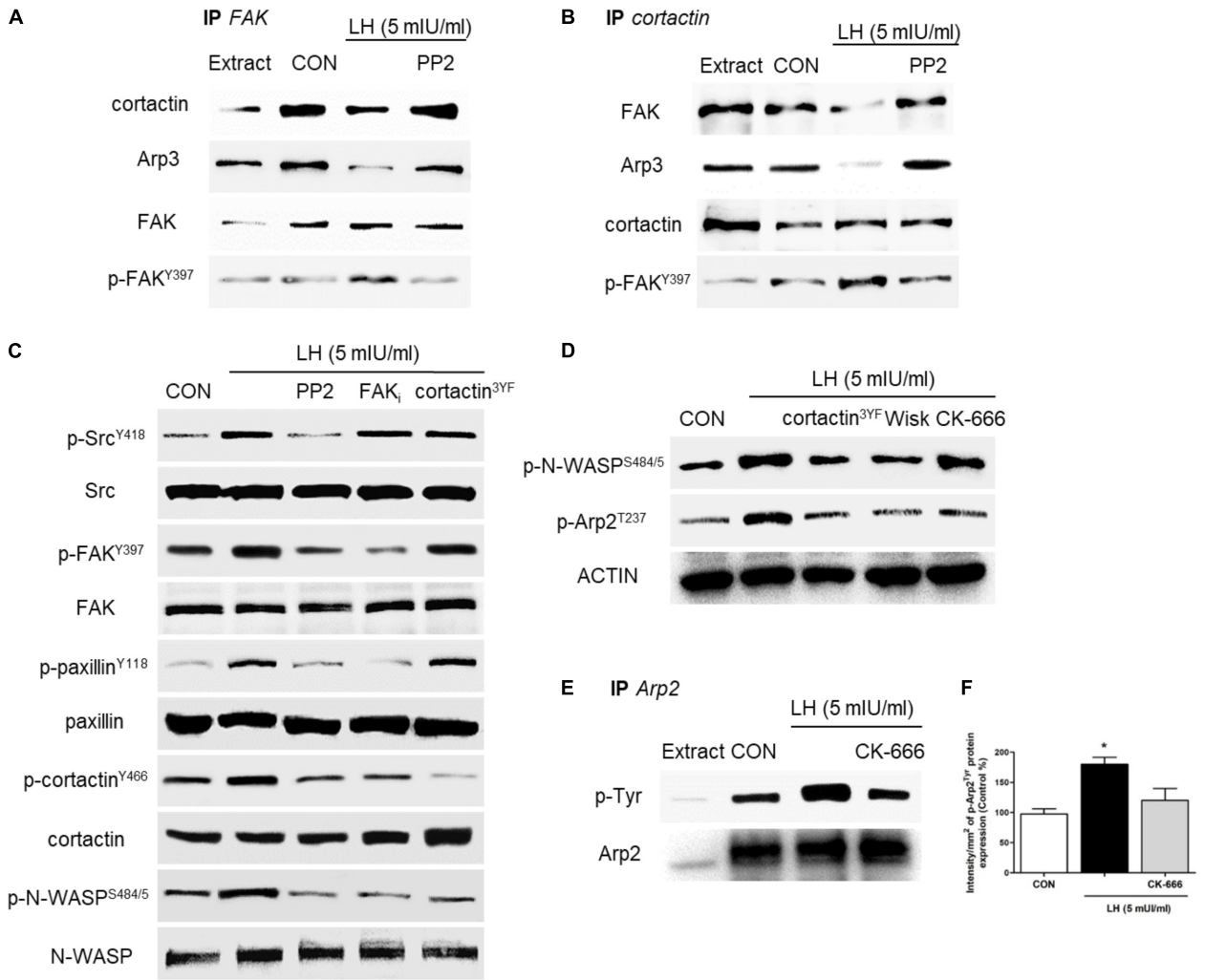

FIGURE 4 | LH stimulates the Src/FAK/paxillin/cortactin/N-WASP and Arp2/3 complexes. **(A,B)** BC cells were exposed to LH (5 mIU/ml) for 20 min in the presence or absence of the c-Src kinase inhibitor PP2 (10 μM). Cell protein extracts were immunoprecipitated with an antibody vs. FAK **(A)** and cortactin **(B)**. The immunoprecipitates (IPs) were assayed for co-immunoprecipitation vs. FAK, p-FAK, cortactin, and the Arp3 subunit. The membranes were re-blotted for the immunoprecipitated protein to show equal input. **(C)** Cells were incubated in the presence of LH (5 mIU/ml) for 20 min with or without PP2 (10 μM), FAK (1 μM), and/or dominant negative constructs vs. cortactin (cortactin^{3YF}). The total cell amounts of wild-type c-Src, FAK, paxillin, cortactin, and N-WASP or p-Src^{Y416}, p-FAK^{Y397}, p-paxillin^{Y118}, p-cortactin^{Y466}, and p-N-WASP$^{S484/485}$, respectively, are shown by western blot. **(D)** BC cells were transfected with the dominant negative constructs of cortactin (cortactin^{3YF}), the specific inhibitor of N-WASP (Wiskostatin, 10 μM), and/or the inhibitor of the Arp2/3 complex (CK-666, 4 μM) and incubated in the presence of LH (5 mIU/ml) for 20 min. Phospho-N-WASP$^{S484/485}$ and phospho-Arp2^{T237} were assayed by western blot analysis. **(E,F)** T-47D cells were exposed to LH (5 mIU/ml, 20 min) in the presence or absence of CK-666. Cell protein extracts were immunoprecipitated (IP) with an antibody vs. Arp2. IP was assayed for co-immunoprecipitation vs. p-Tyr. The membranes were re-blotted for the immunoprecipitated protein vs. Arp2 to show equal input. All experiments were performed in triplicate with consistent results; representative images are shown. *$P < 0.05$ vs. CON, control.

promoted by the recruitment of functional LH receptors that are expressed in these cells (Sanchez et al., 2016, 2018). LH, via LHR, triggers the recruitment of several kinase and scaffold proteins through a non-genomic pathway, leading to an increased phosphorylation and translocation of N-WASP and promoting BC movement. N-WASP is a key nucleation promoter factor; its deregulation has been involved in the invasion, intravasation, and metastasis of mammary tumors (Frugtniet et al., 2015). We have recently described that N-WASP promotes cell migration and invasion of BC cells after being activated by several hormones, such as triiodothyronine (Uzair et al., 2019) and estradiol (Sanchez et al., 2010; Shortrede et al., 2016). Our previous findings highlight the importance of N-WASP's actin branching ability, which could thus be considered a potential therapeutic target for invasion and metastasis inhibition in diverse types of cancers, including BC. Indeed, Hebbrecht et al. (2017) developed nanobodies that target the N-WASP^{VCA} domain responsible for Arp2/3 activation, thus causing a decrease in invadopodia formation in BC cells (Hebbrecht et al., 2017). This approach could lead to the development of a novel anticancer drug that limits the metastatic potential of cancer cells.

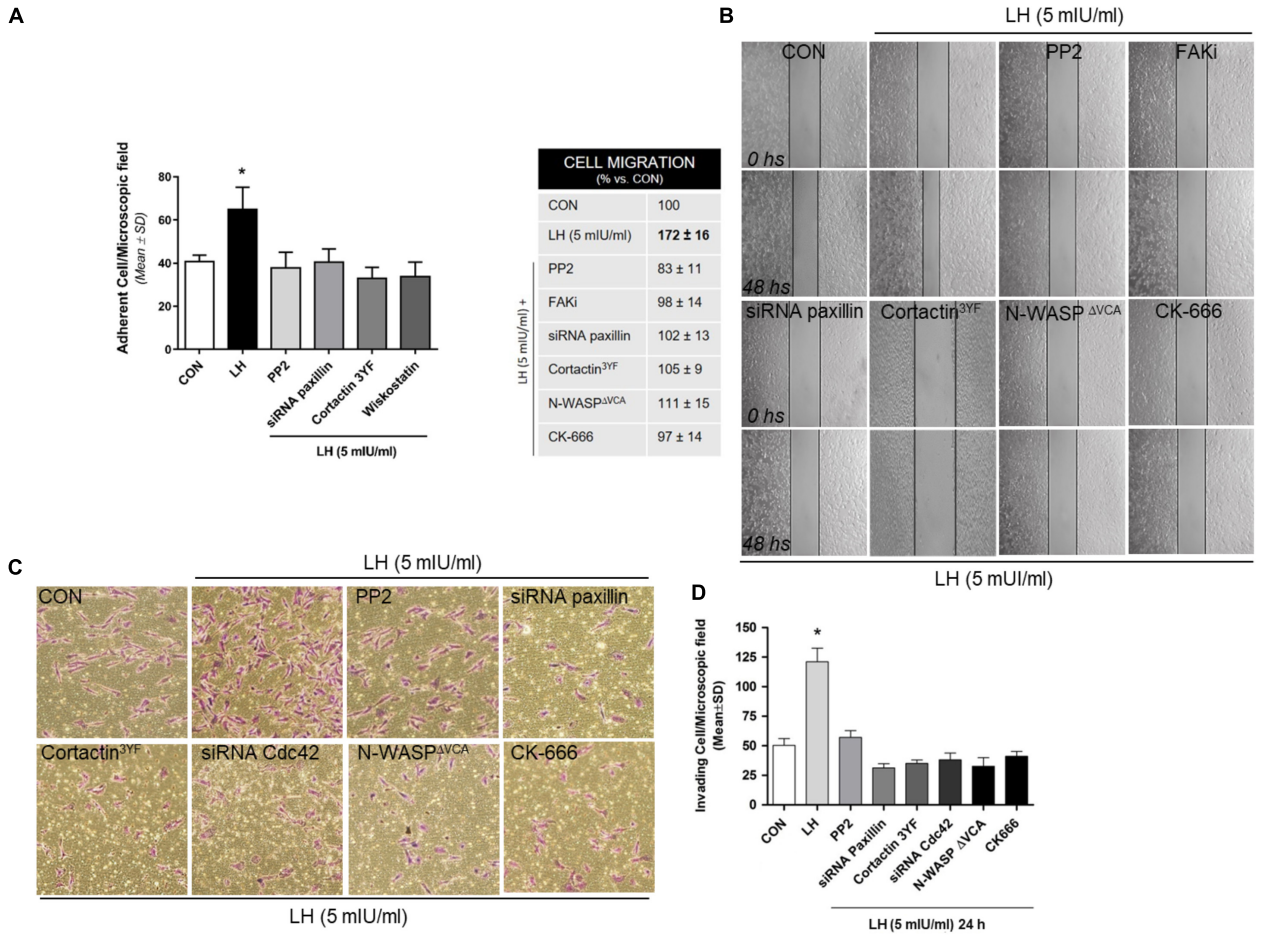

FIGURE 5 | LH modulates cell adhesion, migration, and invasion in BC cells. **(A)** Cells were treated with LH (5 mIU/ml) for 20 min in the presence or absence of the specific inhibitor PP2, Wiskostatin, or CK-666 and transfected with siRNAs vs. paxillin or mutant constructs for cortactin (cortactin^{3YF}). Numbers indicate quantity of attached cells per field. Experiments were performed in triplicate; $^*P > 0.05$ vs. control. **(B)** Cells were treated with LH (5 mIU/ml) for 48 h in the presence or absence of PP2, FAKi, and CK-666. Other cells were transfected with siRNAs toward a paxillin, cortactin^{3YF}, and N-WASP$^{\Delta VCA}$ construct. Representative images are shown. The upper black lines indicate the starting line, and the lower black lines the mean migration distance. Cell migration gap closure was quantified by the use of the NIH ImageJ software. Values are presented as a percentage of control. $^*P < 0.05$ vs. control. The experiments were performed in triplicate. **(C,D)** T-47D cells were treated with LH (5 mIU/ml) in the presence or absence of different inhibitors or siRNAs, as indicated in **(A,B)**, and siRNA Cdc42. BC cell invasion through Matrigel was assayed with invasion chambers. Representative images in chambers with Matrigel are shown. Invading cells were counted in the central field, and the graph indicates the mean number of invading cells ± *SD* from three separate experiments. $^*P < 0.05$ vs. CON, control.

We also evaluated the influence of LH on FA regulation. In T-47D cells, LH rapidly increases FAK and paxillin phosphorylation with the consequent translocation to FA, enhancing the formation of specialized cell membrane structures involved in cellular motility. We have previously reported that several hormones exert a regulatory effect on FA activation. The formation of FA is one of the first steps to impulse actin cytoskeleton reorganization and accomplish molecular motility (Sanchez et al., 2010; Shortrede et al., 2016; Uzair et al., 2019). Paxillin is a crucial component of FA. Once phosphorylated, it serves as a scaffolding molecule that mediates FA assembly and turnover (Lopez-Colome et al., 2017). Furthermore, it plays an important role in neoplastic transformation due to its ability to directly bind to several oncogene proteins, such as Src and FAK kinases, disrupting normal adhesion and growth factor signaling cascades necessary to impulse migration and invasion (Shortrede et al., 2016; Lopez-Colome et al., 2017). Several studies have reported that paxillin overexpression is associated with alterations and malignant progression of breast tumors (Madan et al., 2006; Short et al., 2007). Thus, paxillin may be used as a prognostic biomarker. It could, potentially, also have implications for therapeutic approaches targeted at preventing invasion.

We next explored the role of cortactin, another nucleation-promoting factor (NPF) that regulates the activation of the Arp2/3 complex either alone or synergistically with N-WASP (Takenawa and Suetsugu, 2007; MacGrath and Koleske, 2012). We determined that LH increases cortactin^{Y466} phosphorylation and translocation to FA in a Src/FAK/paxillin-dependent manner. Blockage of this signaling with specific inhibitors, such as siRNAs or mutant constructs, significantly reduces cell adhesion, migration, and invasion, which reveals the fundamental role of actin nucleation proteins in tumorigenesis. In recent years,

great progress has been made in understanding the role of cortactin and its molecular mechanism in cell motility. Cortactin has been considered as an invadopodial marker, as the ability of cancer cells to form invadopodia is often correlated with their invasive and metastatic capabilities (Meirson and Gil-Henn, 2018). miRNAs targeting the cortactin gene have been shown to inhibit invadopodial formation in human lung cancer (Li et al., 2018), whereas its overexpression enhances cell migration in oral cancer (Ramos-Garcia et al., 2019). Treatment with specific inhibitors of NPF could thus be an interesting approach to counteract metastasis. Dasatinib, a drug that disrupts the Src/cortactin signaling, is currently being tested as a therapeutic to block the action of NPF (Meirson and Gil-Henn, 2018).

Cortactin is an actin-binding and adaptor-scaffolding protein with binding sites for diverse target proteins, including Src, FAK, and the Arp3 subunit. It acts as a central molecule between FA formation and actin nucleation, which are key steps in the regulation of cell motility (MacGrath and Koleske, 2012; Tomar et al., 2012). Here, we demonstrate that LH disrupts the basal FAK/cortactin/Arp3 subunit interaction and that this effect can be prevented with the specific Src inhibitor (PP2). Similarly, we recently reported that sex steroid treatment diminishes the association between cortactin and the Arp3 subunit in cortical neuron cells (Uzair et al., 2020). We propose that the specific phosphorylation of these proteins affects their interaction as a consequence of physical impediments involving a cycle of binding, phosphorylation, and subsequent dissociation accompanied by FA turnover and cell movement.

Our results evidence that a tight regulation of Arp2/3 is crucial for cell migration, invasion, and metastasis. Several chemical inhibitors of the Arp2/3 complex, such as CK-666, are currently available; they arrest cell motility by impairing actin branching. Further research on inhibitors of Arp2/3 is needed to better understand the mechanisms of Arp2/3 activity, as its uncontrolled activation is correlated with the onset and progression of many diseases, including BC (Chanez-Paredes et al., 2019).

Gonadotrophins are an important component of the menopausal transition. During the latter, LH and FSH serum levels increase significantly over a period of 3–9 years (Landgren et al., 2004). This variation may lead to physiological changes that impact women's health. Diverse pathologies seem to be associated with elevated gonadotrophin levels. LH has previously been described as a cell enhancing migration and invasion by activating regulator proteins in ovarian (Mertens-Walker et al., 2010), endometrial (Noci et al., 2008), and breast cancer (Sanchez et al., 2016). Casadesus et al. (2007) described that increased LH levels are associated with declines in cognitive performance. Human chorionic gonadotrophin (hCG), which shares the α-subunit with LH and acts through the same LHR receptor (Choi and Smitz, 2014), increases gastric cancer cell proliferation through the PKA/c-Met signaling pathway (Zhao et al., 2018). Regarding LHR, its overexpression in endometrial cancer cells increases invasiveness, tumor development, and distal metastasis (Pillozzi et al., 2013). All this evidence highlights the diverse extragonadal actions of gonadotrophins and their physiological consequences.

Altogether, our experiments evidence the rapid signaling of LH through extragonadal LHR to the Src/FAK/paxillin/cortactin–N-WASP/Arp2/3 complex, enhancing BC cell adhesion, migration, and invasion. Our results highlight that LH could promote BC progression, particularly in postmenopausal

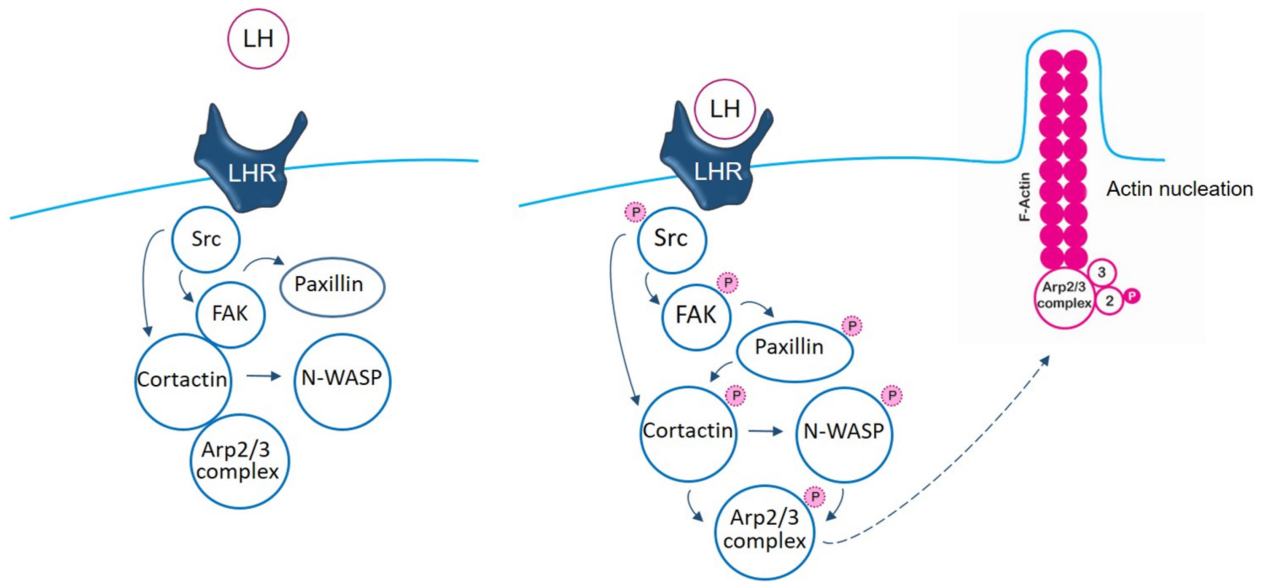

FIGURE 6 | Schematic signaling cascade triggered by LH, promoting BC cell adhesion, migration, and invasion. In control T-47D BC cells, there is an interaction between FAK/cortactin/Arp3 subunit. Binding of LH to LHR led to FAK/cortactin/Arp3 disruption, inducing FAK-Tyr^{397} phosphorylation, via Src kinase. This phosphorylation induces a conformational change that allows FAK protein its complete autophosphorylation. When FAK is phosphorylated, it signals to paxillin to finally modulate the Arp2/3 complex, via cortactin and N-WASP, enhancing actin nucleation and promoting BC cell motility.

women in which the absence of a menstrual cycle leads to an increase in circulating levels of gonadotrophins. Hence, a potential therapeutic approach in BC patients could be to regulate gonadotrophin levels.

There are widely available drugs that reduce the synthesis and release of LH/FSH via GnRH agonists and antagonists. GnRH agonists suppress sex steroid levels and are used as an adjuvant treatment of hormone-sensitive tumors, such as prostate or breast cancer (Chengalvala et al., 2003; Huhtaniemi et al., 2009). Prostate cancer patients are treated with LHR agonists as a first-line therapy to downregulate LHR expression in the pituitary gland, which leads to a reduced androgen synthesis (Liu et al., 2010). The same approach has been proposed to treat patients with LHR + urinary bladder invasive cancer (Szepeshazi et al., 2012). Some studies, however, suggest that patients treated with gonadotrophins to induce ovulation (Pappo et al., 2008) or with drugs that increase their circulating levels (Lerner-Geva et al., 2006; Orgeas et al., 2009) may have a higher BC risk. Assuming that gonadotrophins might promote BC, it would be primordial in postmenopausal women who have higher circulating levels of LH and FSH and the highest BC incidence (Bray et al., 2004).

The initial steps induced by LH to Src/FAK kinases, via LHR, have been previously reported by our group (Sanchez et al., 2016). The main contribution of this manuscript is that we reveal important new aspects based on the analysis of nuclear promoter factors, cortactin and N-WASP, to the control of the actin regulator Arp2/3 complex which participates in the actin nucleation process. We have continued elucidating the signaling pathway where LH triggers, via LHR, the phosphorylation of Src/FAK to the paxillin/cortactin–N-WASP/Arp2/3 complex, controlling BC cell adhesion, migration, and invasion (schematic **Figure 6**). Understanding the molecular mechanisms impulsed by LH in BC pathology is key for the development of original clinical strategies or new drugs that decrease the metastatic potential of gonadotrophin-sensitive cancers.

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.

AUTHOR CONTRIBUTIONS

JM carried out different experiments, cell culture, and treatments. IU performed immunofluorescence and migration assays. AC performed co-immunoprecipitation and invasion assays. MF was instrumental in funding the study and participated to the writing of the manuscript. AS planned and funded the project, supervised the experiments, wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Agency for Scientific and Technological Promotion (The Ministry of Science, Technology and Productive Innovation) PICT-2015-0864 grant to MF; PICT-2015-2175 and ROCHE S.A.Q. e I. 2020 grant to AS; and by Proyectos de Investigación de Unidades Ejecutoras (PUE), Resolución No. 1547/16.

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 09 February 2021
doi: 10.3389/fcell.2021.623809

Androgen-Driven Fusion Genes and Chimeric Transcripts in Prostate Cancer

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Androgens are steroid hormones governing the male reproductive development and function. As such, androgens and the key mediator of their effects, androgen receptor (AR), have a leading role in many diseases. Prostate cancer is a major disease where AR and its transcription factor function affect a significant number of patients worldwide. While disease-related AR-driven transcriptional programs are connected to the presence and activity of the receptor itself, also novel modes of transcriptional regulation by androgens are exploited by cancer cells. One of the most intriguing and ingenious mechanisms is to bring previously unconnected genes under the control of AR. Most often this occurs through genetic rearrangements resulting in fusion genes where an androgen-regulated promoter area is combined to a protein-coding area of a previously androgen-unaffected gene. These gene fusions are distinctly frequent in prostate cancer compared to other common solid tumors, a phenomenon still requiring an explanation. Interestingly, also another mode of connecting androgen regulation to a previously unaffected gene product exists via transcriptional read-through mechanisms. Furthermore, androgen regulation of fusion genes and transcripts is not linked to only protein-coding genes. Pseudogenes and non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs) can also be affected by androgens and *de novo* functions produced. In this review, we discuss the prevalence, molecular mechanisms, and functional evidence for androgen-regulated prostate cancer fusion genes and transcripts. We also discuss the clinical relevance of especially the most common prostate cancer fusion gene *TMPRSS2-ERG*, as well as present open questions of prostate cancer fusions requiring further investigation.

Keywords: androgen receptor, androgens, prostate cancer, fusion gene, fusion transcript, castration-resistant prostate cancer, *TMPRSS2:ERG*, lncRNA

OPEN ACCESS

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

Received: *30 October 2020*
Accepted: *14 January 2021*
Published: *09 February 2021*

Citation:
Scaravilli M, Koivukoski S and Latonen L (2021) Androgen-Driven Fusion Genes and Chimeric Transcripts in Prostate Cancer. Front. Cell Dev. Biol. 9:623809. doi: 10.3389/fcell.2021.623809

INTRODUCTION

Androgens are steroid hormones governing the development of male reproductive tract organs and secondary male sex characteristics, as well as functioning in the regulation of muscle mass, fat deposition, and function of steroid hormone-sensitive neurons (Werner and Holterhus, 2014). Androgens are also critical for normal physiology of the male reproductive tract organs. As such, androgens and the key mediator of their functions, androgen receptor (AR), have a leading role in several diseases such as androgen insensitivity syndrome and prostate cancer (Shukla et al., 2016).

The AR is a ligand-dependent transcription factor (**Figure 1**). In its inhibited form, AR is located in the cytoplasm, bound to HSP90 chaperone protein. The binding of androgens (testosterone and dihydrotestosterone or DHT) induces a conformational change in AR, leading to release of HSP90 and translocation of the receptor to the nucleus. In the nuclear compartment, homodimers of AR recognize and bind to specific DNA motifs termed androgen response elements (AREs). AREs are usually located at the promoter or enhancer regions of androgen-regulated genes, and binding of AR to them usually leads to activation of host gene transcription (**Figure 1**) (Lamb et al., 2014). However, the regulation of target genes by AR is context-dependent, influenced by other transcriptional regulators present at the same time, leading to differences of AR transcriptional output depending on e.g., cell type and disease state (Pihlajamaa et al., 2015).

The major disease where the AR plays a key role is prostate cancer, the second most commonly diagnosed malignancy in men worldwide and the fifth leading cause of cancer-related death (Torre et al., 2015). While radical prostatectomy and/or radiation therapy represent effective treatments for primary cancer that is still confined within the prostate, there currently exists no cure for the advanced form of the disease. Advanced, metastatic prostate cancer is treated with androgen deprivation therapy (ADT), exploiting the dependence of prostate cancer cells on androgen signaling. However, most of these cases inevitably progress to castration-resistant prostate cancer (CRPC) which remains uncurable (Watson et al., 2015).

In prostate cancer, AR is responsible for the activation of specific target genes that promote cancer initiation and progression. Recent investigations revealed that AR binding to its target elements in the genome is reprogrammed during prostate tumorigenesis (Pomerantz et al., 2015). The AR also plays a crucial role in the development of castration-resistant disease (Chen et al., 2004) and the majority of CRPC cases remain dependent on AR signaling. The persistence of AR activity in the low androgen level conditions can be achieved through several AR-dependent mechanisms, including AR overexpression caused by AR gene amplification or transcriptional upregulation, AR gene mutations that increase AR activity, and expression of constitutively active AR splice variants (Coutinho et al., 2016).

At the molecular level, prostate cancer is a heterogeneous disease as revealed by recent high-throughput sequencing studies (Armenia et al., 2018). Primary prostate cancer tends to be more driven by copy number aberrations than small nucleotide variants (Fraser et al., 2017). In addition, prostate cancer commonly harbors fusion genes (Kumar-Sinha et al., 2008). Although gene fusions are found at high frequency in several rare solid cancers, many common solid cancers harbor recurrent gene fusions only at low frequencies (Kumar-Sinha et al., 2015), making prostate cancer a curious exception. In fact, the most common type of genetic alteration in prostate cancer is a structural rearrangement between an androgen-regulated gene and a member of ETS family transcription factor gene. Fusion genes of this type are found in up to ∼70% of prostate cancer cases (Tomlins et al., 2005, 2006). The fusion events usually bring together an androgen-regulated 5′-part of a gene with critical 3′-protein-coding parts of the ETS genes (**Figure 2**). This results in androgen-induced overexpression of the ETS proteins which function as transcription factors regulating expression of genes involved in various cancer-related cellular processes, including proliferation, differentiation, transformation and apoptosis (Seth and Watson, 2005). In addition to the *ETS*-family gene fusions, several other types of fusion events also exist in prostate cancer, many of which are in a similar fashion androgen-regulated. However, not all fusions are androgen-regulated, and several 3′ fusion partners can be found fused to both androgen-regulated and androgen insensitive 5′ partners (Kumar-Sinha et al., 2015). Furthermore, not all fusion events that produce a novel androgen-regulated transcript occur at the chromosomal level. Recently, increasing evidence has revealed the presence of fusion transcripts occurring at the level of transcription and RNA, creating an interesting addition to the pool of androgen-regulated factors in prostate cancer.

MOST COMMON ANDROGEN-DRIVEN FUSION GENES IN PROSTATE CANCER

The most prevalent genetic rearrangement in prostate cancer involves the fusion of the androgen-regulated gene *TMPRSS2* with the ETS transcription factor *ERG*, which is estimated to occur in ∼50% of prostate cancer cases (Tomlins et al., 2005; Kumar-Sinha et al., 2008) being by far the single most common genetic fusion gene in solid tumors (PCAWG Transcriptome Core Group et al., 2020). In particular, more than 50% of these fusion events join the first intron of *TMPRSS2* with the third intron of *ERG* and lead to the most common *TMPRSS2:ERG* mRNA fusion transcript juxtaposing exon 1 of *TMPRSS2* with exon 4 of *ERG* (Weier et al., 2013) (**Figure 2**). Several other *TMPRSS2:ERG* fusion events with different junction sites have also been described to occur in prostate cancer clinical samples with lower frequency (Kumar-Sinha et al., 2008; Weier et al., 2013). The *TMPRSS2:ERG* fusion is present in the VCaP prostate cancer cell line (Tomlins et al., 2005) and has been more recently well-characterized in this model. It harbors an intragenic rearrangement between introns 1 and 4 of *TMPRSS2* and a subsequent intergenic rearrangement with intron 3 of *ERG* (Weier et al., 2013). Androgen stimulation of VCaP cells was found to cause a significant increase in ERG expression, whereas androgens did not affect ERG levels in fusion-negative LNCaP cells, confirming that the androgen regulation of *ERG* is caused by the fusion with *TMPRSS2* (Tomlins et al., 2005). Moreover, siRNA-mediated knock-down of ERG in VCaP cells significantly inhibited invasion without affecting proliferation (Tomlins et al., 2008a). Because of its prevalence and the availability of fusion-positive cell line model, the relevance of *TMPRSS2-ERG* for prostate cancer cells and the clinical manifestations of the disease has been widely studied and will be discussed in more detail in the following chapters.

TMPRSS2 is also involved in a small percentage of rearrangements with the ETS family members *ETV1* (Tomlins et al., 2005, 2007), *ETV4* (Tomlins et al., 2006), and *ETV5* (Helgeson et al., 2008) (**Figure 2**). In the *TMPRSS2-ETV1* fusion

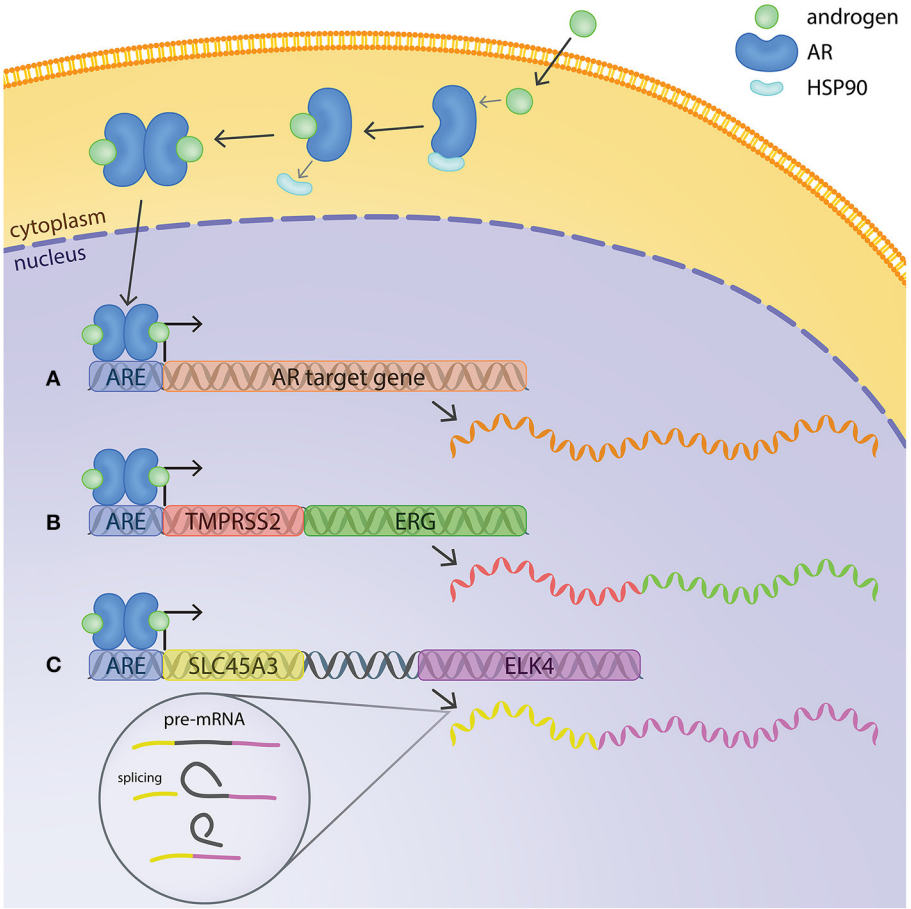

FIGURE 1 | Mechanisms of androgen regulation of gene expression in prostate cancer cells. In its inactive form, AR is located in the cytoplasm bound to HSP90. The binding of androgens induces a conformational change in AR, releasing Hsp90 and enabling translocation of AR to the nucleus. AR binds to androgen response elements (AREs) at the promoter or enhancer regions of androgen-regulated genes and regulates host gene transcription. **(A)** Example of a typical androgen-regulated gene, expression of which is induced when AR binds to the ARE at the promoter region. **(B)** *TMPRSS2:ERG* fusion gene at chromosomal level and mRNA transcript. The ARE on *TMPRSS2* brings *ERG* under transcriptional regulation of AR. **(C)** *SLC45A3* and *ELK4* are located adjacently in the same chromosome. Transcription by RNA-polymerase readthrough and mRNA splicing generates SLC45A3:ELK4 fusion transcripts.

event, exon 1 of *TMPRSS2* joins exon 4 of *ETV1*, resulting in a rearrangement very similar to the *TMPRSS2:ERG* fusion gene. Although LNCaP cells were reported to have a marked overexpression of ETV1 (Tomlins et al., 2005), they were not found to harbor the *TMPRSS2:ETV1* fusion (Tomlins et al., 2007). Lentiviral vector-mediated ETV1 overexpression in the immortalized prostate epithelial cell line RWPE was found to have no effect in cell proliferation, but to increase cell invasion (Tomlins et al., 2007). Moreover, androgen-mediated ETV1 upregulation in LNCaP cells was found to induce the expression of matrix metalloproteinases (MMPs) responsible for the degradation of the extracellular matrix and basal membrane. siRNA knock-down of ETV1 in androgen-dependent LNCaP cells as well as androgen-independent C81 cells was found to significantly reduce invasion and indicates a role of *ETV1* in disease progression (Cai et al., 2007). In the *TMPRSS2:ETV4* fusion event, a short regulatory region 8 Kb upstream of *TMPRSS2* and containing an androgen-regulated enhancer is juxtaposed to an intronic region immediately upstream of exon 3 of *ETV4*. This fusion gene has not been reported in prostate cancer cell lines, but native ETV4 expression is present in RWPE, PC-3 and DU145 cells and its downregulation inhibits proliferation, anchorage-independent growth and migration of prostate cancer cells (Pellecchia et al., 2012). More recently, co-expression of *ETV1* and *ETV4* was found in PC-3 and MDA-PCa-2b prostate cancer cell lines, representing models of advanced disease. Silencing of either ETS family member did not affect proliferation or apoptosis. However, *ETV4* knock-down cells presented a significant decrease in colony formation, whereas *ETV1* knock-down cells showed a significant decrease in cell invasion, confirming a relevant role of *ETV1* in disease progression (Mesquita et al., 2015). In the *TMPRSS2:ETV5* fusion event, exons 1 to 3 of *TMPRSS2* are fused to exon 2 of *ETV5*. Although this rearrangement is also absent in all known prostate cancer cell lines, functional studies of ETV5 overexpression performed on the RWPE model produced very similar results

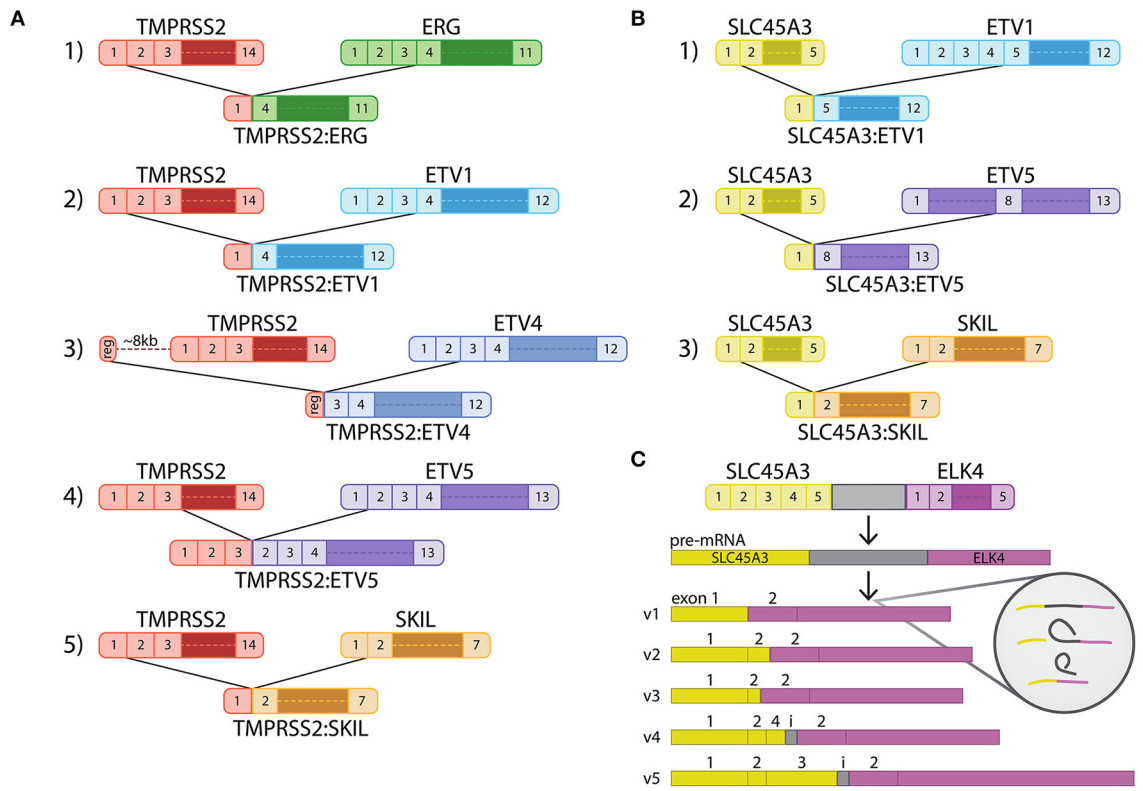

FIGURE 2 | Structure of the most common and relevant androgen-driven fusion genes in prostate cancer. **(A)** Fusion genes involving *TMPRSS2* as the 5′ partner. **(B)** Fusion genes involving *SLC45A3* as the 5′ partner. **(C)** Splice variants of the chimeric SLC45A3:ELK4 fusion transcript.

to those obtained with the RWPE-ETV1 overexpression model (Helgeson et al., 2008).

More recently, a novel fusion event was reported in about 1% of prostate cancer cases, juxtaposing exons 1 or 2 of *TMPRSS2* to exon 2 of the SMAD inhibitor and oncogenic factor *SKIL* and leading to its overexpression. Downregulation of SKIL expression in PC-3 cells was found to reduce cell growth, invasion and colony formation, whereas SKIL overexpression in RWPE cells showed a marked increase in invasive potential (Annala et al., 2015). *ETV1*, *ETV5*, and *SKIL* have also been found to be 3′-prime fusion partners with the prostate-specific, androgen-induced gene solute carrier family 45, member 3 (*SLC45A3*), also referred to as prostein, as a 5′ partner (Tomlins et al., 2007; Helgeson et al., 2008; Annala et al., 2015) (**Figure 2**). In these fusion events, exon 1 of *SLC45A3* is juxtaposed to exon 5 of *ETV1* (Tomlins et al., 2007), exon 8 of *ETV5* (Helgeson et al., 2008) or exon 2 of *SKIL* (Annala et al., 2015). These *SLC45A3-ETS* fusions and the fusions involving *SKIL* have not been reported in prostate cancer cell lines. However, a recent study has shown that concomitant treatment of LNCaP cells with androgens and irradiation induced TMPRSS2:ERG, TMPRSS2:ETV1 and SLC45A3:ETV1 transcript expression. Genomic sequencing confirmed the authenticity of the fusion events at chromosomal level, suggesting a potential role of AR in promoting tumor translocations (Lin et al., 2009).

Recently, Chakravarthi and colleagues identified a fusion occurring in around 30% of primary prostate cancer cases and involving the AR target gene *KLK4* as a 5′ partner and the non-coding pseudogene *KLKP1* (Chakravarthi et al., 2019). Both KLK4 and KLKP1 belong to the kallikrein family of serine proteases, and their genes are located adjacent to each other in a cluster of 15 genes on chromosome 19 (q13.33–q13.41), containing also the well-known *KLK3* (PSA). The KLK4-KLKP1 fusion is formed either by a trans-splicing mechanism or an in-frame fusion due to a microdeletion, leading to the fusion of the first two exons of *KLK4* with exon 4 and 5 of *KLKP1*. The resulting chimeric sequence predicts a 164–amino acid protein, of which the latter third is derived from KLKP1, leading to a conversion of the non-coding pseudogene to a protein-coding gene. Utilizing cell culture and chicken chorioallantoic membrane (CAM) assay, the expression of KLK4-KLKP1 fusion transcript was shown to affect cell proliferation, cell invasion, intravasation, and tumor formation (Chakravarthi et al., 2019).

In addition, transcriptome sequencing of ETS-fusion-negative prostate cancer revealed genetic rearrangements involving RAF-kinase family members, namely SLC45A3-BRAF and ESRP1-RAF1, recurrent in about 2% of advanced PCa cases, the former one being AR-regulated (Palanisamy et al., 2010). Ectopic expression of both chimeras in prostate epithelial cells showed an increase in oncogenic properties, and these RAF-kinase fusion

genes are generally associated with features of advanced disease, such as high Gleason score and castration resistance (Palanisamy et al., 2010; Beltran et al., 2013; Ross et al., 2016; Pederzoli et al., 2020). In addition to the fusion genes mentioned above, a significant number of other prostate cancer fusion genes have been described in clinical material (Tomlins et al., 2007; Kumar-Sinha et al., 2008; Weier et al., 2013). Most of these occur with very low frequencies and/or have not been studied further, often due to lack of cell models expressing them.

ANDROGEN-DRIVEN FUSION TRANSCRIPTS IN PROSTATE CANCER

Recent evidence has shown that, in addition to fusions at the genetic level, also chimeric fusion transcripts may be relevant for prostate cancer. As mentioned already above, the KLK4-KLKP1 fusion, combining sequences of a protein-coding gene and a pseudogene, may potentially be formed by a trans-splicing mechanism (Chakravarthi et al., 2019). So far, the most studied one in prostate cancer is the one where *SLC45A3* is involved in the generation of a chimeric transcript with *ELK4* (**Figure 1**). *ELK4* has been previously described as a growth-promoting androgen receptor target in LNCaP cells and has been shown to be overexpressed in a subset of prostate tumors (Makkonen et al., 2008). SLC45A3-ELK4 mRNA expression was later confirmed in prostate cancer samples, as well as in LNCaP cells, and five different mRNA variants of the chimeric transcript were described (Rickman et al., 2009). The most common form consists of exon 1 of SLC45A3 joined to exons 2 of ELK4, two other forms showed exon 1 and 2 of SLC45A3 joined to exon 2 of ELK4, a fourth variant includes exon 1, 2 and part of exon 4 of SLC45A3 (with a short intergenic sequence) joined to exon 2 of ELK4 and the last variant consists of exon 1–3 of SLC45A3 (including the same short intergenic sequence) fused to exon 2 of ELK4 (Rickman et al., 2009) (**Figure 2**). *SLC45A3* and *ELK4* are located adjacent to each other on chromosome 1 and in this case the generation of the chimeric transcript is not caused by a chromosomal rearrangement, as described for *TMPRSS2:ERG* and *SLC45A3-ETV1*, but rather by *cis*-splicing of adjacent genes/gene read-through (Zhang et al., 2012). Moreover, the SLC45A3-ELK4 transcript was shown to be induced by androgens and the chimeric mRNA, but not the wild-type ELK4, was found to drive androgen-dependent proliferation in prostate cancer cells (Zhang et al., 2012). Other examples of chimeric transcripts generated by a *cis*-splicing of adjacent genes were later described in prostate cancer samples. However, they involve genes that are not androgen regulated and are not specific to cancer but were also found in normal prostate (Qin et al., 2015, 2017), indicating that this mechanism is not unique to cancer cells.

CONNECTIONS OF AR-DRIVEN FUSION GENES AND LONG NON-CODING RNAS IN PROSTATE CANCER

When a fusion gene coding for a transcription factor is present and expressed, the transcriptional program of the prostate cancer cells is affected. For example, when AR drives expression of *ERG* from the common *TMPRSS2:ERG* fusion, both of these factors have been recently described to be involved in the regulation of long non-coding RNAs (lncRNAs) in prostate cancer. LncRNAs are >200 bp long RNAs that do not encode for protein end-products. They are known to play important roles in the regulation of gene expression and to be dysregulated in several types of human malignancies, including prostate cancer (Martens-Uzunova et al., 2014).

In a recent report, the transcriptomes of primary tumors, castration-resistant prostate cancers and benign prostatic hyperplasia controls were deep-sequenced with the aim of identifying prostate cancer-specific lncRNAs associated with more advanced stages of the disease. Interestingly, the expression of a novel lncRNA (*PCAT5*) was shown to be strongly correlated with *ERG* expression in ERG-positive primary tumors, as well as CRPCs (Ylipaa et al., 2015). The expression of PCAT5 was confirmed in the VCaP prostate cancer cell line harboring the *TMPRSS2:ERG* fusion and was significantly decreased as a result of siRNA-mediated knock-down of ERG. In addition, siRNA-mediated knock-down of PCAT5 expression in ERG-positive DuCaP cells significantly reduced cell growth (Ylipaa et al., 2015). Altogether, this study revealed the role of ERG in driving the expression of a growth-promoting ncRNA. In a later investigation, it was shown that several other prostate cancer-associated lncRNAs or PCATs are correlated with *ERG* expression and are significantly down-regulated by ERG knock-down in both VCaP and DuCaP cells (Kohvakka et al., 2020). Moreover, the majority of these PCATs were found to be also regulated by the AR, and analysis of previously published ChIP data (Pomerantz et al., 2015) revealed that most of the sites bound by ERG in PCATs were co-occupied by the AR (Kohvakka et al., 2020), confirming the previous findings by Yu and colleagues who reported the co-occupancy of AR and ERG in prostate cancer cells (Yu et al., 2010). Kohvakka and colleagues further demonstrated that the ERG- and AR-regulated lncRNA EPCART (ERG-positive PC-associated androgen responsive transcript) is functionally relevant for prostate cancer, as knockout of *EPCART* reduces migration and proliferation of LNCaP cells. Moreover, high expression of EPCART was associated with biochemical recurrence in prostatectomy patients and was found to be an independent prognostic marker in primary prostate cancer (Kohvakka et al., 2020).

Fusion events other than the *TMPRSS2:ERG* are also associated with the regulation of lncRNAs in prostate cancer. As described above, the SLC45A3-ELK4 fusion transcript is generated by *cis*-splicing of adjacent genes/gene read-through, rather than by actual genomic rearrangement (Zhang et al., 2012). A later study showed that this fusion transcript functions as a long non-coding chimeric RNA (lnccRNA). SLC45A3-ELK4 lnccRNA was found to be <1% of the expression level of the native ELK4 mRNA and therefore would only contribute to a minor percentage of the total ELK4 protein pool in prostate cancer cells. Selective siRNA-mediated knock-down of the fusion transcript proved effective at reducing cell proliferation rate, whereas ELK4 mRNA knock-down had no such effect. Moreover, a mutant SLC45A3-ELK4 transcript with an early stop codon, and therefore unable to generate a functional ELK4 protein

product, rescued the proliferation of siSLC45A3-ELK4 treated cells, highlighting the functional role of the chimeric RNA. SLC45A3-ELK4 mutant characterization showed that exon1 and exon3 of ELK4 are needed for the chimeric transcript to exert its rescue activity, and functional studies showed that the chimeric transcript represses the expression of *CDKN1A* and therefore promotes cell cycle progression from G1 to S phase (Qin et al., 2017).

MOLECULAR FUNCTIONS OF *TMPRSS2:ERG* FUSION GENE–THE MOST COMMON FUSION GENE IN PROSTATE CANCER

More than 90% of prostate cancer samples that overexpress ERG harbor the *TMPRSS2:ERG* fusion gene (Tomlins et al., 2005; Demichelis et al., 2007). The first investigations on the molecular mechanisms of ERG overexpression driven by the fusion gene were performed by Tomlins and colleagues. They showed that the *TMPRSS2:ERG* positive VCaP prostate cancer cells overexpressed the fusion product when treated with synthetic androgens (Tomlins et al., 2005). Further experiments were performed on RWPE benign, immortalized prostate epithelial cells infected with a lentivirus expressing the truncated ERG product analogous to the one deriving from the *TMPRSS2:ERG* fusion gene. These cells showed increased invasion capabilities, although no changes were observed in cell proliferation. However, the overexpression of ERG was not sufficient to cause transformation of the cells (Tomlins et al., 2008a). Moreover, Tomlins and colleagues generated transgenic mice expressing the same truncated product specifically in the prostate (under a probasin promoter) and showed that about 40% of mice developed PIN lesions, with disruption of the basal cell layer, but not prostatic adenocarcinoma (Tomlins et al., 2008a). Similar findings were also reported by Klezovitch and colleagues (Klezovitch et al., 2008).

Carver and colleagues also showed that *ERG* rearrangements are often associated with loss of the tumor suppressor *PTEN*, which had also previously been reported in almost 50% of HGPIN lesions (Bettendorf et al., 2008). Pten heterozygous mice overexpressing ERG specifically in the prostate (*Pten*$^{+/-}$*;Probasin-ERG*) developed prostatic adenocarcinoma, whereas *Pten*$^{+/-}$ mice only showed HGPIN lesions. Moreover, two genes involved in promoting cell migration and invasion (*CXCR4* and *ADAMTS1*) were found to be upregulated in the context of ERG overexpression (Carver et al., 2009). Another study performed on xenograft models using VCaP cells with knocked-down *ERG* expression (siRNA), showed a significant reduction in tumorigenicity, concomitant reduction in the expression of the oncogene *C-MYC* and upregulation of prostate epithelial differentiation genes *KLK3* and *SLC45A3* (Sun et al., 2008), suggesting that *ERG* overexpression has an oncogenic role in established prostate tumors, by inducing upregulation of *C-MYC* and repressing prostate epithelial differentiation. Later, Yu and colleagues performed chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-Seq) in LNCaP and VCaP cells. The results revealed that ERG and AR can co-occupy the same target genes and ERG functions as a repressor of AR-driven lineage-specific differentiation program. ERG also directly regulates the expression of the histone methyltransferase *EZH2,* by binding to its promoter and activating EZH2-mediated cell de-differentiation program (Yu et al., 2010). Interestingly, the transcriptional role of ERG described by Yu and colleagues is in contrast with *ETV1* transcriptional activity in prostate cancer. A recent study demonstrated that ERG and ETV1 can regulate a common set of AR target genes, but in an opposite fashion. In particular, ERG negatively regulates the androgen receptor (AR) transcriptional program, whereas ETV1 was found to upregulate genes involved in AR signaling and cooperates in its activation.

These findings were confirmed both *in vitro* and *in vivo* and pointed to a role of the ETV1 transcriptional program in the development of more aggressive disease and poorer clinical outcome (Baena et al., 2013).

Several investigations on the role of the fusion gene have been performed using cell line models of prostate cancer and non-tumorigenic prostate epithelial cells. Klezovitch and colleagues used immortal but non-tumorigenic BPH-1 human prostate epithelial cells with overexpression of a truncated form of ERG analogous to the one derived from the most common *TMPRSS2:ERG* fusion gene (*TMPRSS2* exon 1 and *ERG* exon 4). These authors, in contrast to what reported by Tomlins and colleagues (Tomlins et al., 2008a), found that ERG overexpression increased the growth of BPH-1-ERG compared to native BPH-1 cells. Moreover, BPH-1-ERG cells also showed higher invasion rate, but no effect was observed on migration. They reported similar results using RWPE-1 cells and in both cases, the addition of plasminogen activator inhibitor (PAI-1) completely eliminated the difference in invasion rate between native and ERG overexpressing cells (Klezovitch et al., 2008). These latest findings were confirmed by Tomlins and colleagues in VCaP cells. They showed using ChIP that urokinase plasminogen activator (*PLAU*) is a direct target of ERG in VCaP cells and that PAI-1 inhibited the invasion of VCaP cells (Tomlins et al., 2008a).

Cai and colleagues showed that *ERG* and *CXCR4,* which has been previously shown to contribute to the formation of bone metastases (Chinni et al., 2008), are both overexpressed in the fusion-positive VCaP cells, compared to PC-3 cells (Cai et al., 2010). Moreover, ChIP experiments performed in VCaP revealed that ERG binds within the *CXCR4* promoter in VCaP cells. Synthetic androgen (R1881) treatment of VCaP and LNCaP cells showed increased expression of both *ERG* and *CXCR4* in VCaP, but not LNCaP, suggesting that, indeed, the androgen-mediated *ERG* overexpression caused by the *TMPRSS2:ERG* fusion drives *CXCR4* expression in VCaP cells, confirmed by the lack of *CXCR4* induction when siERG-VCaP cells were treated with R1881. Androgen-induced *CXCR4* overexpression was also shown to increase invasiveness of VCaP cells (Cai et al., 2010). These results are in accordance with the data shown earlier by Carver and colleagues (Carver et al., 2009) and altogether reveal a role of the TMPRSS2:ERG fusion in the progression to advanced disease.

More recent efforts have revealed several other downstream effectors of *ERG* in prostate cancer. Stable knock-down of

ERG expression in VCaP cells was shown to lead to increased expression of active β1-integrin and E-cadherin, both responsible for cell adhesion (Gupta et al., 2010), supporting the previous finding of increased invasion in *ERG* overexpressing cells (Tomlins et al., 2008a) and highlighting a role of *ERG* in epithelial to mesenchymal transition (EMT). Moreover, *ERG* overexpression was shown to activate the Wnt signaling pathway via increased expression of the Wnt receptor *FZD4* (Gupta et al., 2010). Subsequently, stable populations of immortalized prostate epithelial cell lines BPH-1, PNT1B, and RWPE-1 overexpressing *ERG* were also shown to undergo EMT and acquire invasive characteristics with downregulation of cell adhesion molecules (E-cadherin) and upregulation of the EMT mediator integrin-linked kinase (ILK) and its downstream effectors Snail and LEF-1 (Becker-Santos et al., 2012). *ERG* expression driven by the *TMPRSS2:ERG* fusion was also associated with increased expression of *SOX9*, a transcription factor required for prostate development and involved in the maintenance of stem/progenitor cells. The correlation between ERG and SOX9 protein levels was verified in clinical samples of prostate cancer by IHC and in androgen-treated VCaP cells (Cai et al., 2013). Transgenic overexpression of *SOX9* in the prostate of mice caused the development of PIN, as observed previously with *ERG* overexpression by Tomlins et al. (2008a). Moreover, overexpression of *SOX9* in LNCaP cells significantly increased cell invasion and the same effect was observed in doxycycline-inducible SOX9-VCaP cells treated with siRNA for *ERG* after *SOX9* induction, suggesting that the invasive phenotype caused by *ERG* overexpression is mediated by *SOX9* activation. ChIP experiments showed that ERG binds and opens the regulatory region for an AR-regulated enhancer of *SOX9* expression (Cai et al., 2013). Another study connected *ERG* to miR-200c, a member of the miR-200 family. ERG was shown to directly repress the expression of miR-200c, by binding an ETS motif in its promoter. Decreased miR-200c expression causes reactivation of its target gene *ZEB1*, an important mediator of EMT (Kim et al., 2014).

CLINICAL IMPLICATIONS OF AR-DRIVEN FUSION GENES IN PROSTATE CANCER

Diagnostic and Prognostic Implications of TMPRSS2:ERG

Due to its high frequency in prostate cancer cases, the *TMPRSS2:ERG* fusion is considered to be relevant for the disease. However, its correlation to prostate cancer development and progression, as well as its clinical significance are not yet fully understood. Since the discovery of the fusion gene and its prevalence in the disease, several studies have also been performed with the aim of assessing its potential use as a diagnostic or prognostic marker with conflicting results.

The *TMPRSS2:ERG* fusion gene is not present in non-neoplastic prostate epithelium, but has been described in high-grade prostatic intraepithelial neoplasia (HGPIN) lesions (Cerveira et al., 2006; Park et al., 2010), suggesting that it might represent an early event in the development of prostate cancer (Perner et al., 2007). Subsequently, a seminal study on the biological role of aberrant ERG expression showed that, in fact, *ERG* rearrangements are not frequently found in HGPIN. Evaluation of HGPIN lesions with adjacent adenocarcinoma revealed that few cases showed rearrangements in the lesions and when present, they were always detected in the adenocarcinoma as well. Conversely, several cases harbored rearrangements in the adenocarcinoma, but not in HGPIN lesions (Carver et al., 2009). This suggests that the *TMPRSS2:ERG* fusion gene is an early event but associated with progression from HGPIN to adenocarcinoma.

Population-based studies using watchful-waiting patient cohorts showed a significant association between the presence of the fusion gene and poorer clinical outcome, defined as development of distant metastases or cancer-related death (Demichelis et al., 2007; Attard et al., 2008). Moreover, investigations performed on retrospective cohorts of prostate cancer patients undergoing radical prostatectomy revealed that the fusion gene is associated with more advanced tumor stage (Perner et al., 2006), earlier biochemical recurrence (Yoshimoto et al., 2008) and lymph node metastases and seminal vesicle invasion (Wang et al., 2006). In contrast, other retrospective studies showed opposite findings. The fusion gene was either associated with significantly longer biochemical recurrence-free survival (Saramaki et al., 2008; Hermans et al., 2009; Boormans et al., 2011), or not significantly associated with clinical outcome (Gopalan et al., 2009; Minner et al., 2011; Toubaji et al., 2011). These findings suggest that ERG overexpression driven by the *TMPRSS2:ERG* fusion might represent a highly diagnostic marker rather than prognostic. More recently, combined detection of urinary prostate cancer gene 3 (*PCA3*) and *TMPRSS2:ERG* has been shown to improve the sensitivity for prostate cancer diagnosis (Robert et al., 2013).

Subsequent investigations have shown that the fusion gene can generate several different TMPRSS2:ERG transcripts via alternative splicing (Hu et al., 2008; Wang et al., 2008). These variants can be grouped into two types. Type I variants encode full length ERG proteins, whereas type II variants encode a shorter version of ERG, lacking the ETS domain (Hu et al., 2008). Interestingly, type II splice variants were found to be more abundantly expressed in prostate cancer clinical samples, as well as in VCaP cells (Hu et al., 2008). The relative amount of type I/type II splice variants has also been found to correlate with clinical features of prostate cancer patients. A higher ratio of type I/type II was correlated with poorer outcome (Hu et al., 2008) and type II variants can function in a dominant-negative fashion by interfering with the transcriptional regulatory function of type I variants (Rastogi et al., 2014). Moreover, a recent study showed an association between increased retention of a 72 bp exon (exon 11) in the ERG transcript and more advanced stages of the disease (Hagen et al., 2014).

Role of TMPRSS2:ERG in Advanced and Metastatic Prostate Cancer

While the *TMPRSS2:ERG* fusion gene is an early event of prostate tumorigenesis and associated with progression from

HGPIN to adenocarcinoma, the role of this genetic alteration in more advanced and metastatic disease has also been recently investigated. Already in the initial report on the identification of the *TMPRSS2:ERG* fusion gene, fusion transcripts where detected in clinical specimens of metastatic, castration-resistant prostate cancer (Tomlins et al., 2005). Later, mouse xenografts derived from primary tumors, local and distant metastases of fusion-positive prostate cancers were used to study the expression of *ERG*. All androgen-dependent, fusion-positive xenografts were shown to overexpress *ERG*, including samples derived from local and distant metastases. In contrast, AR-negative and fusion-positive xenografts, all derived from metastases, did not express *ERG*, consistently with the model of AR-driven *ERG* expression from the fusion gene. These results demonstrate that *ERG* overexpression is also present in more advanced stages of the disease in AR-positive samples, but it is bypassed in androgen-independent tumors (Hermans et al., 2006).

Interestingly, a subsequent study reported that the NCI-H660 cell line, derived from a metastatic site of an extrapulmonary small cell carcinoma arising from the prostate, harbors the *TMPRSS2:ERG* fusion (Mertz et al., 2007). NCI-H660 cells are androgen-independent, as opposed to the androgen-dependent VCaP cell line derived from the vertebral bone metastasis of a hormone-refractory prostate tumor (Korenchuk et al., 2001). Moreover, NCI-H660 cells overexpress *ERG* from the *TMPRSS2:ERG* fusion gene in an androgen-independent fashion, suggesting that the fusion gene might have a role in AR-negative tumors as well (Mertz et al., 2007). *ERG* expression was later examined in samples of fusion-positive, androgen-dependent primary prostate cancers and CRPC samples, as well as in VCaP xenografts before and after castration, with the aim of establishing whether TMPRSS2:ERG fusion transcript expression is reactivated in CRPC after androgen deprivation therapy. The results showed that ERG expression levels were comparable in samples of fusion-positive primary tumors and fusion-positive, AR-overexpressing CRPCs, suggesting that AR overexpression at least partly reactivates TMPRSS2:ERG transcript expression in CRPC samples to levels similar to those present in the primary tumors. This was confirmed in VCaP cells/xenografts showing declining levels of ERG transcripts and protein in response to removal of androgens and reactivation of ERG expression in VCaP xenografts that relapsed and showed AR reactivation (Cai et al., 2009). Attard and colleagues used circulating tumor cells (CTCs), primary prostate tumor and CRPC samples from fusion-positive prostate cancers to study the *ERG* status and expression. The results showed that the *ERG* status in CTC matched the status in tumor samples, both primary tumors and CRPC. Moreover, *ERG* expression was detected and maintained in CRPC samples as well, indicating that hormone regulation of fusion-derived *ERG* expression is retained in the more advanced stages of the disease (Attard et al., 2009).

