BIOLOGY AND PHARMACOLOGICAL EFFECTS OF EXTRACELLULAR VESICLES IN CANCER

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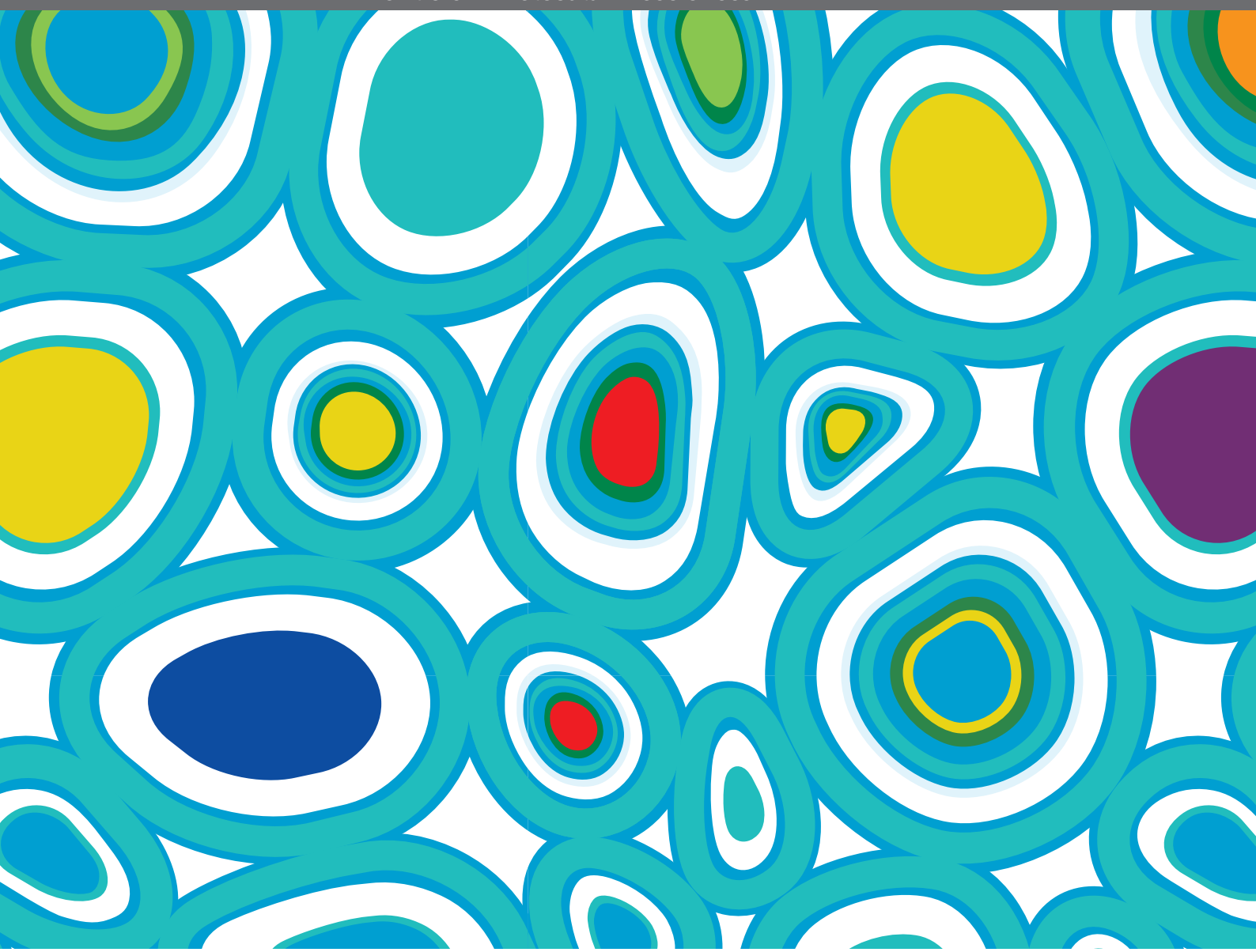

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BIOLOGY AND PHARMACOLOGICAL EFFECTS OF EXTRACELLULAR VESICLES IN CANCER

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EDITORIAL
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Editorial: Biology and Pharmacological Effects of Extracellular Vesicles in Cancer

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Keywords: extracellular vesicles, contents, biology, pharmacological effects, cancer

Editorial on the Research Topic

Biology and Pharmacological Effects of Extracellular Vesicles in Cancer

The term extracellular vesicles (EVs) generally refers to various nanoscale membrane vesicles secreted by most eukaryotic cells into extracellular environment. EVs include exosomes, microvesicles, and apoptotic bodies. EVs have attracted numerous attention of biomedical investigators and their roles in intercellular communication in multiple physiological and pathological processes have been widely studied. This Research Topic collates the research findings which illustrate the biological and pharmacological roles of EVs in cancer. The topic consists of 14 articles, including 8 review articles and 6 original research articles, contributed by more than 128 authors in the fields of cancer pharmacology and therapeutics. Our goal was to reveal the detailed molecular mechanism of EVs in mediating tumorigenesis and development, and to open new approaches for the clinical therapeutics of cancer.

EVs contain various bioactive molecules, including proteins, lipids, mitochondrial DNA, RNAs and metabolites. Among them, non-coding RNA (ncRNA), especially microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have been extensively investigated in cancer migration, metastasis, drug resistance, and immunosuppression. Zheng et al. found that gastric cancer-derived exosomal miR-590-5p inhibited gastric cancer cell migration and invasion *in vitro*. In addition, serum exosomal miR-590-5p expression was significantly low in gastric cancer patients, and the expression of miR-590-5p was strongly associated with the TNM stage and the survival rate of gastric cancer patients. A study performed by Xuan et al. showed that exosomal miR-549a derived from tyrosine kinase inhibitors (TKI)-resistant renal cancer possessed a stronger ability to promote vascular permeability, angiogenesis and tumor lung metastasis in nude mice model, compared with sensitive tumor cells. Their mechanistic studies found that exosomal miR-549a regulated the VEGFR2-ERK-XPO5 pathway mainly by activating HIF1α. In line with the article of Xuan's group, the review of Huang et al. discussed recent reports on tumor-derived EVs and their cargoes, especially ncRNAs and proteins, on tumor angiogenesis and their mechanisms. Xue et al. provided evidence that miR-317b-5b-loaded engineered exosomes could be internalized by tumor cells, subsequently inhibiting cell proliferation, migration, invasion, and inducing cell apoptosis.

In their review articles, Shan et al. discussed the different roles of M1 and M2 subtype macrophages-derived exosomes in tumor progression. M2 macrophages-derived exosomal miRNA could suppress proliferation, migration, invasion, and promote apoptosis of tumor cells, while M1 macrophages-derived exosomal miRNA showed diametrically opposed effects. In addition, Izadirad et al. summarized the roles of EVs-derived miRNAs in the development of acute myeloid

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leukemia and acute lymphoblastic leukemia, and discussed the prognostic value of EVs in the clinical setting of leukemia. Similarly, Zhang et al. focused on the biology and function of EVs in cancer development. These findings suggest that EVs-derived ncRNAs might become novel strategies for cancer therapy.

EVs, particularly exosomes, are considered to be an excellent drug delivery vehicle due to their membrane-enclosed structure and good biocompatibility. Liu et al. illustrated that adeno-associated virus (AAV)-containing exosomes (AAVExo) apparently improved the gene transfer efficiency in a variety of lung cancer cell types both *in vitro and in vivo* compared to conventional AAV vector. Previous studies have shown that AAV-mediated gene transfer is easily blocked by neutralizing antibodies in human serum when used in the treatment of cancer, resulting in unsatisfactory effects. Therefore, AAVExo may enabled the application of a new exosome-based vector to therapeutic treatments of lung cancers. The review of Song et al. discussed the most recent advances in exosomes as natural product delivery carriers in cancer therapy. They gathered evidence that exosomes loaded with natural products such as paclitaxel, curcumin, doxorubicin, celastrol, and β-elemene showed enhanced anti-tumor efficacy. Consistently, in another review, Shan et al. summarized that macrophages-derived exosomes loaded with paclitaxel or adriamycin showed enhanced anti-tumor effects. Wu et al. reviewed that the use of exosomes to control ferroptosis in targeted cells is promising for cancer therapy. The authors summarized an opinion that ferroptosis-inducing drugs such as erastin and newly recognized natural ferroptosis-inducing compounds could be loaded onto exosomes to provide new strategies and approaches for tumor therapy.

Crosstalk between EVs and the tumor microenvironment has important implications for cancer migration and metastasis. The review of Dong et al. highlighted that EVs was critical for the formation of tumor pre-metastatic niche. Tumor-derived EVs promoted tumor cells colonization in distant organs through increasing vascular permeability, extracellular matrix remodeling, angiogenesis and immunosuppression. Similarly, Bao et al. also discussed the biological functions of tumor-derived EVs in reprograming tumor microenvironment.

Interestingly, Sadovska et al. found that exercise-induced EVs could delay the progression of prostate cancer. RNA-sequencing analysis showed substantial changes in the RNA content of EVs collected before and after exercise in rats. Exercise-induced EVs significantly inhibit lung metastasis of prostate cancer cells in rats. Therefore, the research supported the idea that regular physical exercise should be prescribed to prostate cancer patients as a tertiary prevention measure. Bandini et al. focused on potentially useful biomarkers in breast cancer-derived EVs for diagnosis and monitoring. They identified 11 biomarkers in plasma EVs that could be used to significantly distinguish healthy subjects from breast cancer patients, including CD3, CD56, CD2, CD25, CD9, CD44, CD326, CD133/1, CD142, CD45, and CD14.

In summary, the Research Topic of "Biology and Pharmacological Effects of Extracellular Vesicles in Cancer" highlights the important roles of EVs in regulating tumor proliferation, migration, metastasis, immune escape, inflammatory response and drug resistance. With the continuous in-depth study on the mechanism by which EVs regulate the biological behavior of tumors, EVs-based therapy will become a new avenue of cancer treatment.

AUTHOR CONTRIBUTIONS

J-yZ drafted the manuscript. J-JQ, DZ, and D-HY revised the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Exosomal miR-590-5p in Serum as a Biomarker for the Diagnosis and Prognosis of Gastric Cancer

Guo-Dian Zheng[1†], Zhi-Yuan Xu[2†], Can Hu[3], Hang Lv[4], Hua-Xia Xie[5], Ting Huang[6], Yan-Qiang Zhang[2], Gui-Ping Chen[1], Yu-Fei Fu[4] and Xiang-Dong Cheng[2]*

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The purpose of this study is to explore the expression of miRNA-590-5p, an exosome of gastric cancer (GC), and to evaluate the suitability of miR-590-5p, an exosome with its own clinical characteristics. Serum samples from 168 gastric cancer patients and 50 matched controls were collected and exosomal RNAs were extracted. After that, miR-590-5p is analyzed by quantitative polymerase chain reaction (qRT-PCR), which is more related to clinical and pathological parameters and patient monitoring data. MGC-803 and HGC-27 cells were treated by miR-590-5p mimics, and then the changes of cell fluidity and invasiveness were monitored. The results showed that the expression level of miR-590-5p in exosomes of healthy observation group, early stage (I and II) group, and late stage (III) group was 30.34 ± 6.35, 6.19 ± 0.81, and 2.9 ± 0.19, respectively (all $p < 0.05$). ROC (receiver-operating characteristic curve) showed that the AUC (area under the curve) of exosomal miR-590-5p was 0.810 with 63.7% sensitivity and 86% specificity. The expression of exosomal miR-590-5p in serum was related to clinical stage ($p = 0.008$), infiltration depth, and the expression level of ki-67 ($p < 0.001$). In addition, Kaplan-Meier analysis showed that the decrease of explicit level of exosomal miR-590-5p was related to the decrease of overall survival (OS) rate ($p < 0.001$). Cox regression analysis showed that miR-590-5p can be used as an independent predictor. Furthermore, upregulation of miR-590-5p inhibited cell migration and invasion in MGC-803 cells and HGC-27 cells. The serum expression level of exosomal miR-590-5p may be a biomarker, which is potentially useful and noninvasive for early detection and prediction of GC. In addition, miR-590-5p can play a role in eliminating carcinogens by actively regulating the malignant potential of gastric cancer.

Keywords: exosome, miRNA-590-5p, gastric cancer, serum, biomarker

INTRODUCTION

The fourth most common malignant tumor in the world is gastric cancer (GC). GC is still the third leading cause of cancer-related death in the world, because the early diagnosis of gastric cancer is stagnant, there is no improvement, and there is no ideal treatment strategy (Ferlay et al., 2015). Increasing the accuracy of detection of gastric cancer biomarkers can reduce their mortality. Although the development of new biomarkers in blood tests has shown great potential, the development of clinical validation of effective cancer detection markers remains a challenge for a variety of human cancers (Iorio and Croce, 2009). Therefore, looking for a more accurate representation of GC biology features and better diagnostic biomarkers is very important and valuable for the screening of early GC in addition to predicting clinical outcomes, which will allow more patients to receive curative surgery.

MicroRNA (miRNA) is a short-chain noncoding RNA molecule (about 22 nucleotides in length). It regulates protein expression of a particular mRNA by incomplete base pairing, causing inhibition of protein translation of the target gene (Bartel, 2004; Carthew and Sontheimer, 2009). Because miRNAs play a role in carcinogenesis or tumor inhibition during tumor development, research on it has been extended to many types of tumors. Recent studies have shown that abnormal expression of mature miRNAs may be helpful for early detection of gastric cancer (Chen et al., 2014; Fu et al., 2014; Zhang et al., 2015). For example, Rui Liu et al. identified 21 miRNAs which were found differentially expressed in GC. Five kinds of serum miRNAs (miR-20a, miR-34, miR-27a, miR-423-5p, and miR-1) were compared with the control group (Liu et al., 2011).

It is actively secreted by various living cells, and foreign bodies are a group of vesicles of size 50–150 nm. They have physiological functions including immune modulation (Pan et al., 1985; Gross et al., 2012). In the process of inward budding of endosomes in late stage, they develop into intracellular multivesicular endosomes. Besides that, exosomes nucleic acids and proteins exist in exosomes (Trajkovic et al., 2008; Demory Beckler et al., 2013), thereby acting as essential medium for intercellular communication (Bang and Thum, 2012; Waldenstrom and Ronquist, 2014). Separating and identifying specific foreign substances of cancer in body fluids, and subsequently identifying DNA, nucleic acids, and proteins without foreign noncancer pollution, can contribute to the diagnosis and treatment of cancer. It has been found that exosomes prevent their miRNAs from being degraded by RNase (Koga et al., 2011) and remain stable for 5 years at minus 20°C, even after 2 weeks at 4°C. Moreover, they are resistant to freeze-thaw cycles (Weber et al., 2010). Because of its ease of access and stability, exosomal miRNA is considered as a new and slightly invasive cancer diagnosis tool, which may have precalculated value. MiR-590-5p can play a role in carcinogen or tumor inhibitor of vulvar cancer and rectal cancer (Zhou et al., 2016; Yang and Wu, 2016). However, the relationship between the expression of exosomal miR-590-5p and the clinical features of gastric cancer has not been reported, and the potential correlation between the expression of exosomal miR-590-5p and the treatment and prognosis of gastric cancer needs further study.

The purpose of this study is to explore the clinical significance of serum miR-590-5p and its role in metastasis and invasion of infectious cancer cells. Our current research results show that miR-590-5p can limit the spread and invasion of GC cells, so it may be a potential biological indicator of GC cells being attacked.

PATIENTS AND METHODS

Clinical Samples

168 patients with gastric cancer who visited Zhejiang Cancer Hospital from March 2008 to November 2011 were included in the gastric cancer group. The average age of 168 patients with gastric cancer was 61 years (31–86 years). All patients with gastric cancer were confirmed by histopathology, and the tumor stage was determined according to International Union Against Cancers (UIAC) tumor-node-metastasis (TNM) system. Patients who suffered from other cancers were excluded from this study. A healthy control group of 50 volunteers, who visited the hospital for physical examination were enrolled; the average age was 40 years (26–59 years). Volunteers were diagnosed through internal inspection and on-site inspection. None of the patients had received chemotherapy, radiation, or other preoperative tumor treatments. Serum samples were centrifuged for 10 min at 3,000 rpm and then stored at -80°C.

Follow-up

The survival time of all patients was calculated from the date of diagnosis to the deadline for follow-up which was December 31, 2016. The follow-up period was (5.35 ± 1.52) years, and the median follow-up was 5.35 (3.61–7.67) years. During the follow-up period, 38 patients had recurrence and metastasis, where 33 cases died of GC.

Isolation of Exosomes From Serum

All of the frozen serum samples were thawed in a 25°C water bath until they were completely liquid and placed on ice until needed. Then the serum sample was centrifuged at 2000 ×g for 30 min to remove cells and debris. The upper liquid containing clear serum was transferred to a new test tube without affecting precipitation and placed in ice until it is ready for separation. Next, the required volume of clarified serum was transferred to a new tube and 0.2 volumes of the Total Exosome Isolation (from serum) reagent (cat no. 4478360; Invitrogen; Thermo Fisher Scientific, Inc.) was added; at this point the solution was thoroughly mixed by pipetting up and down until made homogenous. Samples were incubated for 30 min at an ambient temperature of 4°C and then at an ambient temperature of 10,000 ×g for 10 min. The waste liquid was inhaled and discarded, and the exosome

pellet was resuspended in 1 × PBS and subsequently stored for a short time at 4°C.

Transmission Electron Microscopy

The 400-mesh carbon coated grid was placed to float on a droplet sample for 15 s. Then, we use clean filter paper to move the grid and drain excess liquid from the edge of the grid. The grid was exposed to a drop of 2% of uranyl or phosphotungstic acid at pH 7.0 for about 8 s and excess liquid was drained off. The mosquito net was dried for 8 min. Samples were monitored under 80 kV using an JEC-1200EX microscope (Akasaka Province, Japan).

Western Blot Analysis

Pellets of exosomes were collected and dissolved in SDS-based buffer. Proteins were quantified by tetracyclic protein detection kit (Beijing Genetically Modified Organisms Technology Co., Ltd., Beijing, China). Protein samples were separated by SDS-PAGE and then transferred to a membrane containing polyvinylidene fluoride (primo bo, Massachusetts, USA). After 1 h, the membrane was separated from the selected first antigen body with 5% skim milk: 1:1,500 diluted anti-CD9 (Abcam, #ab92726) and anti-CD63 (Abcam, #ab134045) overnight at 4°C. Next, they were probed with corresponding secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. An ECL kit (Millipore) was used to reveal the immunoblots.

Exosome Quantification and Purity Assessment

The intensity, volume, and distribution of exosomes were analyzed by dynamic light scattering (DLS). Exosomes were suspended in 1× PBS and analyzed with the DLS instrument of Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). All the data were collected and repeated at least three times.

RNA Extraction From Exosome and qRT-PCR

Total RNAs including miRNAs were extracted from serum exosome using Total Exosome RNA and Protein Isolation Kit (inversion # 4478545). According to the manufacturer's instructions, c-DNA synthesis was performed using the miScript II RT kit (Qiagen, # 218161) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using the miScript SYBR green PCR kit (Qiagen, # 218075). The use of a customized miScript miRNA PCR array in a 384-well array produces the miR-590-5p expression profile (Qiagen, #CM1HS0064C), manufacturer's instructions: use ABI 7900 high-temperature real-time rapid polymerization chain reaction system, circulating at 95°C for 15 min, circulating at 94°C for 15 s, circulating at 55°C for 30 s and circulating at 70°C for 40 cycles. The threshold period data were analyzed by SDS software. And the data is standardized as ce-miR-39 (Takara Bio Company, Tokyo, Japan), a synthetic nonhuman miRNA; at the beginning of RNA isolation, in order to normalize the size of serum and determine whether our miRNA analysis by quantitative polymerase chain reaction (PCR) belongs to linear test range, ce-miR-39 will be referred to in polybrominated biphenyl insulating solution before RNA extraction. A total of 3.5 ul was added to each sample. The relative expression level of miR-590-5p was normalized to ce-miR-39, and the fold change of miR-590-5p expression relative to healthy control group was analyzed by $2^{-\Delta\Delta CT}$ method. ΔC_T and ΔC_T and $\Delta\Delta C_T$ are calculated using the following formula:

$$\Delta C_T = C_{T\,\text{sample}} - C_{T\,\text{ce-miR-39}},$$
$$\Delta\Delta C_T = \Delta C_{T\,\text{case}} - \Delta C_{T\,\text{control}}.$$

Cell Culture

Among the cancer cells listed in MGC-803 and HGC-27, there are cells from Chinese Academy of Sciences. These cells grow in an environment with a carbon dioxide content of 5% at a high temperature of 37°C, and 10% of active bovine serum is added in these environments, in 25 ml culture flasks.

Transfection of miR-590-5p Mimics

On the day before the transformation, MGC-803 and HGC-27 were vaccinated on six wells to ensure that 70% of the cells were integrated during the transformation. MiR-590-5p mimics were purchased from Biomics (Jiangsu, China); according to the manufacturer's instructions, Lipofectamine 2000 (Invitrogen) was used for redyeing. Oligonucleotides were used when the final concentration was 100 nM. For migration and invasion, cells were collected within 24 h after infection. As controls, all cell lines were used in regular culture conditions, incubated with Lipofectamine 2000 (Mock) or negative control (Control).

Scratch-Wound Assay

In 160 μL DMEM medium, cancer cells were inoculated with 4 × 10^5 cells/ml vaccine, and two wells (same as above, Munich, Germany) were cultured for 16 h until polymerization was achieved. After the culture was removed, the cells were washed twice with PBS and closed in DMEM containing 1% fetal bovine serum for 24 h. For each wound, blank area at specific time points after migration was measured. All healing tests were conducted three times and repeated at least five times. The closing rate is calculated using the following formula: closing rate (%) = (initial empty space - empty space after migration)/initial empty space × 100%.

Cell Invasion Assay

100 μL of cell suspension (2×10^4 cells) was added to the top chamber of a 24-well plate with 8 μm pores' membrane (Corning Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower cavity is filled with 500 microliters of DME containing 20% fetal bovine serum and small bubbles. After incubation in the

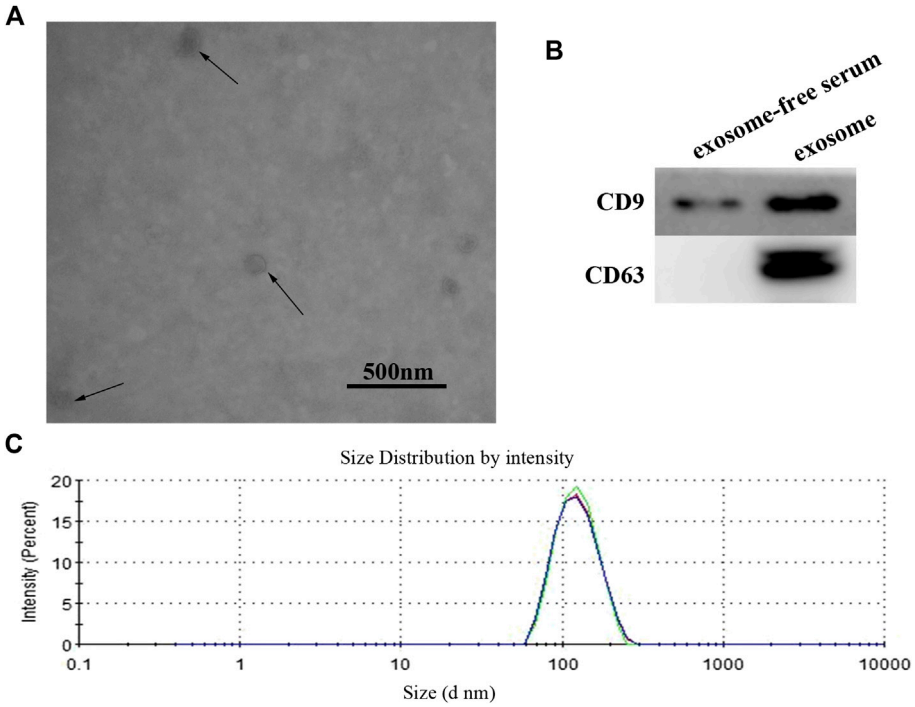

FIGURE 1 | (A) Identification and characterization of exosome. The purified exosome from serum of GC patients was observed under a transmission electron microscope (scale 500 nm). **(B)** Western blot analysis of CD9 and CD63 expression in free-exosome serum and exosomes isolated from GC serum. **(C)** The size of exosomes was determined by using the DLS analysis.

incubator for 24 h, the filter film was stirred into the methanol for 30 min and dyed in crystallized purple for 10 min. Number of cells attached to the lower surface of a polycarbonate film was counted using a high-speed microscope (×100) and averaged by using five random fields.

Statistical Analysis

The significance of exosomal miRNA-590-5p expression level difference between patients with gastric cancer and the heathy control was analyzed using nonparametric Mann-Whitney U test. The relationship between exosomal miRNA-590-5p expression and clinicopathological features was assessed using χ^2 test. Analysis of the ROC, the Kaplan-Meyer survival analysis, and Cox proportional risk model were performed using SPSS (version 24.0) and prism 8 (GraphPad software). The model was applied to the multivariate analysis to determine the independent survival forecast factor, with the p value being double and the $p < 0.05$ being considered significant.

RESULTS

Identification and Characterization of Exosomes

In order to verify the efficacy of serum exosomes separation, we analyzed the characteristics of exosomes by using TEM (transmission electron microscopy) and Western blotting. The serum exosomes showed a circular vesicle with a diameter of about 100 nm (**Figure 1A**). The exosome markers, CD9 and CD63, could be detected in isolated exosomes (**Figure 1B**). The quality of exosome preparation was further verified using dynamic light scattering (DLS), as shown in **Figure 1C**. Our results confirmed successful isolation of exosomes from serum samples.

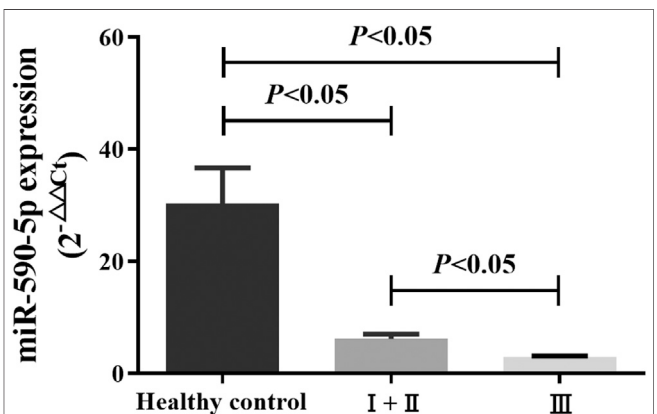

FIGURE 2 | Comparison of serum exosomal miR-590-5p in GC patients and healthy controls. Expression of serum exosomal miR-590-5p was determined by qRT-PCR in 50 healthy controls and 168 GC patients (36 patients in early stages (I and II) and 132 patients in late stages (III)). Ce-miR-39 is used as an internal reference. All data shown were the means ± SEM. $p < 0.05$.

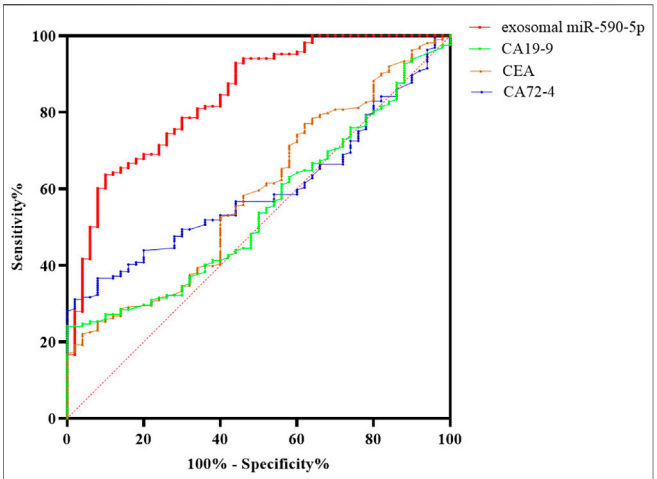

FIGURE 3 | ROC curve analysis of serum exosomal miR-590-5p to discriminate gastric cancer with exosomal miR-590-5p and different tumor biomarkers from healthy controls. The results show that the exosomal miR-590-5p produces 0.810 below the curve, with 63.7% sensitivity and 86.0% specificity. The sensitivity and accuracy of the diagnosis of GC by serum exosomal miR-590-5p were significantly higher than those of any serum tumor markers.

Serum Exosomal miR-590-5p Expression Was Significantly Lower in Gastric Cancer Patients

The level of expression of miR-590-5p in the exosome was assessed on 168 patients with GC and 50 healthy controls. Expression level of the exosome of the healthy control group miR-590-5p, early (I and II) stage group, and late stage (III) group was 30.34 ± 6.35, 6.19 ± 0.81, and 2.9 ± 0.19, respectively. The exosomal miR-590-5p expression levels (relative expression normalized by ce-miR-39) were significantly decreased in GC patients compared to the healthy controls (0.12-fold, $p < 0.05$). Moreover, we found that the level of expression of the exosomal miR-590-5p of the gastric cancer group was significantly lower than that of the healthy control group, in both the early and late stages of GC (0.20-fold, $p = 0.0063$, and 0.10-fold, $p < 0.05$, respectively; **Figure 2**). Of importance, the levels of exosomal miR-590-5p were significantly lower in the late stages than in early stage (0.47-fold, $p < 0.05$).

Diagnostic Value of Exosomal miR-590-5p in Peripheral Serum

ROC curve was plotted according to serum exosomal miR-590-5p expression. As shown in **Figure 3**, the serum exosomal miR-590-5p was worth distinguishing GC patients from healthy controls. As soon as the cutoff value reached 3.47, the ROC showed that serum exosomal miR-590-5p revealed a good classifier with an AUC of 0.810 (95% CI = 0.751–0.860) exhibiting a sensitivity of 63.7% and specificity of 86.0%. According to our results serum exosomal miR-590-5p expression may be a noninvasive diagnostic biomarker of gastric cancer.

TABLE 1 | Clinicopathological correlations of serum exosomal miR-590-5p expression in 168 gastric cancer (GC) patients.

Clinicopathologic factor	miR-590-5p expression		χ^2 value	*p* value
	Low	High		
Age			2.386	0.122
<60 years	45	35		
≥60 years	39	49		
Gender			0.253	0.615
Male	60	57		
Female	24	27		
Tumor size			0.858	0.354
<5 cm	38	44		
≥5 cm	46	40		
Tumor site			2.425	0.489
Cardia	8	4		
Body	14	12		
Antrum	23	30		
More than two parts	39	38		
Venous invasion			0.858	0.354
Absent	38	44		
Present	46	40		
Differentiation			0.869	0.351
Poor	44	50		
Well/Moderate	40	34		
T stage			23.899	<0.001
T1 + T2	5	31		
T3 + T4	79	53		
N Stage			0.136	0.712
N0 + N1 + N2	42	36		
N3	42	48		
TNM stage			6.929	0.008
I + II	11	25		
III	73	59		
Helicobacter pylori				
+	44	38	0.858	0.354
–	40	46		
Ki-67				
<40%	36	51	5.364	0.021
≥40%	48	33		
Her-2				
+	39	40	0.167	0.683
–	45	44		

The Relationship Between the Expression of miR-590-5p and Clinicopathological Factors in Gastric Cancer Patients

In order to better understand the potential role of the exosomal miR-590-5p in the development of gastric cancer, the potential association of serum exosomal miR-590-5p levels with various clinicopathological features of GC was analyzed. The 168 patients with stomach cancer were divided into high expression groups and weak expression groups with the expression value of the intermediate exosomal miR-590-5p as a tangent point. **Table 1** summarizes the relationship between the level of expression of the exosomal miR-590-5p and the clinical characteristics of gastric cancer. The results showed that the serum level of

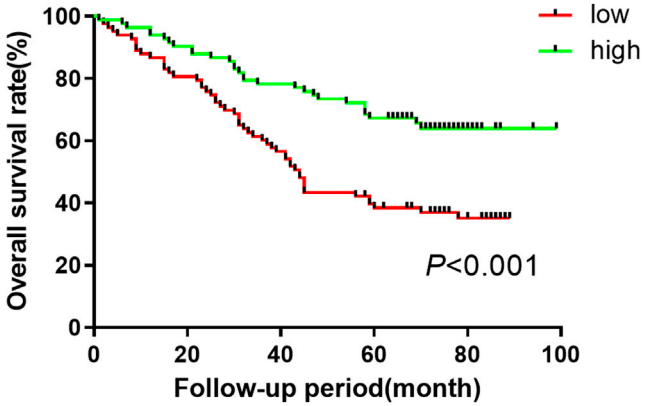

Figure 4 | Kaplan-Meier curves of overall survival for GC patient based on serum miR-590-5p expression. Log-rank tests are used for relatively low and high survival rates of patients in the miR-590-5p expression group based on the median of the miR-590-5p expression.

exosomal miR-590-5p in patients with gastric cancer was strongly related to the TNM phase (p = 0.008), the depth of infiltration, and the expression level of ki-67 (p <0.001). However, there is no significant correlation between expression of exosomal miR-590-5p and other clinical pathological characteristics such as age, sex, tumor size, tumor part, intravenous infiltration, cell differentiation, lymph node transfer, Helicobacter pylori, and the expression level of her-2 (all at p > 0.05).

Exosomal miR-590-5p Expression in Serum Is Related to Survival Rate of Gastric Cancer Patients

In order to assess the precalculated value of exosomal miR-590-5p in patients with GC, patients were dichotomized into two groups of high or low expression level as previously mentioned. The log-rank test suggested that the high expression group appeared to show improvement of overall survival (60.3 months) compared to the low expression group (47.3 months for overall). Moreover, the results showed a significant association between low expression exosomal miR-590-5p and poor survival (p < 0.001, **Figure 4**), indicating that the exosomal miR-590-5p can be used as a potential prognosis indicator for patients with stomach cancer. In addition, as **Table 2** shows, analysis of the Cox proportional risk regression model, a single variable, shows that the OS is strongly related to tumor size (p = 0.003), invasion depth (p < 0.001), clinical stage (p = 0.006), and exosomal miR-590-5p level (p < 0.001). In multivariate analysis, expression level of exosomal miR-590-5p remained significant (p = 0.013). The other independent prognostic factor was the cancer invasion depth (p = 0.005).

Improving the Level of miR-590-5p Expression by Mimics

To monitor the expression of miR-590-5p in gastric cancer cells, miR-590-5p mocks are delivered to HGC-27 and MGC-803 cells. 24 h after the infection, the expression level of miR-590-5p was detected through qRT-PCR. Expression of miR-590-5p among cells transmitted by miR-590-5p mimic increased by about 45 times (**Figure 5**). These results were used as the basis for determining subsequent experiments.

TABLE 2 | Univariate and multivariate analysis of prognostic parameters in patients with GC by Cox regression analysis.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age				
≥60 vs. <60 years	1.01 (0.66–1.56)	0.960		
Gender				
Male vs. female	0.90 (0.56–1.46)	0.669		
Tumor size			1.296	
≥5 cm vs. <5 cm	1.94 (1.26–3.00)	0.003	(0.805–2.087)	0.285
Venous invasion				
Present vs. absent	1.36 (0.88–2.10)	0.168		
Differentiation				
Poor vs. well/ Moderate	1.34 (0.87–2.06)	0.194		
Invasion depth			3.569	
T3+T4 vs. T1+T2	4.73 (1.71–4.52)	<0.001	(1.466–8.692)	0.005
Lymph node status			1.512	
N3 vs. N0+N1+N2	1.36 (0.88–2.10)	0.167	(0.958–2.388)	0.076
TNM stage				
III vs. I+II	2.46 (1.24–3.34)	0.006		
miR-590-5p expression			0.558	
Low vs. high	2.31 (1.51–3.61)	<0.001	(0.351–0.886)	0.013

Abbreviations: CI = confidence interval; HR = relative risk.

MiR-590-5p Inhibited Gastric Cancer Cells Migration and Invasion *In Vitro*

In order to study the role of miR-590-5p in gastric cancer metastasis, the scratch experiment and transwell experiments were performed to see if miR-590-5p is associated with the movement and encroachment of gastric cancer cells. As shown in **Figure 6**, the migration and attack capacity of the MGC-803 and HGC-27 cells was significantly reduced after the conversion of the miR-590-5p mimics. These observations show that high expression of miR-590-5p may play an important role in stemming the spread and invasion of gastric cancer cells.

DISCUSSION

As it is reported that miRNA can be detected in the serum and express itself regularly at room temperature and in many

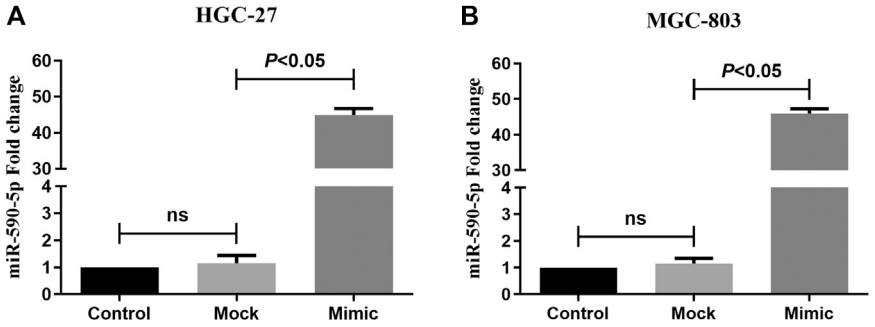

FIGURE 5 | The expression of miR-590-5p in HGC-27 and MGC-803 cells. Compared to mock and control, cells that were transformed with the miR-590-5p simulation showed a higher miR-590-5p expression. ns $p > 0.05$.

freezing cycles, it is widely accepted that circular miRNAs can be used as new noninvasive biological markers of cancer, prostate cancer and colorectal cell carcinoma (Wang et al., 2015; Fabris et al., 2016; Nagata et al., 2016; Schou et al., 2016). More than 100 miRNAs have been found to be aberrantly expressed in GC. However, there is still no description of exosomal miRNAs in GC; here we reveal that high exosomal miR-590-5p expression was significantly better for prognosis of GC. By targeting mRNA translation, microRNA can regulate cell proliferation, invasion, and migration. Due to the diversity of miRNAs and the complexity of the regulatory mechanism, it is difficult to determine whether a particular miRNA is carcinogenic or tumor suppressive (Svoronos et al., 2016). MiR-590-5p is not unique to many types of substances but is also overexpressed in the SW480 cell line and was found to inhibit SMAD3 protein expression (Jafarzadeh and Soltani, 2016). Jiang and others found that the decline in miR-590-5p resulted in an increase in TGF-beta RII and inhibited the proliferation and invasion of HepG2 cells (Jiang et al., 2012). Moreover, it has been shown that miR-590-5p can exert oncogenic activity in cervical carcinoma by targeting the CHL1 gene (Chu et al., 2014). However, in this study, the level of serum expression of miR-590-5p in patients with gastric cancer is significantly lower than that of the healthy control group. This inconsistency may be due to the differences in sample origin and the tumor clinicopathological characteristics. Moreover, the advanced level of miR-590-5p is also significantly lower than in the early stages, suggesting that exosomal miR-590-5p could be a promising biological marker for early gastric cancers, and yet, further validation is required to support this proposal.