More recent studies examined the functional role of the *TMPRSS2:ERG* fusion gene in metastatic prostate cancer. Tian and colleagues used a newly established prostate cancer cell line (PC3c), derived from PC-3, to assess the role of the TMPRSS2:ERG fusion transcript in the formation of bone metastases. They used PC3c clones that overexpress the most common TMPRSS2:ERG transcript variant (TMPRSS2 exon 1 and ERG exon 4) at variable levels and also including the 72 bp exon 11 previously shown to be associated with more advanced stages of the disease (Hagen et al., 2014). PC3c cells, like the parental PC-3 cells, are both AR- and *TMPRSS2:ERG* fusion-negative, but unlike PC-3 cells, can rapidly generate mixed bone lesions *in vivo*, whereas PC-3 cells only generate pure osteolytic bone lesions. Therefore, PC3c represent a better model of prostate cancer bone metastasis as it recapitulates the commonly observed mixed lesions found in advanced prostate cancer clinical cases (Fradet et al., 2013). The results of TMPRSS2:ERG overexpression in PC3c revealed no effect on cell proliferation compared to native PC3c, but a significant increase in both cell migration and invasion in a dose-dependent manner. Moreover, global gene expression analysis of the TMPRSS2:ERG overexpressing clones compared to native PC3c showed a significant upregulation of the genes for the metalloproteinase *MMP9* and transmembrane glycoprotein, semaphorin co-receptor Plexin-A2 (*PLXNA2*). These genes were confirmed to be directly regulated by ERG overexpression. Knock-down experiments confirmed that *PLXNA2* is directly involved in the increased migration and invasion capabilities of prostate cancer cells (Tian et al., 2014), providing insight into the molecular mechanisms of action of the fusion transcript in metastatic disease. ERG binding sites in *MMP9* were also previously shown in ChIP experiments performed in VCaP cells (Yu et al., 2010). Similar functional results were obtained by Deplus and colleagues using the highly metastatic PC-3M cell line with stable luciferase expression (PC3-M-luc) and overexpression of the TMPRSS2:ERG fusion transcript. As previously shown by Tian et al. (2014), the overexpression of the fusion transcript did not affect cell proliferation, but increased cell migration and invasion compared to native PC-3M-luc (Yoshimoto et al., 2008). Moreover, a significant increase in tumor growth was observed when the cells were subcutaneously injected in mice, as well as a significant increase in tumor dissemination with intracardiac injection mimicking the hematogenous dissemination of metastatic cells (Deplus et al., 2017). These results provide further evidence on the role of *TMPRSS2-ERG* in advanced prostate cancer and specifically in tumor cell dissemination into the bone.

A more recent study provides yet more data supporting the involvement of the *TMPRSS2:ERG* fusion gene in bone metastasis progression. The same clones of TMPRSS2:ERG overexpressing PC3c cells described by Tian et al. (2014) were used for direct injection into the tibiae of SCID mice. Compared with native PC3c, the fusion-overexpressing cells generated larger bone formation areas and smaller bone destruction areas, overall larger bone volume and reduced osteoclast surface, indicating an enhanced osteoblastic phenotype and inhibition of osteoclastic destruction *in vivo* (Delliaux et al., 2018). Overexpression of TMPRSS2:ERG was found to induce the expression of the osteoblastic markers Collagen Type I Alpha 1 Chain (*COL1A1*) and Endothelin-1 (*ET-1*), responsible for improved acquisition of a bone-like phenotype in cancer cells (osteomimicry), helping the cancer cells survive in the bone microenvironment (Delliaux et al., 2018). Altogether, the data from these latest studies reveal

an important role of *ERG* in the dissemination of metastatic cells, the seeding to the bone as a preferential metastatic site and the generation of metastatic lesions in prostate cancer.

Fusion Co-occurrence and Multifocal Nature of Prostate Cancer

Prostate cancer is a heterogeneous disease which very often harbors multiple cancer foci within the same gland. It is well-established that different foci are histologically and molecularly heterogeneous, suggesting that they are clonally independent (Wise et al., 2002; Arora et al., 2004). The study of fusion genes in the context of multifocal disease has provided significant insight into tumor clonality [recently reviewed in Pederzoli et al. (2020)]. Assessments of TMPRSS2 rearrangements by fluorescence *in situ* hybridization (FISH) in separated foci of prostate cancers revealed interfocal heterogeneity and intrafocal homogeneity, indicating that individual foci are the result of clonal expansion (Mehra et al., 2007). Similar results were shown by FISH analysis of TMPRSS2-ERG rearrangements in multifocal prostate cancers (Barry et al., 2007). TMPRSS2-ETS rearrangements were later characterized in prostate cancer metastases and different metastatic sites from the same patients were found to harbor the same molecular sub-type of gene fusion events, indicating clonal expansion of advanced disease from a single primary focus (Mehra et al., 2008).

Profiling studies of fusion genes in multifocal disease are also important to evaluate co-occurrence of these alterations. Other FISH analyses of recurrent ETS gene rearrangements in multifocal prostates showed complex patterns of alterations, with both rearranged and un-rearranged foci and multiple ETS rearrangements within the same gland (Clark et al., 2008). Moreover, these fusion events were found to be mostly mutually exclusive between foci and might represent effective clonal markers. However, exceptions were observed with multiple ETS rearrangements within the same tumor focus (Svensson et al., 2011). More recently, several investigations have shown that ETS gene fusion exclusivity or co-occurrence in prostate cancer is associated with several other factors and aberrations. Outlier expression of *SPINK1* had been reported in a subset of ETS-negative prostate cancer samples exclusively (Tomlins et al., 2008b). Later, ERG/SPINK1 immunohistochemistry analyses performed in different foci of prostate cancer samples revealed that *ERG* and *SPINK1* overexpression were mutually exclusive in all tumor foci (Fontugne et al., 2016). In another report, it was found that 17% of prostate cancer cases with multifocal tumors showed both *ERG* and *SPINK1* overexpression within different regions of either the same tumor focus or different foci, but not in the same tumor cells (Lu et al., 2020). Deletions in *CHD1* and *MAP3K7*, and mutations in *SPOP*, *FOXA1*, and *IDH1* were also found to be associated with the ETS-fusion negative subtype (Liu et al., 2007; Barbieri et al., 2012; Grasso et al., 2012; Cancer Genome Atlas Research Network, 2015). Interestingly the *SKIL* fusions described in Annala et al. (2015) and the RAF-kinase fusions described in Palanisamy et al. (2010) were identified in analyses performed on ETS rearrangements-negative cases. As many AR target genes are also regulated by ERG (Yu et al., 2010; Zhang et al., 2019), the ERG fusion positive cancers may have correlating expression of the androgen-driven fusion transcripts due to overexpression of *ERG*. For example, correlative analysis with other ETS gene fusions showed that KLK4-KLKP1 expression is associated with ERG but not ETV1, ETV4, or ETV5 (Chakravarthi et al., 2019). This may be explained by the presence of a strong ERG binding site at the fusion junction, suggesting that the expression of the KLK4-KLKP1 fusion gene is regulated by ERG in addition to AR. The diverse molecular heterogeneity within the ETS fusion-negative subtype, its clinical significance, and implication in designing novel therapeutic strategies has been recently reviewed in Bhatia and Ateeq (2019).

Utility of Androgen-Driven Fusions in Prostate Cancer Diagnostics and Treatment

Tumor-specific gene fusions can serve as diagnostic biomarkers or help define molecular subtypes of tumors. For example, gene fusions involving ETS transcription factors have been utilized in diagnostic applications, such as with detection of TMPRSS2-ERG fusion transcripts in urine samples or CTCs from patients or ERG protein by immunostaining in biopsies [reviewed recently in Kumar-Sinha et al. (2015), Berg (2016), and Garcia-Perdomo et al. (2018)]. Despite the recently increased molecular understanding and array of prostate cancer molecular biomarkers available, molecular subtyping of prostate cancer with clinically relevant treatment stratification based on fusion genes and other genetic aberrations remains a challenge (Kohaar et al., 2019). In general, expression of the AR-driven fusions is inhibited along other AR targets by antiandrogens or androgen deprivation, but specific means to target the fusion products and their effects are rare (Bhatia and Ateeq, 2019; Pederzoli et al., 2020).

As the TMPRSS2-ERG fusion is the most common alteration in prostate cancer, molecular targeting of it has gained attraction as a potential therapeutic strategy. Recent examples include the work of Wang and colleagues, who identified a series of peptides that interact specifically with the DNA binding domain of ERG, leading to proteolytic degradation of the ERG protein, and attenuation of ERG-mediated transcription, chromatin recruitment, protein-protein interactions, cell invasion and proliferation, and tumor growth (Wang et al., 2017). Butler and colleagues identified and characterized a new class of small molecule ERG antagonists through rational *in silico* methods, demonstrating that a small molecule targeting the ERG-ETS domain suppressed its transcriptional activity and reverse transformed the characteristics of prostate cancers aberrantly expressing *ERG* (Butler et al., 2017). Treatment of prostate cancer cells with the USP9X inhibitor WP1130 resulted in ERG degradation both *in vivo* and *in vitro*, impaired the expression of genes enriched in *ERG* and prostate cancer relevant gene signatures, and inhibited growth of ERG-positive tumors in mouse xenograft models (Wang et al., 2014). Mohamed and colleagues screened small-molecule libraries for inhibition of ERG protein in TMPRSS2-ERG harboring VCaP cells and

identified a small molecule that selectively inhibits erg-positive cancer cell growth (Mohamed et al., 2018).

On the basis of the interaction of ERG and other ETS fusions with the DNA repair proteins PARP1 and DNA-PKc, use of PARP inhibitors has shown initial promise and is being tested in ETS fusion-positive prostate cancers [reviewed in Kumar-Sinha et al. (2015) and Pederzoli et al. (2020)]. Going further downstream to find effective targets, characterization of the ERG-regulated kinome identified *TNIK* as a potential therapeutic target in ERG-fusion gene positive prostate cancer (Lee et al., 2019). Another small molecule inhibitor termed YK-4-279 was shown to be effective against ETV1 activity. In xenografts models YK-4-279 significantly reduced both primary tumor growth and metastasis to the lungs (Rahim et al., 2014).

While the therapeutic targeting of transcription factor oncogenes remains challenging, tumors with fusions involving therapeutically targetable genes, most often kinases, often have the strongest implications in personalized treatment of cancer patients. Amongst prostate cancer fusion genes, especially the effects of androgen-regulated SLC45A3-BRAF and a non-androgen-regulated ESRP1-RAF1 are targetable. The effects of ectopic expression of these fusion genes were studied in RWPE benign immortalized prostate epithelial cells and resulted in increased proliferation, invasion and anchorage-independent growth, which were sensitive to RAF and MEK inhibitors (Palanisamy et al., 2010). These results indicate that RAF-fusion-positive patients may respond to these drugs regardless of AR regulation of the fusion gene. Despite the low recurrence (1–2% in Caucasian, 4–6% in an Indian cohort (Ateeq et al., 2015), screening of these actionable RAF alterations could be beneficial in disease management of RAF-fusion-positive patients (Palanisamy et al., 2010; Bhatia and Ateeq, 2019; Pederzoli et al., 2020). Several FGFR inhibitors currently in clinical trials represent potential therapeutics for cancers harboring FGFR fusions [reviewed in Parker et al. (2014) and Krook et al. (2020)]. A rare interchromosomal fusion of *SLC45A3* with *FGFR2* in which the *SLC45A3* non-coding exon 1 is fused to the intact coding region of *FGFR2* has been found from a brain metastasis of a prostate cancer patient (Wu et al., 2013), indicating that there are also prostate cancer patients that likely benefit from these FGFR inhibitors. Further rare and potentially targetable, AR-driven fusions include for example PIK3C family gene fusion ACPP-PIK3CB and R-spondin fusion GRHL2-RSPO2 (Robinson et al., 2015).

Prostate cancer xenografts play a central role in pharmacological testing of potential drugs. The VCaP cell line, due to the TMPRSS2-ERG fusion gene it harbors, has been widely utilized in xenograft drug studies. For example, TMPRSS2-ERG harboring VCaP bone xenograft models were shown to better respond to enzalutamide treatment, suggesting that ERG expression status in tumors could help stratify patients for enzalutamide therapy (Semaan et al., 2019). TMPRSS2-ERG-targeted gene silencing therapy using liposomal nanovectors suppressed tumor growth in a VCaP xenograft model and enhanced the efficacy of docetaxel chemotherapy (Shao et al., 2020). While TMPRSS2-ERG activates NO-cGMP signaling in prostate cancer cells, sGC inhibitor treatment repressed tumor growth in TMPRSS2-ERG-positive VCaP xenograft models and acted in synergy with enzalutamide, the potent AR antagonist (Zhou et al., 2019). In the future, more of the specific marker-driven therapies are likely to be developed, especially through utilization of patient-derived 3D cultures as well as xenografts (PDXs) [recently reviewed in Kato et al. (2020), Palanisamy et al. (2020), and Risbridger et al. (2020)]. Patient-derived 3D cultures include spheroids and organoids, which are applicable in high throughput screening of e.g., drug libraries, while PDX models entail engrafting patient tissue in immunocompromised mice [reviewed in Kato et al. (2020) and Risbridger et al. (2020)]. Although an intact immune system against the tumor is missing from the PDXs, this experimental model retains many other valuable properties of tumor tissue and *in vivo* environment and is thus valuable in developing new drugs and selecting appropriate treatment strategies for prostate cancer patients. In terms of prostate cancer fusion genes, the expression of *ERG* has been shown to be retained in the PDXs along with other molecular, histopathologic, and genomic characteristics (Palanisamy et al., 2020), indicating PDXs to be a valuable strategy to assess fusion-specific therapeutic options in the future.

CONCLUSIONS

The frequent gene fusions in prostate cancer are a curiosity amongst solid tumors. Why and how this particular tumor type benefits so much from these rearrangements for them to be so frequent are still open questions. While the benefit with certain fusions may clearly result from *de novo* expression of a cancer driver protein, for some fusions the advantage seems not as straightforwardly explained nor convincingly supported by functional data. Especially, despite a lot of effort, the field has yet to pinpoint why and how TMPRSS2-ERG fusion is an early event in prostate cancer development, yet the most significant functions of it seem concentrated in the phase of metastatic disease. The PCAWG Consortium recently reported that, amongst their 3,540 fusion events identified in 1,188 pan-cancer samples studied, 82% were associated with specific genomic rearrangements (PCAWG Transcriptome Core Group et al., 2020). For the remaining fusions, it is possible that the relevant genomic rearrangements have not been detected, or that fusions occur at the RNA level. Thus, up to a fifth of chimeric fusion transcript types may result from a trans-splicing or read-through event, which suggests that a significant number of non-genetic fusions are present also in prostate cancer. Furthermore, the existence of transcriptional read-through mechanisms suggests that, in addition to transcriptional deregulation, also splicing and RNA-binding regulatory mechanisms are functionally relevant for fusion transcript expression in prostate cancer.

The case of SLC45A3-ELK4 fusion has proven that it is possible for a chimeric RNA to function as a ncRNA, even though the 3′ fusion partner is initially protein-coding. Considering that chimeric transcripts may have acquired *de*

novo structures and functions, it is possible that also some of the other fusion transcripts may have non-coding functions yet to be discovered. This is supported by the notion that up to 20% of expressed prostate cancer fusion transcripts are non-canonical, with one or both transcripts in antisense orientation (Vellichirammal et al., 2020). Furthermore, according to the data by Dehghannasiri and colleagues, up to 10% of prostate cancer fusions involve lncRNAs as the other partner (Dehghannasiri et al., 2019), making it likely that more AR-driven lncRNA fusions will be discovered. Thus, the fascinating field of prostate cancer fusions will presumably keep us entertained also in the foreseeable future.

AUTHOR CONTRIBUTIONS

MS and LL wrote the manuscript. SK designed the figures. MS, LL, and SK edited the manuscript. All authors have approved the final version of the manuscript.

FUNDING

The work of the authors was supported by grants from Sigrid Juselius Foundation, Cancer Society of Finland, Foundation of the Finnish Cancer Institute, and Academy of Finland (grant no 317871).

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 18 March 2021
doi: 10.3389/fcell.2021.630503

Growth Hormone/Insulin Growth Factor Axis in Sex Steroid Associated Disorders and Related Cancers

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To date, almost all solid malignancies have implicated insulin-like growth factor (IGF) signalling as a driver of tumour growth. However, the remarkable level of crosstalk between sex hormones, the IGF-1 receptor (IGF-1R) and its ligands IGF-1 and 2 in endocrine driven cancers is incompletely understood. Similar to the sex steroids, IGF signalling is essential in normal development as well as growth and tissue homoeostasis, and undergoes a steady decline with advancing age and increasing visceral adiposity. Interestingly, IGF-1 has been found to play a compensatory role for both estrogen receptor (ER) and androgen receptor (AR) by augmenting hormonal responses in the absence of, or where low levels of ligand are present. Furthermore, experimental, and epidemiological evidence supports a role for dysregulated IGF signalling in breast and prostate cancers. Insulin-like growth factor binding protein (IGFBP) molecules can regulate the bioavailability of IGF-1 and are frequently expressed in these hormonally regulated tissues. The link between age-related disease and the role of IGF-1 in the process of ageing and longevity has gained much attention over the last few decades, spurring the development of numerous IGF targeted therapies that have, to date, failed to deliver on their therapeutic potential. This review will provide an overview of the sexually dimorphic nature of IGF signalling in humans and how this is impacted by the reduction in sex steroids in mid-life. It will also explore the latest links with metabolic syndromes, hormonal imbalances associated with ageing and targeting of IGF signalling in endocrine-related tumour growth with an emphasis on post-menopausal breast cancer and the impact of the steroidal milieu.

Keywords: cancer, IGF, sex steroids, endocrine, metabolism

OPEN ACCESS

Edited by:
Isabel R. Schlaepfer, University of Colorado, United States

Reviewed by:
Gabriella Castoria, Second University of Naples, Italy
Haim Werner, Tel Aviv University, Israel
Liou Y. Sun, University of Alabama at Birmingham, United States

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

Received: *17 November 2020*
Accepted: *11 February 2021*
Published: *18 March 2021*

Citation:
Bleach R, Sherlock M, O'Reilly MW and McIlroy M (2021) Growth Hormone/Insulin Growth Factor Axis in Sex Steroid Associated Disorders and Related Cancers. Front. Cell Dev. Biol. 9:630503. doi: 10.3389/fcell.2021.630503

INTRODUCTION

The hypothalamic-pituitary (HP) axis is the central regulator of endocrine action, controlling the function of a number of endocrine glands including thyroid, adrenal, gonadal and the growth hormone (GH)/insulin growth factor-1 (IGF-1) system, which modulate a myriad of physiological processes. The HP axis integrates various stimuli, including those generated internally such as energy levels, stress, inflammation and also in response to external stimuli (dark/light cycles and temperature) (Petrescu et al., 2018) and orchestrates endocrine outputs which control numerous physiological processes. The role of endocrine hormones and growth factors are interlinked, often being involved in the same cellular functions leading to crosstalk between the pathways and demonstrating bi-functional roles. Moreover, alterations in signalling through these pathways leads to a diverse number of diseases including

many cancers, neurodevelopmental disorders and metabolic syndromes, highlighting the critical importance of understanding the precise regulation of these pathways and their interconnectivity. Furthermore, sex steroids crosstalk with several growth factors such as the epidermal growth factor (EGF) (Auricchio et al., 2008), fibroblast growth factor (FGF) (Smith et al., 2002), vascular endothelial growth factor (VEGF) (Hyder, 2006), transforming growth factor (TGF), platelet-derived growth factor (PDGF) (Schmahl et al., 2008), nerve growth factor (NGF) (Luo et al., 2018), and IGF in both normal and cancerous cells (Kenney and Dickson, 1996). This occurs at a number of different levels to influence cellular processes, including the production of steroids (Schmahl et al., 2008; Luo et al., 2018). Many of these growth factors are also known to be involved in mechanisms of resistance to endocrine therapies for the treatment of breast and prostate cancer (Schiff et al., 2003). This review aims to outline the role of the IGF/IGF-1R and steroid hormone interplay during normal growth and development, followed by an in-depth look at how these pathways impact metabolism and the potential consequences of this in the development of endocrine-related cancers.

THE HYPOTHALAMIC PITUITARY GH AND IGF-1 AXIS

The regulation of the GH/IGF-1 system is dependent on the integrity of the hypothalamus, pituitary and liver. The primary source of circulating GH is the somatotrophs of the anterior pituitary gland, however, it is also synthesised in other tissues including reproductive tissues, lymphoid tissues and the gastrointestinal tract (Harvey et al., 2000). The pulsatile secretion of GH from the anterior pituitary is carefully regulated by the stimulatory effect of hypothalamic growth hormone releasing hormone (GHRH), dietary protein and ghrelin (Milman et al., 2016) and the inhibitory effects of somatostatin and glucose. IGF-1 is a GH dependant growth factor produced in a number of tissues but predominantly in the liver in response to GH. IGF-1 circulates attached to a number of IGF binding proteins (IGFBP 1-6), which are regulated by GH to varying degrees. The most biologically important of these binding proteins is IGFBP-3 (Ballard et al., 1989). IGF-1 levels are also dependant on a number of other hormone factors including sex steroids (which may play an important role in age dependant decrease in IGF-1), thyroxine, and glucocorticoids (Sherlock and Toogood, 2007).

Effects of Age and Gender on the GH/IGF-1 System

The GH/IGF-1 system changes over the human lifespan and these alterations are associated with, although not necessarily causative of, metabolic alterations and ageing related disease. GH pulsatile secretion is impacted by a vast array of factors including: gender, sex steroids, age, nutritional status, body composition, visceral adiposity, sleep, physical activity, and metabolic stress (Veldhuis and Iranmanesh, 1996).

During the period of increased growth associated with puberty, GH secretion rates increase resulting in a twofold to threefold increase in serum IGF-1 concentrations (Martha et al., 1992, 1996) with an associated increase in whole body protein synthesis (Mauras et al., 1996). Once growth and development are complete GH levels begin to fall. Several studies have shown a decrease in GH secretion in healthy elderly adults compared to healthy younger adults (Veldhuis et al., 1991). The age-related decrease in GH secretory burst frequency, the half-life of endogenous GH, and the daily secretory rate correlates with increasing adiposity, decreased physical performance and decreased testosterone levels (Ho et al., 1987). In men GH secretion is closely linked to serum testosterone levels, hence in individuals with primary hypogonadism the replacement of testosterone increases serum GH and IGF-1 significantly (Veldhuis et al., 1997).

The effect of age on spontaneous GH secretion is less pronounced in pre-menopausal women (Weltman et al., 1994) with secretion remaining relatively stable until after the menopause, when GH levels fall significantly. GH secretion differs considerably between men and women. Young women have approximately a twofold to threefold increase in GH serum concentration production compared to age matched males (van den Berg et al., 1996). It is now well recognised that estrogen reduces the hepatic production of IGF-1 in response to GH (Ho et al., 2006).

IGF Signalling

The IGF signalling network consists of IGF-1R, IGF-2R, and the insulin receptor (IR). IGF-1R and the IR are classified as receptor tyrosine kinases and share a similar structure. Ligands involved in the IGF signalling pathway include IGF-1 and IGF-2. IGF-1 has highest affinity for the IGF-1R (Steele-Perkins et al., 1988) with much lower affinity for IGF-2R and the IR. IR and IGF-1R display approximately 50% sequence homology (Ullrich et al., 1986). Although they can mediate control of many of the same intracellular pathways with many interconnected physiologic functions, the biological outputs influenced can also be exceptionally distinct as proven though the use of knockout models (Liu et al., 2000; Kitamura et al., 2003; Coan et al., 2008). Furthermore, Cai et al. (2017) proved by mutational analysis that the intracellular domain of the receptors regulates differential gene expression patterns. Normal growth and development are dependent on IGF signalling and perturbations are associated with dwarfism (Lin et al., 2018) and acromegaly (Sata and Ho, 2007), whereas the IR is more associated with the regulation of metabolic processes. However, the considerable overlap of functions between these receptors is exemplified in breast cancer where it has been suggested that inhibition of both IGF-1R and IR may be required for effective antitumour response (Fagan et al., 2012).

As well as the hypothalamic pituitary GH axis, IGF-1 secretion is controlled by autocrine/paracrine signals in peripheral tissues. Interestingly, the paracrine versus endocrine sources of IGF-1 can impact its function. Paracrine IGF-1 has a greater effect on mammary gland branching than IGF-1 from endocrine sources (Richards et al., 2004). Furthermore, the complexity of IGF signalling is enhanced by the myriad of proteins which are activated downstream of it such as the Ras/Raf/MAPK

and PI3K/AKT signalling pathways. These pathways regulate numerous biological processes, alterations in which, are known contributors to carcinogenesis. Additional complexity arises from IGF-1 stimulated signalling exerting differential effects in the mammary gland depending on whether it is pre-pubertal or pubertal, with switching between the activation of PI3K/Akt and Ras/Raf/MAPK signalling pathways (Tian et al., 2012).

Insulin-Like Growth Factor Binding Proteins

The IGF signalling pathway is influenced by a number of different factors. In order to gain more insight into the activity of this pathway studies have also looked at the levels of its regulatory proteins, the IGFBP family (Renehan et al., 2004). IGF-1 bioavailability in the circulation and activity at a tissue level is modulated by its association with six IGFBPs (IGFBP1-6). They can stimulate and inhibit IGF signalling by regulating its half-life, clearance, and modulating receptor interactions. IGF-1 is released from IGFBPs by mechanisms involving extracellular matrix (ECM) binding or proteolysis in the linker domain (Argente et al., 2017). The majority of IGF-1 in circulation is bound to IGFBPs with a low amount of free ligand present (Brahmkhatri et al., 2015). The IGFBP family members share a conserved structure, however, they differ in their functional motifs. Furthermore, IGFBP-2 (Azar et al., 2014), 3, 5 (Schedlich et al., 2000), and 6 (Iosef et al., 2008) contain a nuclear localisation sequence. IGFBPs are also found to be able to induce effects independently of IGF-1/IGF-1R. For example, IGFBP5 is known to interact with cell surface proteins which consequently increases local concentrations of IGF-1, therefore enhancing binding with IGF-1R within the vicinity (Jones et al., 1993). The role of IGFBPs in human health has not yet been fully elucidated. Combinational knockout of IGFBP3, 4, and 5 leads to a reduction in growth and also decreased fat and adipocyte size (Ning et al., 2006). However, evidence suggests that there is some redundancy between the IGFBPs and loss of only one could be compensated for by others. This highlights the importance these proteins are likely to play in the regulation of normal physiology.

Interconnectedness of IGF and Sex Steroids Network During Development and Ageing

Estrogens

In adulthood, estrogen is the primary sex-steroid agonist of GH secretion in both women and men (Veldhuis et al., 2005, 2006). Estrogens are synthesised from androgen substrates via the aromatase enzyme expressed in the brain, skin, ovary, adipose, bone and adrenal cortex. In breast cancer IGF-1 has been shown to increase aromatase activity (Su et al., 2011). The action of estradiol is primarily directed by interaction with the estrogen receptor (ER) nuclear receptors (alpha and beta) in peripheral target tissues further modulated via feedback by GH and IGF-1 (Veldhuis et al., 2006). Hence, whilst estrogen exerts central stimulatory effects on the GH/IGF axis, localised estrogen synthesis in peripheral tissue is inhibitory (Birzniece et al., 2012) (**Figure 1**). Interestingly, there have been mixed reports on the impact of HRT on IGF-1 concentrations in post-menopausal

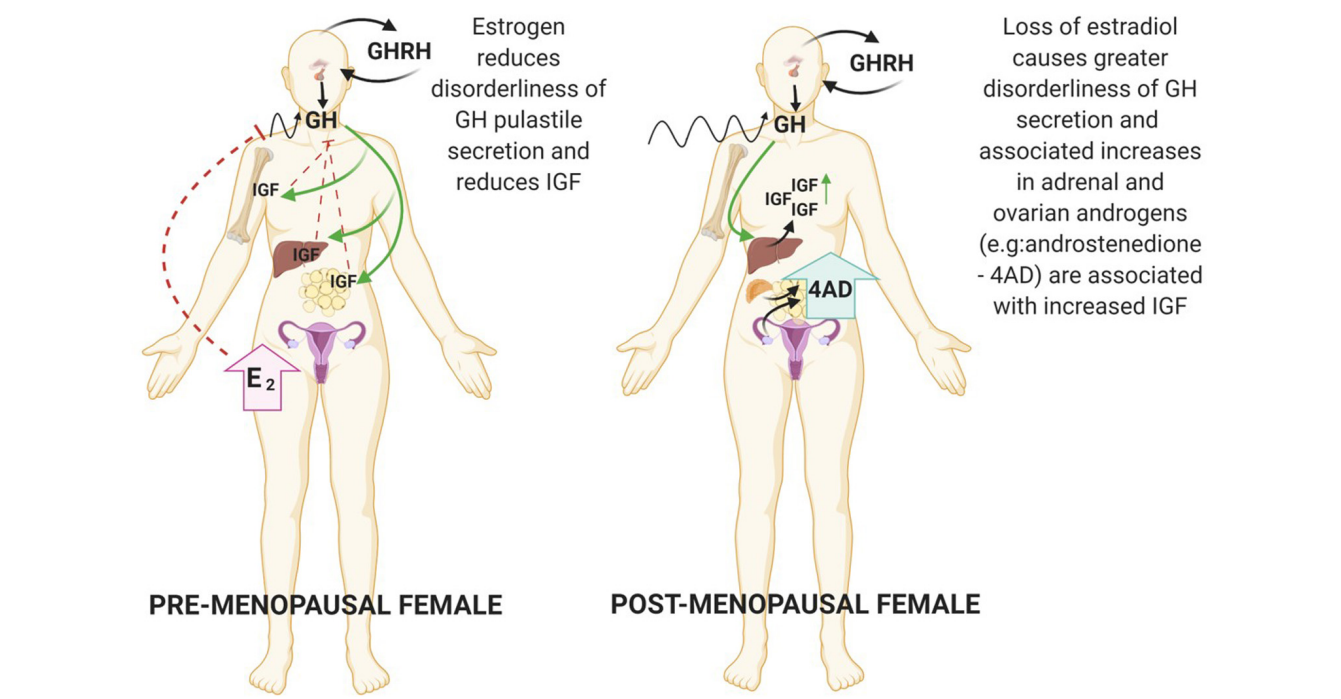

FIGURE 1 | During the female lifespan sex steroid levels impact the pulsatile secretion of GH. In the premenopausal state high E_2 reduces disorderliness of GH secretion. Reduction in E_2 due to ovarian shutdown during menopause accompanies increases in GH pulsatile disorderliness. Created with BioRender.com.

women, confounded by the different formulations (estrogen or estrogen plus progestin) and modes of delivery with oral estradiol decreasing IGF-1 concentrations and the androgenic synthetic progestin causing an increase (Campagnoli et al., 1995; Biglia et al., 2003). Indeed, in the post-menopausal woman loss of estradiol coincides with increased disorderliness of GH secretion and a diminished negative feedback control of IGF secretion.

Androgens

Although androgens are known to stimulate the GH/IGF-1 axis in men there is scant information in the literature about the impact of androgens on the GH/IGF-1 axis in women, which may be a significant factor especially during the post-menopausal period. This is an interesting point when you consider that in prostate cancer it has been shown that estrogen can substitute to upregulate IGF-1R when androgen levels are low (Genua et al., 2009). It is generally accepted that there is an age-related decline in androgens in women due to ovarian failure and involution of the adrenal zona reticularis. However, recent data suggest that 11-oxygenated androgens may not decline with age, which may be relevant here (Lasley et al., 2011; Turcu et al., 2017). How these weaker androgens may impact metabolism is not understood but their levels do coincide with the development of metabolic syndrome in post-menopausal women and those suffering from polycystic ovary syndrome (PCOS) (Azziz et al., 1998; Stefanska et al., 2015; Kempegowda et al., 2020). Utz et al. (2008) took a more focused look at the impact of obesity on the GH/IGF axis, they reported that androgens can maintain elevated IGF peripherally in the absence of estrogen driving GH secretion in obese, post-menopausal women. Whilst DHEA-S levels are the highest in circulation, they identified androstenedione as the most prevalent circulating androgen with a binding affinity for its cognate receptor. Whilst this is by no means a potent androgen they encouraged further investigation into the role of androstenedione as a moderator of the GH-IGF-1 axis. Specifically, they hypothesised that elevated androgens in overweight, postmenopausal women may preserve endogenous IGF even in the absence of GH stimulation.

In men, testosterone concentrations positively correlate with the regularity of GH secretion, with concentrations of both hormones, diminishing with increasing age (Veldhuis and Iranmanesh, 1996). It has been widely reported that decreasing levels of testosterone via the natural ageing process result in increased levels of visceral fat and the development of metabolic syndrome, and importantly this is apparent even in non-obese individuals (Kupelian et al., 2006; Brand et al., 2011).

GH/IGF AND SEX STEROID SIGNALLING IMPACT METABOLISM

Studies have shown that nutritional status is a strong determinant of IGF gene expression, not only in liver, but also in other tissues; with fasting shown to reduce serum and tissue IGF-1 levels (Lowe et al., 1989), although there appears to be gender specific ramifications for GH secretion under fasting conditions (see **Figure 2**). Of interest, increased adrenal androgen production

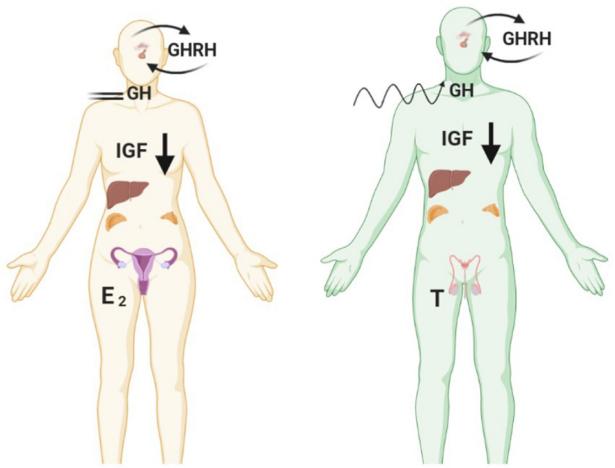

FIGURE 2 | Fasting reduces levels of IGF-1 in both genders but causes opposing effects on pulsatile GH secretion. Female GH levels become constrained in contrast to greater disorderliness of pulses in males. Created with BioRender.com.

is associated with IGF-1 levels only in females, potentially due to subsequent aromatisation and stimulation of the HP-somatotrophic axis (Veldhuis et al., 1997; Guercio et al., 2002, 2003). Further adding to the complexity it is now acknowledged that sex steroids may impact metabolism through non-genomic action independent of classical steroid receptor activation which is an area that requires further elucidation (Liu and Mauvais-Jarvis, 2009; Wong et al., 2010; Navarro et al., 2016). Many diseases associated with metabolic dysregulation are linked with the perturbation of sex hormones, examples of which are outlined in the following sections.

Type 2 Diabetes Mellitus

As alluded to earlier, the importance of the HP axis in the regulation of somatotrophic growth and steroid production and associated feedback undergoes significant changes with age. An insightful study by Rettberg et al. (2014) highlighted this by showing loss of ovarian hormones causes a reduction in glucose uptake by the brain. The authors suggest this may be attributed to myriad impairments in glucose transport and handling within neurons which ultimately results in reduced mitochondrial function (Ding et al., 2013). It is also evident that these changes in glucose metabolism are not restricted to the brain but have a knock-on effect in whole body energy storage, which manifests as increased adiposity (specifically visceral) in postmenopausal women. Whilst there is a paucity of information with regards how androgens impact metabolism and adiposity via alterations in IGF in females, there are some studies which indirectly point to a role for androgens as mediators of type 2 diabetes mellitus (T2DM). IGF signalling is linked to metabolic dysfunction with equivalently low androgen levels in males and excess androgens in females leading to type 2 diabetes and metabolic dysfunction (Navarro et al., 2015). Postmenopausal women with impaired glucose tolerance have higher androgen activity than women

with normal glucose tolerance (Larsson and Ahren, 1996). Furthermore, clinical studies looking at the impact of HRT on the development of insulin resistance and T2DM showed that the homeostatic assessment of insulin resistance (HOMA-IR) score was greatly reduced in women taking hormone replacement (Salpeter et al., 2006). Importantly, this seems very much dependent upon the type of steroid in the formulation with studies in primates indicating that only estrogen could restore insulin sensitivity whereas medroxyprogesterone acetate (MPA) caused increased adiposity and reduced insulin sensitivity (Shadoan et al., 2003, 2007). Since adipose tissue expresses androgen receptor (AR) as well as ER alpha and beta we cannot discount the role that these androgens are playing in regulation of adipogenesis and metabolism particularly in the post-menopausal woman. While there are not many studies evaluating this in men there have been reports of the development of metabolic syndromes manifesting during the initial stage of insulin resistance. Moreover, both insulin and IGF-1 signalling could play an essential role in driving prostate cancer growth (Yanase et al., 2017; Drincic et al., 2018).

Polycystic Ovary Syndrome

Polycystic ovary syndrome may provide useful clinical insights into the relationship between androgen excess, insulin signalling and metabolic dysfunction. PCOS is a common chronic health condition affecting up to 10% of all women, and is defined by androgen excess, anovulation and often polycystic ovarian morphology on imaging (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). It has traditionally been perceived as a predominantly reproductive disorder, but it is increasingly clear that PCOS is a lifelong chronic metabolic disorder of women (Randeva et al., 2012). Population data shows that women with PCOS are at a 2–4-fold increased risk of developing T2DM than the background age- and BMI-matched female population, and that onset precedes diagnosis of T2DM in non-PCOS women by 4 years (Rubin et al., 2017). Androgen excess is a cardinal clinical feature of PCOS, and correlates closely with the severity of metabolic dysfunction, including insulin resistance, T2DM, and non-alcoholic fatty liver disease (NAFLD) (O'Reilly et al., 2014; Kumarendran et al., 2018).

Androgen excess is likely to be directly complicit in metabolic dysfunction in PCOS. Serum testosterone concentrations predict development of hyperglycaemia in population studies (O'Reilly et al., 2019); *in vitro*, androgen excess induces peripheral insulin resistance and pancreatic β-cell insulin hypersecretion in both female human and mouse cell culture models, predisposing to onset of β-cell failure and T2DM (Navarro et al., 2018). Adipose tissue is also a key target organ of androgen action; prenatally androgenised female mice have aberrant adipose tissue function (Roland et al., 2010), while locally generated androgens in female adipose tissue may drive *de novo* lipogenesis and adipocyte hypertrophy (O'Reilly et al., 2017). The net effect of these changes is to fuel systemic insulin resistance and hyperinsulinemia, which in turn drive androgen generation in the ovaries and peripheral tissues (Poretsky et al., 1999).

Hyperinsulinemia augments growth hormone receptor signalling, and increases hepatic IGF-1 production. Limited human *in vivo* data have identified subtle disturbances in the growth hormone-IGF-1 axis in women with PCOS, however studies to date have not elucidated if these changes are linked to hyperinsulinemia, obesity or androgen excess (Wu et al., 2000; de Boer et al., 2004). Women with PCOS are at a significantly increased risk of endometrial carcinoma (Barry et al., 2014); while this has traditionally been attributed to the endometrial hyperplasia and dysplasia in the context of chronic amenorrhoea, increased endometrial expression in women with PCOS of genes associated with the insulin signalling pathway (IGF-1, IGFBP1, and PTEN) hints at a role for IGF-1 and insulin resistance in this process (Shafiee et al., 2016). Data on the risks of other gynaecological or non-gynaecological malignancies, including breast, in PCOS are ambiguous to date, predominantly due to heterogeneity in diagnostic criteria for PCOS and the lack of large-scale prospective data. Given the high population prevalence of PCOS, urgent further studies are needed to understand the complex associations between androgen metabolism, insulin signalling, metabolic risk and malignancy in this patient cohort.

Sarcopenia, Hypogonadism, and the IGF1 Axis

Sarcopenia is the age-related loss of muscle that leads to frailty in the elderly, diminishes their ability to lead active lives, makes them vulnerable to falls and is very detrimental to their quality of life. There are a wide range of endocrine factors that impact muscle mass and function, in particular IGF-1 signalling and androgen anabolic action and efforts to elucidate these are ongoing.

The anabolic properties of GH suggest that the manipulation of the GH/IGF-1 axis may provide a possible therapeutic option for the treatment of many of the adverse changes which occur with ageing and in particular sarcopenia. Studies in ageing populations have used GH alone or in combination with sex steroids or physical training and have examined the effect upon a number of variables including body composition, muscle strength, bone mineral density and physical performance (Sherlock and Toogood, 2007). The results of these studies have been inconsistent and there appears to be a close interplay between GH treatment/exercise and sex steroid therapy on the improvement of muscle mass/strength in sarcopenia with most studies showing that one intervention alone is less likely to lead to increase in muscle mass/strength (Taaffe et al., 1994; Papadakis et al., 1996; Lange et al., 2000; Blackman et al., 2002; Giannoulis et al., 2006). The use of GH in elderly patients (particularly in those with no evidence of GH deficiency) is not advised given the metabolic complications and also the concern regarding cancer risk (Sherlock and Toogood, 2007).

Male androgen deficiency is associated with an accelerated reduction in muscle mass and strength. Men undergoing androgen deprivation therapy for prostate cancer lose significant muscle protein content within 6 weeks of induction of hypogonadism (Lam et al., 2019). The advent of selective androgen receptor modulators (SARMs) promises significant therapeutic potential to ameliorate the sarcopenic effect of

androgen deprivation therapy in the future. SARM therapy as an adjuvant to androgen deprivation therapy in prostate cancer may protect against the adverse musculoskeletal, metabolic and neuro-cognitive impacts of hypogonadism induced by GnRH agonists and other therapies for prostate cancer, while at the same time inhibiting prostate cancer tissue proliferation and growth (Chisamore et al., 2016).

To date, there are no convincing clinical data linking hypogonadism with disturbances in growth hormone secretion or responsiveness to stimulation as a potential exacerbating factor in hypogonadism-induced sarcopenia. In one small proof-of-principle physiology study, untreated hypogonadal men had an intact IGF1 response to GH stimulation, and this response was unchanged by cross-over treatment with both testosterone and dihydrotestosterone administered as transdermal gel (Gleeson and Shalet, 2009). Supporting this finding, acute hypogonadism induced by GnRH agonist therapy in healthy men did not result in blunting of the GH response to dynamic stimulation, although basal GH secretion was marginally reduced (Veldhuis et al., 2009). On the basis of these limited data, it appears unlikely that the GH-IGF1 axis plays a direct role in the mediation of loss of muscle mass associated with hypogonadism.

Insulin-Like Growth Factor Signalling and Adipose Tissue

Although often overlooked, adipose tissue is an endocrine organ which plays a very important role in the secretion of many substances such as steroid hormones and growth factors. Adipose tissue is also a target for the actions of growth factors and hormones. GH and IGF-1 are involved in regulating adipocyte differentiation and proliferation and furthermore, sex hormones influence adipose tissue in numerous different ways such as gene expression and function (Chang et al., 2018). A very interesting study by D'Esposito et al., found that adipocytes from obese individuals had two fold higher levels of IGF-1 release than from lean individuals. Additionally, co-culturing adipocytes with MCF7 breast cancer cells resulted in enhanced growth (D'Esposito et al., 2012). Huang et al. (2013), found that AR knockout bone marrow stromal cells have greater adipocyte formation that their wild-type counterparts. Further studies to explore the mechanism promoting adipogenesis revealed that AR knockout decreased IGFBP3 expression which allowed IGF to activate the Akt signalling pathway.

A recently published study reported that periodic fasting or a fasting-mimicking diet can enhance the anti-cancer activity of anti-estrogen therapies by lowering the circulating levels IGF-1, insulin and leptin and consequently inhibiting the AKT mTOR pathway (Caffa et al., 2020). Conversely, hyperinsulinemia and insulin resistance are associated with increased cancer mortality in both obese and non-obese individuals (Tsujimoto et al., 2017). An important consideration therefore is the type of adipose tissue with visceral adipose deposits appearing to drive metabolic perturbations more so than their subcutaneous counterparts. This is particularly interesting when you consider the role of estrogens and androgens as evident in observed gender differences in adiposity.

THE GH/IGF-1 SYSTEM AND CANCER RISK

For several decades there has been an accumulation of data from epidemiological studies, basic science research and studies related to patient groups with altered levels of GH/IGF-1 which has suggested that the GH/IGF-1 system may be associated with either tumourigenesis or more aggressive behaviour in cancers (Holly et al., 1999). Laron syndrome is associated with insensitivity to GH and results in obesity and very low levels of IGF-1 in serum. However, affected individuals are reported to have reduced risk of developing cancer (Werner et al., 2019). In acromegaly (a condition with elevated GH and IGF-1 concentrations due to a pituitary tumour) (Dineen et al., 2017) some but not all studies have suggested an increased risk of developing cancer (Sherlock et al., 2010; Dal et al., 2018; Dworakowska and Grossman, 2019). Collectively, these data support epidemiological and experimental evidence of a role for GH and IGF-1 in the development of cancer.

Insulin Growth Factors and Cancer

Although oncogenic mutations frequently initiate cancer development, the growth and expansion of tumours can also be mediated by growth factors. Cells that have undergone oncogenic transformation often display overexpression of growth factors and dysregulation of signalling pathways downstream of these growth factors. Local production of growth factors in normal tissue is limited and therefore competition for availability coupled with a balance of pro versus anti-growth signals in the local environment restrains cell growth in a controlled manner. Growth factors are not simply involved in driving growth of the tumour, they can also impact the tumour microenvironment (Zhang et al., 2010), and cancer-cell de-differentiation (Nakano et al., 2019). Growth factors have been found to be involved in all steps of tumour invasion and metastasis (reviewed by Witsch et al., 2010). Additionally although signalling via growth factor receptors can be oncogenic the same receptors can also drive apoptosis within cancer cells (Ali et al., 2018).

IGF mutations do not occur frequently in cancer indicating that it is often not an initial driver of tumourigenesis (Simpson et al., 2017). In the MSK-IMPACT clinical sequencing cohort of over 10,000 cancer patients 2.4% had a genetic alteration in IGF-1R (Cerami et al., 2012; Gao et al., 2013; Zehir et al., 2017). However, artificial overexpression of IGF-1R *in vitro* does result in malignant transformation (Kaleko et al., 1990). IGF-1R activity and expression are frequently increased in malignant tumours showing it to certainly play a role in the progression of tumourigenesis. It has been reported that increased IGF-1R activity in cancers may occur secondary to the loss of tumour suppressor genes such as *TP53*, *BRCA1*, von-Hippel Lindau protein and Wilms' tumour-1 (Werner, 2012). Kruger et al., also reported that IGF-1R activation rather than IGF-1R overexpression is sufficient to induce downstream activation of the MAPK/PI3K signalling pathways and overcome tamoxifen treatment in breast cancer (Kruger et al., 2020).

Of the IGFBP family of proteins IGFBP3 has been extensively investigated and is the most frequently linked to the pathogenesis of cancer (Johnson and Firth, 2014; Cai et al., 2020). However, it has been associated with both pro-tumourigenic and anti-tumourigenic functions due to its ability to inhibit or enhance IGF actions. A collection of evidence now also points to IGF/IGF-IR-independent actions of IGFBP-3. Depending on cell type this has revealed both tumour suppressing and tumour promoting effects. Through its interaction with proteins located on the cell surface and within the cell, IGFBP-3 is involved in several biological processes that are independent of IGF-1/IGF-1R. It is known to interact with nuclear hormone receptors which include the vitamin D receptor (Moreno-Santos et al., 2017) and the retinoid X receptor (RXR) (Schedlich et al., 2007). IGFBP3 was found to mediate anti-tumour activity through its interaction with the RXR. By modulating the translocation of the RXR binding partner (orphan nuclear receptor Nur77), from the nucleus to the mitochondria it inhibits cell growth and induces apoptosis (Lee et al., 2005).

Insulin-Like Growth Factor and Endocrine-Related Cancer

Many studies have demonstrated that in the absence of IGF-1 there is an impairment of gonadal steroidogenesis (Baker et al., 1996). As shown in **Figure 3** there are many levels of crosstalk between the hypothalamic pituitary GH and IGF-1 axis, and endocrine organs. This in turn can have an impact on the development and progression of endocrine-related tumours which express receptors for GH, IGF, androgens and estrogens.

AR, ER, and IGF signalling are involved at multiple ontogenetic stages of life. Crosstalk between the IGF signalling system and the steroid hormone receptor superfamily in endocrine-related cancers may be mediated through genomic or non-genomic signalling cascades that can be ligand dependent or independent, as displayed in **Figure 4**. This can occur at many levels, such as at the cell surface by phosphorylation of the IGFR, through crosstalk with cell signalling cascades, and ultimately converging at the level of transcriptional regulation. Crosstalk between IGF-1 and ER is known to regulate gene expression in breast cancer cells, but the underlying mechanisms are not fully understood. This is further confounded by ligand-dependent or ligand-independent activation of ER. Cascio et al. (2007) found that in MCF7 breast cancer cells estradiol and IGF-1 differentially regulate ER transcription at ERE and AP-1 sites. FOXA1 is a pioneer factor which is a protein that facilitates transcription factor – DNA binding. Nuclear steroid receptor DNA binding and transcriptional activation is hugely dependent upon the presence of the pioneer factor FOXA1 which co-ordinates ER and AR binding (Bernardo and Keri, 2012). FOXA1 is also a known mediator of IGF-1 activity and genes that are regulated by IGF-1 are enriched for FOXA1 binding sites. In addition, IGF-1

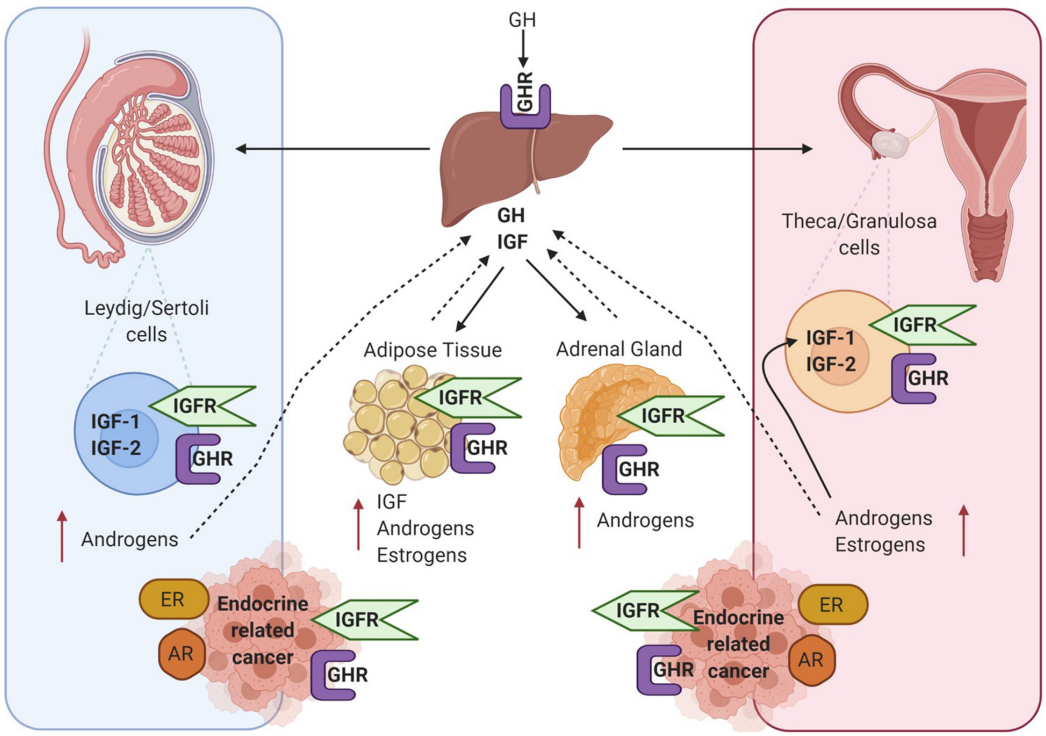

FIGURE 3 | Diagram showing crosstalk between the hypothalamic pituitary GH and IGF-1 axis and endocrine organs – testis in men, ovaries in woman and adipose tissue and adrenal gland in both sexes. All these tissue express the GHR and IGF1R and are also involved in the production of estrogens and androgens. GH, IGF and sex steroid autocrine and paracrine signalling can influence the development of endocrine-related cancers such as breast and prostate. Created with BioRender.com.

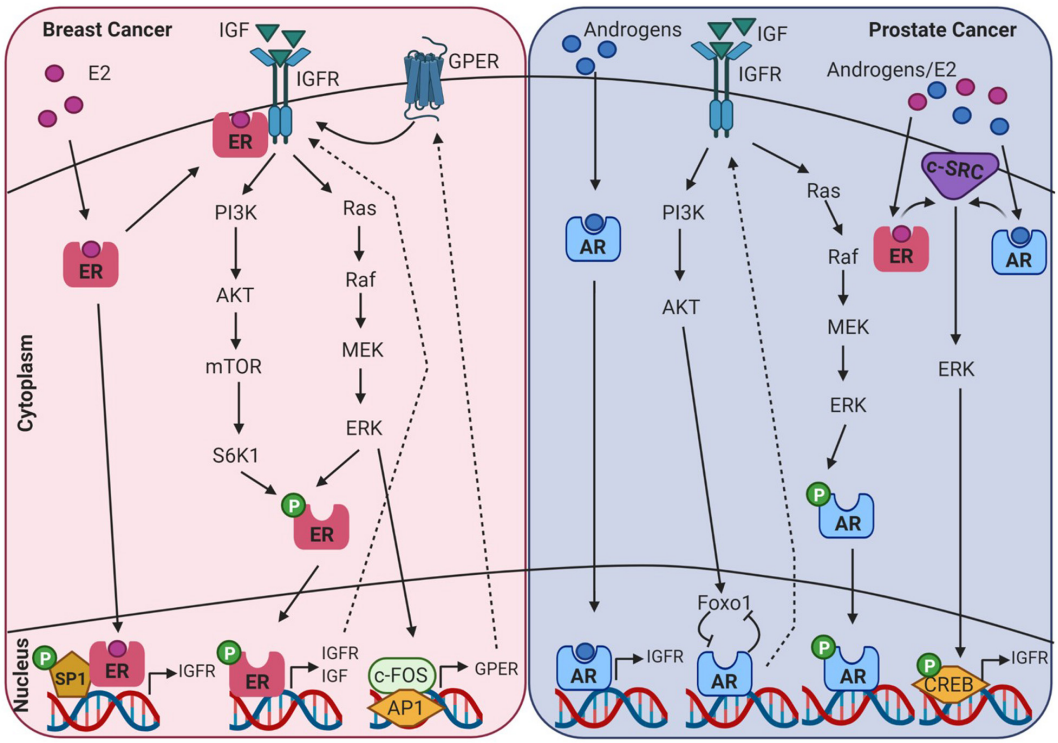

FIGURE 4 | Diagram showing intracellular crosstalk between sex steroid receptors and IGF pathways in breast and prostate cancer. This illustrates the convergence of genomic and non-genomic signalling as mediators of transcriptomic gene expression in endocrine-related cancers. Created with BioRender.com.

stabilises FOXA1 protein expression (Potter et al., 2012). IGFBP3 has also been identified as a gene target of FOXA1 and has been shown to be involved in the regulation of cell proliferation in prostate cancer cells (Imamura et al., 2012).

Prostate Cancer

There are conflicting reports as to the influence of serum IGF-1 levels and the risk of developing prostate cancer. Some studies have shown an increased risk (Chan et al., 2002) while other studies have shown no correlation (Chan et al., 2002). IGF-1 has been shown to initiate growth response in both androgen dependent and androgen-independent prostate cancer cell lines (Orio et al., 2002). Moreover, it is known to enhance AR transactivation in low androgen environments (Orio et al., 2002). An interesting point to note is that the level of androgens present directly influence Src/ERK activation in a parabolic manner. In low physiological androgen levels (0.01–10 nmol/L) the pathway is activated, however, in higher concentrations (100 nmol/L) it is inhibited (Unni et al., 2004). In prostate cancer cells Foxo1 is recruited by ligand activated AR to the promoter of AR target genes. Here it interferes with AR-DNA interactions. Activation of the IGF1/insulin-PI3K/Akt pathway and the subsequent phosphorylation of Foxo1 ameliorates this inhibitory effect. This also results in a positive feedback loop between IGF-1 and AR as androgens activating AR stimulate IGF-1R expression (Fan et al., 2007). The close association between IGF-1 and AR is further evident with two androgen response elements (ARE) located in the upstream promoter of IGF-1 (Wu et al., 2007). In prostate cancer cells androgens have been shown to upregulate the expression of IGF-1R and as a consequence results in increased proliferation and invasion when stimulated with IGF-1 (Pandini et al., 2005). Furthermore, IGF and AR signalling pathways display a feed-back loop and can regulate expression of each other. However, mutational analysis demonstrated that androgen driven upregulation of IGF-1R was not driven by AR genomic activity but involved the activation of the Src-extracellular signal-regulated kinase pathway (Pandini et al., 2005).

Breast Cancer

In hormone-dependent breast cancer cells, the IGF-1R and ERα are frequently co-expressed. IGF-1R mRNA and protein levels are higher in luminal (ER+ve) cell lines compared to non-luminal cell types (Iida et al., 2019). It has been shown that at least part of estrogen induced IGF-IR gene transcription in breast cancer cells is controlled by interactions between ERα and the transcription factor Sp1 (Sharon et al., 2006). Many observational studies have been conducted to investigate if there is a link between IGF-1 signalling and breast cancer risk. Most but not all prospective studies have reported a positive association between IGF-1 and breast cancer risk particularly in ER positive breast cancer however the influence of age and menopausal status remains ambivalent as highlighted in results of studies summarised in **Table 1** (risk highlighted in bold) (Rinaldi et al., 2006; Schernhammer et al., 2006;

TABLE 1 | Observational studies of IGFI levels and breast cancer risk.

Risk		Breast cancer subtype	IGFBP3 concentration stat us	Menopausal	References
IGF1	HR per 5 nmol/l increment = **1.11** (95% CI, 1.07–1.16; $p < 0.0001$)	Not reported	Not reported	No influence on association	Murphy et al. (2020)
IGF1-genetically-predicted	Mendelian randomisation analyses per 5 nmol/l increment OR = **1.05** (95% CI, 1.01–1.10; $p = 0.02$)	ER positive – OR = 1.06	No association with breast cancer risk	Not reported	Murphy et al. (2020)
		ER negative – OR = 1.02			
IGFI	Highest and lowest quartiles, OR = **1.34** (95% CI, 1.00–1.78; $p = 0.03$)	ER positive – OR = 1.41	Not reported	50 years or older	Kaaks et al. (2014)
		ER negative – OR = 1.16			
IGFI	Highest vs. lowest quintile, OR = **1.28** (95% CI, 1.14–1.44; $p < 0.0001$)	CR positive – OR 1.38	No influence on association	No influence on association	Endogenous Hormones and Breast Cancer Collaborative Group et al. (2010)
		ER negative – OR 0.80			
IGF1	Highest vs. lowest quintile, OR = **1.38** (95% CI, 1.02–186; $p = 0.01$)	Not reported	OR 1.44	Age > 50 at diagnosis	Rinaldi et al. (2006) (EPIC)
IGFI	Top vs. bottom quartile, RR **0.98** (95% CI, 0.69–1.39)	Premenopausal:	No association with breast cancer risk	No influence on association	Schernhammer et al. (2006) (Nurses' Health Study II)
		ER negative – RR 1.25			
		ER positive – RR 1.14			

OR = odds ratio, RR = relative risk, CI = confidence interval, HR = hazard ratios.

Endogenous Hormones and Breast Cancer Collaborative Group et al., 2010; Kaaks et al., 2014; Murphy et al., 2020).

A pooled data analysis of 17 prospective studies showed a higher odds ratio for IGF-1 in ER positive breast cancer (Endogenous Hormones and Breast Cancer Collaborative Group et al., 2010). Additionally, IGF-1 and estradiol has been shown to co-regulate the expression of a set of genes associated with breast cancer outcome (Casa et al., 2012). It is widely accepted that earlier onset of menarche is associated with increased breast cancer risk. Supporting evidence which links hormones and IGF to risk in breast cancer was found in a longitudinal study of 183 girls, where-in association of menarche and breast cancer risk may be due to estrone-to-androstenedione ratio and IGF-1 concentrations (Biro et al., 2021). It is interesting to note that it is the activity rather than the level of expression of IGF-1R that may be more relevant to these effects. In a study of 438 breast cancer patients, activated IGF-1R/IR as indicated by phosphorylation status was predictive of reduced survival (Law et al., 2008). One of the mechanisms involved in the crosstalk between the ER and the IGF-1R involves E_2 induced phosphorylation of the IGF-1R and subsequent activation of ERK (Kahlert et al., 2000). Phosphorylation of IGF-1R and subsequent activation of downstream signalling cascades were also found to contribute to tamoxifen resistance and drive cell proliferation in breast cancer (Kruger et al., 2020). The IGF-1/IGF-1R axis can also induce phosphorylation of ER through ribosomal S6 kinase 1 (S6K1), downstream of the PI3K/AKT/mTOR pathway which results in the upregulation of IGF-1, IGF-1R and other ER target genes (Becker et al., 2011). In mammary epithelial cells, constitutively active IGF-1R induces cells to undergo epithelial to mesenchymal transition which is associated with increased migration and invasion (Kim et al., 2007). Also it has been found that a loss of E-cadherin such as is observed in invasive lobular breast carcinoma (ILC) results in increased expression and activity of the IGF-1R pathway (Nagle et al., 2018). Moreover, a recent report by Kang et al., identified a pro-tumourigenic transcriptomic phenotype in normal mammary tissue associated with increased future risk of breast cancer. This microenvironment was characterised by an 80% increase in adipocyte nuclei, larger adipocytes and activation of gene sets associated with adipogenesis including IGF-1 (Kang et al., 2020). When you look to the less well studied G protein estrogen receptor (GPER) in human breast tumour samples, GPER expression correlates with IGF-1R expression (Pandini et al., 2005). IGF-1 can also regulate GPER expression in ER+ breast cancer cells through the IGF-IR/ERK/c-fos/AP1 transduction pathway (De Marco et al., 2013).

Clinical Trials Targeting IGF Overview in Endocrine-Related Cancers

As IGF-1R has been implicated in several cancer types it has garnered a great deal of interest as a therapeutic target. Pre-clinical assessments presented it as a very promising target

and subsequently led to substantial clinical efforts to develop drugs against it. Unfortunately, the clinical trials conducted have failed to produce the anticipated benefits to patients and disappointingly there are now very few active or recruiting clinical trials targeting IGF in cancer. By searching the clinicaltrials.gov database one can see that there are currently no therapeutic agents targeting IGF in phase four clinical trials for endocrine-related cancers.

Initially monoclonal antibodies (mAB) targeting IGF-1R such as dalotuzumab (MK-0646), ganitumab (AMG479), cixutumumab (IMC-A12), and figitumumab (CP-751871) were investigated. These did not show therapeutic benefit, some reasons postulated for this include crosstalk between the IGF-1R and other growth factor pathways such as the IR (Buck et al., 2010) and feedback signalling resulting in increased release of growth hormone. Other confounding issues were side effects such as hyperglycaemia and metabolic disruption (Robertson et al., 2013; Gradishar et al., 2016). The methods for overcoming this feedback mechanism included the use of bispecific antibodies and other small molecule tyrosine kinase inhibitors (TKIs). XGFR is a bispecific antibody that was developed to target EGFR and IGF-1R and has shown some promise in pre-clinical studies of metastatic osteosarcoma (Gvozdenovic et al., 2017). XGFR was developed as a single chain Fab heterodimeric bispecific IgG (OAscFab-IgG) antibody which targets IGF-1R and EGFR by containing one binding site for each target antigen (Schanzer et al., 2014). As for the more general TKIs the sequence homology between IGF-1R and IR-A/B kinase domains presented a major problem and therefore side effects were of great concern (Guha, 2013). None have proceeded to clinical development.