ROC curves are widely used to assess diagnostic performance. In the current data, we compare serum exosomal miR-590-5p with traditional tumor markers CA72-4, CEA, and CA19-9 in serum, in the diagnosis of GC. Yet traditional serum tumor markers show a poor sensitivity and specificity; serum exosomal miR-590-5p achieved good diagnostic efficacy by distinguishing GC patients with exosomal miR-590-5p and different tumor markers from those with health control, with an AUC of 0.810 (sensitivity = 63.7%, specificity = 86%). Furthermore, it showed statistical significance with the clinical stage of GC. From reviewing and analyzing previous studies to our findings, serum exosomal miR-590-5p indicated a better sensitivity and specificity than serum tumor biomarkers which had reportedly low specificities and sensitivities (Schneider and Schulze, 2003; Yang et al., 2016); in addition, we were surprised to observe a negative correlation of exosomal miR-590-5p levels with increased ki-67 protein levels which is recognized to reflect the proliferation of tumor cells. However, this miRNA has no statistically significant correlation with *Helicobacter pylori* and her-2 protein levels which are independent risk factors affecting the prognosis of GC. The Kaplan-Meier analysis and Cox's multivariate regression analysis showed that the low level of exosomal miR-590-5p reflected a much less favorable prognosis ($p < 0.001$). This suggests that the serum exosomal miR-590-5p might be a potential marker of poor prognosis in GC. Although lymph node status revealed a trend association without statistical significance in univariate analysis, after examining the depth of immersion, the level of serum exosomal miR-590-5p is significant as an independent prognostic factor through a Cox multivariate regression analysis, pathological grade, and lymph node status.

Given that the low serum level of miR-590-5p in patients with gastric cancer is related to the depth of infiltration, we further study the role of miR-590-5p in the cell transfer of human gastric cancer. Compared to the control group and the simulated group, after transfer with the miR-590-5p simulation, MGC-803 and HGC-27 cells were detected using the retroviral polymerase chain reaction. MiR-590-5p overexpression correlated with the decreased migration and invasiveness of MGC-803 cells and HGC-27 cells, indicating that it possessed a cancer suppressing role in GC. It is necessary to further explore the biological mechanism of miR-590-5p. Some research reports have shown miR-590-5p affects tumor cell epithelial-mesenchymal-transition (EMT), which plays an important role in tumor progression, and some related marker proteins such as β-catenin, N-cadherin, and Snail1 have changed (Jin et al., 2018; Khandelwal et al., 2019). We need to further study the role of miR-590-5p on the target genes of GC cells, which provides a new way for us to study the

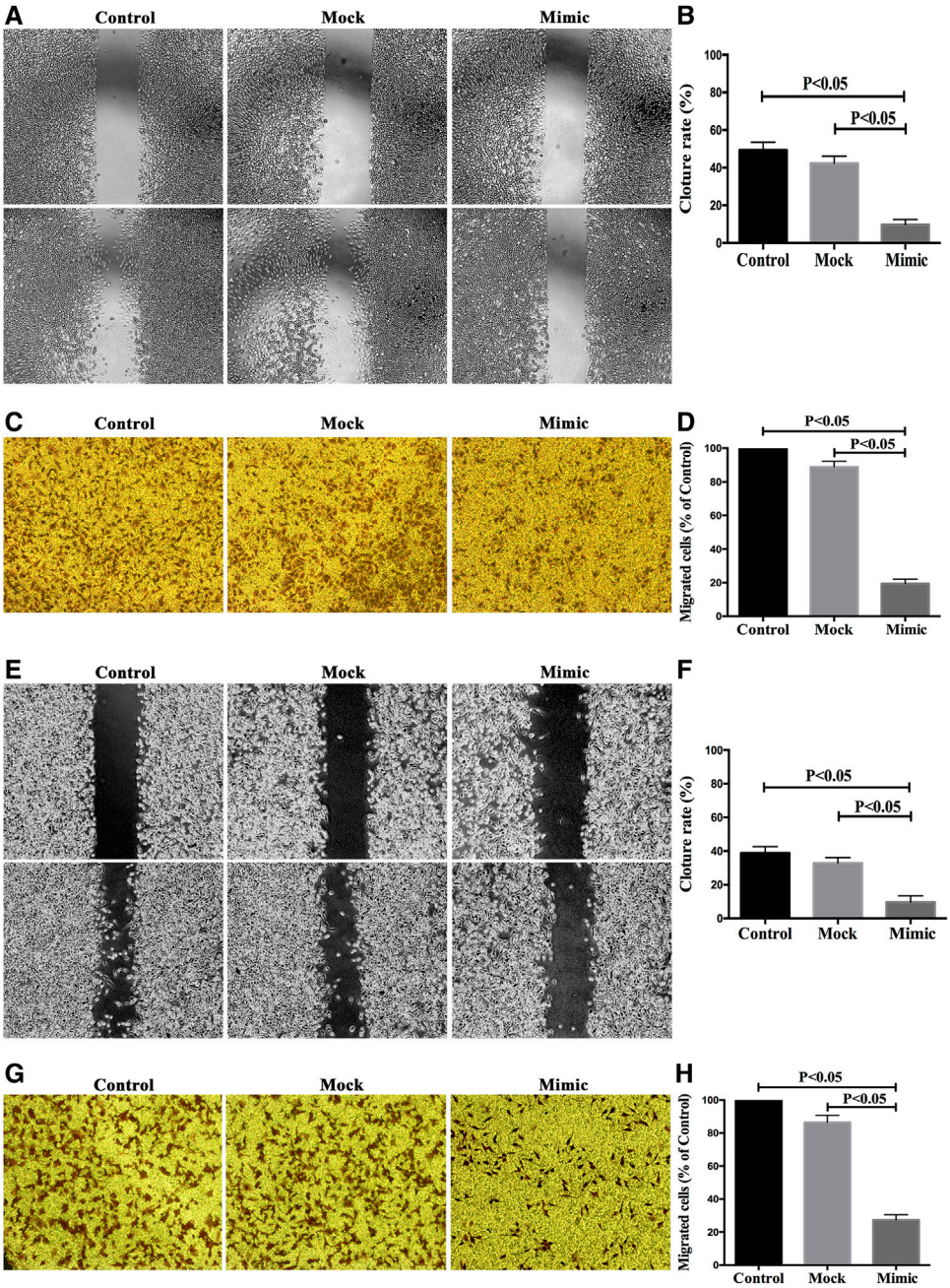

FIGURE 6 | The overexpression of miR-590-5p inhibits *in vitro* the migration and intrusion of MGC-803 and HGC-27 cells. Effect of miR-590-5p on cell mobility and attack capability of MGC-803 and HGC-27 cells. The number of cells was observed under an inverted microscope 100 times larger and counted in each field. Three independent experiments were conducted. The results are indicated by the average standard deviation.

diagnosis, prognosis, and gene therapy of GC. We acknowledge that the study may have been more persuasive if larger samples had been used in the groups. Furthermore, the exact mechanism by which cancer cells secreted exosome-containing specific miRNAs has not been elucidated in detail. In order to clarify the biological mechanism of serum exosomal miR-590-5p for patients with gastric cancer, further research on this topic is needed.

CONCLUSION

The study showed that serum exosomal miR-590-5p might be a potential biological marker for the early detection of GC. Its downregulation may be related to poor prognosis in GC, which suggests that exosomal miR-590-5p could serve as promising biomarkers of further risk analysis for GC. This hopeful result has led to further research into the ambiguous mechanism of

exosomal miR-590-5p as an intercellular messenger to regulate the invasion and transfer of gastric cancer.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Zhejiang Cancer Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception or design of the work was done by X-DC, G-DZ, and Z-YX; drafting the work was done by G-DZ, Z-YX, and CH; data acquisition was done by GZ, ZX, TH, and HL; data analysis was done by G-DZ, Z-YX, H-XX, Y-QZ, Y-FF, G-PC, and CH; supervision or mentorship was done by X-DC, G-DZ, and Z-YX. All the authors contributed important intellectual content for the overall work. X-DC takes responsibility for the honesty and accuracy of the present study.

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REVIEW
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Exosome-Based Delivery of Natural Products in Cancer Therapy

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A rapidly growing research evidence has begun to shed light on the potential application of exosome, which modulates intercellular communications. As donor cell released vesicles, exosomes could play roles as a regulator of cellular behaviors in up-taken cells, as well as a delivery carrier of drugs for targeted cells. Natural product is an invaluable drug resources and it is used widely as therapeutic agents in cancers. This review summarizes the most recent advances in exosomes as natural product delivery carriers in cancer therapy from the following aspects: composition of exosomes, biogenesis of exosomes, and its functions in cancers. The main focus is the advantages and applications of exosomes for drug delivery in cancer therapy. This review also summarizes the isolation and application of exosomes as delivery carriers of natural products in cancer therapy. The recent progress and challenges of using exosomes as drug delivery vehicles for five representative anti-cancer natural products including paclitaxel, curcumin, doxorubicin, celastrol, and β-Elemene. Based on the discussion on the current knowledge about exosomes as delivery vehicles for drugs and natural compounds to the targeted site, this review delineates the landscape of the recent research, challenges, trends and prospects in exosomes as delivery vehicles for drugs and natural compounds for cancer treatment.

Keywords: exosome, natural product, cancer, therapy, delivery

INTRODUCTION

Cancer is one of the major treats to human life worldwide. In the Western world, the mortality of cancer has decreased, but cancer mortality remains high in the developing and underdeveloped countries. In 2012, 64.9% of cancer-related deaths occurred in underdeveloped regions (Ferlay et al., 2013). The cost of cancer care is high, which limits proper cancer treatment. In recent years, natural products have been proven to have various anti-cancer properties, including inhibiting cell proliferation, inducing cell apoptosis or autophagy, interfering with cancer angiogenesis, invasion or metastasis, and modulating epigenetic modifications (Wang et al., 2010; Zhuang et al., 2012). Using natural products for cancer management is an appealing alternative to overcome expensive cancer care, especially in developing or underdeveloped countries.

Numerous studies have shown that natural products have poor solubility, rapid biotransformation and low bioavailability *in vivo*, that limit their pharmacological activities (Brglez Mojzer et al., 2016; Huang et al., 2018; Zhang et al., 2019). For example, cuicumin, one of the natural products shown to have multiple-pharmacological roles, is reported to have a low plasma concentration, extensive and rapid biotransformation, and poor oral bioavailability (Huang et al., 2018). Magnolol, a hydroxylated biphenyl natural compound, was reported to have multiple-pharmacological characteristics including anti-inflammatory, anti-microorganism, anti-oxidative, anti-cancer, neuroprotective, and cardiovascular protective effects. Yet, it also has low water solubility, low bioavailability, and rapid metabolism (Zhang et al., 2019). Polyphenols, as secondary plant metabolites, are reported to have many advantages for anti-cancer effects such as high accessibility, low toxicity, and specificity of response, but have limited usage in clinics because of their poor bioavailability and rapid metabolism (Brglez Mojzer et al., 2016). Therefore, it would be useful to find a new drug delivery system to improve the bioavailability of natural products *in vivo*.

Nanotechnology has been employed for drug delivery for increasing bioavailability of therapeutic agents. Unfortunately, drug nanoformulations often lead to toxicity and are usually rapidly cleared by the mononuclear phagocytic system (MPS) (Peng et al., 2013). Although PEGylation of drug-loaded nanocarriers could reduce the clearance by the MPS, it reduces the biodistribution of drug in disease tissues (Veronese et al., 2002). Moreover, rapid generation of anti-PEG antibodies following repeated injections of PEGylated nanoparticles would result in extended blood clearance and decreased efficacy of nanoformulations (Gabizon, 2001). Furthermore, biological barriers reduce the bioavailability and limit the therapeutic efficacy of nanoformulations (Blanco et al., 2015). Therefore, new targeted deliveries of drugs should be studied to avoid the clearance and overcome the biological barriers.

Exosomes have emerged as drug delivery vehicles. Exosomes deliver nucleic acids (Pan et al., 2012; Wahlgren et al., 2012), proteins (Haney et al., 2015), and small molecule drugs, such as doxorubicin (Tian et al., 2014). As delivery vehicles, exosomes deliver their payload to target cells or tissues, and diminish the MPS-mediated clearance (Wiklander et al., 2015). Moreover, siRNA could be delivered across the blood-brain barrier by exosomes to the central nervous system (Alvarez-Erviti et al., 2011). These results demonstrate that exosomes may be a promising alternative to nanoparticles as drug delivery vehicles (Parodi et al., 2017). The focus of this review is the anti-cancer application of natural products delivered by exosomes.

THE COMPOSITION OF EXOSOMES

Chargaff and West (1946) reported that plasma clotting was inhibited by the removal of the pelleted plasma fraction. Subsequently, Wolf (1967) reported that these clotting suppressors are vesicles in the range of 20–50 nm secreted by platelets. Since then, a number of studies have indicated the existence of extracellular vesicles. Extracellular vesicles include three forms: exosomes, microvesicles, and apoptotic bodies (Gyorgy et al., 2011). Exosomes are 30–150 nm in diameter, and are secreted by various kinds of cells including dendritic cells (Thery et al., 2006), macrophages (Bhatnagar et al., 2007), B cells (Clayton et al., 2005), T cells (Nolte-'t Hoen et al., 2009), mesenchymal stem cells (Lai et al., 2015), endothelial cells (Song et al., 2014), and epithelial cells (Skogberg et al., 2015), and a variety of cancer cells (Benito-Martin et al., 2015).

The contents of exosomes include lipids, nucleic acids, and various proteins such as receptors, enzymes, transcription factors, and extracellular matrix proteins, that are inside or on the surface of exosomes (D'Asti et al., 2012). The lipid content is cell-specific or conserved and can protect the shape of exosomes, joins in the biogenesis of exosomes, and regulates the homeostasis of the recipient cells (Minciacchi et al., 2015). For example, lysobisphosphatidic acid (LBPA) was reported to interact with Alix regulating the invagination of the endosomal membrane (Laulagnier et al., 2004) and result in the formation of exosomes (Chu et al., 2005; Bissig et al., 2013). However, the protein contents of exosomes can be divided into a specific type and a non-specific type (Van Niel et al., 2006). The specific type of proteins include integrins, tetraspanins, adhesion molecules, transferrin receptors, and major histocompatibility complex (MHC) class I and II (Van Niel et al., 2006). The non-specific type of proteins include transferring proteins and fusion, cytoskeleton proteins, and heat shock proteins (Van Niel et al., 2006; Poliakov et al., 2009) (**Figure 1**).

These contents of exosomes can reflect the composition of the donor cell and the mechanism of physiological or pathological changes (Liu and Pilarsky, 2018). For example, antigen-presenting cells secrete the exosomes carrying T cell co-stimulatory molecules, MHC class I and class II molecules on the surface, that play important roles in antigen presentation (Schorey et al., 2015). Endothelial cells secrete exosomes containing high levels of DLL4 (delta-like-4) protein, which can activate the Notch signaling pathway and induce capillary sprouts in the neighboring microvascular endothelial cells (Sharghi-Namini et al., 2014). And miR-222 from tumor-derived exosomes can down-regulate the level of Pdlim2 resulting in enhanced metastatic capacity in breast cancer cells (Ding et al., 2018).

THE BIOGENESIS OF EXOSOMES

Unlike microvesicles budding directly from the plasma membrane, exosomes arise from the invagination of the endosomal membrane (Simons and Raposo, 2009). The first step is the fusion of primary endocytic vesicles forming early endosomes (EEs) (Huotari and Helenius, 2011). EE can either return the cargo to the plasma membrane or change into "late endosomes" (LEs) by inward budding of the membrane with the cargo packed (Mashouri et al., 2019).

The package of proteins into the intraluminal vesicles is dependent on the ESCRT (endosomal-sorting complex required for transport), which includes four complexes: ESCRT-0,

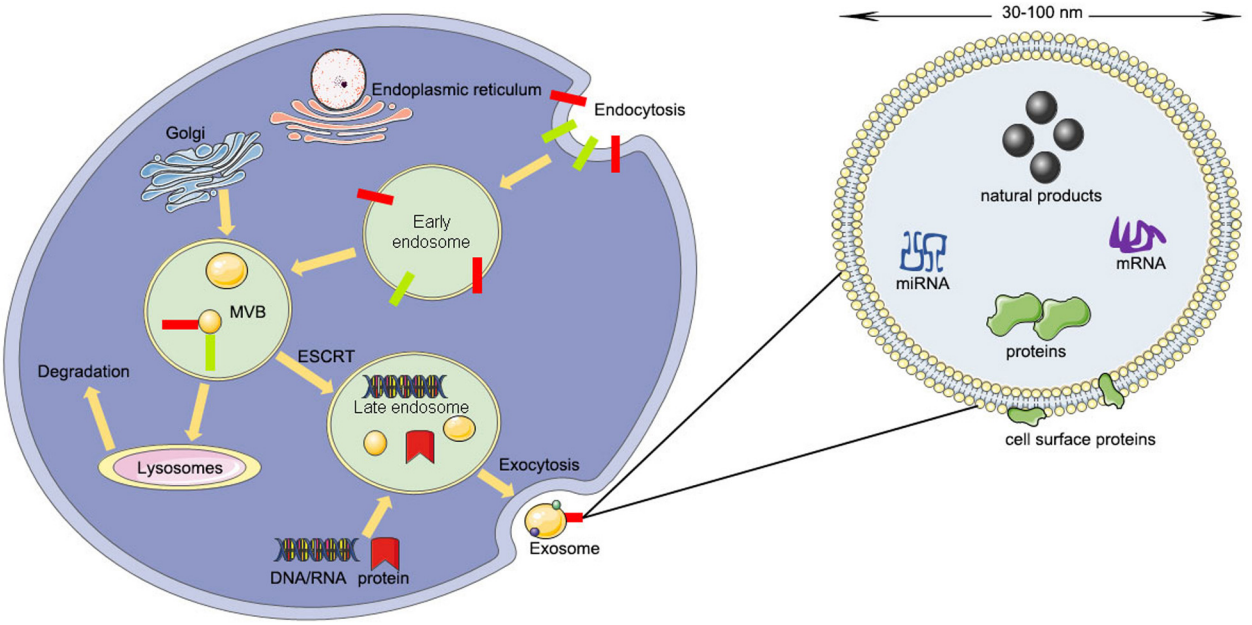

FIGURE 1 | The producing process and structural composition of exosomes (ESCRT, endosomal-sorting complex required for transport; MVB, multivesicular body).

ESCRT-I, ESCRT-II, and ESCRT-III (Mashouri et al., 2019). ESCRT-0 recognizes mono-ubiquitinated proteins with the help of an HRS heterodimer, which recruits clathrin to help ESCRT-0 encounter the ubiquitinated cargo (Ren and Hurley, 2010). Next, ESCRT-I, ESCRT-II, and ESCRT-0 constitute a recognition domain of ubiquitinated substrates (McGough and Vincent, 2016). Subsequently, ESCRT-III joins the complex, pinches off the membrane, and releases the buds into the endosome (Wollert et al., 2009). Then the intraluminal vesicles will be degraded within the lysosome unless de-ubiquitylating enzymes (DUBs) de-ubiquitinated the cargoes (Yeates and Tesco, 2016). The intraluminal vesicles can be released into the extracellular environment by moving to the plasma membrane (Kumar et al., 2016). Rab27A and Rab27B are the crucial mediators to lead the vesicles toward the cell periphery (Ostrowski et al., 2010). Finally, the membrane fusion and exosome secretion are completed by the soluble N-ethylmaleimide (NEM)-sensitive factor attachment protein receptor (SNARE) complex (Kennedy and Ehlers, 2011).

Sometimes the package of proteins into the intraluminal vesicles is carried out by the ESCRT-independent pathway. The ESCRT-independent mechanism occurs in the melanosome of melanocytes. Pmel17 is the crucial mediator in the formation of the intraluminal vesicles in an ESCRT-independent manner, which can connect its luminal domains with lipids (Theos et al., 2006). Tetraspanin CD63 is another mediator for the invagination of the melanosome membrane in an ESCRT-independent manner (Theos et al., 2006; Van Niel et al., 2011). Moreover, proteolipid proteins are delivered from the endosomal membrane to the intraluminal vesicles in an ESCRT-independent manner, which might suppress the formation of the intraluminal vesicles (McGough and Vincent, 2016).

THE FUNCTIONS OF EXOSOMES IN CANCER

Numerous studies reveal that exosomes have a wide variety of functions in cancers. First, tumor microenvironment (TME), endothelial cells, fibroblasts, and infiltrating immune cells interact with tumor cells, and these interactions are determined by the contents of the exosomes (Kohlhapp et al., 2015). Exosomes also activate the extracellular receptor signals and block cell adhesion to modulate the TME and extracellular matrix (Luga et al., 2012; Sung et al., 2015). For example, exosomal integrins take part in the initial colonization of cancer cells and the formation of a pre-metastatic niche (Paolillo and Schinelli, 2017). Exosomal miR-105 can downregulate the level of ZO-1 and destroy the barrier function of endothelial monolayers, resulting in metastasis and vascular permeability in distant organs (Zhou et al., 2014). Exosomes from cancer cells can induce differentiation of TME cells to cancer-associated fibroblasts (CAFs), that are the dominant cell population of the TME in most cancers (Webber et al., 2010) (**Figure 2**).

Second, exosomes can promote angiogenesis and induce EMT (epithelial to mesenchymal transition) (Syn et al., 2016), that favor the motility and dissemination of tumor cells. Exosomes are associated with one of the main mechanisms resulting in angiogenesis. Exosomes carry many kinds of angiogenic stimulatory factors such as VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), TGF-β (transforming growth factor β), and bFGF (basic fibroblast growth factor) (Katoh, 2013). Exosomes also induce reprogramming and modulation of endothelial cells to promote angiogenesis (Ludwig et al., 2018). Furthermore, it

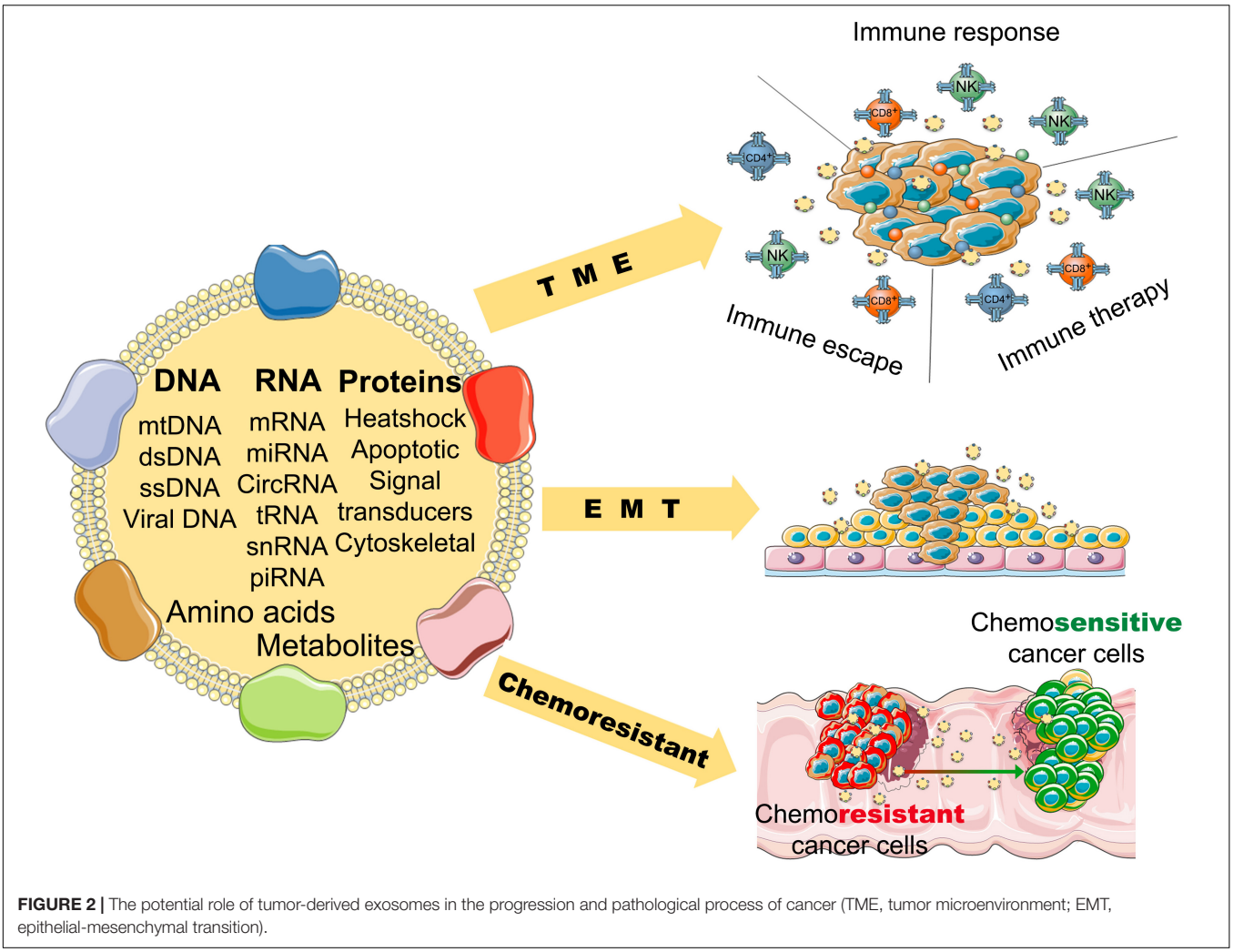

FIGURE 2 | The potential role of tumor-derived exosomes in the progression and pathological process of cancer (TME, tumor microenvironment; EMT, epithelial-mesenchymal transition).

has been reported that exosomes in pivotal position contribute to all process of EMT, form invasive phenotype to distant metastasis (Whiteside, 2017). Matrix metalloproteinase (MMP) 13-loading exosomes promote metastasis by inducing EMT in nasopharyngeal cancer cells (You et al., 2015). Exosomes derived from bladder cancer cells can promote EMT in urothelial cells by increasing the expressions of mesenchymal biomarkers, such as α-SMA, S100A4, and snail, and decreasing the expressions of epithelial biomarkers, including E-cadherin and β-catenin (Franzen et al., 2015).

Moreover, exosomes play an important role in the chemoresistance of cancers. Tumor cells can pack the chemotherapeutic drugs into exosomes and shuttle them out (Safaei et al., 2005). The contents carried by exosomes are associated with tumor drug resistance (Shedden et al., 2003). For example, miR-155 delivered by exosomes can increase EMT biomarkers to induce chemoresistance in breast cancer cells (Santos et al., 2018); miR-32-5p delivered by exosomes can cause multi-drug resistance by promoting angiogenesis and EMT (Fu et al., 2018). Tumor-derived exosomes can inhibit the response of immune effector cells and induce immune suppressor cells to modulate the TME, which results in chemoresistance of cancers (Hellwinkel et al., 2016; Syn et al., 2016). And exosomes can use a decoy to help cancer cells evade the immune effector cells (Battke et al., 2011).

THE ADVANTAGES AND APPLICATIONS OF EXOSOMES FOR DRUG DELIVERY IN CANCER THERAPY

Although exosomes have the capacity to promote the progression of cancers, exosomes show advantages in drug-delivery because of their good biodistribution, biocompatibility, and low immunogenicity. Exosomes have good tolerance because of their similarity to the cell membrane in structure and composition (Bang and Thum, 2012). Some exosomes can evade the immune system (Hood, 2016). For example, Adriamycin-loaded exosomes have minimal immunogenicity and toxicity (Tian et al., 2014). Comparison with liposomes, exosomes permeate tumor cells with higher rate (Kohlhapp et al., 2015). Because exosomes are small, they can pass

through bodily barriers. In 2011, some studies indicated the feasibility of using exosomes for drug delivery for the first time by delivering siRNA across the blood-brain barrier (BBB) using exosomes derived from dendritic cells (Alvarez-Erviti et al., 2011). Also, exosomes could promote targeting efficiency of anti-cancer drugs with easy manipulation (Li et al., 2017).

Recently, the applications of exosomes in the delivery of chemotherapeutic drugs have exhibited enhanced curative effects in cancer therapy. For example, paclitaxel-loaded exosomes can be used to treat prostate, lung, and pancreatic cancers (Saari et al., 2015). Doxorubicin-loaded exosomes also showed great efficiency in breast cancer cells (Tian et al., 2014). However, exosomes from different donor cells play different physiological functions. For example, tumor-derived exosomes can play a role of anti-tumor immunity by carrying tumor-specific antigens, proteins and miRNAs, but they can induce apoptosis of T cells, inhibit monocyte differentiation, and induce a pro-inflammatory microenvironment (Taylor and Gercel-Taylor, 2011). Exosomes from mesenchymal stem cells can regulate immunity and promote tissue repair, but they can promote tumor growth by activating tumor angiogenesis related factors (Zhu et al., 2012). Exosomes from immune cells can avoid the clearance of the immune system and prolong the retention time in the peripheral circulation (Haney et al., 2015). Milk-derived exosomes have no immune exclusion and inflammatory reaction and can improve the oral bioavailability of drugs (Ju et al., 2013). Therefore, it is necessary to select exosomes derived from appropriate donor cells when selecting exosomes for drug-delivery.

THE ISOLATION AND APPLICATION OF EXOSOMES IN THE DELIVERY OF NATURAL PRODUCTS IN CANCER THERAPY

At present, there are many methods to isolate exosomes from bodily fluids or conditioned cell culture media, such as filtration paired with centrifugation, immunoaffinity chromatography, size exclusion chromatography, polymer-based precipitation, differential centrifugation, and microfluidic technologies (Witwer et al., 2013). Among these methods, differential ultracentrifugation and density gradient centrifugation are considered to be the "gold standard" methods (Thery et al., 2006). Each method has its two sides, advantages and disadvantages, and which method is selected is dependent on the user's application. The combination of different methods can maximize advantages and avoid disadvantages compared to a single method (Stremersch et al., 2016).

Because exosomes as drug delivery carriers have good biodistribution, biocompatibility, and low immunogenicity, more researchers have begun to study their applications for enhancing the bioavailability of natural products in cancer therapy (**Figure 3**).

Paclitaxel (PTX)

PTX is a microtubule-stabilizing agent that exhibits anticancer effects in many malignant tumors, such as glioblastoma multiforme (GBM) tumors (Salarpour et al., 2019) and breast cancer (Agrawal et al., 2017). Cisplatin-resistant cancer patients

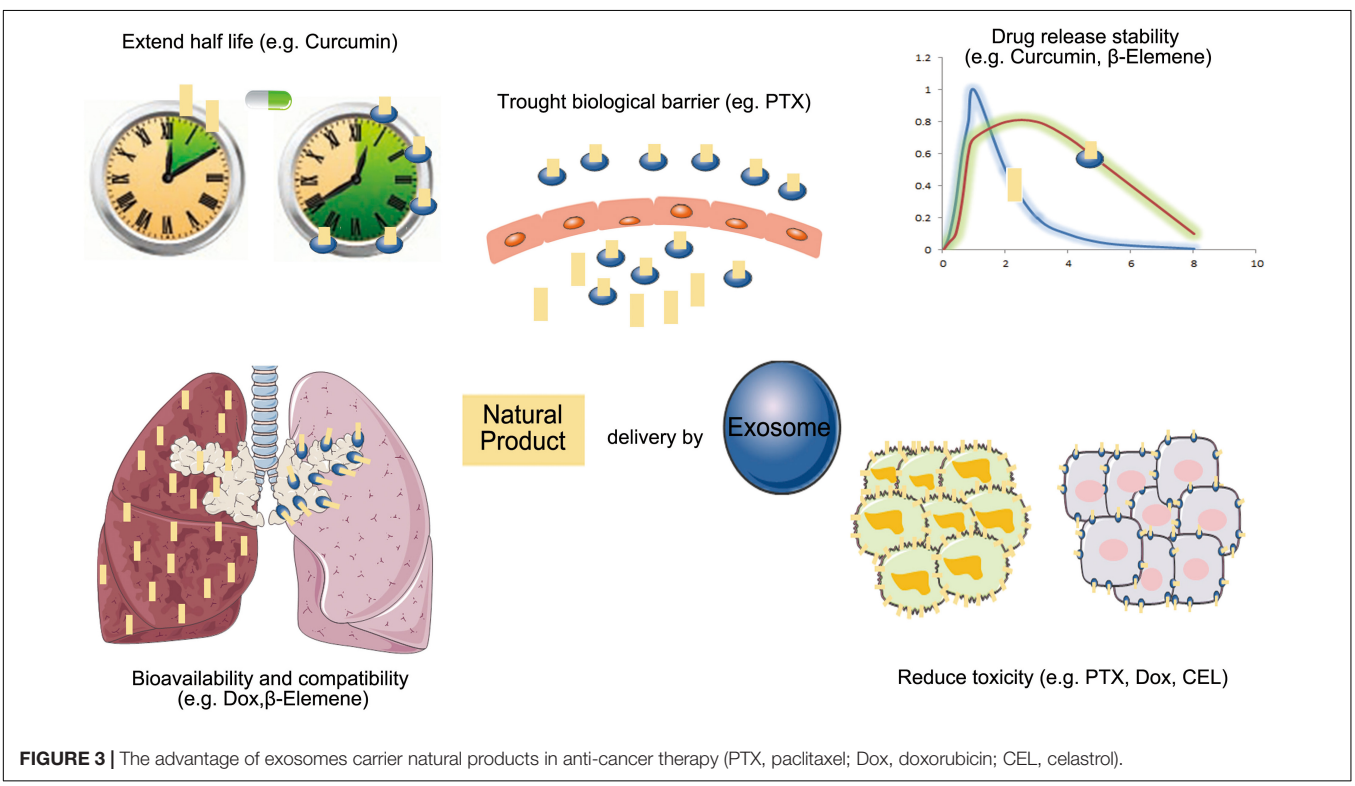

FIGURE 3 | The advantage of exosomes carrier natural products in anti-cancer therapy (PTX, paclitaxel; Dox, doxorubicin; CEL, celastrol).

often retain sensitivity to PTX (Aqil et al., 2017a). However, some studies report that PTX has low bioavailability and cannot pass through BBB (Xin et al., 2012; Mu et al., 2015). It was reported that PTX has a dose-dependent toxic effect, which hamper the application of PTX in clinical trials (Wang et al., 2019).

Research from Italy firstly presented that mesenchymal stromal cells could package PTX into exosomes and exhibit enhanced anticancer effects of PTX (Pascucci et al., 2014). Recently, various studies demonstrated that exosomes used as PTX carriers could enhance the anticancer effects of PTX. American scientist reported that milk-derived exosomes for oral delivery of PTX showed better tumor suppressor properties against human lung tumor xenografts in nude mice, and had lower systemic and immunologic toxicities as compared to i.v. PTX (Agrawal et al., 2017). Some studies reported that exosomes from M1-polarized macrophages enhanced the antitumor effect of PTX by activating macrophage-mediated inflammation in tumor-bearing mice (Wang et al., 2019). It was reported that exosomes from U-87 cells could pass through BBB and enhanced the anticancer effects of PTX in GBM (Salarpour et al., 2019). Another study indicated that embryonic stem cell-derived exosomes could improve the curative effect of PTX via enhanced targeting in GBM (Zhu et al., 2019). American researchers reported that macrophage-derived exosomes could enhance the antitumor effect of PTX in resistant cancer cells (Kim et al., 2016). They further reported that the aminoethylanisamide-polyethylene glycol-vectorized exosomes derived from macrophages possessed a high loading capacity of PTX, an enhanced ability to accumulate in cancer cells upon systemic administration, and better therapeutic outcomes (Kim et al., 2018). Moreover, it was reported that cancer cell-derived exosomes showed potential carrying capacity of PTX to their parental cells. They may bring the drug into the target cells by endocytic pathway to achieve high cytotoxicity (Saari et al., 2015).

Curcumin

Curcumin as a natural polyphenol compound can mitigate the initiation and metastasis of pancreatic, colon, breast, oral, and several other cancers (Ramayanti et al., 2018). Several clinical trials for the treatments of cancers have addressed the safety, pharmacokinetics, and efficacy of using curcumin in humans (Dhillon et al., 2008). The dominant features, inexpensive and low toxicity made curcumin ideal for clinical applications (Chen et al., 2012). However, curcumin has low bioavailability, low solubility in water, short half-life in plasma, and low stability (Salehiabar et al., 2018), which limits its usage in patients.

Previous studies showed that exosomes could enhance the anti-inflammatory activity of curcumin, and the formation of exosome-curcumin complexes could increase the stability of curcumin *in vitro* and its bioavailability *in vivo* (Sun et al., 2010). Scientist used exosomes to encapsulate curcumin and gave the exosomes-curcumin complex to a GL26 brain tumor model via an intranasal route, which significantly delayed brain tumor growth with reduced inflammation and mitigated the dysfunction of the brain endothelial cells (Zhuang et al., 2011). Previous research indicated that although exosomes derived from pancreatic cancer cells increased the proliferation of pancreatic cancer cells, curcumin-loaded exosomes induced the apoptosis of pancreatic cancer cells (Osterman et al., 2015). American scientists reported that milk-derived exosomes could enhance the antitumor activity of curcumin both *in vitro* and *in vivo* without gross or systemic toxicity (Aqil et al., 2017b). A recent study supported that both cow milk-derived and intestinal epithelial cell-derived exosomes could improve cellular uptake and intestinal permeability of curcumin, that confirm the bioavailability of an oral drug can be enhanced by the exosomes-based delivery (Carobolante et al., 2020). Furthermore, Chinese scientists loaded curcumin into exosomes, and conjugated the exosome membrane with neuropilin-1-targeted peptide to obtain glioma-targeting exosomes. These exosomes smoothly crossed the BBB and provided good results for targeted imaging and therapy of glioma (Jia et al., 2018). It has been reported that exosomes loaded with curcumin could increase the levels of claudin-5, occludin, ZO-1, and VE-cadherin, that played important roles in the integrity of cerebral tight junctions and adherent junctions (Kalani et al., 2014). Exosomes loaded with curcumin could attenuate the toxicity induced by homocysteine, a compound capable of disrupting the BBB (Kalani et al., 2014).