The IGF-signalling system has long been an area of therapeutic interest in the treatment of breast cancer which was further supported by the findings of both the European Prospective Investigation into Cancer and Nutrition (Kaaks et al., 2014) and the Endogenous Hormones and Breast Cancer Collaborative group (Endogenous Hormones and Breast Cancer Collaborative Group et al., 2010). Consistent with these studies, in March 2020 the largest single study on the relationship of IGF-1 and breast cancer was published. From the results obtained the authors concluded that there is a probable causal relationship between circulating IGF-1 concentrations and breast cancer regardless of menopausal status. Interestingly this study also reported a positive association between genetically predicted IGF-1 concentrations and breast cancer risk however this was only in ER+ tumours (Murphy et al., 2020). Over the past 20 years there have been approximately 15 clinical trials targeting IGF in breast cancer listed as terminated or completed on the clinicaltrials.gov database. None of these have progressed any further and therefore have not contributed to the clinical management of the disease. That said, a significant point to note is that drugs targeting IGF did work well in some patients, but the limiting factor was the ability to enrich for these patients using validated biomarkers. Therefore, the important questions to be asked here are; why targeting IGF hasn't been significantly beneficial in the clinical setting? What have we learnt from these clinical trials? And are we any closer to identifying a robust biomarker for IGF targeted therapies in cancer?

A more recent approach being investigated is the use of therapies targeting the IGF ligands directly to reduce their bioavailability. Dusigtumab (MEDI-573) and Xentuzumab (BI-836845) are dual IGF-I/IGF-II neutralizing antibodies which have now entered clinical trial. Xentuzumab has a binding affinity for both IGF-1 and IGF-2. The advantage of this drug is that it does not target the isoform B of the IR which is involved in glucose metabolism and therefore does not induce the hyperglycaemia and metabolic toxicity observed with IGF-1R targeted therapies or TKIs (de Bono et al., 2020). Xentuzumab has already been evaluated in combination with enzalutamide for the treatment of castration resistant prostate cancer (NCT02204072), however it failed to improve progression free survival over enzalutamide alone. The authors stated that the treatment was given to patients at an advanced stage of their disease as they had already failed docetaxel and abiraterone; they concluded that investigation at an earlier point in their treatment course is warranted (Hussain et al., 2019). *In vitro* and patient-derived xenograft (PDX) models of prostate cancer have since found evidence that PTEN and the abnormally spliced ligand independent androgen receptor variant 7 (AR-V7) could have potential use as biomarkers for response to this combination of therapy. In prostate cancer cell models xentuzumab failed to inhibit AKT phosphorylation in PTEN-null cells. Regarding AR-V7, its expression was increased in the xentuzumab + enzalutamide group compared with the enzalutamide-only group (Weyer-Czernilofsky et al., 2020). Xentuzumab is also being investigated in a phase 2 trial as a combination with everolimus and exemestane in patients with HR+/HER2- advanced or metastatic breast cancer with recruitment ongoing and expected completion of the trial in 2022 (NCT03659136). Another approach is to target IGFBP proteins, but in order to do so we need a more comprehensive understanding as to their role in both normal physiology and cancer. This is particularly relevant with the emergence of evidence suggesting that IGFBPs may bind directly to multiple growth factors (Wang et al., 2020). An innovative approach to using IGF signalling pathways to target cancer is to use them to direct therapies to the tumour, for example, they can be conjugated to nanoparticles to enhance targeting and penetration of the tumour (Zhou et al., 2015).

Of key importance to the advancement of IGF targeting therapies in cancer is a comprehensive understanding of its role under normal physiological conditions. In normal prostate cells IGF-1 maintained differentiated cellular characteristics, however in prostate cancer cells it induced a mesenchymal phenotype (Mansor et al., 2020). While the IGF pathway has been well studied in cell models of endocrine-related cancers we are undoubtedly lacking clinical evidence. The eligibility criteria for enrolment into clinical trials targeting the IGF pathway was not specific enough to identify patients most likely to have a positive clinical response. However, all is not lost in the potential for use of IGF therapies in the treatment of cancer. A recent study highlighted the benefit of a dual IGF-1R/IR inhibitor linsitinib to restore sensitivity to endocrine therapy in breast cancer. Although a lot of the evidence is based on cell models they did find that p-IGF-1R/IR positivity in ER+ breast cancer

is associated with reduced benefit from adjuvant tamoxifen in postmenopausal patients. Interestingly, they also report that in cell lines, stimulation rather than overexpression of IGF-1R is driving tamoxifen resistance to be abrogated by linsitinib. This highlights the need for biomarkers that indicate activity of the IGF signalling pathway (Kruger et al., 2020).

SUMMARY

The interconnected IGF/IGFR and sex steroid signalling pathways play crucial roles in normal growth and development and their perturbation is often associated with diseases of metabolism. What remains to be understood is how age, estrogen and visceral adiposity jointly regulate the secretion of GH in ageing humans and how this may differ between genders. Both androgens and estrogens influence IGF-1 release, and their respective signalling pathways are intertwined. This has important implications for the many cancers that are linked to the development of metabolic syndrome in ageing adults such as tumours of the breast and prostate. Furthermore, as outlined previously IGF signalling is integrated with nutritional status and metabolism and now more than ever a focus on lifestyle interventions such as diet and exercise is necessary. With future studies in this area a greater understanding of IGF pathway activation will certainly lead to a resurrection of IGF targeted therapies in cancer but critically this time with an ability to identify the patients who will most benefit from it.

AUTHOR CONTRIBUTIONS

RB and MM conceived and co-wrote the main manuscript. MO'R and MS contributed to the sections with particular clinical relevance to endocrine disorders. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Beaumont Hospital Cancer Research and Development Trust.

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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.

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frontiers in Cell and Developmental Biology

MINI REVIEW
published: 22 March 2021
doi: 10.3389/fcell.2021.641449

Role of the IGF-1 Axis in Overcoming Resistance in Breast Cancer

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Over the last two decades, many studies have demonstrated that the insulin-like growth factor-1 (IGF-1) is involved in a number of patho-physiological processes, as well as in the development of different types of solid tumors, including breast cancer (BC). Preclinical and clinical data showed that IGF-1 receptor (R) is overexpressed and hyper-phosphorylated in several subtypes of BCs. The central implications of this pathway in tumor cell proliferation and metastasis make it an important therapeutic target. Moreover, the IGF-1 axis has shown strong interconnection with estrogen regulation and endocrine therapy, suggesting a possible solution to anti-estrogen resistance. IGF-1R might also interfere with other pivotal therapeutic strategies, such as anti HER2 treatments and mTOR inhibitors; several clinical trials are ongoing evaluating the role of IGF-1R inhibition in modulating resistance mechanisms to target therapies. Our aim is to offer an overview of the most recent and significant field of application of IGF-1 inhibitors and relevant therapeutic strategies, weighing their possible future impact on clinical practice.

Keywords: IGF1, IGF-1R, clinical trial, therapy resistance, breast cancer

OPEN ACCESS

Edited by:
Marzia Di Donato, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Leena Hilakivi-Clarke, Georgetown University, United States
Chunchun Han, Sichuan Agricultural University, China

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

Received: *14 December 2020*
Accepted: *04 March 2021*
Published: *22 March 2021*

Citation:
Ianza A, Sirico M, Bernocchi O and Generali D (2021) Role of the IGF-1 Axis in Overcoming Resistance in Breast Cancer. Front. Cell Dev. Biol. 9:641449. doi: 10.3389/fcell.2021.641449

INTRODUCTION

The insulin-like growth factor-1 (IGF-1) is an insulin-like protein with anabolic effects, whose production is stimulated by growth hormone (GH), and is one of the main mediators of GH effects. Its circulating levels vary during childhood and reach its highest levels during puberty (Grimberg et al., 2016). The insulin-like growth factors (IGF-1 and IGF-2), their receptors, and a system of six insulin-growth factor binding proteins (IGFBP-1 to IGFBP-6) form a network involved in the activation of many downstream pathways (Allard and Duan, 2018). Multiple factors might activate IGF-1 receptor (R) tyrosine kinase activity (Llak, 2008) leading to interaction with its substrate, as insulin receptor (IR) substrate and the Drc-homology-2 containing protein SH2 (Radhakrishnan et al., 2011). After phosphorylation, this protein, acting as docking molecules, activates cellular kinases and initiates different downstream signaling pathways. Specifically, IGF-IR activates the PI3K/AKT/mTOR and ras/raf/MEK signaling pathways that promote cell proliferation and, at the same time, inhibits programmed cell death, through the activation of the B-cell lymphoma 2 (Bcl2)/Bcl2 antagonist of cell death (BAD) pathway, leading to carcinogenesis (Hakuno and Takahashi, 2018). The transcription of IGF-1 enables the activation of the STAT3 pathway, which enhances the invasive ability of tumor cells in prostate cancer (Ma et al., 2020); Wang et al. (2020)

demonstrated that IGF-1 activates NFkB signaling inflammation via cytosolic ROS in various cell cultures. An overview of the signaling pathways is described in **Figure 1**.

Insulin-like growth factor-1 and its system of binding proteins and receptors are physiologically involved in the development of many human tissues (Slepicka et al., 2020). It has been suggested that IGF-1 plays a significant role in the ductal and mammary gland formation, function, and maintenance (Christopoulos et al., 2015). Preclinical and clinical data have shown that IGF-IR is overexpressed and hyper-phosphorylated in several subtypes of breast cancers (BCs) (Law et al., 2008), from which its role in BC development has stemmed. High plasma levels of IGF-1 and IGFBP-3 represent a risk factor for the development and recurrence of BC in the general population (Key et al., 2010). This is particularly verified for the incurrence of estrogen receptor positive (ER+) tumors, independent from menopausal status (Key et al., 2010). Whether it constitutes an additional risk for women with a family history of disease is not yet clarified (Monson et al., 2020). However, an Italian study associated an increased risk of BC in patients with BRCA mutation (hereditary BC) with high serum IGF-1 levels (Pasanisi et al., 2011). Moreover, its role and level regulation naturally reveal a strong connection with dysmetabolism and body mass index (BMI), especially being a risk factor in HER2 positive (HER2+) overweight patients (Tong et al., 2020). Furthermore, it has been recently suggested that this factor could hold a negative prognostic significance in BC (Hartog et al., 2013), overall and in patients undergoing endocrine therapy (Duggan et al., 2013; Hartog et al., 2013). Our aim is to offer a focused review of the possible clinical role of IGF-1 as a therapeutic target and/or as part of combination therapy in BC.

PLASMA LEVELS OF IGF-1 AND BREAST CANCER

The concentrations of IG1 in plasma are approximately 150–400 ng/mL, where it is present mostly as protein-bound form (Clemmons, 2007b). The free ligand concentration is less than 1% (Clemmons, 2007b). A family of high affinity IGF binding proteins (IGFBPs) has the role of protecting IGFs from degradation through the formation of the complex IGFBP-IGF (Firth and Baxter, 2002). Even if IGFBPs were originally described as passive circulating transport proteins for IGF-I and IGF-II, now they are recognized as playing an important role in BC and IGF-1 action (Firth and Baxter, 2002). The major IGF transport function might be attributed to IGFBP-3, which is the most abounding IGF binding protein in the blood stream, followed by IGFBP-2 (Firth and Baxter, 2002). Once removed from the circulation, the binary complexes of IGFBP-IGF cross the endothelium to reach the target tissue and to interact with cell surface receptors. As the IGFBPs have a higher affinity for the IGFs than the receptors, they could sequestrate IGFs away from the type I IGF receptor, blocking their interaction. On the other hand, IGFBPs may increase IGF cellular functions in the local microenvironment by acting as a reservoir that could slowly unbind the ligands (Brahmkhatri et al., 2015).

Many different factors affect IGF-1 plasma concentrations: GH activity, nutritional status, sex, estrogen levels, and age (Clemmons and Van Wyk, 1984). Circulating IGF-1 is one of the major risk factors associated with increased BC risk (Lann and LeRoith, 2008). Previous *in vitro* studies demonstrated that IGF-1 stimulates the growth of human BC cell lines (Sasi et al., 2014; de Groot et al., 2020) and the *in vitro* blocking of IGF-1 system inhibits the response of human BC cell lines (Zha and Lackner, 2010). In the 1980s, the initial report by Furlanetto and DiCarlo (1984) highlighted the possible role of IGF-1 in the development of BC.

Later, many epidemiological and prospective studies have reported a positive correlation between circulating IGF-1 levels and BC development. A case-controlled study reported higher IGF-1 plasma concentrations in women with BC than patients without (Bruchim et al., 2009). Additionally, Werner and Laron (2020) reported a positive association between circulation concentrations of IGF-1 and BC risk for premenopausal women, but not for postmenopausal women. In the meta-analysis conducted by Renehan et al. (2004), high concentrations of IGF-1 and IGFBP3 were associated with an increased risk of incident premenopausal BC but not with postmenopausal BC. A pooled data analysis of 4790 cases from 17 prospective studies from 12 countries clearly showed that women with relatively high circulating IGF-1 had a 30% higher risk of BC than women with relatively low circulating IGF-1. This positive association was found in ER+ but not estrogen-receptor negative (ER−) tumors. In addition, this correlation was independent of IGFBP3 and menopausal status (Key et al., 2010). Murphy et al. (2020) in their observational and Mendelian randomization analyses with 430,000 women found evidence that supports a probable causal relationship between circulating IGF-1 concentrations and BC.

Mammographic density is another BC risk factor. With regard to the association between mammographic density and serum IGF-1, there are controversial findings: Diorio et al. (2005) found a positive association in premenopausal women, but other studies did not support this result (Rice et al., 2012; Rinaldi et al., 2014). Recently, Hada et al. (2019) demonstrated a positive association between circulating IGFBP2 and mammographic density particularly among women with lower BMI, but no strong correlation with IGF-1.

Studies investigating the association between the IGF system and BC prognosis are limited and controversial. Some findings suggest a positive correlation (Duggan et al., 2013), others an inverse (Kalledsøe et al., 2019), or no clear association of the biomarkers of the IGF system with all causes of mortality or BC-specific mortality and recurrence (Al-Delaimy et al., 2011; Hartog et al., 2013). Zhu et al. (2020) in their large prospective study showed an inverse and independent association between circulating IGF-1 and all-cause mortality in invasive BC patients, with association being consistent across all clinical risk factors.

IGF AS A TARGET OF THERAPY

In hormone-responsive BC cells, IGF-1R function is crucially linked with ER action. In particular, both the IGF-1R and the

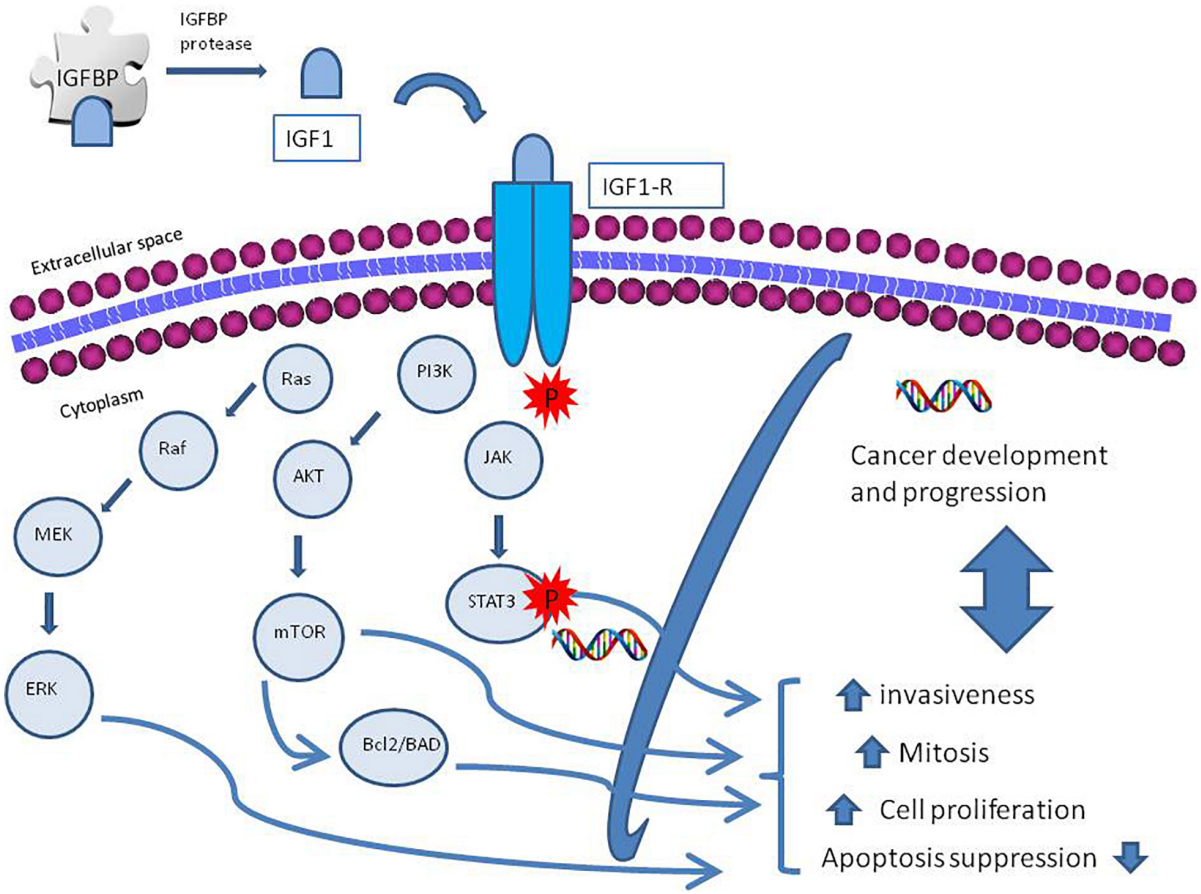

FIGURE 1 | A schematic diagram of insulin growth factor-1 receptor (IGF-IR) activation and regulation. The IGF axis consists of ligands as insulin, insulin-like growth factor 1 and 2 (IGF-1, IGF-2), receptor, IGF binding proteins (IGFBPs) 1–7, and IGFBP proteases. The IGF ligands bind their receptors and binding proteins with high affinity. IGFBPs bind tightly to IGF ligands, influencing binding to their receptors; IGFBP proteases cleave the IGFBPs into fragments with lower affinity for the IGF ligands, thereby increasing free IGF-1 and IGF-2 bioavailability. Activation of IGF-1R promotes cellular growth, proliferation, survival, and metastasis via activation of molecular pathways downstream; among them the phosphatidylinositol 3-kinase (PI3K)-AKT and RAS-extracellular signal-regulated kinase (ERK) pathways.

ER are expressed and act in synergy with estrogen steroid hormone to increase cell proliferation (Radhakrishnan et al., 2011). Otherwise in ER-BC cells, a more aggressive subtype of BC, the levels of the IGF-1R and IRS-1 are often low and IGF is not mitogenic, although IGF-1R is still required for metastatic spread (Radhakrishnan et al., 2011). Additionally, in ER+ cells, estrogens stimulate the expression of the IGF-IR and its major signaling substrate, IR substrate-1 (IRS-1), that promotes estrogen-independence for growth and transformation (Skandalis et al., 2014). Furthermore, IGF-1R and its substrate IRS-1 might induce drug and radio resistance of BC, cells leading to relapse (Pollak, 2012). Besides, high IGF-1R levels in primary tumor samples have been reported to be predictors of shorter disease-free survival, but data on the prognostic value of the IGF-1R for overall survival are contradictory (Pollak, 2012; Yerushalmi et al., 2012). Regarding these evidences, several strategies used to target the IGF axis have been clinically developed for cancer prevention and treatment.

IGF activities are mediated through substrate binding and subsequent activation of IGF-1R (Weroha and Haluska, 2012). The role of the IGF-1R pathway in promoting tumor growth and survival is well established. Targeting the IGF signaling pathway represents a promising approach in the development of novel anti-cancer therapy. The rationale for targeting the IGF-1R is derived widely from cell culture experiments that demonstrate the importance of IGF-IR signaling in promoting proliferation, inhibiting apoptosis, and its involvement and impact on BC cells that are resistant to radiation and chemotherapy (Jones et al., 2009). In BC, specifically the expression of IGF-1R is at least 50% (Ekyalongo and Yee, 2017), much more compared to HER2+ positive BC, which represents 20–25% BC (Wang and Xu, 2019); besides, there is a broader potential group of patients that could be candidates for targeted therapy. In the last few years, different therapeutic strategies have been evaluated to inhibit the IGF-1R signaling pathway. These can be divided into three categories: monoclonal anti-IGF1R antibodies, small molecule tyrosine kinase inhibitors (TKIs), and IGF ligand antibodies. Based on preclinical data, these classes of drug have different profiles of selectivity, efficacy, and toxicity which might have some implications in clinical

practice (Burtrum et al., 2003; Maloney et al., 2003). The main clinical trials targeting IGF-1 axis in solid tumors are detailed in **Table 1**.

IGF-1R Antibodies

Monoclonal antibodies that target IGF-1R have shown benefit in early-stage clinical trials (Gualberto and Pollak, 2009). IGF-1 antibodies block ligand binding, inducing receptor internalization and degradation. A few IGF-1R-specific antibodies can also partially affect the IR-A signaling pathway by targeting IGF-1R/IRA hybrid receptors (Wang et al., 2005; Gualberto, 2010). However, they do not inhibit IGF-II activation of IR-A homodimers. One example is MEDI-573 (AstraZeneca), a fully humanized antibody able to neutralize both IGF-I and IR-A pathways *in vitro* and in mice. However, compared to the other human monoclonal antibodies, MEDI-573 selectively inhibits the activation of both the IGF-1R and the IR-A signaling, without cross-reactivity with insulin, sparing the insulin/IR pathway; besides glucose metabolism remains stable (Gao et al., 2011). However, after the completion of phase 2 study in metastatic BC AstraZeneca discontinued the investigation. More recently, another novel IGF ligand neutralizing antibody, Xentuzumab (BI836845) (Boehringer Ingelheim Pharmaceuticals) showed preclinical antitumor efficacy of rapamycin by suppressing IGFs' bioactivity and inhibiting rapamycin-induced PI3K AKT activation (Adam et al., 2012).

Receptor Tyrosine Kinase Activity

Another strategy, employed with several agents, is tyrosine kinase inhibition (TKI). This kind of therapy is able to inhibit the kinase domains of the β-subunits of both immunoglobulin and IRs, as their primary sequences share 84% identity in the kinase domains maintaining a relatively intact ATP binding pocket (Munshi et al., 2003). There are only two exceptions to this, NVP-AEW541 and NVD-ADW742. NVP-AEW541 is a small molecular weight pyrrolo-[2,3]-pyrimidine derivative kinase inhibitor of IGF-IR (García-Echeverría et al., 2004), while NVP-ADW742 is an ATP-competitive inhibitor that prevents IGF-IR phosphorylation (Warshamana-Greene et al., 2005). These two inhibitors have 15–30 fold increased potency for IGF-1R kinase inhibition compared to IR kinase inhibition in cellular assay, but they are able to distinguish between the IGF-IR and the closely related InsR (Mitsiades et al., 2004; Serra et al., 2008). TKIs' lack of selectivity might have some benefit—upregulated serum levels of insulin after IGF-1R monoclonal antibody treatment may not have as much effect on the tumor if both IGF-I1 and IR are blocked. Several studies demonstrated that these TKIs inhibit IGF-IR/IR phosphorylation and AKT activation,

TABLE 1 | Key clinical trial targeting IGF-1 axis in solid tumors.

Title	ID number	Drug regimen	Phase and design	Primary outcome	Status
A dose escalating clinical trial of the IGF-1 receptor inhibitor AXL1717 in patients with advanced cancer	NCT01062620	AXL1717	Ia/b Single arm, open label	RPTD, MTD	Completed, results published
A phase I study of the oral mTOR inhibitor ridaforolimus (RIDA) in combination with the IGF-1R antibody dalotozumab (DALO) in patients (pts) with advanced solid tumors.	NCT00730379	Ridaforolimus plus dalotozumab	I Single arm, open label	Optimal dose, MTD	Completed, results published
A phase 2 study of ridaforolimus (RIDA) and dalotuzumab (DALO) in estrogen receptor positive (ER+) breast cancer	NCT01605396	Ridaforolimus + dalotozumab VS examestane	II Randomized, parallel assignment, open label	PFS	Completed, results published
A phase I trial of the IGF-1R antibody ganitumab (AMG 479) in combination with everolimus (RAD001) and panitumumab in patients with advanced cancer	NCT01061788	AMG 479 + RAD001 VS AMG 479 + RAD001 + panitumumab	I Single center, dose escalation trial	MTD, RPTD	Completed
Phase I study of everolimus (E, RAD001) and ganitumab (GANG 479) in patients (pts) with advanced solid tumors	NCT01122199	Everolimus + ganitumab	I Single arm, open label	MTD, RPTD	Completed
A phase Ib/II study of the combination of BYL719 plus AMG 479 in adult patients with selected solid tumors	NCT01708161	BYL719 (alpelisib) and AMG 479 (ganitumab)	I/II Multicenter, open label, single arm	DLT, ORR	Terminated
The XENERA™ 1 study tests xentuzumab in combination with everolimus and exemestane in women with hormone receptor positive and HER2-negative breast cancer that has spread	NCT03659136	Everolimus + exemestane VS everolimus + exemestane + xentuzumab	II Two arm, open label	PFS	Recruiting
Capecitabine and lapatinib ditosylate with or without cixutumumab in treating patients with previously treated HER2-positive stage IIIB-IV breast cancer	NCT00684983	Capecitabine plus lapatinib ± cixutumumab	II Randomized, parallel assignment, open label	PFS	Completed

MTD, maximum tolerated dose; RPTD, recommended phase II dose; PFS, progression free survival; DLT, dose limiting toxicities.

and consequently lead to increased apoptosis, decreased *in vitro* cell proliferation, and tumor suppression in xenografts models (Serra et al., 2008; Carboni et al., 2009). However, the potential benefit of TKIs over antibody therapies targeting IGF-I1 might be their capacity to block also IR, which comes at the expense of metabolic alterations such as hyperglycemia and evidence of insulin resistance (Haluska et al., 2006). In 2015 Simon Ekman, in his phase 1a/b study, showed that the oral small molecule IGF-1-receptor pathway modulator had an acceptable safety profile and demonstrated promising efficacy in this heavily pretreated patient cohort, especially in patients with NSCLC (Ekman et al., 2016).

CROSSTALK AND COMBINATION THERAPIES

Chemotherapy

Cancers have the capacity to develop resistance to traditional therapies, and the increasing prevalence of these drug resistant cancers necessitates wider research and treatment development (Housman et al., 2014). Chemo-resistance is a common problem in the treatment of cancer patients, as cancer cells become resistant to chemical substances used in treatment, limiting the efficiency of chemotherapeutic agents (Phi et al., 2018). When tumor cells are treated with cytotoxic chemotherapy, susceptible cells die, while a subset of resistant cells will continue to proliferate (Weroha and Haluska, 2008). The IGF-pathway is implicated in the chemotherapy resistance process. For instance, IGF-I attenuated the response of theMCF-7 BC cell line to doxorubicin and paclitaxel by at least two mechanisms: induction of proliferation and inhibition of apoptosis (Clemmons, 2007a). Therefore, inhibition of IGF-I action could be useful to cytotoxic chemotherapy in BC. Moreover, it has been evaluated that also the timing of IGF-1R inhibition influences responses to chemotherapy. Zeng et al. showed that the administration of IGF1R inhibitors prior to doxorubicin therapy resulted in the best therapeutic responses registered in BC cell lines. The optimal dosage sequence was doxorubicin followed by an anti-IGF-1R antibody, while the opposite sequence decreased doxorubicin effects (Zeng et al., 2009). Therefore, the timing of IGF-IR inhibition should be considered in the design of future clinical trials, combining IGF-IR blockade and chemotherapy. However, unlike other solid tumors (Goto et al., 2012), in BC, there are no results from clinical trials supporting the hypothesis of whether IGF-1R inhibition will enhance the activity of cytotoxic chemotherapy.

IGF-1R and Hormonal Therapies

As discussed above, the crosstalk between IGF/IS pathway and estrogen receptors has been widely evaluated for potential new target drugs in ER + BC (Yee and Lee, 2000). ER + BC is the most common subtype, constituting almost 70% of all diagnosed BCs. Therefore, many trials have been performed to verify the efficacy of the combination between anti-IGF-1R and anti-estrogen directed therapies. The overall effect of hormonal agents on the IGF/insulin system is to regulate positively signaling. However, resistance to anti-estrogen therapies is still a pivotal clinical problem (Nahta and Esteva, 2006; Abderrahman and Jordan, 2018). Drug resistance might be partially related to the crosstalk between the ER and the IGF pathways (Fagan et al., 2002). For instance, HBL100 cells, under tamoxifen therapy, are not able to proliferate, but if they are treated concomitantly with IGF, they could survive (Christopoulos et al., 2018). However, the majority of clinical trials evaluating the combination of anti-IG-1R and anti-ER therapies in endocrine-resistance BC have yielded disappointing results, as they did not lead to any improvement in clinical outcome (Kaufman et al., 2010). Most of the women enrolled in these trials had already developed resistance and anti-IG-1R strategies were tested as the second and third line of therapy. The lack of clinical success of these trials implies that targeting just IGF-1R is not enough to overcome tumor growth. It has been reported that the continuous exposure of MCF-7 cells to tamoxifen resulted in the eventual emergence of resistant cells, called MCF-7 Tam-R, which lose IGF-IR expression but maintain IR expression for their growth (Fagan et al., 2002). Considering these results, targeting the IR pathway could be an alternative option to treat TamR BC.

IGF-1R and PI3K/Akt/mTOR Axis

Insulin-like growth factor-1 signaling is involved in complex cross-talk with other receptor tyrosine kinases (RTLs) and their downstream effector which could likely confer resistance to inhibitors of a single class of receptor (Wilson et al., 2012). As known, the PI3K/AKT and RAS-MAPK axes are two well-established downstream pathways of IGF/insulin signaling. It is understood that the AKT pathway could be reactivated despite IGF-1R downregulation, mediated by anti-IGF-1R antibody or TKIs, leading to tumor progression (Cao et al., 2008). Based on this evidence, PI3K inhibitor such as LY294002 (Clark et al., 2002), S6K1 inhibitor H89 (Becker et al., 2011), MAPK inhibitor U0126 (Becker et al., 2011; Casa et al., 2012), and dual PI3K/mTOR inhibitor NVP-BEZ235 (Brachmann et al., 2009) have been studied in pre-clinical and clinical studies supported by the hypothesis that combinations of AKT and IGF-IR/InsR inhibitors would be an effective treatment against hormone-independent ER + BC (Fox et al., 2013). Several studies have also shown that the dual inhibition of IGF-IR and m-TOR increased antitumor activity both *in vitro* and in BC. Di Cosimo et al. (2010), in a phase 1 clinical trial, have demonstrated clinical benefit in 21.7% of BC patients combining ridaforolimus (a small molecule inhibitor of mTOR) and IGF-1R antibody dalotuzumab. The combination was feasible and well tolerated and a phase 2 was initiated, but accrual was prematurely interrupted due to a higher than expected incidence of stomatitis in the treated patients (Rugo et al., 2017).

Vlahovic et al. have evaluated the clinical benefits of combining ganitumab, a monoclonal antibody directed versus IGF-1R, with everolimus (Ev) and panitumumab in patients with advanced cancers. However, the triplet regimen of ganitumab, Ev, and panitumumab was associated with unacceptable toxicity, and clinical activity has been demonstrated only in NSCLC and sarcoma (Vlahovic et al., 2018). Moreover, another phase I study of Ev and ganitumab in patients with advanced solid tumors has shown that this combination is safe; nevertheless, prolonged

clinical benefit [stable disease (SD) ≥ 20 weeks] was noted only in refractory fibrolamellar HCC, neuroendocrine, GIST, and urachal cancers (Jalal et al., 2013). A phase Ib/II study (NCT01708161) investigated the maximum tolerated dose (MTD) and response rate of the combination of ganitumab with alpelisib, a small molecule inhibiting the subalpha of PI3-kinase, in patients with ovarian and hormone receptor positive cancer carrying the somatic PIK3CA mutation. However, the recruitment has been stopped due to inconclusive results.

Recently, a new IGF-1 monoclonal antibody, Xentuzumab (Xen) has been investigated in the phase II XENERA-1 trial in combination with Ev and exemestane (Ex) in post-menopausal women with ER+ and HER2− metastatic BC (Crown et al., 2019). Crown et al., at San Antonio Breast Cancer Symposium in 2018, showed that in the overall (randomized) population, progression-free survival (PFS) was not significantly improved in patients treated with Xen + Ev + Ex compared with Ev + Ex (Schmid et al., 2019). However, a pre-specified subgroup analysis showed that in the non-visceral metastases subgroup, the Xen + Ev + Ex arm demonstrated favorable PFS compared with the Ev + Ex arm. Specifically, an ongoing Phase II study (NCT03659136) is investigating the use of Xen + Ev + Ex in post-menopausal women with HR+/HER2− LA/mBC and non-visceral disease (Nahta et al., 2005).

IGF-IR and HER2/erbB Receptor Therapy

Most of the patients who obtain an initial response to trastuzumab-based therapy develop resistance within 1 year after commencing treatment (Albanell and Baselga, 2001). The possible existence of bi-directional crosstalk between the erbB family of receptors and IGF-1R may be implicated in resistance to targeted therapies including these receptors pathways (Weroha and Haluska, 2008). In BC cell models that overexpress HER2, an increased level of IGF-IR signaling might interfere with the action of trastuzumab (Lu et al., 2001). Moreover, BC cell lines, cultured in combination with an anti IGF-1 antibody, showed an increased cytotoxic effect when treated with trastuzumab (Albanell and Baselga, 2001). Thus, strategies that co-target HER-2 and IGF-1R may prevent or postpone development of resistance to trastuzumab (Browne et al., 2012). In BC, an IgG1 monoclonal antibody that binds IGF-1R, cixutumumab (IMC-A12) is being investigated in combination with lapatinib in a phase II trial (Haluska et al., 2014). The mechanisms related to IGF-1R-driven HER-2 therapy are not well known; nevertheless, some studies showed that HER-2 therapy resistance may be associated with the downregulation of the PTEN/PI3K/AKT signaling pathway (Gallardo et al., 2012). Despite this, it has been shown that HER-2 overexpressing cancers treated with PI3K inhibitors developed AKT-mediated activation of other tyrosine kinase growth factors such as IGF1-R, Ins-R, and HER3 treatment. Besides, PI3K inhibitors should be combined with HER-2 targeted therapies including trastuzumab or lapatinib, in order to avoid AKT signaling activation (Chakrabarty et al., 2012). Moreover, in trastuzumab-resistant tumors, IGF-1R cell motility is related to the stimulation of FAK signaling and Forkhead box protein M1 (FoxM1). Furthermore, trastuzumab-resistant cancer cells might be the best candidates for anti-HER2 and anti-IGF-1R combined therapies (Sanabria-Figueroa et al., 2015).

FUTURE DIRECTIONS

Novel approaches to target IGF/insulin systems are related to small interfering RNA (siRNA) and microRNA (miRNA) in order to reduce IGF-IR expression and function (Jung and Suh, 2012). Durfort et al. showed that silencing IGF-IR using synthetic siRNA bearing 29-O-methyl nucleotides could induce cell-cycle arrest and decrease cell proliferation. Moreover, this study suggested that the crosstalk between the IGF-I axis and antitumor immune responses can mobilize pro-inflammatory cytokines, offering a new clinical approach for treatment of mammary tumors expressing IGF-IR (Durfort et al., 2012). Nevertheless, this approach has two main problems: the first one is that siRNA formulations for systemic application face a series of hurdles *in vivo* before reaching the cytoplasm of the target cell (Whitehead et al., 2009) and the second is the transient inhibition of the IGF pathway. However, preclinical *in vivo* studies showed that it might be possible to overcome at least the second obstacle, with the development of stable *in vivo* and inducible long-term expression of target short hairpin RNA using dimerizing drugs such as doxycycline or tetracycline (Jones et al., 2009). Furthermore, other miRNAs were investigated in the past few years (Guo et al., 2013). For instance, decreased levels of miR-139, which targets IGF-IR in colorectal cancer (CRC), were associated with disease progression and metastasis. This re-expression of miR-139 might suppress CRC cell invasion and metastasis by targeting IGF-IR (Shen et al., 2012). In esophageal squamous cell carcinoma (ESCC), it has been shown that miR-375 inhibits tumor growth and metastasis through repressing IGF-1 receptor (Kong et al., 2012). Maybe in the future, siRNAs targeting IGF-IR will be modified in order to improve the effect of IGF-IR downregulation and consequently modulate antitumor immune responses with the aim to offer a new clinical approach for treatment of mammary tumors expressing IGF-IR.

CONCLUSION

The IGF system has been involved in the oncogenesis of the majority of solid tumors. The central implications of this pathway in tumor cell proliferation and metastasis makes it an important therapeutic target. In BC, the IGF pathway has been implicated in resistance to the three cornerstones of BC therapy: hormonal agents, HER receptor targeting agents, and cytotoxic chemotherapy. Therefore, several clinical trials are currently evaluating the efficacy of IGF-1R inhibition to overcome these resistance mechanisms. The competitive landscape for anticancer therapies in BC and the difficulty to recruit a sufficient number of patients limited *de facto* the continuation and validation of research with IGF-1R and GF inhibitors. That is why, even considering the encouraging initial results that we have illustrated, combined with the enormous potential clinical impact of the IGF axis, there is not yet an optimal combination therapy paradigm.

AUTHOR CONTRIBUTIONS

AI, MS, and OB drafted the manuscript. DG conceptualized the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. This article was published with the contribution of MEDnOTE.

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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.

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frontiers in Cell and Developmental Biology

REVIEW
published: 01 April 2021
doi: 10.3389/fcell.2021.660853

Androgen Receptor-Related Non-coding RNAs in Prostate Cancer

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Prostate cancer (PCa) is the second leading cause of cancer-related death among men in the United States. Androgen receptor (AR) signaling is the dominant oncogenic pathway in PCa and the main strategy of PCa treatment is to control the AR activity. A large number of patients acquire resistance to Androgen deprivation therapy (ADT) due to AR aberrant activation, resulting in castration-resistant prostate cancer (CRPC). Understanding the molecular mechanisms underlying AR signaling in the PCa is critical to identify new therapeutic targets for PCa patients. The recent advances in high-throughput RNA sequencing (RNA-seq) techniques identified an increasing number of non-coding RNAs (ncRNAs) that play critical roles through various mechanisms in different diseases. Some ncRNAs have shown great potentials as biomarkers and therapeutic targets. Many ncRNAs have been investigated to regulate PCa through direct association with AR. In this review, we aim to comprehensively summarize recent findings of the functional roles and molecular mechanisms of AR-related ncRNAs as AR regulators or targets in the progression of PCa.

Keywords: prostate cancer, androgen receptor, non-coding RNA, microRNA, lncRNA, circRNA

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

Received: *29 January 2021*
Accepted: *12 March 2021*
Published: *01 April 2021*

Citation:
Yang Y, Liu KY, Liu Q and Cao Q (2021) Androgen Receptor-Related Non-coding RNAs in Prostate Cancer. Front. Cell Dev. Biol. 9:660853. doi: 10.3389/fcell.2021.660853

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second-highest cause of cancer death among men in the United States, with an estimated 248,530 new cases and 34,130 deaths expected in 2021 in the United States (Siegel et al., 2021). The growth and survival of PCa are mainly dependent on the sex steroid hormone, androgens (Folkerd and Dowsett, 2010). The androgen receptor (AR) is a ligand-activated transcription factor that is vital for both normal prostate development and tumorigenesis. Upon binding by androgen in the cytoplasm, AR dimerizes and translocates to the nucleus, stimulating target gene transcription through association with androgen response elements (AREs) within promoter and enhancer sequences. AR and its downstream signal cascades are critical for the initiation and progression of both localized and advanced metastatic PCa (Scher and Sawyers, 2005). Advances in screening and therapeutic strategies promoted successful treatment of PCa by surgery and/or radiation. The testing of prostate-specific antigen (PSA), a prototypic AR target, has been used for years as a diagnostic biomarker for the disease (Lilja et al., 2008). Androgen deprivation therapy (ADT) by AR antagonists and chemical castration is the standard treatment for patients with biochemical recurrence after primary therapy or with locally advanced or metastatic disease (Feldman and Feldman, 2001). Patients with metastasis-free PCa have a 100% 5-year survival rate (Brawley, 2012). Unfortunately, the majority of primary cancers will eventually acquire ADT resistance and progress to castration-resistant prostate cancer (CRPC) (Kirby et al., 2011). Patients with

metastatic PCa have a low 5-year survival rate (Kirby et al., 2011; Brawley, 2012). Generally, CRPC is caused by AR aberrant activation with enhanced AR expression, hypersensitivity to androgens (Waltering et al., 2009), intra-tumoral steroidogenesis (Locke et al., 2008), and abnormal AR splicing variant expression (Sun et al., 2010). Recent studies revealed that the frequency of AR-negative neuroendocrine prostate cancer (NEPC) and AR-Null and Neuroendocrine-Null Prostate Cancer (Double-Negative PCa, DNPC) is elevated due to the application of potent AR antagonists such as enzalutamide (ENZ) and abiraterone (Aparicio et al., 2011; Hu et al., 2015; Bluemn et al., 2017; Labrecque et al., 2019). Therefore, there is an urgent need to elucidate the molecular mechanisms contributing to the development of AR-dependent CRPC as well as advanced NEPC and DNPC for developing alternative therapeutic options for advanced PCa.

Next-generation RNA sequencing (RNA-seq) advances have become overly convenient to measure gene expression levels and explore new transcriptional units across the transcriptome. 2% of the human genome encodes approximately 20,000 protein-coding genes (Lander et al., 2001; Venter et al., 2001; Alexander et al., 2010; Consortium, 2012) and up to 80% of the human genome encodes a large number of non-coding RNAs (ncRNA) (Consortium, 2012; Djebali et al., 2012). Ribosomal RNA (rRNA) (about 80% of the total RNA weight) and transfer RNA (tRNA) (about 15% of the total RNA weight) are two of the most abundant ncRNA types in cells (Palazzo and Lee, 2015). The other ncRNAs are categorized as short ncRNAs (sncRNAs) and long ncRNAs (lncRNAs) based on whether their size is longer than 200 bases (Cech and Steitz, 2014). SncRNAs include microRNA (miRNA) (Lagos-Quintana et al., 2001), small interfering RNA (siRNA) (Hamilton and Baulcombe, 1999), piwi-interacting RNA (piRNA) (Siomi et al., 2011), small nucleolar RNA (snoRNA) (Kiss, 2001; Matera et al., 2007), small nuclear RNA (snRNA) (Matera et al., 2007; Guiro and Murphy, 2017), and tRNA-derived fragments (tRF) (Schimmel, 2018). Unlike linear lncRNAs, circular RNAs (circRNAs) are single-stranded circularized ncRNAs commonly generated from the precursor mRNA (pre-mRNA) back-splicing process (Memczak et al., 2013; Salzman, 2016). Functionally, rRNA, tRNA, snoRNA, and snRNA are housekeeping ncRNAs, while miRNA, siRNA, piRNA, tRF, lncRNA, and circRNA are regulatory transcripts.

Over recent decades, ncRNAs have been emerged as critical regulators instead of junk RNAs in different disease processes, including cancer (Du et al., 2013; Hayes et al., 2014; Vo et al., 2019). Notably, many ncRNAs (miRNA, lncRNA, and circRNA) are aberrantly expressed with significant contribution to PCa initiation and/or progression (Bonci et al., 2008; Prensner et al., 2011; Pickl et al., 2014; Fredsoe et al., 2018; Hua et al., 2018; Chen S. et al., 2019). MiR-101 negatively regulates *EZH2* expression by binding to *EZH2* 3′ untranslated region (UTR), and has a strong negative correlation with PCa progression from benign to localized disease to metastasis (Varambally et al., 2008). LncRNA *SChLAP1* is critical for PCa cell invasiveness and metastasis through antagonizing the genome-wide localization and regulatory functions of the SWI/SNF chromatin-modifying complex (Prensner et al., 2013). CircRNA 0005276 (circ0005276), a circular RNA stem from *XIAP*, is highly expressed in PCa tissues with advanced tumor stage and metastasis. Circ0005276 interacts with FUS to regulate the transcription of *XIAP* in PCa, thus promoting the tumorigenesis and development of PCa (Feng et al., 2019). Aside from miRNAs, lncRNAs and circRNAs are relatively new players in the ncRNA field and are less well understood. ncRNAs are gaining widespread attention for their abundance in number, expression specificity, functional roles in diseases, and potential clinical applications. Given the critical role of AR in PCa initiation and progression, we will focus on the impact of miRNA, lncRNA, and circRNA, on AR's function in PCa in this review.

ncRNAs AS AR REGULATORS

AR Is Repressed by miRNAs

MicroRNAs are a family of small untranslated RNAs with ∼21–25 nucleotides in size that control gene expression by mediating target mRNA degradation (He and Hannon, 2004; Bartel, 2009), and repressing (Pillai et al., 2005; Petersen et al., 2006; Mathonnet et al., 2007) or promoting (Vasudevan et al., 2007; Truesdell et al., 2012) target mRNA translation. These regulations often occur through the association of miRNAs with 3′ UTRs of transcripts (Bartel, 2009; Krol et al., 2010), and some miRNAs may also target 5′ UTR and coding regions of transcripts. Many tools have been developed to predict miRNA targets (Chen L. et al., 2019). *AR* mRNA is comprised of a 1.1 kb 5′ UTR, a 2.7 kb open reading frame (ORF), and an exceptionally long 3′ UTR with a length of approximately 6.8 kb (Ostling et al., 2011; Ebron and Shukla, 2016). Thus *AR* mRNA is the most miRNA targeted transcript in PCa cells (Hamilton et al., 2016; **Table 1**).

Several groups have systemically explored AR modulatory miRNAs through different strategies (Ostling et al., 2011; Hamilton et al., 2016; Kumar et al., 2016; Fletcher et al., 2019). Ostling et al. (2011) and Kumar et al. (2016) used pre-miR libraries to perform gain-of-function screening for AR-modulatory miRs in PCa cells. Östling et al. identified and validated 13 miRNAs that interact with the *AR* 3′ UTR region and could significantly reduce *AR* 3′ UTR activity: miR-135b, miR-185, miR-297, miR-299-3p, miR-34a, miR-34c, miR-371-3p, miR-421, miR-449a, miR-449b, miR-634, miR-654-5p, and miR-9. Among these miRNAs, miR-185 and miR-34a were consistently reported by other groups to show the regulatory function on PCa aggressiveness through directly targeting *AR* (Kashat et al., 2012; Qu et al., 2013). Similarly, Kumar et al. (2016) identified 15 miRNAs (miR-101-3p, miR-138-5p, miR-149-3p, miR-30b-3p, miR-30c-5p, miR-30d-5p, miR-411-3p, miR-425-5p, miR-488-5p, miR-541-3p, miR-635, miR-646, miR-650, miR-654, and miR-9-5p) that significantly suppressed *AR* 3′ UTR reporter activity, especially miR-9-5p, miR-30b-3p and miR-541-3p, highlighting the critical role of miR-30 family members in inhibiting AR activity through 3′ UTR association. The study also revealed that 3 miRNAs, miR-371-3p, miR-193-3p, and miR-646 could suppress *AR* transcriptional activity through binding sites within the coding region of *AR* mRNA (Kumar et al., 2016). The role of miR-488 in inhibiting AR expression in

TABLE 1 | MiRNAs targeting *AR* mRNA.

MiRNAs (References)	Expression in PCa	Target region of *AR* mRNA	Cancer types	Function in cancer cells
miR-124 (Shi et al., 2013, 2015; Xiong et al., 2017)	↓	3′UTR	PCa, bladder cancer	Inhibits cell growth and increases apoptosis
miR-135b (Ostling et al., 2011; Aakula et al., 2015; Bao S. X. et al., 2020)	↓	3′UTR	PCa, hepatocellular carcinoma (HCC), breast cancer	Inhibits cell growth
miR-181c-5p (Wu et al., 2019)	–	3′UTR	PCa	Increases ENZ sensitivity of PCa cells and represses cell invasion
miR-185 (Ostling et al., 2011; Qu et al., 2013; Liu C. et al., 2015; Kalinina et al., 2020)	↓	3′UTR	PCa, breast cancer	Suppresses cell growth, migration, invasion, and tumorigenicity
miR-193a-3p (Kumar et al., 2016; Liu et al., 2017)	↓	Coding region	PCa	Suppresses cell growth
miR-197-3p (Fletcher et al., 2019; Huang et al., 2020)	↓	3′UTR	PCa	Inhibits cell growth and colony formation
miR-205 (Boll et al., 2013; Hagman et al., 2013; Coarfa et al., 2016; Kalinina et al., 2020)	↓	3′UTR	PCa, breast cancer	Suppresses cell growth, migration, and invasion
miR-297 (Ostling et al., 2011; Fang et al., 2016)	↓	3′UTR	PCa	Suppresses cell growth
miR-299-3p (Ostling et al., 2011; Ganapathy et al., 2020)	↓	3′UTR	PCa	Suppresses cell growth, migration, induces cell cycle arrest, and apoptosis
miR-30b-3p (Kao et al., 2014; Kumar et al., 2016)	↓	3′UTR	PCa	Suppresses EMT phenotypes and inhibits cell migration and invasion
miR-31 (Lin et al., 2013; Coarfa et al., 2016; Wang et al., 2016)	↓	Coding region	PCa	Suppresses cell growth, migration and invasiveness
miR-320a (Okato et al., 2016; Sato et al., 2016; Lieb et al., 2018)	↓	3′UTR	PCa	Suppresses cell growth, migration and invasiveness
miR-320b (Hsieh et al., 2013; Lieb et al., 2018; Dai et al., 2019)	↓	3′UTR	PCa	Suppresses cell growth
miR-346 (Fletcher et al., 2019; Lin et al., 2020)	↑	3′UTR	PCa	Promotes cell growth and invasion
miR-34a (Liu et al., 2011; Ostling et al., 2011; Kashat et al., 2012; Leite et al., 2015)	↓	3′UTR	PCa	Inhibits prostate cancer stem cell regeneration and metastasis
miR-34c (Hagman et al., 2010; Ostling et al., 2011; Walter et al., 2013; Fang et al., 2016)	↓	3′UTR	PCa	Suppresses cell growth
miR-361-3p (Fletcher et al., 2019; Liu B. et al., 2020)	↓	3′UTR	PCa	Suppresses cell growth and increases ENZ sensitivity of PCa cells
miR-371-3p (Ostling et al., 2011; Leite et al., 2015; Kumar et al., 2016)	↓	Coding region, 3′UTR	PCa	Suppresses cell growth
miR-421 (Ostling et al., 2011; Meng et al., 2016)	↓	3′UTR	PCa	Suppresses cell growth, induces cell cycle arrest, reduces glycolysis, and inhibits migration
miR-449a (Ostling et al., 2011; Zheng et al., 2015; Chen W. et al., 2017; Guo et al., 2018)	↓	3′UTR	PCa, bladder cancer	Suppresses cell growth, invasion, and angiogenesis
miR-449b (Ostling et al., 2011; Mortensen et al., 2014)	↑	3′UTR	PCa	Suppresses cell growth
miR-488* (Sikand et al., 2011; Ebron and Shukla, 2016)	-	3′UTR	PCa	Suppresses cell growth, increases apoptosis
miR-541-3p (Kumar et al., 2016; He et al., 2021)	↓	3′UTR	PCa	Suppresses cell growth and enhances the radiosensitivity of PCa cells
miR-634 (Ostling et al., 2011; Kumar et al., 2016)	–	3′UTR	PCa	Suppresses cell growth
miR-646 (Kumar et al., 2016)	–	Coding region 3′UTR	PCa	Suppresses cell growth
miR-654-5p (Ostling et al., 2011; Kumar et al., 2016)	–	3′UTR	PCa	Suppresses cell growth
miR-92a-2-5p (Liu G. et al., 2020)	–	3′UTR	HCC	Increases liver cancer cell invasion
miR-9-5p (Ostling et al., 2011; Kumar et al., 2016; Moazzeni et al., 2017; Bandini et al., 2020)	–	3′UTR	PCa, breast cancer	Suppresses cell growth

PCa cells was also confirmed by Sikand et al. (2011). Hamilton et al. (2016) applied photoactivatable ribonucleoside-enhanced cross-linking immunoprecipitation of the Argonaute protein and sequencing (AGO-CLIP-Seq) to broadly explore interactions between miRNAs and miRNA target sites in a panel of PCa cells (Hamilton et al., 2016). Among 22 PCa driver genes, *AR* 3′ UTR has the most abundant miRNA target sites (71 unique miRNA families at 147 seed sites), including the miR-135,

miR-185, miR-34, miR-421, and miR-9 families reported by Ostling et al. (2011).

Recently, using a library of LNA-modified antisense inhibitors against 983 human miRNAs, Fletcher et al. (2019) systematically identified microRNAs modulating AR activity in PCa cells. The application of the miR inhibitors limits off-target or non-specific effects by avoiding targeting endogenous miRNA processing or effector complexes. 78 miRNA inhibitors were found to significantly modulate AR reporter activity, including inhibitors of miR-135b, miR-421, miR-449a, miR-634, and miR-654-5p, which is consistent with Ostling et al.'s (2011) report. Interestingly, inhibition of miR-346, miR-361-3p, and miR-197 significantly reduced AR activity in a dose-dependent manner. Upregulation of *AR* 3′ UTR activity by miR-346, miR-361-3p, and miR-197 was also confirmed by *AR* 3′ UTR reporter assay combined with miRNA mimics. Mimics of miR-346, miR-361-3p, and miR-197 also prevented Actinomycin D-induced loss of *AR* transcript. Previous studies demonstrated that miR-346 binds to 3′ UTR of *AGO2* and *hTERT*. Upon miR-346 binding to the 3′ UTR of *AGO2* and *hTERT*, the middle sequence motif (CCGCAU) of miR-346 recruits G-rich RNA sequence binding factor 1 (GRSF1) to form a "bulge loop," thus facilitating the recruitment of *AGO2* mRNA and *hTERT* mRNA to ribosomes to promote translation (Guo et al., 2015; Song et al., 2015). MiR-346, miR-361-3p, and miR-197 may enhance *AR* mRNA stability and promote AR expression through a similar mechanism. In Fletcher et al.'s (2019) study, miR-197 inhibitor increased caspase activity and suppressed cell growth, indicating miR-197 may act as a tumor promoter. However, miR-197's expression was lower in prostate tumor tissues than in normal tissues (Fletcher et al., 2019), which is consistent with the miR-197 inhibitor's role in promoting PCa cell growth reported by Huang et al. (2020). Besides, the miR-361-3p inhibitor also suppressed PCa cell growth (Fletcher et al., 2019), while another report showed that miR-361-3p enhanced ENZ sensitivity of PCa cells and inhibited PCa cell growth by suppressing AR splice variant 7 (*AR-V7*) expression (Liu B. et al., 2020). Thus, the function of miR-197 and miR-361-3p in PCa cell growth from different studies are conflicted. Some miRNAs may be able to either activate or repress mRNA translation, and whether they act as translation activators or repressors highly depends on cell cycle and RNA binding factors (Vasudevan et al., 2007; Truesdell et al., 2012). Further studies are necessary to disentangle these contradictory results.

Many miRNAs targeting *AR* mRNA might be not reflected in the result from the above systematic analysis (**Table 1**). MiR-205 is mainly expressed in prostate basal epithelial cells (Zhang et al., 2010; Gandellini et al., 2012), and PCa patients with low expression of miR-205 have poor survival (Hagman et al., 2013). MiR-205 can suppress AR expression by binding to *AR* 3′ UTR, and also interfere with MAPK and IL-6 signaling pathways in PCa cells (Boll et al., 2013; Hagman et al., 2013), while the promoter region of the miR-205 gene contains ARE and the expression of miR-205 is increased after AR activation by R1881 treatment (Hagman et al., 2013). MiR-212 is downregulated in prostate tumor cells, and it suppresses the transcription of *AR* and *AR-V7* through direct targeting *hnRNPH1*, which may regulate *AR* mRNA transcription or splicing. Interestingly, hnRNPH1 can also interact with AR protein and modulate AR binding to target genes (Yang et al., 2016). MiR-31 is regulated by promoter hypermethylation in both triple-negative breast cancer (Augoff et al., 2012) and PCa (Lin et al., 2013), and its expression is negatively correlated with the aggressiveness of the PCa. MiR-31 directly inhibits AR expression through binding to the coding region of *AR* mRNA. Subsequently, genes related to cell cycle regulation are also repressed by miR-31 as its direct targets. Interestingly, miR-31 can be suppressed by AR as a transcriptional target, forming a negative regulation loop between miR-31 and AR (Lin et al., 2013). MiR-124 was downregulated in PCa (Shi et al., 2013, 2015), breast cancer (Feng et al., 2016), and bladder cancer (Xiong et al., 2017). Intravenous delivery of miR-124 in combination with ENZ sufficiently inhibited prostate tumor growth and increased cell apoptosis (Shi et al., 2015). Mechanistically, miR-124 directly represses *AR* along with *EZH2* and *SRC* through binding to the 3′ UTR regions of these mRNAs (Shi et al., 2015). HDAC inhibitor, OBP-801, induced miR-320a mediated suppression of AR expression through binding to the 3′UTR of *AR* (Sato et al., 2016). Both miR-181c-5p and miR-361-3p could regulate the expression of *AR-V7* but not wild-type *AR* in PCa cells via binding to the specific target sequence in the *AR-V7* 3′UTR (Wu et al., 2019; Liu B. et al., 2020). Besides, exosome transportation of miR-92a-2-5p from macrophages to liver cancer cells could suppress AR expression by directly targeting *AR* 3′UTR and enhance the invasion capacity of liver cancer cells (Liu G. et al., 2020).

AR Is a Target of lncRNAs

The size of lncRNAs is normally longer than 200 bases, and thus they can fold into complex structures to carry out various functions through interaction with protein, chromatin, and RNA (Schmitt and Chang, 2016; Goodall and Wickramasinghe, 2020). LncRNAs have been reported to regulate gene transcription by recruiting transcription regulators or direct interaction with chromatin, affect protein and mRNA stability through direct binding, and act as sponges for miRNAs (Goodall and Wickramasinghe, 2020). With these diverse regulatory mechanisms, lncRNAs regulate numerous signal pathways and play critical roles in different cellular processes and disease progression. Therefore, lncRNAs have great potentials as biomarkers or therapeutic targets. Currently, many lncRNAs are characterized to participate in PCa progression through direct association with AR protein, DNA, or mRNA (**Table 2**).

LncRNA *HOTAIR* was first studied in breast cancer and identified its functionality to reprogram chromatin state by affecting the chromatin occupancy of Polycomb repressive complex 2 (PRC2) and altering histone H3K27 methylation (Wu et al., 2015). Zhang et al. (2015) showed that *HOTAIR* is upregulated in advanced PCa and could reduce AR degradation through directly binding to AR protein which blocked the interaction between AR and MDM2. Interestingly, miR-34a was reported to suppress *HOTAIR* expression through direct binding (Chiyomaru et al., 2013). Coincidentally, AR is a target of miR-34a (Ostling et al., 2011), suggesting *HOTAIR* may also modulate *AR* mRNA expression by acting as a sponge of miR-34a. LncRNA *PCAT1* is a prostate-specific regulator correlated with PCa

TABLE 2 | LncRNAs regulating AR.

LncRNAs (References)	Expression in PCa	Molecular mechanisms	Cancer types	Function in cancer cells
ARLNC1 (Zhang et al., 2018)	↑	Stabilizes the *AR* transcript via RNA-RNA interaction	PCa	Promotes cell growth
GAS5 (Kino et al., 2010; Pickard et al., 2013; Hudson et al., 2014; Luo et al., 2017)	↓	Interacts with AR protein and suppresses its transcriptional targets	PCa, breast cancer	Promotes cell apoptosis and decreases viability
HOTAIR (Zhang et al., 2015)	↑	interacts with AR protein and protects it from degradation	PCa	Promotes cell growth and invasion
HOXA11-AS-203 (Schmidt et al., 2020)	–	Interacts with AR protein	Melanoma	-
HOXC-AS1 (Takayama et al., 2020)	↑	Interacts with U2AF2 and promotes *AR* mRNA splicing	PCa	Promotes cell growth
LBCS (Gu et al., 2019)	↓	Interacts with hnRNPK and *AR* mRNA to suppress AR translation	PCa	Suppresses cell growth
MALAT1 (Dai et al., 2019)	↑	Sponges miR-320b and activates AR signaling	PCa	Promotes cell growth, metastasis and invasion
PCAT1 (Guo et al., 2016)	↑	Interacts with AR protein and regulates its chromosome binding	PCa	Promotes cell growth
PCGEM1 (Yang et al., 2013; Hung et al., 2014; Prensner et al., 2014c; Zhang et al., 2016)	↑	Interacts with AR protein and enhances its transactivation	PCa	Promotes cell growth
PlncRNA-1 (Cui et al., 2013; Fang et al., 2016)	↑	Sponges miR-34c and miR-297 and protects *AR* mRNA	PCa	Suppresses apoptosis, promotes cell growth and migration
PRKAG2-AS1 (Takayama et al., 2020)	↑	Interacts with U2AF2 and promotes *AR* mRNA splicing	PCa	Promotes cell growth
PRNCR1 (Yang et al., 2013; Prensner et al., 2014c)	↑	Interacts with AR protein and enhances its transactivation	PCa	Promotes cell growth
SARCC (Zhai et al., 2016, 2017)	–	Interacts with AR protein and destabilizes it	RCC	Suppresses cell growth
SLNCR1 (Schmidt et al., 2016, 2020)	–	Interacts with AR protein and regulates its chromosome binding	Melanoma	Promotes melanoma invasion
SOCS2-AS1 (Misawa et al., 2016)	↑	Interacts with AR protein and regulates its cofactor recruitment	PCa	Promotes cell growth, migration, and suppresses apoptosis
SRA (Lanz et al., 1999)	–	Interacts with AR protein and enhances its transactivation	Breast cancer	–
YY1BM (Wu et al., 2020)	–	Blocks the interaction between YY1 and AR protein	Esophageal squamous cell carcinoma	Suppresses cell growth

progression, which was first showed to activate AKT and NF-κB signaling in CRPC through reconfiguring FKPB51-IKKα-PHLPP complex after a direct interaction with FKBP51 (Shang et al., 2019), and suppress BRCA2 expression (Prensner et al., 2014b) and regulate MYC stabilization at the post-transcriptional level (Prensner et al., 2014a). Furthermore, *PCAT1* was reported to interact with AR and LSD1 (Guo et al., 2016). This interaction alters the genomic occupancy of the AR-LSD1 complex, which mainly regulates the transcription of AR target genes through interaction with chromatin (Metzger et al., 2005; Guo et al., 2016). *AR* has also been investigated as an oncogene in human renal cell carcinoma (RCC) (He et al., 2014; Huang Q. et al., 2017), which is consistent with the incidence that RCC is more frequently diagnosed in men than women (Siegel et al., 2021). LncRNA *SARCC* was reported to suppress RCC through binding and destabilizing AR protein and thus concealing AR's downstream transcriptional targets (Zhai et al., 2016, 2017). *PCGEM1* is a well-known prostate tissue-specific lncRNA associated with high-risk PCa patients (Srikantan et al., 2000; Petrovics et al., 2004; Xue et al., 2013). However, the exact mechanisms of how *PCGEM1* is associated with PCa are conflicting. Yang et al. (2013) reported that *PCGEM1*, together with *PRNCR1* could bind to AR protein and increase its activity through forming an AR-bound enhancer-promoter loop, and both contribute to castration resistance in PCa. However, Prensner et al. (2014c) failed to verify the binding of *PCGEM1* and *PRNCR1* to AR, and they also suggested that neither gene is a component of AR signaling. Then Hung et al. (2014) showed that *PCGEM1* regulates PCa metabolism partially through AR activation, but mainly through promoting chromatin recruitment of c-MYC and activating c-MYC signaling via physical interaction between *PCGEM1* and c-MYC. Interestingly, Zhang et al. (2016) reported that *PCGEM1* could pull down heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and splicing factor U2AF65. The *PCGEM1*-hnRNP A1 interaction could suppress hnRNP A1 interaction with *AR* pre-mRNA, while *PCGEM1*-U2AF65 interaction could promote U2AF65 interaction with *AR* pre-mRNA, indicating that *PCGEM1* may participate in the AR signaling by regulation of *AR*

mRNA splicing (Zhang et al., 2016). The relationship between *PCGEM1* and AR, and the clinical significance of *PCGEM1* in PCa need further studies.