Doxorubicin (Dox)

Dox is one of the most effective anticancer agents and is used in a wide variety of cancers including solid tumors, transplantable leukemia, and lymphomas. However, the clinical usage of Dox is limited because of its low bioavailability and severe side effects, such as bone marrow suppression and cardiotoxicity. Although nanoparticles have been used as deliveries of Dox to increase its anti-tumor effects, nanoparticles can cause adverse effects such as immune responses and oxidative stress (Yang et al., 2015).

Recently, exosomes as natural nanoparticles have been studied to deliver Dox. Studies proved that exosomes from mesenchymal stem cells could enhance cellular uptake efficiency and anti-tumor effects of Dox in osteosarcoma MG63 cells (Wei et al., 2019). Scientists designed targeted exosomes from mesenchymal stem cells with a chimeric protein against HER2-positive breast cancer, which was used to deliver Dox to HER2-positive cancer cells, resulting in the selective distribution and enhanced antitumor effect of Dox (Gomari et al., 2019). Furthermore, researcher form China designed exosomes with disintegrin and metalloproteinase 15 expressing on exosomal membranes, and packed Dox and cholesterol-modified miRNA 159 into the modified exosomes, resulting in improved anticancer effect of Dox without adverse effects (Gong et al., 2019).

Celastrol (CEL)

CEL is a plant-derived triterpenoid and has anticancer effect against a wide variety of cancers (Li et al., 2018, 2019; Jiang et al., 2019). It can induce apoptosis of vincristine-multidrug-resistant oral cancer cells via JNK1/2 signaling pathway (Lin et al., 2019). However, due to its poor bioavailability and off-site toxicity, the clinical usage of CEL is limited (Freag et al., 2018).

CEL was packed into exosomes derived from milk and the effect of CEL-loading exosomes on lung cancer cells was studied (Aqil et al., 2016). It was found that exosomes enhanced the

anticancer effects of CEL on lung cancer *in vitro* (Aqil et al., 2016). CEL-loading exosomes are stable and could be delivered orally, exhibiting enhanced biological efficacy without gross or systemic toxicity *in vivo* (Aqil et al., 2016).

β-Elemene

β-Elemene, a natural compound extracted from Zedoary, has effects against a wide variety of tumors (Zhang et al., 2014; Gong et al., 2015; Jiang et al., 2017). It can reverse multidrug resistance and increase the sensitivity of chemotherapeutic drugs (Guo et al., 2014).

There are studies showed that β-elemene could promote the release of exosome to inhibit the growth of lung cancer cells, demonstrating that exosomes are involved in the anticancer effects of β-elemene (Li et al., 2014). Researchers used β-elemene-loaded exosomes to treat drug-resistant breast cancer cells, and found that β-elemene-loaded exosomes reverse the drug-resistance of breast cancer by down-regulating the expression of P-gp (Zhang et al., 2015).

Natural compounds can modify the contents of exosomes. For example, docosahexaenoic acid (DHA) can promote the secretion of exosomes and increase the levels of small RNA in the exosomes to inhibit pro-angiogenetic mRNAs, resulting in the suppression of tumor angiogenesis in breast cancer cells (Hannafon et al., 2015). Tea polyphenol epigallocatechin gallate (EGCG) can up-regulate miR-16 in the exosomes from murine breast cancer cells, resulting in decreased levels of CSF-1 and CCL2, two growth factors associated with tumor promoting associated macrophages (M2) (Jang et al., 2013).

CONCLUSION

Although the use of natural products can reduce the cost of cancer care, the applications are limited because of their poor solubility, rapid biotransformation, and low bioavailability. For improving the therapeutic index of natural products, their delivery should be improved. Conventional drug delivery has some disadvantages including low therapeutic index and adverse side effects. Various biological barriers prevent drugs from reaching the tumor site with an efficacious therapeutic dose. Efficient delivery of natural products should have these features, including circulation in the bloodstream without opsonization, escaping surveillance of the immune system, preserving their contents, delivering a drug into the targeted site of tissues, overcoming the biological barriers, penetrating the membranes of target cells, and minimizing accumulation at undesired sites. There are many progress in drug delivery by nanotechnology. Unfortunately, nanoparticles have some disadvantages, such as toxicity and rapid clearance. Exosomes are a promising alternative to nanoparticles because of their advantages in drug delivery, such as a high drug-carrying capacity, non-cytotoxic effects, and a low immunogenic profile. Exosomes can prolong time of circulation in the blood, reduce the levels of clearance, and protect contents from degradation or inactivation. However, the technological, functional and safety features of exosome-based drug formulations need to be further elucidated. Deficiencies in our knowledge for the molecular mechanisms of exosome biogenesis, and no method to interfere with the package of contents or with vesicle release, still hampers the identification of their physiological relevance *in vivo*.

It is a meaningful and feasible way to explore the exosome-like vesicles for delivering natural products in targeting and penetrating solid tumor with effectively therapeutic doses in clinical cancer therapy. In this review, we summarized the advantages of exosomes and showed that exosomes offer new possibilities for cancer treatment, potentially as drug delivery vehicles for the natural products. We also discuss the problems in the research of exosomes. However, exosome will still be an attractive method for delivering the natural products in the cancer treatments.

AUTHOR CONTRIBUTIONS

HS, BL, and JW participated in the design of this review and revised manuscript. BL, BD, HS, and LL wrote the manuscript. JX, HZ, SN, YL, YP, and FC collected literature and made a preliminary summary. All authors contributed to the article and approved the submitted version.

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TKI-Resistant Renal Cancer Secretes Low-Level Exosomal miR-549a to Induce Vascular Permeability and Angiogenesis to Promote Tumor Metastasis

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Tyrosine kinase inhibitors (TKI)-resistant renal cancer is highly susceptible to metastasis, and enhanced vascular permeability promotes the process of metastasis. To evaluate the effect of cancer-derived exosomes on vascular endothelial cells and clarify the mechanism of metastasis in TKI-resistant renal cancer, we studied the crosstalk between clear cell renal cell carcinoma (ccRCC) cells and human umbilical vein endothelial cells (HUVECs). Exosomes from ccRCC cells enhanced the expression of vascular permeability-related proteins. Compared with sensitive strains, exosomes from resistant strains significantly enhanced vascular endothelial permeability, induced tumor angiogenesis and enhanced tumor lung metastasis in nude mice. The expression of miR-549a is lower in TKI-resistant cells and exosomes, which enhanced the expression of HIF1α in endothelial cells. In addition, TKI-resistant RCC cells reduced nuclear output of pre-miR-549a via the VEGFR2-ERK-XPO5 pathway, and reduced enrichment of mature miR-549a in cytoplasm, which in turn promoted HIF1α expression in RCC, leading to increased VEGF secretion and further activated VEGFR2 to form a feedback effect. miR-549a played an important role in the metastasis of renal cancer and might serve as a blood biomarker for ccRCC metastasis and even had the potential of becoming a new drug to inhibit TKI-resistance.

Keywords: TKI-resistant, clear cell renal cell carcinoma, exosome, microRNA, HIF1α, vascular endothelial permeability, metastasis

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INTRODUCTION

Tyrosine kinase inhibitors (TKI) is the main treatment for advanced renal cancer, but most patients will eventually develop TKI-resistant RCC then metastasis occurs after 6–15 months, in which hematogenous metastasis is the main route (Wyler et al., 2014). Sorafenib is the first multi-targeted TKI drug for the treatment of metastatic renal cell carcinoma (mRCC) to inhibit Raf/MEK/ERK signaling pathway and VEGFR to achieve multiple anti-tumor effects (Wilhelm et al., 2004). In recent years, sunitinib has been recommended as first-line treatment for ccRCC and systemic

treatment for non-clear cell RCC according to National Comprehensive Cancer Network (NCCN) guidelines. However, sunitinib is more toxic than sorafenib. In Asian patients, sorafenib may be more appropriate than sunitinib (Deng et al., 2019). Therefore, we conducted a study on sorafenib-resistant clear cell renal cancer. Tumor metastasis is a complex multistep process. Tumor cells detach from the primary foci, migrate and invade the extracellular matrix, enter the blood vessel, survive in the blood, exude and disseminate in the target organ, grow and form metastatic foci (Reymond et al., 2013; Paul et al., 2017). The increase of vascular permeability is tightly associated to the tumor cells entering the blood circulation and then colonizing at the distal organ to form metastatic foci (García-Román and Zentella-Dehesa, 2013; Harrell et al., 2014). Exosomes are a subset of extracellular vesicles (EVs) with an average diameter of about 100 nm, and their components include nucleic acids, proteins, lipids, amino acids, and metabolites (Xu et al., 2018; Kalluri and LeBleu, 2020). After being released from tumor cells, exosomes are ingested by adjacent or distal cells, then the contained miRNAs regulate tumor immunity and microenvironment (Sun et al., 2018; Meng et al., 2019). Studies have found that exosomal miRNAs from breast, liver and colon cancer affect endothelial cells to promote metastasis (Zhou et al., 2014; Fang et al., 2018; Zeng et al., 2018). However, the mechanism by which TKI-resistant renal cancer cells induce vascular endothelial cell permeability changes to promote metastasis remains unclear. Here, we found that TKI-resistant cells of renal clear cell carcinoma had lower expression level of miR-549a than sensitive cells. miR-549a was delivered to vascular endothelial cells via exosomes to inhibit the expression of HIF1α. Therefore, compared with sensitive strain, resistant strain with lower levels of miR-549a had weaker effects on HIF1α, which enhanced permeability of vascular endothelium, and promoted angiogenesis, which in turn promoted tumor metastasis. In addition, in exploring the upstream regulatory mechanism of miR-549a in TKI-resistant renal cancer cells, we found that there was a positive feedback in renal cancer cells. Activation of the VEGFR2-ERK-XPO5 pathway inhibited the nuclear export of pre-miR-549a, and reduction of mature miR-549a in the cytoplasm promoted HIF1α expression to enhance VEGF secretion and then activated VEGFR2. The above results demonstrated the key role of miR-549a in promoting vascular permeability and angiogenesis in TKI-resistant renal cancer, and provided a new idea for the treatment.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human umbilical vein endothelial cells (HUVEC), renal clear cell carcinoma cells (786-O) and human renal epithelial cells (293T) were purchased from the American Type Culture Collection (ATCC). 786-O-SR was induced from 786-O with sorafenib (Solarbio, China). HUVECs were cultured in the ECM medium (ScienCell, United States) supplemented with 15% fetal bovine serum (ScienCell, United States). 786-O and 786-O-SR cell lines were cultured in 1640 medium (GIBCO, United States) supplemented with 10% fetal bovine serum (GIBCO, United States), and 15 μm sorafenib was added to 786-O-SR culture to maintain drug resistance. 293T cells were cultured in DMEM medium (GIBCO, United States) containing 10% fetal bovine serum. The cells were cultured in humidified air at 37°C and 5%CO_2 with 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, United States). For hypoxia culture, cells were placed in a hypoxic incubator at 37°C in a humidified 1% O2, 5% CO2 environment, with the balance provided by N2 for 24 h. FBS, used for CM collection, exosome separation and endothelial cell treatment, was centrifuged overnight at 100,000 *g* at 4°C.

Preparation of Conditioned Medium (CM)

Conditioned medium of renal cell carcinoma cells cultured in ECM were collected and stored at −80°C. When incubated with HUVEC, the collected CM was supplemented with 10% exosome-depleted FBS.

Isolation, Characterization, and Quantification of Exosomes

Exosomes were isolated from CM of renal carcinoma cells, which were cultured in 1640 supplemented with 10% exosome-depleted FBS, analyzed by transmission electron microscopy (TECNAI spirit, Fei, Netherlands) and particle size analyzer (Nicomp 380 N3000, PSS). The number of cells was counted to appropriately correct the CM volume used to separate the exosomes. The protein concentration of the harvested exosomes was detected by BCA Protein Quantification Kit (Yeasen, China). For cell treatment, exosomes collected from 5×10^6 cells (equivalent to 2 μg exosomes) were added to 2×10^5 endothelial cells.

Cellular Internalization of Exosomes

The exosomes were labeled with BODIPY TR ceramide (Thermo Fisher Scientific, United States) and then resuspended in 10% exosome-depleted FBS-ECM, added to HUVECs at 80% confluence, incubated for 4 h, and imaged under fluorescence microscope.

Real Time PCR

Total RNA was extracted from the cells using TRIzol RNA Isolation Reagents (ThermoFisher, United States). All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, United States) was used for miRNA reverse transcription. PrimeScript™ RT Master Mix (Clonech Laboratories, United States) was used for universal gene. Real time PCR was performed by SYBR Green PCR Master Mix (Applied Takara, Japan) and CFX96 deep hole real-time PCR detection system (BioRad, United States). The sequences of all primers are listed in **Supplementary Figure 1F**.

Gel Electrophoresis

RT-PCR samples were electrophoretic on 1% agarose gel and photographed under ultraviolet light using BioRad imager. BioRad quantity one imaging software was used to quantify the strip strength.

Protein Extraction and Western Blotting

Cells and exosomes were prepared in RIPA buffer (KeyGEN BioTECH, China) and quantified by BCA Protein Quantification Kit (Yeasen, China). Nuclear protein was extracted by CelLytic^{TM} NuCLEAR^{TM} Extraction Kit (Sigma-Aldrich, United States). Then, the lysate was transferred to the PVDF membrane (Millipore, United States) by SDS-PAGE. Then the membrane were incubated with primary antibodies at 4°C overnight. The following antibodies were used: vimentin (Cell Signaling, 5741t, 1:1000 dilution), β-catenin (Cell Signaling, 8480t, 1:1000 dilution), *E*-cadherin (Cell Signaling, 3195t, 1:1000 dilution), *N*-cadherin (Cell Signaling, 13116t, 1:1000 dilution), claudin1 (Cell Signaling, 13255t, 1:1000 dilution), ZO-1 (Cell Signaling, 8193t, 1:1000 dilution), β-actin (Cell Signaling, 4970s, 1:1000 dilution), CD81 (Cell Signaling, 10037s, 1:1000 dilution), TSG101 (Invitrogen, ma1-23296, 1:2000 dilution), HIF1α (Cell Signaling, 36169s, 1:1000 dilution), Xpo5 (Cell Signaling, 12565s, 1:1000 dilution), VEGFR2 (Cell Signaling, 9698s, 1:1000 dilution), ERK 1/2 (Cell Signaling, 4696s, 1:2000 dilution), Hillstone H3 (Cell Signaling, 14269s, 1:1000 dilution). After incubation with HRP linked antibody (Cell Signaling, 7076s or 7074s, 1:3000 dilution), the chemiluminescence signal was detected using the hypersensitive ECL Western blotting detection reagent (Seville Biology).

RNA Oligoribonucleotides and Vectors

miR-549a-3p/miR-549a-5p mimics and miR-549a-3p/miR-549a-5p inhibitors and their negative controls (NC and anti-NC, respectively) were provided by GenePharma, China. The sequences of the above miRNA mimics and inhibitors are listed in **Supplementary Figure 1H**. GFP plasmid and pcDNA plasmid were purchased from Sino Biological, China. The sea cucumber firefly dual luciferase reporter system (GenePharma, China) was used to verify whether the 3′-UTR of HIF1α mRNA was targeted by miR-549a.

Cell Transfection

Lipofectamine 3000 (ThermoFisher, United States) was used for the transfection of miR-549a-3p/miR-549a-5p mimics, miR-549a-3p/miR-549a-5p inhibitors and their negative controls (NC and anti-NC, respectively) and co-transfection of RNA duplexes with plasmid DNA.

Transendothelial Invasion Assay, Migration Assay and Angiogenesis Assay

For transendothelial invasion assays, *in vitro* endothelial permeability was assessed by counting the amount of 786-O-GFP that passed through a single layer of HUVECs with or without exosome treatment. For migration assays, exosome-treated HUVECs were suspended in serum-free medium and seeded into transwell chamber with 8-micron pore size (BD Biosciences, United States). The medium containing 15% FBS was placed in the bottom chamber. After 12 h, cells that migrated through the membrane and adhered to the submucosal surface were stained with hematoxylin and counted under light microscopy in four random fields of view (200x). For tube formation assays, the Matrigel matrix (Corning, United States) was laid in a 24-well plate and incubated at 37°C for 30 min to polymerize the matrix. The treated HUVECs were seeded on the matrix gel-coated holes. The plates were then incubated at 37°C in a 5%CO_2 humidified atmosphere. Tube formation was observed with a microscope after 12 h. The tube forming ability is determined by measuring the number of tubes. Each experiment was repeated three times.

Luciferase Activity Assay

The HIF1α 3′UTR plasmid was co-transfected into cells with either miR-549a-3p or miR-549a-3p mimics. Luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, United States) 48 h after transfection. All assays were performed in triplicate and each experiment was repeated three times.

Animal Models

Six-week-old male athymic BALB/c nude mice were purchased from the Laboratory Animal Center of Xiamen University (Xiamen, China) and raised in a pathogen-free environment. All protocols for animal research were reviewed and approved by the Laboratory Animal Center of Xiamen University (Ethics No. XMULAC20200039). For tumor metastasis assay, 2×10^6 786-O cells were injected into nude mice via tail vein. Six-week-old nude mice was injected into the tail vein with 5 μg exosomes every other day for 2 weeks. The control group was injected with equal volume PBS. After 15 and 30 days, respectively, the mice were sacrificed and the lungs were removed for examination.

Immunohistochemistry

Paraffin-embedded tissue blocks were cut into 2.5-μm sections and transferred to slides. Sections were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity and incubated with primary antibody overnight at 4°C. Subsequently, horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark) were applied and incubated at room temperature for 1 h. CD34 expression was visualized by using DAB and counterstained with hematoxylin. The following primary antibodies were used: CD34 (Abcam, ab81289, 1:200 dilution).

Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were rinsed twice with PBS. Blocking buffer (DakoCytomation, Glostrup, Denmark) was added for 30 min, and then stained with primary antibodies and fluorescent second antibody. The following antibodies were used: VEGFR2 (Cell Signaling, 9698S, 1:800 dilution), ERK 1/2 (Cell Signaling, 4696S, 1:100 dilution). Anti-mouse IgG (Alexa Fluor #594 Conjugate) (Cell Signaling, 8890, 1:2000 dilution), Anti-rabbit IgG (Alexa Fluor #488 Conjugate) (Cell Signaling, 4412, 1:2000 dilution).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. Quantitative values of all experiments were expressed

as mean standard deviation. Differences between sample groups were analyzed by one-way ANOVA or independent sample *T*-test. $P < 0.05$ was considered statistically significant. Adobe Illstrator CC, Adobe Photoshop CC and Image J software were used for the figure.

RESULTS

Renal Clear Cell Carcinoma TKI Resistant Cell Strain 786-O-SR Is Resistant to Sorafenib

To derive sorafenib resistant cell strain, we continuously cultured renal clear cell carcinoma cells (786-O), which is sensitive to sorafenib, in a stepwise manner by increasing the concentration of sorafenib, the concentration of sorafenib started at 5 μM and increased by 2.5 μM per generation to finally reach a concentration of 15 μM, from which a sorafenib resistant renal clear cell carcinoma cell line (786-O-SR) was induced (**Figure 1A**). 786-O-SR was cultured at 15 μM sorafenib to maintain its drug resistance.

To identify the TKI resistant renal clear cell carcinoma cell strain (786-O-SR) derived from 786-O induced by sorafenib, we assessed cell viability of 786-O versus 786-O-SR at different sorafenib concentrations by CCK8 assay, and the results showed that the cell viability of 786-O-SR did not alter significantly at low concentrations of sorafenib and decreased when sorafenib concentrations exceeded 10 μM, whereas the TKI sensitive strain 786-0 exhibited a dramatic inhibition of cell viability under the treatment of low concentrations of sorafenib (**Figure 1B**). Furthermore, we examined the apoptosis of 786-O and 786-O-SR at different sorafenib concentrations using annexin-V in combination with PI, and the results showed that 786-O exhibited a higher apoptosis rate with sorafenib treatment and positively correlated with sorafenib concentration, while the apoptosis of 786-O-SR was unaffected under sorafenib treatment at a range of concentrations (**Figure 1C**). Under fluorescence microscopy, an increasing trend of 786-O early apoptotic cells (annexin-V single positive), late apoptotic cells (annexin-V and PI double positive) as well as necrotic cells (PI single positive) could be observed with increasing sorafenib concentration, while the change of 786-O-SR counterpart was less obvious (**Figure 1D**).

Exosomes Derived From Clear Cell Renal Cell Carcinoma Cells Increase the Permeability of the Endothelial Cells

To understand the effect ccRCC exert on endothelial cells and whether sorafenib-sensitive (786-O) and TKI-resistant (786-O-SR) cells have differential effects, HUVECs were cultured with CM of 786-O or 786-O-SR. After CM treatment, HUVECs showed decreased expression of β-catenin, Vimentin, ZO-1 and Claudin and up-regulated expression of *E*-cadherin, and the change was more significant with treatment of CM from 786-O-SR than 786-O (**Figure 2A**). Vimentin is a type III intermediate filament protein which plays a role in stabilizing and enhancing endothelial matrix adhesion (Tsuruta and Jones, 2003). β-catenin inhibits VE-cadherin hydrolysis (Komarova and Malik, 2010), promotes the formation and maintenance of adherent junctions. ZO-1 and Claudin are tight junction proteins. *N*-cadherin inhibits vascular protective repair in epithelial cells (Jian et al., 2016). The above changes indicated that the permeability of HUVECs was enhanced after CM treatment, and the effect of 786-O-SR was more obvious.

Exosome is an important tool for intercellular communication with diameters from tens to hundreds of nanometers. We extracted and identified the exosomes of 786-O and 786-O-SR. Vesicle-like structures (**Figure 2B**) were observed under the electron microscopy, and the expression of CD81 and TSG101 (**Figure 2C**) was detected by WB. The particle size of 786-O exosomes was slightly larger than that of 786-O-SR, but all were within the diameter range of exosomes (**Figure 2D**). After co-incubation with exosomes, the changes of β-catenin, Vimentin, ZO-1, Claudin and *N*-cadherin in HUVECs were the same as those after CM treatment (**Figure 2F**). Transendothelial invasion assay showed that the number of 786-O-GFP crossing monolayer HUVECs increased after exosome treatment, and the effect of 786-O-SR exosome was more significant (**Figure 2G**). To confirm the absorption of exosomes derived from 786-O/786-O-SR by HUVECs, HUVECs were incubated with exosomes labeled with BODIPY TR ceramide, and red fluorescence signal was transferred to HUVEC (**Figure 2E**), but not to control group. Thus, ccRCC exosomes have an impact on vascular endothelial cell permeability, and TKI-resistant renal cancer has a greater impact on vascular permeability.

Exosomal miR-549a Affects Vascular Permeability

We sequenced the VEGF pathway of HUVEC cells treated with exosomes from 786-O and 786-O-SR cells and found that there were a series of differentially expressed proteins (**Supplementary Figure 1A**). 17 proteins were over-expressed in 786-O-SR treatment group and four proteins were under-expressed (**Supplementary Figure 1B**), among which HIF1α expression level was the most significant after excluding the influence of oxygen conditions during cultivation process (**Supplementary Figure 1B**). MiRNAs are a class of single-stranded small RNAs about 22 NT long, processed from hairpin structural transcripts produced endogenously in cells (Kim, 2005).The main function of miRNAs is to inhibit the expression of downstream genes then weaken or eliminate their function. Moreover, miRNAs achieve intercellular communication through exosomes. Nine upstream miRNAs of HIF1α were preliminarily screened by combining the predicted results of Targetscan, miRDB, and miRNAMap databases (**Figure 3A**). Further examination of the expression of these miRNAs in 786-O and 786-O-SR revealed that miR-17-5p, miR-199, miR-626, and miR-18a were not expressed in ccRCC. No significant difference was observed in the expression of miR-767 and miR-126 between sensitive and resistant strains. miR-302, miR-640, and miR-549a showed differential expression, among which the difference of miR-549a was the most significant (**Figure 3B**).

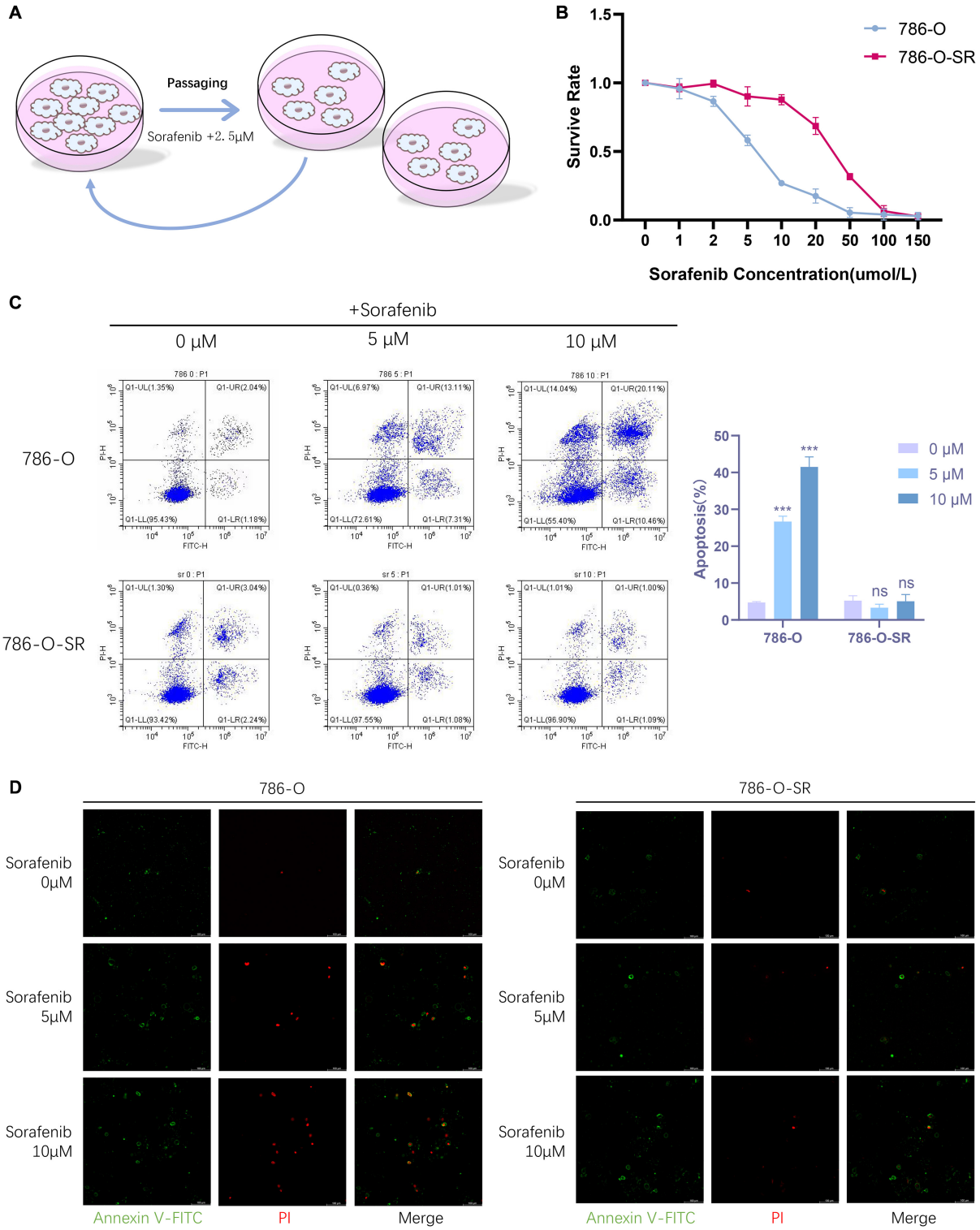

FIGURE 1 | Renal clear cell carcinoma TKI resistant cell strain 786-O-SR is resistant to sorafenib. **(A)** Induction process of TKI-resistant cell line 786-O-SR. **(B)** CCK8 assay of cell viability of 786-O and 786-O-SR at different sorafenib concentrations. **(C)** Flow cytometry assay of apoptosis of 786-O and 786-O-SR in response to different sorafenib concentrations (detected by annexin-V combined with PI). **(D)** Apoptosis situations of 786-O and 786-O-SR at different sorafenib concentrations observed by fluorescence microscopy. Mean ± SEM are provided ($n = 3$). ***$P < 0.001$; ns, not significant according to two-tailed Student's t-test.

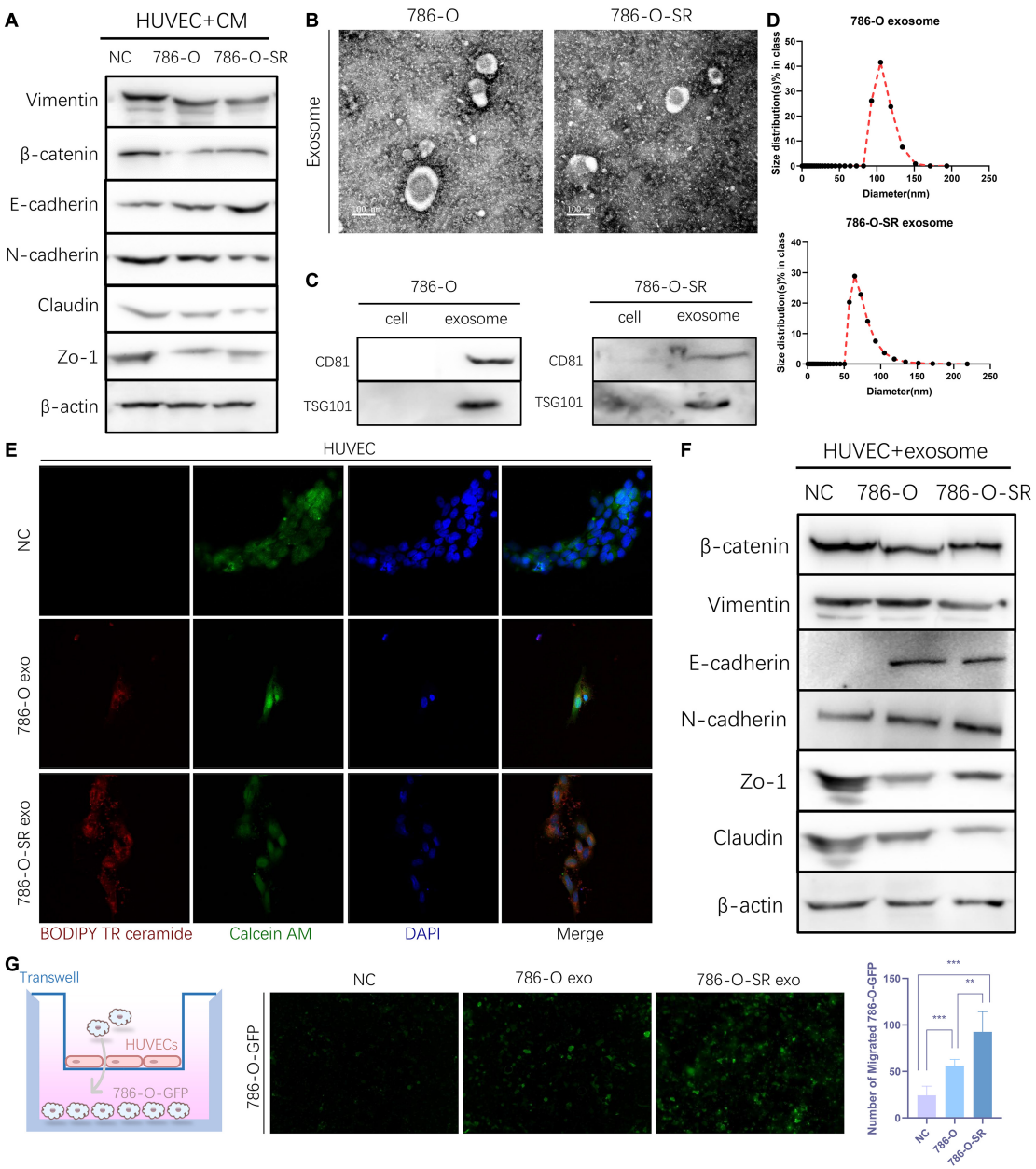

FIGURE 2 | Exosomes derived from Clear cell renal cell carcinoma cells increase the permeability of the endothelial cells. **(A)** Western blot analysis of Vimentin, β-catenin, *E*-cadherin, *N*-cadherin, Zo-1, Claudin expression in HUVECs incubated with CM of 786-O and 786-O-SR. **(B)** Transmission electron microscopy of exosomes derived from 786-O and 786-O-SR. Scale bar, 100 nm. **(C)** Western blotting analysis of CD81 and TSG101 in 786-O, 786-O-SR and their exosomes. **(D)** Nanoparticle tracking analysis of the size distribution and median diameter of particles per μg exosomes from 786-O and 786-O-SR. **(E)** The presence of BODIPY TR ceramide fluorescence in HUVECs after adding dye-labeled exosomes derived from 786-O and 786-O-SR cells for 48 h. HUVECs incubated with PBS were used as a negative control. Red: BODIPY TR ceramide; Green: Calcein AM; Blue: DAPI. **(F)** Western blot analysis of β-catenin, Vimentin, *E*-cadherin, *N*-cadherin, Zo-1, Claudin expression in HUVECs incubated with exosomes of 786-O and 786-O-SR. **(G)** Transendothelial invasion assay analysis of the number of GFP-expressing 786-O cells that invaded through HUVECs monolayers cultured with exosome derived from 786-O or 786-O-SR. Mean ± SEM are provided ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$, according to two-tailed Student's *t*-test. exo, exosomes; NC, negative control.

The expression level of miR-549a-3p/miR-549a-5p of 786-O was higher than 786-O-SR (**Figure 3C**), and same trend difference was observed in its exosomes (**Figure 3D**). Treated with CM or exosomes of 786-O-SR/786-O, the level of miR-549a-3p/miR-549a-5p of HUVEC increased, and 786-O had a greater effect (**Figures 3E,F**).

To verify whether miR-549a affects HUVEC permeability, a transendothelial invasion assay was performed. Transfected with NC, mimics or inhibitor, the number of 786-O-GFP was significantly different. miR-549a mimics resulted in a reduction, and the effect of miR-549a-5p mimics was more obvious (**Figure 3G**). Opposite result was obtained in miR-549a inhibitor

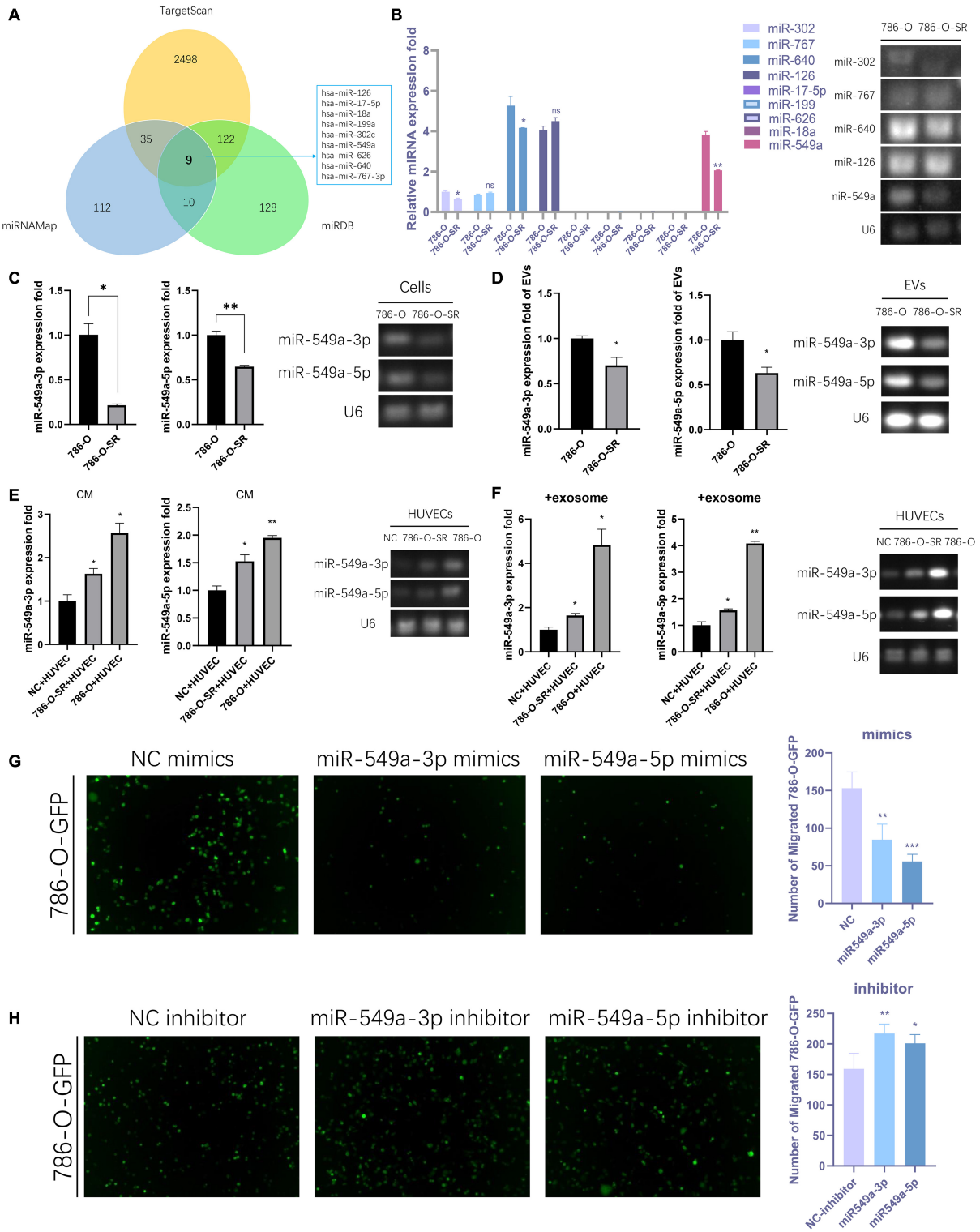

FIGURE 3 | Exosomal miR-549a affects vascular permeability. **(A)** The Wayne figures of overlapping and different miRNAs which target HIF1α according to TargetScan, miRNAMap and miRDB. **(B)** RT-PCR analysis of 9 miRNAs (which target HIF1α) expression in 786-O/786-O-SR cells and gel electrophoresis of PCR products. **(C–F)** RT-PCR and gel electrophoresis of PCR products analysis of miR-549a-3p/miR-549a-5p expression in 786-O/786-O-SR cells **(C)**, exosomes **(D)**, HUVECs treated with 786-O/786-O-SR CM **(E)** or exosomes **(F)**. **(G,H)** Transendothelial invasion assay analysis of the number of GFP-expressing 786-O cells that invaded through HUVECs monolayers transfected with miR-549a-3p/miR-549a-5p mimics **(G)** or miR-549a-3p/miR-549a-5p inhibitor **(H)**. Mean ± SEM are provided ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, according to two-tailed Student's *t*-test. ns, not significant.

treated group (**Figure 3H**). This suggested that miR-549a-3p and miR-549a-5p inhibited the permeability of HUVEC, which explained why TKI-resistant renal cancer cells (786-O-SR) with low expression of miR-549a exerted stronger permeability-promoting effect on HUVECs, whereas sensitive strains (786-O) with higher expression of miR-549a had weaker effect.

However, the permeability of HUVECs treated with CM or exosomes of renal cancer cells was enhanced compared with that of the control group (i.e., HUVEC without exogenous input of miR-549a) (**Figures 2A,F,G**), suggesting that tumor-derived exosomes had some factors that positively regulated vascular permeability. HUVEC naturally expressed low level of *E*-cadherin, a key molecule in cell-cell adhesions (van Roy and Berx, 2008), which increased after treatment with renal cancer exosomes (**Figure 2F**). It was reported that *E*-cadherin localized on the surface of exosome membrane was transported to endothelial cells to promote angiogenesis (Tang et al., 2018). *E*-cadherin was expressed both in 786-O/786-O-SR cells and their exosomes, and 786-O-SR expression was higher (**Supplementary Figure 1C**). This suggested that renal cancer exosomes transmitted *E*-cadherin to endothelial cells. Studies have suggested that *E*-cadherin regulated HIF1α (Maroni et al., 2015; Liang et al., 2016), which may be one of the mechanisms by which renal cancer exosomes promote vascular permeability.

Exosomal miR-549a Affects Angiogenesis and Endothelial Cell Migration

Angiogenesis plays a key role in tumor progression. In the primary lesion, angiogenesis ensures the nutrient supply of tumor cells. Formation of secondary metastatic foci by tumor cells requires the formation of pre-metastatic niche, in which angiogenesis is the key step. In addition, neovascularization is characterized by high vascular permeability, so the enhancement of angiogenesis also leads to the increase of overall vascular permeability. To test the effect of miR-549 expression upon angiogenesis, we performed a tube-formation assay of HUVEC *in vitro*. The results showed that miR-549a-3p mimics significantly reduced the lumen structure formed by HUVEC, while miR-549a-3p inhibitor enhanced the tube-forming ability of HUVEC (**Figure 4A**). Similarly, miR-549a-5p mimics led to a decrease in the tubulogenic capacity of HUVEC, whereas inhibitor did the opposite (**Figure 4B**).

Since endothelial cell migration is also essential for angiogenesis and leads to increased vascular leakage, we evaluated the effect of miR-549a on HUVEC migration. After treating HUVECs with mimics and inhibitors of miR-549a-3p, miR-549a-5p, we found that, miR-549a-3p and miR-549a-5p significantly attenuated the migration ability of HUVECs, while the migration ability of HUVECs was improved after inhibiting miR-549a-3p and miR-549a-5p (**Figure 4C**). These results indicate that miR-549a weakens angiogenesis and endothelial cell migration.

Renal Cancer Exosomes Promote ccRCC Metastasis

To determine whether exosomes promote renal cancer cell metastasis *in vivo*, we injected ccRCC cells into mice via the tail vein, daily treated with 786-O/786-O-SR exosomes, and monitored tumor metastasis. Mice were sacrificed on day 15, and the lungs were removed and subjected to histological examination (**Figure 5A**). HE showed metastasis sites were not formed in lung (**Figure 5A**), but the microvessel density (MVD) of the lung tissue had changed. Both 786-O/786-O-SR exosomes lead to an increase of CD34-positive cell rate, of which 786-O-SR exosomes had a greater impact (**Figure 5B**). This suggests that before tumor metastases, exosomes modify the microenvironment of distal organs, inducing enhanced angiogenesis to form a pre-metastatic niche which is conducive to tumor colonization.

After the occurrence of metastasis, the lungs of mice were removed on day 30. The representative gross morphology of lung metastasis was displayed. More metastasis foci were observed after exosome treatment, and the effect of 786-O-SR exosome was more significant (**Figure 5C**). Moreover, the metastases of 786-O-SR exosome-treated tumors had invaded the mediastinum and pleura. HE showed that the exosome-treated group had more metastases (**Figure 5D**). Tumor MVD was higher in the exosome-treated group than in the control group, with 786-O-SR group having the highest MVD (**Figure 5E**).

miR-549a Silences HIF1α in HUVECs

To validate the regulatory effect of miR-549a on HIF1α, we compared their sequences by BLAST. A base match was found between HIF1α mRNA 3′-UTR and miR-549a-3p as well as miR-549a-5p, respectively (**Figure 6A**).

There are three main effects of miRNA: transcriptional repression and cleavage or degradation of mRNA, while the miRNA *in vivo* generally does not match the target gene well, so this regulation is not common in animals (Mohr and Mott, 2015). The possibility of miRNA-mediated mRNA degradation is higher. When silenced, the decapping and tail removal reaction of the mRNA are triggered, then cause the degradation (Jonas and Izaurralde, 2015). RT-PCR showed that miR-549a-5p reduced the HIF1α mRNA level in HUVECs, while miR-549a-3p had not effect (**Figure 6B**). Mimics of miR-549a-3p/miR-549a-5p inhibited HIF1α protein level, while their inhibitor effect was opposite (**Figure 6C**). The above results indicated that both miR-549a-3p and miR-549a-5p reduced HIF1α protein level. MiR-549a-3p inhibits the translation process of HIF1α mRNA but does not decrease its mRNA level, while miR-549a-5p induces the degradation of HIF1α mRNA.

To verify the binding effect of miR-549a to HIF1α mRNA 3′-UTR, a dual-luciferase reporter gene assay was performed. The luciferase activity of 3′ UTR of HIF1α was suppressed notably by miR-549a-3p, while mutant HIF1α had no such effect (**Figure 6D**). Similarly, miR-549a-5p had also been proved to bind to the 3'-UTR region of HIF1α mRNA (**Figure 6E**).

Since HIF1α is regulated by environmental oxygen levels, in order to verify the regulatory effect of miR-549a on HIF1α

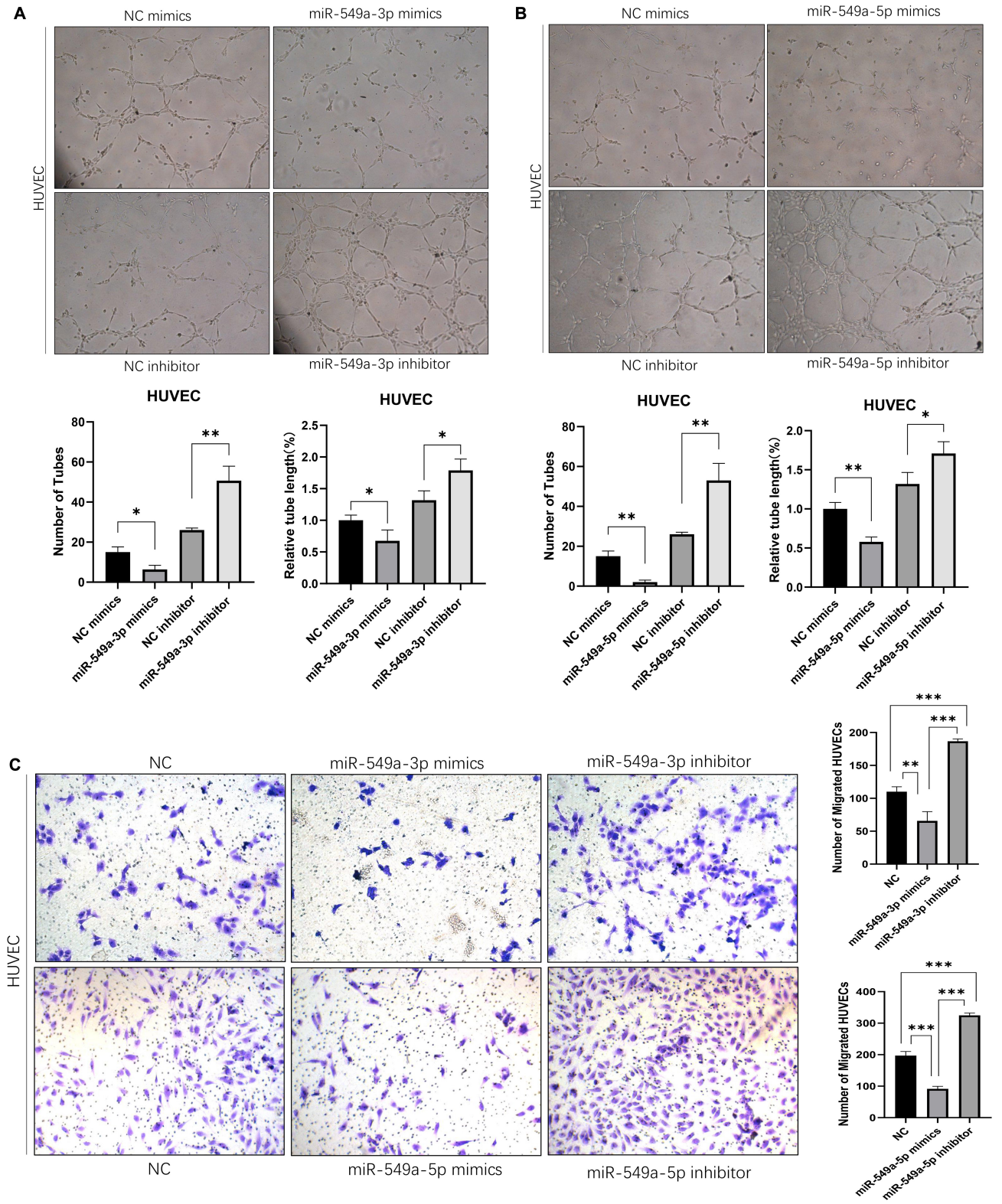

FIGURE 4 | Exosomal miR-549a affects angiogenesis and endothelial cell migration. **(A,B)** Effect of miR-549a-3p mimics/inhibitor **(A)**, miR-549a-5p mimics/inhibitor **(B)** on tube formation ability of HUVECs by tube formation assay. Mean ± SEM are provided ($n = 3$). **(C)** Effect of miR-549a-3p/miR-549a-5p mimics and inhibitor treatment on migration of HUVECs. Mean ± SEM are provided ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ according to two-tailed Student's *t*-test.

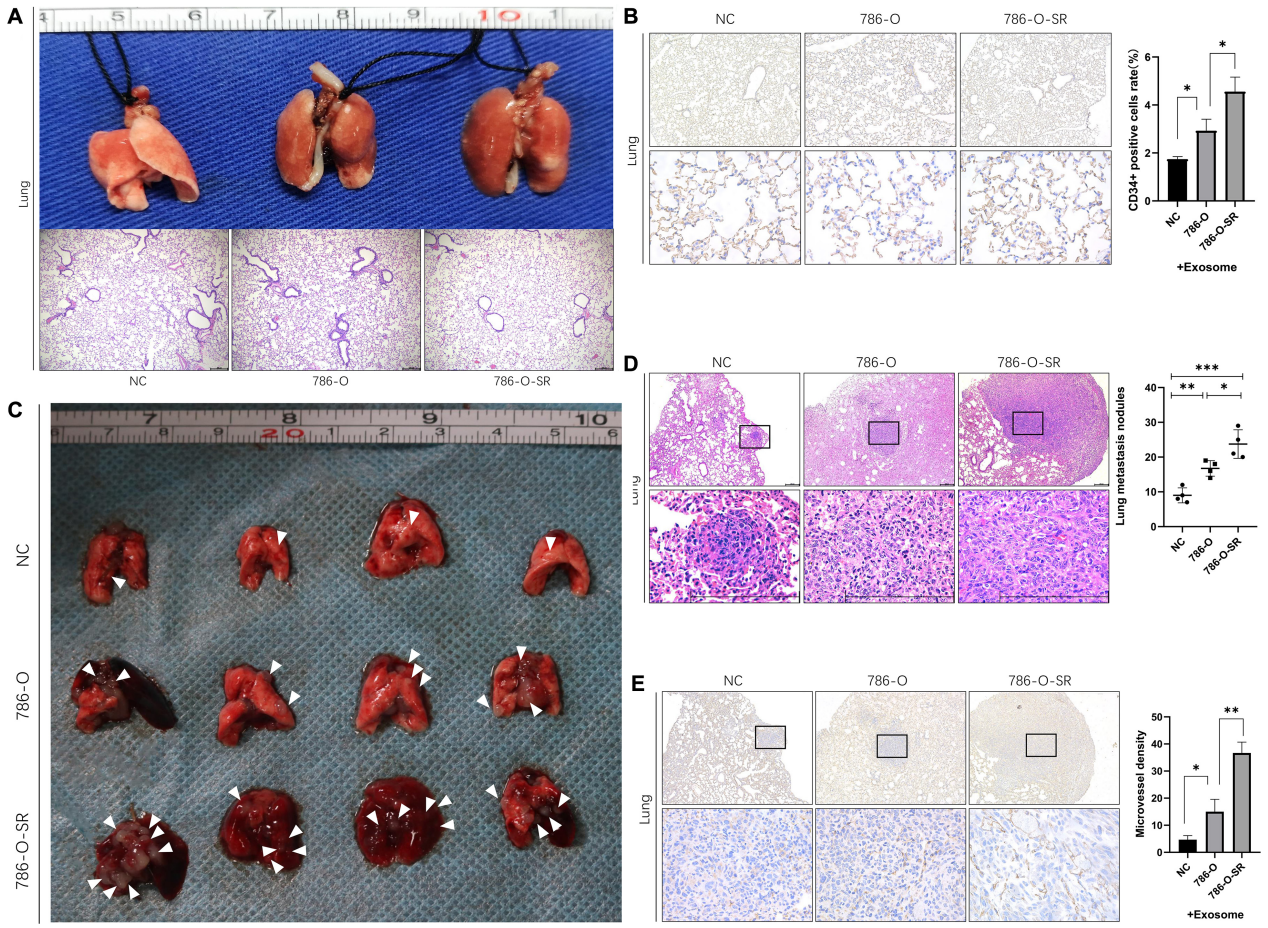

FIGURE 5 | Renal cancer exosomes promote ccRCC metastasis. **(A)** The mice were injected with 786-O cells via tail vein after exposure to 786-O or 786-O-SR exosomes. The lungs were removed for H&E staining after 15 days. **(B)** IHC staining for CD34-positive cells. Mean ± SEM are provided (n = 3). **(C)** The lungs were removed after 30 days. Lung metastatic sites were indicated by arrows. **(D)** The number of lung metastatic sites was counted under the microscope. Mean ± SEM are provided (n = 4). Scale bar 200 μm. **(E)** Effect of exosomes of 786-O/786-O-SR on introtumoral microvessel density. The number of microvessel was counted under the microscope. Mean ± SEM are provided (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ according to two-tailed Student's t-test.

under hypoxia, we detected HIF1α levels in HUVECs cultured under hypoxia. The results showed that the changes in HIF1α RNA and protein levels were consistent with those in normoxia (**Supplementary Figure 1E**). Moreover, HIF1α was not detected in exosomes derived from renal cancer, excluding the possibility that renal cancer derived exosomes carrying HIF1α protein to recipient endothelial cells to achieve regulation (**Supplementary Figure 1I**).

The above results show that miR-549a binds to the 3′-UTR region of HIF1α mRNA to inhibit its translation process, in which miR-549a-5p leads to a decrease in HIF1α mRNA levels while miR-549a-3p does not, but ultimately both lead to a significant reduction in HIF1α protein levels in target cells, and the process is universal under different oxygen conditions.

Erk2 Regulates the Output of miR-549a via XPO5

Exportin-5 (XPO5) is a miRNA transport protein present in the nucleus. In the nucleus, primary microRNAs are sheared by nuclease Drosha to form pre-miRNAs with stem-loop structure of about 70 nucleotides. XPO5 transports the pre-miRNAs from the nucleus to the cytoplasm. Sheared by the nuclease, Dicer, in the cytoplasm, pre-miRNAs become mature miRNAs with about 20–25 nucleotides and get bioactivity (Clancy et al., 2019). Given the critical role of nuclear export of pre-miRNAs in the biological functions of miRNAs, any changes affecting XPO5 affect the expression of miRNAs, thus having a profound impact on tumorigenesis and progression (Wu et al., 2018). Erk (mainly Erk2) decreases the binding ability of p-XPO-5 to pre-miRNA by phosphorylating XPO5 at T345/S416/S497, resulting in a decrease in the extranuclear export of pre-miRNA (Sun et al., 2016).

To verify whether the differential expression of miR-549a between TKI-sensitive and resistant strains of ccRCC is also regulated by the above pathways, we examined the nuclear and cytoplasmic proteins of 786-O and 786-O-SR. Compared with 786-O, the nuclear XPO5 expression of 786-O-SR was higher, while the cytoplasmic expression was less (**Figure 7A**). Moreover, Erk2 (44 kd) expression of 786-O-SR was significantly

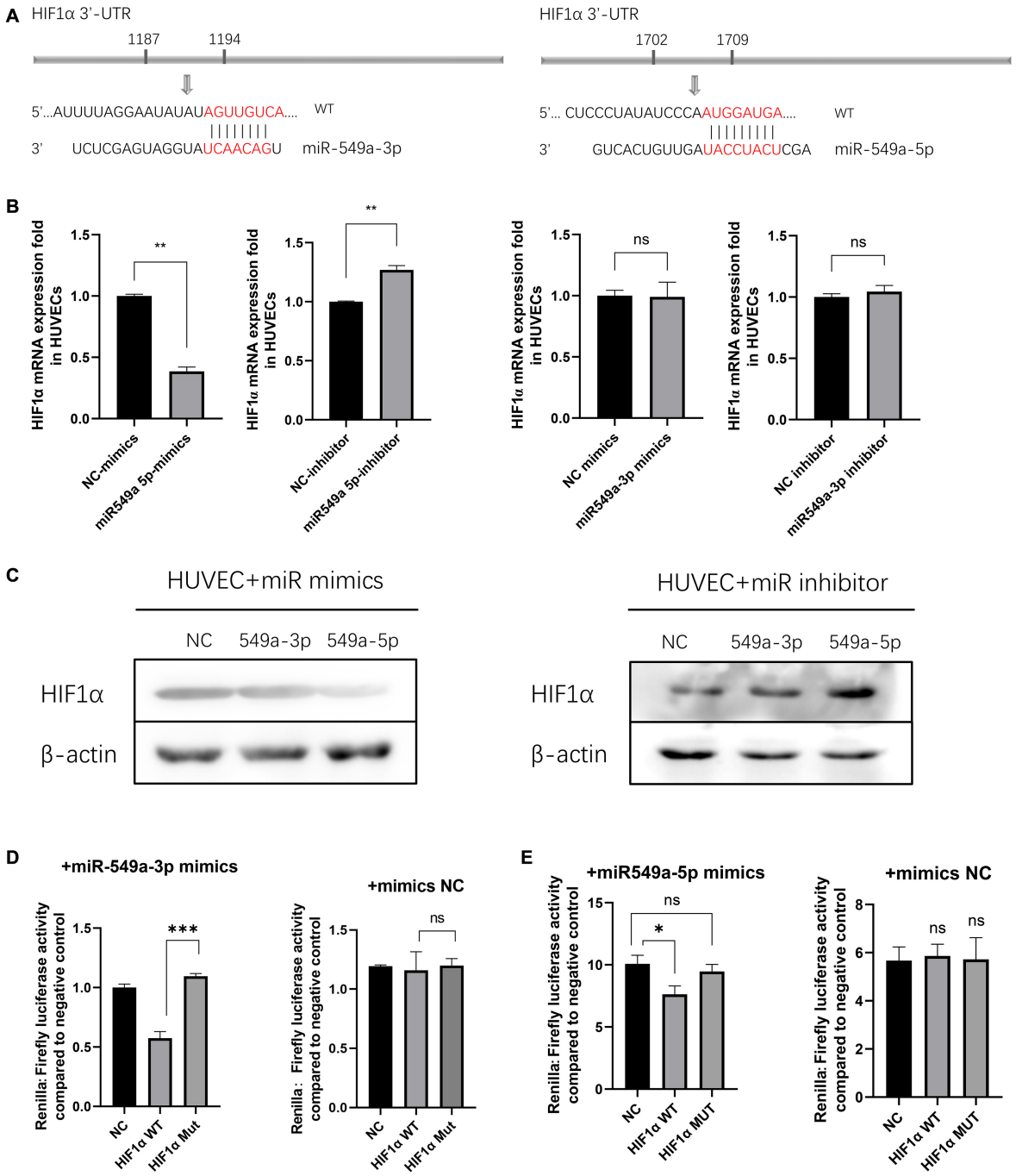

FIGURE 6 | miR-549a silences HIF1α in HUVECs. **(A)** The miR-549a-3p and miR-549a-5p binding sites in the 3′-UTR of HIF1α were predicted. **(B)** RT-PCR analysis of HIF1α RNA expression in HUVECs treated with miR-549a-3p/miR-549a-5p mimics or inhibitor. **(C)** Western blot analysis of HIF1α expression in HUVECs treated with miR-549a-3p/miR-549a-5p mimics or inhibitor. **(D,E)** Dual-luciferase reporter gene assay showed that miR-549a-3p/miR-549a-5p inhibited the luciferase activity of reporter containing wild-type but not mutant 3′-UTR of HIF1α. Mean ± SEM are provided ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant according to two-tailed Student's *t*-test.

higher than that of 786-O, while Erk1 (42 kd) was not (**Figure 7B**). Subsequently, we detected pre-miR-549a levels of 786-O and 786-O-SR. Pre-miR-549a levels of 786-O-SR were significantly lower than 786-O (**Figure 7C**). Erk2 (**Figure 7B**) was overexpressed in 786-O, the nuclear expression of XPO5 increased, and the cytoplasmic expression decreased (**Figure 7D**). This indicates that Erk2 affect the transport of pre-miR-549a via XPO5 in ccRCC.

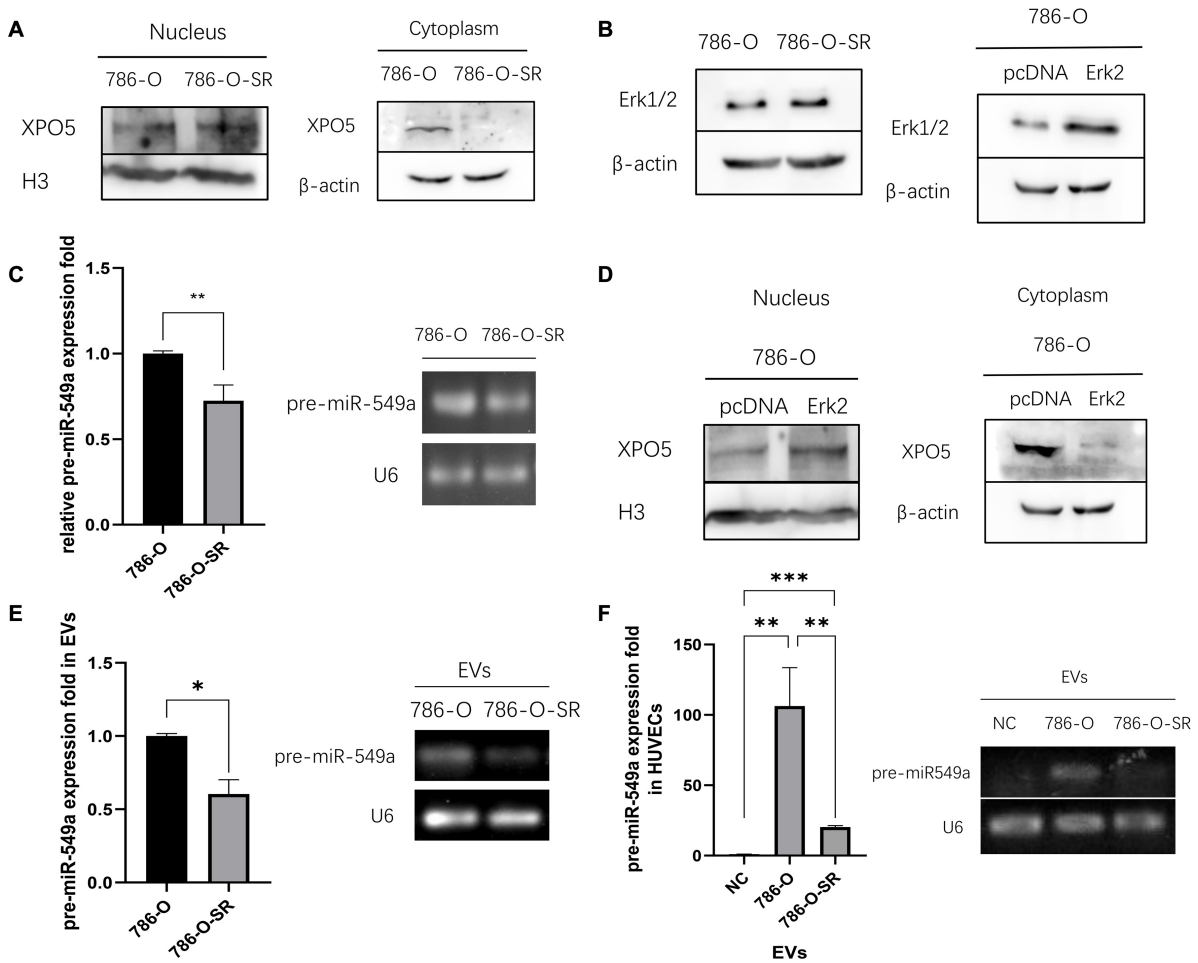

FIGURE 7 | Erk2 regulates the output of miR-549a via XPO5. **(A)** Cytosolic and nuclear protein were prepared and analyzed for XPO5 expression in 786-O and 786-O-SR by Western blot. **(B)** Western blot analysis of Erk1/2 expression in 786-O and 786-O-SR and the detection of overexpression efficiency of Erk2 in 786-O. **(C)** RT-PCR and gel electrophoresis of PCR products analysis of pre-miR-549a expression in 786-O/786-O-SR cells. **(D)** Cytosolic and nuclear XPO5 expression in 786-O and Erk2 overexpressing 786-O by Western blotting. **(E,F)** RT-PCR and gel electrophoresis of PCR products analysis of pre-miR-549a expression in 786-O/786-O-SR exosomes **(E)** and HUVECs treated with exosomes **(F)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ according to two-tailed Student's *t*-test.

In addition, exosomal pre-miR-549a levels of 786-O and 786-O-SR showed a consistent trend with donor cells (**Figure 7E**). The pre-miR-549a levels of HUVECs after exosome treatment also changed differentially (**Figure 7F**). This suggests that not only mature miR-549a, but also pre-miRNAs are transported to recipient cells via exosomes. In fact, XPO5, Dicer and Argonaute-2 are all expressed in exosomes (Clancy et al., 2019), which means that pre-miR-549a can be processed and matured not only in the cytoplasm of donor cells, but also in exosomes and even in recipient cells, resulting in increased levels of miR-549a-3p/miR-549a-3p in recipient cells.

miR-549a Achieved Positive Feedback Regulation of VEGF-VEGFR2-Erk2 Pathway in Tumor Cells via HIF1α

To evaluate whether the miR-549a regulatory mechanism is involved in HIF1α gene expression of ccRCC, we transfected 786-O-SR (**Figure 8A**) with miR549a-3p/5p mimics. After miR-549a-5p mimics transfection, both HIF1α mRNA and protein level decreased, while miR-549a-3p only had an inhibitory effect on HIF1α protein (**Figure 8B**), which was consistent with the experimental results of HUVEC. This indicates that there is conservation of this regulatory mechanism across different cells. Moreover, the difference of HIF1α expression between 786-O and 786-O-SR persisted after changing the cell culture medium species (**Supplementary Figure 1J**).

HIF1α has been demonstrated to promote the expression and secretion of VEGF (Chen et al., 2015), and the higher expression of HIF1α in 786-O-SR leads to its secretion of higher levels of VEGF than 786-O. After VEGF binding to VEGFR2 on the cell membrane of ccRCC, the multiprotein complexes, including neuropilin-1, synectin and Ephrin-B2, is initiated (Gutiérrez-González et al., 2019). Subsequently, the internalization process is initiated, and the complex is encapsulated into the membrane by intracellular endocytosis (Tian et al., 2018). After stimulation

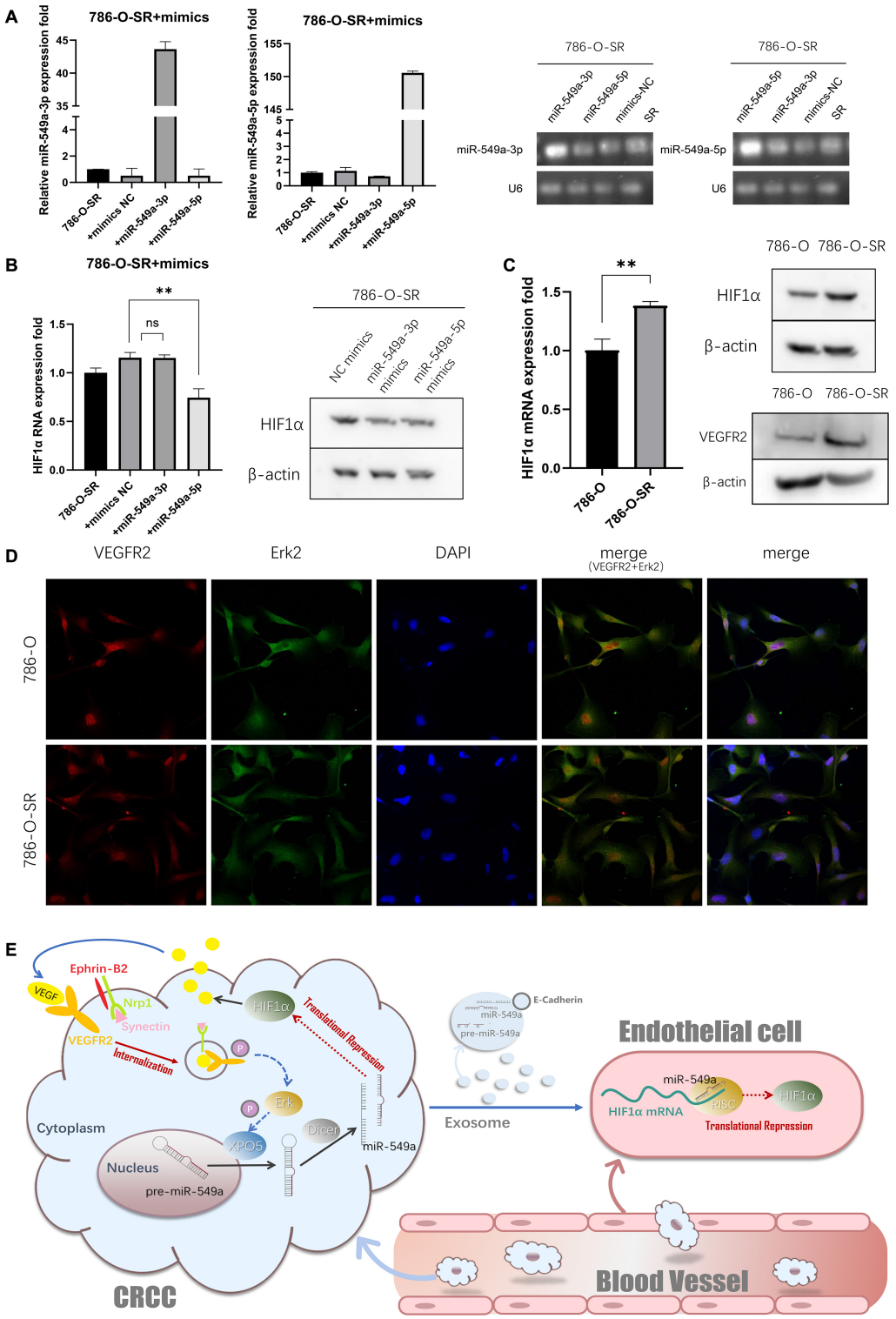

FIGURE 8 | miR-549a achieved positive feedback regulation of VEGF-VEGFR2-Erk2 pathway in tumor cells via HIF1α. **(A)** RT-PCR and western blotting analysis of HIF1α expression in 786-O and 786-O-SR. VEGFR2 expression in 786-O and 786-O-SR by western blot. $^{**}P < 0.01$; ns, not significant according to two-tailed Student's *t*-test. **(B)** RT-PCR and western blotting analysis of HIF1α expression in 786-O-SR, 786-O-SR + mimics NC, 786-O-SR + miR-549a-3p mimics and 786-O-SR + miR-549a-5p mimics. **(C)** The detection of transfection efficiency of miR-549a-3p/miR-549a-5p mimics in 786-O-SR by RT-PCR and gel electrophoresis of PCR products analysis. **(D)** The subcellular localization of VEGFR2 and Erk2 was examined by confocal microscopy analysis. **(E)** Schematic diagram of the role of exosomal miR-549a in tumor metastasis and TKI resistance.

by VEGF, the subcellular localization of VEGFR2 of 786-O and 786-O-SR was located in the cytoplasm (**Figure 8D**), and the expression of VEGFR2 in the cytoplasm of 786-O-SR was higher than that of 786-O (**Figure 8C**), which confirmed that 786-O-SR had stronger VEGFR2 complex endocytosis with higher levels of VEGF. After autophosphorylation of VEGFR2, the downstream signaling pathway is activated (Clegg and Mac Gabhann, 2015). Erk, as a downstream signaling molecule of the classical VEGF pathway, is activated. A coincidence (**Figure 8D**) between VEGFR2 and Erk2 subcellular localization was indicated. Activated Erk2, which in turn phosphorylates XPO5, results in a decrease in the output of miR-549a. The pathway centered on miR-549a not only changes vascular permeability by exosomes acting on endothelial cells, but also affects the tumor microenvironment leading to further activation of the tumor cell VEGF pathway, forming a positive feedback regulation (**Figure 8E**).

DISCUSSION

This study revealed that exosomal miR-549a regulated the expression of HIF1α of vascular endothelial cells to promote angiogenesis, enhance vascular permeability, then promote tumorigenesis and metastasis after ccRCC TKI resistance. The regulatory effect of miR-549a on HIF1α also exists in ccRCC, which affects the secretion of VEGF, then increases the nuclear output of pre-miR-549a through the VEGFR2-ERK-XPO5 axis, forms a positive feedback. Overall, our study revealed a novel function of exosomal miR-549a and its clinical significance in TKI-resistant renal cancer.

Studies have shown that miRNAs effectively silence stromal cell mRNA via tumor cell exosomes and affect their functions (Chen et al., 2012; Zhou et al., 2014). For example, exosomal miR-103 secreted by hepatoma cells targets connexins to increase vascular permeability and promote metastasis (Fang et al., 2018). Colon cancer exosome miR-25-3p targets KLF2 and KLF4 of vascular endothelial cell, affecting their function (Zeng et al., 2018). At present, there are still few studies on miR-549a, and this study is the first to report the role of miR-549a in tumor progression. Tumor metastasis is closely related to the increase of tumor vascular permeability (Reymond et al., 2013). On the one hand, the increase of tumor vascular permeability *in situ* is conducive to the penetration of tumor cells into blood (Harney et al., 2015). On the other hand, the enhancement of vascular permeability and angiogenesis of secondary metastatic foci in distal organs provide the 'soil' for tumor cell colonization (Liu and Cao, 2016; Peinado et al., 2017).

As mentioned above, although the influence of tumor exosomes on vascular permeability has been proven. Whether metastasis-prone renal cancer after TKI resistance is associated with this effect remains unclear. This study revealed the interaction mechanism between tumor cells and endothelial cells mediated by exosomes. Our results showed that a series of permeability-related proteins in vascular endothelial cells were altered after co-incubation with the culture supernatants or exosomes of TKI-resistant renal cancer strains, and the degree of permeability enhancement was greater than that of sensitive strains. Therefore, we hypothesized that renal cancer cells affected vascular endothelial permeability via exosomes. After co-incubation of membrane dye-labeled exosomes with HUVEC, fluorescent signals were observed on HUVEC membranes, demonstrating the absorption of renal cancer-derived exosomes by HUVEC. Moreover, after exosome treatment, the ability of HUVEC to allow tumor cells to penetrate was enhanced. *In vivo* analyses demonstrated that renal cancer exosomes promoted tumor metastasis, and CD34-positive cell rate in tumor foci significantly increased. Array gene expression analysis of the VEGF pathway of HUVECs treated with renal cancer exosomes revealed that the change of HIF1α was significant. HIF1 is a transcription factor with helix-loop-helix structure, which is a heterodimer composed of HIF1α and HIF1β. HIF1α strongly activate the transcription and secretion of VEGF to achieve the promotion of vascular permeability and angiogenesis (Damert et al., 1997; Semenza, 2001). Our results showed that miR-549a bound to the 3′-UTR region of HIF1α mRNA to inhibit its translation process. TKI-resistant renal cancer had a weaker inhibitory effect on HIF1α due to its low expression level of miR-549a, resulting in greater vascular permeability and angiogenesis of endothelial cells. In addition, renal cancer exosomes delivered *E*-cadherin to endothelial cells to promote vascularization, but the mechanism required further studied. At present, there are many reports on the mechanism of exosomes acting on recipient cells, but there are still few studies on donor cells. This research studied the effect of miR-549a on RCC, and penetrated the signaling pathway from donor cells to receptor cells. Further studies revealed that the effect of miR-549a on HIF1α was also present in RCC cells, which in turn affected the secretion of VEGF in RCC cells. In renal cancer resistant strains, low-expression of miR-549a leads to increased secretion of VEGF by HIF1α, which induce its internalization after binding of VEGF to membrane protein VEGFR2 (Gutiérrez-González et al., 2019). Inbound VEGFR2 activates ERK, which phosphorylates XPO5 resulting in reduced pre-miR-549a output, forming a positive feedback regulation.

The enrichment in sphingolipids, phosphatidylserine, and cholesterol of exosomes has protective effect on their cargo (Skotland et al., 2019). The nucleic acid signal encapsulated by the exosome is also not easily cleared by plasma (Kamerkar et al., 2017). Changes in nucleic acid, proteins, metabolites and lipids of cancer cells are reflected in their secreted exosomes (LeBleu and Kalluri, 2020), and can be used as biomarkers for cancer early diagnosis (Jalalian et al., 2019), process monitoring (Maisano et al., 2020), and drug-resistance prediction (Liu et al., 2019; Augimeri et al., 2020). Therefore, exosomal miR-549a, which plays an important role in TKI-resistance and metastasis of RCC, may also be of value as a biomarker. Exosomes have great potential as drug delivery nano mediators due to their natural properties derived from cells (Wu et al., 2008; Schulz and Binder, 2015). Studies have shown that MSC-derived exosomes transfer miR-133b to nerve cells and can be used to treat neurodegenerative diseases (Xin et al., 2012; Yang et al., 2017). Exosomal miR-9 derived from MSC is transferred to glioblastoma multiforme and reverse its

chemoresistance (Munoz et al., 2013). Overexpressed miR-let7c in MSCs is delivered into damaged renal cells, reducing renal damage in unilateral ureteral obstruction (Wang et al., 2016). Various evidences have shown that exosomes can overcome the problem that exogenous siRNAs are easy to degrade and difficult to penetrate the cell membrane, and deliver specific functional siRNAs to target cells to regulate gene expression and achieve therapeutic value. We believe that the delivery of miR-549a to TKI-resistant renal cancer cells using exosomes as carriers can effectively reduce its impact on vascular permeability and reverse its own TKI resistance.