Growth arrest-specific 5 (*GAS5*) is a lncRNA firstly identified in growth-arrested mammalian cells (Schneider et al., 1988), and overexpression of *GAS5* could induce cell apoptosis and cell cycle arrest in PCa cells (Pickard et al., 2013; Luo et al., 2017; Sun et al., 2017). Intriguingly, *GAS5* was shown to interact with some steroid receptors which share similar response sequences, including glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and AR, at their DNA binding domains through *GAS5* contained hairpin RNA glucocorticoid receptor response element (GRE)-mimic and, thereby, inhibit the association of these receptors with their DNA recognition sequence and thus repress the transcriptional activity of these steroid receptors (Kino et al., 2010). These interactions are in a conserved, sequence-specific manner (Hudson et al., 2014). LncRNA steroid receptor RNA activator (*SRA*) could selectively enhance the transactivation of steroid receptors, such as PR, GR, estrogen receptor (ER), and AR, through interaction with their N-terminal, regulatory domain (NTD) (Lanz et al., 1999). Schmidt et al. (2016, 2019) reported another lncRNA, steroid receptor RNA activator-like non-coding RNA (*SLNCR1*), recruits AR to *MMP9* and EGR1-bound genomic loci to regulate melanoma invasion and proliferation. Interestingly, several confirmed AR bound lncRNAs, such as *HOTAIR*, *SRA*, *SLNCR1*, and even *PCGEM1*, all include a conserved region with a similar sequence ($SLNCR1^{609-637}$) which is required for AR-lncRNA interaction (Yang et al., 2013; Zhang et al., 2015; Schmidt et al., 2016). Further investigation revealed that AR NTD binds with short, pyrimidine-rich RNA containing at least one CYUYUCCWS motif, and lncRNA *HOXA11-AS-203* which contains such motif was validated to bind with AR NTD (Schmidt et al., 2020). These studies strongly suggested that some lncRNAs containing specific sequences may bind to AR protein and other steroid receptors at the DNA binding domain to compete with the target response elements and suppress their transcriptional activity, or at the N-terminal regulatory domain to modulate their transactivation.

A specific type of lncRNAs called enhancer RNAs (eRNAs) are derived from super-enhancers and have been proven to control mRNA transcription through facilitating enhancer-promoter interaction (Kim et al., 2010; Wang et al., 2011). *KLK3* eRNA (*KLK3e*) is an eRNA produced from the upstream enhancer regions of Kallikrein-related peptidase 3 (*KLK3*) (Hsieh et al., 2014), a well-known AR regulated gene encoding the protein product PSA. *KLK3e*'s expression is induced by AR, and *KLK3e* could scaffold the AR-associated protein complex, the *KLK3* enhancer, and the *KLK2/3* promoter, resulting in enhanced transcriptional activation of nearby *KLK3* and long-distance *KLK2*. daSilva et al. (2018) further identified numerous lncRNAs bound by AR (ARA-lncRNAs), and many of them are also transcriptionally regulated by AR. Further analysis revealed that protein-coding genes adjacent to these ARA-lncRNAs had a significantly greater androgen-induced change in expression than protein-coding genes neighboring lncRNAs not associated with AR, and suppressing the expression of ARA-lncRNA attenuates androgen-induced expression change of protein-coding genes adjacent to the ARA-lncRNA. These ARA-lncRNAs' transcription start sites (TSSs) are enriched with epigenetic signatures of active enhancers, highlighting hundreds of AR-bound lncRNAs act as cis-regulatory RNA enhancers to control the androgen regulatory program of PCa cells (daSilva et al., 2018). The exact regulation mechanisms and functions of AR-associated eRNAs in PCa cells are still waiting to be fully discovered.

Additionally, several lncRNAs can affect AR signaling through RNA-RNA interaction. LncRNA *ARLNC1* was reported to directly bind to *AR* mRNA 3′ UTR, stabilize *AR* mRNA, and increase the cytoplasmic fraction of *AR* mRNA, thus regulating PCa cell growth and apoptosis (Zhang et al., 2018). LncRNA *LBCS* was also shown to interact with *AR* mRNA and hnRNPK, forming a complex and suppressing AR translation efficiency (Gu et al., 2019). LncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), one of the most studied lncRNAs, was previously reported to bind to EZH2 and enhance EZH2-mediated repression of Polycomb-dependent target genes (Hirata et al., 2015; Wang et al., 2015). *MALAT1* could also function by activating AR signaling through sponging miR-320b which targets *AR* 3′ UTR (Sato et al., 2016; Dai et al., 2019).

Apart from *PCGEM1*, several AR-regulated lncRNAs (*CRPC-lncs*) which are highly expressed in CRPC tissues, were also reported to participate in *AR* mRNA splicing through association with splicing factors (Takayama et al., 2020). Among them, *FAM83H-AS1*, *PRKAG2-AS1*, *HOXC-AS1*, *ELFN1-AS1*, and *ERVK3-1* could interact with U2AF2, which is a component of the U2 complex in spliceosome and regulates *AR* mRNA splicing (Liu et al., 2014; Takayama, 2019; Takayama et al., 2020). Silencing these lncRNAs reduced the nuclear enrichment of U2AF2 and suppressed the association of U2AF2 with *AR* pre-mRNA, resulting in decreased AR expression and inhibited PCa cell growth (Takayama et al., 2020).

A few lncRNAs have been reported to encode short peptides which may play roles as proteins (Anderson et al., 2015; Huang J. Z. et al., 2017). Recently, Wu et al. reported that a Y-linked lncRNA, *LINC00278*, could encode a Yin Yang 1 (YY1)-binding micropeptide, YY1BM. YY1BM could suppress the interaction between YY1 and AR and thus downregulate eEF2K expression and induce apoptosis in human esophageal squamous cell carcinoma (Wu et al., 2020).

The Regulation of AR by circRNAs

CircRNAs have been recognized as regulatory RNAs (Memczak et al., 2013; Guarnerio et al., 2016), and exhibit critical roles through mechanisms like lncRNAs. CircRNA could inhibit miRNA target degradation as sponges (Hansen et al., 2013; Zheng et al., 2016), bind to proteins, RNAs, and DNAs to affect gene transcription (Li et al., 2015; Yang et al., 2017), RNA splicing (Conn et al., 2017), and translation (Li et al., 2020), and serve as protein scaffold containing different binding sites (Du et al., 2017). CircRNA also has its specific regulatory mechanism distinct from lncRNA. CircRNAs' unique circularization structure lacking open ribonucleotide end may resist the RNA cleavage by miRNA recruited exonuclease, thus stabilizing miRNAs after binding (Piwecka et al., 2017;

Chen S. et al., 2019). CircRNA profiling through ribosomal-depleted RNA sequencing has identified many circRNAs that are differentially expressed between normal and cancerous prostate tissues (Zheng et al., 2016; Chen S. et al., 2019). Among them, circRNA-17 is lower expressed in higher grade PCa tissues, and suppressing circRNA-17 could increase the expression of AR-V7, and enhance the resistance to anti-AR therapy. Further investigation revealed that circRNA-17 could bind and stabilize miR-181c-5p, which targets the 3′UTR of AR-V7 (Wu et al., 2019). Since circRNA is a relatively new research field of ncRNAs, more investigations about circRNAs are needed to explore and elucidate their exact roles in tumorigenesis.

ncRNAs AS AR TARGETS

As a critical hormonal transcription factor, AR can exhibit its function through direct binding to ARE located at enhancers and promoters of its targets. Genomic occupation of AR and profiles of androgen-responsive genes have been defined through Chromatin Immunoprecipitation (ChIP)-on-chip, ChIP-seq assays (Jin et al., 2013). AR directly targeted miRNAs (Takayama et al., 2011; Pasqualini et al., 2015) and lncRNAs (Zhang et al., 2018; Takayama et al., 2020) in PCa cells have also been systematically identified by combined analysis of androgen dysregulated miRNA and lncRNA expression data from microarray or RNA sequencing with AR genome-wide binding information (**Table 3**).

Takayama et al. (2011) integrated 5′-cap analysis of gene expression (CAGE) and ChIP-on-chip analysis and identified a cluster of androgen-inducible miRNAs in LNCaP cells, including miR-100, miR-125b, miR-21, miR-218-1, miR-218-2, miR-221, miR-222, and let-7c, which are all located adjacent to androgen receptor binding sites (Takayama et al., 2011). Among them, miR-21 has been verified as one of 16 AR-responsive miRNAs (Ribas et al., 2009). Interestingly, miR-21 may indirectly increase AR expression by decreasing PTEN, forming a positive regulation loop between AR and miR-21 (Mishra et al., 2014). MiR-125b was shown to be induced by AR and partially involved in AR's downregulation of MUC1 by targeting *MUC1* 3′ UTR (Rajabi et al., 2011). Sun et al. (2014) reported that miR-125b-2, let-7c, and miR-99a are a cluster of miRNAs from the same host gene, and were all repressed following androgen activation in LNcaP cells, which is in contrast to Takayama et al.'s result probably due to different conditions of androgen treatment and tissue culture. Pasqualini et al. (2015) performed AR ChIP-seq and miRNA host gene array analysis after AR stimulation in DUCaP cells, and successfully identified 32 miRNA host genes that were significantly regulated and bound by AR. MiR-22 and miR-29a are significantly increased by AR activation in a time-dependent manner, and both of them are higher expressed in benign prostate tissues when compared to tumor tissues. MiR-125b, miR-22, and miR-29a/b were later examined to mediate AR's repression of TET2 in PCa cells (Takayama et al., 2015). Besides, AR-induced miR-26a together with miR-101 both target *EZH2* at the 3′ UTR region and thus are involved in AR's regulation of EZH2 (Cao et al., 2010). Murata et al. (2010) identified androgen-responsive miRNAs in LNCaP cells through short RNA sequencing and the expression of miR-148a, miR-141, and miR-200a along with miR-125b, miR-22, and miR-29b were all increased after R1881 treatment in LNCaP cells (Murata et al., 2010). The function of miR-148a is complex in prostate cancer progression. MiR-148a was highly expressed in PCa patients and significantly correlated with biochemical recurrence of PCa independent of PSA values (Al-Qatati et al., 2017), and it was shown to promote LNCaP cell growth through targeting *CAND1* 3′ UTR (Murata et al., 2010). However, overexpression of miR-148a precursor suppressed androgen-refractory PC3 cell growth (Fujita et al., 2010). Another report also suggested miR-148a exhibited tumor suppressor roles in several common cancers (Lujambio et al., 2008). As more AR-regulated miRNAs in PCa and other types of cancers were reported, it is clear that AR's function is partially mediated by AR-induced oncogenic miRNAs and -inhibited tumor-suppressive miRNAs (**Table 3**).

Similarly, many AR-regulated lncRNAs have also been identified in PCa cells (**Table 3**). Misawa et al. (2016) identified 5 lncRNAs induced by androgen through RNA sequencing. Surprisingly, one of these 5 lncRNAs, *SOCS2-AS1*, was shown to interact with AR protein and modulate AR activity by regulating cofactor recruitment, leading to a positive regulation loop in PCa cells. Zhang et al. (2018) performed an integrative transcriptomic analysis in PCa tissues combined with AR ChIP-seq, resulting in the identification of AR-regulated clinically relevant lncRNAs and *ARLNC1* was identified as one of the AR-regulated lncRNAs that regulates *AR* mRNA stability (Zhang et al., 2018). Another lncRNA in the list is *PRCAT38*, which was later proven to share enhancers with *TMPRSS2*, and both of them are activated by AR/FOXA1 binding (Chen Z. et al., 2019). *PCAT29* is a PCa-associated lncRNA suppressed by DHT and knocking down *PCAT29* increases PCa cell proliferation and migration (Malik et al., 2014). Takayama et al. (2020) identified AR-regulated lncRNAs which are highly expressed in CRPC tissues. Among the list, *PRKAG2-AS1* was suppressed by AR activation, while *HOXC-AS1* was induced by AR activation, and both lncRNAs play essential roles in *AR* mRNA splicing through interaction with *AR* splicing factor, U2AF2.

Several circRNAs directly regulated by AR have also been reported (**Table 3**). CircRNA-ZMIZ1 is upregulated in PCa patients' plasma samples than in corresponding normal samples (Jiang et al., 2020). CircRNA-ZMIZ1 expression is increased by androgen activation, and silencing circRNA-ZMIZ1 induces PCa cell growth inhibition and cell cycle arrest. ChIP-seq and luciferase assay confirmed that AR suppresses the expression of circRNA-HIAT1 in clear cell renal cell carcinoma (ccRCC). CircRNA-HIAT1 serves as a "reservoir" to stabilize miR-195-5p/29a-3p/29c-3p that target *CDC42*, thus indicating AR promotes ccRCC through regulating circHIAT1/miR-195-5p/29a-3p/29c-3p/*CDC42* axis (Wang k. et al., 2017). Interestingly, *AR* can be transcribed into several circRNAs due to alternative RNA splicing (Cao et al., 2019; Luo et al., 2019a). The expression of these *AR*-transcribed circRNAs are positively correlated with linear *AR* transcripts and can be detected in plasma samples from metastatic castration-resistant

TABLE 3 | AR regulated ncRNAs.

ncRNAs (References)	Regulation by AR	Molecular mechanisms	Cancer types	Function in cancer cells
miRNAs		**Targeted genes**		
Let-7d (Ramberg et al., 2011)	↑	*PBX3*	PCa	–
miR-1 (Liu Y. N. et al., 2015; Chen W. Y. et al., 2017)	↑	*ZBTB46*, *SRC*	PCa	Inhibits metastasis
miR-101 and miR-26a (Cao et al., 2010)	↑	*EZH2*	PCa	Inhibits proliferation and invasiveness
miR-125b (Rajabi et al., 2011)	↑	*MUC1*, *BAK1*	PCa	Promotes cell growth
miR-135a (Kroiss et al., 2015)	↑	*ROCK1*, *ROCK2*	PCa	Inhibits invasiveness
miR-141 (Waltering et al., 2011)	↑	-	PCa	Promotes cell growth
miR-148a (Fujita et al., 2010; Murata et al., 2010)	↑	*CAND1* (Murata et al., 2010), *MSK1* (Fujita et al., 2010)	PCa	Promotes LNCaP cell growth (Murata et al., 2010), Suppresses PC3 cell growth, and invasiveness (Fujita et al., 2010)
miR-185-5p (Huang Q. et al., 2017; Zhu et al., 2019)	↑	*CSF-1*, *VEGFC*, *HIF2α*	RCC	Increases RCC cell metastases to lung and liver, while suppresses the lymph nodes metastases
miR-193a-3p (Jia et al., 2017)	↑	*AJUBA*	PCa	Promotes cell migration
miR-21 (Ribas et al., 2009; Mishra et al., 2014)	↑	*TGFBR2*	PCa	Promotes cell growth
miR-216a (Chen et al., 2012)	↑	*TSLC1*	HCC	Promotes cell growth and migration
miR-22, miR-29a, and miR-17-92 cluster (Pasqualini et al., 2015)	↑	*LAMC1*, *MCL1*	PCa	Deceases cell viability and migration
miR-29 (Takayama et al., 2015)	↑	*TET2*	PCa	Promotes cell growth and migration
miR-31 (Lin et al., 2013)	↓	*AR*	PCa	Suppresses cell growth
miR-32 and miR-148a (Jalava et al., 2012)	↑	*BTG2*, *PIK3IP1*	PCa	Reduces apoptosis or promotes cell growth
miR-99a/let7c/125b-2 cluster (Sun et al., 2014)	↓	*IGF1R*	PCa	Suppresses cell growth
miR-421 (Ostling et al., 2011; Meng et al., 2016)	↓	*AR* (Ostling et al., 2011), *NRAS*, *PRAME*, *CUL4B*, and *PFKFB2* (Meng et al., 2016)	PCa	Suppresses cell growth, induces cell cycle arrest, reduces glycolysis, and inhibits migration
lncRNAs				
ARNILA (Yang et al., 2018)	↓	Sponges miR-204 to facilitate Sox4 expression	Breast cancer	Promotes migration, invasion and EMT
AR-Associated lincRNAs (daSilva et al., 2018)	↑	Scaffolds AR-dependent looping complex	PCa	–
CTBP1-AS (Takayama et al., 2013)	↑	Represses CTBP1 by recruiting PSF together with histone deacetylases	PCa	Promotes cell growth
DANCR (Jia et al., 2016)	↓	Represses TIMP2/3 expression by mediating the binding of EZH2 on their promoters	PCa	Promotes cell invasion and metastasis
DRAIC (Sakurai et al., 2015)	↓	-	PCa	Suppresses cell migration and invasion
KLK3e (Hsieh et al., 2014)	↑	Scaffolds AR-dependent looping complex	PCa	Promotes cell growth
Linc00304 (Zhang et al., 2019)	↓	Promotes CCNA1 expression	PCa	Promote cell growth and cell cycle progression
Linc00844 (Lingadahalli et al., 2018)	↑	Indirectly modulates AR binding to chromatin	PCa	Suppresses cell migration and invasion
Linc01503 (He et al., 2020)	↑	Recruits SFPQ and activates FOSL1	Nasopharyngeal carcinoma (NPC)	Promotes cell growth, migration, and invasion
LncRNA-p21 (Luo et al., 2019b)	↓	Interacts with EZH2 and enhances STAT3 methylation	PCa	Promotes ENZ induced neuroendocrine differentiation (NED)
PCAT18 (Crea et al., 2014)	↑	–	PCa	Promotes cell growth, migration, and invasion
PCAT29 (Malik et al., 2014; Sakurai et al., 2015)	↓	–	PCa	Suppresses cell migration and invasion
POTEF-AS1 (Misawa et al., 2017)	↑	Represses Toll-like receptor signaling	PCa	Promotes cell growth and suppresses apoptosis
SOCS2-AS1 (Misawa et al., 2016)	↑	Interacts with AR protein and regulates its cofactor recruitment	PCa	Promotes cell growth and suppresses apoptosis
TMPO-AS1 (Huang et al., 2018)	↓	–	PCa	Promotes cell growth and migration
circRNAs				
circAR3 (Luo et al., 2019a)	↓	Encoded by *AR* gene	PCa	No effect on cell growth and invasion
AR-circRNAs (Cao et al., 2019)	↓	Encoded by *AR* gene	PCa	–
circHIAT1 (Wang k. et al., 2017)	↓	Stabilizes miR-195-5p/29a-3p/29c-3p	ccRCC	Suppresses cell migration and invasion
circRNA7 (Bao S. et al., 2020)	↓	Sponges miR-7-5p and increases VE-cadherin and Notch4	HCC	Promotes vasculogenic mimicry formation
circZMIZ1 (Jiang et al., 2020)	↑	Increases expression of AR and AR-V7	PCa	Promote cell growth and cell cycle progression

PCa (mCRPC) patients and may serve as biomarkers of high-risk primary PCa (Cao et al., 2019; Luo et al., 2019a).

DISCUSSION

Crosstalk Between ncRNAs in AR Regulatory Network

Some lncRNAs and circRNAs share similar RNA sequences as the miRNA targeted mRNA, and then they could act as miRNA sponges to diminish miRNA-induced mRNA degradation (Hansen et al., 2013; Goodall and Wickramasinghe, 2020). For example, lncRNA *MALAT1* could decrease miR-320b mediated *AR* mRNA degradation through competitively binding to miR-320b (Dai et al., 2019; **Figure 1**). Some circRNA-miRNA interactions may form RNA duplex resistant to RNA cleavage, thus stabilizing miRNAs (Piwecka et al., 2017; Chen S. et al., 2019; Wu et al., 2019). CircRNA-17 suppresses the expression of AR-V7 by binding and stabilizing miR-181c-5p which induces the degradation of *AR-V7* through targeting its 3′ UTR region (Wu et al., 2019; **Figure 1**). Both lncRNAs and circRNAs can also serve as scaffolds to mediate interactions between proteins and RNAs. LncRNAs and circRNAs might also mutually affect the binding with the same targets, which may be due to the RNA sequence similarity between lncRNAs and circRNAs, or RNA structure-induced protein conformational change.

Feedbacks Between AR and ncRNAs

As one of the most important regulators in PCa, the expression of AR is precisely controlled by various factors through different mechanisms in different stages, including feedback regulation loops between AR and ncRNAs. Many ncRNAs regulate AR expression at transcription and post-transcription levels, while they are also regulated by AR. MiR-31 inhibits AR expression by directly targeting the *AR* mRNA coding region, while miR-31 itself is suppressed as an AR repressive target, thus forming a negative feedback loop to promote PCa (Lin et al., 2013; **Figure 2A**). AR increases the expression of miR-21, which in turn increase AR expression and activity probably via the down-regulation of PTEN (Ribas et al., 2009; Mishra et al., 2014). Both AR-induced *HOXC-AS1* and AR-repressed *PRKAG2-AS1* can regulate *AR* mRNA splicing and promote AR expression (Takayama et al., 2020). The expression of *ARLNC1* is increased after AR binding to the *ARLNC1* promoter region. *ARLNC1* further stabilizes *AR* mRNA and promotes AR expression through binding to *AR* mRNA 3′ UTR, thus forming a positive regulation loop in PCa cells (Zhang et al., 2018; **Figure 2B**).

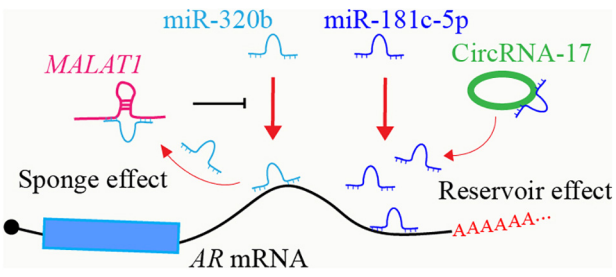

FIGURE 1 | Crosstalk between ncRNAs in AR regulatory network. LncRNA *MALAT1* acts as a sponge to inhibit miR-320b-targeted *AR* mRNA degradation. CircRNA-17 binds to and stabilizes miR-181c-5p, enhancing miR-181c-5p-targeted *AR* mRNA degradation.

Steroid Receptors and lncRNAs

Several lncRNAs have been shown to regulate steroid receptors, including GR, MR, PR, AR, and other nuclear receptors, through directly binding in a conserved sequence-specific manner (Kino et al., 2010; Hudson et al., 2014; Liu et al., 2016). These lncRNAs bind to these receptors at two protein domains: DNA binding domain, and the N-terminal, regulatory domain (**Figure 3A**). *GAS5* contained a GRE-mimic hairpin RNA sequence and thus can bind to GR DNA domain, block GR's binding to GRE DNAs, and suppress GR-induced gene transcription (Kino et al., 2010; Hudson et al., 2014; Schmidt et al., 2016; **Figure 3B**); **Figure 3B**). On the other hand, several AR-bound lncRNAs share short, pyrimidine-rich RNA motif (CYUYUCCWS) that are required for the interaction with steroid receptors at N-terminal, regulatory domain, such as *HOTAIR*, *SRA*, *SLNCR1*, *HOXA11-AS-203*, and *PCGEM1* (Lanz et al., 1999; Yang et al., 2013; Zhang et al., 2015; Schmidt et al., 2016; **Figure 3C**); **Figure 3C**). The discovery of ncRNAs including circRNAs containing steroid receptor responsive element (SRE) mimic RNA sequences, or the pyrimidine-rich RNA motif (CYUYUCCWS) is of great interest to identify new mechanisms in various diseases that ncRNAs regulate steroid receptors through binding to steroid receptors. These regulation mechanisms may also apply to the ncRNAs that bind to other transcription factors.

Clinical Implications of ncRNAs in PCa

Increasing research of ncRNAs has greatly revolutionized our understanding of RNA biology. More and more evidence showed that ncRNAs have critical functions in diverse diseases. MiRNA, lncRNA, and circRNA are ubiquitously expressed throughout the body, and they can readily be measured from various human samples, including serum, saliva, and urine (Weber et al., 2010; Iyer et al., 2015; Vo et al., 2019). Many ncRNAs play important roles in PCa and their expressions are correlated with different clinicopathological characteristics of PCa patients. These ncRNAs hold great promises as biomarkers and therapeutic targets in clinical applications. Several single miRNA and panels of miRNAs combinations from plasma or tissue samples of PCa patients have shown more extraordinary diagnostic performance than PSA (Kachakova et al., 2015; Kelly et al., 2015). At the same time, serum miR-210 level is notably correlated with the change in PSA level during treatment among metastatic CRPC patients (Cheng et al., 2013). MRX34, a synthetic miRNA mimic of miR-34a that directly regulates at least 24 known oncogenes including AR, is the first miRNA mimic in clinic application (Bouchie, 2013). In phase 1 clinical trial (NCT01829971), MRX34 was delivered in patients with advanced solid tumors by a liposome technology named Smarticles, which demonstrated exciting proof-of-concept for miRNA-based cancer treatment but unfortunately failed due

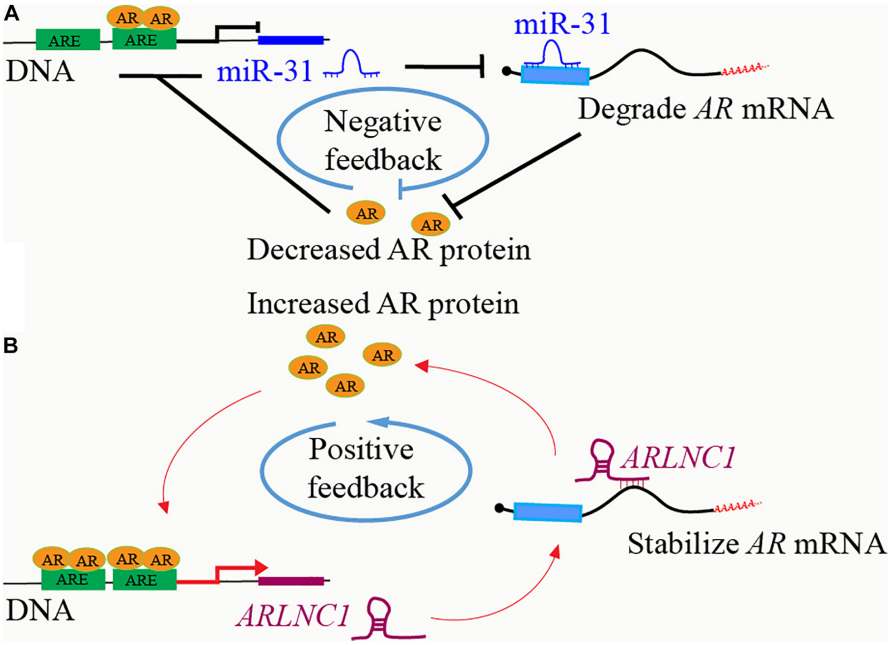

FIGURE 2 | Feedbacks between AR and ncRNAs. **(A)** AR binds to the promoter region of lncRNA *ARLNC1* and induces its expression. *ARLNC1* binds to and stabilizes *AR* mRNA, which increases AR expression and further activates the transcription of itself, *ARLNC1,* forming a positive feedback regulation loop. **(B)** AR binds to the promoter region of miR-31 and suppresses its expression. MiR-31 induces *AR* mRNA degradation via targeting the coding region of *AR* mRNA, thus forming a negative feedback regulation loop.

to serious adverse events (Hong et al., 2020). On the other hand, lncRNA *PCA3* is specifically overexpressed in most PCa cancer patients (Bussemakers et al., 1999) and has been approved by the FDA as a PCa diagnostic marker in the urine of PCa patients (de Kok et al., 2002; Deras et al., 2008), but its use for assessing response to ADT in advanced PCa is limited (Martinez-Pineiro et al., 2014). Some other lncRNAs have been identified as biomarkers for metastatic PCa, such as *PCAT18* (Crea et al., 2014) and *SChLAP1* (Prensner et al., 2014d). Candidate circRNAs were also identified and detected in urine to serve as biomarkers for PCa (Vo et al., 2019).

Antisense oligonucleotides (ASOs) as an RNA-based therapeutic approach can induce gene silencing through RNase H-mediated degradation of target RNAs. It has shown improved target specificity and stability as well as tolerated toxicity after significant advancements in the design, chemical modifications, and delivery (Verma, 2018). Several ASO-based drugs have been approved by the FDA for the treatment of different human diseases (Dhuri et al., 2020). For PCa treatment, ASOs targeting *Bcl-2* mRNA (Oblimersen- G3139) (NCT00085228) and *Clusterin* mRNA (Custirsen- OGX011) (NCT01188187) had been evaluated in PCa human patients in Phase II and III clinical trials, but both failed due to major toxic events or no significant survival improvement (Sternberg et al., 2009; Beer et al., 2017). Additionally, ASOs targeting *Hsp27* mRNA (Apatorsen- OGX-427) (NCT01120470) and *AR* mRNA (ARRx-AZD5312) (NCT03300505) are currently under Phase I clinical trials. In addition to targeting the protein-coding mRNAs, ASO targeting lncRNA *MALAT1* dramatically prevented lung cancer metastasis in a pulmonary metastatic mouse model (Gutschner et al., 2013), showing attractive potentials for developing ASO drugs targeting functional ncRNAs to treat PCa. More preclinical investigations for ASO targeting ncRNAs are needed to enable ASO-based prostate cancer treatment in the near future.

LncRNA Studies Through *in vivo* Mouse Models

Most lncRNAs' functions and mechanisms were revealed through knocking down strategies from *in vitro* study until to date, and several lncRNAs are proven to be necessary for life and brain development through the studies in the knockout mouse model (Sauvageau et al., 2013; Nakagawa et al., 2014). On the other hand, accumulating evidence showed that inactivating the same lncRNAs in mouse models resulted in no phenotype, and even opposite effects for some lncRNAs (Bassett et al., 2014; Sun and Ma, 2019). Bassett et al. (2014) summarized the results of *in vivo* studies of 30 lncRNAs through different inactivation strategies from 17 groups. Among them, lncRNA *MALAT1* was inactivated through 3 different strategies: deleting 3kb genomic region covering the 5′ end of *MALAT1* and its promoter (Zhang et al., 2012), removing the entire 7kb *MALAT1* gene (Eissmann et al., 2012), and premature transcriptional termination by inserting *lacZ* and polyadenylation sequences downstream of the transcriptional start site of *MALAT1* (Nakagawa et al., 2012). All these *MALAT1*-deficient mice from these 3 studies were viable and fertile without significant changes in mice development and growth, and global gene expression, which argues against the in vitro and xenograft studies that

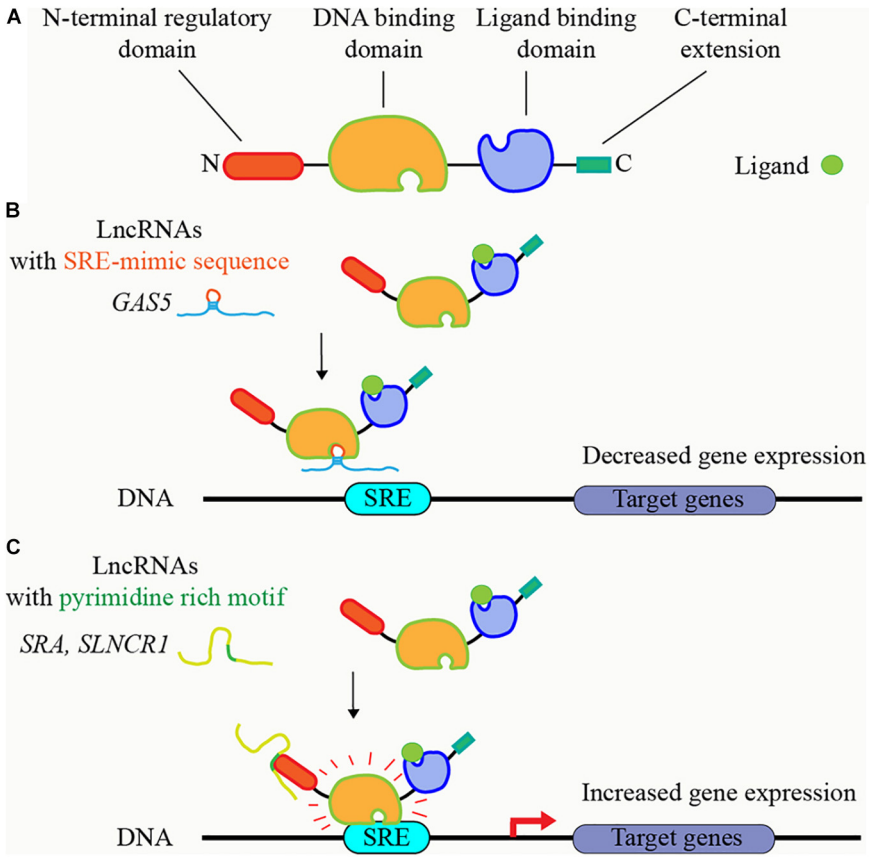

FIGURE 3 | LncRNAs bind to steroid receptors and regulate their activities. **(A)** Principal functional domains of steroid receptors. The steroid receptors have N-terminal regulatory domain, central DNA binding domain, ligand-binding domain, and C-terminal extension. **(B)** LncRNAs containing steroid receptor responsive element (SRE) mimic RNA sequence, such as *GAS5*, block the binding of steroid receptors to SRE DNA sequence, and suppress steroid receptors' transcriptional activity. **(C)** LncRNAs containing short, pyrimidine-rich RNA motif, such as *SRA* and *SLNCR1*, bind to steroid receptors at N-terminal, regulatory domain, and increase steroid receptors' transcriptional activity.

demonstrated *MALAT1*'s role in promoting cell proliferation and metastases through regulating pre-mRNA splicing (Tripathi et al., 2010), coordinating gene transcription (Yang et al., 2011; Wang et al., 2015), and acting as competitive endogenous RNA (Wang y. et al., 2017; Dai et al., 2019). Recently, Kim et al. (2018) investigated *MALAT1*'s role in a transgenic mouse model of breast cancer in which the *MALAT1* gene was inactivated through premature transcriptional termination by inserting lacZ and polyadenylation sequences as Nakagawa et al. (2012). Targeted inactivation of *MALAT1* in this breast cancer mouse model doesn't affect breast tumor growth, but surprisingly promotes breast cancer lung metastasis (Kim et al., 2018), which is consistent with some other reports that suggest *MALAT1* functions as a tumor suppressor (Xu et al., 2015; Cao et al., 2016; Han et al., 2016; Latorre et al., 2016; Kwok et al., 2018). Importantly, the metastatic-promoting effect of *MALAT1* insertional inactivation can be reversed by genetical re-expression of *MALAT1*, and targeted transgenic overexpression of *MALAT1* in mice inhibits breast cancer metastasis (Kim et al., 2018), strongly suggesting that the lncRNA *MALAT1* suppresses breast cancer metastasis.

There are several possible reasons to explain why some *in vivo* mouse models failed to validate lncRNAs' function discovered from *in vitro* and xenograft studies. First, most lncRNAs were studied in cells through the knocking down methods mediated by short-hairpin RNA (shRNA) or siRNA without appropriate rescue assays. Silencing nuclear lncRNAs requires the nuclear enrichment of AGO2 and RNA interference (RNAi) factors Dicer, TRBP, and TRNC6A/GW182 (Gagnon et al., 2014), while AGO2's nuclear distribution depends on cell type and tissue context (Sharma et al., 2016), and thus results from knocking down lncRNAs in cells lacking nuclear distribution of AGO2 are questionable. Secondly, several lncRNAs were silenced in cells and mouse through ASO treatment. However, the delivery of ASO to the targeted cells and organs is still a challenge, and it is not clear whether ASO could efficiently degrade the nascent RNAs. Besides, the potential off-target effects of ASO may lead to non-specific results (Deleavey and Damha, 2012). Thirdly, loss of function approaches in cells and mouse models through large size gene deletion or clustered regularly interspaced short palindromic repeats (Crispr)-Cas9 knocking out may also delete the neighboring genes and destroy the

regulatory elements for other genes. Some lncRNAs function through *cis* mechanisms to regulate their neighboring genes. The effect of destructing these regulatory elements located in the lncRNA genome loci and neighboring genes prevails against the effect of lncRNA loss (Yin et al., 2015). LncRNA inactivation induced by transcriptional terminator insertion abrogates lncRNA transcription with minimal disruption of genomic sequences and mice phenotypes induced by insertional inactivation of lncRNAs can be rescued by re-expression of lncRNAs (Bond et al., 2009; Berghoff et al., 2013; Grote et al., 2013). Fourthly, even though lncRNA is inactivated through the same method, different mouse models may display different phenotypes. Insertional inactivation of *MALAT1* in MMTV-PyMT mouse which is a transgenic model of metastatic breast cancer, induced significantly increased lung metastasis of breast cancer cells (Kim et al., 2018), while insertional inactivation of *MALAT1* in mouse with normal physiological condition showed no apparent phenotype (Nakagawa et al., 2012), suggesting *MALAT1* is dispensable for development but plays important roles in suppressing breast cancer metastasis. Taken together, it is critical to choose the proper method to generate lncRNA depleting cells and mouse model based on lncRNA's cellular distribution, genome localization and its function mechanism, and it is also important to take rescue experiments into consideration when investigating lncRNAs' function through loss of function methods (Bassett et al., 2014; Kopp and Mendell, 2018).

CONCLUSION

A growing number of novel discovered ncRNAs and various research has revealed the crucial roles of ncRNAs in different disease processes. Many ncRNAs have been verified to participate in PCa initiation or progression by regulating or mediating AR signaling. These ncRNAs hold great potentials as diagnostic biomarkers and therapeutic targets. Given that ncRNAs consist of the majority of the human transcriptome, a long journey in the understanding of ncRNAs, especially lncRNAs, circRNAs, and other ncRNAs, is yet to be achieved. Further investigations based on high-throughput sequencing technology and integrative bioinformatics analysis will enable the discovering of new functional ncRNAs and their regulation mechanisms, and these works will further promote the development of effective therapeutic strategies for PCa.

AUTHOR CONTRIBUTIONS

YY and QC developed the concept of the review. YY and KL collected the related publications and drafted this manuscript. QL and QC helped to revise this manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported in part by grants from the United States Department of Defense (W81XWH-17-1-0357, W81XWH-19-1-0563, and W81XWH-20-1-0504 to QC), American Cancer Society (RSG-15-192-01 to QC), NIH/NCI (R01CA208257 and Prostate SPORE P50CA180995 Developmental Research Program to QC), and Northwestern University Polsky Urologic Cancer Institute (to QC).

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 17 May 2021
doi: 10.3389/fcell.2021.642307

Steroid Hormone Biosynthesis Metabolism Is Associated With Fatigue Related to Androgen Deprivation Therapy for Prostate Cancer

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OPEN ACCESS

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

Received: 15 December 2020
Accepted: 06 April 2021
Published: 17 May 2021

Citation:
Feng LR, Barb JJ, Allen H, Regan J and Saligan L (2021) Steroid Hormone Biosynthesis Metabolism Is Associated With Fatigue Related to Androgen Deprivation Therapy for Prostate Cancer. Front. Cell Dev. Biol. 9:642307. doi: 10.3389/fcell.2021.642307

Background: Androgen deprivation therapy (ADT) is a cornerstone treatment for prostate cancer. Despite the clinical benefits, ADT is associated with multiple adverse effects including fatigue. The goal of the study was to examine metabolomic changes to better understand cancer-related fatigue specific to ADT treatment.

Methods: A total of 160 plasma samples collected from participants with (+ADT, n = 58) or without neoadjuvant ADT (−ADT, n = 102) prior to radiation therapy for treatment of non-metastatic localized prostate cancer were included in the study. Fatigue and sleep-related impairment were measured using the Patient Reported Outcomes Measurement Information System. Plasma metabolites were identified and measured using untargeted ultrahigh-performance liquid chromatography/mass spectrometry metabolomics analyses. Partial least square discriminant analysis was used to identify discriminant metabolite features, and the diagnostic performance of selected classifiers was quantified using AUROC curve analysis. Pathway enrichment analysis was performed using metabolite sets enrichment analyses.

Findings: Steroid hormone biosynthesis pathways, including androstenedione metabolism as well as androgen and estrogen metabolism, were overrepresented by metabolites that significantly discriminated samples in the +ADT from the −ADT group. Additional overrepresented metabolic pathways included amino acid metabolism, glutathione metabolism, and carnitine synthesis. Of the metabolites that were significantly different between the groups, steroid hormone biosynthesis metabolites were most significantly correlated with fatigue severity. Sleep-related impairment was strongly correlated with fatigue severity and inversely correlated with ADT-induced reduction in androsterone sulfate.

Conclusions: Patients with non-metastatic prostate cancer receiving neoadjuvant ADT prior to radiation therapy reported relatively more severe fatigue. Increased fatigue in this population may be attributable to sleep-related impairment associated with alterations in steroid hormone biosynthesis. Findings in this study provide a basis for further research of changes in sleep patterns and their role in this specific subcategory of cancer-related fatigue caused by the treatment.

Keywords: cancer-related fatigue, radiation therapy, prostate cancer, androgen deprivation therapy, metabolomics, steroid hormone biosynthesis, androgen metabolism

INTRODUCTION

Androgen-deprivation therapy (ADT) is considered a cornerstone treatment option for prostate cancer, the second leading cause of cancer mortality among North American men (Duchesne et al., 2016). Nearly 50% of all patients with prostate cancer will undergo ADT at some point after diagnosis, either as primary, neoadjuvant, or secondary therapy (Alibhai et al., 2010). The effects of hormonal ADT in suppressing tumor growth and delaying metastasis was first reported in 1941 and is thought to be related to the ubiquitous expression of androgen receptors in prostate cancer and the dependence of tumor cell growth on the transcription of specific pro-survival genes downstream from androgen receptor signaling (Harris et al., 2009).

Despite the significant survival advantage it confers, ADT is associated with numerous adverse effects including increased risk for cardiovascular diseases, diabetes, sexual dysfunction, cognitive, and mood dysfunction, and sleep disturbance (Gonzalez et al., 2018; Siddiqui and Krauss, 2018). One of the most common and debilitating symptoms of both cancer and ADT is fatigue, reported by up to 80% of oncology patients and 40% of patients receiving ADT (Nelson et al., 2016). Cancer-related fatigue is among the most debilitating symptoms related to cancer or cancer treatment and a common cause for falls leading to physical disability, inability to work, and feelings of hopelessness and despair (Vande Walle et al., 2014; Wolvers et al., 2019). There is an urgent need to understand the underlying mechanisms of cancer-related fatigue to find ways to better manage this common condition (Berger et al., 2015).

Metabolomic profiling refers to the comprehensive identification and quantification of endogenous or exogenous small-molecule metabolites (Lanznaster et al., 2018). This method is particularly well-suited for biomarker discovery because the metabolomic status in a biospecimen directly reflects the chemical transformation during metabolism, depicting both the steady-state equilibrium and dynamic responses to physiological stimuli (Tolstikov et al., 2020). The targeted approach refers to quantitation of chemically annotated metabolites using isotope labeling; the untargeted metabolomics, on the other hand, is the unbiased and comprehensive approach that first detects distinct chromatographic features, such as mass-to-charge ratio, and subsequently identifies metabolites using the reference spectral library (Ribbenstedt et al., 2018). In recent years, metabolomics has gained popularity as a powerful tool for biomarker discovery and mechanistic investigations that complement other -omics methodologies, providing invaluable information on tissue specificity and temporal dynamics (Armstrong et al., 2012).

Cancer-related fatigue is a clinical condition that likely encompasses a multitude of subcategories with different pathogenic mechanisms that lead to the same subjective experience (Berger et al., 2015). For example, previous studies have shown that cancer-related fatigue that lasts up to a year may be due to unresolved inflammation (Feng et al., 2017, 2018a), whereas acute fatigue during radiation therapy with neoadjuvant ADT may be related to anemia and mitochondrial dysfunction (Feng et al., 2015, 2018b). The complexity of the symptom and the heterogeneity of underlying mechanisms make metabolomics particularly well-suited for studying cancer-related fatigue. Our goal in this study was to focus specifically on mechanisms of fatigue related to androgen deprivation in men with non-metastatic localized prostate cancer. We utilized an unbiased comprehensive approach to examine metabolic changes associated with this specific ADT-related subcategory of cancer-related fatigue. We further explored the contribution of ADT-induced sleep impairment to increased fatigue reported by patients who received ADT.

MATERIALS AND METHODS

Study Participants

This study was approved by the National Institutes of Health (NIH) Institutional Review Board. All participants were men with confirmed diagnoses of localized non-metastatic adenocarcinoma of the prostate who were scheduled to receive external-beam radiation therapy. Exclusion criteria included progressive illnesses, psychiatric diseases within the past 5 years, uncorrected hypothyroidism, anemia, a second malignancy, and use of sedatives, steroids, or non-steroidal anti-inflammatory agents. At the time of the study, participants in the +ADT group received on average 52 days of ADT prior to starting external-beam radiation therapy. The ADT treatment included 22.5 mg leuprolide acetate, a gonadotropin-releasing hormone (GnRH) agonist, and a daily dose of 50 mg bicalutamide, an androgen receptor antagonist (Sharifi et al., 2005). Participants were recruited at the NIH Magnuson Clinical Research Center, Bethesda, MD, United States. Signed written informed consents were obtained prior to study participation.

Instruments

Sleep quality was measured using the Patient Reported Outcomes Measurement Information System (PROMIS™) v1.0 – Sleep Related Impairment (PROMIS-SRI) Short Form 8b, an eight-question form that quantifies the extent to which sleep impairment impacts daily life (Buysse et al., 2010; Yu et al., 2012). Raw scores ranging from 8 to 40 are converted to a *T*-score with a mean of 50 (Yu et al., 2012). A lower PROMIS-SRI *T*-score indicates better sleep, and a higher *T*-score indicates increased impairment due to reduced sleep (Yu et al., 2012). Fatigue was quantified with the PROMIS v1.0 – Fatigue (PROMIS-Fatigue) Short Form 7a, which measures the impact in the last 7 days that fatigue has on daily life (Ameringer et al., 2016). Raw scores range from 7 to 35, and the *T*-score ranges from 29.4 to 83.2, with a mean of 50 (Cook et al., 2012). Similar to PROMIS-SRI, higher scores on the PROMIS-Fatigue scale indicate higher fatigue symptom severity (Rothrock et al., 2010). The PROMIS *T*-score metric is anchored to the United States general population, and a cutoff *T*-score of 50 differentiates clinically meaningful fatigue in the oncology population (Cella et al., 2014).

Metabolomics

Untargeted metabolomics analysis was performed at Metabolon, Inc., (Durham, NC, United States), as previously described (Collet et al., 2017). Briefly, individual plasma samples were subjected to methanol extraction and divided into aliquots for analysis. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove proteins, dissociate small molecules bound to proteins, or trapped in the precipitated protein matrix, and recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse-phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample reserved for backup. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolutions. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA, and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water; however, with 6.5 mM ammonium bicarbonate at pH 8. The fourth aliquot was analyzed *via* negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70–1,000 m/z.

Raw data were extracted, peak-identified, and QC-processed using Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. The reference library consists of authenticated standards that contain the retention time/index (RI), mass-to-charge ratio (m/z), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library ± 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities and to remove those representing system artifacts, mis-assignments, and background noise. Library matches for each compound were checked for each sample and corrected if necessary, and peaks were quantified using the area under the curve.

Statistical Analysis

Metabolite concentrations were normalized to sample volume utilized for extraction and rescaled to set the median equal to 1. Metabolite concentrations were subsequently interquantile range (IQR) filtered and analyzed using univariate ANOVA and *t*-tests (unpaired, unequal variance assumed) to generate the volcano plots. IQR-filtered data were further log transformed, autoscaled, and analyzed using partial least square-discriminant analysis (PLS-DA) to determine the variance importance in projection (VIP). Multiple comparisons were adjusted with the Benjamini–Hochberg false discovery rate (FDR) method (Benjamini and Hochberg, 1995). LOOCV cross-validation and permutation tests were performed to test the model with Q^2 and R^2 being used to assess the robustness of the model. Metabolites were considered significant features for further analysis at VIP > 1.5, | log2 fold change| > 1.5, and FDR ≤ 10% (Newell et al., 2016). Diagnostic performance of selected classifiers was quantified using the Area under the

Receiver Operating Characteristics (AUROC) curve analysis. Metabolite pathway analysis metabolite set enrichment analysis (MSEA) was performed in MetaboAnalyst 4.0 as previously described (Xia and Wishart, 2016). Statistical significance was defined as $p < 0.05$. Data analyses were performed using a combination of JMP Pro^{TM} Statistical Discovery Software 15 15.0.0 (SAS Institute, Cary, NC, United States).

RESULTS

A total of 160 plasma samples collected from participants with (+ADT, $n = 58$) or without neoadjuvant ADT (−ADT, $n = 102$) prior to radiation therapy for treatment of non-metastatic localized prostate cancer were included in the current study (**Table 1**). All study participants were older men with an average of 66 ± 7.07 years of age (**Table 1**). Participants who had received ADT exhibited higher body mass index (BMI) as compared to those without ADT (**Table 1**; +ADT: 29.65 ± 4.83; −ADT: 28.00 ± 4.30). There was no statistically significant difference in education or ethnicity between the two groups (**Table 1**). A larger proportion of participants exhibited higher Gleason scores in the +ADT group, whereas no significant difference was observed in the *T*-stage between +ADT and −ADT groups (**Table 1**).

Using untargeted LC/MS, we found 1,120 compounds of known identity. After applying an interquartile range filter, a total of 661 metabolites remained: 315 lipids, 140 xenobiotics, 120 amino acids, 27 cofactors and vitamins, 13 carbohydrates, 13 nucleotides, 12 peptides, and 18 partially characterized molecules.

First, we compared the overall metabolomic profiles of the +ADT vs. −ADT groups. A total of 28 metabolites were found to be significant features for further analyses based on a log2 fold change cutoff of 1.5 at 10% false-discovery rate (**Figure 1A**; see **Table 2** for detailed chemical information), and a PLSDA VIP score of ≥1.5 (**Figures 1B,C**). The receiver operator characteristic curve analysis (AUROC) demonstrated good discriminating power of the classification model to distinguish the +ADT from the −ADT group [**Figure 1D**: AUC = 0.839, 95% CI (0.725, 0.903)]. As expected for the intended effect of ADT, metabolite set enrichment analysis

TABLE 1 | Demographics and clinical characteristics of sample population.

	Total (n = 160)	ADT (n = 58)	No ADT (n = 102)	*P*-value
Age (years)	66 ± 7.07	67.31 ± 7.15	64.30 ± 8.70	0.027
BMI (kg/m2)	28.6 ± 4.56	29.65 ± 4.83	28.00 ± 4.30	<0.0001
Race				
African American	21.25%	24.14%	19.61%	0.751
Asian	5.00%	3.45%	5.88%	
Caucasian	72.50%	72.41%	72.55%	
Unknown	1.25%	0.00%	1.96%	
Ethnicity				
Hispanic/Latino	2.50%	1.70%	2.94%	0.64
Non-Hispanic/Latino	96.25%	98.30%	95.10%	
Unknown	1.25%	0.00%	1.96%	
Education				
Eighth grade or less	0.63%	1.72%	0.00%	0.058
High school	11.88%	12.07%	11.76%	
Vocational/Technical Degree	18.13%	8.62%	23.53%	
Associate degree/some college	5.00%	10.34%	1.96%	
Bachelor's degree	17.50%	20.69%	15.69%	
Advanced degree	45.63%	44.83%	46.08%	
Unknown	1.25%	1.72%	0.98%	
***T*-stage**				
T0	5.00%	0.00%	7.84%	0.15
T1c	36.88%	34.48%	38.24%	
T2a-c	34.38%	34.48%	34.31%	
T3a-c	20.00%	25.86%	16.67%	
T4	3.75%	5.17%	2.94%	
Gleason score				
6	21.90%	0.00%	34.31%	<0.0001
7	38.10%	39.66%	37.25%	
8	25.00%	32.76%	20.59%	
9	13.10%	24.14%	6.86%	
10	1.90%	3.45%	0.98%	

BMI, body mass index; ADT, androgen deprivation therapy.

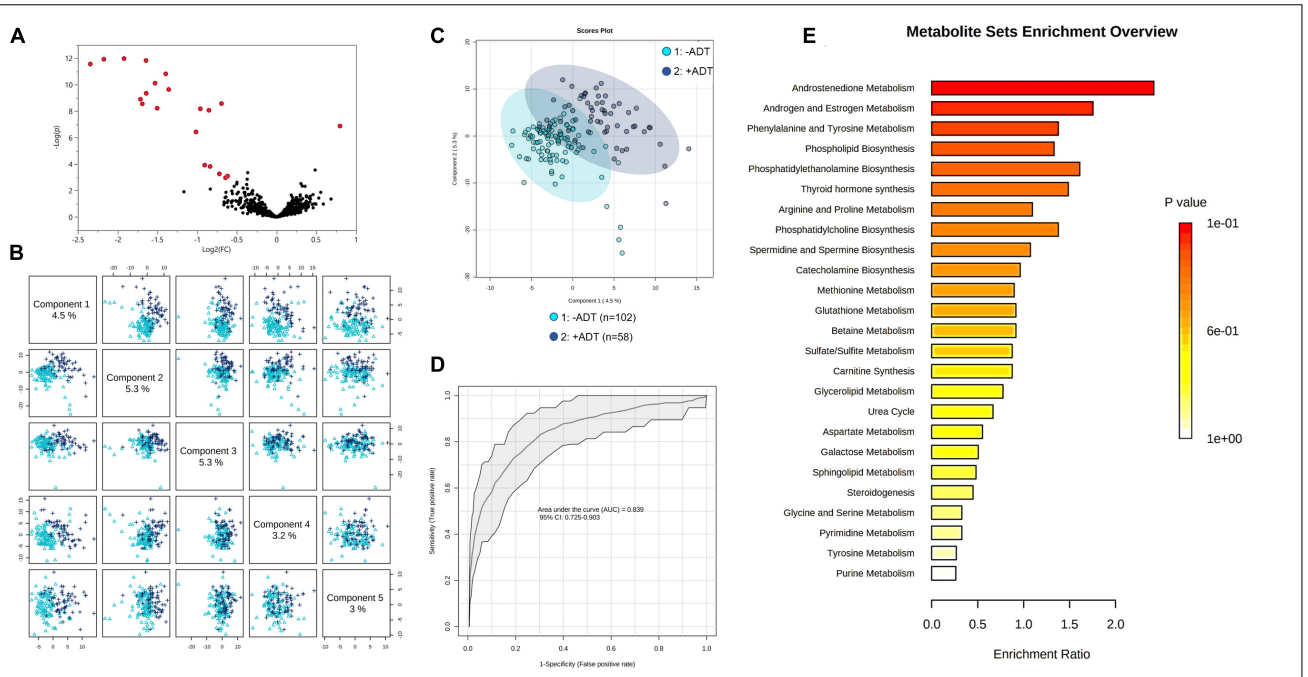

FIGURE 1 | Metabolic profiles of patients with non-metastatic prostate cancer with (+ADT, n = 58) or without androgen deprivation therapy (−ADT, n = 102). **(A)** Volcano plot of metabolites of the +ADT group compared to −ADT. The y axis represents p-value converted to negative log 10 scale, and the x axis represents log2 fold change. Significant metabolites (fold change > 1.5, FDR ≤ 0.1) were highlighted in red. **(B)** Pairwise PLSDA score plot of the top five components. **(C)** PLSDA two-dimensional plot ellipses representing 95% confidence intervals. **(D)** ROC curve demonstrating the specificity and sensitivity of the PLSDA model discriminating the +ADT group from the −ADT group. AUC = 0.839, 95% CI (0.725, 0.903). Excellent classification is indicated by an AUC > 0.90. **(E)** Metabolite set enrichment analysis (MSEA) of significant metabolites.

(MSEA) of the significantly different metabolites revealed steroid hormone biosynthesis pathways, including androstenedione metabolism as well as androgen and estrogen metabolism, to be overrepresented by metabolites that were significantly different between +ADT and −ADT groups (**Figure 1E**). Box plots of individual metabolites related to steroid hormone biosynthesis are shown in **Figure 2**. Sulfated (**Figures 2A–H**) as well as the glucuronide steroid hormone metabolic products (**Figures 2I–L**) were decreased by ADT, demonstrating the effectiveness of ADT. Although the length of treatment in the +ADT group varied [mean = 52.53 days, 95% CI (44.65, 60.43)], reductions in androsterone sulfate levels were not significantly correlated with the length of ADT treatment ($p = 0.10$). Notably, the various degrees to which ADT decreased levels of androsterone sulfate illustrated the heterogeneity of the response to treatment in the +ADT group (**Figure 2A**). Additional top enriched pathways (**Figure 1E**) also included amino acid metabolism pathways (**Figure 3A**), glutathione metabolism (**Figure 3B**), and carnitine synthesis (**Figure 3C**).

Second, we examined the associations between PROMIS-Fatigue T-scores and metabolites that significantly distinguished the +ADT group from the −ADT group. A PROMIS T-score of 50 best differentiates clinically meaningful fatigue in the oncology population (Cella et al., 2014). Fatigue (T-score ≥ 50) was reported by 40% of participants in the +ADT group (PROMIS T-score: 47.92 ± 7.28) compared to 24% of the −ADT group (PROMIS T-score: 44.34 ± 7.81) (**Figure 4A**, $p = 0.0064$). Correlations of fatigue severity and metabolites that significantly distinguished the two groups are shown in **Figure 4B**. Metabolites that significantly correlated with PROMIS-Fatigue T-scores were overrepresented by the steroid hormone biosynthesis pathway (KEGG ID: M00107), including androgen and estrogen metabolism (SMPDB ID: SMP0000068) and androstenedione metabolism (SMPDB ID: SMP0030406) (**Figure 4C**), which included androsterone sulfate ($r = -0.26$, $p = 0.0009$), epiandrosterone sulfate ($r = -0.25$, $p = 0.0016$), etiocholanolone glucuronide ($r = -0.19$, $p = 0.018$), and dehydroepiandrosterone sulfate (DHEA-S) ($r = -0.18$, $p = 0.023$). PROMIS-Fatigue T-scores were also correlated with sulfated metabolites of androgen including androstenediol (3beta,17beta) monosulfate ($r = -0.26$, $p = 0.0008$), 5alpha-androstan-3beta,17beta-diol disulfate ($r = -0.21$, $p = 0.0083$), 5alpha-androstan-3beta,17beta-diol monosulfate ($r = -0.20$, $p = 0.012$), 5alpha-androstan-3alpha,17alpha-diol monosulfate ($r = -0.22$, $p = 0.0062$), and 5alpha-androstan-3beta,17alpha-diol disulfate ($r = -0.17$, $p = 0.028$) (**Figure 5**).

Third, we investigated whether the increased fatigue in the +ADT group was related to sleep impairment, measured by PROMIS-SRI. Interestingly, participants in the +ADT group also reported higher levels of sleep-related impairment at 41% (T-score ≥ 50), compared to 25% of the −ADT group (**Figure 6A**, $p = 0.0053$). Self-reported sleep-related impairment

(PROMIS-SRI T-score) was significantly correlated with cancer-related fatigue (**Figure 6B**, $r = 0.75$, $p = 1.28 \times 10^{-29}$). Of all 28 metabolites that were significantly different between +ADT and −ADT groups, only androsterone sulfate levels significantly correlated with PROMIS-SRI sleep impairment T-scores (**Figure 6C**, $r = -0.19$, $p = 0.020$), which serves as a metabolic endpoint indicator of the effectiveness of ADT in reducing circulating androgens (Chi et al., 2020).

TABLE 2 | Chemical information of metabolites of interest.