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 animal study was reviewed and approved by The Laboratory Animal Center of Xiamen University (Ethics No. XMULAC20200039).

AUTHOR CONTRIBUTIONS

ZX and HS contributed to conception and design of the study. ZX and CC organized the database. WT performed the statistical analysis. ZS wrote the first draft of the manuscript. JZ, YZ, ZS, and LZ wrote sections of the manuscript. CS was responsible for review. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.689947/full#supplementary-material

Supplementary Figure 1 | Supplemental results. **(A)** RT^2 profiler-PCR array gene expression analysis of the VEGF pathway of HUVEC cells treated with exosomes from 786-O and 786-O-SR cells. **(B)** The Table about the over-expressed and under-expressed proteins in 786-O-SR treatment group Compared with the 786-O treatment group. **(C)** Western blot analysis of *E*-cadherin expression. **(D)** The detection of transfection efficiency of miR-549a-3p/ miR-549a-5p mimics/inhibitors in HUVECs by RT-PCR and gel electrophoresis of PCR products analysis. **(E)** RT-PCR and western blotting analysis of HIF1α expression. **(F)** The sequences of all primers. **(G)** The sequences of reverse transcription primers. **(H)** The sequences of miRNA mimics and inhibitors. **(I)** Western blot analysis of HIF1α expression of exosomes from 786-O and 786-O-SR. **(J)** Western blot analysis of HIF1α expression of 786-O and 786-O-SR treated by different media. $^{**}P < 0.01$, $^{***}P < 0.001$ according to two-tailed Student's *t*-test.

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CORRECTION
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Corrigendum: TKI-Resistant Renal Cancer Secretes Low-Level Exosomal miR-549a to Induce Vascular Permeability and Angiogenesis to Promote Tumor Metastasis

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Keywords: TKI-resistant, clear cell renal cell carcinoma, exosome, microRNA, HIF1α, vascular endothelial permeability, metastasis

A Corrigendum on

TKI-Resistant Renal Cancer Secretes Low-Level Exosomal miR-549a to Induce Vascular Permeability and Angiogenesis to Promote Tumor Metastasis

by Xuan, Z., Chen, C., Tang, W., Ye, S., Zheng, J., Zhao, Y., et al. (2021). Front. Cell Dev. Biol. 9:689947. doi: 10.3389/fcell.2021.689947

In the original article, there were errors. **"E-cadherin" is mistakenly stated as "N-cadherin." Figure 2E, Figure 2F and Figure 2G are incorrectly matched to figure legends.**

A correction has been made to ***Exosomes Derived From Clear Cell Renal Cell Carcinoma Cells Increase the Permeability of the Endothelial Cells, Paragraph: 1 and 2. Exosomal miR-549a Affects Vascular Permeability. Paragraph: 4.***

To understand the effect ccRCC exert on endothelial cells and whether sorafenib-sensitive (786-O) and TKI-resistant (786-O-SR) cells have differential effects, HUVECs were cultured with CM of 786-O or 786-O-SR. After CM treatment, HUVECs showed decreased expression of β-catenin, Vimentin, ZO-1 and Claudin and up-regulated expression of E-cadherin, and the change was more significant with treatment of CM from 786-O-SR than 786-O (Figure 2A). Vimentin is a type III intermediate filament protein which plays a role in stabilizing and enhancing endothelial matrix adhesion (Tsuruta and Jones, 2003). β-catenin inhibits VE-cadherin hydrolysis (Komarova and Malik, 2010), promotes the formation and maintenance of adherent junctions. ZO-1 and Claudin are tight junction proteins. N-cadherin inhibits vascular protective repair in epithelial cells (Jian et al., 2016). The above changes indicated that the permeability of HUVECs was enhanced after CM treatment, and the effect of 786-O-SR was more obvious.

Exosome is an important tool for intercellular communication with diameters from tens to hundreds of nanometers. We extracted and identified the exosomes of 786-O and 786-O-SR. Vesicle-like structures (Figure 2B) were observed under the electron microscopy, and the expression of CD81 and TSG101 (Figure 2C) was detected by WB. The particle size of 786-O exosomes was slightly larger than that of 786-O-SR, but all were within the diameter range of exosomes (Figure 2D). After co-incubation with exosomes, the changes of β-catenin, Vimentin, ZO-1, Claudin and N-cadherin in HUVECs were the same as those after CM treatment (Figure 2F).

Transendothelial invasion assay showed that the number of 786-O-GFP crossing monolayer HUVECs increased after exosome treatment, and the effect of 786-O-SR exosome was more significant (Figure 2G). To confirm the absorption of exosomes derived from 786-O/786-O-SR by HUVECs, HUVECs were incubated with exosomes labeled with BODIPY TR ceramide, and red fluorescence signal was transferred to HUVEC (Figure 2E), but not to control group. Thus, ccRCC exosomes have an impact on vascular endothelial cell permeability, and TKI-resistant renal cancer has a greater impact on vascular permeability. However, the permeability of HUVECs treated with CM or exosomes of renal cancer cells was enhanced compared with that of the control group (i.e., HUVEC without exogenous input of miR-549a) (Figures 2A,F,G), suggesting that tumor-derived exosomes had some factors that positively regulated vascular permeability.

HUVEC naturally expressed low level of E-cadherin, a key molecule in cell-cell adhesions (van Roy and Berx, 2008), which increased after treatment with renal cancer exosomes (Figure 2F). It was reported that E-cadherin localized on the surface of exosome membrane was transported to endothelial cells to promote angiogenesis (Tang et al., 2018). E-cadherin was expressed both in 786-O/786-O-SR cells and their exosomes, and 786-O-SR expression was higher (S1C). This suggested that renal cancer exosomes transmitted E-cadherin to endothelial cells. Studies have suggested that E-cadherin regulated HIF1α (Maroni et al., 2015; Liang et al., 2016), which may be one of the mechanisms by which renal cancer exosomes promote vascular permeability.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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The Biogenesis, Biological Functions, and Applications of Macrophage-Derived Exosomes

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Macrophage-derived exosomes have been implicated on the modulation of inflammatory processes. Recent studies have shown that macrophage-derived exosomes contribute to the progression of many diseases such as cancer, atherosclerosis, diabetes and heart failure. This review describes the biogenesis of macrophage-derived exosomes and their biological functions in different diseases. In addition, the challenges facing the use of macrophage-derived exosomes as delivery tools for drugs, genes, and proteins in clinical applications are described. The application of macrophage-derived exosomes in the diagnosis and treatment of diseases is also discussed.

Keywords: macrophage-derived exosomes, formation mechanisms, polarization, biological functions, applications

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INTRODUCTION

Exosomes are lipid bilayer particles that are actively secreted out of the cell. Pan et al. reported a small vesicle from the supernatant of sheep reticulocytes, which was initially thought to be a cell-secreted waste product (Pan et al., 1985). Further studies on exosomes reported that vesicles comprise several components including cell-specific proteins, lipids and RNA (mRNA, miRNA and other non-coding RNA) (Llorente et al., 2017; Tomasetti et al., 2017). Exosomes can be secreted by a variety of cells, for example, in 1996, Raposo et al. reported that B lymphocytes secrete antigen-presenting vesicles (Raposo et al., 1996). Notably, exosomes secreted by immune cells such as dendritic cells (DCs) modulate the immune response, therefore, these membranous vesicles are being explored as potential immunotherapeutic reagents (Greening et al., 2015). Natural killer (NK) cells exhibit rapid immunity to metastatic or hematological malignancies, and clinical studies are being conducted to explore the antitumor properties of NK cells. A study by Zhu et al. reported that exosomes derived from NK cells (NK-Exos) exert cytotoxic effects on melanoma cells (Zhu et al., 2017). Mast cells are important effector cells of the immune system. Mast cell-derived exosomes carrying RNAs play a role in immune regulation (Liang et al., 2018). Exosomes are widely distributed in various body fluids including blood, urine, peritoneal fluid, synovial fluid and breast milk. They affect the physiological and pathological state of the target cells by carrying and transmitting important signaling molecules to these cells. Extracellular vesicles are grouped into three main categories based on size, biological properties, and formation process. These include exosomes (30–150 nm), microvesicles (200–1,000 nm), and apoptotic bodies (500–2000 nm) (Shao et al., 2018). Exosomes are formed by the intranuclear body system. Formation, sorting of the encapsulated contents, and release of exosomes are regulated by a series of precise regulatory mechanisms. Microvesicles are formed by outgrowth of the cell membrane. Formation is mainly induced stimulation of the redistribution of

the phospholipid bilayer from the cell membrane by inward flow of Ca^{2+}, leading to outgrowth of the cell membrane (Akers et al., 2013). However, the molecular mechanism of microvesicle formation has not been fully elucidated. Apoptotic bodies are formed when the cell membrane crumples, and invaginates during apoptosis, shedding organelles, and nuclear debris with wrapped cytoplasm (Gurunathan et al., 2019). Exosomes are the smallest extracellular vesicles and play key biological roles (Kalluri and LeBleu, 2020). Therefore, exosomes have been explored as novel potential therapeutic tools owing to their ability to modulate various biological processes, including immune response, cell proliferation, cell invasiveness, synapsis plasticity, angiogenesis and tubule formation. Moreover, high levels of macrophage-derived exosomes in blood makes them potential biomarkers for minimally invasive liquid biopsies for diagnosis and prognosis in cancer patients (Ismail et al., 2013; Jin et al., 2015; Lin and Dihua, 2019).

Macrophages are multifunctional cell types presenting in most vertebrate tissues. They form the first line of defense against pathogens through phagocytosis of microbial infections, particles and dead cells (Verdeguer and Aouadi, 2017). Macrophages are heterogeneous cells, and the phenotypes and functions are regulated by the surrounding microenvironment (Shapouri-Moghaddam et al., 2018; Yang et al., 2019b). Macrophages are classified into classically activated (M1) and alternatively activated (M2) macrophages based on whether they mediate anti-inflammatory or pro-inflammatory responses (Olefsky and Glass, 2010; Murray et al., 2014; Verdeguer and Aouadi, 2017). Metabolites associated with microbial infections, such as lipopolysaccharide (LPS) and interferon-gamma (INF-γ), induce secretion of inflammatory factors by macrophages, such as tumor necrosis factor-alpha (TNF-α) and interleukin-12 (IL-12). Therefore, they stimulate the body's immune response by triggering a typical pro-inflammatory response. Notably, macrophages are polarized to M2a in response to IL-4 or IL-13, M2b in response to immune complexes and M2c in response to the anti-inflammatory cytokine, interleukin-10 (IL-10). This polarization induces macrophages to secrete anti-inflammatory factors, such as Arginase-1 (Arg-1) and transforming growth factor-β (TGF-β), thus reducing inflammatory response and promoting wound healing (Atri et al., 2018; Funes et al., 2018). Polarization of macrophages is implicated in development and progression of several diseases and study on macrophages enables understanding of macrophage-derived exosomes (Lee et al., 2014). Exosomes carry biological information on macrophages and play an important regulatory role in several diseases, such as tumors, inflammations, and atherosclerosis (Théry et al., 2002). Macrophage-derived exosomes are more than exosomes from other cell sources, and are biocompatible thus they can be used as drug carriers for drug delivery (Kim et al., 2018). In the current review, the mechanisms of formation, classification, and function of macrophage-derived exosomes were explored. In addition, application of macrophage-derived exosomes as delivery tools of drugs, genes, and proteins was reviewed. Deeper understanding of macrophage-derived exosomes may provide possible therapeutic targets for various diseases.

FORMATION MECHANISMS OF MACROPHAGE-DERIVED EXOSOMES

Formation of macrophage-derived exosomes, similar to that of most cell-derived exosomes, takes place in three main stages including exosome biogenesis, sorting of cargo into exosome and exosome release (Kalluri and LeBleu, 2020). This process is precisely regulated and involving multiple proteins. The cytoplasmic membrane of the macrophage initially invaginates to form endocytic vesicles, and multiple endocytic vesicles fuse to form early endosomes. The early endosomes then invaginate, encapsulating intracellular material in the process, thus forming multiple intracellular vesicles (ILVs) and further transforming into late endosomes, which are known as multivesicular bodies (MVBs). MVBs then fuse with the cytoplasmic membrane and release ILVs into the extracellular space as exosomes. ESCRT (endosomal sorting complex required for transport) pathway is the most explored mechanism for ILV and MVB formation. ESCRT machinery comprises four multimeric complexes and associated proteins that assemble in an ordered manner at the endosome (Friand et al., 2015). ESCRTs comprise approximately twenty proteins that assemble into four complexes (ESCRT-0, -I, -II, and -III) with associated proteins including VPS4, VTA1 and ALIX (Colombo et al., 2013). ESCRT-0, -I, and -II complexes recognize and sequester ubiquitinated membrane proteins at the endosomal delimiting membrane, whereas ESCRT-III complex plays a role in membrane budding and actual scission of ILVs (Raposo and Stoorvogel, 2013). In addition to controlling exosome unharness, ESCRTs are implicated in packaging of biomolecules into exosomes. (Pipe and Katzmann, 2006). Heparanase is a modulator of the syndecan-syntenin-ALIX pathway that induces endosomal membrane budding. This leads to formation of exosomes by trimming the heparan sulphate chains on syndecans (Roucourt et al., 2015). However, studies report that the cargo is segregated into distinct subdomains on the endosomal membrane (Hunt et al., 2013). In addition, transfer of exosome-associated domains into the lumen of the endosome does not depend on the function of the ESCRT, however, it is induced by sphingolipid ceramide. Purified exosomes comprise ceramide, and release of exosomes is reduced by inhibition of neutral sphingomyelinases (Trajkovic et al., 2008). This implies that in addition to proteins, lipids play a regulatory role during release of exosomes. Furthermore, protein sorting in MVBs is mediated by ESCRT-dependent and independent pathways (**Figure 1**).

Macrophage secretion of exosomes is stimulated by the extracellular environment. During the process of cargo sorting, the macrophage P2X7 signaling pathway is activated through stimulation of extracellular adenosine triphosphate (ATP) and the intracellular calcium concentration increases, promoting entry of proteins such as IL-1β into exosomes (Qu et al., 2007). Rab GTPases, the largest family of small GTPases, regulate several steps of membrane trafficking, including vesicle budding, transport of vesicles along actin and tubulin, and membrane fusion (Stenmark, 2009). LPS induces the release process of macrophage exosomes, however, this effect can be reversed by IL-25, which also downregulates expression of

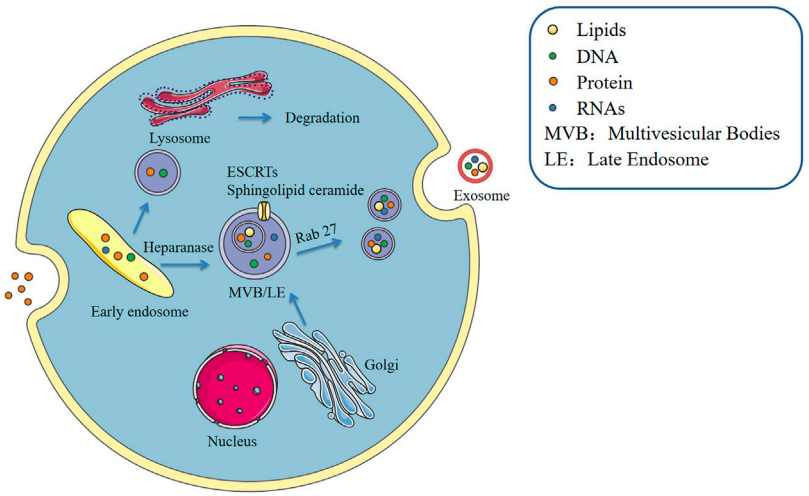

FIGURE 1 | Formation of exosomes.

Rab27a and Rab27b in macrophages. IL-25 thus inhibits exosome release (Li et al., 2018). Pretreatment of RAW264.7 macrophages with the exosome secretion inhibitor GW4869, followed by stimulation with LPS, causes a reduction in macrophage exosome secretion and reduction in inflammatory factor secretion. Therefore, blocking exosome production in sepsis can inhibit sepsis-induced inflammatory response thus improving cardiac function and survival (Essandoh et al., 2015). In addition, most mature MVB are broken down by lysosomes and the products are released into the extracellular environment as extracellular bodies with the help of Rab proteins and small GTPases by cytosolic spitting. This indicates that the function of lysosomes is closely linked to secretion of exosomes. In alcoholic liver disease, alcohol decreases lysosomal function in hepatocytes and macrophages, and expression of lysosome-associated membrane protein 1 (LAMP1) and LAMP2 is downregulated. Amphisome in macrophages does not bind to lysosomes, resulting in increase in the level of exosome release (Liu et al., 2020a). These findings have significant implications in understanding the role of different cell types and different cellular environments in modulating exosome release. Increase or decrease in secretion of macrophage-derived exosomes has different implications for different diseases.

DIFFERENT PHENOTYPES OF MACROPHAGE-DERIVED EXOSOMES

Macrophage Metabolism and Polarization

Macrophages are heterogeneous and their phenotype and functions are regulated by the surrounding micro-environment. An IL-4-mediated macrophage phenotype known as alternatively activated (M2) macrophages was reported in the early 1990s. This phenotype was characterized by high clearance of mannosylated ligands, enhanced expression of MHC II antigens, and reduced secretion of pro-inflammatory cytokines compared with the classically activated M1 macrophages induced by IFN-γ (Martinez and Siamon, 2014). This classification is based on the phenotypic changes observed *in vitro* after stimulation by different cytokines (Stein et al., 1992). Different typologies of macrophages and their cell expression markers are presented in **Figure 2**. Reprogramming of intracellular metabolism is necessary for effective polarization and function of activated macrophages. M1 macrophages increase glucose consumption and lactate release, whereas M2 macrophages predominantly promote the oxidative glucose metabolic pathway. In the tumor microenvironment, glucose metabolism of tumor-associated macrophages (TAMs) mainly takes place through aerobic glycolysis. Inhibition of aerobic glycolysis of TAMs can convert the tumor-promoting M2-TAMs to the tumor-inhibiting M1-TAMs, thus inhibiting tumor development (Linnan et al., 2015). Mills et al. reported that oxidized succinate and mitochondrial membrane potential in the mitochondria of macrophages is increased by LPS stimulation, and succinate dehydrogenase (SDH) promotes mitochondrial reactive oxygen species (ROS) production (Mills et al., 2016). This implies that the macrophage function shifted from oxidative phosphorylation to ATP production to glycolysis, resulting in increased succinate levels. Moreover, succinate promotes LPS-induced glycolysis in macrophages and promotes and maintains expression of endogenous pro-inflammatory genes and inhibits expression of anti-inflammatory genes (O'Neill and Pearce, 2015). The carbohydrate kinase-like protein (CARKL) induces macrophage polarization by regulating glucose metabolism (Haschemi et al., 2012). Succinic acid regulates the pro-inflammatory IL-1β-HIF-1α axis, whereas itaconate exerts anti-inflammatory effects by inhibiting succinate dehydrogenase-mediated oxidation of succinate thus regulate macrophage metabolism (Lampropoulou et al., 2016). These studies report an interactive relationship between metabolic reprogramming and macrophage polarization. Understanding

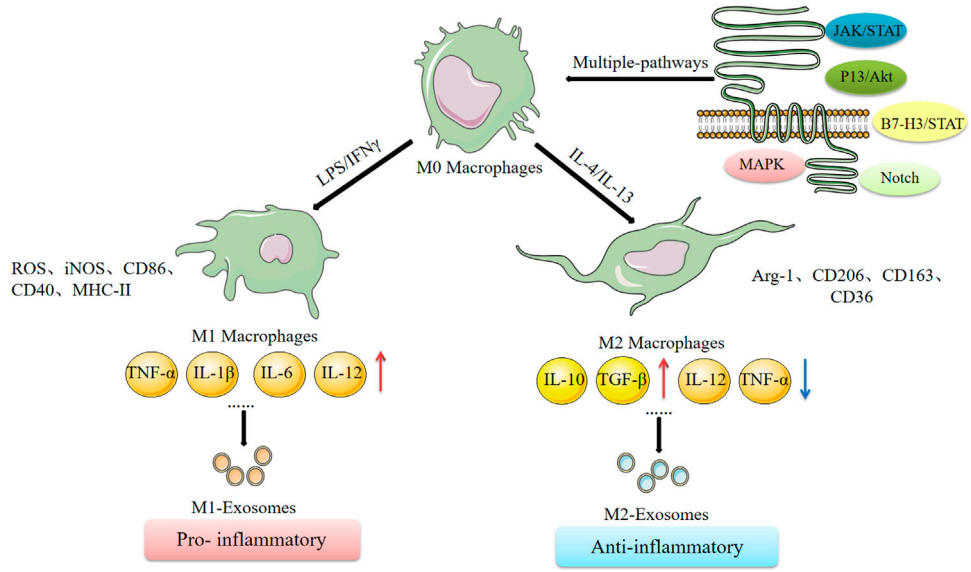

FIGURE 2 | Macrophage polarization and its related mechanisms.

the relationship between cellular metabolism and macrophage polarization provides an insight into the molecular mechanisms underlying functions of exosomes in cancer development.

Differences Between Macrophage-Derived Exosome Subtypes

Three types of macrophage-derived exosomes including unpolarised M0 macrophage-derived exosomes (M0-Exos), polarised M1 and M2 macrophage-derived exosomes (M1-Exos and M2-Exos) have been explored (Funes et al., 2018). Variations between exosomes derived from completely different phenotypes of macrophages have been reported which reflect the parental cell properties. For example, M2-Exos contain more miR-365 than M1-Exos, and blocking this miRNA restores sensitivity of cancer cells to gemcitabine (Binenbaum et al., 2018). M1-Exos have high levels of miR-326 and suppress proliferation, migration, and invasion, and promote apoptosis of hepatocellular carcinoma cells (HCC), through downregulation of NF-κB expression in HCC by miR-326 (Bai et al., 2020). Long non-coding RNAs (lncRNAs) play key roles in multiple diseases. Wu et al. reported that the lncRNA PVT1 carried by M2-Exos acts as a miR-21-5p sponge to upregulate the cytokine signaling repressor protein, SOCS5 and inactivates the JAKs/STAT3 pathway (Wu et al., 2020b). M1-Exos containing miR-16-5p inhibit gastric cancer progression by activating T-cell immune responses through PD-L1 (Li et al., 2020b). Furthermore, M0 macrophage-derived extracellular transfer of miR-223 induces resistance to adriamycin in gastric cancer (Gao et al., 2020). In summary, these findings indicate that different phenotypes of macrophage-derived exosomes contain different biological information, thus perform different functions. Several studies on exosomes have nit explored whether macrophages are polarized or not and the origin of macrophage exosomes is not fully elucidated in these studies. Macrophage-derived exosomes increases MMP-2 expression in vascular smooth muscle cells through activation of JNK and p38 pathways, thus promoting abdominal aortic aneurysm (Wang et al., 2019a). Macrophage-derived exosomes can directly inhibit pro-inflammatory enzymes and cytokines such as IL-6 and TNF-α in diabetic wound dysfunction to achieve anti-inflammatory effects and further induce endothelial cell proliferation and migration to accelerate the wound healing process (Li et al., 2019b). Exosomes are “nanospheres”, which contain proteins and lipids from parental cells, mainly including tetraspanin (CD9, CD63 and CD81), proteins involved in biosynthesis of multivesicles (such as Alix and TSG101), heat shock proteins (HSP70 and HSP90) and membrane translocation and fusion proteins (GTPases and membrane coupling proteins). These protein markers of exosomes were originally identified by mass spectrometry during purification of exosomes as highly abundant proteins present in extracellular vesicles, thus these proteins were used as markers of extracellular vesicles. However, currently there are no biomarkers that can distinguish M1-Exos from M2-Exos (**Table 1**). Application of new technologies, such as neighborhood coding techniques (Wu et al., 2019), may facilitate differentiation of exosomes from different origins.

THE BIOLOGICAL FUNCTIONS OF MACROPHAGE-DERIVED EXOSOMES

Role of Macrophage-Derived Exosomes in the Tumor Microenvironment

Tumor microenvironment is a local homeostatic environment comprising tumor cells, macrophages, fibroblasts, and extracellular matrix. It plays an important role in development, recurrence, metastasis, and chemotherapy

TABLE 1 | Biomarkers of macrophage-derived exosomes.

Protein type	Protein name	References
Tetraspanin	CD9, CD63, CD81	(Jin et al., 2015; Deng et al., 2018)
ESCRT proteins	Alix, TSG101	(Diaz et al., 2018; Jeppesen et al., 2019)
Heat shock proteins	HSP60, HSP70, HSPA5, CCT2, HSP90	(Mathivanan et al., 2010; Deng et al., 2018)
Membrane translocation and fusion proteins	GTPases, membrane coupling proteins, annexins, flotillin	(Théry et al., 2002; Liu et al., 2017)

resistance of cancer (Perrin et al., 2019; Vitale et al., 2019). Macrophage-derived exosomes are one of the independent components of the tumor microenvironment once they are released into the extracellular environment and they play their functions in the tumor microenvironment (Liu et al., 2020b). Tumor-associated macrophages (TAMs) are similar to M2-polarized macrophages, which are activated by Th2 cytokines (IL-4, IL-10, and IL-13) (Lan et al., 2019). MiR-501-3p in M2-Exos promotes tumor development by activating the transforming growth factor-β signaling pathway and inhibiting the tumor suppressor gene TGFBR3 (Yin et al., 2019). M2-Exos transfer LncRNA AFAP1-AS1, down-regulate miR-26a and up-regulate activating transcription factor 2 (ATF2), thus promoting esophageal cancer invasion and metastasis. Targeting M2 macrophages and the lncRNA AFAP1AS1/miR-26a/ATF2 signaling axis is a potential therapeutic strategy for esophageal cancer (Mi et al., 2020). Apolipoprotein E (ApoE) is a highly specific protein in M2-Exos. M2-Exos mediate intercellular transfer of the ApoE-activated PI3K-Akt signaling pathway within recipient gastric cancer cells thus it can remodel cytoskeleton-supporting migration (Zheng et al., 2018). Similarly, Lan et al. reported that M2-Exos exhibit a regulatory effect on BRG1 through delivery of miR-21 and miR-155-5p, thus downregulating BRG1 to promote colorectal cancer metastasis (Lan et al., 2019). miRNAs carried by M2-Exos are important targets for reversing tumor migration whereas altering the phenotype of macrophages can be used to regulate the tumor microenvironment. Exosomes derived from M1 macrophages repolarize M2 macrophages into M1 macrophages, thus they are used to enhance anti-cancer effects of immune checkpoint inhibitors such as aPD-L1 (Choo et al., 2018). Notably, study reports that M1-Exos can polarize macrophages into M1 macrophages. M1-Exos activate the macrophage NF-κB pathway through a caspase-3-mediated pathway, promoting release of inflammatory cytokines, thus establishing a local inflammatory environment and enhancing their anti-tumor activity (Wang et al., 2019b).

Role of Macrophage-Derived Exosomes in Atherosclerosis

Macrophage-derived exosome-mediated cell-cell communication plays an important role in atherosclerotic processes. Oxidized low-density lipoprotein (ox-LDL) promotes dysregulation of the metabolism of lipoproteins and deposition of lipoproteins in the arterial wall. In addition, ox-LDL is implicated in initiation and development of atherosclerosis (AS). ox-LDL stimulates macrophage-derived exosomes and mediates endothelial cell growth and tube-forming capacity. Notably, blocking exosome secretion rescues endothelial cell growth and tube-forming capacity (Huang et al., 2018). Nguyen et al. reported that the expression profile of ox-LDL stimulated macrophage-derived exosomal miRNAs and exosomal miRNAs, mainly miR-146a, may accelerate development of atherosclerosis by reducing cell migration and promoting macrophage capture in the vessel wall (Nguyen et al., 2018). Further studies report that miR-146a is enriched in serum-derived exosomes from atherosclerotic patients and ox-LDL-treated macrophage-derived exosomes. Exosomal miR-146a secreted by ox-LDL-treated macrophages accelerates AS by targeting superoxide dismutase 2 (SOD2) and promoting release of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) (Zhang et al., 2019). Moreover, increased expression of macrophage-derived exosomal miRNA-21-3p exhibits similar activities to those of miR-146a (Zhu. et al., 2019). MSC-derived exosomes attenuate atherosclerotic progression through miR-let7-mediated infiltration and polarization of M2 macrophages (Li et al., 2019a). Wu et al. electroporated M2-Exos with hexyl 5-aminolevulinate hydrochloride (HAL) (Wu et al., 2020a). After systemic administration, the molecularly engineered M2-Exos exhibited good chemotactic and anti-inflammatory effects, which promoted release anti-inflammatory cytokines from anti-inflammatory M2 macrophages by binding to surface-bound chemokine receptors. Furthermore, encapsulated HAL can produce anti-inflammatory carbon monoxide and bilirubin through endogenous biosynthesis and metabolism of hemoglobin, thus further promoting anti-inflammatory effect and ultimately reducing AS. Although the role of macrophage-derived exosomes in atherosclerosis has received mixed reviews, exosomal miRNA and lncRNA are more stable compared with serum RNA, and altering their levels in exosomes may be more valuable in treatment of AS.

Role of Macrophage-Derived Exosomes in Diabetes

Obesity is a risk factor for diabetes and is correlated with intracellular stress, low-grade inflammation, over-activation of the inflammatory response, and imbalance in M1-M2 polarization of macrophages (Bouloumié et al., 2005). A previous study reports that adipose tissue macrophage (ATM) secreted exosomes from obese mice cause abnormal glucose tolerance and insulin resistance when administered to lean mice. On the contrary, ATM exosomes harvested from lean mice improved glucose tolerance and insulin sensitivity when administered to obese recipients. Further, *in vivo* and *in vitro*

studies of ATM-secreted exosomes report that miRNAs in the exogenous genes cause modulate insulin signaling (Ying et al., 2017). Exosomes secreted by macrophages exhibit no effect on differentiation from preadipocytes to adipocytes, fat storage, and insulin-mediated glucose uptake. However, miRNAs in LPS-activated macrophage exosomes are highly variable, for instance miR-530, chr16_34840, and chr9_22532 are highly expressed in these exosomes (De et al., 2018). miRNA-mediated pathogenesis of diabetes contained in macrophage-derived exosomes can be explored as a new target for development of diabetes diagnosis approaches and clinical therapy. Tian et al. reported that miR-210 in adipose tissue macrophages regulates glucose uptake and mitochondrial complex IV (CIV) activity by targeting ubiquinone 1 alpha subcomplex 4 (NDUFA4) gene expression, thus promoting development of obesity diabetes in mice (Tian et al., 2020). Diabetic foot disease is a major complication of diabetes. Macrophage-derived exosomes significantly reduce secretion of pro-inflammatory cytokines and promote proliferation and migration of endothelial cells, thus improving angiogenesis and re-epithelialization of diabetic wounds (Li et al., 2019b). However, the study did not elucidate the type of macrophages implicated in this role (Li et al., 2019b). Furthermore, a previous study reported that M2 macrophages improve high glucose (HG)-induced podocyte apoptosis and epithelial-mesenchymal transition by secreting miR25-3p in exosomes and confirmed that dual-specificity protein phosphatase 1 (DUSP1) was the downstream target. MiR25-3p acts by inhibiting DUSP1 expression to activate cellular autophagy (Huang et al., 2020). In summary, the ability of macrophage-derived exosomes to modulate the inflammatory microenvironment and to express miRNAs implies that it is a potential therapeutic strategy for treatment of diabetes-related metabolic diseases.

Role of Macrophage-Derived Exosomes in Heart Disease

A fine-tuned balance between M1 and M2 macrophage states is important for myocardial repair. Although M1 macrophages play a key role in the immune response of the heart, they promote pro-inflammatory state and degradation of extracellular matrix and cell death. Stimulation of macrophage polarization towards M2 phenotype promotes regression of inflammation and facilitates infarct healing after acute myocardial infarction (Zhou et al., 2015). M2-Exos carrying miR-148a alleviates myocardial ischemia/reperfusion (MI/R) injury by down-regulating thioredoxin-interacting protein (TXNIP) and through inactivation of the TLR4/NF-κB/NLRP3 inflammasome signaling pathway (Dai et al., 2020). MiR-155 is a specific marker for M1 macrophage differentiation and a mediator of miRNA, and is one of the most abundant miRNAs in M1-Exos (Jablonski et al., 2016). Recent studies explored the role of miR-155 in myocardial injury. Wang et al. reported high expression levels of miR-155 in exosomes of activated macrophages (Wang et al., 2017). Notably, miR-155-enriched exosomes suppressed fibroblast proliferation and promoted fibroblast inflammation (Wang et al., 2017). Furthermore, miR-155 downregulation significantly decreased incidence of cardiac rupture and improved cardiac function after acute myocardial infarction (AMI) (Wang et al., 2017). However, the study did not explore whether the stimulated macrophages were M1 macrophages. A previous study reported that M1-Exos inhibit Sirt1/AMPKα2 endothelial nitric oxide synthase and RAC1-PAK2 signaling pathways by targeting five molecular nodes (genes) through delivery of miR-155 to endothelial cells. These events reduce the angiogenic capacity of endothelial cells, exacerbate myocardial injury and inhibit cardiac healing (Liu et al., 2020b). Fusion of released exosomes with the plasma membrane results in release of miR-155 into the cytosol and translational repression of forkhead transcription factors of the O class (FoxO3a) in cardiomyocytes. Macrophage-derived miR-155-containing exosomes promote cardiomyocyte pyroptosis and uremic cardiomyopathy changes by directly targeting FoxO3a in uremic mice (Wang et al., 2020). These findings indicate that inhibition of secretion of miR-155-containing macrophage-derived exosomes, or targeted inhibition of miR-155 gene expression is a novel strategy for treatment of cardiomyopathies.

Role of Macrophage-Derived Exosomes in Inflammation

Macrophage-derived exosomes are highly correlated with inflammation. A previous study explored the effects of different types of M2 macrophage-derived exosomes (M2a, M2b, and M2c macrophage-derived exosomes) on inflammatory bowel disease (IBD) induced by dextran sodium sulfate (DSS). The findings showed that although all types of M2 macrophage-derived exosomes reduced severity of IBD, M2b macrophage-derived exosomes were more effective compared with M2a and M2c macrophage-derived exosomes. M2b macrophage-derived exosomes carry Chemokine (C-C Motif) Ligand 1 (CCL1) protein to the colon, which interacts with its ligand C-C chemokine receptor 8 (CCR8), and promotes Th2 cells polarization, thus increases levels of Treg cells and reduces production of pro-inflammatory cytokines in the colon (IL-1β, IL-6, and IL-17A) (Yang et al., 2019a). Diabetic wound dysfunction is a severe, chronic complication of diabetes, and is characterized by continuous inflammatory response leading to impaired wound healing. Li et al. reported that exosomes inhibited activation of the AKT (P-AKT) signaling pathway, down-regulated expression of MMP-9, reduced secretion of inflammatory factors, improved pathophysiological status of diabetic wounds, and accelerated healing process in diabetic rats after administration of macrophage-derived exosomes Li et al., 2019b). Moreover, MSCs can release several exosomes with superior regulatory and regenerative abilities thus maintaining the balance of macrophages and improving the resolution of chronic inflammation after LPS treatment. LPS pretreated MSC-derived exosomes promote conversion of macrophages to an M2-like phenotype by shuttling let-7b (Ti et al., 2015). Macrophages infiltrate blood vessels and release exosomes that interact with endothelial cells during hypertension thus increasing inflammation by increasing expression of

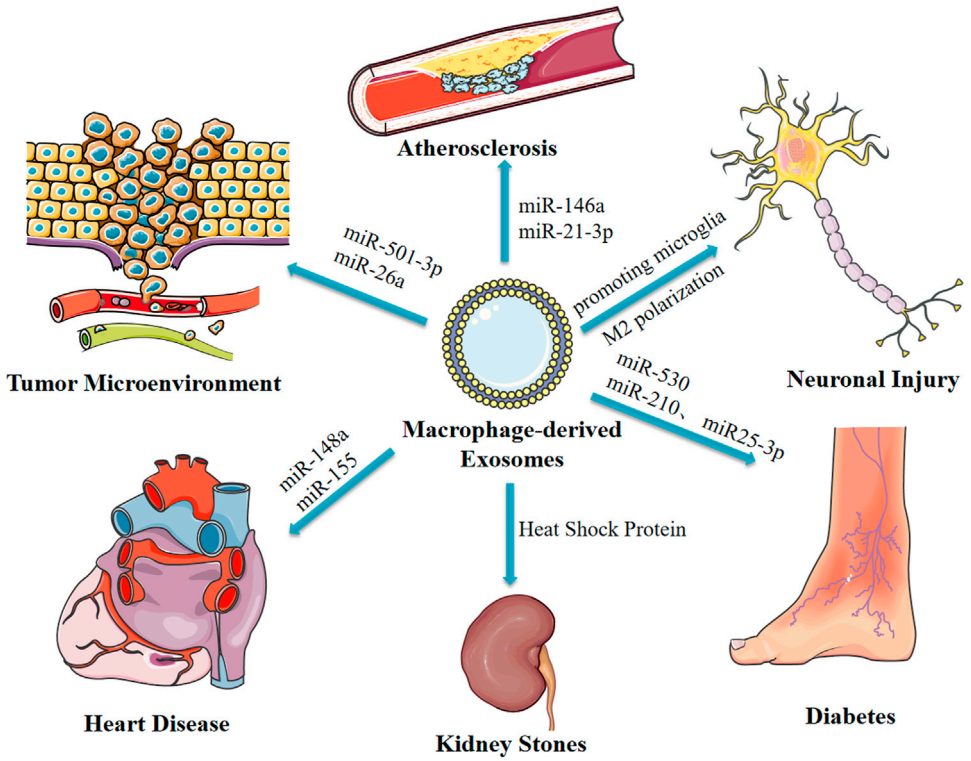

FIGURE 3 | Macrophage-derived exosomes affect disease progression through delivery of miRNAs and other pathways.

endothelial cell adhesion factor-1 (ICAM-1) and fibrinogen activator inhibitor-1 (PAI-1) (Osada-Oka et al., 2016). Macrophage-derived exosomes play anti-inflammatory roles and regulate homeostasis in organisms (Mcdonald et al., 2014). Holder et al. reported that the human placenta takes up macrophage-derived exosomes in a time- and dose-dependent manner through clathrin-dependent endocytosis. Moreover, macrophage-derived exosomes induce the placenta to produce pro-inflammatory cytokines thus activating a response to maternal inflammation and infection and preventing damage to the fetus (Holder et al., 2016). Ye et al. reported that macrophage-derived exosomes are the main early secretors of pro-inflammatory cytokines in severe acute lung injury (ALI) and may activate neutrophils to produce several pro-inflammatory cytokines and IL-10. The IL-10 may then polarize macrophages to M2c, which may cause fibrosis after ALI (Ye et al., 2020).

Other Diseases

Spasmolytic polypeptide-expressing metaplasia (SPEM) is the initial step of gastric precancerous lesions, which can progress to heterogeneous hyperplasia or even carcinoma with chronic inflammatory stimulation. Macrophages may be involved in this inflammatory process. Xu et al. reported that Deoxycholic acid-stimulated exosomes secreted by macrophages promote cellular communication between macrophages and gastric epithelial cells, thus facilitating development of SPEM, however, the study did not elucidate the mechanism (Xu et al., 2020). Macrophages are implicated in pathogenesis of kidney stones as they are involved in the immune response through the exosomal pathway after exposure to calcium hydroxalate (COM) crystal crystals. Although other proteins are involved, the main protein implicated in this process is the heat shock protein (Nilubon et al., 2018). LPS-stimulated exosomes secreted by macrophages inhibit neuronal inflammation in acute ischemia-induced neuronal injury by promoting microglia M2 polarization (Zheng et al., 2019). In addition, macrophage-derived exosomes from syphilis spirochete infection promote adhesion and permeability of human umbilical vein endothelial cells. Although the study did not explore the mechanisms underlying the activity, it reported that macrophage-derived exosomes are implicated in the pathogenesis of syphilis (Xu et al., 2019). These findings indicate the significance of macrophage-derived exosomes in development and progression several diseases. Therefore, these findings provide a basis for identification of new targets for treatment of different diseases (**Figure 3**).

APPLICATIONS OF MACROPHAGE-DERIVED EXOSOMES

Engineering Macrophage-Derived Exosomes

Modification of exosomes through genetic or non-genetic approaches can enhance cytotoxicity and targeting of

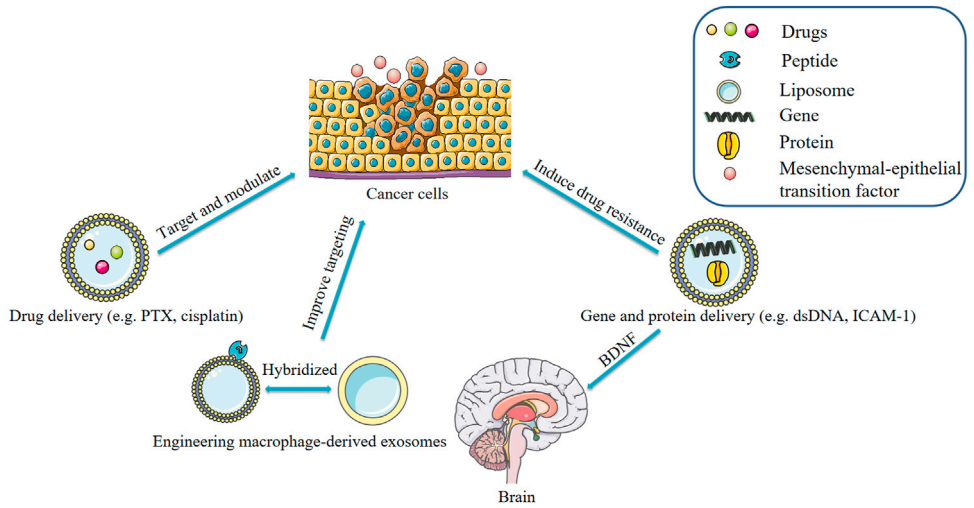

FIGURE 4 | Applications of macrophage-derived exosomes.

therapeutic agents, thus improving their effectiveness in killing cancer cells (Luan et al., 2017; You et al., 2018). Macrophage-derived exosomes can be packaged with various molecules to target tumor sites. Kim et al. developed and optimized a formulation of macrophage-derived exosome-loaded paclitaxel incorporating an aminoethylbenzamide-polyethylene glycol (AA-PEG) carrier fraction to target the overexpressed sigma receptor in lung cancer cells (Kim et al., 2018). The AA-PEG carrier exosome carrying PTX- (AA-PEG-exoPTX) exhibited a high drug loading capacity and high accumulation in cancer cells after systemic administration (Kim et al., 2018). Chemotherapy and surgery are the conventional treatments for triple-negative breast cancer (TNBC) due to a lack of effective therapeutic targets. However, limitations such as poor targeting and toxicity of chemotherapeutic agents limit the efficacy of chemotherapy and surgery. To circumvent this limitation, Li et al. developed a nano-delivery system of macrophage-derived exosome-encapsulated poly (lactic acid-hydroxyacetic acid) (Li et al., 2020a). The group modified the surface of the exosome with a peptide to target the mesenchymal-epithelial transition factor (c-Met), which is overexpressed by TNBC cells (Li et al., 2020a). A15 is the only ADAM protein containing an Arg-Gly-Asp (RGD) motif in its disintegrin-like domain. A15-rich exosomes with integrin αvβ3 increases affinity to tumor cells in an RGD-dependent manner (Chen et al., 2008; Ungerer et al., 2010). Gong et al. designed an A15-modified exosome-encapsulated adriamycin with a cholesterol-modified delivery system which exhibited synergistic anticancer effects *in vitro* and *in vivo* without adverse effects (Gong et al., 2019). Rayamajhi et al. combined macrophage-derived exosomes with synthetic liposomes and the bionic exosome improved the yield of exosomes, and improved targeting of tumor sites through encapsulation of adriamycin (Rayamajhi et al., 2019). These engineered macrophage-derived exosomes significantly improve targeting, however their safety should be evaluated (Luan et al., 2017).

Application of Macrophage-Derived Exosomes as Drug Delivery Tools

In recent years, the use of exosomes as drug delivery systems has gained strong interest from researchers (Wei et al., 2017; Zhang et al., 2018). Previously, three cell-derived exosomes: pancreatic cancer cells (PCCs), pancreatic stellate cells (PSCs), and macrophages were used to deliver adriamycin. It was found that among the three types of exosomes, PCCs-derived exosomes had the highest drug loading efficiency whereas macrophage-derived exosomes loaded with adriamycin yielded the highest anti-tumor effect (Kanchanapally et al., 2019). Similarly, M1 macrophage-derived exosomes loaded with paclitaxel inhibited tumors by activating macrophage-mediated inflammation (Wang et al., 2019b). One factor that significantly limits the efficacy of chemotherapeutical medicine is multidrug resistance (MDR). Among the mechanisms that lead to MDR include overexpression of drug outflow transporter P-glycoprotein (Pgp) (Krishna et al., 2001; Sui et al., 2012). One study used ultrasound to encapsulate paclitaxel into exosomes (exoPTX) for delivery. The exoPTX inhibited the activity of p-gp thereby overcoming MDR in tumors. However, further investigations are needed to unravel the mechanism involved (Kim et al., 2016). Cisplatin is a platinum-containing anticancer drug that causes apoptosis primarily by damaging DNA and inhibiting replication and mitosis (Florea and Busselberg, 2011). A previous study (Zhang et al., 2020) found that umbilical cord-derived macrophages differentiated into M1 and M2 cells under the action of cytokines. M1 and M2 exosomes leaded with cisplatin fused with ovarian cancer cell line A2780 and cisplatin-resistant A2780/DDP leading to its accumulation in the cytoplasm near the nucleus, and reduce cisplatin IC50 (half maximal inhibitory concentration) of A2780 and A2780/DDP. Through this mechanism, it inhibits proliferation and promotes apoptosis of A2780 cells. In

comparison, M1 exosomes loaded with cisplatin showed stronger anti-tumor effect than M2 exosomes. Nevertheless, molecules on the surface of exosomes that facilitate exosomal binding to target cancer cells should be further elucidated. In summary, macrophage-derived exosomes acquire macrophage properties such as the ability to target and modulate the tumor microenvironment and are therefore a promising vehicle for drug delivery. However, the application of exosomes as drug delivery systems is limited by low yields of exosomes from many tissues. This problem may be solved by constructing exosome-mimetic vesicles or genetic engineered exosomes (Xia et al., 2020).

Use of Macrophage-Derived Exosomes as Gene and Protein Delivery Vehicles

Exosomes contain genes and proteins derived from parental cells. Recent research has found that macrophage-derived exosomes significantly decreased the sensitivity of PDAC (pancreatic ductal adenocarcinoma) cells to gemcitabine. In the study by Yoav et al., artificial dsDNA (barcode fragments) was transfected into mouse peritoneal macrophages and injected into mice bearing PDAC tumors. The concentration of barcode fragments was 4-fold higher in primary tumors and liver metastases than in normal tissue. This effect was mediated by the transfer of miR-365 in macrophage-derived exosomes. MiR-365 impaired activation of gemcitabine by upregulation of the triphospho-nucleotide pool in cancer cells and the induction of the enzyme cytidine deaminase; the latter inactivates gemcitabine (Yoav et al., 2018). Elsewhere, it was found that macrophage-derived exosomes contain integrin lymphocyte function-associated antigen 1 (LFA-1) acquired from parental cells (Yuan et al., 2017). These exosomes interact with intercellular adhesion molecule 1 (ICAM-1) and transport a brain-derived neurotrophic factor (BDNF) to the brain. TAMs are characterized by M2-polarized phenotype and have been shown to promote the migration of gastric cancer cells. A recent study suggested that M2 macrophage-derived exosomes mediate an intercellular transfer of ApoE-activating PI3K-Akt signaling pathway in recipient gastric cancer cells to remodel the cytoskeleton-supporting migration. Because ApoE is a highly specific and effective protein in M2 macrophages-derived exosomes. Of note, exosomes derived from M2 macrophages of $ApoE^{-/-}$ mice did not affect the migration of gastric cancer cells (Zheng et al., 2018). The delivery of a therapeutic miRNA or protein to its target tissue or cell has been a challenging task (Zhang et al., 2018). Recent studies have confirmed that macrophage-derived exosomes have great potential in the treatment of diseases by serving as delivery vehicles for genes and proteins. It is possible to modify parental cells and use transgenesis to make such cells secrete exosomes containing the desired therapeutic protein (**Figure 4**).

CONCLUSION AND PERSPECTIVES

In summary, macrophage-derived exosomes have important role in the treatment of diseases such as tumors, atherosclerosis, and diabetes. When used as delivery vehicles, they bind to receptors on target cells thereby delivering loaded drugs such as proteins and nucleic acids. The function of macrophage-derived exosomes is influenced by macrophage polarization, which regulated by the surrounding inflammatory environment. Inflammation is usually an alternating process, thus the role of macrophage-derived exosomes should be viewed in a dynamic light.

Compared to artificially targeted nanocarriers, macrophage-derived exosomes are safer and can be easily modified for application in gene therapy. The following issues also need to be resolved in future studies: 1) Currently, there is no uniform protocol to isolate, purify, and preserve exosomes. Moreover, some of the existing isolation and purification methods are not effective. 2) The low yields of exosomes from specific donor cells limits their application as targeted drug carrier systems. 3) There are few pharmacokinetic studies on the use of macrophage-derived exosomes as drug delivery tools, which limits their development as biopharmaceuticals. It is believed that with the development of biotechnology, macrophage-derived exosomes will play a key role in the diagnosis, prevention, and treatment of diseases in the future.

AUTHOR CONTRIBUTIONS

XS, CZ, YX, CM and LW participated in the design of this review and revised manuscript. XS, CZ, XH, and WC wrote the manuscript. CM, DP, XS, XH, NC, YX and LW collected literature and made a preliminary summary. All authors contributed to the article and approved the submitted version.

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AAV-Containing Exosomes as a Novel Vector for Improved Gene Delivery to Lung Cancer Cells

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Lung carcinoma is the most common type of cancer and the leading cause of cancer-related death worldwide. Among the numerous therapeutic strategies for the treatment of lung cancer, adeno-associated virus (AAV)-mediated gene transfer has been demonstrated to have the potential to effectively suppress tumor growth or reverse the progression of the disease in a number of preclinical studies. AAV vector has a safety profile; however, the relatively low delivery efficacy to particular subtypes of lung carcinoma has limited its prospective clinical translation. Exosomes are nanosized extracellular vesicles secreted from nearly all known cell types. Exosomes have a membrane-enclosed structure carrying a range of cargo molecules for efficient intercellular transfer of functional entities, thus are considered as a superior vector for drug delivery. In the present study, we developed a novel strategy to produce and purify AAV-containing exosomes (AAVExo) from AAV-packaging HEK 293T cells. The cellular uptake capacity of exosomes assisted and enhanced AAV entry into cells and protected AAV from antibody neutralization, which was a serious challenge for AAV *in vivo* application. We tested a list of lung cancer cell lines representing non-small-cell lung cancer and small-cell lung cancer and found that AAVExo apparently improved the gene transfer efficiency compared to conventional AAV vector. Our *in vitro* results were supported *in vivo* in a lung cancer xenograft rodent model. Additionally, we evaluated the gene delivery efficiency in the presence of neutralizing antibody on lung cancer cells. The results demonstrated that AAVExo-mediated gene transfer was not impacted, while the AAV vectors were significantly blocked by the neutralizing antibody. Taken together, we established an efficient methodology for AAVExo purification, and the purified AAVExo largely enhanced gene delivery to lung cancer cells with remarkable resistance to antibody neutralization.

Keywords: AAV-exosome, AAV, lung cancer, neutralizing antibody, gene therapy, exosome, extracellular vesicles

INTRODUCTION

Lung carcinoma is the most common type of cancer and remains in the top rank of cancer-related mortality (Bray et al., 2018). Based on the cell size and appearance in histopathology, lung cancer is commonly categorized into two types including the non-small-cell lung cancer (NSCLC) and the small-cell lung cancer (SCLC), with the former accounts for more than 80% of lung cancers. Although chemotherapy and radiotherapy remain the standard treatment, they have serious side effects (Singh and Dhindsa, 2007). Gene therapy is defined as a therapeutic strategy introducing genetic materials into target cells. Up to date, the majority of clinical trials of gene therapy have targeted tumor tissues with delivered nucleotides expressed antiangiogenic factor, tumor suppressors, or immune stimulators for cancer treatment (Santiago-Ortiz and Schaffer, 2016). However, the selection of an ideal vector for gene delivery is still a major challenge. Adeno-associated virus (AAV) is a promising gene delivery vector for its safety, low toxicity, and multiple serotypes with preferred tropism to distinct tissue and cell types. Chen et al. (2017) observed suppression of NSCLC *in vivo* through delivery of Ang-(1–7) via AAV8, while another group suggested an AAV5-mediated strategy targeting mice bearing a xenograft A549 cancer (Wu et al., 2007). However, it was difficult to develop a best recombinant AAV for diverse lung cancer types, and the reported highest transduction efficiency was achieved between 30 and 50% at multiplicity of infection (MOI) of 100 (Chen et al., 2013), which was relatively low for an efficient clinical translation. The other major challenge that limits the efficacy of AAV-mediated gene therapy is the presence of serum-neutralizing antibody (Nab) that binds to AAV and blocks AAV infection (Nonnenmacher and Weber, 2012; Louis Jeune et al., 2013; Chaanine et al., 2014; Greenberg et al., 2015; Rapti et al., 2015). In fact, more than 90% of the human population is naturally infected with AAV, and about half has neutralizing antibody (Nab) against the virus (Louis Jeune et al., 2013). Nab binds to AAV capsid epitopes and inhibits their interaction with target cells (Nonnenmacher and Weber, 2011, 2012; Nonnenmacher et al., 2015), thereby reducing AAV transduction efficiency.

Exosomes are nanosized extracellular vesicles secreted from almost all cell types. Cargos carried within exosomes include specific proteins and RNAs that are transferred to recipient cells in the vicinity or at a distance (Liang and Sahoo, 2015). Recent studies indicate exosomes as a natural carrier for virus including hepatitis A and hepatitis C virus (Feng et al., 2013; Ramakrishnaiah et al., 2013). Interestingly, AAVs are also naturally secreted via exosomes (Maguire et al., 2012; György et al., 2014). Exosomes with their efficient rate of uptake by multiple cell types can assist the delivery of AAVs to target cells, and the naturally enveloped arrangement would shield the AAV vector from the neutralizing antibody. Therefore, AAV-containing exosomes (AAVExo) could be superior agents for delivering genes to lung cancer cells.

In this study, we developed a method of AAVExo purification based on iodixanol density gradient ultracentrifugation. Our purification methodology was able to effectively isolate AAVExo with minimal free AAV contamination. We compared the transduction rate of AAVExo with conventional AAV in several NSCLC cell lines (A549; H1299; adenocarcinoma HCC827, H23, and H1975; large-cell carcinoma H460; and H661) and SCLC cell lines (H446). We found that AAVExo has significantly higher transduction efficiency across all these cell types compared to the conventional AAV. Remarkably, AAVExo has superior resistance to Nab as compared to free AAV for lung cancer cell gene delivery. Our data support the improvement of AAVExo-mediated gene transfer both *in vitro* and *in vivo* and present a positive clinical prospect for lung cancer treatment in the future.

MATERIALS AND METHODS

Cell Culture

HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA, United States) and cultured in Dulbecco's modified Eagle's medium (Cat. No. C11995500BT, Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (Cat. No. F8318, Sigma-Merck, United States) and 1% streptomycin–penicillin (Cat. No. V900929, Sigma-Merck, Shanghai Warehouse, China) in a humidified atmosphere with 5% CO_2 at 37°C. A549, H1299, HCC827, H23, H1975, H460, H661, and H446 cell lines were obtained from the American Type Culture Collection (Manassas, VA, United States) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Cat. No. SH30809.01, HyClone Laboratories, South Logan, UT, United States) with the same supplements as that of HEK 293T cell culture.

AAV and AAVExo Production and Purification

AAVs were produced by double transfection of HEK 293T cells as described previously (Rapti et al., 2012). Briefly, cells were cultured in a T175 flask with culture medium. When 60–70% confluency was achieved, cell culture medium was replaced with transfection reagent, which was made by mixing 50 μg of the helper plasmid, 17 μg of the transgene plasmid, and 233 μl of polyethylenimine (1 mg/ml, linear, MW ∼25,000; Cat. No. MX2202; Maokang, Shanghai, China) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and streptomycin–penicillin. The cells were collected 3 days later at 300 *g* for 10 min (with cell-free supernatant saved for AAVExo purification) and resuspended in 10 ml of lysis buffer (150 mmol/l sodium chloride, 50 mmol/l Tris–HCl, pH = 8.5), subjected to three freeze–thaw cycles and treated with 1,500 U of benzonase nuclease (Cat. No. MP1509-25KU; Maokang, China) in the presence of 1 mmol/l magnesium chloride for 1 h at 37°C. Cellular debris was removed by centrifugation for 10 min at 5,000 *g* (Avanti J-E with a JA-25.50 rotor, Beckman Coulter, Brea, CA, United States). The virus was purified by a four-step iodixanol gradient centrifugation [5.8 ml of 15%, 3.9 ml of 25%, 3.1 ml of 40%, and 3.1 ml of 60% iodixanol (Optiprep, Cat. No. D1556; Sigma-Aldrich), overlayed with 10 ml of cell lysate in lysis buffer] in a 70Ti rotor (Beckman Coulter, Brea, CA) at 68,000 rpm for 1 h using polycarbonate bottles (Cat. No. 355618; Beckman Coulter). The 40–60% interphase of the gradient was collected, and the buffer was exchanged using a

Vivaspin20 column with 100,000 MWCO (Sartorius, Göttingen, Germany) in sterile phosphate-buffered saline (PBS).

AAVExo were purified from cell culture medium by a combination of ultracentrifugation and Optiprep density gradient (Optiprep, Cat. No. D1556; Sigma-Aldrich). Specifically, a cell-free supernatant was sequentially centrifuged at 2,000 *g* and 10,000 *g* to remove cell debris and large vesicles. The supernatant from 10,000 *g* was ultracentrifuged under 100,000 *g* to obtain the crude exosome pellet, which was resuspended in 5 ml PBS and loaded on top of a four-step iodixanol gradient (5 ml of 15%, 10 ml of 25%, 3 ml of 40%, and 2 ml of 60%) and centrifuged at 250,000 *g* for 3 h using polycarbonate bottles (Cat. No. 355618; Beckman Coulter) and a 70Ti rotor (Beckman Coulter, Brea, CA, United States). Two milliliters of fractions from the top to the bottom of the gradient was collected. Fraction 6 that contained AAVExo was diluted in PBS and centrifuged at 100,000 *g* for 1 h. AAVExo pellet was resuspended in PBS for *in vitro* and *in vivo* experiments.

The titers of AAV and AAVExo were determined by quantitative PCR (qPCR) using the SYBR qPCR premix (PerfectStart Green qPCR SuperMix, Cat. No. AQ601-01, TransGen Biotech, Beijing, China) with an Applied Biosystems QS6 real-time PCR system (Applied Biosystems, Carlsbad, CA, United States) with primers against the CMV sequence (forward: 5′-TCAATTACGGGGTCATTAGTTC-3′; reverse: 5′-ACTAAT ACGTAGATGTACTGCC-3′). To test the plasmid contamination, we used the primers targeting the Ampicillin resistance (AmpR) region (forward: 5′- CTCACCAGTCACAGAAAAGC -3′; reverse: 5′- AATGCTTAATCAGTGAGGCACC -3′).

Nanoparticle Tracking Analysis for Exosome Size and Concentration

The ZetaView® PMX 110 (Particle Metrix, Meerbusch, Germany) NTA instrument was employed to evaluate the exosome and AAVExo used in this study. Polystyrene nanoparticle standard (102 nm; Cat. No. 3100A, Thermo Fisher Scientific Inc., Waltham, MA, United States) was used for instrument calibration prior to each day's analyses. Purified exosome or AAVExo or raw condition medium was serially diluted in PBS to provide optimal initial ZetaView® instrument readings (10^6–10^9 particles/ml) and then evaluated for consistency over three measurement cycles. A set of parameters for data acquisition was standardized throughout the experiments: temperature of 23°C, sensitivity of 85, video frame rate of 30 frames per second, and a capture shutter speed of 100. Postacquisition parameters for exosome/AAVExo analysis included minimum brightness of 25, maximum size of 200 pixels, and a minimum size of 5 pixels (with pixel size not correlating to an equivalent nanometer diameter value). The data for nanoparticle diameters and concentration (particles/ml) from the ZetaView® were analyzed using a proprietary software package (ZetaView® 8.02.28) and graphically displayed and further analyzed via Excel.

Electron Microscopy

AAV-producing HEK 293T cells were washed with PBS and fixed on the flask with 1% glutaraldehyde for 20 min. Then, cells were gently scrapped off the flask and washed with PBS, followed by 1% osmium tetroxide for 40 min at room temperature. Cells were then embedded in EPON resin (Electron Microscopy Sciences, Hatfield, PA, United States). Thin and ultrathin sections were cut on an ultramicrotome (Leica HistoCore MULTICUT) and stained with uranyl acetate and lead citrate. Samples were observed using a JEOL JEM-2100 plus microscope operating at 40–50 kV.

In vitro Transduction

HEK 293T or lung cancer cell lines were seeded on a 48-well plate and cultured in DMEM or RPMI 1,640, respectively, with 10% FBS and penicillin/streptomycin. Cells were ready for AAVExo or AAV infection when they reached ∼70% confluency. Dilutions of mice serum (neutralizing antibody positive) from 1/5 to 1/160 or equal volume of PBS were mixed with AAVExo or AAV for 30 min at 37°C and then added to the cell culture. Three days later, cells were ready for fluorescence microscopy imaging.

Fluorescence Imaging and Quantification

The fluorescence of mCherry/EGFP expression and 4′,6-diamidino-2-phenylindole (DAPI) staining was imaged by Zeiss Axio Observer 7. Five fields of view with four from corners and one from the center were snapped from each well. All images were captured at the same exposure setting. The transduction efficiency was expressed by the normalized intensity, which was extracted from the original grayscale images using ImageJ. Normalized intensity was calculated by normalizing the corrected mCherry/EGFP intensity (the mean intensity from mCherry/EGFP channel subtracting the mean intensity of the background) normalized to the corrected DAPI intensity (the mean intensity from DAPI channel subtracting the mean intensity of the background). The background intensity was defined by averaging the pixels within a selected region without fluorescence. The normalized intensities were averaged from five fields for each well, and three independent biological replicates were performed.

Animals and Lung Tumor Xenograft Model

All animal experiments were approved by the Ethics Committee of Anhui University of Chinese Medicine and was in compliance with the institutional and governmental regulations. Male C57BL/6 and NOD SCID mice of 4–6 weeks old were purchased from Cyagen Biosciences, Guangzhou, China. For Nab-positive serum collection, C57BL/6 mice were intravenously injected with AAV6-EGFP (1E9 g.c.). Blood was collected after 48 h, and serum was obtained by clotting at room temperature and centrifuging at 2,000 *g* for 10 min. For tumor cell injection, 5×10^6 A549 cells were subcutaneously injected into both dorsal flanks or one ventral site (in the following trial for other mice) of NOD SCID mice with a 21-G needle. Tumor growth was checked every week, and mice were ready to randomize into three groups ($n = 1$ for a preliminary trial, and $n = 4$ for the following reproducing trial) after 4 weeks. Equal titer (5E9 g.c. in total) and volume of AAV6Exo-luciferase, AAV6-luciferase, or saline was directly injected into multiple (three to four) sites of the tumor tissue. One week later, luciferase

gene transfer and expression was examined through IVIS® Spectrum optical imaging system (Lumina III, PerkinElmer, Waltham, MA, United States). Mice were anesthetized and then injected intraperitoneally with D-luciferin resuspended in PBS (150 μg/g body weight; Sigma). Postinjection mice were imaged for luciferase expression using an IVIS100 charge-coupled device imaging system every 2 min until the signal reached a plateau. Data analysis for signal intensities and image comparisons were performed using Living Image® software (Caliper Life Sciences, Waltham, MA, United States). To calculate total flux in photons per second for each animal, regions of interest were carefully drawn and quantified around tumor areas.

Statistics

All data were presented as mean ± standard deviation. One-way ANOVA was used to evaluate statistical significance in the mouse experiment by GraphPad. A p value was considered to be significance when < 0.05.

RESULTS

Strategies for Purification of AAVExo With Minimal Contamination of Free AAVs

HEK 293T cells, which are widely used to generate AAVs, are known to secrete exosomes (Ban et al., 2015; Kanada et al., 2015). To test whether they also secrete AAV-containing exosomes (Maguire et al., 2012), HEK 293T cells were transfected with standard plasmids reported previously (Kho et al., 2011) to produce double-stranded AAV-EGFP. Consistent with the literature report (Vandenberghe et al., 2010), we found that the extracellular secretion of AAV6 was significantly higher compared to most other serotypes (data not shown). Therefore, we chose to use AAV6 in our subsequent experiments.

In addition to AAVExo, free AAV is released to culture medium as well (Vandenberghe et al., 2010). To isolate AAVExo without significant contamination with free AAV, we meticulously designed sequence of steps as shown in **Figure 1A**. We obtained crude AAVExo plus copelleted free AAV in Step 1 and subsequently collected pure AAVExo pellet by designing Step 2 with 15–60% iodixanol density gradient based on the flotation density of exosomes, AAVEXo, and free AAV. We compared the purity of AAVExo before and after the isolation using nanoparticle tracking analysis (ZetaView® by Particle Metrix). The small and large particles and protein aggregates were observed in the raw conditioned medium population of AAVExo (∼100 nm) (**Figure 1B**), while after a two-step isolation clean population of AAVExo was obtained (**Figure 1C**).

Furthermore, we evaluated the purification efficiency of our density gradient-based isolation strategy by including sophisticated controls. Purified free AAVs were loaded as control 1 (**Figure 2A**) to demonstrate absence of free AAV in the AAVExo fraction. In addition, we included control 2, which was a mixture of empty wild-type exosomes and purified free AAVs (preincubated for 1 h, at 37°C before loading on the gradient) to demonstrate that free AAVs do not bind or stick to the surface of exosomes non-specifically (**Figure 2B**). Control 1, control 2, and AAVExo (**Figure 2C**) had equal amount of AAVs in genome copy (1E10 g.c.) number. Control 2 and AAVExo had equal amount of exosomes in vesicle number (characterized by ZetaView). After density gradient ultracentrifugation, we observed a white layer of exosomes floating at ∼20% in both the AAVExo and control 2 gradient but not in the control 1 gradient, which is in the location consistent with reported density of exosomes. Separate fractions with equal volume were collected from the top, and the layer with white band was precisely collected as fraction 6 (F6). Each fraction was analyzed for the presence of exosomes [(1) size by ZetaView and (2) exosomes marker protein CD81 by Western blot (WB)] and for the g.c. number of AAV by qPCR (**Figures 2A–C**). The ZetaView and WB data indicate that exosomes were primarily located in F6 (boxed in red) and F7. Additional exosome marker CD63 and flotillin-1 were confirmed within F6 and F7 (**Supplementary Figure 1**). Furthermore, a significant level of AAV genome was detected in F6 and F7 of AAVExo, while the free AAVs as shown in controls mainly presented downward in denser gradients. Since the primers that we used to detect the viral genome target the CMV sequence, it could be possible that the positive qPCR readings for AAVExo fractions came from the trace of original plasmid/PEI complex. To rule out this possibility, we designed a pair of primers targeting the ampicillin resistance (AmpR) region in the plasmid and performed qPCR for AAVExo fractions (**Supplementary Figure 2**). We did not collect positive signals from the AmpR amplification, confirming the specificity of the earlier qPCR detection. Taken together, our data suggested that free AAV was not residing in and around F6, and more importantly, free AAVs did not show tendency of stickiness to exosomes. These results demonstrated that that our purification process successfully separated the AAVExo from free AAVs secreted by HEK 293T cells.

As both F6 and F7 contained AAVExo, we analyzed them using ZetaView to determine the size and quantity of exosomes. AAVExo had comparable sizes in F6 and F7 (**Figure 3A**), while for quantity, F6 from both control 2 and AAVExo had 1.5–2.5-fold more exosomes than F7 (by particles/ml, **Figure 3B**). Moreover, the transduction efficiency of AAV6Exo-EGFP from F6 was significantly higher compared to that from F7 using HEK 293T cells (**Figure 3C**). In parallel, F9, F10, and F11 with pure AAV fractions were tested and showed moderate capacity of HEK cell infection (**Supplementary Figure 3**). Next, we confirmed AAVs packed in exosomes using transmission electron microscopy (TEM). AAV-producing HEK 293T cells were fixed and embedded in resin, of which the sections were imaged by TEM. Within the cytoplasm, we observed exosomes in the multivesicular body, a membrane-bound structure that fuses with plasma membrane and releases exosomes into extracellular environment. Notably, viral particles with viral DNA densely stained were found within exosomes, as indicated by arrows in **Figure 3D**. The morphology of purified AAVExo was further confirmed by TEM (**Supplementary Figure 4**). These data suggested that AAVExo purified from F6 was relatively pure without significant contamination of free AAVs. AAVExo from

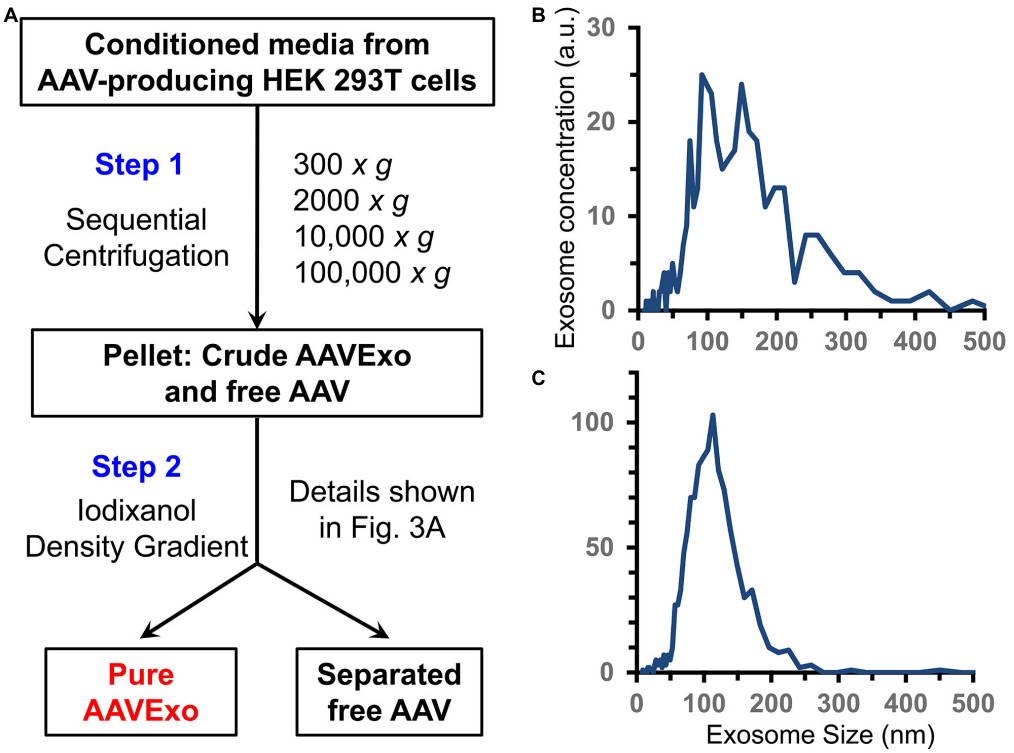

FIGURE 1 | Exosomes are secreted and isolated from the conditioned medium of AAV-transfected HEK 293T cells. **(A)** Methodology flow chart of 2-step AAVExo purification. **(B)** Conditioned medium of AAV-producing HEK 293T cells analyzed by ZetaView. Five biological replicates were performed, and a representative was shown. **(C)** Purified AAVExo analyzed by ZetaView. Three biological replicates were performed, and a representative was shown here.

F6 had higher transduction efficiency compared to that from F7. Thus, we chose F6 as AAVExo for all our subsequent studies.

AAVExo Has Higher Gene Delivery Efficiency Compared to AAV *in vitro*

To compare the gene transfer efficiency of AAV and AAVExo, we produced double-stranded AAV6-mCherry and AAV6Exo-mCherry using the above protocol. A list of cell lines representing human lung cancer was selected from the NCI-60 panel as *in vitro* cell models to test AAVExo-mediated gene delivery. Specifically, the extensively used A549 and H1299 were included to represent common NSCLC; H460 and H661 were included as models for large-cell carcinoma in NSCLC; HCC827, H23, and H1975 were chosen to represent adenocarcinoma in NSCLC; and finally, H446 were incorporated into the experiment to represent SCLC. We covered a total of eight cell types for multiple cancer subtypes because the molecular mechanism of exosome uptake was not well known, particularly for the AAVExo uptake by lung cancer cells. Distinct cell types may display different surface protein profiles with varied preference for exosome docking, endocytosis, or membrane fusion. Therefore, we would like to verify the gene delivery efficiency of AAVExo within multiple types of lung cancer types. As stated above, AAV6Exo was selected due to the high yield of exosome-enveloped AAVs harvested from the cell culture supernatant. Equal tier of pure AAVs was set as a control. The intensity of mCherry expression was quantified and normalized to the DAPI intensity to represent the efficiency for gene delivery (**Figure 4**). We noted that the fluorescence expression was dependent on the total titer of administration (or multiplicity of infection, MOI) and the cell culturing time. Therefore, for all experiments, we fixed the MOI to 100 (equal to 3E6 g.c. virus per well) and the incubation time to 3 days. Remarkably, we found that AAVExo had a higher capacity for gene delivery across the common NSCLC cell lines (**Figures 4A,B**), large-cell carcinoma cell lines (**Figures 4C,D**), adenocarcinoma cell lines (**Figures 4E,F**), and a SCLC cell line (**Figures 4G,H**). The transfection enhancement was around two- to fourfold presenting in A549 and H1975. Collectively, the result demonstrated that AAVExo had significantly higher transduction efficiency than AAV to a variety of lung cancer cell types *in vitro*.