Metabolite ID	Super pathway	Sub pathway	Compound ID	Platform	Chemical ID	RI (retention time/index)	Mass/Charge ratio
epiandrosterone sulfate	Lipid	Androgenic Steroids	33973	LC/MS Neg	100001287	4855	369.1741
androsterone glucuronide	Lipid	Androgenic Steroids	61846	LC/MS Neg	100002761	4953	465.2494
androsterone sulfate	Lipid	Androgenic Steroids	31591	LC/MS Neg	100001073	5022	369.1741
5alpha-androstan-3alpha,17beta-diol monosulfate (2)	Lipid	Androgenic Steroids	37185	LC/MS Neg	100006005	5080	371.1898
5alpha-androstan-3beta,17alpha-diol disulfate	Lipid	Androgenic Steroids	37187	LC/MS Neg	100002021	4215	225.0697
etiocholanolone glucuronide	Lipid	Androgenic Steroids	47112	LC/MS Neg	100005403	4915	465.2494
androstenediol (3alpha, 17alpha) monosulfate	Lipid	Androgenic Steroids	37207	LC/MS Neg	100002026	4712	369.1741
androstenediol (3beta,17beta) disulfate	Lipid	Androgenic Steroids	37203	LC/MS Neg	100001994	4065	224.0624
androstenediol (3beta,17beta) monosulfate	Lipid	Androgenic Steroids	37210	LC/MS Neg	100002029	4500	369.1741
N,N,N-trimethyl-5-aminovalerate	Amino Acid	Lysine Metabolism	57687	LC/MS Pos Early	100015962	2186	160.1332
glycosyl-N-(2-hydroxynervonoyl)-sphingosine (d18:1/24:1(2OH))	Lipid	Hexosylceramides (HCER)	57444	LC/MS Pos Late	100015752	3839	826.6767
behenoyldihydrosphingomyelin (d18:0/22:0)	Lipid	Dihydrosphingomyelins	57331	LC/MS Pos Late	100009026	3150	789.6844
malonylcarnitine	Lipid	Fatty Acid Synthesis	37059	LC/MS Pos Early	100001526	2086	248.1129
N-methylproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	37431	LC/MS Pos Early	100001956	1335	130.0863
sphingomyelin (d18:0/20:0, d16:0/22:0)	Lipid	Dihydrosphingomyelins	57476	LC/MS Pos Late	100015786	2600	761.6531
cys-gly, oxidized	Amino Acid	Glutathione Metabolism	18368	LC/MS Neg	1224	925	353.0595
homostachydrine	Xenobiotics	Food Component/Plant	33009	LC/MS Pos Early	100001550	1750	158.1176
chiro-inositol	Lipid	Inositol Metabolism	37112	LC/MS Polar	100001859	3191.2	225.0616
5alpha-androstan-3alpha,17beta-diol 17-glucuronide	Lipid	Androgenic Steroids	47132	LC/MS Neg	100005396	4930	467.265
pregnanediol-3-glucuronide	Lipid	Progestin Steroids	40708	LC/MS Neg	100003470	5145	495.2963
dehydroepiandrosterone sulfate (DHEA-S)	Lipid	Androgenic Steroids	32425	LC/MS Neg	100000792	4745	367.1585
spermidine	Amino Acid	Polyamine Metabolism	485	LC/MS Pos Late	50	700	146.1652
cysteinylglycine	Amino Acid	Glutathione Metabolism	35637	LC/MS Pos Early	278	2132	179.0485
phenylalanine	Amino Acid	Phenylalanine Metabolism	64	LC/MS Pos Early	460	2878	166.0863
guanidinoacetate	Amino Acid	Creatine Metabolism	43802	LC/MS Polar	344	2884	116.0466
proline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1898	LC/MS Pos Early	480	1603	116.0706
deoxycarnitine	Lipid	Carnitine Metabolism	36747	LC/MS Pos Early	100001662	2052	146.1176
succinylcarnitine (C4-DC)	Energy	TCA Cycle	37058	LC/MS Pos Early	100001948	2291	262.1285

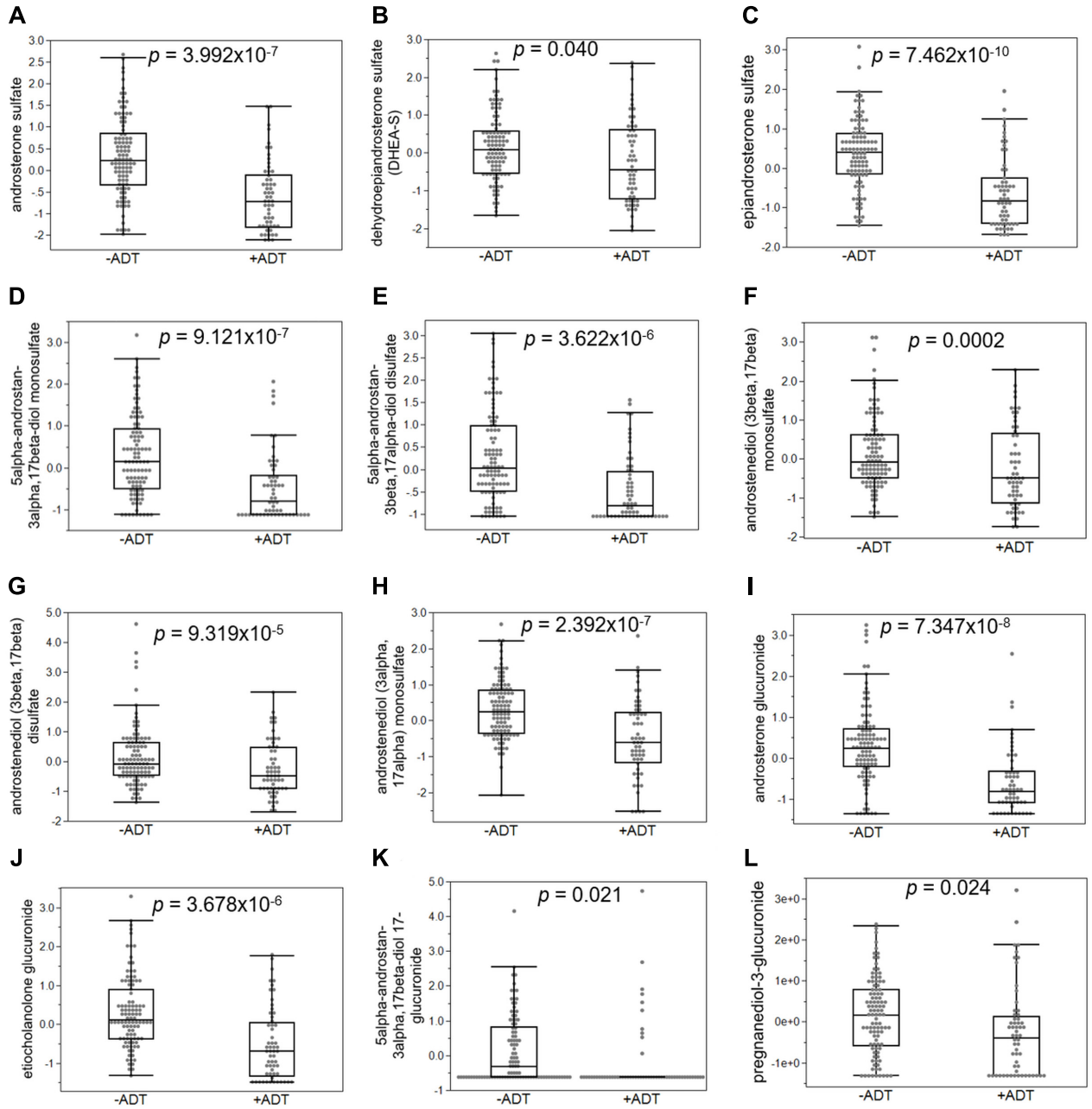

FIGURE 2 | Androgen deprivation therapy broadly affected metabolites related to steroid hormone biosynthesis. **(A)** Androsterone sulfate was significantly decreased in patients with ADT. Sulfated **(B–H)** and glucuronidated androgen metabolites **(I–L)** were significantly decreased in the +ADT group. $^{*}p < 0.05$ and false-discovery rate $\leq$ 10%.

DISCUSSION

Our goal in this study was to focus on mechanisms of a subcategory of cancer-related fatigue specifically related to ADT in patients with non-metastatic localized prostate adenocarcinoma. We examined metabolomic profile changes that may explain increased fatigue severity in response to receiving ADT (**Figure 7**). We found that metabolites related to steroid hormone biosynthesis best correlated with self-reported fatigue severity, which may be explained by sleep-related impairment as a result of ADT. To our knowledge, this is the first study that used the unbiased comprehensive metabolome profiling to examine the underlying mechanisms of ADT-induced increase in fatigue in men with non-metastatic prostate cancer.

Previous work showed that patients receiving ADT reported significant sleep disturbances manifested as difficulties with falling asleep and maintenance of sleep due to nocturia and hot flashes (Gonzalez et al., 2018). This is consistent with our findings

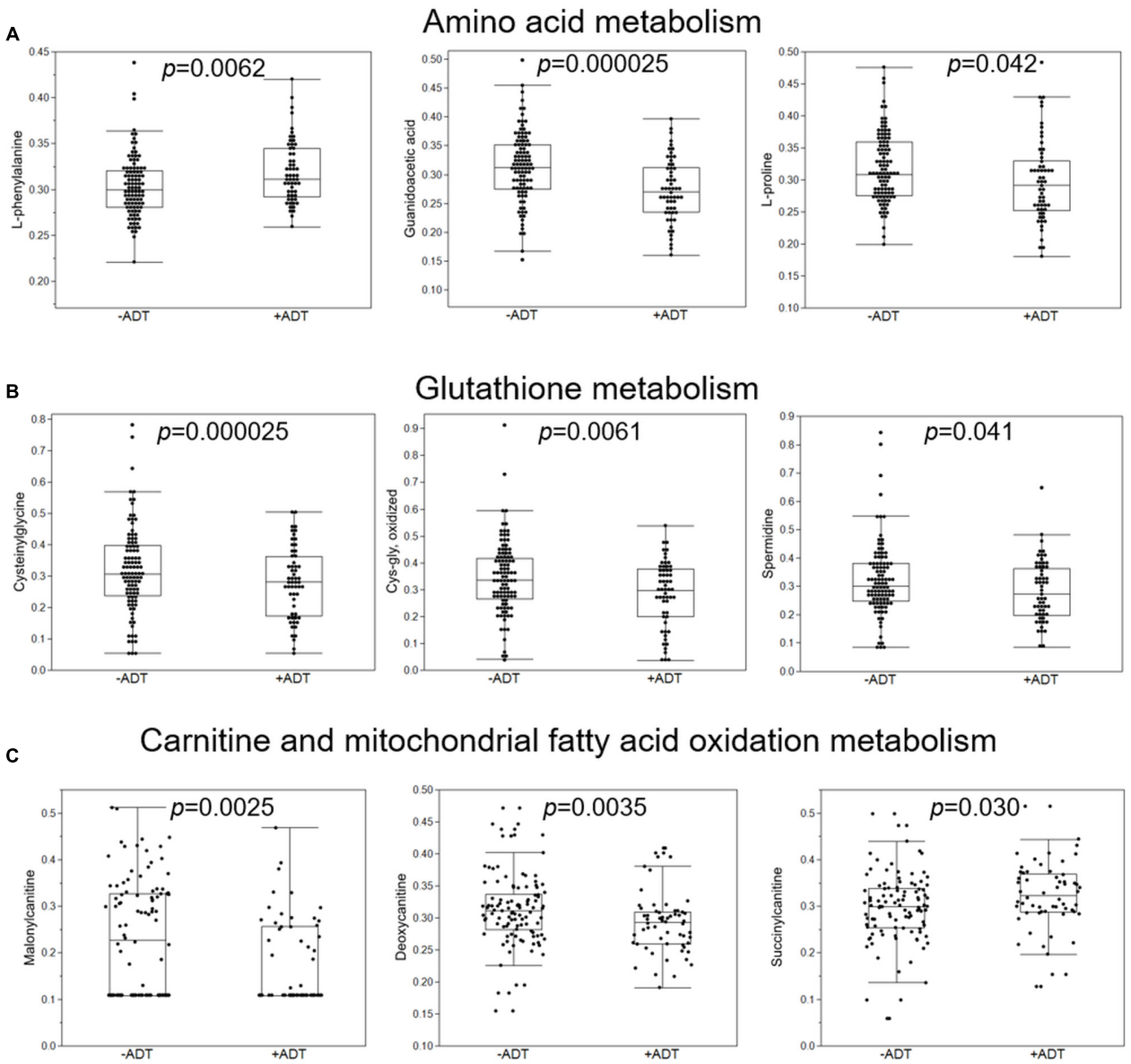

FIGURE 3 | Additional metabolic pathways that were overrepresented by metabolites significantly distinguished the +ADT from the −ADT group. **(A)** Box plots of individual metabolites related to amino acid metabolism. **(B)** Box plots of individual metabolites related to glutathione metabolism. **(C)** Box plots of individual metabolites related to carnitine and mitochondrial fatty acid oxidation metabolism.

that prostate cancer patients receiving ADT reported higher incidences of fatigue and sleep impairment. Of all metabolites that significantly distinguished the two groups, metabolites enriched in androstenedione metabolism as well as androgen and estrogen metabolism pathways most significantly correlated with PROMIS-Fatigue *T*-scores. The strong association between sleep-related impairment and self-reported fatigue severity suggests that the increased fatigue in the +ADT group may be due to sleep dysfunction. Importantly, of all the metabolites that significantly distinguished +ADT from −ADT group, the only metabolite that significantly correlated with PROMIS-SRI sleep impairment *T*-scores was androsterone sulfate, which is a metabolic endpoint marker of the effectiveness of ADT in reducing circulating androgens (Chi et al., 2020). This finding suggests that cancer-related fatigue specific to non-metastatic cancer patients undergoing ADT may be related to sleep impairment caused by the treatment itself. Besides physical discomfort caused by hot flashes and nocturia, the exact mechanism by which ADT causes sleep dysfunction is poorly understood (Gonzalez et al., 2018). Interestingly, androgen receptors are abundant in the suprachiasmatic nucleus (SCN) and play an important role in the neuroendocrine modulation of circadian rhythm (Mong et al., 2011). It is possible that ADT may cause sleep dysfunction, and subsequently fatigue, *via* alterations in the androgenic action on

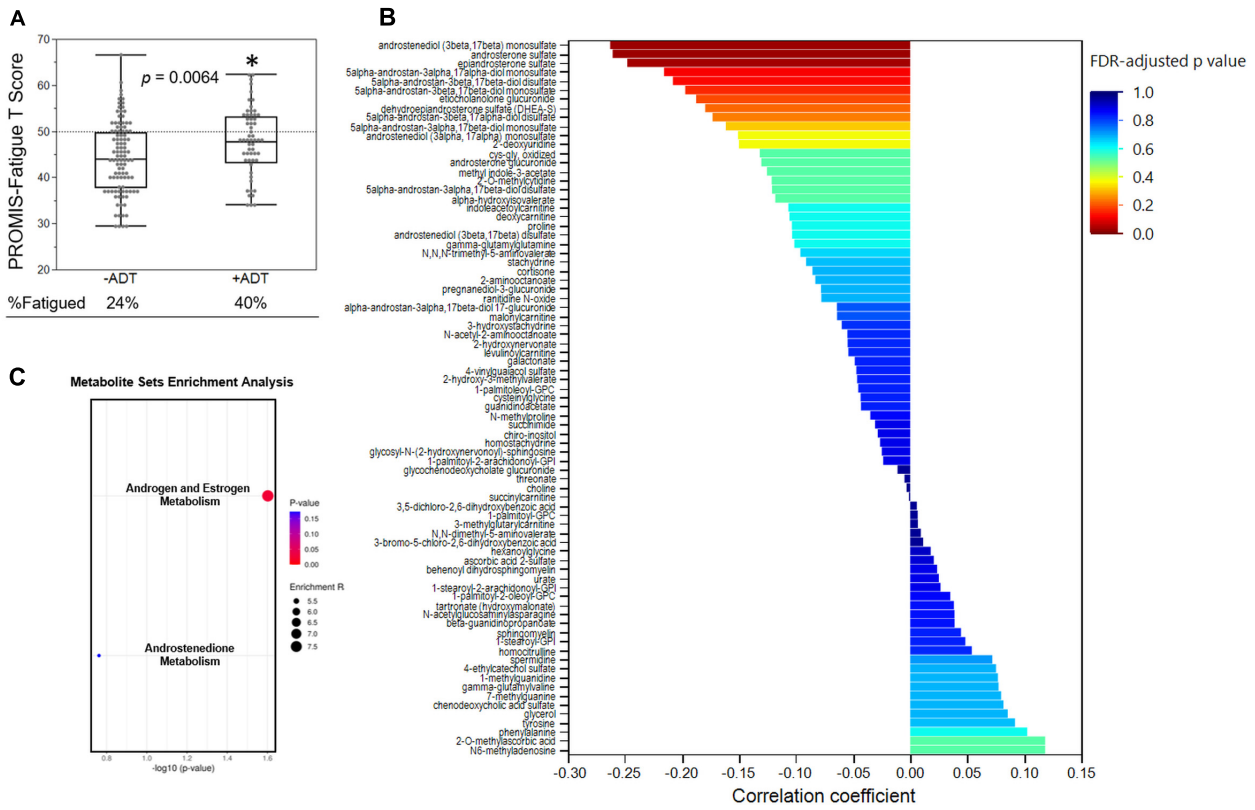

FIGURE 4 | Steroid hormone biosynthesis was associated with fatigue severity. **(A)** Box plot showing the PROMIS-fatigue *T*-scores of the −ADT PROMIS-Fatigue *T*-scores of the −ADT group (44.34 ± 7.81) and the +ADT group (47.92 ± 7.28). *Indicates statistical significance ($p = 0.0064$). Scores above the dotted lines are considered fatigued (24% of −ADT, 40% of +ADT). **(B)** Correlations of PROMIS-Fatigue *T*-score and metabolites that was significantly different between the two groups. X axis indicates the correlation coefficient. Colors of the bars indicate FDR-adjusted *p*-values. **(C)** Metabolite set enrichment analysis of metabolites that significantly correlated with PROMIS-Fatigue *T*-scores. Steroid hormone biosynthesis (KEGG ID: M00107), including androgen and estrogen metabolism (SMPDB ID: SMP0000068) as well as androstenedione metabolism (SMPDB ID: SMP0030406), were significantly overrepresented.

SCN circuitry and circadian rhythmicity. Although it is beyond the scope of this study, future research is needed to explore specific changes in sleep patterns caused by reduced androgen metabolites using more precise tools for measuring sleep, such as polysomnography and/or actigraphy.

Related to the natural history nature of the study design, the length of ADT treatment varied among participants in the +ADT group. Since reductions in levels of androsterone sulfate did not appear to depend on the length of treatment in the +ADT group, it is possible that the wide distribution of androsterone sulfate concentrations in the +ADT group reflected the heterogeneity in individual responses to hormonal ADT, rather than variability in treatment itself. Therefore, in analyses regarding fatigue and sleep impairment, we chose to use levels of androsterone sulfate, an indicator for the effectiveness of ADT (Chi et al., 2020), instead of a binary classification (+ADT vs. −ADT), to take into account the individual metabolic response to hormonal androgen suppression. In addition to steroid hormone biosynthesis pathways, we found preliminary indications that ADT may also affect metabolites related to carnitine homeostasis (**Figures 1E**, **3**). Interestingly, carnitine is important for the shuttling long-chain fatty acids across the mitochondrial inner membrane and β-oxidation (Longo et al., 2016). In fact, mitochondrial dysfunction is often secondary to a disruption of carnitine homeostasis (Sharma and Black, 2009). ADT-induced changes in metabolites related to carnitine synthesis and mitochondrial fatty acid oxidation may help explain our previous observation of mitochondrial dysfunction in fatigued patients (Feng et al., 2020). Another pathway of interest that was associated with metabolites that significantly discriminated between the two groups was glutathione metabolism (**Figure 1E**), including cysteinylglycine and oxidized cys-gly (**Figure 3B**). Cysteinylglycine (cys-gly) is produced from the hydrolysis of glutathione (glutamyl-cysteinyl-glycine), one of the most important endogenous free radical scavengers (Garibotto et al., 2003). Both cysteinylglycine and oxidized cys-gly are indicators of the redox state, which can be influenced by androgen receptor signaling (Chettimada et al., 2018; Cruz-Topete et al., 2020). Lastly, androgen signaling has been shown to affect amino acid metabolism (Putluri et al., 2011; Saylor et al., 2012; Chi et al., 2020). For example, certain oncogenic mutations result in a preference for particular amino acids; the metabolic microenvironment, in turn, helps shape the genetic landscape of the tumor (Tang et al., 2015; Chi et al., 2020), although

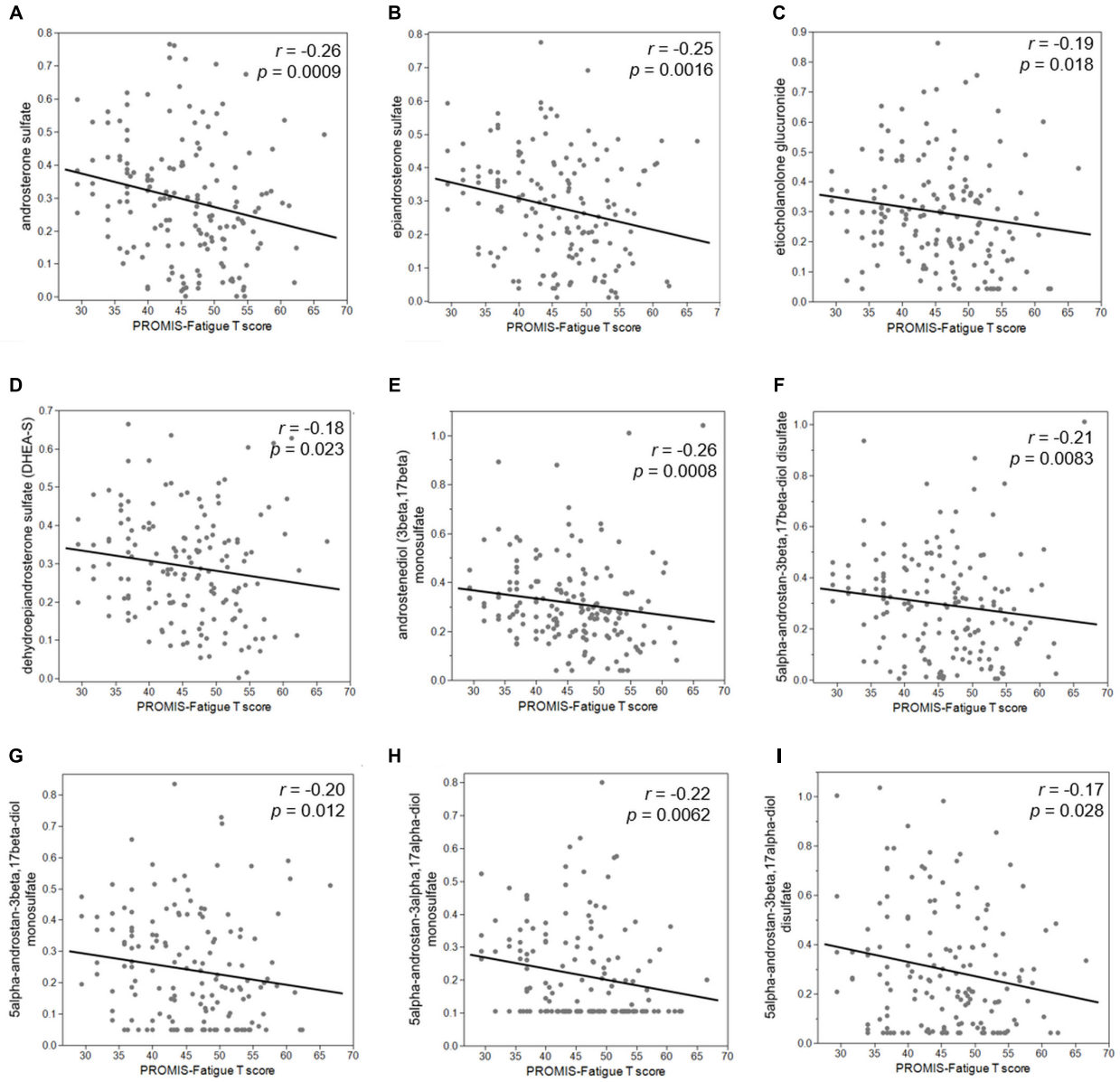

FIGURE 5 | Reduced steroid hormone biosynthesis metabolites were associated with increased severity of cancer-related fatigue. PROMIS-Fatigue *T*-scores significantly correlated with major androgen metabolites including **(A)** androsterone sulfate ($r = -0.26$, $p = 0.0009$), **(B)** epiandrosterone sulfate ($r = -0.25$, $p = 0.0016$), **(C)** etiocholanolone glucuronide ($r = -0.19$, $p = 0.018$), and **(D)** dehydroepiandrosterone sulfate (DHEA-S) ($r = -0.18$, $p = 0.023$). PROMIS-Fatigue *T*-scores also correlated with sulfated metabolites of androgen including **(E)** androstenediol (3beta,17beta) monosulfate ($r = -0.26$, $p = 0.0008$), **(F)** 5alpha-androstan-3beta,17beta-diol disulfate ($r = -0.21$, $p = 0.0083$), **(G)** 5alpha-androstan-3beta,17beta-diol monosulfate ($r = -0.20$, $p = 0.012$), **(H)** 5alpha-androstan-3alpha,17alpha-diol monosulfate ($r = -0.22$, $p = 0.0062$), and **(I)** 5alpha-androstan-3beta,17alpha-diol disulfate ($r = -0.17$, $p = 0.028$).

less clear is the role of the specific amino acid metabolites in fatigue pathogenesis in the +ADT group (**Figures 1E**, **3A**). It is possible that dysregulation in both oxidative stress response and mitochondria fatty acid trafficking may affect sleep and the consequent fatigue in these patients.

One caveat of the study is that the groups were not matched in sample size and lacked in randomization. This is because the participants were part of an exploratory prospective study instead of a clinical trial and treatment decisions were made by the patients in collaboration with their oncologists. Future studies with a larger sample size, particularly in the +ADT group, will be needed to validate findings in this study. Participants in the +ADT group exhibited higher BMI compared to the −ADT group (**Table 1**). However, based on our unpublished data, there was no significant daily physical activity difference between the groups ($p = 0.22$, unpublished Actigraphy data), suggesting that the difference in BMI was not attributable to

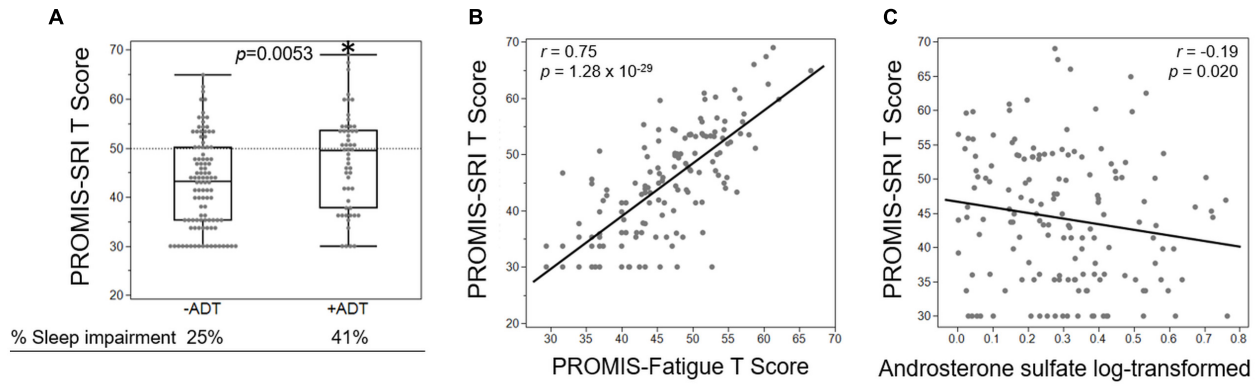

FIGURE 6 | Fatigue severity was associated with ADT-related increase in sleep impairment. **(A)** Box plot showing the PROMIS-SRI *T* scores of the −ADT PROMIS-Fatigue *T*-scores of the −ADT group (42.67 ± 9.24) and the +ADT group (47.2 ± 9.95). *Indicates statistical significance ($p = 0.0053$). Scores above the dotted lines are considered fatigued (25% of −ADT, 41% of +ADT). **(B)** PROMIS-SRI *T*-score was highly correlated with PROMIS-Fatigue *T*-score ($r = 0.75$, $p = 1.28 \times 10^{-29}$). **(C)** Androsterone sulfate levels significantly correlated with PROMIS-SRI *T*-scores ($r = -0.19$, $p = 0.020$).

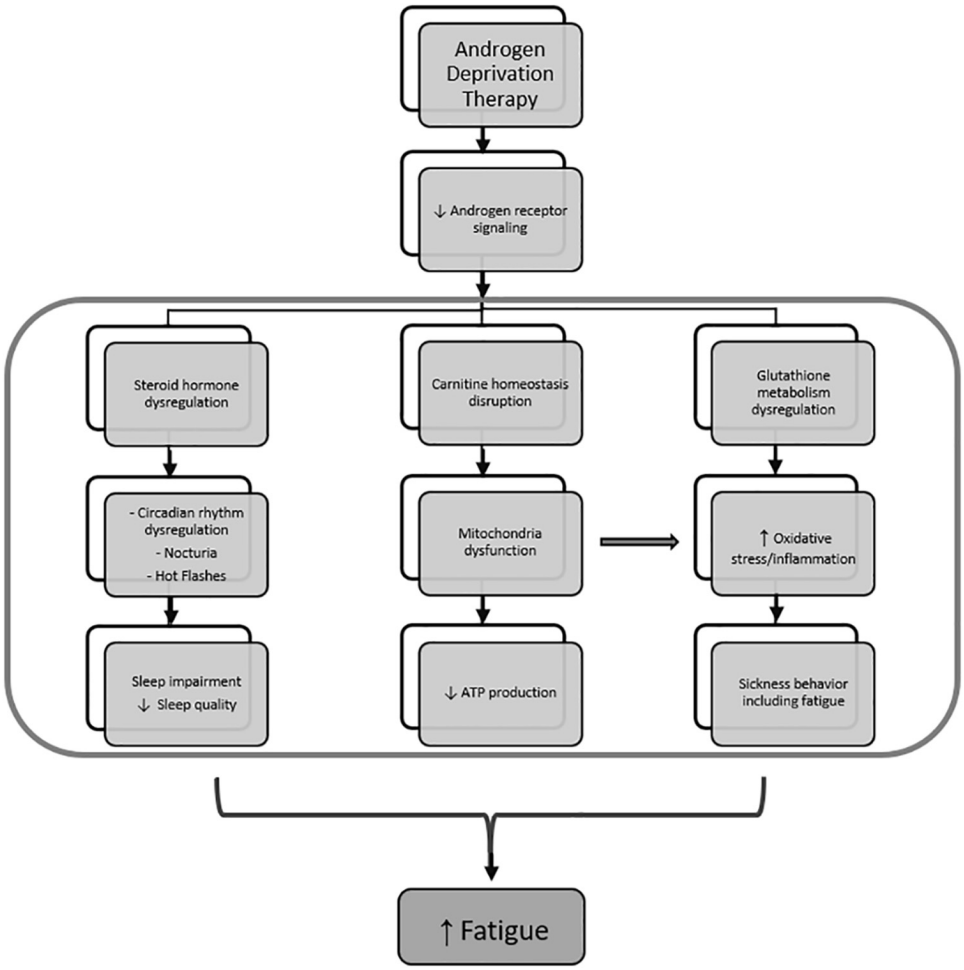

FIGURE 7 | Mechanism of fatigue related to androgen deprivation therapy. Androgen deprivation therapy, which inhibits androgen receptor signaling, results in steroid hormone metabolism dysregulation and leads to sleep impairment by affecting circadian rhythm regulation, nocturia, and hot flashes. At the same time, androgen deprivation also results in dysregulated carnitine homeostasis and glutathione metabolism, leading to mitochondrial dysfunction and oxidative stress, respectively. Mitochondrial dysfunction further increase oxidative stress and contributes to inflammation-induced sickness behavior that includes fatigue.

lifestyle differences between the groups. Although previous work has demonstrated an association between BMI and plasma levels of steroid hormones, future studies will employ more accurate measures of lean/fat mass, such as DEXA scan, to further examine the correlation between obesity and fatigue (He et al., 2018). In addition, the untargeted approach was chosen to allow for the simultaneous measurement of as many metabolites as possible to map out the ADT-related global metabolomic profile without requiring any *a priori* hypotheses. While different experimental platforms were used in this study to ensure optimized detection coverage (total number of detected metabolites of known identity: 1,120; total number of detected steroids: 42), future studies are needed to examine steroid hormones more closely using more targeted analyses. Relatedly, the pathway "androgen and estrogen metabolism" (pathway ID: SMP0000068) is an annotated metabolic pathway identified using pathway analysis (Metabolite Sets Enrichment Analysis), performed to discover biologically meaningful patterns within the data. However, the statistical significance of this pathway was likely attributable to androgen metabolites, as we did not see any difference in estrogen metabolites. Since the goal of the current study was to profile ADT-related metabolomic changes, the untargeted approach was preferable and allowed for a more global metabolomic profiling. However, it is possible the global untargeted approach is not sufficiently sensitive for estrogen measurements, particularly in male study participants. We plan to further examine androgen and estrogen metabolites in future studies using a more targeted approach. In addition, sleep impairment was measured by PROMIS-SRI, a self-report questionnaire. Future studies will be needed to assess specific changes in sleep patterns caused by ADT. Furthermore, we included participants with non-metastatic localized prostate cancer receiving neoadjuvant ADT prior to radiation therapy. Future studies will also investigate the effects of ADT as a primary therapy for treatment of advanced prostate cancer. Additionally, the effects of ADT were examined by cross-sectional comparisons in this study at one timepoint. As we continue to follow these patients, post−ADT samples will be collected to allow for measurements of longitudinal changes in hormone levels before and after ADT completion. Finally, we did not detect any significant difference between the two groups in levels of inflammatory metabolites, such as prostaglandin ($p = 0.78$) and leukotriene ($p = 0.39$). While the specific role of inflammation is beyond the scope of the current study, we hope to continue to follow these patients carefully examine the role of inflammation in this ongoing clinical protocol.

CONCLUSION

In conclusion, we found that patients with non-metastatic prostate cancer receiving neoadjuvant ADT prior to radiation therapy reported increased fatigue severity compared to those without ADT. Cancer-related fatigue in patients receiving ADT may be specifically attributable to sleep-related impairment related to alterations in steroid hormone biosynthesis. These findings provide a basis for further research of changes in sleep patterns and their role in the specific subcategory of cancer-related fatigue caused by alterations in steroid hormones as a result of the treatment. Although ADT is considered an effective therapy that confers survival advantage, undesirable side effects should be taken into consideration when designing the optimal treatment strategy. As individuals may place different values on different treatment-related toxicities, knowledge of anticipated adverse effects is vitally important in designing individualized treatment plans (Loblaw et al., 2004). It is our hope that findings in this study will help patients and clinicians make more precise cost/benefit analyses when considering incorporating ADT into the treatment plan. Finally, mechanistic investigations aimed at understanding the heterogeneous pathogenic origins of cancer-related fatigue will help clinicians devise personalized and evidence-based treatment strategies.

DATA AVAILABILITY STATEMENT

All metabolomics data for "Steroid hormone biosynthesis metabolism is associated with fatigue related to androgen deprivation therapy for prostate cancer" was deposited in the Open Science Framework database (https://osf.io/) under the DOI number 10.17605/OSF.IO/SVEQK.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Institutes of Health (NIH) Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LF and LS designed the study. LF, JB, HA, JR, and LS contributed to writing and editing of the draft. LF and JB contributed to data analysis. All authors edited and approved the final draft.

FUNDING

The present study is fully supported by the Division of Intramural Research of the National Institute of Nursing Research of the National Institutes of Health, Bethesda, MD, United States (grant number: ZIA NR000020-06).

ACKNOWLEDGMENTS

The authors would like to thank the National Institutes of Health Library for assistance with manuscript editing. The authors would also like to thank the clinical staff for assistance with clinical sample collection.

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 29 June 2021
doi: 10.3389/fcell.2021.676568

Targeting the Nerve Growth Factor Signaling Impairs the Proliferative and Migratory Phenotype of Triple-Negative Breast Cancer Cells

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OPEN ACCESS

Edited by:
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Consejo Superior de Investigaciones Científicas (CSIC), Spain

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

Received: 05 March 2021
Accepted: 27 May 2021
Published: 29 June 2021

Citation:
Di Donato M, Galasso G, Giovannelli P, Sinisi AA, Migliaccio A and Castoria G (2021) Targeting the Nerve Growth Factor Signaling Impairs the Proliferative and Migratory Phenotype of Triple-Negative Breast Cancer Cells. Front. Cell Dev. Biol. 9:676568. doi: 10.3389/fcell.2021.676568

Triple-negative breast cancer is a heterogeneous disease that still lacks specific therapeutic approaches. The identification of new biomarkers, predictive of the disease's aggressiveness and pharmacological response, is a challenge for a more tailored approach in the clinical management of patients. Nerve growth factor, initially identified as a key factor for neuronal survival and differentiation, turned out to be a multifaceted molecule with pleiotropic effects in quite divergent cell types, including cancer cells. Many solid tumors exhibit derangements of the nerve growth factor and its receptors, including the tropomyosin receptor kinase A. This receptor is expressed in triple-negative breast cancer, although its role in the pathogenesis and aggressiveness of this disease is still under investigation. We now report that triple-negative breast cancer-derived MDA-MB-231 and MDA-MB-453 cells express appreciable levels of tropomyosin receptor kinase A and release a biologically active nerve growth factor. Activation of tropomyosin receptor kinase by nerve growth factor treatment positively affects the migration, invasion, and proliferation of triple-negative breast cancer cells. An increase in the size of triple-negative breast cancer cell spheroids is also detected. This latter effect might occur through the nerve growth factor-induced release of matrix metalloproteinase 9, which contributes to the reorganization of the extracellular matrix and cell invasiveness. The tropomyosin receptor kinase A inhibitor GW441756 reverses all these responses. Co-immunoprecipitation experiments in both cell lines show that nerve growth factor triggers the assembly of the TrkA/β1-integrin/FAK/Src complex, thereby activating several downstream effectors. GW441756 prevents the complex assembly induced by nerve growth factor as well as the activation of its dependent signaling. Pharmacological inhibition of the tyrosine kinases Src and FAK (focal adhesion kinase), together with the silencing of β1-integrin, shows that the tyrosine kinases impinge on both proliferation and motility, while β1-integrin is needed for motility induced by nerve growth factor in triple-negative breast cancer cells. The present data support the key role of the nerve growth factor/tropomyosin receptor kinase A pathway in triple-negative breast cancer and offer new hints in the diagnostic and therapeutic management of patients.

Keywords: triple-negative breast cancer, NGF, TrkA, NGF signaling, new therapy

INTRODUCTION

Despite the significant progress in diagnosis and treatment, breast cancer (BC) still represents a global challenge. Additionally, a specific BC subtype lacking estrogen or progesterone receptor (ER or PR, respectively) and not exhibiting HER2 overexpression/amplification has attracted the attention of oncologists. This subtype is commonly defined as triple-negative breast cancer (TNBC) and accounts for approximately 10–20% of all BCs. TNBC can be considered as a heterogeneous disease, often associated with a worse prognosis. Specific treatments for this cancer are still lacking, and chemotherapy represents the main therapeutic option in the early as well as advanced stages of the disease (Marra et al., 2020). This scenario has been made even more intricate by the discovery of a specific TNBC subtype, characterized by the expression of the androgen receptor (AR) (Lehmann et al., 2011). These findings, together with the identification of various "druggable" biomarkers (e.g., the signaling effectors of the PI3-K- or Ras-dependent pathways), have paved the way for the use of AR- or PI3-K- or MEK-targeted agents in monotherapy or combinatorial therapy for TNBC. TNBC patients, however, often exhibit intrinsic resistance to therapies or acquire drug resistance (Bianchini et al., 2016). The identification of new predictive response biomarkers and therapeutics is needed for the clinical management of TNBC patients.

The neurotrophin β-nerve growth factor (β-NGF, referred to as NGF hereafter) activates two structurally unrelated receptors: the p75 neurotrophin receptor ($p75^{NTR}$, also called NGF receptor), which binds all the neurotrophins, and the receptor tyrosine kinase A (TrkA), which shows high-affinity binding to NGF (Huang and Reichardt, 2003). The last years have seen intense investigations on the role of NGF and its receptors in human cancers. As such, many compounds targeting Trk receptors have been designed and studied for their effects in cultured cancer cells as well as mouse models (Vaishnavi et al., 2015; Drilon et al., 2018; Konicek et al., 2018; Meldolesi, 2018; Smith et al., 2018).

NGF and its receptors play a role in BC. TrkA levels have a prognostic value in BC patients (Descamps et al., 2001a), and secretory BCs are driven by oncogenic *ETV6–NTRK3* gene fusions (Lee et al., 2014). NGF signaling fosters the survival and proliferation of BC cells (Descamps et al., 1998, 2001b), and the anti-estrogen tamoxifen inhibits this effect (Chiarenza et al., 2001). These findings support a role for NGF signaling in the pathogenesis and progression of BC. Scant evidence, however, has been so far reported on the role of NGF signaling in TNBC.

In this manuscript, we investigated the role of NGF signaling on the aggressiveness of two TNBC cell lines and the resulting effects of NGF signaling inhibition in these cells. We have employed the MDA-MB-231 and MDA-MB-453 cell lines, which represent the mesenchyme and luminal phenotypes of TNBC-derived cells, respectively (Cailleau et al., 1978; Doane et al., 2006). Albeit at different extents, both cell lines express TrkA and secrete significant amounts of NGF, whose biological activity is neutralized by a specific anti-NGF antibody. Challenging of TNBC cells with NGF activates TrkA and its dependent downstream signaling. Such activation results in mitogenesis, motility, invasion, and a significant increase in the TNBC cell spheroid size. Molecular analysis indicates that NGF challenge triggers the assembly of the TrkA/β1-integrin/FAK/Src complex in TNBC cells. Pharmacological inhibition of TrkA prevents the TrkA/β1-integrin/FAK/Src complex assembly and reverses the mitogenesis and motility in NGF-treated TNBC cells. Similar data were detected using Src or FAK tyrosine kinase inhibitors, while somatic knockdown of β1-integrin only impairs the NGF-elicited motility in TNBC cells.

Taken together, our results dissect the molecular mechanism of NGF action in TNBC cells and indicate that pharmacological inhibitors against TrkA and humanized anti-NGF antibodies might profitably be used as therapeutic tools in TNBC.

MATERIALS AND METHODS

Chemicals

NGF (Millipore, Burlington, MA, United States) and GW441756 (Selleckchem, Munich, Germany) were used at 100 ng/ml and 1 μM, respectively, throughout the manuscript. The Src tyrosine kinase inhibitor SU6656 (Cayman Chemical, Ann Arbor, MI, United States) was used at 5 μM. The FAK inhibitor defactinib (VS-6063, Selleckchem) was used at 10 μ M.

Cell Cultures

The human TNBC-derived cells MDA-MB-231 and MDA-MB-453 and the human prostate cancer-derived C42-B cells were from the Cell Bank Interlab Cell Line Collection (ICLC; Genoa, Italy). Rat pheocromocytoma-derived PC12 cells were from the European Collection of Authenticated Cell Culture (ECACC; Public Health England, London, United Kingdom). The suppliers authenticated the cell lines for DNA profiles using short tandem repeat (STR) analysis. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Unless otherwise stated, the media and supplements were from Gibco (Thermo Fisher Scientific, Waltham, MA, United States). MDA-MB-231 cells were cultured in phenol red Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM glutamine. MDA-MB-453 cells were grown in phenol red DMEM/F12 containing 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, and 10 μg/ml insulin (Roche, Basel, Switzerland). Twenty-four hours before stimulation, growing MDA-MB-231 and MDA-MB-453 cells at 70% confluence were made quiescent using phenol red-free DMEM containing 0.1% charcoal-stripped serum (CSS), 100 U/ml penicillin, and 100 U/ml streptomycin. PC12 cells were cultured in Corning plates using F12K medium (ATCC) supplemented with 2.5% FBS, 15% horse serum, streptomycin at 100 μg/ml, and penicillin at 100 U/ml. The cells were made quiescent using DMEM containing 0.1% FBS, antibiotics, and L-glutamine (Gibco) at 2 mM. C42-B cells were cultured as reported (Di Donato et al., 2019). All the cell lines were routinely monitored for mycoplasma contamination. Cell quiescence was evaluated by fluorescence-activated cell sorting (FACS) analysis, as reported (Castoria et al., 2014). It indicates

that a large number (almost 85%) of TNBC cells were in G0/G1 (not shown). Cell quiescence was also monitored by 5-bromo-2′-deoxyuridine (BrdU) incorporation analysis, as reported in the subsequent section.

Phase-Contrast Microscopy, Immunofluorescence, DNA Synthesis, WST-1, and Cyto 3D Live–Dead Assays

PC12 (3×10^4) cells were made quiescent for 24 h and embedded in 250 μl of phenol red-free growth factor-reduced Matrigel (10 mg/ml; BD Biosciences, San Jose, CA, United States). Conditioned medium (CM) derived by TNBC cells unchallenged or challenged for 10 days with anti-NGF neutralizing antibody (1,600 pg/ml) was collected and added to PC12 cells. After 6 days, different fields were analyzed using a Leica DMIRB (Leica, Wetzlar, Germany) microscope equipped with C-Plan ×40 or HCX PL Fluotar ×63 objective (Leica). Images were captured using a DFC 450C camera (Leica). TNBC cells on coverslips were made quiescent and after 72 h were rinsed with phosphate-buffered saline (PBS), fixed for 10 min with paraformaldehyde (4%, *w*/*v*, in PBS; Merck, Saint Louis, MO, United States), permeabilized for 10 min with Tween (0.1%, *v*/*v*, in PBS; Bio-Rad, Hercules, CA, United States), and incubated for 1 h with PBS containing FBS (1%, vol/vol). Cells on coverslips were then incubated with the anti-NGF (1:100, ab6199; Abcam, Cambridge, United Kingdom) antibody overnight at 4°C. After extensive washings in PBS, the coverslips were incubated for 1 h at 37°C with diluted (1:200 in PBS containing 0.01% BSA) fluorescein-conjugated AffiniPure anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA, United States). When indicated in the figures, the nuclei were stained for 5 min with Hoechst 33258 (1 μg/ml; Merck) and the plasma membrane for 10 min with red fluorescent Alexa Fluor®594 wheat germ agglutinin (WGA; 5 μg/ml) (Molecular Probes, Invitrogen Ltd., Paisley, United Kingdom). The number of cells positive for NGF (NGF-positive cells) was determined using the formula: percentage of NGF-positive cells = (No. of NGF or pro-NGF-positive cells/No. of total cells) × 100. DNA synthesis was analyzed by BrdU incorporation. To this end, quiescent cells on coverslips were left unchallenged or challenged with NGF in the absence or presence of the indicated compounds for 18 h. After *in vivo* pulse with 100 μM BrdU (Sigma-Aldrich, St. Louis, MO, United States), BrdU incorporation into the newly synthesized DNA was analyzed as reported (Pagano et al., 2004) using a DMLB (Leica, Wetzlar, Germany) fluorescent microscope equipped with HCX PL Apo ×63 oil and HCX PL Fluotar ×100 oil objectives. Images were captured using a DC480 camera (Leica) and acquired using the Leica Suite software. BrdU incorporation was calculated using the formula: percentage of BrdU-positive cells = (No. of BrdU-positive cells/No. of total cells) × 100. Only PC12 cells that, under basal conditions, incorporated <10% BrdU were used in the indicated experiments. WST-1 reagent (Roche) was used to analyze TNBC cell proliferation, as reported (Di Donato et al., 2019). The resulting values were expressed as the fold increase over the basal level. The Cyto3D live–dead assay kit (TheWell Bioscience, North Brunswick, NJ, United States) was used to detect apoptotic TNBC cells. The kit was used according to the manufacturer's instructions. Dead cells were visualized by using a DMIRB inverted microscope (Leica) equipped with N-Plan ×10 or HCX PL Fluotar ×40 objective (Leica), and the percentage of dead cells was determined using the formula: [No. of propidium iodide (PI)-positive cells/No. of acridine orange (AO)-positive cells] × 100.

Enzyme-Linked Immunosorbent Assay

TNBC cells (8×10^4 in six-well plates in **Figures 1B,C,G,H**; 55×10^4 in 100-mm plates in **Supplementary Figure 1E** and the corresponding experiments in **Figure 2**) were made quiescent. The cell culture media were collected at the times indicated in the figures and the corresponding legends. Enzyme-linked immunosorbent assay (ELISA) kits for β-NGF (EHNGF; Thermo Fisher Scientific) and pro-NGF (MBS706083; MyBioSource, San Diego, CA, United States) were used for the quantitative determination of β-NGF and pro-NGF in the cell culture media. The resulting data were analyzed using the curve-fitting statistical software GraphPad Prism.

Wound Scratch Assay, Boyden Chamber Migration Assay, and Invasion Assay

In the wound scratch analysis, 1.8×10^5 cells were seeded in a 24-well plate. The cells were made quiescent, wounded using 10-μl sterile pipette tips, and left unstimulated or stimulated for 12 h with NGF in the absence or presence of the indicated compounds. To avoid cell proliferation, cytosine arabinoside (Sigma-Aldrich) at 50 μM (final concentration) was included in the cell medium. Different fields were analyzed using a DMIRB inverted microscope (Leica) equipped with N-Plan ×10 objective (Leica), as reported (Giovannelli et al., 2019). Phase-contrast images were captured using a DFC 450C camera (Leica) and acquired using the Application Suite software (Leica). Images are representative of at least three different experiments. The wound gap was calculated using ImageJ software and expressed as the percentage of decrease in the wound area. Migration and invasion assays were done as reported (Giovannelli et al., 2019) using quiescent MDA-MB-231 or MDA-MB-453 cells in collagen (for migration assay) or Matrigel (for invasion assay) pre-coated Boyden chambers with 8 μm polycarbonate membrane (Corning, Corning, NY, United States). The indicated compounds were added and cytosine arabinoside (Sigma-Aldrich) was included (at 50 μM final concentration) in the cell medium. The cells were allowed to migrate or invade for 7 or 18 h, respectively. Migrating or invading cells were finally stained with Hoechst 33258 and scored (Giovannelli et al., 2019).

Gelatine Metalloproteinase Zymography

Zymography assay was done using MDA-MB-231 cells at 80% of confluence. The cells were made quiescent, left in serum-free media, and then unstimulated or stimulated for 30 h with NGF in the absence or presence of GW441756. CM was collected and centrifuged, while the cells were detached by trypsin and counted. CM was normalized to 1×10^6 MDA-MB-231 cells and MMP-9

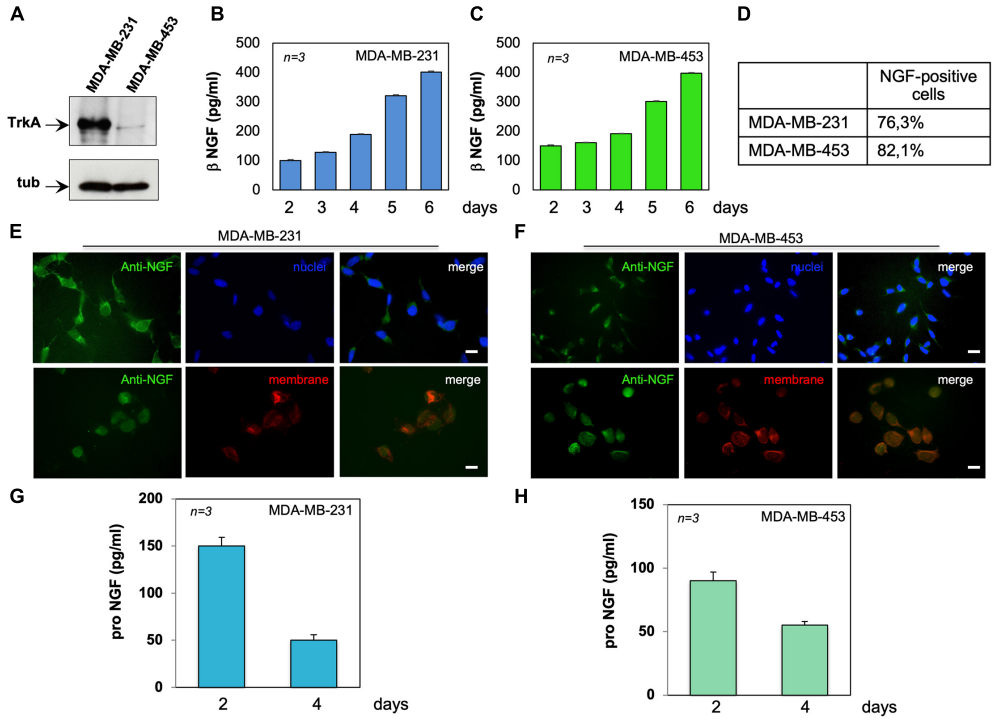

FIGURE 1 | MDA-MB-231 and MDA-MB-453 cells express tyrosine kinase A (TrkA) and release active neurotrophin β-nerve growth factor (NGF). **(A)** Lysates from the indicated cell lines were prepared and the lysate proteins analyzed by Western blotting (WB) using the antibodies against the indicated proteins. **(B,C)** MDA-MB-231 **(B)** and MDA-MB-453 **(C)** cells were made quiescent. Conditioned media (CM) were collected at the indicated times and the amount of β-NGF (in picograms per milliliter) was analyzed. Quiescent MDA-MB-231 and MDA-MB-453 cells on coverslips were stained for NGF. **(D)** Quantification of the cells positive for NGF immunostaining. **(E,F)** Representative immunofluorescence (IF) images from three different experiments (each in duplicate) were captured for MDA-MB-231 cells **(E)** and MDA-MB-453 **(F)** cells. For each experiment, at least 300 cells were scored. NGF (*green*), nuclei (*blue*), and the plasma membrane (*red*) were stained. *Right panels* show the merged images. *Scale bar*, 10 μM. **(G,H)** CM from MDA-MB-231 **(G)** and MDA-MB-453 **(H)** cells were collected at the indicated times and the amount of pro-NGF (in picograms per milliliter) was analyzed by ELISA. Means and SEMs are shown. *n* represents the number of experiments.

proteolytic activity was assayed in CM as reported (Di Donato et al., 2021). It appeared as a clear band migrating at ≈92 kDa on a blue background.

3D Cultures and Spheroid's Viability by MTT Assay

Spheroids were generated as reported (Di Donato et al., 2021). MDA-MB-231 and MDA-MB-453 cells (3×10^4) were mixed in each well with 250 μl of phenol red-free growth factor-reduced Matrigel (10 mg/ml; BD Biosciences) and 50 μl of spheroid plating medium. It was made using phenol red-free DMEM/F12 medium containing 7% CSS, 100 U/ml penicillin, 100 U/ml streptomycin, GlutaMAX 100× (Gibco), 10 mM HEPES, 1 M nicotinamide (Merck), 500 mM *N*-acetylcysteine (Sigma-Aldrich), and 10 μM Y-27632 (Merck). After 3 days, the spheroid plating medium was replaced with a similar medium in the absence of *N*-acetylcysteine and Y-27632. On day 4, the spheroids were untreated or treated with the indicated compounds. Unless otherwise stated, the medium was changed every 2 days. In **Figures 3D,E**, the media were not changed until the 9th day. Different fields were analyzed using Leica DMIRB (Leica) microscope equipped with C-Plan ×40 objective (Leica) and phase-contrast images were acquired using a DFC 450C camera (Leica). The relative spheroid size was calculated using the Application Suite software (Leica) and expressed as a fold increase over the basal spheroid size, which was measured on the 3rd day. After 15 days, spheroid viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). Briefly, MDA-MB-231 and MDA-MB-453 spheroids were incubated with a 2% (*w*/*v*) SDS solution to solubilize the Matrigel. After 2 h at 37°C, the MTT solution (final concentration of 500 μg/ml) was added to the spheroids at 37°C and 5% CO_2. Two hours later, DMSO (100 μl) was added and the mixture was incubated for 1 h at 37°C. The optical density (OD) from duplicate samples was measured at 562 nm using an EnSpire plate reader (PerkinElmer, Waltham, MA, United States).

Transfections and siRNA Experiments

Growing MDA-MB231 cells were transfected using Lipofectamine 2000 (Invitrogen). For β1-integrin small interfering RNA (siRNA), a pool of three to five target-specific 19–25 siRNAs (Santa Cruz, Dallas, TX, United States) was used. Non-targeting siRNA [control (ctrl) siRNA], containing a scrambled sequence, was from Santa Cruz. The cells were co-transfected with 2 μg eGFP-cDNA (Lonza, Milan, Italy) to help

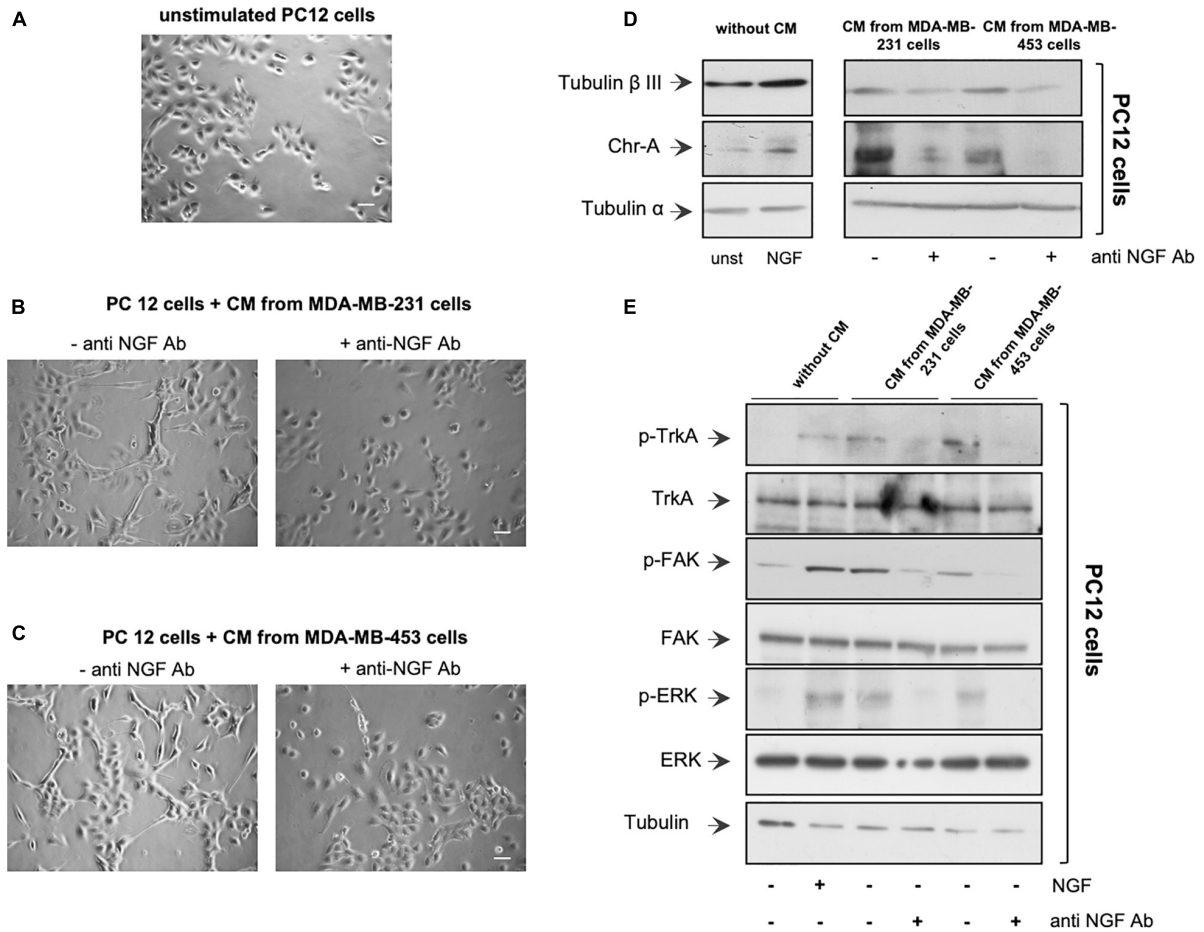

FIGURE 2 | Neurotrophin β-nerve growth factor (NGF) secreted by triple-negative breast cancer (TNBC) cells is biologically active. **(A,B)** PC12 cells were embedded in Matrigel and unchallenged **(A)** or challenged with conditioned media (CM) from MDA-MB-231 **(B)** or from MDA-MB-453 **(C)** cells (*left panels*) in the absence or presence of anti-NGF neutralizing antibody (anti-NGF; *right panels*) for 6 days. Phase-contrast images are representative of three different experiments, each in duplicate. *Scale bar*, 10 μM. **(D,E)** PC12 cells were unstimulated or stimulated for 72 h with 100 ng/ml NGF or CM from MDA-MB-231 and MDA-MB-453 cells unchallenged or challenged with anti-NGF. Lysate proteins from PC12 cells were analyzed by Western blot (WB) using the antibodies against the indicated proteins. WB are representative of three different experiments.

in the identification of transfected cells. After 6 h, transfected cells were made quiescent for 24 h and then used.

Lysates, Immunoprecipitation, Co-immunoprecipitation, and Western Blot

All these were done as reported (Di Donato et al., 2019). The following reagents were used: mouse monoclonal anti-p75 (B-1, sc-271708; Santa Cruz), anti-FAK (610088; BD Transduction Laboratories), or anti P-Tyr 397 FAK (611722; BD Transduction Laboratories); anti-Src (sc-8056; Santa Cruz), anti-p42 extracellular signal-regulated kinase (ERK) (sc-1647; Santa Cruz), or anti p44 and p42 P-ERK (sc-7383; Santa Cruz); anti-tubulin (T5168; Sigma-Aldrich) antibodies; and rabbit polyclonal anti-TrkA (06-574; Millipore), P-Tyr490 TrkA (#9141; Cell Signaling, Danvers, MA, United States), β1-integrin (Ab1952; Millipore), P-Thr 638/641 PKCα/β II (#9375; Cell Signaling), and P-Ser 643/676 PKCδ/θ (#9376; Cell Signaling) antibodies. The ECL system (GE Healthcare, Chicago, IL, United States) was used to reveal immunoreactive proteins.

Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Two-tailed unpaired Student *t*-tests and one-way or two-way ANOVA (Bonferroni's *post-hoc* test) were used, where appropriate.

RESULTS

TNBC Cells Express TrkA and Release Biologically Active NGF

The expression of the NGF receptor TrkA was analyzed by Western blot (WB) technique in lysate proteins from MDA-MB-231 and MDA-MB-453 cells. The anti-TrkA antibody revealed

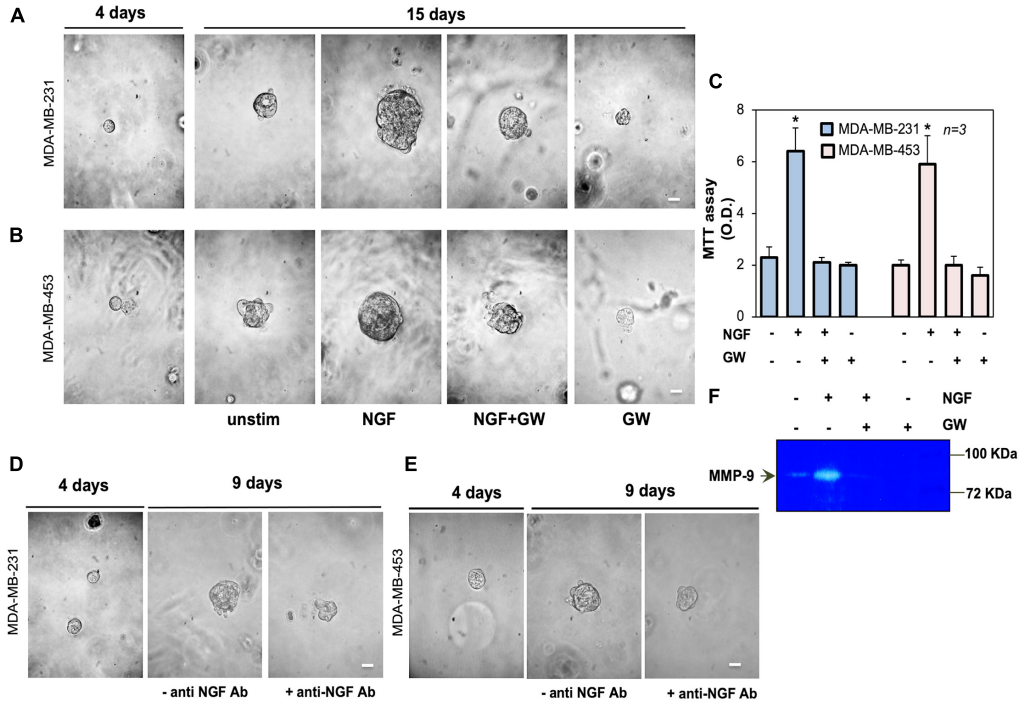

FIGURE 3 | Neurotrophin β-nerve growth factor (NGF) challenge increases the size of triple-negative breast cancer (TNBC) cell spheroids and the MMP-9 release. **(A,B)** MDA-MB-231 **(A)** and MDA-MB-453 **(B)** cells embedded in Matrigel were used. Four days later, representative images were acquired. Cells were left untreated or treated with 100 ng/ml NGF in the absence or presence of GW441756 (GW; 1 μM) for 15 days. Shown are phase-contrast images, representative of three different experiments, captured on the 15th day. *Scale bar*, 100 μm. **(C)** Spheroid viability was analyzed by the MTT assay after 15 days of treatment. Optical density (OD) was measured at 562 nm and the obtained results are shown in the graph. Means and SEMs are shown. *n* represents the number of experiments. **(D,E)** MDA-MB-231 **(D)** and MDA-MB-453 **(E)** cells were used to generate spheroids, as in **(A,B)**. Spheroids were left untreated for 9 days, avoiding refreshing the medium, in the absence or presence of the anti-NGF antibody (anti-NGF Ab). Shown are phase-contrast images, representative of three different experiments, captured at day 9. *Scale bar*, 100 μm. **(F)** MDA-MB-31 cells were treated for 30 h with 100 ng/mL NGF in the absence or presence of GW441756 (GW; 1 μM). The release of MMP-9 in conditioned media was analyzed by zymography. $^*p < 0.05$ for the indicated experimental points vs. the corresponding untreated controls.

an immunoreactive band migrating at 140 kDa, the expected molecular weight of TrkA. The amount of immunoreactive bands in MDA-MB-231 cells was much higher than that observed in MDA-MB-453 cells (**Figure 1A**), and similar results were obtained from three different experiments (**Supplementary Figure 1**). Despite significant levels of the neurotrophin receptor family member p75 have been recently detected in a lung metastatic clone from modified MDA-MB231 cells (Wu et al., 2021), we did not observe in the WB analysis robust levels of p75 (**Supplementary Figures 2A,B**), regardless of the TNBC cell line. These apparent discrepancies might be due to the quite different clones of TNBC cells used. Nevertheless, other critical factors, such as the stromal microenvironment as well as the signal- and context-dependent interactions between BC metastatic cells and the lung cellular components, might influence the re-expression of p75 in the lung metastatic clone of MDA-MB-231.