AAVExo Is More Resistant to Antibody Neutralization Compared to AAV in Lung Cancer Cells *in vitro*

Pre-existence of neutralizing antibody (Nab) against AAV is prevalent in the human serum. Nab binds to AAV, blocks its infection, and impairs AAV-mediated gene delivery. AAVExo has AAV enveloped inside the exosome compartment, therefore may have the potential to protect the AAV from antibody neutralization. To determine whether AAVExo is resistant to Nab neutralization, we generated Nab-positive serum by

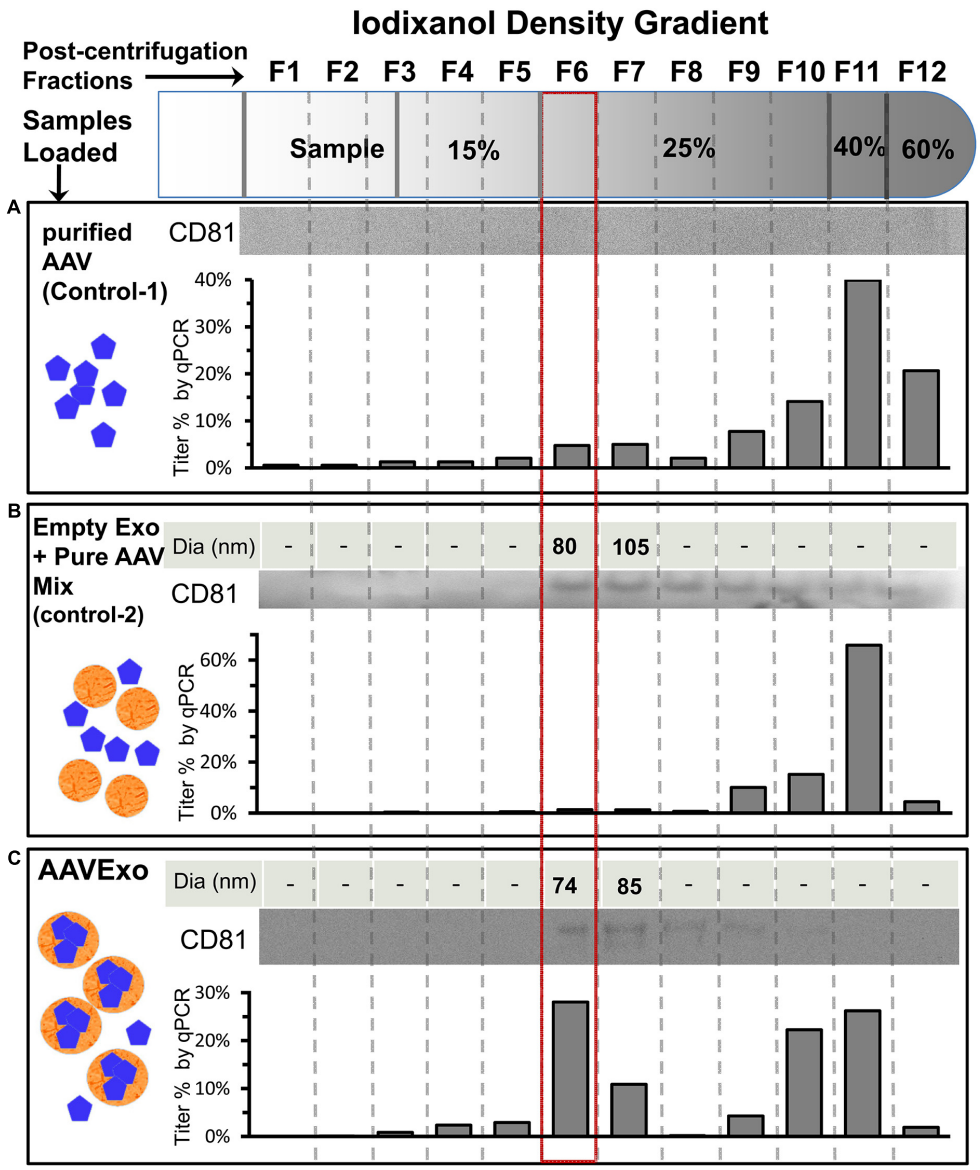

FIGURE 2 | Strategies and characterization of AAVExo purification. Equal titer of pure AAV (**A**, control 1), a mixture of empty exo and pure AAV (**B**, control-2), and AAVExo **(C)** crude prep loaded on top of an Optiprep density gradient and ultracentrifuged at 250,000 *g* for 3 h. Separated fractions were collected from the top as indicated and analyzed for the presence of exosomes [(1) size, by ZetaView; (2) exosomes marker protein, CD81 by WB] and for the presence of AAV (by qPCR). The experiment was biologically reproduced for three times, and the representative results were shown.

intravenously injecting AAV6-EGFP to C57BL/6 mice and collecting blood 48 h thereafter. The Nab-positive serum was shown to sufficiently suppress the AAV infection *in vitro* (data not shown). Equal titer of AAV6Exo-mCherry or AAV6-mCherry was preincubated with dilutions of Nab-positive serum or PBS control at 37°C for 30 min before applied on A549 or H446 cells. After 3 days, mCherry expression was examined by fluorescence microscopy, and the relative transfection efficiency was plotted from the quantified mCherry intensity that was normalized to the DAPI intensity (**Figures 5A,B** for A549 and **Figures 5C,D** for H446). As expected, we observed a significant reduction of mCherry expression for Nab serum-incubated AAV (∼0 at 1/40 dilution). However, there was no significant decrease in AAVExo-mCherry expression in the presence of Nab in selected lung cancer cell models. These data indicated that AAVExo, but not AAVs, can resist neutralization by the Nab.

AAVExo Has Higher Capacity of Gene Delivery Efficiency to Lung Cancer Compared to AAV *in vivo*

Furthermore, we explored the AAVExo-mediated gene delivery in a rodent lung cancer xenograft model. In brief, A549 cells were subcutaneously implanted into the dorsal flank areas or ventral

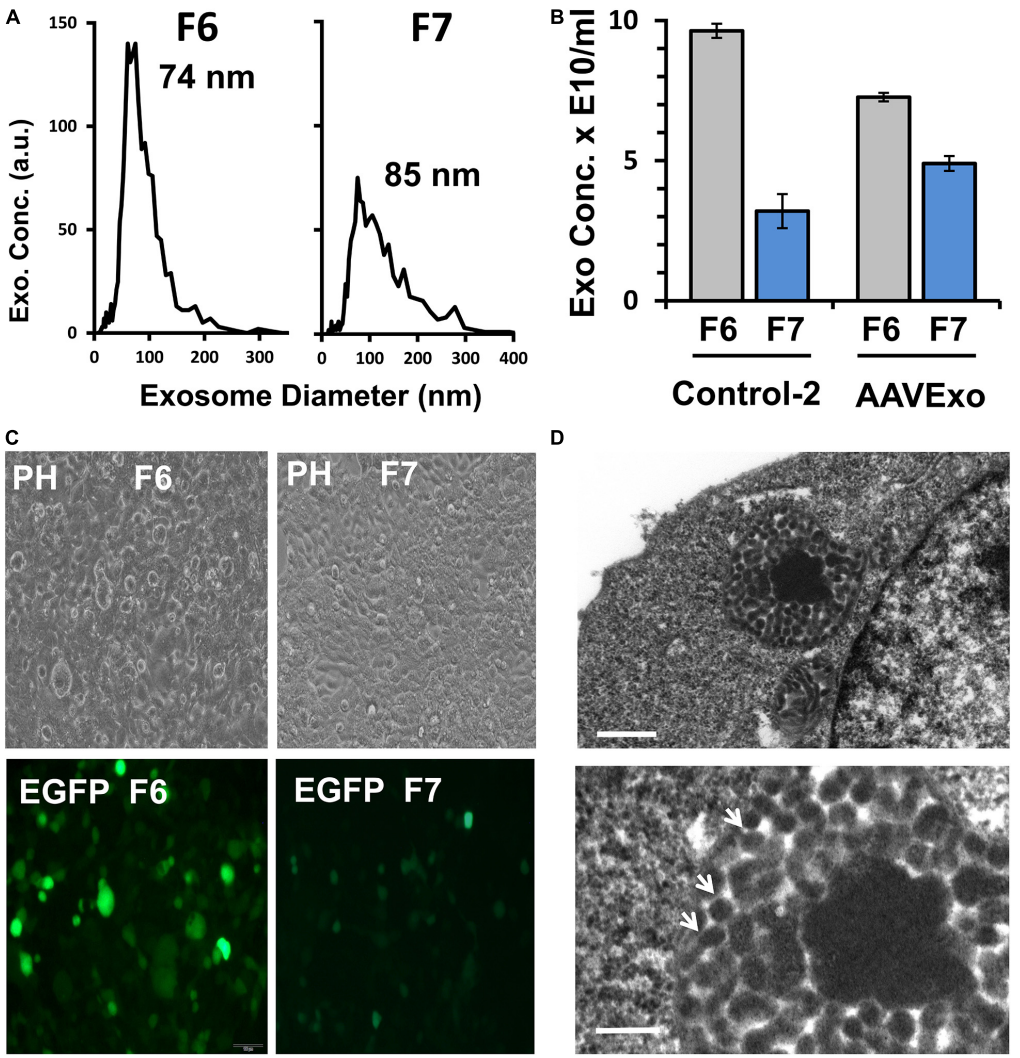

FIGURE 3 | Fraction 6 has the major amount of clean AAVExo compared to fraction 7. **(A)** Exosome size distribution in F6 and F7 of AAVExo measured by ZetaView. Three replicates were conducted, and a representative was shown. **(B)** Exosome concentration of F6 and F7 in control 2 and AAVExo measured by ZetaView. The graph was plotted by the mean of three biological replicates with bars showing the standard deviations. **(C)** F6 but not F7 has effective transduction capacity *in vitro*. HEK 293T cells were treated with the same titer of AAV6Exo-EGFP F6 or F7 for 5 days. EGFP was detected by fluorescence microscopy. Three biological replicates were performed, and a representative was shown. **(D)** AAV-containing exosomes in multivesicular body. AAV-producing cells were imaged by TEM, and AAV particles were observed in exosomes prior to secretion. Lower panel represents the zoom-in area of upper panel. Scale bar: upper panel, 500 nm; lower panel, 200 nm.

areas. The tumors were allowed to grow for 4 weeks followed by direct intratumoral injection of AAV6Exo-luciferase, AAV6-luciferase, or saline as a control. One week later, live mice were imaged for firefly luciferase expression through bioluminescent imaging. Mice enrolled in the first trial with tumors implanted on both sides of the dorsal flank areas are shown in **Figure 6**. Other mice administrated in a follow-up trial with ventral xenograft are shown in **Supplementary Figure 5**. Consistently, AAV6 administration exhibited a certain level of gene transfer and expression within tumor tissues as expected; however, AAV6Exo treatment demonstrated a significantly higher efficiency of gene delivery to the xenografts, which was consistent with the *in vitro* data presented above. We did not observe luciferase expression within other tissues, most likely due to the local injection performed, suggesting a rapid uptake of vectors by the tumor cell instead of dispersing in the systematic circulation. Taken together, these data demonstrated that AAVExo was a superior vector for enhanced gene delivery to the lung carcinoma than the conventional AAV vector.

DISCUSSION

AAV vectors have demonstrated their high safety profiles among over a hundred of early phase clinical trials worldwide (Kuzmin et al., 2021). Although a few AAV-mediated gene therapies have finally been approved (European Medicines Agency, 2012; Administration, U.S.F.a.D, 2017, 2019), effective gene treatment

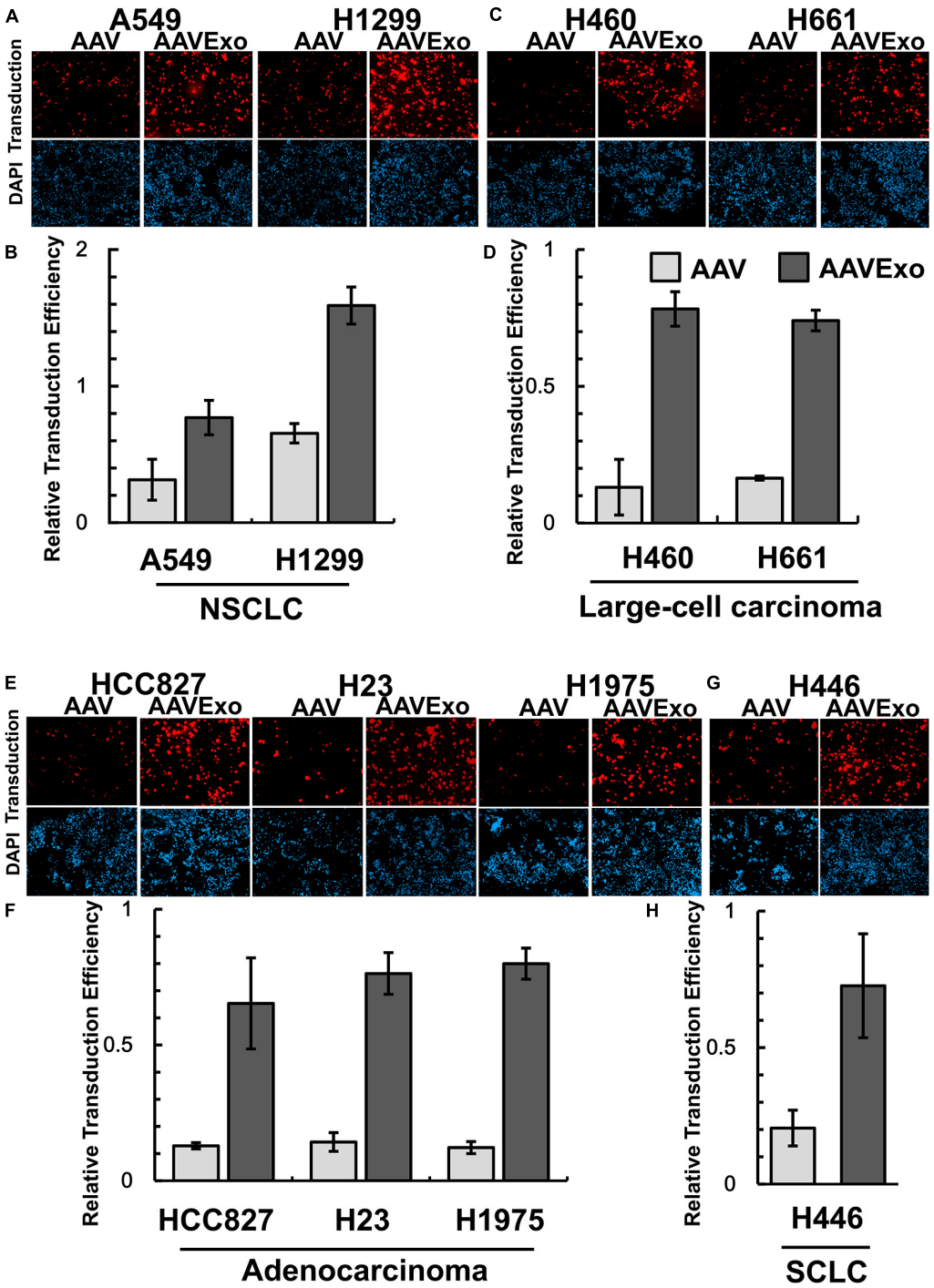

FIGURE 4 | AAVExo has high capacity for gene delivery *in vitro*. A couple of selected lung cancer cell lines representing **(A,B)** common NSCLC, **(C,D)** large-cell carcinoma, **(E,F)** adenocarcinoma, or **(G,H)** SCLC were infected by equal titer (3E6 g.c. for a 48-well plate) of AAV6Exo-mCherry or pure AAV6-mCherry for 3 days. Cells were stained with DAPI before fluorescence microscopy imaging. The mCherry intensity was quantified and normalized to DAPI and plotted via Microsoft Excel. This experiment was reproduced biologically for three times, and the mean with standard deviation was graphed.

for patients with cancers is still not well developed. It comes to our attention that one of the reasons could be the low efficiency of tumor delivery for most AAV-based gene therapy, due to the weak tropism to tumor tissues, and the prevalence of AAV neutralizing antibody existed in human serum (Weber, 2021). Thus, it has become essential to improve the current gene transfer systems and reinforce delivery tools and methods for future success.

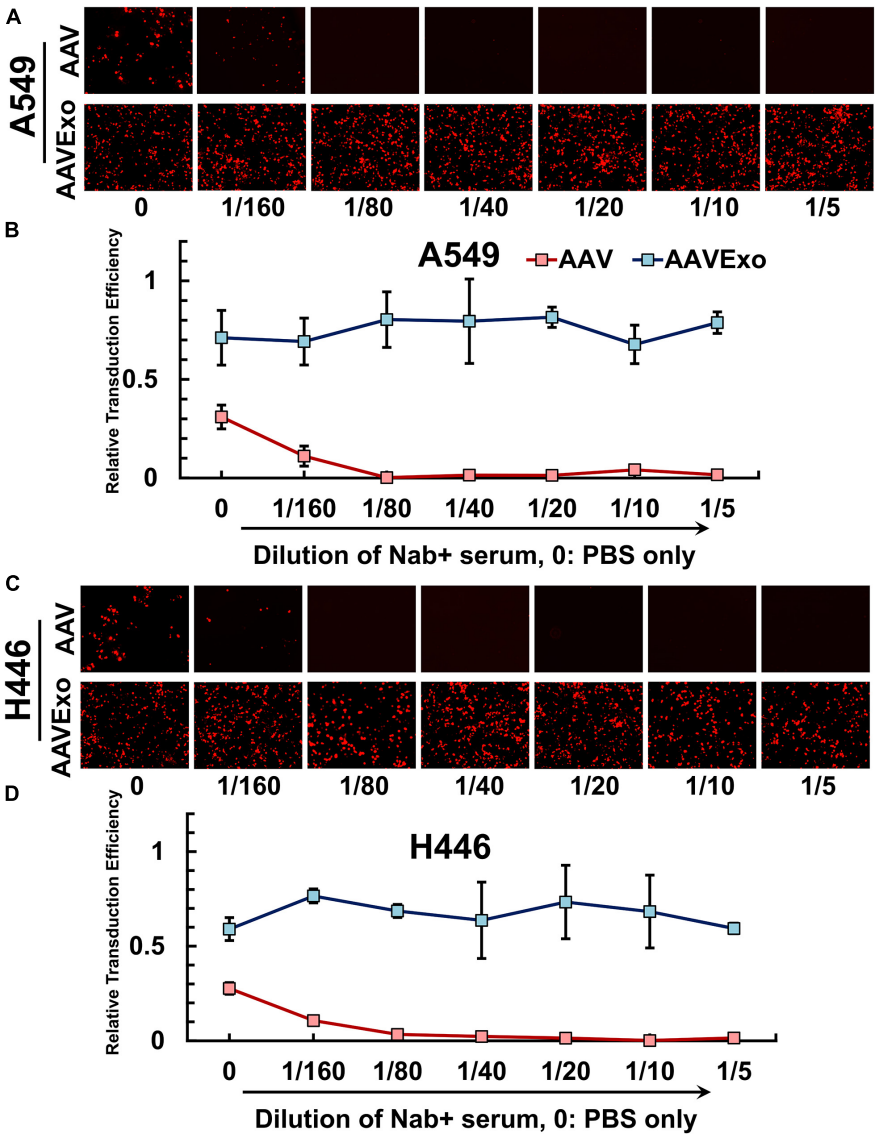

FIGURE 5 | AAVExo has excellence resistance to neutralizing antibodies *in vitro*. Dilutions (1/5–1/160) of Nab-positive serum or PBS were incubated with AAV6Exo-mCherry or AAV6-mCherry for 30 min. The mixture with equal titer of genome copy was added to the culture of **(A,B)** A549 or **(C,D)** H446, and cells were ready for imaging 3 days later. Fluorescence intensity was quantified and normalized to DAPI for compiled plotting. The plot showed the mean with the standard deviation for three biological replicates.

Recently, Maguire et al. (2012) and György et al. (2014) reported that AAVs were associated with extracellular vesicle (EV) and therefore acquired protection against inhibition by its neutralizing antibody. However, although it outperformed conventional AAV, EV-associated AAV was inhibited by higher concentration of Nab. Because EVs are a mixture of highly heterogeneous vesicles in their biogenesis, functions, and sizes, inadequate isolation could be a reason that lowers the effect of EV-AAV. Additional pitfall of compromised evasion from Nab might be the failure of excluding free AAV vectors from the final EV-AAV isolates. This is important because, while free AAVs count for the total titer of target genes, it will be eventually eliminated by Nab, thus impairing the overall effects of resistance to Nab. In our study, we devised a strategy to optimize the combination of ultracentrifugation and iodixanol density gradients and developed a novel method for isolating EV-AAVs with high purity and minimal contamination of free AAV vectors. More importantly, our study focused on exosomes, which are a population of EV with robust structure and smaller size ranging from 70 to 150 nm. To prove the effective purification of AAV–exosomes, we designed a meticulously controlled experiment, which included pure AAV vectors and premixture of wild-type exosomes and pure AAVs. The purpose of pure AAV control is to verify its distinct floating density against AAVExo, while the setup of premixture control is to address the concern that AAVs may potentially bind to the surface of exosomes non-specifically.

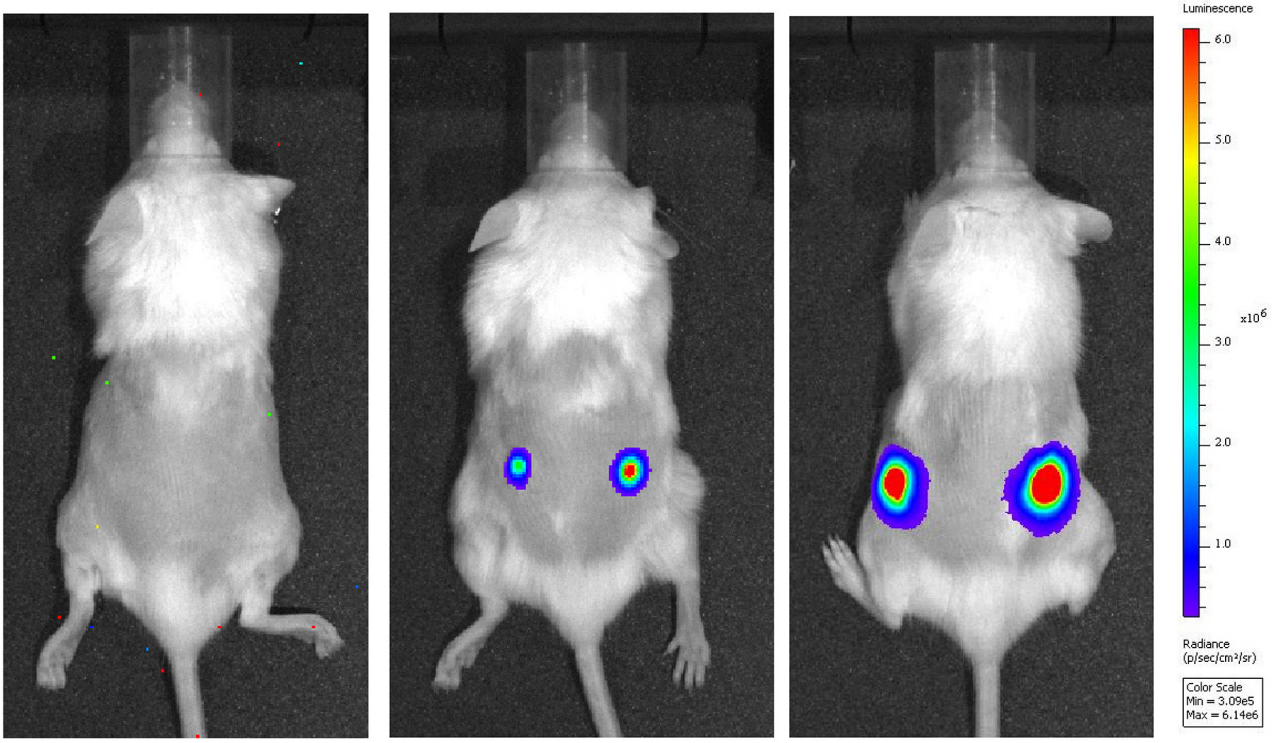

FIGURE 6 | AAVExo has stronger gene-transfer efficacy *in vivo*. Male NOD SCID mice aged 4–6 weeks were subcutaneously implanted with A549 cancer cells. Four weeks later, equal titer (5E9 g.c.) of AAV6Exo-luciferase, AAV6-luciferase, or saline was directly injected into the tumor. One week later, bioluminescence was recorded by IVIS system.

With the examination of exosomal protein marker and viral genome, our data supported that nonspecific binding of AAV to exosome was minimal in our experiments. More importantly, only the AAVExo fraction with lowest possible contamination of free AAVs was successfully isolated. The development of effective purification method for AAVExo enabled the application of the new exosome-based vector to therapeutic treatments of lung cancers.

We characterized the purified AAVExo using several different *in vitro* systems to determine its performance in aspects of transduction and evasion from Nab. First, we thoroughly compared the transduction efficiency of AAVExo and conventional AAV vectors among a panel of lung cancer cell types. Remarkably, we observed highly enhanced efficiency of gene delivery and expression through AAVExo when compared to conventional AAVs. This result was robustly confirmed in multiple cultured lung cancer cell lines and in a xenograft mouse model. It is not fully understood why AAVExo could notably increase the transduction efficiency in the molecular level, and more work is needed to unveil the entry of AAVExo into recipient cells and its intracellular pathways in the future. Nevertheless, as a promising vector for cancer cell gene transfer, AAVExo has great potential to improve the strategies of gene transfer for the treatment of lung cancer.

Currently, one of the top challenges for AAV-based gene delivery is to overcome antibody neutralization, which is prevalent in the human body. There has been continuous effort to limit neutralization of AAV in multiple dimensions, including viral capsid engineering (Maheshri et al., 2006), pretreatment with anti-CD4 antibodies (Manning et al., 1998), or empty capsid decoys (Mingozzi et al., 2013). However, although promising, all of those approaches have limitations and drawbacks (Louis Jeune et al., 2013). AAVExo, as a novel vector, is thought to have virus protected by the exosome and is expected to evade Nab binding. Thus, we designed *in vitro* experiments to test the resistance of AAVExo to a dilution of increasing concentrations of Nab. We chose A549 and H446 as cell models for the transduction with AAVExo that had been preincubated with increasing concentration of Nab. We observed sustained AAVExo transduction without significant influence from increasing Nab, whereas infection of conventional AAVs dramatically reduced and eventually muted completely. These data demonstrated the superior profile of AAVExo as a novel gene-transfer vector with good resistance to Nab in an *in vitro* system, although further *in vivo* study using a Nab-positive rodent model may be essential to thoroughly characterize AAVExo transduction and resistance to Nab.

As an innovative approach, AAV-associated exosomes have been reported to efficiently deliver genes to the central nervous system (Hudry et al., 2016; Volak et al., 2018), immune cells (Breuer et al., 2020), retina (Wassmer et al., 2017), cochlea (György et al., 2017), and liver cells (Meliani et al., 2017). Our study has shown great potential that AAVExo may enhance the existing gene therapies for cancer treatment. In addition to the improvement in gene delivery efficacy and prevention of Nab neutralization that are presented in our study, exosome-based therapeutic platform has other substantial benefits. Published data from our and other laboratories demonstrated that exosomes could be engineered for selected cargo loading and the surface display of tumor-targeting entities (Ohno et al., 2013; Villarroya-Beltri et al., 2013; Liang et al., 2014; Kooijmans et al., 2016; Yim et al., 2016). On the other hand, AAVExo shields the natural tropism of AAV serotypes, and how serotypes affect AAVExo production and transduction warrants further exploration. On the other hand, although iodixanol density gradient provides the pure isolate, the potential scaling up in future manufactures should be considered. Despite the challenges, the future effort is worthwhile in designing an exosome-based gene therapy system that combines recombinant AAVs and vesicle surface engineering for lung-cancer-specific targeting.

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 animal study was reviewed and approved by Ethics Committee of Anhui University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

YL conceived the study and the entire research plan. FC and SHu led and designed the animal experiment. BL conducted the *in vitro* and *in vivo* experiments. BY and SHe performed cell culture and AAV and AAVExo production and purification. YL and ZL wrote the manuscript and prepared the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Ferroptosis and Cancer: Complex Relationship and Potential Application of Exosomes

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Cell death induction has become popular as a novel cancer treatment. Ferroptosis, a newly discovered form of cell death, features regulated, iron-dependent accumulation of lipid hydroperoxides. Since this word "ferroptosis" was coined, numerous studies have examined the complex relationship between ferroptosis and cancer. Here, starting from the intrinsic hallmarks of cancer and cell death, we discuss the theoretical basis of cell death induction as a cancer treatment. We review various aspects of the relationship between ferroptosis and cancer, including the genetic basis, epigenetic modification, cancer stem cells, and the tumor microenvironment, to provide information and support for further research on ferroptosis. We also note that exosomes can be applied in ferroptosis-based therapy. These extracellular vesicles can deliver different molecules to modulate cancer cells and cell death pathways. Using exosomes to control ferroptosis occurring in targeted cells is promising for cancer therapy.

Keywords: ferroptosis, apoptosis, cancer, cell death, exosomes

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INTRODUCTION

ROS, A Hint for Cancer Therapy

Cancer has become one of the major threats to human health. A report estimated that in 2021 in the United States, there will be 1,898,160 new cancer cases and 608,570 cancer deaths (Siegel et al., 2021). Although the cancer mortality rate has decreased in recent years, access to healthcare has also decreased due to the COVID-19 pandemic, which has led to hampered cancer diagnosis and treatment (Siegel et al., 2021). As widely applied chemoradiotherapy is showing its drawbacks, such as frequent resistance and toxic side effects, cell death induction is becoming increasingly popular for developing novel cancer treatment.

Common forms of cell death, such as apoptosis, autophagy, and necroptosis, are all related to reactive oxygen species (ROS) and are regulated by ROS. For example, ROS can facilitate the extrinsic apoptosis pathway through negative regulation of the cellular FLICE-inhibitory protein (Wang et al., 2008) and can induce intrinsic apoptosis pathways by triggering quick release of Cyt-c (Madesh and Hajnoczky, 2001) and regulating the Bcl-2 protein family (Burlacu, 2003). Evidence shows that ROS generated from ETC and NOX can regulate several pathways that mediate autophagy induction (Li et al., 2011), and AMPK can be activated by AMPK kinase after H_2O_2 treatment, which also results in autophagy induction (Choi et al., 2001). In addition, ETC- and NOX-derived ROS are involved in necroptosis facilitation (Dixon and Stockwell, 2014). Evidence

of cell death regulation, mediated by ROS, can also be found in ferroptosis and chemosensitization (Galadari et al., 2017).

Given the strong relationship between ROS and cell death, regulating ROS generation upward or controlling oxidative defense downward has become central for new cancer treatments, which have been enhanced by the finding that cancer cells process a higher level of ROS than do healthy cells (Galadari et al., 2017). A higher level of ROS, partly attributed to defective mitochondrial oxidative metabolism (Tafani et al., 2016), can lead to two opposite outcomes: the promotion of cancer and its suppression. Cancer suppression occurs because an elevated ROS level promotes various cell death processes, as mentioned above. Cancer promotion occurs because an elevated ROS level does the following:

(a) facilitates tumorigenesis through damaging or modifying cellular proteins, DNA, and lipids, leading to activation or inhibition of various tumorigenesis related signaling cascades (Tafani et al., 2016);

(b) promotes angiogenesis by mediating the proliferation, migration, and tube formation of endothelial cells (Potente et al., 2011) or by modulating various vascular endothelial growth factors;

(c) contributes to invasion and metastasis through active involvement in essential events including modulating signaling kinases and the cytoskeleton (Tochhawng et al., 2013); and

(d) participates in chemoresistance (Ledoux et al., 2003).

Cancer cells exhibiting a greater ROS level display increased activity of antioxidant enzymes, which help create a homeostasis for cell surviving. Therefore, it would be valuable to develop therapeutic strategies to break the redox homeostasis in cancer cells and activate cell death pathways to limit cancer progression. There are two possible approaches: the first is to decrease intracellular ROS. This can be done by, for example, hindering mitochondrial ETC and the activation of NOX, thus inhibiting ROS generation. This technique has been demonstrated in several cancer cell lines and has been proven to be beneficial. A study induced apoptosis in PANC-1 pancreatic cancer cells using diphenylene iodonium, which suppressed ROS generation through inhibiting NOX4 (Mochizuki et al., 2006). The opposite strategy consists of increasing the ROS to a toxic level and thus triggering cell death pathways. Researchers report that piperlongumine, a natural small molecule, can selectively induce ROS-dependent cell death in cancer (Chen et al., 2014). Moreover, glucose metabolism is thought to be related to ROS elimination, and a study has shown that glucose deprivation can induce cytotoxicity in MCF-7/ADR human multidrug-resistant breast cancer cells (Gupta et al., 1997; Lee et al., 1997).

In This Review

Cancer therapy based on cell death induction has become an important research topic, and ferroptosis, a newly discovered form of cell death, has gained general attention. In this paper, we review the literature on ferroptosis and its relationship with cancer from different perspectives, including proto-oncogene and tumor suppressor gene, epigenetics, cancer stem cells (CSCs), and the tumor microenvironment (TME). Based on the new insights into cancer treatments using cell death induction, we believe ferroptosis to be a promising candidate for cancer treatment. As numerous molecules, ranging from RNAs to plant-derived natural compounds, have been demonstrated to have a therapeutic effect on cancer *via* the induction of ferroptosis-like cell death, drug delivery, which is a critical step in the application of ferroptosis as a cancer treatment, is still being discussed. Given various advantages, such as easy tissue penetration, low toxicity, and low immunogenicity, exosomes are believed to be a reliable drug delivery system able to selectively target specific cells (**Figure 1**). Here, we provide a simple overview of exosomes and their potential applications in ferroptosis-based therapy.

FERROPTOSIS AND CANCER

A newly discovered form of cell death, different from apoptosis, autophagy, and necroptosis, called ferroptosis has recently gained recognition for use in cancer treatment. Ferroptosis, a word coined in 2012 (Dixon et al., 2012), is a form of regulated cell death characterized by iron-dependent accumulation of lipid hydroperoxides to lethal levels. It was first used to describe a cell death process induced by a small molecule called erastin, which inhibits the intake of cystine, resulting in glutathione depletion and inactivation of the phospholipid potentially toxic lipid peroxidase 4 (GPX4) (Yang et al., 2014). GPX4 converts lipid hydroperoxide, which is potentially toxic, to a non-toxic form of lipid alcohol (Ursini et al., 1982). Therefore, inactivation or inhibition of the enzyme GPX4 triggers overwhelming lipid peroxidation that causes iron-dependent cell death. Regulation of ferroptosis can be achieved generally by interfering with iron metabolism and ROS metabolism, and the ferroptosis process can be suppressed by iron chelators, lipophilic antioxidants, lipid peroxidation, and the depletion of polyunsaturated fatty acids and correlates with the accumulation of lipid-peroxidation markers (Stockwell et al., 2017).

For the successful application of ferroptosis in cancer treatment, a more concrete understanding of ferroptosis and cancer is needed. The following section contains a review of recent research on ways in which ferroptosis interacts with cancer, especially as regards cancer-related genes, epigenetics, the TME, and so on, and provides a short review of research on ferroptosis regulation and its application to cancer treatment.

FERROPTOSIS AND CANCER GENES

RAS

The RAS family of small GTPase, including HRAS, NRAS, and KRAS, is closely related to ferroptosis since the two most well-known ferroptosis inducers, erastin (eradicator of RAS and ST) and RSL3 (RAS-selective Lethal 3), are technically oncogenic RAS-selective lethal small molecules (Yagoda et al., 2007; Yang and Stockwell, 2008). The relationship between ferroptosis and RAS has been carefully investigated by numerous studies (**Table 1**). For example, researchers have found that $HRAS^{V12}$ expressing cancer cells are electively sensitive to ferroptosis, and KRAS silencing in KRAS mutant Calu-1 cells strongly reduces

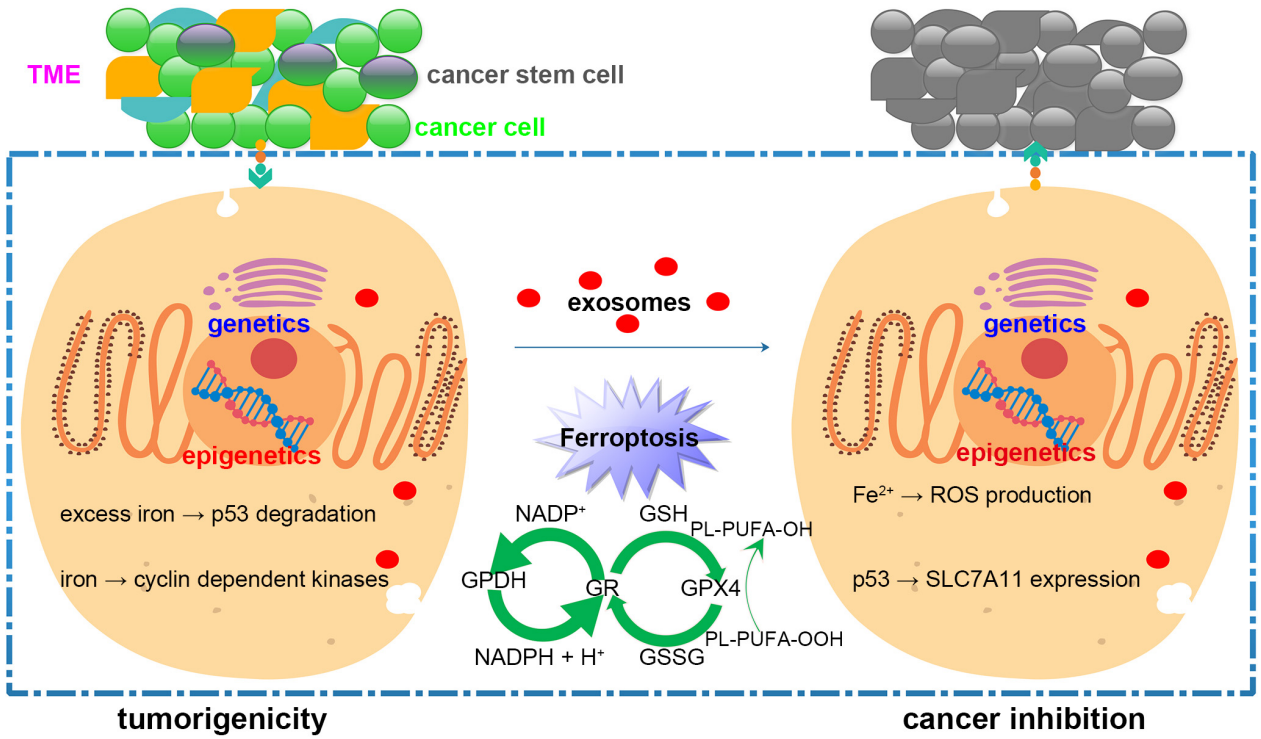

FIGURE 1 | The schematic diagram of modulating ferroptosis in cancer inhibition based on an exosome delivery system. Cancer development and treatment efficacy are impacted by genetic factors, epigenetic modifications, cancer stem cells, and the tumor microenvironment (TME). Ferroptosis is a double-edged sword in cancer survival because it is regulated by different signaling pathways. Cancer treatment could benefit from using an exosome delivery system to control the onset of ferroptosis.

erastin sensibility (Yagoda et al., 2007). A possible explanation might be that constitutive RAS pathway activity promotes TFRC (a gene related to iron metabolism) expression while suppressing the expression of iron storage proteins. However, more evidence for the relation between RAS mutation and erastin sensibility cannot be found in some cancer cell lines (Yang et al., 2014). In contrast, RMS13 rhabdomyosarcoma cells that overexpress HRAS, KRAS, or NRAS are resistant to erastin and RAL3 (Schott et al., 2015), which means RAS does affect erastin sensitivity, while the oncogenic RAS pathway is not the sole determinant of ferroptosis sensitivity (Dixon and Stockwell, 2019). Other research has shown that, in an $NRAS^{Q61L}$ expressing HL-60 cell line, high mobility group box 1 (HMGB1) is an essential regulator of erastin-induced ferroptosis (Ye et al., 2019). ADP Ribosylation Factor 6 (ARF6), a part of the RAS superfamily, facilitates high sensitivity to RSL3-induced lipid peroxidation (Ye et al., 2020). Reports have shown that oncogenic RAS induces rapid increase of ROS partly through upregulating NOX1 (Irani et al., 1997; Mitsushita et al., 2004). In mice with KRAS-driven pancreatic ductal adenocarcinoma (PDAC), high iron diets and GPX4 depletion, which results in 8-OHG release, lead to macrophage infiltration and activation (Dai E. et al., 2020).

p53

Because of its role in cell cycle arrest, senescence, and apoptosis, as well as for its interesting role in metabolism, oxidative responses, and ferroptosis, p53 has long been an important focus of research. p53's essential function of survival promotion is confirmed by the fact that cells are more sensitive to ferroptosis after p53 depletion through CRISPR/Cas9 (Tarangelo et al., 2018). By antagonizing p53 activity, such as the O-GlcNAcylated c-Jun (the first discovered oncogenic transcription factor), cell death can be prevented (Eferl et al., 2003). Recently, p53-related signal pathways have been shown to modulate ferroptosis in the following ways.