Since BC cells release NGF (Adriaenssens et al., 2008), we measured the NGF content in CM from MDA-MB-231 (**Figure 1B**) or MDA-MB-453 (**Figure 1C**) cells. Data from ELISA in **Figures 1B,C** show that both TNBC cell lines release appreciable amounts of NGF, already after 2 days to reach elevated levels (about 400 pg/ml) after 6 days of cell culture. Immunofluorescence (IF) quantification (**Figure 1D**) and images (**Figures 1E,F**) reveal that almost 77% of MDA-MB-231 (**Figures 1D,E**) and 82% of MDA-MB-453 (**Figures 1D,F**) cells were positive for NGF immunostaining. In both cell lines, NGF staining was prevalently seen in the extranuclear compartment (upper panels in **Figures 1E,F**), close to the plasma membranes (red; lower panels in **Figures 1E,F**), and the specificity of the IF approach was confirmed by the absence of fluorescence in the control staining, obtained from the secondary antibody alone (**Supplementary Figures 2C,D**).

Because of previous findings on the role of pro-NGF in BC aggressiveness (Lévêque et al., 2019), we also analyzed the release of pro-NGF from TNBC cells. After 2 days of culture, MDA-MB-231 (**Figure 1G**) and MDA-MB-453 (**Figure 1H**) cells secreted almost 150 and 100 pg/ml of pro-NGF, respectively. Such amounts decreased over the time, to reach almost undetectable levels (<50 pg/ml) after 4 days of culture. Thus, a low amount of pro-NGF is released by TNBC cells in our setting, suggesting that NGF, rather than pro-NGF, might sustain the aggressiveness of TNBC cells through an autocrine loop in our conditions.

We then verified whether the NGF secreted by TNBC cells is biologically active. CM from a robust number of TNBC cells (see also "Materials and Methods") was collected after 10 days of cell culture. About 4,867 and 6,923 pg/ml of NGF were

found in CM collected from MDA-MB-231 and MDA-MB-453 cells, respectively (**Supplementary Figure 2E**). Therefore, we verified whether CM from MDA-MB-231 or the MDA-MB-453 cell line induces a neuronal phenotype in rat adrenal pheochromocytoma PC12 cells (Greene and Tischler, 1976), which undergo differentiation on NGF stimulation (Marshall, 1995). The cells were embedded in Matrigel to simulate the complexity of the extracellular matrix (ECM), and the effect of CM addition was evaluated after 6 days. Contrast-phase images show that CM from MDA-MB-231 (**Figure 2B**) or MDA-MB-453 (**Figure 2C**) cells induced the acquisition of a stellate shape and the development of neurites (left panels) in PC12 cells when compared with the unstimulated PC12 cells (**Figure 2A**). Notably, when the neutralizing anti-NGF antibody was added to CM from TNBC cells, PC12 cells remained in a round and undifferentiated shape (right panels). Since we have observed that NGF treatment of PC12 cells upregulates β-tubulin III and chromogranin A (Chr-A) expressions (Di Donato et al., 2015), we also evaluated the levels of these proteins. In the absence of CM, challenging of PC12 cells with NGF upregulated the β-tubulin III and Chr-A levels after 3 days (left section in **Figure 2D**). A significant expression of β-tubulin III and a robust bulk of Chr-A were detected by adding to PC12 cells the CM derived from TNBC cells. Here, again, CM treatment with the anti-NGF neutralizing antibody resulted in a decrease in the levels of both β-tubulin III and Chr-A (right section in **Figure 2D**), with a more robust effect detectable on this latter marker. Thus, NGF contained in the CM from TNBC cells seems to be responsible for the observed effects in PC12 cells. To strengthen this finding, we investigated the effect of CM from TNBC cells on the activation of various effectors involved in NGF signaling. In the absence of CM, NGF challenging of PC12 cells increased the Tyr-490 TrkA phosphorylation as well as FAK and p44-p42 ERK activation. In the presence of CM, we still observed these effects in PC12 cell lysate proteins. The addition of neutralizing anti-NGF antibody to CM inhibited the activation of various NGF signaling components (**Figure 2E**).

Taken together, the data in **Figures 1**, **2** and **Supplementary Figure 2** indicate that TNBC cells express TrkA and release biologically active NGF. As such, an autocrine loop might sustain the proliferation and aggressiveness of TNBC cells.

NGF Treatment Increases the Size of TNBC Cell Spheroids

We next established a three-dimensional culture system in MDA-MB-231 and MDA-MB-453 cells using growth factor-reduced Matrigel. This condition allows the formation of spheroids after 4 days of culture, as shown by the phase-contrast microscopy images in **Figures 3A,B**. On day 4 of culture, the spheroids were left untreated or treated with NGF in the absence or presence of the TrkA specific inhibitor GW441756 (Wood et al., 2004). The cell medium was refreshed every 2 days and changes in the spheroid size were monitored for 15 days. Phase-contrast microscopy images were captured (**Figures 3A,B**) and the quantification of data was also done (**Supplementary Figure 1F**). After 15 days, NGF increased by about 11- and 10-fold the sizes of the MDA-MB-231 and MDA-MB-453 spheroids, respectively. GW441756 significantly ($p < 0.05$ in **Figures 3A,B** and **Supplementary Figure 2F**) reduced the NGF effect, leaving almost unaltered the spheroid size when used alone, as a control. We also analyzed the effect of NGF on the spheroid's viability with the MTT assay. **Figure 3C** shows that NGF increased by about sixfold the viability of spheroids from both TNBC cell lines and that GW441756 decreased such effect. The inhibitor did not modify the spheroid viability when used alone, as a control. Since the NGF-mediated autocrine loop may be already involved in the "basal" conditions, we generated TNBC cell spheroids avoiding refreshing the medium. On day 4 of culture, the spheroids were left untreated in the absence or presence of the anti-NGF antibody. Changes in the spheroid size were then monitored for 9 days and phase-contrast microscopy images were captured. The images in **Figures 3D,E**, together with the quantification of data (**Supplementary Figure 2G**), show that the sizes of the TNBC cell spheroids increased by almost fourfold. The addition of the anti-NGF antibody reduced such effect. Taken together, these findings demonstrate for the first time a role for NGF and TrkA activation in fueling the size of TNBC-derived spheroids.

The release of matrix metalloproteinases (MMPs) is crucial for ECM remodeling, cell invasion, and tumor progression (Bonnans et al., 2014; Chatterjee et al., 2018; Yuzhalin et al., 2018). Since NGF induces MMP-9 release (Khan et al., 2002), we analyzed the effect of NGF on MMP-9 release by MDA-MB-231 cells. NGF robustly increased the secretion of MMP-9, as assessed using the results from zymography in **Figure 3F**. GW441756 inhibited this effect. Untreated cells, which were maintained in serum-free medium for 30 h, released a low MMP-9 amount, likely because of the scant quantity (<95 pg/ml) of NGF secreted by MDA-MB231 cells at that time (not shown). By affecting such a basal condition, GW441756 slightly perturbed the MMP-9 release, when used alone (**Figure 3F**). The data in panel F support a role for the NGF-induced MMP-9 release in ECM remodeling and the consequent increase in TNBC cell spheroid size.

NGF Treatment Induces DNA Synthesis and Proliferation in TNBC Cells

To evaluate the mitogenic effect of NGF in TNBC-derived cell lines, BrdU incorporation and proliferation assays were done. NGF stimulation increased by about 2.5- and 1.8-fold the number of MDA-MB-231 (**Figure 4A**) and MDA-MB-453 (**Figure 4B**) cells incorporating BrdU as compared to unstimulated cells. GW441756 impaired the NGF-elicited effect, indicating that TrkA activity is required for this response. The inhibitor did not significantly modify the BrdU incorporation when used alone in both cell lines (**Figures 4A,B**). The effect of NGF on cell proliferation was further evaluated by the WST-1 assay. NGF treatment stimulated the proliferation in both TNBC cell lines, with an effect already evident after 24 h, while GW441756 inhibited the NGF-elicited effect, which persists until 72 h of treatment (**Figures 4C,D**). Since the neurotrophin receptor TrkA drives the death of developing neurons *in vitro* and *in vivo* (Nikoletopoulou et al., 2010), we used a live–dead assay to

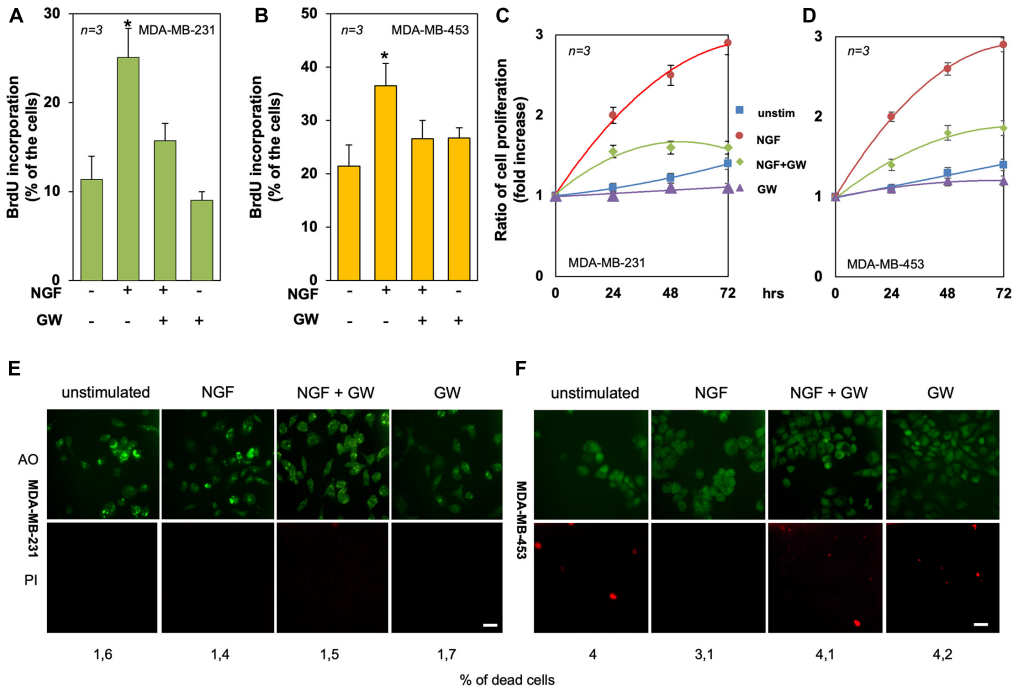

FIGURE 4 | Tyrosine kinase A (TrkA) mediates the neurotrophin β-nerve growth factor (NGF) mitogenic effect in triple-negative breast cancer (TNBC) cells. **(A,B)** Quiescent MDA-MB-231 **(A)** and MDA-MB-453 **(B)** cells were left untreated or treated for 18 h with the indicated compounds. Cells were pulsed *in vivo* with 100 μM BrdU and its incorporation into DNA was analyzed by immunofluorescence (IF) and expressed as the percentage of total cells. **(C,D)** Quiescent MDA-MB-231 **(C)** and MDA-MB-453 **(D)** cells were left untreated or treated for 24, 48, and 72 h with the indicated compounds. Cell proliferation was assayed using the WST-1 reagent. Graphs represent the ratios of proliferation, which was expressed as the fold increase over the basal absorbance. NGF stimulation induced a significant ($p < 0.05$) increase in cell proliferation as compared with the untreated cells. **(E,F)** MDA-MB-231 **(E)** and MDA-MB-453 **(F)** cells were untreated or treated for 72 h with the indicated compounds and stained with the Cyto3D reagent. Shown are IF images of the total (*green*) and dead (*orange*) cells, representative of three different experiments. *Scale bar*, 10 μm. The percentages of dead cells for each point are indicated in the figure. In **(A–F)**, NGF was used at 100 ng/ml and GW441756 (GW) was used at 1 μM. Means and SEMs are shown. *n* represents the number of experiments. *$p < 0.05$ for the indicated experimental points *vs*. the corresponding untreated control.

address this issue. Irrespective of cell treatment, a negligible effect on the number of TNBC dead cells was detectable only after 3 days (**Figures 4E,F**).

In summary, TrkA activation by NGF drives the DNA synthesis and proliferation in both TNBC cell lines.

NGF Treatment Induces Migration and Invasion in TNBC Cells

We next evaluated the effect of NGF on the motility and invasion of TNBC cells. In a first attempt, MDA-MB-231 cells were wounded and allowed to migrate in the absence or presence of the indicated compounds. Phase-contrast images from the wound scratch assay show that a significant number of cells migrated in the wound area upon NGF treatment, while GW441756 inhibited the NGF-induced effect. Images captured at time 0 or from untreated cells were also captured and presented for comparison (**Figure 5A**). Data from three different experiments are graphically shown in **Figure 5B**. They indicate that the wound width was significantly ($p < 0.05$) reduced in cells treated with NGF as compared with the control untreated cells. GW441756 reverted the effect elicited by NGF while exhibiting a negligible effect when used alone. We avoided the wound scratch assay in MDA-MB-453 cells since they are semi-adherent and not rightly available in this approach (Giovannelli et al., 2019). Finally, we studied the NGF effect on the migration and invasiveness of TNBC cells by using collagen- and Matrigel-coated Boyden chambers. NGF increased by ∼2- and 2.7-fold the number of migrating (**Figure 5C**) or invading (**Figure 5D**) MDA-MB-231 cells, respectively. The latter results on cell invasion are consistent with the finding that MDA-MB-231 cells release MMP-9 on NGF treatment (see **Figure 3**). NGF also increased by ∼2.6- and 2.7-fold the number of migrating (**Figure 5E**) or invading (**Figure 5F**) MDA-MB-453 cells, respectively. Throughout this set of experiments, GW441756 inhibited the NGF-induced effects while leaving almost unaffected the migration or invasion of TNBC cells when used alone.

NGF Treatment Triggers the TrkA/FAK/β1-Integrin/Src Complex Assembly and Activates the TrkA-Dependent Signaling Network

In a preliminary time course experiment in MDA-MB-231 cells challenged for different times (from 5 to 30 min) with NGF, we detected a robust co-immunoprecipitation (Co-IP) of TrkA

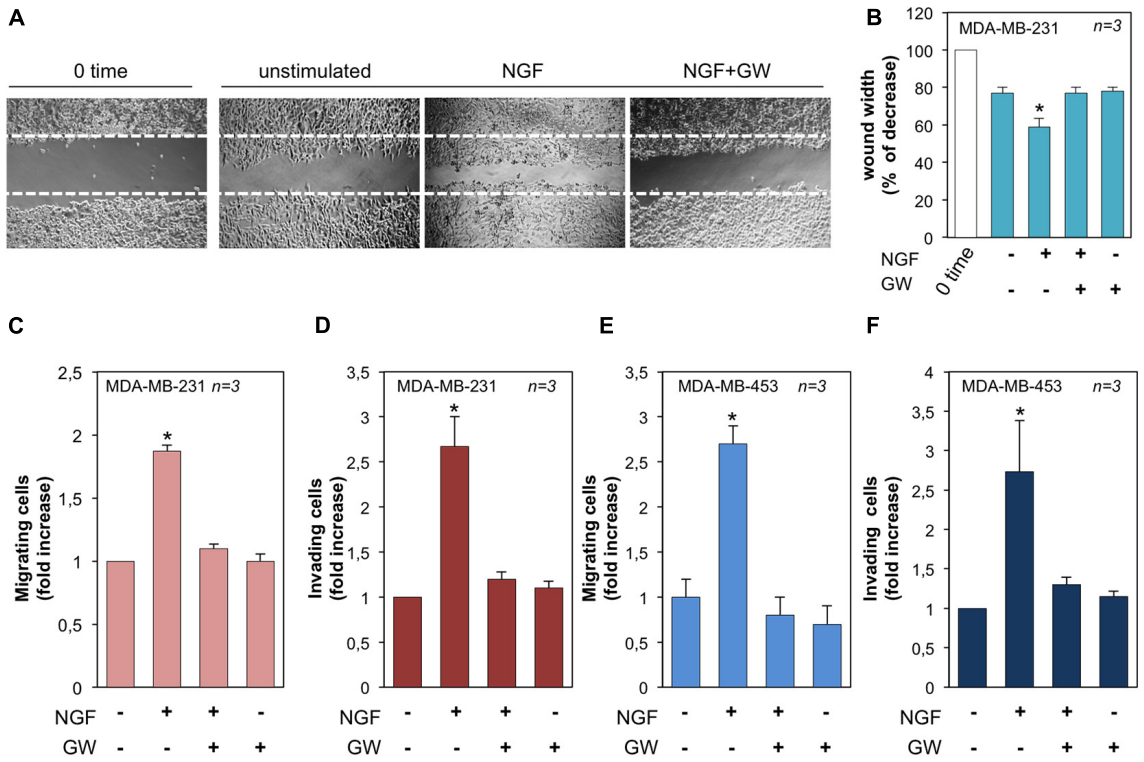

FIGURE 5 | Neurotrophin β-nerve growth factor (NGF) challenge induces migration and invasiveness in triple-negative breast cancer (TNBC) cells. **(A)** Quiescent MDA-MB-231 cells were wounded and left unstimulated or stimulated with NGF for 18 h in the absence or presence of GW441756 (GW). Phase-contrast images are representative of three different experiments, each in duplicate. **(B)** Wound area calculated using Leica Suite software. Data are presented as the percentage of decrease in wound width over the control cells, analyzed at time 0. **(C–F)** Quiescent MDA-MB-231 **(C)** and MDA-MB-453 **(E)** cells were used for migration assays in Boyden chambers pre-coated with collagen. Quiescent MDA-MB-231 **(D)** and MDA-MB-453 **(F)** cells were used for invasion assays in Boyden chambers pre-coated with Matrigel. The indicated compounds were added to the upper and the lower chambers and the cells were counted as reported in "Materials and Methods." Results from three different experiments were collected and expressed as fold increase. In **(B–F)**, means and SEMs are shown. *n* represents the number of experiments. $^*p < 0.05$ for the indicated experimental points *vs*. the corresponding untreated control.

with FAK at 15 min of cell treatment (not shown). Therefore, we selected this time point in the subsequent analysis. MDA-MB-231 cells were then challenged for 15 min with NGF in the absence or presence of GW441756 and the lysates immunoprecipitated with anti-TrkA antibodies. The WB analysis of the Co-IP proteins shows that TrkA was phosphorylated at Tyr490 within 15 min. Simultaneously, a significant Co-IP of TrkA, FAK, β1-integrin, and Src tyrosine kinase was detected upon NGF stimulation (right panels in **Figure 6A**). GW441756 perturbed the NGF-induced complex assembly. Similar TrkA amounts were detected in the immunocomplexes regardless of the experimental condition, and the Co-IP approach is specific since no proteins were detected in the lysates immunoprecipitated with the control antibodies (middle panels in **Figure 6A**). Lastly, WB of lysate proteins with the indicated antibodies shows that similar protein amounts were loaded in our approach (left panels in **Figure 6A**).

NGF binding to TrkA induces the receptor dimerization and phosphorylation of its tyrosine residues. Once phosphorylated, TrkA provides docking sites for the effector molecules, which in turn recruit and activate several signaling effectors, thus propagating different downstream signaling cascades (Biarc et al., 2013). Expectedly, 15 min of NGF stimulation leads to an increase in Tyr-490 TrkA phosphorylation in MDA-MB-231 cells, and GW441756 abolishes such effect, leaving almost unaffected the TrkA phosphorylation when used alone (**Figure 6B**). As readout of TrkA phosphorylation, we then analyzed the activation of several downstream effectors involved in NGF signaling. NGF robustly increases the activation of FAK as well as p44-p42 ERK phosphorylation. NGF also triggers protein kinase C (PKC; α/β and δ/θ) phosphorylation. GW441756 reverses all the effects induced by NGF, leaving unaltered the activation state of various signaling effectors when used alone, as a control (**Figure 6B**).

We next analyzed the NGF signaling activation in MDA-MB-453 cells (**Figure 6C**). As we detected a significant Tyr-490 TrkA phosphorylation within 15 min of NGF stimulation in MDA-MB-231 cells (not shown), we used this time point for the subsequent analysis. A significant increase in FAK as well as p44-p42 ERK activation was detected in NGF-challenged cells, together with PKC phosphorylation (p-PKC; α/β). GW441756 prevented the effects induced by NGF without affecting the activation state of the signaling effectors when used alone (**Figure 6C**).

In summary, NGF rapidly induces the activation of TrkA and its consequent complexation with β1-integrin as well as the Src and FAK tyrosine kinases. The assembly of this complex

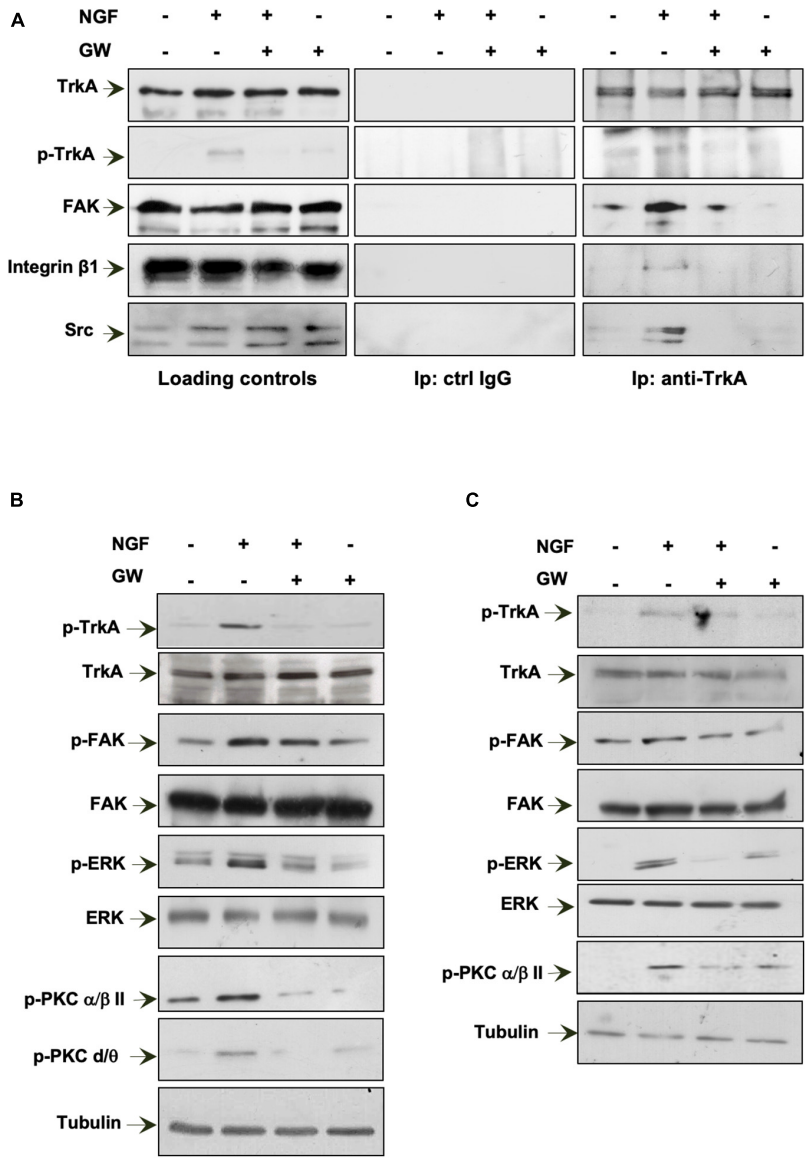

FIGURE 6 | Neurotrophin β-nerve growth factor (NGF) challenge induces the TrkA/FAK/β1-integrin/Src complex assembly and the NGF-dependent signaling activation. **(A,B)** Quiescent MDA-MB-231 cells were left unchallenged or challenged for 15 min with NGF in the absence or presence of GW441756 (GW). In **(A)**, lysate proteins were immunoprecipitated using the anti-TrkA antibody (anti-TrkA; *right panels*) or control immunoglobulin G (ctrl IgG; *middle panels*). Loading controls are shown in *left panels*. Western blot (WB) with the indicated antibodies was done to detect proteins in the immunocomplex. In **(B)**, lysate proteins were analyzed using the antibodies against the indicated proteins. **(C)** Quiescent MDA-MB-453 cells were left unchallenged or challenged for 15 min with NGF in the absence or presence of GW. Lysate proteins were analyzed using the antibodies against the indicated proteins. The filter was stripped and re-probed using anti-tubulin antibody as a loading control. All results are representative of three different experiments. *p-TrkA*, Tyr 490-p-TrkA; *p-FAK*, Tyr 397-p-FAK; *p-ERK*, p44 and p42 ERK; *p-PKC α/βII*, Thr 638/41-p-PKC α/βII; *p-PKC δ/θ*, Ser643/676.

leads to the activation of the downstream NGF signaling pathway in TNBC cells.

Role of TrkA-Dependent Signaling in Biological Responses Elicited by NGF Treatment in TNBC Cells

Given the findings that Src and FAK represent key drivers of mitogenesis and invasion in solid cancers (Irby and Yeatman, 2000; Mitra and Schlaepfer, 2006), we investigated their role in NGF-elicited effects. The effect of the FAK inhibitor VS-6063 (Kang et al., 2013) was exploited on motility and mitogenesis induced by NGF in MDA-MB-231 cells. Phase-contrast images from the wound scratch assay show that a significant number of cells migrated in the wound area upon NGF treatment. VS-6063 inhibited the NGF effect. Images captured at time 0 or from unstimulated cells are also shown (**Figure 7A**). Shown below the images is the corresponding percentage of

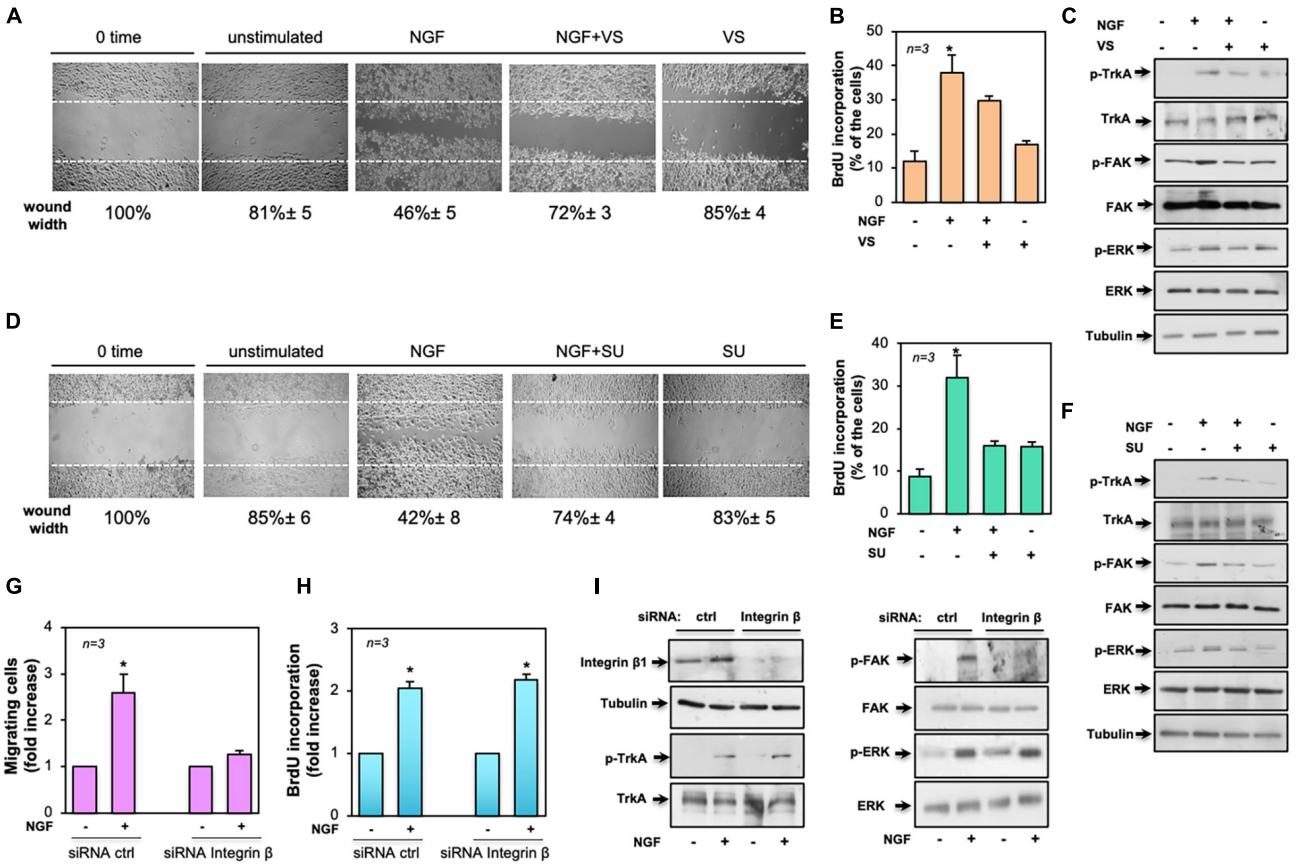

FIGURE 7 | Role of focal adhesion kinase (FAK), Src, and β1-integrin in the biological effects elicited by neurotrophin β-nerve growth factor (NGF) treatment of MDA-MB-231 cells. In **(A–F)**, quiescent MDA-MB-231 cells were used. In **(A–C)**, the FAK inhibitor VS-6063 (VS) was used at 10 μM. In **(D–F)**, the Src inhibitor SU6656 (SU) was used at 5 μM. In **(A–I)**, NGF was used at 100 ng/ml. **(A,D)** Cells were wounded and left unstimulated or stimulated with NGF in the absence or presence of the indicated compounds. Phase-contrast images are representative of three different experiments, each in duplicate. The wound area was calculated using Leica Suite software and data are reported *below each image*. Data are expressed as the percentage of decrease in wound width over the control cells (analyzed at time 0). **(B,E)** Cells were left untreated or treated for 18 h with the indicated compounds. After *in vivo* pulse with 100 μM BrdU, incorporation of BrdU by the cells was analyzed by immunofluorescence (IF) and expressed as the percentage of total cells. **(C,F)** Cells were left unchallenged or challenged for 15 min with NGF in the absence or presence of the indicated compounds. Lysate proteins were analyzed by Western blot (WB) using the antibodies against the indicated proteins. **(G–I)** MDA-MB-231 cells were transfected with β1-integrin or control (ctrl) siRNA in the presence **(G,H)** or absence **(I)** of eGFP-cDNA to help in the identification of transfected cells. **(G)** Quiescent cells were left unstimulated or stimulated with NGF for 7 h and used for the migration assay. Migrated cells were scored using a fluorescent microscope and the data expressed as the relative increase in the number of migrating cells. Data from several independent experiments were collected and analyzed. **(H)** Quiescent cells were left untreated or treated with NGF for 18 h. After *in vivo* pulse with 100 μM BrdU, the DNA synthesis was analyzed by IF and calculated using the formula: percentage of BrdU-positive cells = (No. of transfected BrdU-positive cells/No. of transfected cells) × 100. Data are presented as the fold increase over the basal level. In **(G,H)**, for each plasmid, the data are derived from at least 500 transfected cells. The results of three independent experiments have been averaged. Means and SEM are shown. *n* represents the number of experiments. **(I)** Transfected cells were left unstimulated or stimulated with NGF for 15 min and lysate proteins were analyzed by WB using the antibodies against the indicated proteins. Abbreviations used in **(C,F,E)**: *p-TrkA*, Tyr 490-p-TrkA; *p-FAK*, Tyr 397-p-FAK; *p-ERK*, p44 and p42 ERK. The filter was stripped and re-probed using anti-tubulin antibody as a loading control.

wound width decrease. FAK activation, however, also plays a role in DNA synthesis, as assessed by the inhibitory effect of VS-6063 on the NGF-induced BrdU incorporation (**Figure 7B**). VS-6063 did not affect the NGF-induced Tyr-490 Trk phosphorylation, while it inhibited FAK and p44-p42 ERK activation in NGF-treated cells (**Figure 7C**). Superimposable results in terms of motility (**Figure 7D**), DNA synthesis (**Figure 7E**), and signaling activation (**Figure 7F**) were observed using the Src tyrosine kinase inhibitor SU6656 (Blake et al., 2000). The effect of Src inhibition on NGF-elicited BrdU incorporation was more robust as compared to that observed by FAK inhibition, likely because other members of the Src tyrosine kinase family are engaged by NGF (Dey et al., 2005) to convey its mitogenic signaling, and SU6656 also inhibits these kinases (Blake et al., 2000).

To further address the molecular mechanism underlying NGF signaling in TNBC cells, we silenced β1-integrin, as assessed by the WB analysis in **Figure 7I** (left panel). The NGF-induced motility (**Figure 7G**) and DNA synthesis (**Figure 7H**) of cells were then analyzed. Of note is that β1-integrin knockdown only affected the migratory properties of the NGF-treated cells while leaving unaltered the BrdU incorporation. Again, β1-integrin knockdown did not affect the NGF-induced Tyr-490 TrkA (left

panel in **Figure 7I**) or p44-p42 ERK phosphorylation (right panel in **Figure 7I**), while it almost completely abolished the NGF-induced FAK activation (right panel in **Figure 7I**).

Taken together, our findings indicate that NGF-induced TrkA tyrosine phosphorylation controls a plethora of signaling components. In this plot, β1-integrin behaves as a bridge linking FAK to TrkA. Such a connection seems to be required for motility induced by NGF.

DISCUSSION

Many findings have highlighted the role of TrkA as a driver of cell transformation. They have also suggested that derangement of the NGF circuit is involved in drug resistance, survival, and metastatic spreading of solid tumors (Demir et al., 2016), including the so-called hormone-dependent cancers (Descamps et al., 1998, 2001a,b; George et al., 1998; Pflug and Djakiew, 1998; Sigala et al., 1999; Krygier and Djakiew, 2001, 2002; Miknyoczki et al., 2002; Dollé et al., 2003, 2004; Festuccia et al., 2007; Papatsoris et al., 2007; Anagnostopoulou et al., 2013). Specific targeting of NGF inhibits the proliferation and metastatic events in BC (Adriaenssens et al., 2008). Furthermore, TrkA overexpression has been detected in over 20% of BCs, and it has been linked to their proliferation and spreading (Lagadec et al., 2009; Snowman et al., 2020). Silencing of TrkA enhances chemosensitivity in BC cultured cells and inhibits their spreading in a mouse model (Zhang et al., 2015). These findings point to the role of the NGF/TrkA pathway in BC.

In this study, we have analyzed the role of NGF signaling activation in TNBC cell aggressiveness. MDA-MB-231 and MDA-MB-453 cells both express significant amounts of TrkA and undergo mitogenesis and motility on NGF challenge. Such effects require TrkA activation, as the specific inhibitor GW441756 reverses both the responses. Simultaneously, NGF rapidly triggers the association of TrkA with β1-integrin, FAK, and Src in MDA-MB-231 cells. These effectors are involved in mitogenesis, focal adhesion complex assembly, and migration induced by growth factors, cytokines, neurotrophins, and ECM in various cell types (Roche et al., 1995; Sieg et al., 2000; Bromann et al., 2004). The NGF-triggered TrkA/β1-integrin/FAK/Src complex assembly induces the activation of several downstream effectors, including the p44-p42 ERK and PKCs. GW441756 disrupts the NGF-induced complex assembly and inhibits the TrkA-dependent signaling activation. By this way, the inhibitor reverses the mitogenesis and motility induced by NGF in these cells. Pharmacological inhibition of the Src and FAK tyrosine kinases shows that both the effectors are required for the NGF-elicited proliferation and motility in TNBC cells. Notably, findings from transient knockdown of β1-integrin support the conclusion that its recruitment to the TrkA/FAK/Src complex is needed for the locomotion elicited by NGF in TNBC cells.

Tyrosine kinase A activation is also needed for the NGF-induced increase in TNBC cell spheroid size and viability, as its inhibition by GW441756 results in a significant reduction of these effects. The inhibitory action of GW441756 is consistent with the observed effect on NGF-elicited mitogenesis. However, since NGF treatment also increases the release of MMP-9 in MDA-MB-231 cells, it might be conceived that the NGF-triggered TrkA/β1-integrin/FAK/Src complex assembly constitutes a signaling module relevant to ECM remodeling in TNBC cells. Perturbing the NGF-induced complex assembly and TrkA tyrosine phosphorylation by GW441756 impairs the MMP-9 release and, as consequence, the invasive ability of NGF-treated TNBC cells.

Previous findings have reported that TNBC cells might release NGF. As these cells express TrkA, an autocrine loop might sustain their survival (Dollé et al., 2003; Pundavela et al., 2015; Chakravarthy et al., 2016). Our data confirm these results and also indicate that a very low amount of pro-NGF is released by TNBC cells. Although the amount of secreted NGF is lower (almost 4,867 and 6,923 pg/ml for MDA-MB-231 and MDA-MB-453, respectively) than that used (100 ng/ml) to stimulate TNBC cells, it might be argued that a persistent release of NGF, rather than pro-NGF, self-sustains *in vivo* the growth and aggressiveness of TNBC. By a functional assay, we have also verified that CM induces the differentiation of neuronal PC12 cells. Such effect is actually caused by the NGF present in CM since blocking the activity of the secreted NGF by a specific neutralizing antibody reverses the differentiation as well as the NGF-signaling activation of PC12 cells. Thus, once released by TNBC cells, NGF might play a role in the autonomic innervation of the tumor and its aggressiveness. Infiltration of the tumor microenvironment by nerve fibers involves NGF production by BC cells and is associated with BC aggressiveness (Pundavela et al., 2015). Overall, our data might have implications in the brain metastasis of TNBC since neurotrophic factors released by BCs control the interaction between microglial and metastatic BC cells, allowing their growth in the brain (Louie et al., 2013). In this context, the results observed in MDA-MB-231 cells are particularly relevant since they represent a brain-tropic TNBC cell type (Yoneda et al., 2001).

The findings here reported using neutralizing antibodies against NGF or the TrkA inhibitor GW441756 deserve additional comments. Targeting NGF/TrkA signaling by blocking antibodies and/or inhibitors has gained great attention in the last years. Several drugs, including neutralizing antibodies, small inhibitors, and peptides have been synthesized to shut down the NGF circuit in neurological disorders (Longo and Massa, 2013 and references therein). A neutralizing anti-NGF antibody has been used to reduce the pain related to autoimmune diseases and was successfully tested in preclinical models of prostate cancer. A similar approach might be used to alleviate cancer-related pain. Additionally, small molecules targeting NGF signaling are currently used in ongoing clinical trials for the treatment of many solid tumors (Griffin et al., 2018).

The present study, together with our previous findings in prostate cancer (Di Donato et al., 2018, 2019), further points to the relevance of NGF signaling in "gender-related cancers" and paves the way for new therapeutic opportunities in the clinical management of TNBC patients, who often exhibit or develop drug resistance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MDD contributed to the conceptualization, data curation, formal analysis, validation, investigation, and methodology. GG and PG helped with the investigation and methodology. AS reviewed and helped with funding acquisition. AM did the review and editing and helped with funding acquisition. GC contributed to the conceptualization, supervision, funding acquisition, writing original draft, review, and editing. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Italian Ministry of University and Scientific Research (P.R.I.N. 2017EKMFTN_002 to GC) and VALERE (Vanvitelli per la Ricerca Program; GoMAGIC to AM and AdipCARE to GC). MDD was supported by the iCURE Project (B21C17000030007—Regione Campania). AS, GG, and PG were supported by VALERE (Vanvitelli per la Ricerca) Program.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.676568/full#supplementary-material

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 07 July 2021
doi: 10.3389/fcell.2021.707970

C1GALT1, Negatively Regulated by miR-181d-5p, Promotes Tumor Progression via Upregulating RAC1 in Lung Adenocarcinoma

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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 2021
Accepted: 17 June 2021
Published: 07 July 2021

Citation:
Dong X, Liu Y, Deng X, Shao J, Tian S, Chen S, Huang R, Lin Z, Chen C and Shen L (2021) C1GALT1, Negatively Regulated by miR-181d-5p, Promotes Tumor Progression via Upregulating RAC1 in Lung Adenocarcinoma. Front. Cell Dev. Biol. 9:707970. doi: 10.3389/fcell.2021.707970

Glycosyltransferases are frequently dysregulated in lung cancer. Core 1 β 1, 3-galactosyltransferase 1 (C1GALT1), an enzyme highly expressed in various cancers, is correlated with tumor initiation and development. However, the role of C1GALT1 in lung cancer remains poorly understood. In this study, through bioinformatic analysis and clinical validation, we first discovered that C1GALT1 expression was upregulated in lung adenocarcinoma (LUAD) tissues and was closely related to poor prognosis in patients with LUAD. Gain- and loss-of-function experiments showed that C1GALT1 promoted LUAD cell proliferation, migration, and invasion *in vitro*, as well as tumor formation *in vivo*. Further investigation demonstrated that RAC1 expression was positively regulated by C1GALT1 in LUAD, whereas silencing Rac1 could reverse C1GALT1-induced tumor growth and metastasis. Moreover, miR-181d-5p was identified as a negative regulator for C1GALT1 in LUAD. As expected, the inhibitory effects of miR-181d-5p on LUAD cell proliferation, migration, and invasion were counteracted by restoration of C1GALT1. In summary, our results highlight the importance of the miR-181d-5p/C1GALT1/RAC1 regulatory axis during LUAD progression. Thus, C1GALT1 may serve as a potential therapeutic target for LUAD.

Keywords: lung cancer, C1GALT1, miR-181d-5p, RAC1, growth, metastasis

INTRODUCTION

According to the global cancer statistics in 2018, lung cancer remains the most frequent cancer and the leading cause of cancer death worldwide (Bray et al., 2018). Based on pathological types, lung cancer can be divided into small cell lung cancer (SCLC, 15% of the cases) and non-small cell lung cancer (NSCLC, 85% of the cases). NSCLC is further classified into three types, namely, lung squamous cell carcinoma (LUSC), lung adenocarcinoma (LUAD), and large cell carcinoma. Lung cancer is a highly aggressive disease and has a poor prognosis, with a 5-year survival rate of less than 20% (Siegel et al., 2020). Although therapeutic strategies (surgery, chemotherapy,

Abbreviations: NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; GT, glycosyltransferase; TCGA, The Cancer Genome Atlas; C1GALT1, Core 1 β1, 3-galactosyltransferase 1; RAC1, Rac Family Small GTPase 1; miR, microRNA; qPCR, quantitative Real-Time PCR; IHC, immunohistochemistry; CCK-8, Cell Counting Kit-8; 3′UTR, 3′-untranslated region; GSEA, Gene set enrichment analysis; shRNA, short hairpin RNA; siRNA, small interfering RNA.

radiotherapy, and immunotherapy) have been applied for the treatment of lung cancer, some patients still develop postoperative recurrence and metastasis. Frequent recurrence or metastasis remains a major obstacle for lung cancer treatment (Wang et al., 2019; Zhan et al., 2019). Thus, it is essential to clarify the mechanism underlying the pathogenesis and progression of lung cancer, which may help to improve therapeutic outcomes.

All living cells typically decorate their surfaces with diverse glycans linked to proteins or lipids (Jaiman and Thattai, 2020). These glycans participate in numerous biological events, such as cellular communication, protein folding, and immune regulation (Narimatsu et al., 2019). Cells in different tissues and developmental stages usually express distinct glycans on their surfaces. Unlike RNA transcription and protein translation, glycans are not directly synthesized from a genome-encoded template (Moremen and Haltiwanger, 2019). Glycan biosynthesis is mainly controlled by a series of glycosyltransferases (GTs). GTs are the key enzymes that catalyze the formation of complex glycans by adding one sugar to specific acceptors at a time (Park et al., 2018). Until now, over 200 GTs have been identified in the human genome (Moremen et al., 2018). GTs are frequently altered in various diseases, including cancer (Gloster, 2014; Wu et al., 2019). Remarkably, aberrant expression or dysregulation of GTs also occurs in lung cancer (Reticker-Flynn and Bhatia, 2015; Park et al., 2020). Therefore, elucidating the relationship between GTs and lung cancer is particularly important.

Core 1 β1, 3-galactosyltransferase 1 (C1GALT1), an exclusive T-synthase in mammalian cells, catalyzes the transfer of galactose (Gal) from UDP-Gal to the extant GalNAc forming core 1 O-glycans (Galβ1, 3GalNAcα-O-Ser/Thr) (Liu et al., 2020). Studies have shown that C1GALT1 is abnormally expressed in a variety of malignant tumors, such as gastric cancer (Lee et al., 2020), head and neck cancer (Lin et al., 2018), pancreatic ductal adenocarcinoma (Kuo et al., 2021), laryngeal carcinoma (Dong et al., 2018b), ovarian cancer (Chou et al., 2017), and hepatocellular carcinoma (Wu et al., 2013). However, the role of C1GALT1 in lung cancer remains unclear.

In this study, we comprehensively assessed the biological function of C1GALT1 in lung cancer using bioinformatics tools and clinical samples. We found that C1GALT1 was overexpressed in LUAD tissues and high C1GALT1 expression was associated with poor prognosis in LUAD patients. Through a series of functional experiments *in vitro* and *in vivo*, we confirmed that C1GALT1 could facilitate LUAD progression. Further mechanistic studies demonstrated that C1GALT1 expression was negatively regulated by miR-181d-5p in LUAD and the promotive effects of C1GALT1 on LUAD progression were mediated by Rac Family Small GTPase 1 (RAC1). These findings may provide novel therapeutic targets for the treatment of lung cancer.

MATERIALS AND METHODS

Clinical Specimens and Cell Lines

Sixty paired LUAD tissues and adjacent normal tissues were collected from patients who underwent surgical resection at Taihe Hospital, Hubei University of Medicine. Samples were immediately frozen in liquid nitrogen and stored at −80°C. This study was approved by the Ethics Committee of the Hubei University of Medicine. Informed consent was obtained from all subjects.

LUAD cell lines (A549, H1299, H1975, and H441) and a normal lung epithelial cell line (BEAS-2B) were purchased from the Procell (Wuhan, China). All cells were cultured in DMEM (Gbico, Detroit, MI, United States) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, United States). Cells were maintained at 37°C, 5% CO_2.

Bioinformatics Analysis

The miRNA and mRNA expression datasets (level 3) were downloaded from The Cancer Genome Atlas (TCGA[1]). Data visualization was performed using the ggplot2 R package. Survival analysis was conducted using the "survminer" and "survival" R packages. Correlation matrices were constructed using the R package "corrplot." ROC curves were plotted using the "pROC" package. The prognostic nomogram was generated by the "rms" package. Venn diagram was made using the VennDiagram R package. Gene set enrichment analysis (GSEA) was performed with the GSEA software[2]. $p < 0.05$ was considered statistically significant and $p > 0.05$ was considered no significant.

Cell Transfection

The C1GALT1-overexpressed plasmid (OV) or empty pcDNA3.1 plasmid (Mock), lentiviral vectors expressing C1GALT1 short hairpin RNAs (shRNAs) or negative control shRNA (shNC), RAC1 small interfering RNA (siRNA) or non-silencing siRNA (siNC), miR-181d-5p inhibitor, inhibitor negative control (inhibitor NC), miR-181d-5p mimic and mimic negative control (mimic NC) were purchased from GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States) or RNAi-mate reagent (GenePharma), following the manufacturer's guidelines. Stably transfected cells were selected by G418 or puromycin. Sequences of shRNAs and siRNAs are listed in **Supplementary Table 1**.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from clinical samples or cell lines using RNAiso Plus (Takara, Kusatsu, Japan), according to the manufacturer's instructions. For quantification of mRNA, Reverse transcription and PCR amplification was performed using PrimeScript RT Master Mix (Takara) and SYBR Green PCR Master Mix (Takara). For miRNA analysis, TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) and TaqMan miRNA Assay Kit (Applied Biosystems) were used. Data were normalized to GAPDH or U6 using the $2^{-\Delta\Delta Ct}$ method. Primers for qPCR are listed in **Supplementary Table 2**.

[1]https://tcga-data.nci.nih.gov/tcga/

[2]http://www.broadinstitute.org/gsea/index.jsp

Western Blot

Total protein was extracted using RIPA lysis buffer (Beyotime, Shanghai, China). After quantification, an equal amount of protein was run on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, United States). After blocking with 5% BSA, the membrane was probed with the indicated antibodies. Primary antibodies used in this study were as follows: C1GALT1 (Abcam, Cambridge, United Kingdom, ab237734), RAC1 (ab155938), and GADPH (ab9485). Reactive bands were visualized using an ECL system (Pierce, Rockford, IL, United States). RAC1 activity was determined using the Rac1 activation assay kit (Cytoskeleton, Denver, CO, United States). GTP-RAC1 was detected by Western blot using an anti-RAC1 antibody.

Immunohistochemistry (IHC)

Tumor tissues were embedded in paraffin using standard techniques. IHC was carried out as previously described (Dong et al., 2018a,b). The IHC score (range 0–12) was calculated by multiplying the intensity and the percentage of staining. A total score > 5 was defined as high expression, and ≤ 4 was regarded as low expression. Images were captured using an Olympus BX53 microscope (Olympus, Tokyo, Japan).

Proliferation Analysis

Cell Counting Kit-8 (CCK-8, Dojindo, Japan) and colony formation assays were applied for determining cell proliferation. For the CCK-8 assay, cells were seeded into 96-well plates at a density of 3×10^3 per well. Absorbance at 450 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA, United States). For colony formation assay, cells (1×10^3 per well) were plated in 6-well plates. After 2 weeks, colonies (>50 cells) were fixed with methanol and stained with 1% crystal violet (Beyotime).

Migration and Invasion Analysis

Cell migratory and invasive abilities were determined using uncoated or Matrigel-coated Transwell chambers (Corning Inc., Corning, NY, United States), respectively. Protocols were identical to those described previously (Dong et al., 2018b). After 24 h of incubation, the migrated and invaded cells in lower chambers were counted under a light microscope (magnification, 100×). The wound-healing assay was also done to assess cell migration. Artificial wounds were created by scraping using a sterile 200 μl pipette tip. The wound areas were photographed at 0 and 24 h.

Cell Cycle and Apoptosis Analysis

Cell Cycle Detection Kit (KeyGene, Nanjing, China) was utilized to monitor the cell cycle distribution. Cells were stained with PI staining solution containing RNase. Annexin V-PE/7AAD Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, United States) was used to analyze apoptotic cells (Annexin-V + and 7AAD-). Data were acquired on a FACScan flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR, United States).

Luciferase Assay

The pmirGLO vectors with wild-type or the mutant miR-181d-5p binding site in C1GALT1 3'UTR were designed and constructed by GenePharma. Subsequently, miR-181d-5p mimic or NC mimic were co-transfected with the above reporter vectors into cells using Lipofectamine 3000. After 48 h of transfection, the luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States).

Mice Xenograft Models

Female BALB/c nude mice (3–4 weeks) were obtained from the Animal Center of Hubei University of Medicine. Stably transfected cells (5×10^6) were subcutaneously injected into nude mice. Tumor volumes were evaluated every week. Tumor size was calculated according to the formula tumor volume = (length $\times$ width^2)/2. All mice were sacrificed after 4 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Hubei University of Medicine.

Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, United States) and presented as mean $\pm$ SD from at least three independent experiments. Statistical significances were determined using the Student's *t*-test, one-way analysis of variance (ANOVA), Pearson correlation analysis, or Kaplan-Meier survival analysis. $p < 0.05$ was considered statistically significant.

RESULTS

C1GALT1 Is Overexpressed in LUAD and Predicts Poor Prognosis

To explore the expression pattern of C1GALT1 in lung cancer, we performed bioinformatics analysis using the TCGA database. The results showed that C1GALT1 expression was higher in the majority of tumors than normal tissues, particularly in the stomach, lung, and esophageal (**Figures 1A–C**). Notably, C1GALT1 was highly expressed in both LUAD and LUSC tissues, two major subtypes of NSCLC. Kaplan-Meier survival analysis demonstrated that high C1GALT1 expression was significantly negatively correlated with overall survival and disease-specific survival in LUAD patients but not in LUSC patients (**Figure 1D** and **Supplementary Figure 1**). Thus, we mainly focused on the role of C1GALT1 in LUAD. Univariate and multivariate Cox regression analysis confirmed that C1GALT1 overexpression was an independent prognostic factor for overall survival in LUAD patients (**Figure 1E** and **Table 1**). Using the multivariate Cox proportional hazard models, we successfully constructed a prognostic nomogram to predict 1-, 3-, and 5-year survival probability in LUAD (**Figure 1F**). We then used ROC curve analysis to assess the diagnostic value of C1GALT1. We found that C1GALT1 was able to distinguish LUAD patients from healthy controls, with the AUC value of 0.808 (**Figure 1G**). Subsequently,

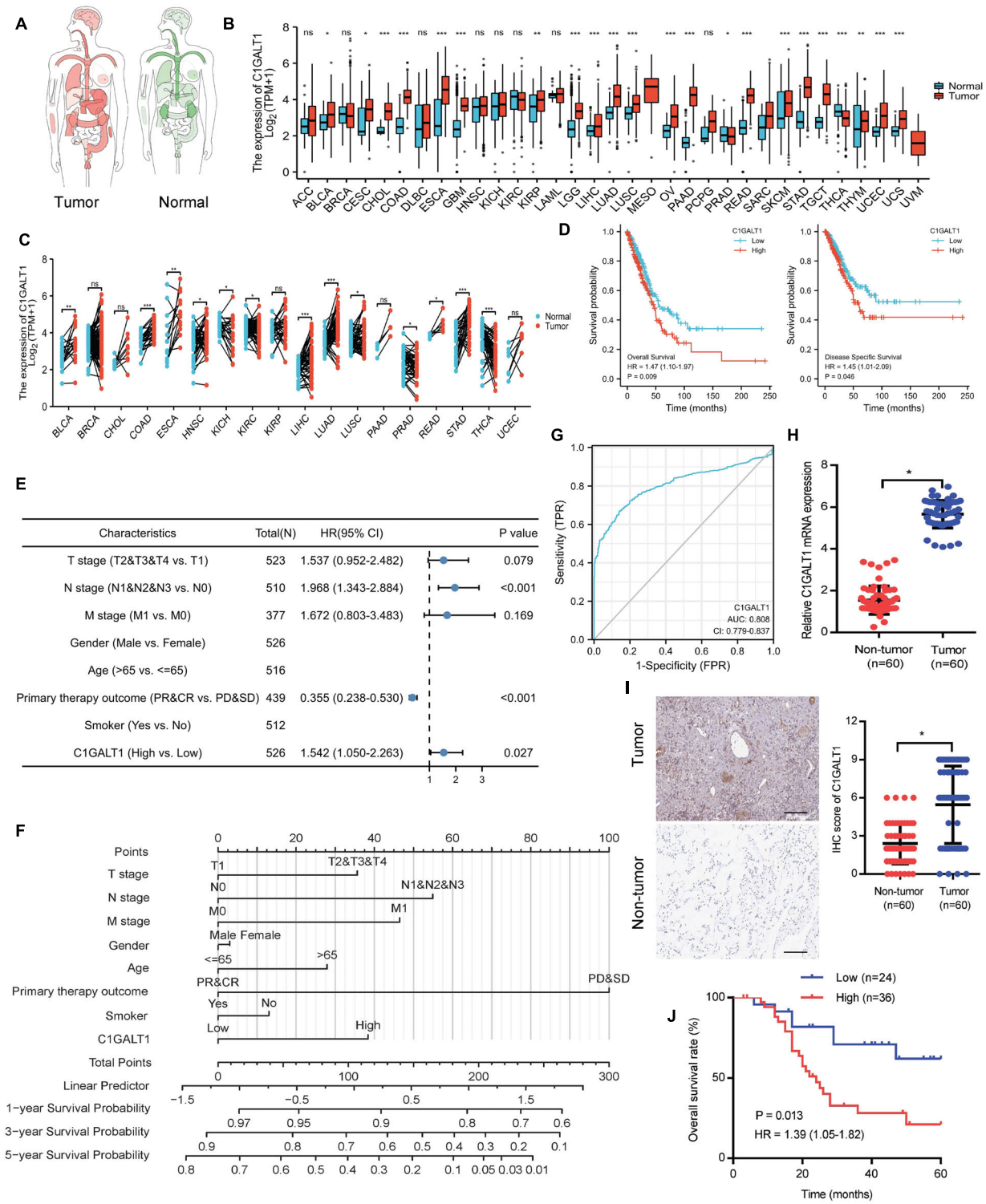

FIGURE 1 | C1GALT1 is overexpressed in LUAD and associated with poor prognosis. **(A)** Pan-cancer analysis of C1GALT1 using the TCGA database. **(B)** C1GALT1 expression in unpaired tumor and normal tissues from the TCGA database. **(C)** C1GALT1 expression profiles across all tumor samples and paired normal tissues in the TCGA database. **(D)** Kaplan-Meier curves for overall survival and disease-specific survival according to C1GALT1 expression in the TCGA-LUAD cohort. **(E)** Forest plot of multivariate Cox regression analysis. **(F)** Nomogram for predicting 1-, 3- and 5-year survival probability of LUAD patients. **(G)** ROC curve of C1GALT1 in LUAD. **(H)** Analysis of C1GALT1 expression in LUAD tissues and adjacent non-tumor tissues by qPCR. **(I)** IHC analysis of C1GALT1 expression in LUAD samples. Scale bar: 100 μm. **(J)** Overall survival analysis of LUAD patients based on differential expression levels of C1GALT1. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns* = not significant.

qPCR and IHC were utilized to validate the data obtained from TCGA. We observed that C1GALT1 expression was upregulated in LUAD tissues compared with adjacent non-tumor tissues (**Figures 1H,I**). LUAD patients with high C1GALT1 expression had shorter overall survival times than those with low C1GALT1 expression (**Figure 1J**). Moreover, C1GALT1 expression was closely related to lymph node metastasis and TNM stage (**Table 2**). Collectively, the above findings highlighted the importance of C1GALT1 in LUAD development and progression.

C1GALT1 Promotes the Growth of LUAD *in vitro*

To investigate the biological function of C1GALT1 in LUAD, the expression levels of C1GALT1 in four LUAD cell lines (A549, H1299, H1975, and H441) and one normal lung epithelial cell line (BEAS-2B) were examined by qPCR and Western blot. We found that C1GALT1 expression was higher in LUAD cells than in normal lung cells (**Figures 2A,B**). Considering the cell cultured condition and growth state, A549 and H1299 cells were used in the following experiments.

TABLE 1 | Univariate and multivariate Cox proportional analysis for overall survival.

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
T stage (T2&T3&T4 vs. T1)	523	1.728 (1.229–2.431)	0.002	1.537 (0.952–2.482)	0.079
N stage (N1&N2&N3 vs. N0)	510	2.601 (1.944–3.480)	< 0.001	1.968 (1.343–2.884)	< 0.001
M stage (M1 vs. M0)	377	2.136 (1.248–3.653)	0.006	1.672 (0.803–3.483)	0.169
Gender (Male vs. Female)	526	1.070 (0.803–1.426)	0.642		
Age (>65 vs. ≤ 65)	516	1.223 (0.916–1.635)	0.172		
Primary therapy outcome (PR&CR vs. PD&SD)	439	0.377 (0.268–0.530)	< 0.001	0.355 (0.238–0.530)	< 0.001
Smoker (Yes vs. No)	512	0.894 (0.592–1.348)	0.591		
C1GALT1 (High vs. Low)	526	1.473 (1.103–1.966)	0.009	1.542 (1.050–2.263)	0.027

TABLE 2 | Relevance analysis of C1GALT1, RAC1, and miR-181d-5p expression in LUAD patients.

Variables	C1GALT1		*P*-value	RAC1		*P*-value	miR-181d-5p		*P*-value
	Low (24)	High (36)		Low (20)	High (40)		Low (37)	High (23)	
Age									
<65	10	18	0.167	9	19	0.386	20	8	0.249
≥65	14	18		11	21		17	15	
Gender									
Male	11	16	0.305	14	13	0.181	18	9	0.063
Female	13	20		6	27		19	14	
Tumor size (cm)									
<5	7	12	0.108	10	9	0.072	14	5	0.059
≥5	17	24		10	31		23	18	
Smoking status									
No	12	15	0.225	7	20	0.144	15	12	0.077
Yes	12	21		13	20		22	11	
Lymph node metastasis									
N0	19	6	0.023	13	12	0.018	12	13	0.011
N1-3	5	30		7	28		25	10	
TNM stage									
I + II	18	13	0.008	12	19	0.005	13	18	0.004
III + IV	6	23		8	21		24	5	

Next, C1GALT1 was stably knocked down or overexpressed in A549 and H1299 cells (**Figures 2C,D**). CCK-8 assay showed that C1GALT1 overexpression strengthened cell viability, whereas C1GALT1 knockdown weakened cell viability (**Figure 2E**). Colony formation assay revealed that the number of colonies was increased by C1GALT1 overexpression, and decreased by knockdown of C1GALT1 (**Figure 2F**). Usually, cell proliferation is regulated by the cell cycle and apoptosis. To determine whether C1GALT1 knockdown-mediated inhibition of cell proliferation was associated with cell cycle arrest or apoptosis induction, the flow cytometry assay was performed. PI staining demonstrated that the cell cycle was arrested at the G0/G1 phase upon C1GALT1 knockdown (**Figures 2G,H**). Annexin V-PE/7AAD staining showed that knockdown of C1GALT1 could increase the percentage of cells undergoing apoptosis (**Figure 2I**). These results indicated that C1GALT1 played a critical role in controlling the growth of LUAD cells.

C1GALT1 Facilitates LUAD Cell Migration and Invasion *in vitro*

To evaluate the impact of C1GALT1 on LUAD cell metastasis, wound-healing, Transwell migration, and Matrigel invasion assays were carried out. We found that C1GALT1 overexpression accelerated the scratch repair of A549 and H1299 cells, whereas C1GALT1 knockdown had the opposite effects (**Figure 3A**). Moreover, the migratory and invasive abilities of A549 and H1299 cells were greatly enhanced by overexpression of C1GALT1 and significantly reduced by knockdown of C1GALT1 (**Figures 3B,C**). It has been reported that itraconazole is a specific inhibitor of C1GALT1 (Lin et al., 2018). We noticed that C1GALT1 protein expression in A549 and H1299 cells was suppressed by itraconazole in a dose-dependent manner (**Figure 3D**). Additionally, Transwell migration and Matrigel invasion assays showed that itraconazole pretreatment could inhibit the migration and invasion of A549 and H1299 cells (**Figures 3E,F**). The above-mentioned data suggested that C1GALT1 may function as an oncogene by affecting LUAD cell migration and invasion.

C1GALT1 Drives LUAD Tumor Growth *in vivo*

To determine whether C1GALT1 could affect tumor growth *in vivo*, we conducted a mouse xenograft experiment. The C1GALT1-overexpressing, C1GALT1-knockdown, or respective control cells were subcutaneously injected into the nude mice. All mice were sacrificed on day 28. We found that C1GALT1 overexpression enhanced the tumorigenic abilities of A549 and H1299 cells, as manifested by increased tumor size, volume, and weight (**Figures 4A–C**). On the contrary, C1GALT1 knockdown in A549 and H1299 cells inhibited the growth of xenograft tumors (**Figures 4D–F**). The C1GALT1 shRNA1 was selected for subsequent experiments due to its higher efficiency. These *in vivo* findings strengthened and confirmed our *in vitro* results that C1GALT1 contributed to LUAD tumorigenesis.

C1GALT1 Expression Is Negatively Regulated by miR-181d-5p in LUAD

To probe the mechanism behind C1GALT1 upregulation, we first evaluated genomic alterations of this gene through the cBioportal online platform and found that C1GALT1 displayed a very low mutation frequency in LUAD (**Supplementary Figure 2**). DNA methylation analysis revealed that the C1GALT1 promoter was almost unmethylated in LUAD (**Supplementary Figure 3**). Hence, we turned our attention to miRNAs, the major regulators of gene expression. Using four publicly available algorithms (PITA, TargetScan, miRWalk, and RNAhybrid), we identified 14 miRNAs that were predicted to target C1GALT1 (**Figure 5A**). Moreover, we downloaded the miRNA expression profiles of LUAD patients from the TCGA database. The top 50 most significant miRNAs negatively correlated with C1GALT1 in LUAD were visualized by the heat map (**Figure 5B**). By intersecting the potential target miRNAs and negatively correlated miRNAs, miR-148b-3p, and miR-181d-5p were selected for validation. Although TCGA dataset analysis showed that these two miRNAs were downregulated in LUAD, only the miR-181d-5p expression had a significant correlation with poor prognosis in patients with LUAD (**Figures 5C–E** and **Supplementary Figures 4A–D**). LUAD patients with low miR-181d-5p expression had shorter overall survival times than those with high miR-181d-5p expression. These observations were further confirmed by qPCR analysis in our samples (**Figures 5F–H** and **Table 2**). Therefore, miR-181d-5p was considered a functional regulator of C1GALT1 in LUAD. We next explored whether C1GALT1 was directly modulated by miR-181d-5p in LUAD cells. The results demonstrated that transfecting miR-181d-5p mimics or inhibitor to A549 and H1299 cell lines could significantly downregulate or upregulate the expression of C1GALT1 in these two cells, respectively (**Figures 5I,J** and **Supplementary Figure 5**). Based on the bioinformatics prediction, we identified a putative binding site for miR-181d-5p in the C1GALT1 3'UTR (**Figure 5K**). As expected, miR-181d-5p mimics inhibited the luciferase activity of wild-type C1GALT1 3'UTR but did not affect the luciferase activity of mutant C1GALT1 3'UTR (**Figure 5L**). Altogether, these findings indicated that miR-181d-5p was the upstream regulatory factor of C1GALT1, and downregulation of miR-181d-5p resulted in increased C1GALT1 expression in LUAD.

miR-181d-5p Suppresses the Proliferation, Migration, and Invasion of LUAD Cells by Targeting C1GALT1

To assess the role of miR-181d-5p in LUAD, we performed loss- or gain-of-function experiments. Cell proliferation, migration, and invasion abilities were determined by CCK-8, Transwell migration, and Matrigel invasion assays, respectively. We found that miR-181d-5p mimics repressed the proliferation, invasion, and migration of A549 and H1299 cells. Conversely, miR-181d-5p inhibitor promoted LUAD cell proliferation, invasion, and migration (**Figures 6A–C**). Moreover, A549 and H1299 cells were co-transfected with miR-181d-5p mimics and C1GALT1 overexpression plasmid. The re-expression efficiency

FIGURE 2 | C1GALT1 promotes LUAD cell growth *in vitro*. **(A,B)** Analysis of C1GALT1 expression in four LUAD cell lines and one normal lung cell line by qPCR **(A)** and Western blot **(B)**. **(C,D)** C1GALT1 transfection efficiency was determined by qPCR **(C)** and western blot **(D)**. **(E,F)** Cell proliferation was assessed by CCK-8 assay **(E)** and colony formation assay **(F)**. **(G)** Flow cytometry analysis of cell cycle. **(H)** Quantitative analysis of cell cycle distribution. **(I)** Analysis of apoptosis by flow cytometry. Mock, cells transfected with empty plasmid; OV, cells transfected with C1GALT1 overexpression plasmid; shNC, cells infected with negative control shRNA; shRNAs, cells infected with C1GALT1 shRNA. Data are expressed as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.001$.

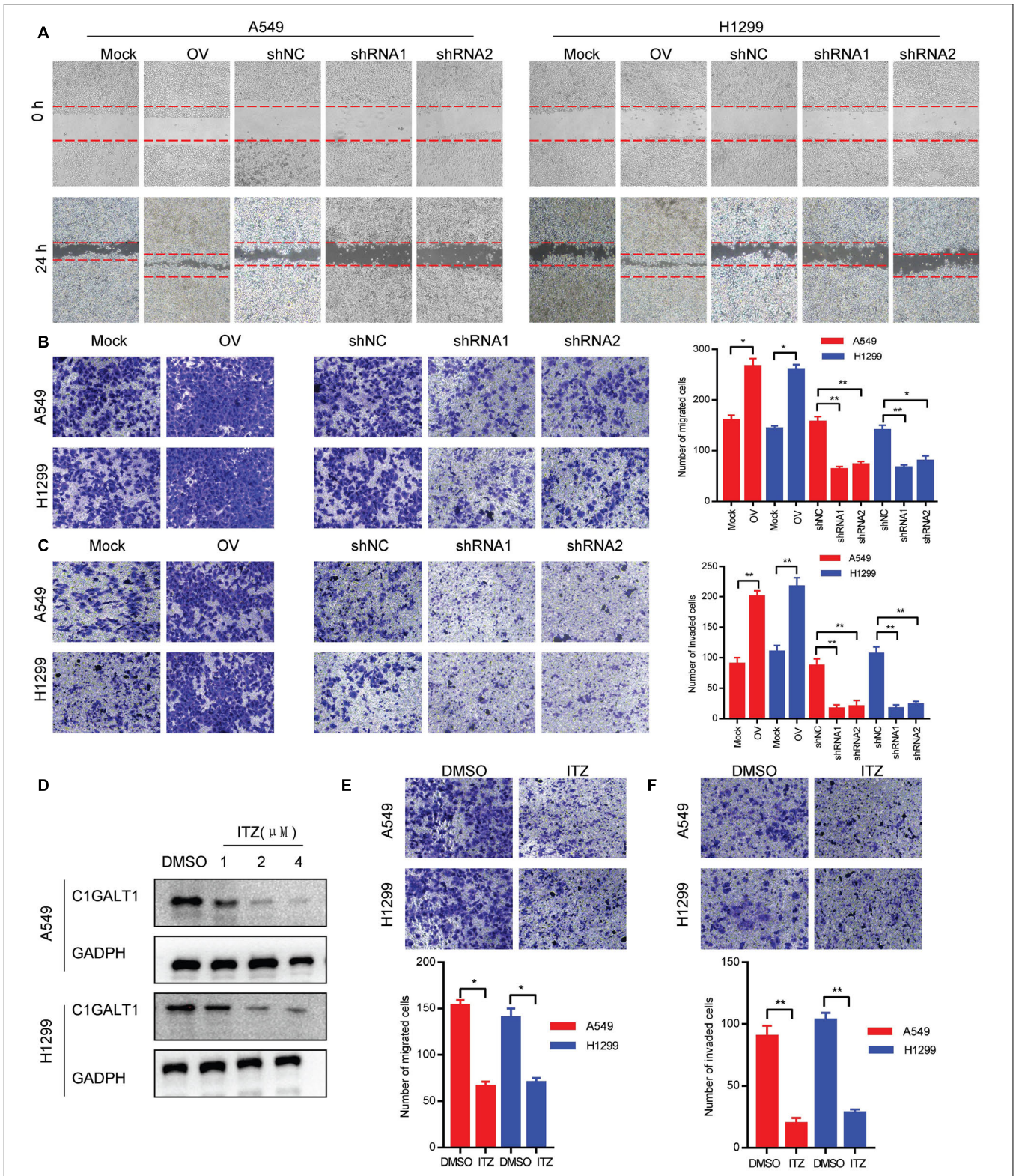

FIGURE 3 | C1GALT1 promotes LUAD cell migration and invasion *in vitro.* **(A,B)** The effect of C1GALT1 on cell migration was measured by wound-healing assay **(A)** and Transwell migration assay **(B)**. **(C)** The effect of C1GALT1 on cell invasion was determined by Matrigel invasion assay. **(D)** Analysis of C1GALT1 expression by Western blot after treatment with itraconazole (ITZ) for 48 h. **(E,F)** The effect of itraconazole (2 μM) on cell migration and invasion was assessed by Transwell migration assay **(E)** and Matrigel invasion assay **(F)**. Mock, cells transfected with empty plasmid; OV, cells transfected with C1GALT1 overexpression plasmid; shNC, cells infected with negative control shRNA; shRNAs, cells infected with C1GALT1 shRNA. Data are expressed as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.001$.

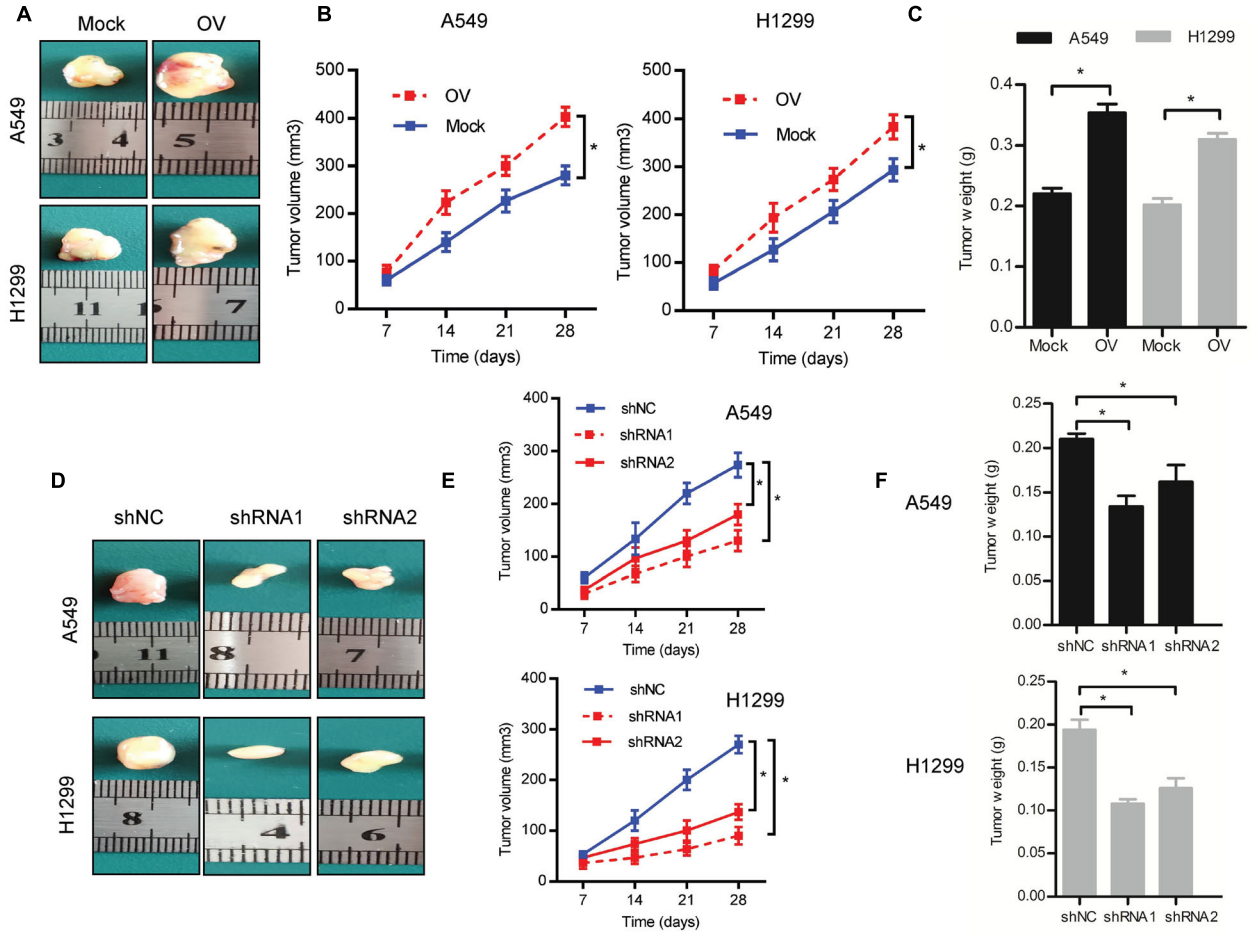

FIGURE 4 | C1GALT1 promotes LUAD tumor growth *in vivo*. **(A)** Representative images of excised tumors from nude mice injected with C1GALT1-overexpressing cells. **(B,C)** Analysis of tumor volume **(B)** and weight **(C)** after C1GALT1 overexpression. **(D)** Representative images of excised tumors from nude mice injected with C1GALT1-knockdown cells. **(E,F)** Analysis of tumor volume **(E)** and weight **(F)** after C1GALT1 knockdown. $n = 3$ mice per group. Mock, cells transfected with empty plasmid; OV, cells transfected with C1GALT1 overexpression plasmid; shNC, cells infected with negative control shRNA; shRNAs, cells infected with C1GALT1 shRNA. Data are expressed as mean ± SD. $^*p < 0.05$.

of C1GALT1 was verified by Western blot (**Figure 6D**). Subsequent functional assays showed that the inhibitory effects of miR-181d-5p mimics on cell proliferation, invasion, and migration were attenuated by restoration of C1GALT1 (**Figures 6E–G**). These results provided further evidence that C1GALT1 was directly regulated by miR-181d-5p in LUAD.

C1GALT1 Positively Regulates the Expression of RAC1 in LUAD

To understand how C1GALT1 influenced LUAD progression, the genes co-expressed with C1GALT1 in LUAD tissues were analyzed using the TCGA database. The top 50 genes positively and negatively correlated with C1GALT1 were shown in the heat maps (**Figure 7A** and **Supplementary Figure 6**). Among these candidates, RAC1 exhibited the highest correlation coefficient (**Figure 7B**). RAC1 is a member of Rho family small GTPases (Nguyen et al., 2018). GSEA analysis indicated that Rho GTPases signaling was closely associated with C1GALT1 expression (**Figure 7C**). Meanwhile, RAC1 is a driver of tumor growth and metastasis (De et al., 2019). Therefore, we speculated that C1GALT1 might facilitate LUAD cell proliferation, invasion, and migration by regulating RAC1. To test this hypothesis, we first examined the expression of RAC1 in LUAD through publicly available TCGA data. We found that RAC1 expression in LUAD tissues was significantly higher than that in normal tissues (**Figure 7D**). We then evaluated the prognostic value of RAC1 in LUAD. We found that high RAC1 expression was associated with poor overall survival (**Figure 7E**). IHC staining in our samples also confirmed that RAC1 was upregulated in LUAD (**Figure 7F** and **Table 2**), and correlated with poor clinical outcome (**Figure 7G**). Additionally, a significant positive correlation was observed between C1GALT1 and RAC1 expression in clinical samples (**Figure 7H**). We subsequently investigated whether C1GALT1 could affect RAC1 expression and activation in LUAD cells. We discovered that RAC1 expression and activity in A549

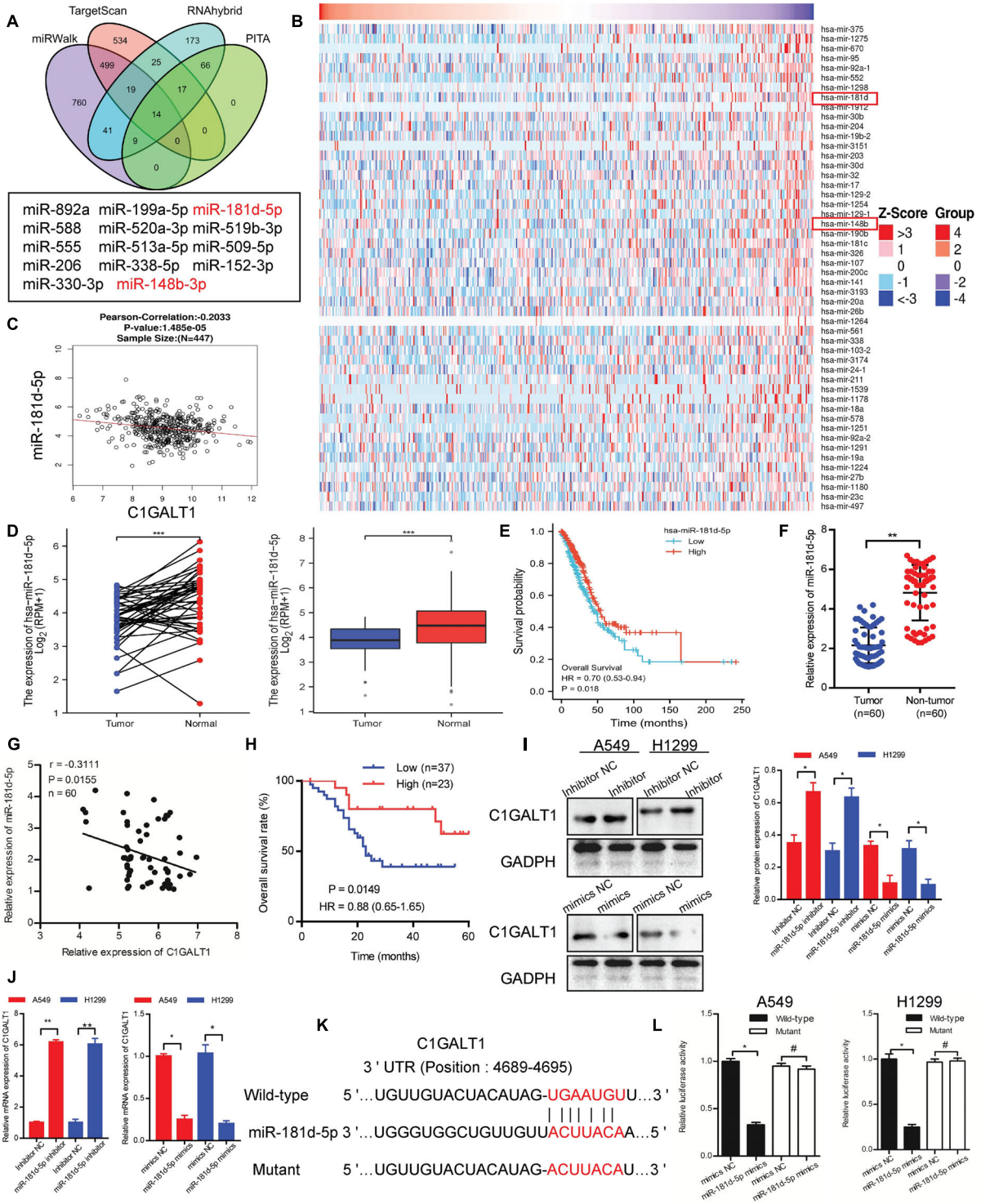

FIGURE 5 | C1GALT1 is directly downregulated by miR-181d-5p in LUAD. **(A)** Venn diagrams showing the number of potential miRNAs targeting the 3' UTR of C1GALT1. **(B)** Heat maps showing the top 50 miRNAs negatively correlated with C1GALT1 in LUAD. **(C)** Correlation between miR-181d-5p and C1GALT1 in TCGA-LUAD samples. **(D)** miR-181d-5p expression in paired (left) or unpaired (right) LUAD tissues and normal lung tissues from the TCGA database. **(E)** Survival analysis of miR-181d-5p in LUAD from the TCGA database. **(F)** Analysis of miR-181d-5p expression by qPCR in clinical LUAD samples. **(G)** Pearson correlation analysis of miR-181d-5p with C1GALT1 expression in clinical LUAD samples. **(H)** Overall survival analysis of LUAD patients based on miR-181d-5p expression in clinical samples. **(I,J)** Analysis of C1GALT1 expression in LUAD cell lines after transfection with miR-181d-5p mimics or inhibitor by Western blot **(I)** and qPCR **(J)**. **(K)** The potential binding site of miR-181d-5p in the 3'UTR of C1GALT1. **(L)** Relative luciferase activity detection. Data are expressed as mean ± SD.$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p > 0.05$.

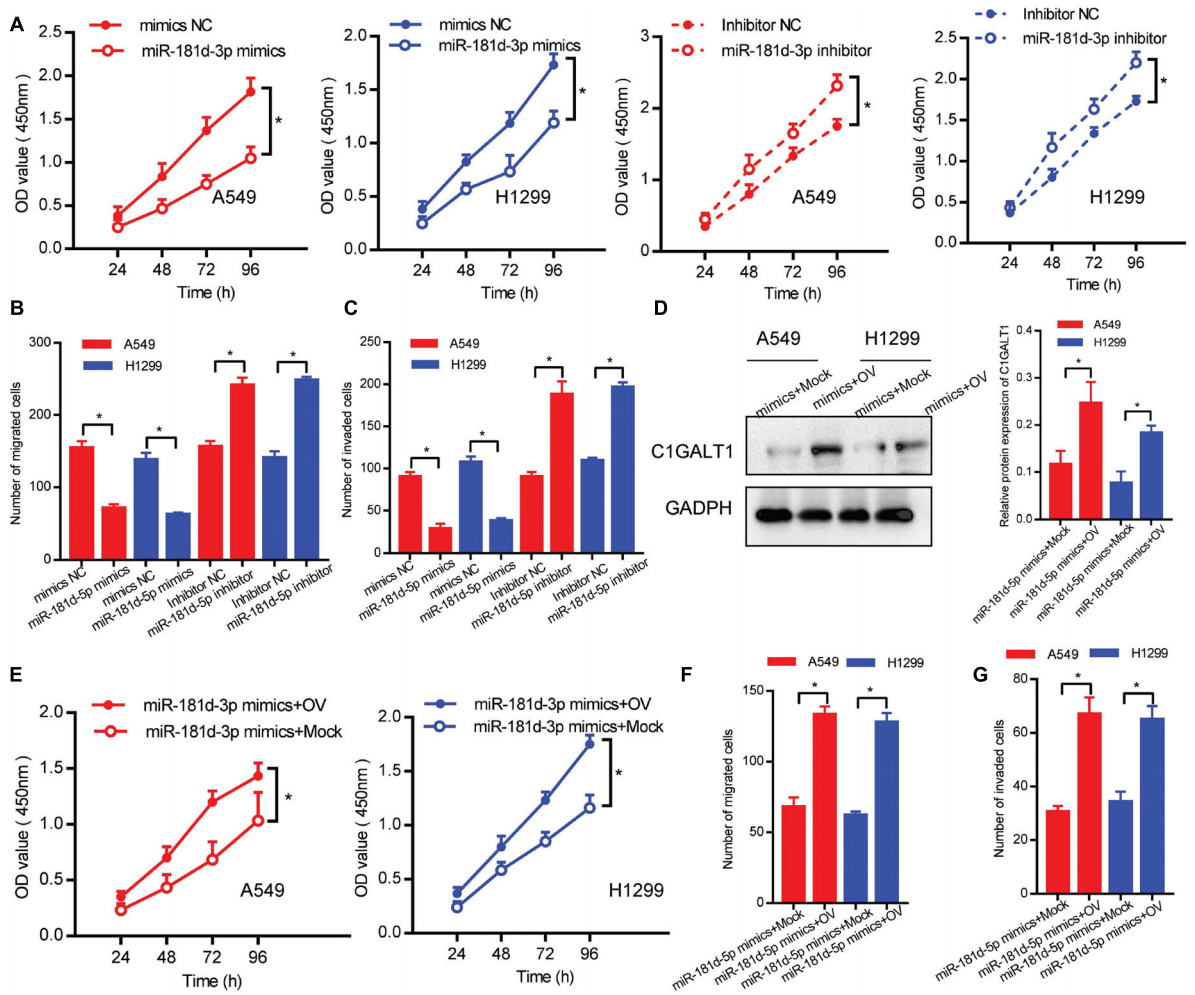

FIGURE 6 | miR-181d-5p functions as a negative regulator of C1GALT1 in LUAD. **(A–C)** The effect of miR-181d-5p mimics or inhibitor on cell proliferation, migration, and invasion was determined by CCK-8 assay **(A)**, Transwell migration assay **(B)**, and Matrigel invasion assay **(C)**. **(D)** Western blot analysis of C1GALT1 expression in cells co-transfected with miR-181d-5p mimics and C1GALT1 overexpression plasmid. **(E–G)** The inhibitory effect of miR-181d-5p mimics on cell proliferation **(E)**, migration **(F)**, and invasion **(G)** were antagonized by overexpression of C1GALT1. Mock, cells transfected with empty plasmid; OV, cells transfected with C1GALT1 overexpression plasmid. Data are expressed as mean ± SD. $^*p < 0.05$.

and H1299 cells was augmented by C1GALT1 overexpression and attenuated by C1GALT1 knockdown (**Figure 7I** and **Supplementary Figure 7**). Moreover, a gene regulatory network was generated by GeneMANIA to determine the interactive relationship between C1GALT1 and RAC1 (**Figure 7J**). Taken together, these results suggested that RAC1 was a downstream effector of C1GALT1 in LUAD.

RAC1 Silencing Reverses C1GALT1-Induced LUAD Growth and Metastasis

To confirm the important role of RAC1 in C1GALT1-mediated LUAD progression, we silenced RAC1 with specific siRNA in C1GALT1-overexpressing cells. Silencing efficiency was determined by qPCR and Western blot (**Figures 8A,B**). Then CCK-8, Transwell migration, and Matrigel invasion assays were used to examine the malignant phenotypes of A549 and H1299 cells. We found that the increased cell proliferation, migration, and invasion induced by C1GALT1 overexpression were weakened by RAC1 silencing (**Figures 8C–E**). Moreover, the promotion of tumor growth by C1GALT1 overexpression was mitigated by silencing of RAC1 (**Figures 8F–H**). Our data indicated that RAC1 was a major contributor to the function of C1GALT1 in LUAD growth and metastasis.

DISCUSSION

Accumulating studies have demonstrated that tumorigenesis and development are closely related to the abnormal expression of GTs (Meech et al., 2019; Akella et al., 2020; Bastian et al., 2021). C1GALT1 is one of the important members of the GT family. Although C1GALT1 is associated with the occurrence,

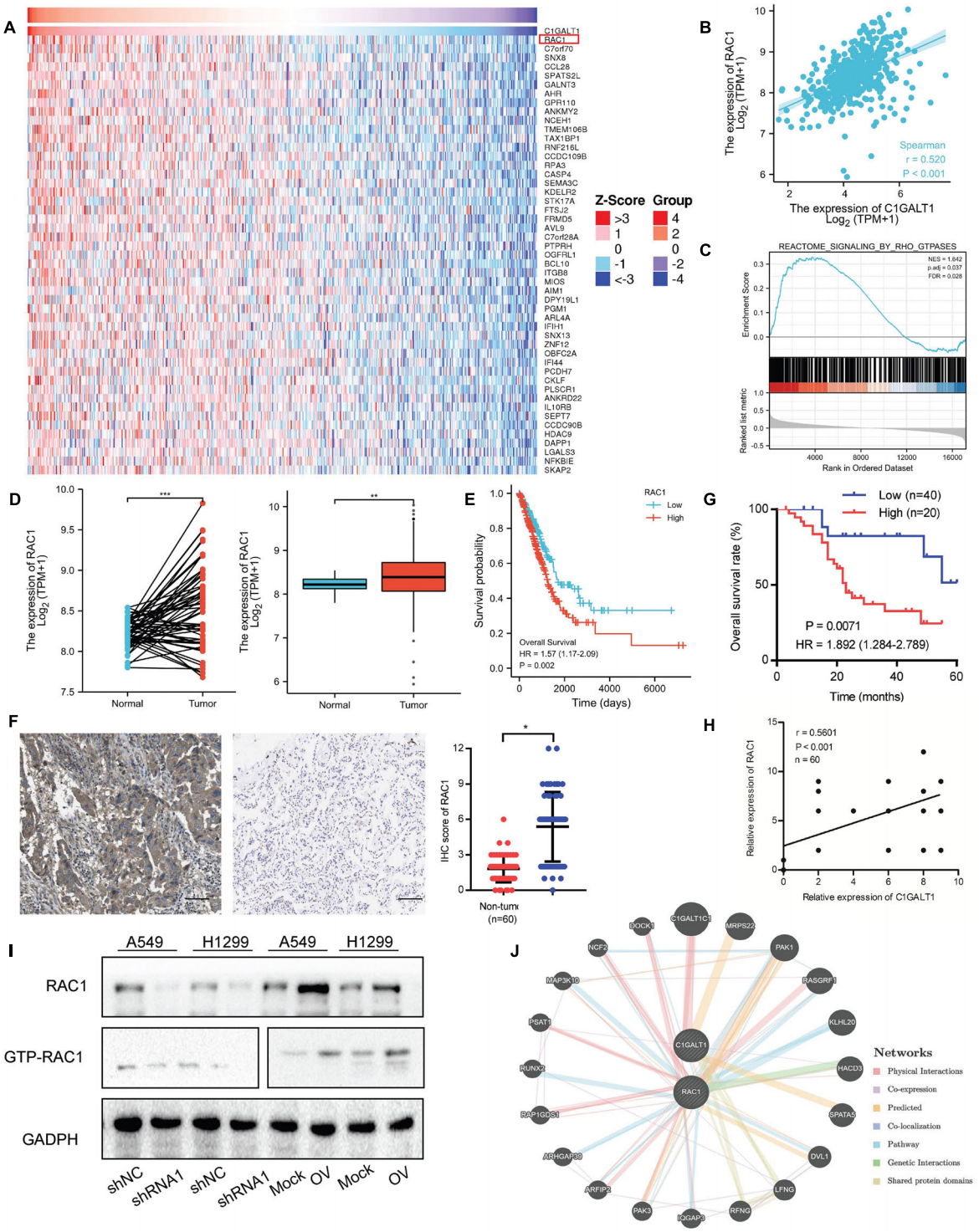

FIGURE 7 | RAC1 expression is positively regulated by C1GALT1 in LUAD. **(A)** Heat maps showing the top 50 genes positively correlated with C1GALT1 in TCGA-LUAD samples. **(B)** Correlation between RAC1 and C1GALT1 in TCGA-LUAD samples. **(C)** GSEA analysis of C1GALT1-associated genes in TCGA-LUAD samples. **(D)** RAC1 expression in paired (left) or unpaired (right) LUAD tissues and normal lung tissues from the TCGA database. **(E)** Survival analysis of RAC1 in LUAD from the TCGA database. **(F)** Analysis of RAC1 expression by IHC in clinical LUAD samples. Scale bar: 100 μm. **(G)** Pearson correlation analysis of RAC1 with C1GALT1 expression in clinical LUAD samples. **(H)** Overall survival analysis of LUAD patients based on RAC1 expression in clinical samples. **(I)** The effect of C1GALT1 on RAC1 expression in LUAD cells was detected by Western blot. **(J)** The network between RAC1 and C1GALT1 was predicted by the online GeneMANIA tool. Mock, cells transfected with empty plasmid; OV, cells transfected with C1GALT1 overexpression plasmid; shNC, cells infected with negative control shRNA; shRNA1, cells infected with C1GALT1 shRNA1. Data are expressed as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

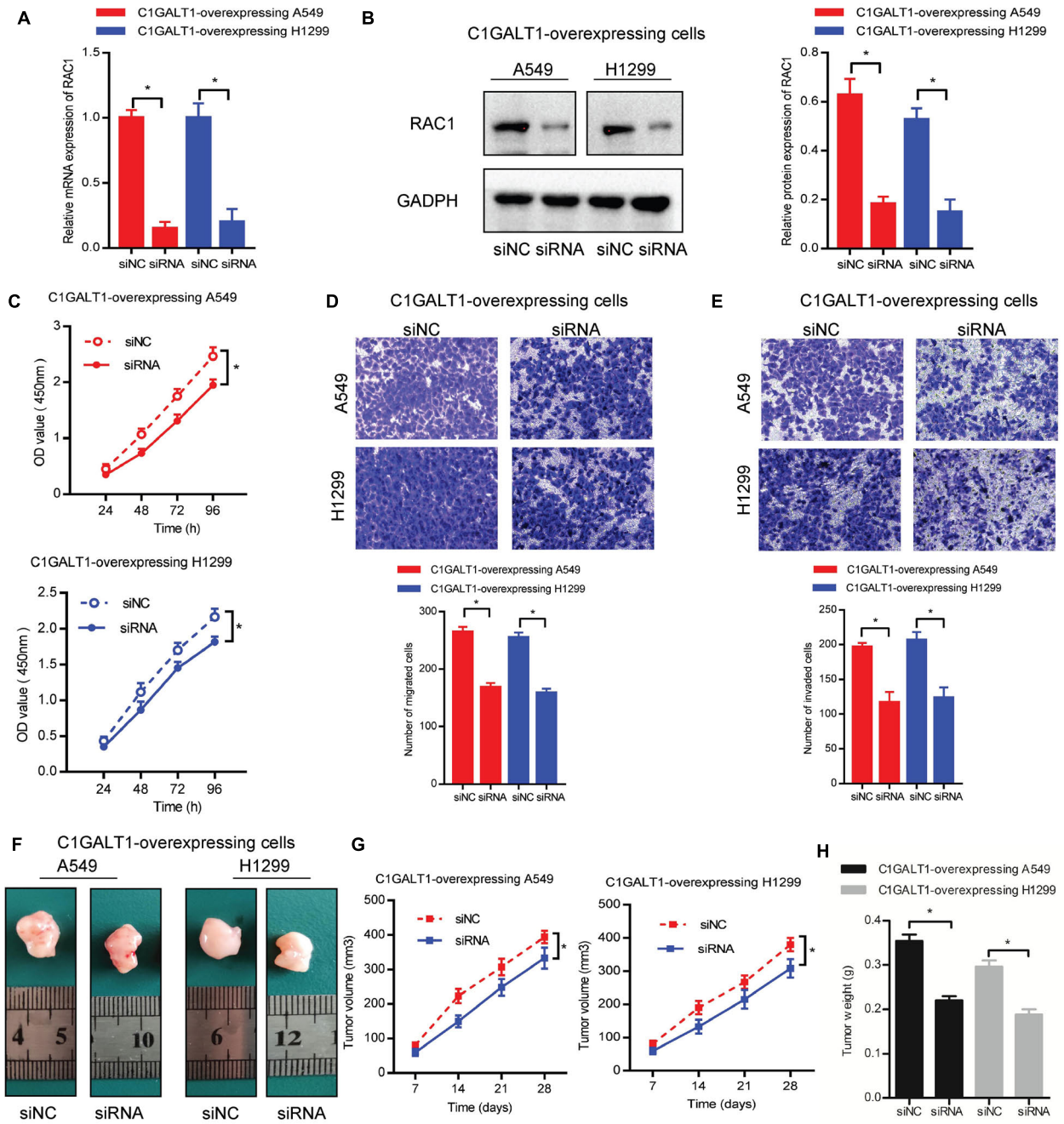

FIGURE 8 | RAC1 serves as a downstream effector of C1GALT1 in LUAD. **(A,B)** Analysis of RAC1 expression in C1GALT1-overexpressing cells after transfection with RAC1 siRNA by qPCR **(A)** and Western blot **(B)**. **(C–E)** The proliferation, migration, and invasion of cells co-transfected with C1GALT1 overexpression plasmid and RAC1 siRNA were determined by CCK-8 assay **(C)**, Transwell migration assay **(D)**, and Matrigel invasion assay **(E)**. **(F)** Representative images of excised tumors from nude mice ($n = 3$ per group). **(G,H)** Tumor volume **(G)** and tumor weight **(H)** were measured. siNC, cells transfected with non-silencing siRNA; siRNA, cells transfected with RAC1 siRNA. Data are expressed as mean ± SD. $^*p < 0.05$.

metastasis, and prognosis of various malignancies, the clinical significance, biological function, and regulatory mechanism of C1GALT1 in lung cancer are poorly understood. This study reported for the first time that C1GALT1 was upregulated in LUAD tissues and cell lines. Moreover, C1GALT1 expression was negatively correlated with overall survival and disease-specific survival in patients with LUAD. Our study further confirmed that C1GALT1 could serve as a useful diagnostic and prognostic indicator for LUAD patients. C1GALT1, negatively regulated by miR-181d-5p, was able to promote LUAD progression via

upregulating RAC1. Thus, C1GALT1 may play an important role in LUAD progression.

CCK-8, colony formation, wound-healing, Transwell migration, and Matrigel invasion assays were used in experiments involving C1GALT1 overexpression or knockdown. The results showed that the proliferation, migration, and invasion of LUAD cells were enhanced by C1GALT1 overexpression but attenuated by C1GALT1 knockdown. Flow cytometry analysis further revealed that loss of C1GALT1 inhibited the proliferation of LUAD cells via arresting the cell cycle at the G0/G1 phase and inducing apoptosis. In addition, overexpression or knockdown of C1GALT1 could facilitate or suppress tumor growth *in vivo*. It has been reported that C1GALT1 is essential for processes such as cell proliferation, migration, and invasion in a variety of malignancies (Lee et al., 2020; Kuo et al., 2021). Our findings are consistent with previous reports, suggesting a pro-oncogenic role for C1GALT1 in LUAD.

As we all know, miRNAs are a class of small non-coding RNAs that modulate gene expression at the post-transcriptional level (Shin et al., 2020). miRNAs are involved in numerous cellular processes including cell growth, development, differentiation, and apoptosis (Saliminejad et al., 2019; Condrat et al., 2020). Dysregulation of miRNAs in cancer cells has been widely reported (Rupaimoole and Slack, 2017). We, therefore, sought to explore how miRNA affects C1GALT1 expression in LUAD. Using bioinformatic analysis and luciferase activity assays, we identified miR-181d-5p as a negative regulator of C1GALT1. Our gain- and loss-of-function experiments confirmed that miR-181d-5p directly regulated C1GALT1 expression via interaction with its 3'UTR. Meanwhile, a significant negative correlation between miR-181d-5p and C1GALT1 was observed in LUAD tissues. As expected, the inhibitory effects of miR-181d-5p on LUAD cell proliferation, migration, and invasion were counteracted by restoration of C1GALT1. There is some evidence that miR-181d-5p participates in tumorigenesis and malignant transformation via different signaling pathways (Chen et al., 2018; Wang et al., 2020). Of note, miR-181d-5p has been proven to have a tumor-suppressive role in NSCLC (Gao et al., 2019). In this study, we demonstrated that miR-181d-5p acted as a tumor suppressor in LUAD by targeting C1GALT1. To the best of our knowledge, miR-181d-5p was first uncovered to be involved in the regulation of C1GALT1 expression in LUAD.

The endogenous small GTPase, Rac1, is implicated in many cellular activities, such as phagocytosis, adhesion, migration, motility, and proliferation (Zou et al., 2017). Recently, many studies have shown that RAC1 plays a critical role in multiple physiological and pathological processes, including cancer (De et al., 2019). Aberrant expression of RAC1 is recognized as a hallmark of cancer and contributes to the tumorigenic and metastatic phenotypes of cancer cells (Kazanietz and Caloca, 2017). A growing body of evidence suggests that RAC1 can be regulated by various factors. For example, lncRNA NR2F2-AS1 overexpression mediated the upregulation of Rac1 in clear cell renal cell carcinoma (Chen et al., 2020). Knockdown of RhoGDI2 reduced the mRNA expression of Rac1 in gastric cancer (Zeng et al., 2020). DDX3 depletion suppressed the protein expression of RAC1 in medulloblastoma (Chen et al., 2015). In the present study, we found that RAC1 expression was augmented by C1GALT1 overexpression and attenuated by C1GALT1 knockdown in LUAD cells. Rescue experiments proved that RAC1 silencing partially abolished C1GALT1-induced LUAD growth and metastasis. Hence, RAC1 was a downstream effector of C1GALT1 in LUAD. Previous studies have mainly focused on the relationship between C1GALT1 and glycoproteins (Lee et al., 2020; Kuo et al., 2021). Besides its direct function of producing aberrant glycoproteins, our current study provided new insights into the regulatory network of C1GALT1.

Our study provides a theoretical basis for C1GALT1 to be designed as a drug target and offers a novel direction for the treatment of LUAD. Indeed, there are several limitations of the current work. First, the physiological meaning and clinical application of C1GALT1 require further investigation. Second, the detailed mechanisms underlying the interaction between C1GALT1 and RAC1 need to be further investigated. Moreover, further studies are required to explore the downstream signaling pathways mediating the oncogenic effects of C1GALT1.

In summary, our study illustrated that C1GALT1 was overexpressed in LUAD tissues and facilitated the growth and metastasis of LUAD cells by upregulating RAC1. Besides, C1GALT1 expression was negatively regulated by miR-181d-5p, and decreased miR-181d-5p further contributed to C1GALT1 upregulation in LUAD. Our study highlights the importance of the miR-181d-5p/C1GALT1/RAC1 regulatory axis during LUAD progression. Accordingly, C1GALT1 may serve as a valuable prognostic biomarker for LUAD. Furthermore, targeting C1GALT1 may be an attractive therapeutic method against LUAD.

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 studies involving human participants were reviewed and approved by the Ethics Committee of the Hubei University of Medicine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of the Hubei University of Medicine.

AUTHOR CONTRIBUTIONS

XDo and YL designed the experiments. XDe, JS, and ST conducted the functional experiments. SC, RH, and ZL analyzed the data. CC wrote the manuscript. LS supervised the research. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (Nos. 81902494 and 81802997), the Hubei Provincial Department of Science and Technology Innovation Group program (No. 2019CFA034), the Free Exploration Foundation of the Hubei University of Medicine (No. FDFR201802), the Initial Project for Post-Graduates of the Hubei University of Medicine (No. 2016QDJZR10), and the National Undergraduate Training Program for Innovation and Entrepreneurship (No. 202013249002).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.707970/full#supplementary-material

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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.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 06 August 2021
doi: 10.3389/fcell.2021.663130

Androgen Activity Is Associated With PD-L1 Downregulation in Thyroid Cancer

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Thyroid cancer is the most prevalent endocrine malignancy in the United States with greater than 53,000 new cases in 2020. There is a significant gender disparity in disease incidence as well, with women developing thyroid cancer three times more often than men; however, the underlying cause of this disparity is poorly understood. Using RNA-sequencing, we profiled the immune landscape of papillary thyroid cancer (PTC) and identified a significant inverse correlation between androgen receptor (AR) levels and the immune checkpoint molecule PD-L1. The expression of PD-L1 was then measured in an androgen responsive-thyroid cancer cell line. Dihydrotestosterone (DHT) treatment resulted in significant reduction in surface PD-L1 expression in a time and dose-dependent manner. To determine if androgen-mediated PD-L1 downregulation was AR-dependent, we treated cells with flutamide, a selective AR antagonist, and prior to DHT treatment to pharmacologically inhibit AR-induced signaling. This resulted in $a > 90\%$ restoration of cell surface PD-L1 expression, suggesting a potential role for AR activity in PD-L1 regulation. Investigation into the AR binding sites showed AR activation impacts NF-kB signaling by increasing IkBα and by possibly preventing NF-kB translocation into the nucleus, reducing PD-L1 promoter activation. This study provides evidence of sex-hormone mediated regulation of immune checkpoint molecules *in vitro* with potential ramification for immunotherapies.

Keywords: PD-L1 pathway, androgen receptor, immune surveillance, gender disparity, thyroid cancer

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Edited by:
Marzia Di Donato, University of Campania Luigi Vanvitelli, Italy

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

Received: *02 February 2021*
Accepted: *23 June 2021*
Published: *06 August 2021*

Citation:
O'Connell TJ, Dadafarin S, Jones M, Rodríguez T, Gupta A, Shin E, Moscatello A, Iacob C, Islam H, Tiwari RK and Geliebter J (2021) Androgen Activity Is Associated With PD-L1 Downregulation in Thyroid Cancer. Front. Cell Dev. Biol. 9:663130. doi: 10.3389/fcell.2021.663130

INTRODUCTION

Thyroid cancer is the most common endocrine cancer and accounts for 3.8% of all cancers (Nguyen et al., 2015). According to the Surveillance, Epidemiology, and End Results Program (SEER), the incidence of thyroid cancer has increased by threefold over the past three decades (Kilfoy et al., 2009) though stabilized during this past decade (Morris et al., 2016). Of the 53,000 new thyroid cancer cases that occurred in 2020, approximately 40,000 occurred in women and 13,000 occurred

in men (Siegel et al., 2020). Age-adjusted SEER incidence rates show a three to fourfold increase in incidence of thyroid cancer in women aged 20–49 as compared to men aged 20–49. A study of 566 papillary thyroid cancer (PTC) patients similarly concluded that women have a higher incidence of PTC than males, and also found that men tended to present with larger carcinomas (Yorke et al., 2016). Previously, it was shown that female gender is associated with a high prevalence of RET/PTC1 and RET/PTC3 fusions in PTC (Su et al., 2016). Changes in estrogen receptor (ER) and androgen receptor (AR) have been observed in PTC, with increases in ERα, decreases in ERβ, and decreases in AR being associated with a more aggressive phenotype (Magri et al., 2012). Rajoria et al. (2010) showed that a metastatic thyroid cancer phenotype is regulated by estrogen and functional ER which enhance mitogenic, migratory, and invasive properties of thyroid cancer cells. ER and AR expression changes may be acting as a cause or a result of the onset of PTC (Magri et al., 2012). Additionally, it was previously suggested that underlying inflammatory processes may predispose healthy females toward developing thyroid cancer (Manole et al., 2001; Tafani et al., 2014).

The gender disparity in thyroid cancer incidence is a unique phenomenon, whereas nearly all other cancers without obvious anatomical bias are neutral or have a bias toward higher incidence in men. Additionally, males and females differ in their immunological responses and show unique innate and adaptive immune responses (Klein and Flanagan, 2016). It has been shown that females in general have a more active humoral and cellular immune response than men (Ansar Ahmed et al., 1985). Women are more prone to autoimmune and inflammatory thyroid diseases such as Graves' Disease or Hashimoto's Thyroiditis (Ngo et al., 2014). Therefore, underlying inflammatory processes in healthy females may predispose them toward developing thyroid cancer (Manole et al., 2001; Tafani et al., 2014). Despite the well-established characterization of sex disparity in thyroid cancer incidence and that inflammation plays a key role in the gender disparity of some cancers (Naugler et al., 2007), the underlying molecular factors that mediate this difference are poorly understood (Rahbari et al., 2010).

Recently, evidence has emerged showing sex differences in the expression of immune checkpoint molecules on tumors (Özdemir and Dotto, 2019; Wilms et al., 2020) and the response to checkpoint inhibition (Nosrati et al., 2017). A comprehensive analysis of the immune landscape of cancer across The Cancer Genome Atlas (TCGA) dataset specifically found the Programmed Death Receptor Ligand 1 (PD-L1) was more highly expressed among women than in men in thyroid carcinoma and three other cancer types (head and neck squamous cell, renal cell carcinoma, and lung adenocarcinoma) (Thorsson et al., 2019). Furthermore, PD-L1 has consistently been shown to be highly expressed in the most aggressive forms of thyroid cancer (Ahn et al., 2017; Chintakuntlawar et al., 2017).

Given the role gender may play in the incidence of thyroid cancer, we hypothesized that sex hormones may modulate the expression of immune checkpoint molecules, and explaining the sex disparity in their expression. In this study, we present data that suggest that AR activation attenuates PD-L1 expression in a thyroid cancer cell line, potentially by inhibiting NF-kB signaling by increasing the IkBα inhibitory subunit. Our study associates the role of androgens in the gender-specific expression patterns of PD-L1 and suggests the possibility of targeting sex hormone pathways in tandem with immunotherapeutics.

MATERIALS AND METHODS

Patient Specimens

This study was approved by the institutional review boards of New York Eye and Ear Infirmary and New York Medical College (NYMC). Written informed consent was obtained from each patient. All TCGA data used in this study were downloaded from publicly available sources. Specimens were obtained from 44 patients undergoing thyroidectomy and fresh frozen thyroid tissue were collected between 2009 and 2013. All tumors had corresponding matched normal adjacent tissue and diagnosis of PTC was validated by pathological examination.

RNA Sequencing

Total RNA was extracted from frozen specimens using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, United States) and RNA cleanup was performed using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA quality, concentration, and fragment size distribution were assessed on a 2100 Bioanalyer (Agilent Inc., Palo Alto, CA, United States). Ribosomal RNA was depleted using the Illumina Ribo-Zero rRNA removal kit. Samples were aligned to the human genome using STAR software version 2.4 (Dobin et al., 2013) and quantified using RSEM version 1.2.14 (Li and Dewey, 2011). KEGG Pathway and gene ontology (GO) enrichment analyses were performed on Advaita's iPathwayGuide[1] Platform using DE genes [abs(log2FC) > 1.5 and q-value < 0.05]. Statistical tests of pathway and GO term enrichment were adjusted using FDR correction.

TCGA Data

Level three RNA-Sequencing data from TCGA project were downloaded from the UCSC Xena Browser (Goldman et al., 2018).

Cell Culture

The 8505C cell line was purchased from MilliporeSigma (ECACC Cat# 94090184, RRID:CVCL_1054) and the K1 cell line was graciously provided as a gift from the Schweppe lab. 84e7, K1-lentiAR, and 8505C-lentiAR cell lines were maintained in phenol-red free RPMI 1640 supplemented with 10 mM L-Glutamine, 100 U/mL penicillin/streptomycin (Corning, 30002CI, Manassas, VA, United States) and 5% United States origin, heat-inactivated fetal bovine serum (FBS) (Genesee Scientific, 25–514 H, El Cajon, CA, United States). 84e7 cells also were supplemented with 50 μg/mL G418 sulfate solution (Corning, 30–234-CR, Manassas, VA). Thyroid cancer

[1]http://www.advaitabio.com/ipathwayguide

cell line gene expression data was obtained from the Cancer Cell Line Encyclopedia (CCLE)[2].

Lentiviral Transduction

HEK293T cells were purchased from ATCC (ATCC Cat# CRL-3216, RRID:CVCL_0063) and cultured in DMEM supplemented with 10 mM L-Glutamine, 100 U/mL penicillin/streptomycin and 10% heat inactivated FBS. The pLENTI6.3/AR-GC-E2325 plasmid (kindly shared by the Kalland lab, Addgene plasmid # 85128[3]; RRID:Addgene_85128) along with 0.4 ug of psPAX2 packaging and 0.15 ug of VSV-G envelope plasmids were transfected into HEK293T cells using the TransIT-LT1 reagent (Mirus, MIR 2304, Madison, WI, United States). Virus-containing supernatants were collected at 48 h post-transfection and filtered using a 0.45 um PES filter. 8505C and K1 cells were treated with 40 ng/mL polybrene (MilliporeSigma #TR-1003-G, St. Louis, MO, United States) and filtered virus for 24 h followed by replacement with 10 ug/mL Blasticidin containing media. After 72 h of Blasticidin selection, cells were then sub-cultured and frozen for future experiments.

Western Blotting

Cultured cells were lysed in RIPA Buffer (50 mM *Tris–Hcl* pH 7.4, 150 mM NaCl, 0.25% sodium deoxcholate, and 1.0% NP-40) supplemented with HALT Protease Inhibitor Cocktail (Thermo Fisher Scientific/ Pierce, P/N 78430). 15 ug of total protein from each cell extract were added to 5 uL of sample buffer (10% β-mercaptoethanol in 4× Laemmli buffer) followed by SDS-PAGE electrophoresis performed under reducing conditions with a 4% stacking gel and 10% separating gel. Proteins were transferred from the gel to an Immobilon-P PVDF Membrane (MilliporeSigma, P/N IPVH00010). Membranes were incubated with primary antibodies diluted in 5% milk (total protein) or 1% milk (phosphorylated protein) in 1× TBST overnight at 4°C. Secondary goat anti-rabbit Horseradish Peroxidase (HRP) conjugated secondary antibody (Abcam Cat# ab6721, RRID:AB_955447) was diluted 1:5,000 in 5% milk with 1× TBST and incubated with membrane for 2 h at room temperature. Quantitative analysis of optical density was performed with ImageJ. Antibodies used for western blotting can be found in **Supplementary Table 1**.

Flow Cytometry

A 0.5–1 × 10^6 cultured cells were collected using cell scrapers and resuspended in 100 μL incubation buffer (0.25 g bovine serum albumin dissolved in 50 mL 1 × PBS) with 20 μL of FITC-conjugated mouse anti-human primary PD-L1 monoclonal antibody (BD Pharmingen, P/N 558065) for 1 h at room temperature. Cells were rinsed 2× in incubation buffer and finally resuspended in 0.45 mL ice cold 1× PBS and strained into polystyrene flow cytometry tubes. Flow Cytometry was conducted using the BD FACScan Flow Cytometer and each experiment was conducted in triplicate. Examples for gating parameters can be found in **Supplementary Figure 1**. Relative fluorescence intensity (RFI) was calculated by normalizing mean fluorescence intensity (MFI) measurements of treated samples to vehicle control:

$$RFI = \frac{MFI_{treatment}}{MFI_{control}} \quad (1)$$

Cleavage Under Targets and Tagmentation (CUT&Tag) and Sequencing

For CUT&Tag, 10^6 8505C-AR cells treated with either DHT or vehicle control for 48 h were used. CUT&Tag DNA were prepared according to Kaya-Okur et al. (2019). The AR primary Rabbit mAb (Cell Signaling #5153) and Guinea Pig anti-Rabbit secondary antibody (Novus Biologicals #NBP1-72763, Centennial, CO, United States) were used as well as the CUTANA^{TM} pAG-Tn5 (Epicypher #15-1017, Durham, NC, United States).

Samples were multiplexed and sequenced on a Nextseq 500 in paired-end mode (38/37) with a high-output, 75-cycle reagent kit. 3-prime read ends containing Nextera Mate Pair adapters were trimmed using cutadapt (Martin, 2011), filtering reads < 20 bp (-m 20) or with an average phred quality score < 20 (-q 20). Trimmed FASTQ files were aligned to the hg38 genome assembly using BWA mem (Li, 2013), marking duplicate reads (-M). Aligned SAM files were compressed to BAM format, sorted, merged, and indexed using samtools (Li et al., 2009). Duplicate reads were removed from BAM files using Picard Toolkit MarkDuplicates (Broad Institute, 2019).

To compare AR binding of genomic loci across biological conditions, we first used deeptools bamCoverage (Ramírez et al., 2014) to generate normalized BigWig files from de-duplicated BAMs. Parameters selected include counts-per-million normalization (–normalizeUsing CPM) with a bin size of 10 bp (-*bs* = 10). Next, we calculated the aggregate normalized accessibility signal at known murine adult *cis*-regulatory elements (CREs) using UCSC bigWigAverageOverBed; these elements are subclassified as promoters, proximal enhancers and distal enhancers, and assigned a unique accession by ENCODE (ENCODE Project Consortium Moore et al., 2020). We used a Z-scoring method to identify high-confidence AR peaks in promoters. Total signal across *cis*-regulatory elements was recorded for each replicate using bigWigAverageOverBed. Only elements ranked in the top 10 percent ($Z > 1.64$) by signal were retained. The intersect of each replicate's top elements constitutes our high-confidence element list.

Statistical Analysis

Data analysis was performed on Prism version 8.1.2 (GraphPad Software, San Diego, CA, United States) and R version 3.6.2. Unless otherwise indicated, all experimental data were expressed as the mean ± SD of experiments performed in triplicate. For analysis of paired NYMC tumor and normal tissue, a two-tailed, paired Student's *t*-test was performed. For comparison of tumor and normal TCGA data, a two-tailed Welch's *t*-test was used for statistical analysis. For all culture experiments, statistical significance of results was analyzed by two-tailed, and paired

[2]http://www.broadinstitute.org/ccle/home
[3]http://n2t.net/addgene:85128

Student *t*-tests. For immunohistochemistry (IHC), Fisher's exact test was performed.

RESULTS

Inflammation and AR Expression in Male and Female PTC

To assess the difference in inflammatory and immune responses between male and female thyroid cancer, we performed RNA-sequencing on 44 PTC with matched-paired normal thyroid tissue in the NYMC biobank (33 female and 11 male). A total of 1532 protein-coding genes and 756 non-coding RNAs underwent $\geq$ 1.5-fold change between tumor and matched normal tissue (q-value $\leq$ 0.05). Principal component analysis (PCA) on all specimens demonstrated distinct separation on the PC2 axis between PTC and normal adjacent tissue, indicating that 10% of transcriptomic variance was sufficient to distinguish tumor samples from normal tissue (**Supplementary Figure 2A**). KEGG pathway enrichment analysis of differentially expressed genes (DEGs) identified cell adhesion molecules and ECM-receptor interaction as well as pathways related to host immune function (Cytokine-cytokine receptor interactions and allograft rejection) (**Supplementary Figure 2B**). These findings are consistent with previous transcriptomic studies of PTC (Song et al., 2018).

Next, we examined GO terms enriched in DEGs from male and female PTC. A subset of GO biological processes related to adaptive immunity and inflammation were significantly enriched among female PTC but not male PTC (**Figure 1A**). Furthermore, genes that mediate T-cell activation such as CD40L, ICOS, and IL-2 were downregulated in female PTC (**Supplementary Figure 3A**).

Given the distinct gene expression patterns among immune-related genes between female and male thyroid tissue, we next examined the expression of AR and its relationship to these gene expression patterns. AR expression in both the NYMC RNA-seq dataset and TCGA Thyroid Carcinoma project's RNA-sequencing dataset showed significant ($p < 0.0001$) reduction in AR expression in tumors compared to normal tissue (**Figure 1B**). We performed a co-expression analysis between AR and a set of immune regulatory molecules, including CD28, CD80, CD86, CTLA-4, PD-1, PD-L2, TIM-3, TIM-3L, 4-1BB, 4-1BBL, OX40, and OX40L, in the NYMC dataset and found the expression of the immune checkpoint molecule *CD274* had a significant inverse correlation (Pearson rho = -0.60) with AR and this finding was replicated with TCGA data (**Figure 1C**). Furthermore, males have a higher rate (70%) of exhibiting an inverse relationship between AR and PD-L1 than females (35%) ($p < 0.05$, **Supplementary Figure 3B**).

Androgen Downregulates PD-L1 in an AR-Dependent Mechanism

Given the correlation between PD-L1 and AR expression in primary PTC tissue, we designed a series of cell culture experiments to test whether androgens regulate the expression of PD-L1 in thyroid cancer. The undifferentiated thyroid cancer cell line, 8505C, was used due to the high surface level expression of PD-L1 (Cantara et al., 2019) and higher AR expression relative to other thyroid cancer cell lines based on mRNA-sequencing and protein array data in the CCLE (**Supplementary Figure 4**). However, androgen treatment of the 8505C cell line failed to increase expression of the AR-responsive gene FKBP5 (**Supplementary Figure 6A**). We therefore used a transfected clone, termed 84e7 (Jones et al., 2021), that constitutively expresses human AR in a pcDNA3.1 vector under a CMV promoter. We initially measured PD-L1 levels by Western blot and detected an approximately 30% reduction ($p < 0.01$) in PD-L1 expression after 72 h of treatment with the potent androgen DHT but no change at 24 h (**Supplementary Figure 5**). Next, we quantified surface expression of PD-L1 using flow cytometry in 8505C and 84e7 cells treated with DHT or vehicle control at the 72 h timepoint. Cell surface PD-L1 levels decreased in 10 nM DHT-treated 84e7 by approximately 60% ($p < 0.005$) as compared to vehicle-treated 84e7 while no change was observed between vehicle- and DHT-treated 8505C (**Figure 2A**). Notably, the DHT-mediated downregulation of PD-L1 was replicated in another thyroid cancer cell line, K1, and stably expressing the pLENTI6.3/AR-GC-E2325 construct (**Supplementary Figure 6B**). A dose response curve that encompassed all human physiological ranges of total androgens was used, from 0.1 nM which is five times below the low end of the female physiological range, to 100 nM which is three times above the high end of the male physiological range (Salameh et al., 2010; **Figure 2B**). Interestingly, the greatest impact of DHT occurred in the male physiological range, with a 50% reduction in PD-L1 expression as compared to the female dose range. Next, we performed a time course of 10 nM DHT treatment in 84e7 cells and found that the 60% reduction in PD-L1 surface expression was achieved by 48 h of treatment (**Figure 2C**).

To confirm that the cell surface decrease in PD-L1 observed in DHT-treated 84e7 was specific to AR signaling, 8505C, and 84e7 were treated with varying concentrations of flutamide, a selective AR antagonist (Goldspiel and Kohler, 1990), prior to DHT treatment to pharmacologically inhibit AR-induced signaling. Flutamide was shown to inhibit the ability of DHT to decrease PD-L1 in 84e7, resulting in a dose-dependent and > 90% restoration of cell surface PD-L1 expression at 50 uM concentration (**Figure 2D**), but not in 8505C cells (**Supplementary Figure 6C**) indicating that AR activity is necessary for DHT-mediated PD-L1 downregulation.

CUT&Tag Identifies AR Binding to Regulators of PD-L1

To begin identifying potential mechanisms by which androgen signaling downregulates PD-L1 expression, we profiled AR binding genome wide using cleavage under targeted nuclease and tagmentation (CUT&Tag) (Kaya-Okur et al., 2019). Since the 84e7 cell line required growth media supplemented with the G418 antibiotic that could confound results of genome-wide sequencing experiments, we generated an 8505C cell line stably expressing AR without requiring constant antibiotic selection via

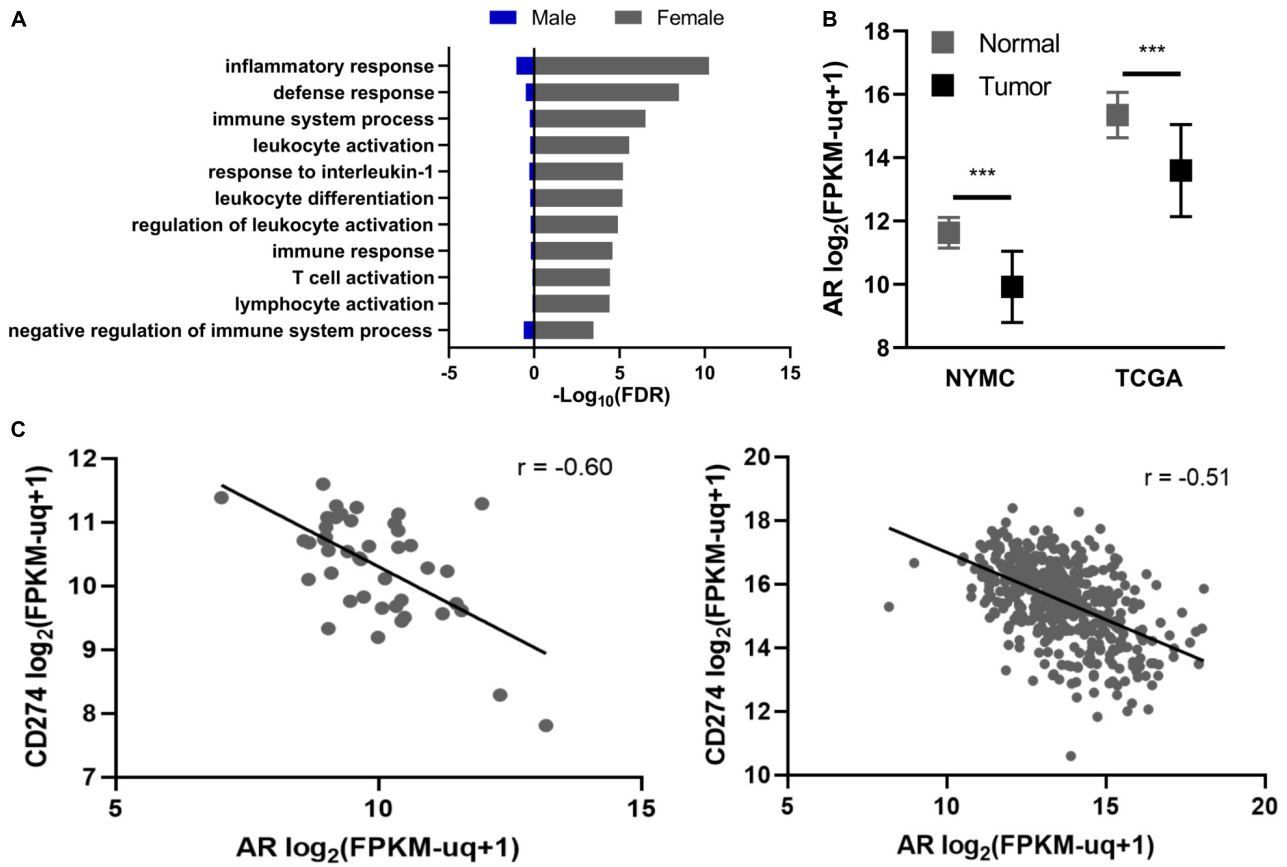

FIGURE 1 | Relationship between androgen receptor (AR), gender, and *CD274* expression. **(A)** Enrichment of GO biological processes incorporating inflammation and immune responses. **(B)** AR expression in primary PTC tissue (Tumor) vs. normal adjacent thyroid tissue (Normal) in the New York Medical College (NYMC) and The Cancer Genome Atlas (TCGA) datasets. AR expression was compared using Student's and Welch's paired *t*-tests for the NYMC and TCGA datasets, respectively. **(C)** Regression analysis of *CD274* and AR mRNA levels in NYMC (left) and TCGA (right) tumors. r represents Pearson's correlation coefficient. FPKM-UQ, Fragments per kilobase million-upper quartile normalized. ***$p < 0.0005$.

lentiviral transduction (Ryu et al., 2017). CUT&Tag sequencing of this cell line (named 8505C-lentiAR) after treatment with 10 nM DHT for 48 h revealed direct AR binding to the PD-L1 gene body (**Figure 3A**), as well as potential regulatory elements downstream of the PD-L1 open reading frame.

PD-L1 expression is regulated by various signaling pathways including NFκB, MAPK, PI3K, mTOR, and JAK/STAT (Ritprajak and Azuma, 2015). We therefore surveyed our AR binding sites for promoter binding to molecules involved in these pathways and identified an AR binding signal at the NFKBIA promoter (**Figure 3B**). The NFKBIA gene encodes the IkBα protein that sequesters NFkB in the cytoplasm, reducing NF-kB translocation to the nucleus. We confirmed that DHT increased both total and cytoplasmic IkBα proteins levels via western blot (**Figure 3C** and **Supplementary Figure 7**).

We next aimed to determine if androgen-mediated IkBα upregulation contributes to the downregulation of PD-L1. 84e7 cells were treated with a selective and potent inhibitor of IKK, BMS-345541. Inhibition of IKK maintains IkBα in the unphosphorylated state, resulting in sequestration of NF-κB in the cytoplasm (Burke et al., 2003). PD-L1 levels in 84e7 treated with BMS-345541 decreased significantly, but did not decrease PD-L1 surface expression to the same extent as 1 nM or 10 nM DHT (**Figure 3D**). When combined with DHT, there was no additive or synergistic effect on PD-L1 levels (**Figure 3D**). Thus, inhibition of IKK does indeed impact PD-L1 expression but does not account entirely for the decrease in PD-L1 observed with DHT, supporting a model where PD-L1 expression is modulated via AR activity at multiple regulatory elements.

DISCUSSION

Despite the fact that thyroid cancer has a relatively quiet mutagenic landscape (Lawrence et al., 2013), there is increasing evidence that immune checkpoint molecules, specifically PD-L1, and play a role in thyroid cancer pathogenesis (Chowdhury et al., 2016; Chintakuntlawar et al., 2017). Inflamed thyroid follicular cells express PD-L1 (Álvarez-Sierra et al., 2019) and immune checkpoint induced thyrotoxicity is a common adverse effect of PD1/PD-L1 blockade (D'Andréa et al., 2021). Furthermore, PD-L1 inhibitor-induced thyroiditis has been

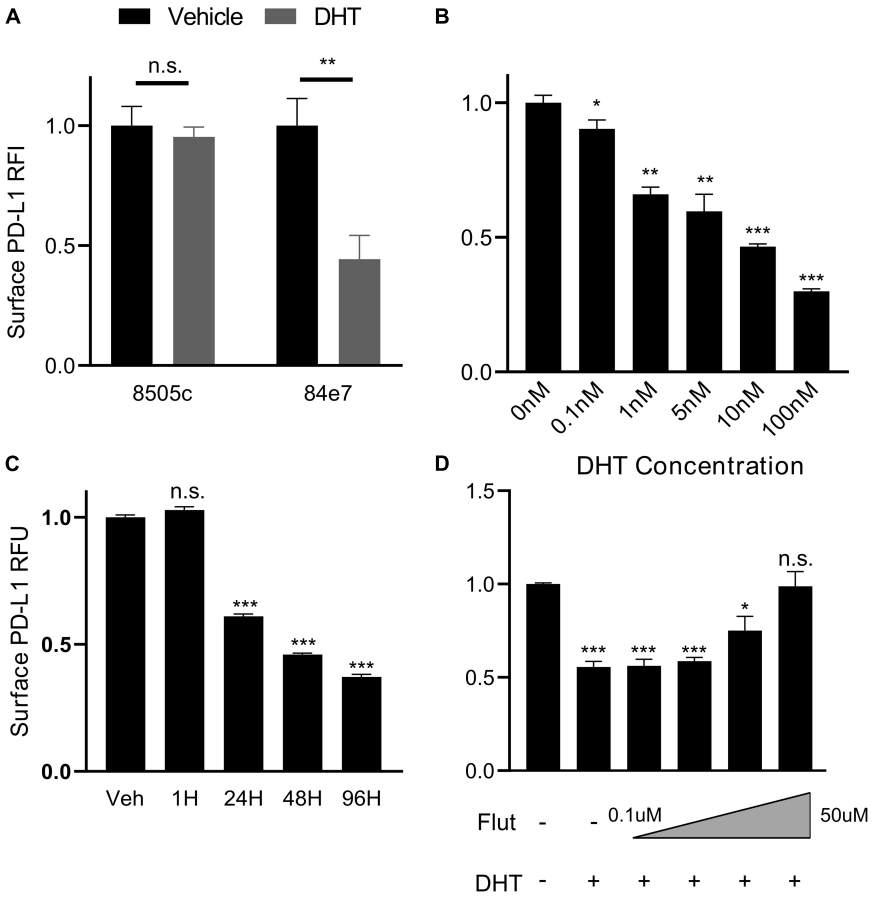

FIGURE 2 | PD-L1 is downregulated by androgen in an AR-dependent manner. **(A)** Surface expression of PD-L1 determined by flow cytometry in AR-lacking (8505C) or AR-expressing (84E7) thyroid cancer cell line after treatment with Vehicle (ethanol) or Dihydrotestosterone (DHT) (10 nM) for 72 h. **(B)** Dose-response to variable DHT concentrations in 84E7 cells at 72 h. **(C)** Time course of variable treatment lengths with 10 nM DHT. **(D)** Surface PD-L1 expression in 84E7 cell lines treated with 10 uM DHT and increasing concentrations of flutamide (Flut) at concentrations of 0.1, 1, 10, and 50 uM. RFI, relative fluorescence intensity. Student *t*-tests paired to control conditions $^{*}p < 0.05$; $^{**}p < 0.005$; and $^{***}p < 0.0005$. Barplots represents mean ± SEM of experiments performed in triplicate. *ns*, not significant.

shown to be an indicator of the drug's efficacy (Kotwal et al., 2020), suggesting functional PD-L1 expression in native thyroid glands. The expression of PD-L1 in thyroid cancers has been extensively studied using IHC (Aghajani et al., 2018). PD-L1 positivity is correlated with lower overall survival in anaplastic thyroid cancer (Chintakuntlawar et al., 2017) and worse clinicopathologic characteristics in PTC including reduced progression-free survival, increased lymphovascular invasion, tumor size, and TNM stage (Chowdhury et al., 2016; Shi et al., 2017; Gillanders and O'Neill, 2018). While the prevalence and severity of thyroid cancer differs between males and females, PD-L1 IHC in PTC exhibits no sex-based changes in the percentage of positively staining cells (Aghajani et al., 2018). The bulk RNA-sequencing performed in this study aggregates PD-L1 expression in the entire tissue while IHC indicates a proportion of cells that express PD-L1 without providing the degree of expression for the individual cell. Furthermore, our data indicates that AR is significantly downregulated in both male and female thyroid tumors compared to normal tissues. This may partially explain why PD-L1 IHC shows no sex-related differences as androgen-mediated PD-L1 attenuation is blunted by the downregulation of AR in male tumors.

To our knowledge, this is the first study identifying a relationship between AR activity and PD-L1 expression in thyroid cancer. Recently, Jiang et al. (2020) characterized AR suppression of PD-L1 in hepatocellular carcinoma. In combination, these findings point to a previously unappreciated role AR has on PD-L1 expression in multiple tissue types and, given that the PD-1/PD-L1 axis is an actionable immunotherapeutic target for various cancers, indicate that AR activity can impact the response to immunotherapy. An ongoing phase II clinical trial is investigating the efficacy of atezolizumab, a humanized PD-L1 monoclonal antibody, in combination with small molecule inhibitors or cytotoxic agents in the most aggressive forms of thyroid cancer (NCT03181100). Preliminary results for this trial were recently reported at the 2020 ASCO Annual Meeting and showed that compared to the historical ATC OS median of 5 months, patients in all three atezolizumab cohorts had a median OS of over 18 months.

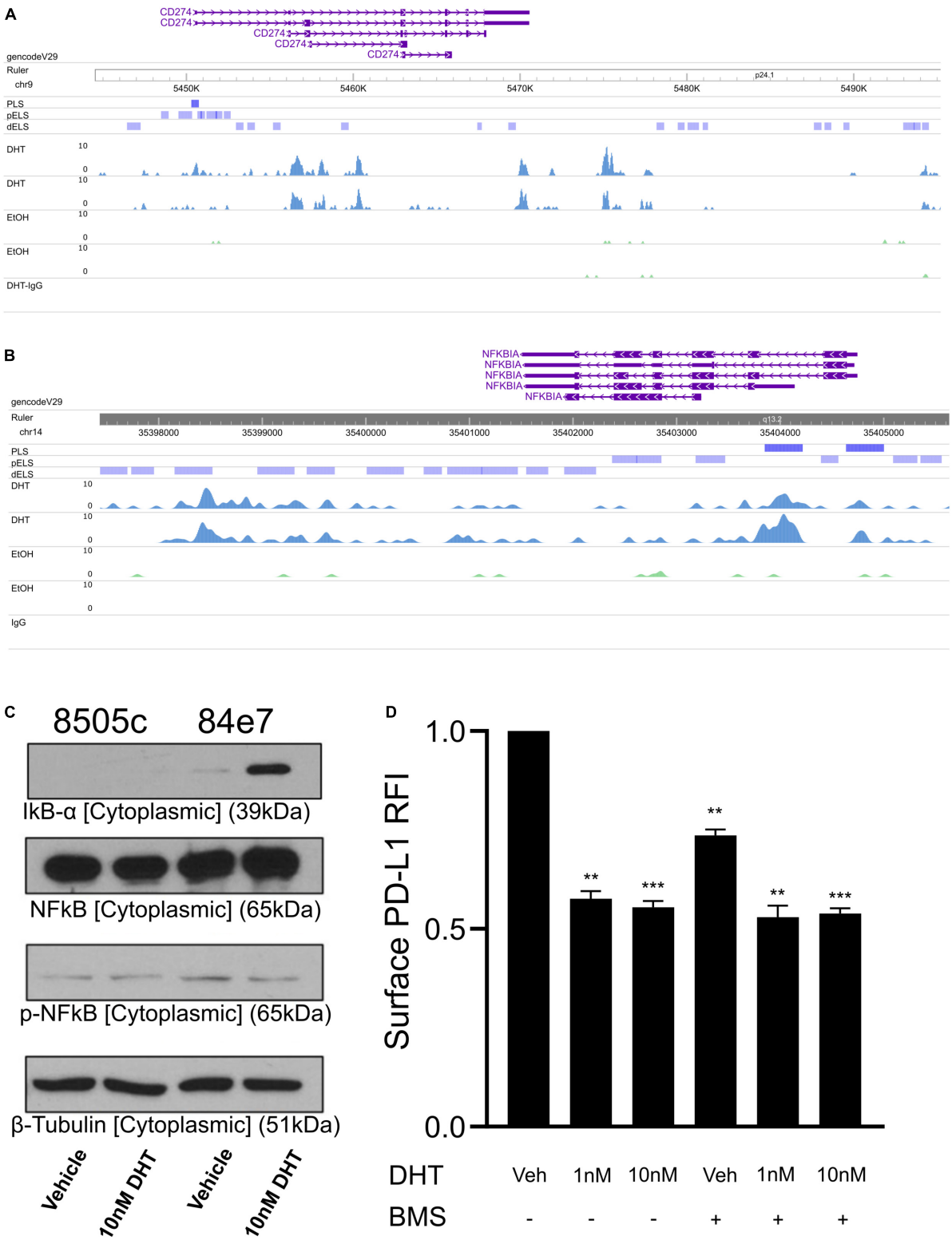

FIGURE 3 | Profiling AR binding genome-wide in 8505C-AR cells. CUT&Tag AR signal at the *CD274* gene body **(A)** and the NFKBIA gene promoter **(B)**. **(C)** Western blot analysis of cell cytoplasmic fraction from 72 h vehicle and 10 nM DHT-treated 8505C and 84E7. **(D)** Surface PD-L1 expression in 84E7 cells co-treated with 10 nM DHT for 48 h and the IKK inhibitor BMS-345541 (BMS) at 10 μM for 24 h. PLS, promoter-like signal; pELS, predicted enhancer-like signal; IgG, isotype control IgG CUT&Tag. Student *t*-tests paired to control conditions $^{**}p < 0.005$ and $^{***}p < 0.0005$.

Overall, 10 of 38 patients had complete tumor resection after therapy and seven of those patients were alive at the time of the report (Cabanillas et al., 2020).

Similar to Jiang et al. (2020), we identified a direct interaction between AR and the *CD274* gene body using CUT&Tag. However, since we were able to profile AR binding sites genome-wide, we also identified an AR binding signal at the NFKBIA promoter. AR activation may impact NF-kB signaling by increasing IkBα which thereby prevents NF-kB translocation into the nucleus. A decrease in nuclear NF-kB results in a direct reduction in PD-L1 expression due to the complex's involvement in key enhancer-promoter interactions (Chen et al., 2018). However, inhibition of NF-kB signaling using BMS-345541 did not reduce PD-L1 expression to the same level as DHT, suggesting AR likely represses PD-L1 via multiple mechanisms. This may include direct inhibition of PD-L1 promoter and enhancer elements as well as modulation of signaling pathways responsible for regulating PD-L1 expression, such as the IFNγ pathway.

There is a well-established gender disparity in thyroid cancer incidence. Underlying inflammatory processes in healthy females may predispose them toward developing thyroid cancer (Manole et al., 2001; Tafani et al., 2014). Klein and Flanagan (2016) have stated that sex is a biological variable that should be considered in immunological studies. We postulate that reduced levels of PD-L1 in males allows for continuous immunosurveillance and elimination of nascent tumors, whereas higher levels of PD-L1 in females results in immuno-evasive phenotypes (Mould et al., 2017). In addition to the role of AR in modulating the PD-L1 expression, ERα activation by 17β-Estradiol upregulates PD-L1 protein expression, and perhaps contributing to the immune inhibitory environment in women (Yang et al., 2017). Given the gender difference in inflammatory thyroiditis, which can contribute to elevated PD-L1 levels due to expression of cytokines such as IFNγ, constitutively active immunological processes, lower AR levels and/or estrogen receptor activation in females may lead to elevated PD-L1 levels, thereby contributing to the thyroid cancer gender disparity. However, the present study is limited to only providing correlative data in primary human thyroid carcinomas to suggest the relationship between AR activity and PD-L1. Furthermore, our cell culture model is limited by the use of exogenous expression of AR in an immortalized thyroid cancer cell line which may not represent the physiologic role of AR signaling *in vivo*. While the 8505C cell line expresses relatively high levels of AR compared to other thyroid cancer cell lines, it is evident from our results that these cells do not respond to DHT stimulation. Further investigation into the role of AR in primary thyroid cancer cells, particularly PTC cells, is warranted to establish a potential underlying mechanism and its role in the disparity in incidence.

In summary, this study suggests that PD-L1 is downregulated by androgens in thyroid cancer cells in an AR-dependent mechanism. This putative mechanism is corroborated by the inverse correlation of AR and PD-L1 expression in primary human tissues and the higher frequency of PD-L1 expression in female PTC tissues. Genome-wide profiling of AR binding sites reveals both direct activity at the PD-L1 locus as well as indirect regulatory mechanisms via inhibition of NF-kB through upregulation of IkBα. These findings provide a foundation for investigating the role PD-L1 expression has in the pathogenesis of thyroid cancer and the disparity in incidence between the genders.

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 below: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA698545, PRJNA698545.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at New York Medical College, New York Eye, and Ear Infirmary. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TO'C, RKT, and JG conceived of the study and designed the experiments. TO'C, SD, AG, and MJ performed the experiments. TO'C, SD, and TR analyzed and interpreted the data. ES, AM, CI, and HI provided clinical expertise and primary tissue. TO'C and SD wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the New York State Empire Clinical Research Investigator Program (RKT awardee), Department of Otolaryngology at New York Medical College, and the 2019 AHNS Endocrine Surgery Section Eisai Research Award (SD awardee).

ACKNOWLEDGMENTS

We would like to thank Zbigniew Darzynkiewicz, Jiangwei Li, and Dorota Halicka for their assistance with flow cytometry experiments and Anne Solas of the New York Medical College Histopathology core for assistance with IHC.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.663130/full#supplementary-material

Supplementary Table 1 | Primary antibodies used for western blotting.

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frontiers in Cell and Developmental Biology

REVIEW
published: 17 November 2021
doi: 10.3389/fcell.2021.740783

To B (Bone Morphogenic Protein-2) or Not to B (Bone Morphogenic Protein-2): Mesenchymal Stem Cells May Explain the Protein's Role in Osteosarcomagenesis

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OPEN ACCESS

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

Received: 13 July 2021
Accepted: 11 October 2021
Published: 17 November 2021

Citation:
Xu C, Wang M, Zandieh-Doulabi B, Sun W, Wei L and Liu Y (2021) To B (Bone Morphogenic Protein-2) or Not to B (Bone Morphogenic Protein-2): Mesenchymal Stem Cells May Explain the Protein's Role in Osteosarcomagenesis. Front. Cell Dev. Biol. 9:740783. doi: 10.3389/fcell.2021.740783

Osteosarcoma (OS), a primary malignant bone tumor, stems from bone marrow-derived mesenchymal stem cells (BMSCs) and/or committed osteoblast precursors. Distant metastases, in particular pulmonary and skeletal metastases, are common in patients with OS. Moreover, extensive resection of the primary tumor and bone metastases usually leads to bone defects in these patients. Bone morphogenic protein-2 (BMP-2) has been widely applied in bone regeneration with the rationale that BMP-2 promotes osteoblastic differentiation of BMSCs. Thus, BMP-2 might be useful after OS resection to repair bone defects. However, the potential tumorigenicity of BMP-2 remains a concern that has impeded the administration of BMP-2 in patients with OS and in populations susceptible to OS with severe bone deficiency (e.g., in patients with genetic mutation diseases and aberrant activities of bone metabolism). In fact, some studies have drawn the opposite conclusion about the effect of BMP-2 on OS progression. Given the roles of BMSCs in the origination of OS and osteogenesis, we hypothesized that the responses of BMSCs to BMP-2 in the tumor milieu may be responsible for OS development. This review focuses on the relationship among BMSCs, BMP-2, and OS cells; a better understanding of this relationship may elucidate the accurate mechanisms of actions of BMP-2 in osteosarcomagenesis and thereby pave the way for clinically safer and broader administration of BMP-2 in the future. For example, a low dosage of and a slow-release delivery strategy for BMP-2 are potential topics for exploration to treat OS.

Keywords: osteosarcoma, bone morphogenetic protein-2, mesenchymal stem cells, osteogenic differentiation, osteogenesis, tumor heterogeneity, bone-marrow-derived mesenchymal stem cell

INTRODUCTION

Although osteosarcoma (OS), a primary bone neoplasm, is rare, with an incidence of only one to three confirmed cases per 1 million people in the world each year, it comprises ∼20% of newly diagnosed bone tumors (Dorfman and Czerniak, 1995; Klein and Siegal, 2006; Mirabello et al., 2009b). Epidemiologically, OS presents in children, the youth, and the elderly with high frequency (Kansara et al., 2014); the morbidity of OS increases to 8–11 per million annually in 15–19-year-olds (Stiller et al., 2006; Mirabello et al., 2009a; Anfinsen et al., 2011). OS most often initiates in the metaphysis of long bones (Ritter and Bielack, 2010), implying a correlation with impaired bone growth. Currently, bone-marrow-derived mesenchymal stem cells (BMSCs) and/or committed osteoblast precursors with genomic mutations (e.g., *TP53*, *RB1*), chromosomal deletion, and chromosomal rearrangements are recognized as the cellular origins of OS (Chandar et al., 1992; Walkley et al., 2008; Mohseny et al., 2009; Rubio et al., 2013; Chen et al., 2014; Deng et al., 2019; Han et al., 2019). As an aggressive tumor, OS is insensitive to some chemotherapy agents (Pavlou et al., 2019; Belisario et al., 2020); MAP (methotrexate, doxorubicin, and cisplatin) is still the first-line drug for OS chemotherapy (Marina et al., 2016). To date, the OS has a 5-year survival rate of ∼50% (Smeland et al., 2019); the leading cause of death in OS is pulmonary metastasis (Bhattasali et al., 2015). Skeletal metastasis is also common in patients with OS and precipitates severe bone erosions. Extensive resection to remove OS is also responsible for voluminous bone defects, which may induce dysfunction and disfiguration. The rehabilitation of bone tissue is a huge challenge in clinical OS therapy. Although bone morphogenic protein-2 (BMP-2) has been widely used in bone repair and has shown promising results, its application in OS has not been reported because of its potential role in tumorigenesis.

BMP-2 was discovered by Urist (1965), and its cDNA was first cloned by Wozney et al. (1988). This growth factor is a member of bone morphogenic proteins (BMPs) belonging to the transforming growth factor-beta (TGF-β) superfamily that is important for diverse cellular processes (e.g., cell proliferation, differentiation, apoptosis, angiogenesis, migration, and extracellular matrix remodeling) (Bierie and Moses, 2006; Massagué, 2008). More than 20 BMPs have been identified in human tissues (Wozney and Rosen, 1998; Reddi, 2005). As the most well-studied one, BMP-2 has been widely used in bone formation because of its potent osteoinductivity and has been approved by the U.S. Food and Drug Administration for orthopedic and dental applications (Burkus et al., 2002; US Food and Drug Administration, 2002; Alonso et al., 2014). After continued clinical use, the adverse effects of BMP-2 (e.g., inflammatory, ectopic bone formation, infection, and potential tumorigenicity) have come into focus; the high dose and off-label application of BMP-2 have also aroused concern (Cahill et al., 2009; Tian et al., 2017; Pardali et al., 2018; Hashimoto et al., 2020; Hsu et al., 2020). Whether BMP-2 suppresses or stimulates tumor development remains a contentious issue (Weiss, 2015), and this controversy still challenges researchers (Kendal et al., 2020; **Table 1**). Using an orthotopic mouse model, Xiong et al. (2018) revealed that 2.5 μg of recombinant human BMP-2 (rhBMP-2) applied for 14 days not only induced bone formation but also suppressed OS growth and pulmonary metastasis in OS-bearing mice. Similar research also documented that rhBMP-2 constrained the tumorigenicity of cancer stem cells in human OS cell lines *in vitro* and *in vivo* (Wang et al., 2011; Gill et al., 2017). Conversely, opposite results from other studies have suggested that BMP-2 promotes OS migration and epithelial–mesenchymal transition (Sotobori et al., 2006; Tian et al., 2019). Because of this discrepancy in results, the use of BMP-2 in those at high risk of OS must be discreet and individualized based on the latest research.

Mesenchymal stem cells (MSCs) are identified as the origin of OS and are capable of differentiating into osteoblasts, a process that can be accelerated by BMP-2. However, BMP-2 is also involved in the progression of OS, suggesting that complicated crosstalk may exist among OS, BMP-2, and MSCs. As multipotent mesenchymal stromal cells, MSCs are universally found in almost all connective tissues (Horwitz et al., 2005; da Silva Meirelles et al., 2006). They possess the ability to differentiate into various mature somatic cells (e.g., osteoblasts, adipocytes, and chondrocytes) with appropriate stimulation (Pittenger et al., 1999) and the capacity to self-renew. BMSCs were first isolated by Owen and Friedenstein (1988) from bone marrow. These heterogeneous cells are involved in osteoblast differentiation through the spatiotemporal expression of osteogenesis-related genes (*RUNX2, COL1A1, ALPL, SP7, BGLAP*, etc.) (Ducy et al., 1997; Nakashima et al., 2002; Twine et al., 2014). A few signal pathways have proven to have pivotal roles in BMSC-induced osteogenesis; the canonical BMP-2 pathway (**Figure 1**) is a well-known example. A great body of research has focused on the effect of MSCs on or toward osteoblastic differentiation and OS progression; to date, though, the effect of BMP-2 on normal BMSCs and on mutated BMSC–induced osteosarcomagenesis is still elusive.

MSCs play contradictory roles in copious cancer types (Devarasetty et al., 2017; Gyukity-Sebestyén et al., 2019; Xu et al., 2019; Zhang et al., 2020; Jia et al., 2021). In OS, MSCs are reportedly involved in not only chemoresistance, proliferation, and pulmonary metastases but also OS recession (Cortini et al., 2017). Thus, the effect of MSCs on OS might be converted according to the relevant OS niche. Herein, we summarize the literature and present the potential mechanism of the contradictory effects of MSCs on OS to provide direction for additional studies.

BONE MORPHOGENIC PROTEIN-2 INHIBITS OSTEOSARCOMA PROGRESSION *VIA* MESENCHYMAL STEM CELLS

MSCs can suppress sarcoma progression. Gauthaman et al. (2012) found that umbilical cord-derived MSCs from Wharton's jelly suppressed the proliferation and migration of MG-63 cells (a human OS cell line) *in vitro*; in a Kaposi sarcoma model, MSCs also inhibited tumor progression (Khakoo et al., 2006). BMP-2 also has inhibited OS progression, although the potential mechanism was not discussed (Xiong et al., 2018). Given the close

TABLE 1 | Studies of bone morphogenic protein 2 on tumor progression.

References	Animal model	Cell lines	
Xiong et al. (2018)	Mice	143B	Lung metastasis↓, Ki-67 ↓, ALDH^{br}↓
Rampazzo et al. (2017)	NA	GBM- derived cells	Ki67↓, drug susceptibility↑, differentiation of GSCs↑
Wang et al. (2012)	Mice	ACHN, Caki-2	Tumor proliferation↓, Runx2↑, tumor volume↓, bone formation↑
Nishimori et al. (2012)	NA	LNCaP, MC3T3-E1	FGF-2↑, EGF↑ LNCaP cells proliferation↑
Kang et al. (2011)	NA	AGS, SNU-638	Cell migration and invasion↑, NF-κB activity↑, MMP-9↑
Wu J. B. et al. (2011)	NA	SMMC7721	Cell invasion↑, MMP-2 and MMP-9↑, p-ERK↑

ALDH^{br}, aldehyde dehydrogenase bright; EGF, epidermal growth factor; FGF-2, fibroblast growth factor (FGF)-2; GBM, glioblastoma; GSCs, glioblastoma stem cells; NA, not available.

link between MSCs and BMP-2 in osteoblastic differentiation and OS etiology, BMP-2 might suppress OS through BMSCs. We reviewed the literature to explore the ability of BMP-2 to inhibit OS through BMSCs and present three assumptions (**Figure 2**): (1) BMP-2 induces proliferation of BMSCs with the capacity to suppress OS; (2) BMP-2 induces differentiation of mutated BMSCs and/or OS cells to normal osteoblasts; (3) BMSC polarization shifts.

Proliferation of Specific Mesenchymal Stem Cells

BMSCs are heterogeneous populations comprising various subpopulations with diverse properties (Horwitz et al., 2005). Except for Wharton's jelly MSCs, BMSCs from rats and mice have demonstrated dose-dependent cytotoxicity to tumor cells (Otsu et al., 2009). Thus, specific BMSCs with anticancer capacity exist and may function according to the altered expression of some cytomembrane receptors (Ridge et al., 2017). It would make sense that BMP-2 could suppress OS through the proliferation of these specific BMSCs and that a BMP-2 and Wnt pathway autocrine loop (**Figure 3**) may be capable of explaining this process. The Wnt pathway is involved in diverse cellular events, including mitogenic stimulation, cell fate determination, differentiation, and proliferation (Huang and Niehrs, 2014; Yao et al., 2016; Steinhart and Angers, 2018). It is not surprising that the Wnt pathway, in particular the canonical Wnt pathway (i.e., the beta-catenin–dependent pathway), plays crucial roles in osteoblastic differentiation and osteogenesis (Liu et al., 2008; Wang et al., 2014b; Lerner and Ohlsson, 2015). Although the Wnt pathway is thought to inhibit MSC proliferation (Moon et al., 2018), an activated Wnt pathway facilitating BMSC proliferation has also been reported (Zhu et al., 2014). After the canonical Wnt pathway is activated, beta-catenin translocates from the cytoplasm into nuclei. In the nuclei, a complex consisting of beta-catenin and some transcription factors—for example, lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF)—modulates the expression of target genes, including *BMP2*, *RUNX2*, and proliferation-related genes (Zhang R. et al., 2013). Conversely, BMP-2 can stimulate the accumulation of beta-catenin in nuclei (Yang et al., 2006; Hiyama et al., 2011), thereby activating the canonical Wnt pathway in turn. BMP-2–induced cell proliferation has been reported in murine preosteoblasts, rat BMSCs, and human pulmonary artery epithelial cells (de Jesus Perez et al., 2009; Rosen, 2009; An et al., 2017). The OS suppression properties of BMP-2 might result from the positive feedback of this loop *via* expansion of the specific BMSCs in the OS niche. In addition, aberrant activation of Wnt/beta-catenin signaling in OS cells has been detected (Chen et al., 2015). The identification of specific BMSCs in the OS niche is a precondition for OS suppression. Unfortunately, few studies about these specific BMSCs have been conducted in OS settings, so detailed information about their characteristics is still lacking.

Induced Osteoblastic Differentiation

Cancer is a disease arising from failed cell differentiation (Honma and Akimoto, 2007). Thus, differentiation-inducing treatments have been proposed. With this strategy, tumor cells differentiate back into normal cells instead of being eliminated by chemotherapeutics and/or radiation. One well-known differentiation-inducing treatment is all-trans-retinoic acid in acute promyelocytic leukemia (Huang et al., 1988). Notably, OS is recognized as an osteoblast differentiation disruption disease (Tang et al., 2008). OS cells have characteristic properties that resemble undifferentiated osteoblasts (Carpio et al., 2001; Postiglione et al., 2003; Haydon et al., 2007), and activating *RB1* transcription has reversed the disrupted osteoblastic differentiation (Thomas et al., 2001). In addition, BMP-2 has been tested for its efficacy as a differentiation-inducing treatment. Rampazzo et al. (2017) successfully induced astroglial differentiation of glioblastoma stem cells using a BMP-2 mimicking peptide. Moreover, BMP-2 has suppressed tumors and promoted bone formation simultaneously: Wang et al. (2012) indicated that renal cell cancer was inhibited and bone formation was induced with the application of BMP-2. Furthermore, BMP-2 has reduced tumor volume, attenuated OS-induced pulmonary metastases, and stimulated bone formation (Xiong et al., 2018). Applying 30 μg of BMP-2 to OS-bearing mice also increased the transcription of osteogenic genes and promoted osteogenesis (Wang et al., 2013). Taken together, these data suggest that BMP-2 may play a therapeutic role in OS by inducing osteogenic differentiation of mutated BMSCs and/or OS cells.

Mesenchymal Stem Cell Polarization

The polarization of macrophages in inflammatory conditions suggests that the effect of BMSCs on OS may also transform

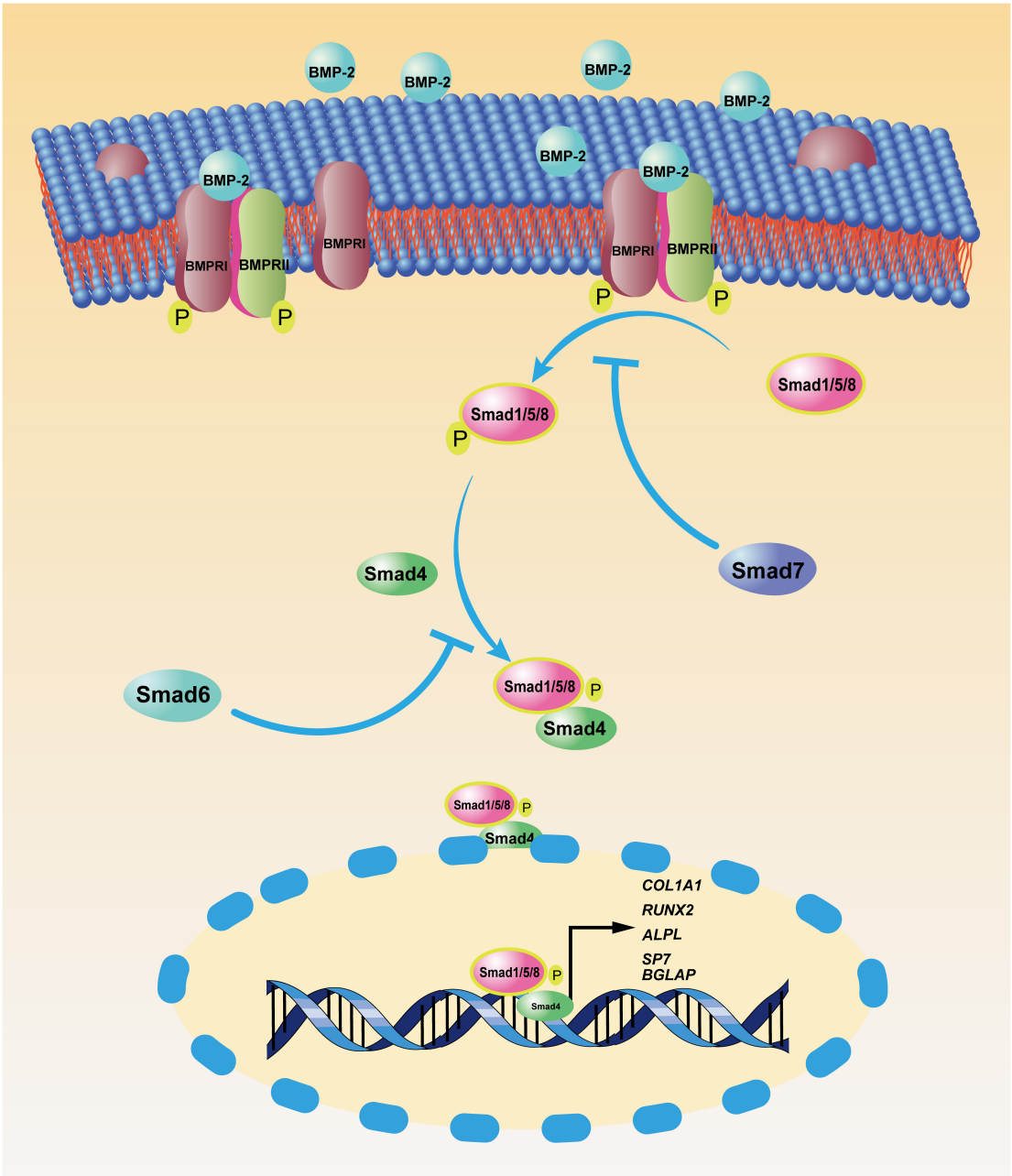

FIGURE 1 | Canonical bone morphogenic protein-2 (BMP-2) signaling pathway. After BMP-2 binds to its transmembrane receptors [bone morphogenic protein receptor (BMPR)I and BMPRII], these phosphorylated receptors facilitate the phosphorylation of mothers against decapentaplegic and the *Caenorhabditis elegans* protein 1/5/8 (Smad1/5/8) in the cytoplasm. Then, the complex of pSmad1/5/8 and Smad 4 translocates to the nucleus, where phosphorylated Smad1/5/8 (pSmad1/5/8) and Smad 4 function as transcription factors, enhancing the transcription of osteoblastic genes, including *COL1A1, RUNX2, ALPL, SP7,* and *BGLAP*. As negative feedback, Smad7 inhibits the phosphorylation of Smad1/5/8, and Smad6 prevents the nucleus translocation of the complex of pSmad1/5/8 and Smad4.

mutually between tumor promotion and tumor suppression (Ridge et al., 2017). This hypothesis has been verified by Waterman et al. (2012) in a study that activated different cytomembrane receptors. The researchers claimed that activation of toll-like receptor-4 (TLR-4) conferred an antitumor effect on human BMSCs, which were named MSC1; after TLR-3 activation, however, the human BMSCs were converted to MSC2, which promoted tumor growth and metastasis (Waterman et al., 2012). Although myriad studies have indicated that TLR-2 and TLR-4 can enhance the expression of BMP-2 in BMSCs and accelerate bone formation (Yang et al., 2009; Su et al., 2011; Oliveira et al., 2017; Zhou et al., 2019), the effect of BMP-2 on the expression of TLRs is still equivocal. The dosage of BMP-2 and the state of BMSCs in the tumor niche may draw contrasting conclusions.

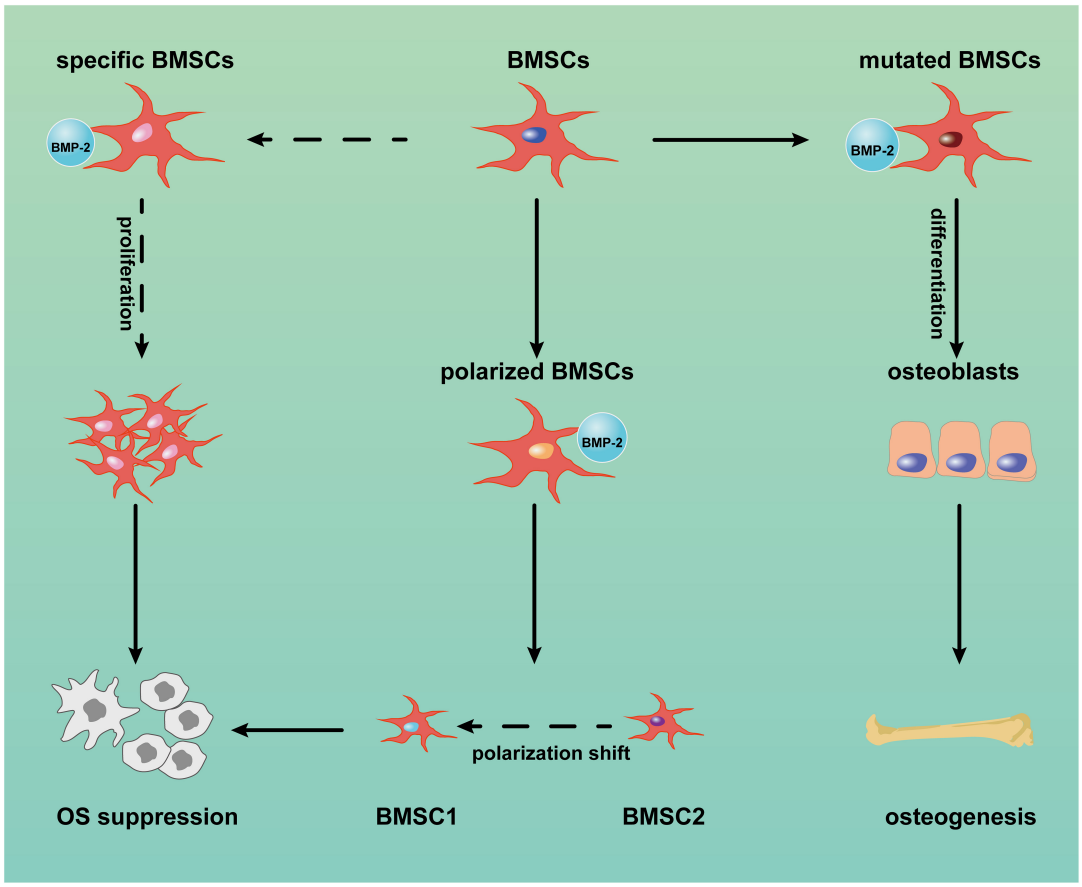

FIGURE 2 | Potential mechanism of bone morphogenic protein-2 (BMP-2) induced tumor suppression *via* bone marrow-derived mesenchymal stem cells (BMSCs). BMP-2 may induce mutated BMSC differentiation into normal osteoblasts. Conversely, BMP-2 may promote the proliferation of specific BMSCs with anticancer capacity and the shift of BMSC polarization from MSC2 (tumor promotion) to MSC1 (tumor inhibition). OS: osteosarcoma.

Thus, the hypothesis that BMP-2 suppresses OS by affecting TLRs must be explored in more detail.

BONE MORPHOGENIC PROTEIN-2 PROMOTES OSTEOSARCOMA PROGRESSION *VIA* MESENCHYMAL STEM CELLS

Aberrant Activation of *RUNX2* and *SP7*

More research has reported that BMPs, especially a supra-physiological dose of BMP-2, induces tumorigenesis, not tumor suppression (**Figure 4**; Jin et al., 2001; Kang et al., 2011; Wu J. B. et al., 2011; Nishimori et al., 2012; Tian et al., 2017; Zhang et al., 2018). The physiological concentration of BMP is ∼2 ng/g of bone. In most clinical trials, supra-physiological doses (mg concentrations) of BMP-2 have been applied, and these doses may disturb the normal BMP-2 signal pathway (Arrabal et al., 2013; Oryan et al., 2014). After BMP-2 binds to its receptors on the cell surface, the BMP-2 signaling pathway is activated. In the canonical BMP-2 pathway, transcription of osteogenic genes, including *RUNX2,* and *SP7* (*OSTERIX*), is upregulated. Although these two genes are vital for bone formation, an increasing body of evidence implies that they are also engaged in tumorigenesis. Normally, *RUNX2* expresses during the cell cycle in healthy osteoblasts to disturb cell growth and induce osteoblast maturation (Pratap et al., 2003). Overexpression of *RUNX2* has been found in patients with OS and is correlated to poor prognosis (Pereira et al., 2009; Sadikovic et al., 2010; Gupta et al., 2019). van der Deen et al. (2012) used chromatin immunoprecipitations to detect *RUNX2* target genes in U2OS cells; results indicated that some motility-related genes were downstream of *RUNX2* and that cell motility decreased after *RUNX2* depletion. Furthermore, an elevated RUNX2 protein level may also be responsible for pulmonary metastasis. After *RUNX2* activates *SPP1* (*OPN*), the *RUNX2* target gene encodes a secreted matricellular protein, thereby remodeling the bone matrix, which leads to tumor metastasis (Villanueva et al., 2019). *RUNX2* may also account for the chemotherapeutic resistance of OS. When *RUNX2* was silenced by si/shRNA, OS cells were more sensitive to doxorubicin (Roos et al., 2015). Another osteogenic gene, *SP7*, has not been associated with osteosarcomagenesis, but it has been described as a stimulus in other tumors (Dai et al., 2015; Yao et al., 2019; Ricci et al., 2020). This finding suggests that sustained activation of the BMP-2

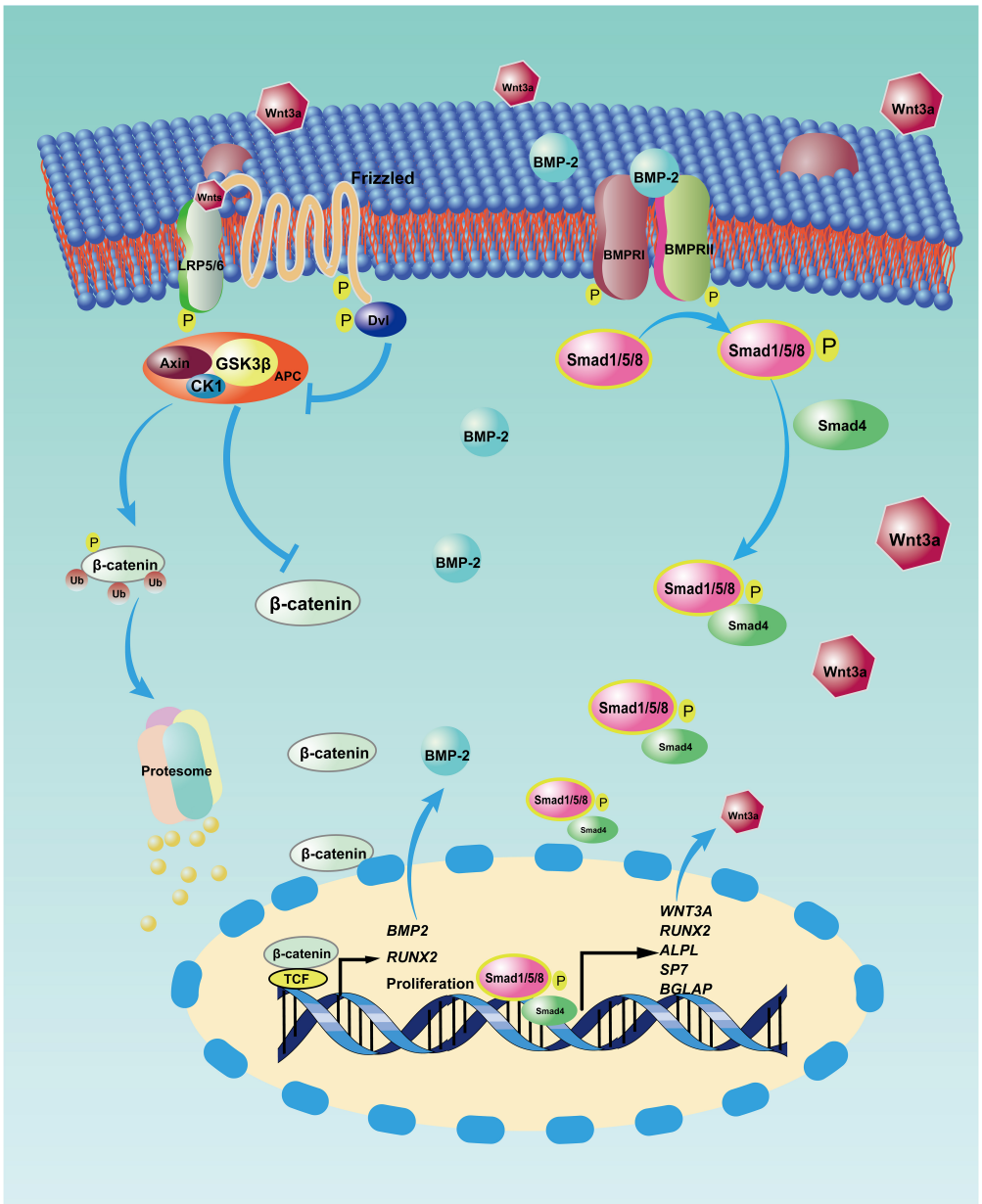

FIGURE 3 | Reciprocal activation between canonical bone morphogenic protein-2 (BMP-2) and canonical Wnt signaling pathway. After BMP-2 binds to its receptors on the cell surface, phosphorylated Smad1/5/8 and Smad4 translocate into nuclei, where this complex modulates the transcription of some target genes, including *WNT3A* (coding the Wnt3a protein) and some osteoblastic differentiation genes. However, with the autocrine signaling, Wnt3a binds to its receptors (i.e., LRP5/6 and Frizzled) on the membrane, so β-catenin accumulates in the nuclei and incorporates with T cell-specific transcription factor (TCF) to upregulate the transcription of target genes, including *BMP2, RUNX-2,* and proliferation-related genes. APC: adenomatous polyposis coli, CK1: casein kinase 1, Dvl: Disheveled, GSK3β: glycogen synthase kinase3β, LRP5/6: low-density lipoprotein receptor-related protein 5.

pathway causing increased *SP7* transcription may also precipitate OS in bone tissues.

Modulation of the Tumor Microenvironment

BMP-2 may promote OS progression through modulation of the tumor microenvironment (TME), which plays an indispensable role in tumor progression (Hui and Chen, 2015; Yang et al., 2020). The bone microenvironment where OS grows is composed of hematopoietic stem cells, lymphoid progenitors, mature immune cells, bone cells, MSCs, mineralized extracellular matrix, and more (Tsukasaki and Takayanagi, 2019; Corre et al., 2020). The crosstalk in these items modulates the OS TME, which affects OS progression. Cancers are identified as "wounds that never heal" (Dvorak, 1986), so it is not surprising that MSCs

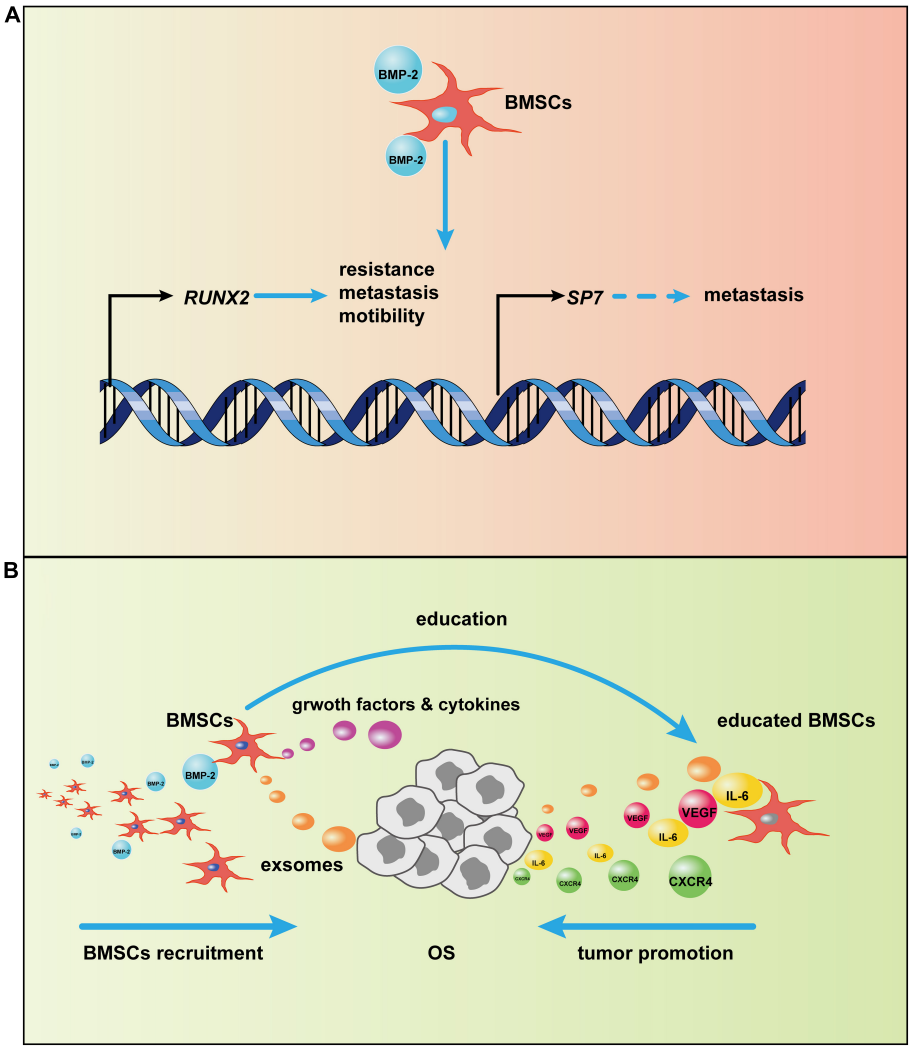

FIGURE 4 | Potential mechanism of bone morphogenic protein-2 (BMP-2)–induced tumor progression *via* bone marrow-derived mesenchymal stem cells (BMSCs). **(A)** After the canonical BMP-2 pathway is activated, *RUNX2* and *SP7* transcription initiate. The overexpression of *RUNX2* and *SP7*, as a result of continuous activation of the canonical BMP-2 pathway, may promote osteosarcoma (OS) progression. **(B)** BMSCs are recruited to OS by BMP-2. Then, BMSCs adapt to OS *via* OS-related cytokines and exosomes; in turn, the tumor-centered BMSCs will secrete growth factors and cytokines, such as vascular endothelial growth factor (VEGF) and CXC chemokine receptor 4 (CXCR4), to promote OS development.

are involved in tumor development, given the central role of MSCs in repairing wounds by altering the local inflammatory environment and secreting growth factors, immunoregulatory factors, and chemokines (Caplan and Correa, 2011; Wang et al., 2014a; Shi et al., 2017) after the tumor-specific tropism of MSCs (Kidd et al., 2009). However, MSCs are not always beneficial for healing; the fluctuation of their function depends on the milieu where they reside (Wang et al., 2014a). In the TME, MSCs can be converted into tumor-associated MSCs that have vast differences from normal MSCs (Le Nail et al., 2018) and that can promote tumor proliferation, migration, immunosuppression, and angiogenesis through extracellular vesicles (Quante et al., 2011; Baglio et al., 2017; Shi et al., 2017; Whiteside, 2018). In the OS niche, interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) secreted from BMSCs have been involved in OS progression (Tu et al., 2012; Zhang P. et al., 2013); BMSCs promoted pulmonary metastasis of OS by increasing the expression of CXC chemokine receptor 4 (CXCR4) and VEGF (Fontanella et al., 2016). Furthermore, extracellular vesicles, such as exosomes from BMSCs, are loaded with certain miRNAs involved in OS aggression and development (Xie et al., 2018). BMP-2, as a member of the TGF-β superfamily with the ability to recruit MSCs to inflammatory surroundings and the TME (Spaeth et al., 2008), may recruit BMSCs to OS, and BMP-2-induced chemotaxis has been reported in other conditions (Hiepen et al., 2014; Simões Sato et al., 2014; Pardali et al., 2018). BMP-2, particularly at high doses, induces inflammation (James et al., 2016), which may cause MSC homing as a result of inflammatory cytokines; in addition, MSCs have been recruited by BMP-2 through CXCR4, accelerating bone

formation (Zwingenberger et al., 2014). Thus, BMP-2 might recruit BMSCs toward the OS phenotype. Together, these results suggest a tentative hypothesis. After BMSCs are recruited by BMP-2 to the OS niche, they will be educated directly or indirectly by OS cells. Afterward, the emergence of the educated BMSCs that can secrete some cytokines and growth factors will promote OS proliferation, migration, angiogenesis, and more.

REASONS FOR CONTRADICTORY CONCLUSION

The debate about BMP-2 is an obstacle to its clinical application, despite the potential value for those at high risk of OS and for patients with OS and bone defects. Illustrating the reasons for these controversies can deepen our understanding of the function of BMP-2 in OS and guide its clinical administration.

Differences in Osteosarcoma Cell Lines

Diverse OS cell lines applied in the research contribute to the confusion about results. Histologically, several OS subtypes with distinct characteristics have been confirmed. At the cellular level, various *in vitro* OS cell lines have been used in research; great differences in these cell lines have been verified. Saos2 cells appear more identical to normal osteoblasts than other OS cell lines, as osteoblastic markers can be detected in these cells. Conversely, osteocalcin, an important marker in bone mature, was hardly expressed in MG-63 and U2OS cells. However, matrix metalloproteinase-9 (MMP-9), a well-known cytokine for tumor migration and metastasis (Deryugina and Quigley, 2006), was positive in most MG-63 cells (Pautke et al., 2004). In other research, researchers (Mohseny et al., 2011) compared differences in differentiation, tumorigenesis, and protein expressions among 19 OS cell lines. Only OSA, IOR/OS9, and IOR/OS18 could differentiate into osteoblasts, chondrocytes, and adipocytes; 13 of the 19 cell lines could differentiate toward osteoblasts. This finding may explain why some researchers claimed that OS cell lines could not be induced into osteoblasts by BMP-2, whereas other studies reported opposite results (Haydon et al., 2007). Moreover, in these 19 OS cell lines, HOS-14B cells had the greatest capacities of tumorigenesis and metastasis. These inherent disparities between various OS cell lines, to some extent, account for the conflicting conclusions about the role of BMP-2 in OS progression.

Heterogeneity of Mesenchymal Stem Cells

Variations in MSCs are also ubiquitous. MSCs are heterogeneous populations consisting of a few subtypes with diverse characteristics; the differences may come from individual differences and species differences (Peister et al., 2004). The proposed definition of MSCs suggests that they must (1) adhere to plastic, (2) express special surface markers, and (3) differentiate along the osteogenic, chondrogenic, and adipogenic lineages (Dominici et al., 2006; Lindsay and Barnett, 2017). Commonly, CD34, CD31, and CD45 are negative on both human and mouse MSCs (Dominici et al., 2006); some markers, such as STRO-1 and CD271, are only detected on human MSCs (Lv et al., 2014); these are specific and can be found on other cell types (Kuhn and Tuan, 2010). CD29, CD51, CD73, CD90, CD105, and CD146 are universal in human and mouse MSCs (Sacchetti et al., 2007; Zhang et al., 2019). BMSCs are the most used MSCs in research; they are heterogeneous as well, which complicates the research and weakens the conclusions. Although some specific isolation kits based on the cell surface markers have been applied to clarify results, it remains hard to purify the homogeneous BMSCs, as MSCs share cell-surface markers and localization with pericytes (Crisan et al., 2008). With the development of biotechnology, the function and characteristic identification of a single cell are practicable. Single-cell RNA sequencing has been used to detect immune cell heterogeneity (Papalexi and Satija, 2018); Zhou et al. (2020) assayed the intratumoral heterogeneity and immunosuppressive microenvironment in advanced OS and demonstrated the complex variations in OS.

Different Doses and Delivery Strategies of Bone Morphogenic Protein-2

Furthermore, the dose and the delivery strategy of BMP-2 affect the research conclusions (Wu G. et al., 2011). Most of the reported disadvantages of BMP-2 result from overdosage. The effective dose of BMP-2 in osteoblastic differentiation of MSCs, which is dose-dependent, is just 25–100 ng/mL *in vitro* (Rickard et al., 1994; Lecanda et al., 1997). However, the working concentration of BMP-2 for *in vitro* or *in vivo* research is not distinguished, and most doses are supra-physiological, which may confound the results and cause adverse effects. The delivery pattern of BMP-2 is also crucial. A continuous and slow release, rather than a burst stimulation, is more bionic and more closely resembles physiological conditions. Most recent research has administered rhBMP-2 protein directly into the culture medium or intravenously, which may cause stress conditions for cells and tissues. The advantages of a sustained, low-dose release of BMP-2, including less inflammation and ectopic ossification, have been verified (Wildemann et al., 2004; Ji et al., 2010; Seo et al., 2017; Berkmann et al., 2020; Xin et al., 2020). The mitigatory inflammatory surroundings can reduce the risk of tumorigenesis as well, which makes low-dose BMP-2 application more reasonable.

LIMITATIONS IN PRESENT STUDIES

Deficiency of *in vitro* Research

Currently, most *in vitro* studies are carried out on traditional two-dimensional (2D) culture models (i.e., flask- and petri-dish-based cultures). However, these 2D models hardly mimic tumor cell biology because of tumor heterogeneity and different responses to secreted cytokines, growth factors, and methylation states of the cells. Moreover, the 2D cell culture systems cannot sufficiently simulate a three-dimensional (3D) physiological microenvironment, so they fail to provide physiologically relevant information regarding cell–cell interactions, cell–extracellular matrix interactions, growth factor synthesis, or physical and chemical cues to oncogenesis

(Hickman et al., 2014; Berkmann et al., 2020). Furthermore, the results obtained from gene expression analysis and drug resistance also differ substantially between 2D and 3D cell culture models (Zhao et al., 2014; Costa et al., 2016; Henriksson et al., 2017; Zhou et al., 2017; Fontoura et al., 2020; Mao et al., 2020; Sun et al., 2020; Xie et al., 2021). The disadvantages of the 2D culture reduce attempts to understand the authentic role of BMP-2 that may play in the formation and pathology of OS.

Inappropriate Animal Models

In most OS studies, rodents, such as mice or rats, have been used as experimental animal models in addition to the patient-derived xenograft or cell line-derived xenograft models. Normally, OS is rare in mice and rats, and these models may present limited information or misinformation. The OS incidence in dogs is ∼27-fold higher than in humans, which makes the canine model a more useful model to the human OS for research (Simpson et al., 2017). To date, preclinical research using dogs as animal models has suggested that a combination of canine BMSCs together with rhBMP-2 treatment suppressed OS by increasing p53 and some other pro-apoptotic proteins (Rici et al., 2012, 2018). However, using dogs as animal models to study the effects of BMP-2 on OS development is not well accepted in Western countries because of social and cultural reasons.

Lack of High-Quality Evidence

Large-scale and multicenter cohort studies for evaluating BMP-2 treatment effects on OS progression remain unavailable. Although some clinical retrospective studies have suggested that BMP-2 used in spine fusion surgery was not involved in tumorigenesis (Fahim et al., 2010; Cooper and Kou, 2013; Lad et al., 2013), these studies were performed with small sample sizes and had insufficient follow-up times. Large-scale and multicenter cohort studies are needed to draw a scientific conclusion and establish the effects of the BMP-2 on patients living with cancer.

SUMMARY

To date, the exact role of BMP-2 in osteosarcomagenesis is still equivocal, although abundant studies have been carried out. This uncertainty is attributed to the intricacy of the OS genome, differences between OS subtypes, the complex TME, and the multifunctionality of BMP-2 activation of several signal transduction pathways. The response of MSCs, which have a pivotal effect on osteogenesis and osteosarcomagenesis, to BMP-2 remains a key to understanding this mystery. This review represents research focused on the BMP-2 effect on OS cell lines and OS animal models and the relevant potential mechanisms involved, and it provides some clues for additional research about OS biology and safe application of BMP-2 in clinical settings. For current clinical application, we recognize that a low-dose and slow-release strategy of BMP-2 applied in bone regeneration is acceptable, even in the tumor-caused bone defects, while in the OS treatment, we still maintain a prudent stand to the employment of BMP-2.

As a growth factor, BMP-2 plays a crucial role in various cell biology activities. BMP-2 use in populations with genetic mutation diseases may promote OS progression; mutations of some genes, particularly *TP53* and *RB1*, and genomic alterations have been associated with osteosarcomagenesis. Likewise, using BMP-2 in patients with some bone metabolic diseases might increase the occurrence of OS, because aberrant activities of osteogenesis-related signaling pathways in these patients are very common; these pathologic activities may enhance the expression of *RUNX2* and *SP7*, the latter of which is overexpressed in patients with OS and is correlated with poor prognosis.

However, BMP-2 is highly likely to be used in OS treatments because of the BMP-2-induced proliferation of specific BMSCs with anticancer capacity. This strategy is based on the isolation and identification of these specific BMSCs. However, to our knowledge, no research on the isolation and identification of characteristics of these specific BMSCs has been carried out. Moreover, BMP-2 may inhibit OS through the osteoblastic differentiation of OS cells and/or mutated BMSCs. In addition, in line with the current consensus, although an overdose of BMP-2 could lead to over-proliferation of cells, which may increase the risk of neoplasm formation and tumorigenesis, using a low dose and a slow-release delivery pattern of BMP-2 appears safe for oncogenesis-related research.

For additional investigations, researchers should pay attention to the differences between various OS cell lines and the diverse OS subtypes. These differences are responsible for the contradictory roles of BMP-2 in OS development. Caution is needed to interpret data about the function of BMP-2 in OS progression when only one subtype of OS cell line is investigated. Because of the various limitations and factors involved, the relationship between BMP-2—in particular, the supra-physiological concentration of BMP-2—and OS has not been determined thoroughly; more research in this field is necessary.

AUTHOR CONTRIBUTIONS

CX contributed to the conception of this work and drafted the manuscript. MW performed literature search. BZ-D made important revisions. WS revised this manuscript and polished language. LW revised this manuscript and supplemented it with important information. YL edited and revised this manuscript and ultimately approved the publication. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Dutch NWO grant (No. 729001041) and Dutch ZonMW grant on LSH 2TREAT (No. 436001004).

ACKNOWLEDGMENTS

We thank Prof. Changsheng Liu for the constructive suggestions in the completion of this review. We are also grateful for the contribution from the Shandong Taishan Scholar Program to YL.

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