Research has shown that p53 can suppress the expression of SLC7A11, a key component of the cystine/glutamate antiporter (X_c^- system), leading to the inhibition of cystine uptake and sensitization to ferroptosis. For example, the p53 mutants $p53^{R237H}$ and $p53^{R175H}$ promote sensitivity to ferroptosis-like cell death, most likely through the combination of p53 mutants with NRF2 and through the suppression of the NRF2-dependent transactivation of SLC7A11 together with other antioxidant genes that oppose ferroptosis (Sasaki et al., 2002; Habib et al., 2015; Liu D.S. et al., 2017). Moreover, overexpression of SLA7A11 in human tumor suppresses ROS-induced ferroptosis and inhibits $p53^{3KR}$-mediated tumor growth suppression in xenograft models. Though mutant $p53^{3KR}$ effectively downregulates SLC7A11, it does not affect other p53 target genes involved in cell cycle regulation or apoptosis (Jiang et al., 2015). In contrast, mutant $p53^{4KR98}$ is unable to reduce SLC7A11 expression (Wang et al., 2016).

TABLE 1 | Summary of ferroptosis associated oncogenes and tumor suppressors.

Gene	Target	Regulatory direction for target	Effect to ferroptosis (references)
$HRAS^{V12}$	iron metabolism related genes	activation	reduce erastin sensibility (Yagoda et al., 2007; Yang et al., 2014)
$HRAS^{V12}$	iron storage proteins	inhibition	enhance ferroptosis (Yagoda et al., 2007; Yang et al., 2014)
$HRAS^{V12}$	GSH system	activation	reduce erastin sensibility (Schott et al., 2015)
$NRAS^{V12}$	GSH system	activation	reduce erastin sensibility (Schott et al., 2015)
$KRAS^{V12}$	GSH system	activation	reduce erastin sensibility (Schott et al., 2015)
ARF6	ACSL4	inhibition	enhance RSL3 sensibility (Ye et al., 2020)
$p53^{R237H}$	NRF2/SLC7A11	regulation	suppress cystine/glutamate antiporter (Sasaki et al., 2002; Habib et al., 2015; Liu D.S. et al., 2017)
$p53^{R175H}$	NRF2/SLC7A11	regulation	suppress cystine/glutamate antiporter (Sasaki et al., 2002; Habib et al., 2015; Liu D.S. et al., 2017)
$p53^{3KR}$	SLC7A11	inhibition	suppress cystine/glutamate antiporter (Jiang et al., 2015)
p53	SLC7A11	inhibition	suppress cystine/glutamate antiporter (Sasaki et al., 2002; Habib et al., 2015; Liu D.S. et al., 2017)
p53	p53-SAT1-ALOX15 axis	activation	enhance ferroptosis (Chu et al., 2019)
p53	GLS2	activation	enhance GSH generation (Hu et al., 2010)
p53	DPP4	regulate the localization and activity	inhibit ferroptosis (Xie et al., 2017)
p53	CDKN1A	activation	delay the onset of ferroptosis (Tarangelo et al., 2018)
MDM2 and MDMX	p53	inhibition	enhance ferroptosis (Venkatesh et al., 2020)
Myc	EGLN1-HIF-1α-LSH-WDR76 axis	activation	inhibit ferroptosis (Jiang et al., 2017)
Myb	CDO1-GPX4 axis	suppress CDO1, and promote GPX4	inhibit ferroptosis (Prouse and Campbell, 2012; Hao et al., 2017)
Src	GPXs	Src acts as the target for GPXs	participate in ferroptosis regulation (Wei J. et al., 2020)
Src	ACSL4	inhibition	inhibit ferroptosis (Brown et al., 2017)

As a transcription target of p53, the activity of spermidine/spermine N^1-acetyltransferase 1 (SAT1), the rate-limiting enzyme in polyamine catabolism, induces lipid peroxidation and sensitizes cells to undergo ferroptosis and the deletion of SAT1 suppresses p53 and $p53^{3KR}$-mediated ferroptosis. However, while p53 modulates SLC7A11 expression, the expression and activity of SLC7A11 and GPX4 are not associated with SAT1, and only ferrostatin-1 can inhibit ROS-induced ferroptosis in Tet-on cells (Ou et al., 2016). Research has also found that SAT1 induction is associated with the expression of arachidonate 15-lipoxygenase (ALOX15), which is essential in p53-mediated ferroptosis (Chu et al., 2019). Unfortunately, the p53–SAT1–ALOX15 axis has not been fully explained.

The metabolism of glutamine, one of the essential components of ferroptosis, is catalyzed by cytosolic glutamine aminotransferases or by mitochondrial glutaminases (Gao et al., 2015; Altman et al., 2016). The expression of glutaminase 2 (GLS2), which has been identified as a transcriptional target of p53, mediates oxygen consumption, mitochondrial respiration, and ATP generation in cancer cells. Based on the evidence that GLS2 facilitates GSH production in several cancer cell lines, GLS2 is recognized as a negative regulator of ferroptosis (Hu et al., 2010).

Colorectal cancer (CRC) caused by a number of genetic disorders, including KRAS mutation, p53 mutation, and p53 depletion, which provides additional evidence for the survival-promoting function of p53. Interestingly, this p53 function might partly be achieved by modulating ferroptosis. Research has found that p53 can inhibit ferroptosis by modulating the localization and activity, but not expression, of dipeptidyl peptidase-4 (DPP4), leading to survival promoting functions. This process occurs through a post-translational interaction with protease DPP4, which strengthens membrane lipid peroxidation in a protease-independent way *via* interaction with an ROS-generating NOX (Xie et al., 2017).

Cyclin dependent kinase inhibitor 1A (CDKN1A/p21), also known as $p21^{WAF1/Cip1}$, is a key mediator of p53-dependent cell cycle arrest after DNA damage (Abbas and Dutta, 2009). A recent study has shown that the expression of CDKN1A, mediated by p53, delays the onset of ferroptosis in response to subsequent cystine deprivation in cancer cells (Tarangelo et al., 2018). As two negative regulators of p53, MDM2 and MDMX facilitate

ferroptosis with or without p53, most likely by altering the lipid profile of cells (Venkatesh et al., 2020), which is confirmed by evidence that with the treatment of MDM2 inhibitor nutlin-3, p53 expression increases and leads to the suppression of X_c^- system inhibitor-induced ferroptosis in HT-1080 cells (Tarangelo et al., 2018). The function of CDKN1A in cell cycle arrest, which is unable to trigger ferroptosis, is primarily achieved by binding to and suppressing the kinase activity of the cyclin-dependent kinases (CDKs) (Abbas and Dutta, 2009).

Interestingly, a recent study reported that retention of p53 in the nucleus, mediated by the interaction of long non-coding RNA (lncRNA) P53RRA, and Ras GTPase-activating protein-binding protein 1 (G3BP1), leads to cell cycle arrest, apoptosis, and ferroptosis. This is because p53 is displaced from the G3BP1 complex (Mao et al., 2018).

Myc

Studies tend to view Myc proteins as transcriptional factors that exert tumorigenesis functions by activating and suppressing target genes (Lutz et al., 2002). Evidence has shown a relationship between Myc and ferroptosis. A recent study reported that egl nine homolog 1 (EGLN1) and Myc activate lymphoid-specific helicase (LSH) expression through HIF-1α, and that LSH suppresses ferroptosis through the interaction with WDR76, leading to the activation of lipid metabolism-associated genes (Jiang et al., 2017). Another study reported that the depletion of VHL, a major tumor suppressor of clear cell renal cell carcinoma (ccRCC), leads to the stabilization of the hypoxia inducible factors HIF-1α and HIF-2α. This paper also found that exogenous expression of pVHL can revert ccRCC cells to an oxidative metabolism and a state of insensitivity to ferroptosis induction. Myc-dependent tumor growth in mouse models can be inhibited by GSH synthesis suppression (Miess et al., 2018). A newly identified oncogene, DJ-1, displays ferroptosis resistance and can synergistically transform mouse NIH3T3 cells together with activated GTPase HRAS and MYC proto-oncogene (c-Myc) (Jiang et al., 2020).

Myb

Members of the Myb family are found in all eukaryotic lineages, the function of which is to regulate fundamental cellular processes, metabolism, and cellular differentiation (Prouse and Campbell, 2012). Evidence shows that c-Myb is involved in ferroptosis through a cysteine dioxygenase 1 (CDO1)–GPX4 axis. Silencing CDO1 leads to suppression of erastin-induced ferroptosis *in vitro* and *in vivo*, and inhibition of CDO1 restores cellular GSA levels, which prevents ROS generation. This paper demonstrates that c-Myb transcriptionally regulates CDO1 and inhibition of CDO1 expression upregulates GPX4 (Hao et al., 2017).

SRC

Cellular SRC (c-SRC), the product of the SRC gene, is involved in tumorigenesis, invasion, and the metastatic phenotype (Alper and Bowden, 2005). A recent study has found that the SRC gene is one of the targeting sites of GPX4, the differential expression of which regulates cell proliferation, cancer progression, apoptosis, and ferroptosis (Wei J. et al., 2020). Another report demonstrated that, mediated by α6β4 integrin, the activation of SRC and STAT3 could inhibit ACSL4 expression, leading to the protection of adherent epithelial and carcinoma cells form erastin-induced ferroptosis (Brown et al., 2017). This is partly because ferroptosis cannot be triggered while there is a lack membranes enriched by ACSL4-mediated long polyunsaturated fatty acids. It was also proved that matrix-detached epithelial and cancer cells cluster spontaneously through a pathway involved with Nectin-4 (also known as cell adhesion protein PVRL4), the process of which sustains GPX4 expression and buffers against lipid peroxidation by stimulating the PVRL4/α6β4/Src axis signal pathway (Brown et al., 2018).

Rb

The retinoblastoma (Rb) protein is the founding member of a protein family that exerts a strong regulatory function on the transcription of various genes in eukaryotes (Knudsen and Knudsen, 2008). Ferroptosis in hepatocellular carcinoma can be promoted, resulting in two or three times more cell death, by sorafenib treatment combining with Rb knockdown using RNA interference (Louandre et al., 2015).

FERROPTOSIS AND EPIGENETICS

Non-coding RNA

Non-coding RNAs (ncRNAs) are RNAs in the transcriptome and will not be translated into proteins. They are identified as several subfamilies based on their molecular size and shape, including long non-coding RNAs (lncRNAs), microRNAs (miRNAs), small nuclear RNAs (snRNAs), and small interfering RNAs (siRNAs) (Hombach and Kretz, 2016). Non-coding RNAs are increasingly regarded as essential regulators of ferroptosis in cancer and a better understanding of them can provide novel ideas for cancer treatment.

MiRNAs exhibit functions by binding to the 3′-untranslated regions of their target mRNAs and thus prevent the expression process (Majidinia et al., 2020). Studies have demonstrated that miRNAs regulate ferroptosis through direct and indirect approaches. For example, miR-7-5p inhibits ferroptosis by downregulating mitoferrin and reducing iron levels in radio-resistant cells (Tomita et al., 2019). miR-6852, which is regulated by lncRNA linc00336, can inhibit lung cancer progression by promoting ferroptosis. Besides direct regulation, evidence shows that miRNAs affect the metabolism of GSH, a scavenger of ROS that protects lipid membrane (Hsu et al., 2019). For instance, miR-18a and miR-218 downregulate GSH levels in hepatocellular carcinoma and bladder cancer separately by targeting GCL (Anderton et al., 2017; Li P. et al., 2017), while miR-152 and miR-155 decrease GSH levels in hepatocellular carcinoma and lung cancer separately by targeting GST (Huang et al., 2010; Lv et al., 2016), the general pathway by which miRNAs modulate GSH level. GST can be targeted and modulated by various miRNAs, including miR-92b-3p, miR-124, miR129-5P, miR-130b, miR-133a/b, miR-144, miR-153-1/2, miR-186, miR-302c-5p, miR-513a-3p, miR-590-3p/5p, miR-36645p, miR-3714, and let-7a-5p

(Zhang et al., 2020e). In the meantime, iron metabolism mainly includes the interaction between transferrin (TF) and TF receptor (TFR), which can also be regulated by miRNAs. For example, in CRC and hepatocellular cancer, TFR can be targeted by miRNAs including miR-22, miR-31, miR-141, miR-145, miR-152, miR-182, miR-200a, miR-320, miR-758, and miR19463–65, resulting in a disruption between TF and TFR and the following iron importing process (Zhang et al., 2020e). Moreover, iron can regulate miRNA levels. Levels of miR-107 and miR-125b can be suppressed by iron in hepatocellular carcinoma (Lobello et al., 2016; Zou et al., 2016), while levels of miR-146a and miR-150 can be increased by iron (Sriramoju et al., 2015; Lobello et al., 2016), which might be due to iron's induction of excess ROS (Zhang et al., 2020e). Moreover, miRNAs regulate the NRF2 pathway through by targeting Kelch-like ECh-Associated Protein 1 (KEAP1) and NRF2 mRNAs (Zhang et al., 2020e).

LncRNAs generally serve as regulators of transcription factors in the nucleus or as sponges of miRNAs in the cytoplasm (Wu et al., 2020). The silence of lncRNA ZFAS1, which acts as a ceRNA and sponge for miR-150-5p, suppresses ferroptosis by downregulating SLC38A1 (Yang Y.N. et al., 2020). Besides the relationship between linc00336/miR-6852 and lncRNA P53rra/G3BP1 mentioned above, lncRNAs modulate ferroptosis indirectly by targeting ferroptosis-associated factors (**Table 2**). A study reported that the reduction of lncRNA ROR leads to reduced GST expression in breast cancer (Li Y.H. et al., 2017), and silencing lncRNA Neat1 contributes to an increase of GST (Wang et al., 2018). Other studies have shown that lncRNAs are associated with iron metabolism and that silencing lncRNA PVT1 suppresses TFR expression and obstructs iron intake *via* miR-150 (Xu et al., 2018). Evidence also shows that lncRNAs affect the expression of NRF2 by directly and indirectly modulating KEAP1 levels, while NRF2 is associated with lncRNA regulation (Zhang et al., 2020e). Besides the above factors, ROS levels can be regulated by lncRNAs. For instance, decreased expression of lncRNA H19 increases ROS *via* the MAPK/ERK signaling pathway (Ding et al., 2018), while the reduction of lncRNA growth arrest specific 5 in melanoma enhances intracellular ROS (Chen et al., 2019). Increased levels of lncRNA GABPB1-AS1 downregulate the peroxiredoxin-5 peroxidase gene and ultimately inhibits the antioxidant capacity of cells (Qi et al., 2019).

Other ncRNAs, such as circRNAs RNAs, rRNAs, piRNAs, snRNAs, and snoRNAs, also interact with ferroptosis in various cancer types (**Table 3**). For circRNAs, circIL4R facilitates tumorigenesis and prevents ferroptosis by regulating the miR-541-3p/GPX4 axis (Xu et al., 2020a). The reduction of circ-TTBK2 delays proliferation and invasion of glioma cells by regulating the miR-761/ITGB8 axis and triggering ferroptosis (Zhang et al., 2020b). Another study reports that circRNA clARs regulate ferroptosis through interacting with the RNA binding protein ALKBH5 (Liu et al., 2020). A recent study has shown that reduction of circ0008035 enhances the anticancer effects of erastin and RSL3 by increasing iron accumulation and lipid peroxidation (Li C. et al., 2020). Moreover, studies revealed that tRNA upregulates ferroptosis by suppressing GSH biosynthesis in a GPX4-independent pattern. However, in contrast, tRNAs can also downregulate ferroptosis by enhancing the antioxidant defense system (Zhang et al., 2020e). Moreover, rRNAs, piRNAs, snRNAs, and snoRNAs were recently found to be involved in ferroptosis-associated pathways (Zhang et al., 2020e).

Methylation

Various studies have revealed the function of DNA or protein methylation in tumor progression, ROS metabolism, and iron metabolism; however, despite being one of the most common molecular modification in epigenetics, the direct relationship between methylation and ferroptosis has not been fully discussed.

Some studies demonstrated the indirect regulation of ferroptosis *via* DNA and protein methylation. For example, lymphoid-specific helicase (LSH), a DNA methylation modifier, can activate lipid metabolism-associated genes to inhibit ferroptosis by interacting with WDR76 (Jiang et al., 2017), and together with another W40 protein DCAF8, they function as a crucial nexus in epigenetic regulation of ferroptosis, controlling

TABLE 2 | lncRNAs participate in the regulation of ferroptosis.

lncRNA	Target	Regulatory direction for target	Effect to ferroptosis (references)
ZFAS1	SLC38A1	activation	enhance ferroptosis (Yang Y.N. et al., 2020)
PVT1	TFR	inhibition	block iron intake (Xu et al., 2018)
H19	MAPK/ERK signaling	regulation	modulate ROS production (Ding et al., 2018)
GABPB1AS1	peroxiredoxin-5 peroxidase	inhibition	decrease antioxidant capacity (Qi et al., 2019)
OIP5-AS1	miR-128-3p/SLC7A11 signaling	sponge	inhibit ferroptosis (Zhang Y. et al., 2021)
NEAT1	ACSL4	regulation	regulate ferroptosis and ferroptosis sensitivity (Wu and Liu, 2021)
LINC00618	lymphoid-specific helicase (LSH)	attenuate LSH to recruit to the promoter regions of SLC7A11	increase ROS and iron, accelerate ferroptosis (Wang Z. et al., 2021)
MT1DP	miR-365a-3p/NRF2 axis	stabilize miR-365a-3p to modulate NRF2 expression	increase intracellular ferrous iron (Gai et al., 2020)
LINC00336	ELAVL1	binding	inhibit ferroptosis (Wang M. et al., 2019)
P53RRA	G3BP1	binding	cytosolic P53RRA-G3BP1 interaction displaces p53 from a G3BP1 complex, induce ferroptosis (Mao et al., 2018)

TABLE 3 | circRNAs modulate the induction of ferroptosis in cancer.

circRNA	Target	Regulatory direction for target	Effect to ferroptosis (references)
IL4R	miR-541-3p/GPX4 axis	sponge	inhibit ferroptosis (Xu et al., 2020a)
TTBK2	miR-761/ITGB8 axis	sponge	inhibit ferroptosis (Zhang et al., 2020b)
clARs	ALKBH5	interaction	regulate ferroptosis (Liu et al., 2020)
KIF4A	circKIF4A-miR-1231-GPX4 axis	sponge	inhibit ferroptosis (Chen W. et al., 2021)
circ0097009	circ0097009/miR-1261/SLC7A11 axis	sponge	regulate ferroptosis (Lyu et al., 2021)
RHOT1	miR-106a-5p/STAT3 axis	sponge	inhibit ferroptosis (Zhang H. et al., 2020a)
EPSTI1	miR-375/409-3P/515-5p-SLC7A11 axis	sponge	regulate ferroptosis (Wu et al., 2021)
ABCB10	miR-326/CCL5 axis	sponge	regulate ferroptosis (Xian et al., 2020)
TTBK2	miR-761/ITGB8 axis	sponge	regulate ferroptosis (Zhang et al., 2020b)

LSH degradation by adapted oxidative damage sensing through DNA hydroxymethylation (Huang et al., 2020). The silencing of the DNA methylation of the elongation of very long-chain fatty acid protein 5 (ELOVL5) and fatty acid desaturase 1 (FADS1) leads to ferroptosis resistance, and these two enzymes are usually upregulated in mesenchymal-type gastric cancer cells (Lee J.-Y. et al., 2020). Besides, GPX4 methylation is also reported to be related to ferroptosis regulation. For example, homocysteine (Hcy), an amino acid involved in DNA methylation, facilitates GPX4 methylation that leads to upregulation of oxidative stress and ferroptosis in nucleus pulposus (Zhang et al., 2020c). Another study reported that the increased expression of GPX4 in cancer tissues might be partly attributed to a lower level of DNA methylation and histone acetylation (Zhang et al., 2020d). A report has shown that KDM3B, a histone H3 lysine 9 demethylase, can protect against erastin-induced ferroptosis and is thus considered a potential epigenetic regulator of ferroptosis (Wang et al., 2020). Meanwhile, the expression of iron metabolism-associated genes, including TRFC, FTH1, and FTL, can be modulated by the epigenetic silencing of the iron-responsive element binding protein 2 (IREB2) (Dixon et al., 2012), while other perturbations of mechanisms, including acetylation and methylation, have been observed to regulate iron metabolism in cancer cells by controlling transcript encoding proteins (Manz et al., 2016).

Some ferroptosis regulation pathways have been found recently in which tumor-associated factors are usually involved. For instance, in head and neck cancer cells, diminution of the hypermethylation of CDH1 results in increased E-cadherin expression and decreased ferroptosis susceptibility (Lee J. et al., 2020); this work also provides evidence that epithelial–mesenchymal transition (EMT) promotes ferroptosis *via* epigenetic regulation pathways. The lower promoter methylation of GPX1, a member of the GPX family that interact with oxidative stress, results in high expression levels of GPX1 in some cancer cell lines (Wei R. et al., 2020). Another study shows that JQ1 can inhibit BRD4 expression and ultimately induce ferroptosis through two pathways, either by inhibiting the histone methylase G9a or by activating the histone deacetylase SIRT1, which can recognize the acetylation site and recruit transcriptional factors (Sui et al., 2019).

Acetylation

A widely occurring post-translational modification, acetylation plays a role in ferroptosis mainly through direct and indirect interaction with ferroptosis regulators. The acetylation of genes and proteins involved in ferroptosis is reported to regulate iron-dependent cell death. For example, an acetylation defect is observed in mutant $p53^{3KR}$, which indirectly inhibits cysteine absorption and reduces GSH consumption, leading to lipid peroxidation and ferroptosis (Jiang et al., 2015). Acetylation absence in the mouse p53 K98 site and on other positions in the DNA-binding domain can result in the loss of tumor suppression functions in xenografts and ferroptosis (Wang et al., 2016). Another study reported that RSL3 promotes the protein expression and acetylation of ALOX12, the key protein in initiating membrane phospholipid oxidation (Wang Y. et al., 2021). Indirect regulation is also observable. It has been reported that suppression of EMT mediated by histone deacetylase SIRT1 gene silencing or pharmacological inhibition consequently decreases ferroptosis, which further suggests that EMT promotes ferroptosis through epigenetic regulation pathways (Lee J. et al., 2020). Moreover, acetylation of HMGB1, a damage-associated molecular pattern molecule (DAMP), is released by ferroptosis cells in an autophagy-dependent manner (Wen et al., 2019).

Ubiquitination

Ubiquitination is a post-translational modification involved in essential host processes that has been reported to regulate ferroptosis epigenetically. The most common regulation pathway involves interaction with SLC7A11, which is essential in the X_c^- system. Evidence suggests that the deubiquitinase OTUB1, usually overexpressed in cancers, replicates the ferroptosis process and promotes tumor development by stabilizing the cystine transporter SLC7A11 (Gan, 2019). Once deubiquitinase is suppressed, caspase-dependent apoptosis and GPX4-degradation-dependent ferroptosis is activated, contributing to the accumulation of ubiquitination proteins that facilitates cell death (Yang L. et al., 2020). The tumor suppressor BAP1, an H2A deubiquitinating enzyme, can reduce SLC7A11 expression by inhibiting H2A ubiquitination (H2Aub) on the SLC7A11 promoter, thus controlling ferroptosis (Zhang Y.L. et al., 2019). Another study shows that p53 may also be involved in ubiquitination-dependent regulation of ferroptosis. For

example, p53 decreases H2B ubiquitination occupancy in the SLC7A11 gene regulatory domain and represses its expression (Wang Y. et al., 2019). Ubiquitination also regulates ferroptosis by modulating ferritin degradation. In iron deficiency, nuclear receptor coactivator 4 (NCOA4) specifically binds iron-rich ferritin to autophagosomes through FTH1 and transports it to the lysosome for iron release, while NCOA4 can be degraded through ubiquitination, which affects that stability of ferritin. Therefore, suppressing NCOA4 can inhibit the degradation of ferritin and the occurrence of ferroptosis (Capelletti et al., 2020).

FERROPTOSIS AND CANCER STEM CELLS

Hallmarks of CSCs

CSCs are a small section of tumor cells that possesses the ability to self-renew, initiate tumors, and cause resistance to conventional anticancer agents. Different from regular cancer cells, CSCs have a lower level of ROS, which might contribute to a slower growth rate, reduced oxidative metabolism, and elevated expression of the ROS scavenging system (Bystrom et al., 2014; Ding et al., 2015; Hyewon and Navdeep, 2018). Lipid intake pathways are upregulated in CSCs, providing energy essential for survival, which explains why interference with GPX4 pathways seems to render CSCs sensitive to ferroptosis (Recalcati et al., 2019; Visweswaran et al., 2020). Higher iron levels are another characteristic of CSCs, such that ferroptosis may be a good method for eliminating CSCs, which are less susceptible to classical anticancer apoptosis-inducing agents. Indications of higher iron levels consist of the expression levels of TFR1 and its ligand iron-loaded TF is upregulated in glioblastoma CSCs compared to non-CSCs (David et al., 2015). Furthermore, cellular iron, TFR1, and TF uptake are more robust in breast CSCs compared to non-CSCs (Mai et al., 2017). TFR1 and ferritin are essential for propagation and formation of tumors *in vivo*. On the other hand, forced reduction of intracellular iron reduces the proliferation and tumorigenicity of ovarian CSCs (Basuli et al., 2017). Evidence points to multiple roles of intracellular iron in CSC proliferation and stemness maintenance (Recalcati et al., 2019). For instance, in breast cancer cells, low iron levels are associated with a lower expression of EMT markers (Guo et al., 2015). Iron also mediates the downregulation of E-cadherin, a hallmark of EMT (Brookes et al., 2008).

Ferroptosis-Based Treatment of Cancer Stem Cells

Higher iron levels do not necessarily relate to ROS levels and ferroptosis, but it has been proven that CSCs are highly sensitive to ferroptosis due to increased expression levels of TFR1, and thus ferroptosis-based treatment and therapeutic interference of iron homeostasis can have a curing effect on cancer (Mai et al., 2017). Notably, recent studies indicate that triggering ferroptosis may specifically kill CSCs; for example, salinomycin can drive ferroptosis-based cell death in breast CSCs (Zhao et al., 2019), and ironmycin, a derivative of salinomycin, can specifically trigger iron accumulation in lysosomes, activating cell death pathways consistent with ferroptosis (Mai et al., 2017). Some small-molecule ferroptotic agents also have the potential to selectively kill breast CSCs (Taylor et al., 2019). The blocking of the lysosomal iron translocation of CSCs by inhibiting the divalent metal transporter 1 (DMT1) leads to iron accumulation and cell death with features of ferroptosis (Turcu et al., 2020). In colorectal CSCs, knockdown or inhibition of SLC7A11 significantly and specifically kills cancer cells and thus attenuates chemoresistance in CRC (Xu et al., 2020c). Besides, two nitroimidazoles (Koike et al., 2020), itraconazole (Xu et al., 2021), and dichloroacetate (Sun et al., 2021) are also proven to have therapeutic potential through promoting ferroptosis in CSCs.

FERROPTOSIS AND THE TUMOR MICROENVIRONMENT

The TME functions as a cradle for tumorigenesis and cancer progression. Understanding the TME and ferroptosis interaction may provide novel and effective anticancer strategies.

A recent study reports that ferroptosis can promote tumor growth by driving macrophage polarization in the TME (Dai E.Y. et al., 2020). Hypoxia is one of the known characteristics of the TME, which is controlled by the hypoxia-inducible factor (HIF) (Labiano et al., 2015). Researchers have found that hypoxia is an essential positive trigger for ferroptosis, and HIF-2α enhances lipid peroxidation while the depletion of HIF-1α decreases sensitivity to ferroptosis (Zou et al., 2019). Moreover, iron metabolism-associated genes, including FTH, TFR1, and SLC11A2, are regulated by hypoxia-responsive elements (HREs) in the promotor region (Li et al., 2019).

Antitumor Immunity

Ferroptosis is thought to be linked to antitumor immunity. This was first proved by the study that immunotherapy-activated $CD8^+$ T lymphocytes can induce ferroptosis in cancer cells by downregulating SLC7A11 and SLC3A2, encoding subunits of system X_c^-. Technically, this study has shown that tumor cell coculture with IFN-γ-rich supernatant obtained from activated T cells induces lipid peroxidation and ferroptosis (Wang W.M. et al., 2019). Overexpression of ferroptosis suppressor protein 1 (FSP1) or cytosolic GPX4 stimulates the genesis of ferroptosis-resistant $CD8^+$ T cells without compromising their function, while the depletion of ferroptosis sensitivity-promoting enzyme acyl-CoA synthetase long-chain family member 4 (ACSL4) protected $CD8^+$ T cells from ferroptosis but impaired antitumor $CD8^+$ T cell response (Drijvers et al., 2021).

Other studies have demonstrated that cancer cells that have undergone ferroptosis can release high mobility group Box 1 (HMGB1) in an autophagy-dependent manner (Yu et al., 2015; Wen et al., 2019). When HMGB1 is released into the TME because of cancer cell death, it can stimulate the innate immune system by interacting with several pattern recognition receptors (Sims et al., 2010; Yamazaki et al., 2014). Evidence shows that during ferroptosis, tumor cells supply arachidonic acid for

eicosanoid synthesis, which can strengthen antitumor immunity (Angeli et al., 2019). Moreover, ferroptosis induction in tumor cells is thought to be related to the release of prostaglandin E2 (PGE2), which facilities the evasion from immune surveillance (Yang et al., 2014).

Nanoparticles and Immunotherapy

The synergism between ferroptosis and immunomodulation in cancer has been widely investigated in recent decades. On the one hand, TME immunomodulation can trigger macrophage polarization from alternately activated macrophages M2 to classically activated macrophages M1, offering intertumoral H_2O_2 for the Fenton reaction (Zanganeh et al., 2016), which effectively generates ROS and triggers lipid peroxidation (Yang and Stockwell, 2016; Sun et al., 2017). On the other hand, ferroptosis in tumor cells can release tumor antigens and generate an immunogenic TME, thus enhancing the immunomodulation response (Zhang F. et al., 2019). Nanoparticles (NPs), which can passively infiltrate tumor tissues because of the enhanced permeability and retention, act as a drug-loading platform with high loading efficiency, and release specific cargos in tumor issues, are gaining recognition in immunotherapy.

Some metal elements are especially popular for their inherent physicochemical properties, and metal-containing nanomaterials are designed for ferroptosis-driven therapy. They can function in different manners, including facilitating Fenton-like reactions, providing hydrogen peroxide, damaging the reducing system, and disturbing cellular communication (Fei et al., 2020). For example, biomimetic magnetosome, composed of an Fe_3O_4 magnetic nanocluster with a TGF-β inhibitor loaded inside and a PD-1 antibody anchored on the membrane surface, was developed to promote ferroptosis/immunomodulation synergism in cancer (Zhang F. et al., 2019). MnO_x nanospikes, as TME-responsive nano-adjuvants and immunogenic cell death drugs, were also designed for cancer nanovaccine-based immunotherapy (Ding et al., 2020). In another study, in which ultrasmall CaO_2 and Fe_3O_4 were co-loaded on to dendritic mesoporous silica NPs, researchers showed that these particles can achieve tumor specialized localization and induction of Fenton reaction, thus triggering ferroptosis (Li and Rong, 2020). The Fe_3O_4-PLGA-Ce_6 nanosystem, which dissociates in acidic TME, and the Fe^{2+}-based metal–organic framework, which delivers Fe^{2+} to cancer cells, can also promote the Fenton reaction and facilitate ferroptosis (Xu et al., 2020b; Chen Q. et al., 2021).

Although nanotechnology is increasingly used in cancer treatment, the application of NP-based therapy faces various issues, such as intrinsic immunogenicity and residual cytotoxicity (Shen et al., 2018). In a new approach that has high biocompatibility, low immunogenicity, preferred tumor homing, and high efficiency in cargo delivery, the 30- to 120-nm endocytic lipid bilayer membrane-derived vesicles is attracting attention as a novel drug carrier for ferroptosis induction (Qin and Xu, 2014; Kibria et al., 2018). Attempts have been made to use exosomes as carriers for ferroptosis-inducing drugs to trigger cell death among cancer cells. For example, engineered M1 macrophages, with CCR2 overexpression, are employed as Fe_3O_4 NP carriers (Li et al., 2021). Moreover, a well-known ferroptosis inducer, erastin, can be loaded into exosomes labeled with folate and delivered to cancer cells that express the folate receptor to generate ROS and glutathione depletion (Yu et al., 2019).

EXOSOMES

Generated from the plasma membrane (Simons and Raposo, 2009), exosomes were first used for carrying clotting suppressors (Wolf, 1967). Since then, these extracellular vesicles have been shown to be secreted by various kinds of cells, including dendritic cells, macrophages, T cells, B cells, mesenchymal stem cells, endothelial cells, epithelial cells, and various cancer cells (Song H. et al., 2021).

Biogenesis and Composition

Exosomes are generated from late endosomes through several different pathways. Endosomal-sorting complexes required for transport (ESCRTs), which recognize ubiquitylated proteins, are the most characterized one among genesis pathways, while others may involve sphingomyelinases (Trajkovic et al., 2008), sphingosine-1-phosphate, and tetraspanin-enriched domains (Brinton et al., 2015). Four ESCRTs, numbered from 0 to 3, consist of many proteins able to recognize ubiquitinated cargoes. Technically, ESCRT-0 subunits recruit proteins for internalization, such as ubiquitinated proteins and clathrin. ESCRT-1 and ESCRT-2 control the initiation of the budding process and facilitate the enzymatic de-ubiquitination of cargo proteins before the formation of intraluminal vesicles (ILVs). ILVs then gather to form larger membranous vesicles in the intracellular compartment. ESCRT-3 drives membrane invagination and separation (Ha et al., 2016; McGough and Vincent, 2016). According to the genesis process, the composition pattern of exosomes faithfully reflects their parent cells. For proteins displayed on the surface, adhesion molecules, which belong to the tetraspanin and integrin families, are the most abundant. These proteins, which are generally membrane crossing, include CD9, CD63, CD81, and CD82 and regulate processes like fusion, migration, and adhesion. They usually attach to each other or associate with nearby proteins, such as integrins, to form a tetraspanin membrane domain (Farooqi et al., 2018). The major histocompatibility complex II (MHC-II) may be present on the surface of exosomes and is involved in promoting certain T-cell responses (Elena and Myung Soo, 2015). Moreover, tumor-derived exosomes are able to promote cancer cell migration and metastasis, containing various kinds of integrin, such as exosomal integrins $\alpha_v\beta_6$ for prostate cancer (Carmine et al., 2015), $\alpha_6\beta_4$ and $\alpha_6\beta_1$ for lung cancer, and $\alpha_v\beta_5$ for liver cancer metastasis (Hoshino et al., 2015). Other protein molecules, such as annexins, flotillin, and GTPases, are associated with lipid fractions on exosomes and serve transportation and fusion functions (Colombo et al., 2014). Besides proteins, lipids are another main component of exosomes, which depend on the type of parent cell plasma

membrane. Phosphatidylethanolamines, phosphatidylcholines, phosphatidylinositols, phosphatidylserines, sphingomyelins, lysobisphosphatidic acid (bis-monoacylglycerol phosphate), phosphatidic acid, cholesterol, lysophosphatidylcholines, ceramide, and phosphoglycerides have been found in these membranes (Caroline et al., 2007). The intraluminal composition of the exosomal membrane also depends on the parent cells and particularly on their cytoplasmatic content. Exosomes shuttle through the body, allowing the horizontal transfer of their cargo while fusing with target cells and releasing their content *via* an endocytosis process, and thus participate in various regulation pathways (Escrevente et al., 2011). A wide range of molecules have been found in different cell-derived exosomes, such as heat shock proteins, cytoskeletal proteins, lipids, and enzymes, along with nucleic acid molecules, such as miRNAs, mRNAs, ncRNAs, mitochondrial DNA, and single-strand DNA (Farooqi et al., 2018).

Exosomes in Cancer and Ferroptosis Regulation

Among the numerous biological roles played by exosomes, their function in cancer is becoming increasingly apparent. A number of studies have revealed that exosomes can regulate the function of target cells by secreting their contents into the TME, using crosstalk, and/or influencing major tumor-related pathways, including EMT, CSCs, angiogenesis, and metastasis involving several cell types (Ha et al., 2016; Wu et al., 2017). Moreover, drug resistance is partly attributed to exosomes, for cancer cells can encapsulate therapeutic drugs in exosomes and transport them out of tumor cells (Arrighetti et al., 2019). Evidence shows that exosomes also overlap with ferroptosis modulation. For example, mesenchymal stromal cells (MSCs) derived from human umbilical cord blood (HUCB-MSCs) tend to significantly inhibit the expression of DMT1 by miR-23a-3p to inhibit ferroptosis (Song Y. et al., 2021). The miR-522 inside exosomes, generated from cancer-associated fibroblasts (CAFs), can block lipid-ROS accumulation by targeting ALOX15 and thus inhibit ferroptosis (Zhang H. et al., 2020a). In a recent study, researchers found that ferroptosis promotes tumor growth by driving macrophage polarization in the TME. One kind of common KRAS mutant, $KRAS^{G12D}$, is secreted into the TME from tumor cells after succumbing to autophagy-dependent ferroptosis. This extracellular protein is then packaged into tumor-derived exosomes and is absorbed by macrophages, leading to the switch from the M1 phenotype to the M2 phenotype and accelerating cancer progression (Dai E.Y. et al., 2020). Prominin 2 is a pentaspanin protein involved in lipid dynamics regulation. It promotes the formation of ferritin-containing multivesicular bodies (MVBs) and exosomes that transport iron out of the cell and thus inhibits ferroptosis (Brown et al., 2019). Exosomes themselves are also proved to have some curing functions; for instance, rat plasma-derived exosomes can enhance cell proliferation and radio-resistance-related genes and yet downregulate ferroptosis in irradiated fibroblasts (Gan et al., 2021).

Delivery of Protein and Small RNAs

Despite the therapeutic potential of nucleic acid and protein drugs, their clinical application has been limited partly by a lack of appropriate delivery systems. Proteins and small RNAs can be loaded onto exosomes and delivered to target cells, interfering with various pathways. For example, a research team engineered human embryonic kidney (HEK) cells to produce exosomes able to target breast cancer cells overexpressing epidermal growth factor receptor (EGFR). In order to achieve elective targeting, researchers have engineered donor HEK cells to express the transmembrane domain of platelet-derived growth factor receptors fused to the GE11 peptide. Let-7a miRNA was introduced into GE11-positive exosomes using the lipofection method and HEK cells. Results show that miRNA exosomes have a curing effect on breast cancer (Ohno et al., 2013). As with ferroptosis, we reviewed a number of proteins and RNAs regulating ferroptosis-based cell death in the previous section. These molecules can be easily introduced to donor cells, and tumor targeting exosomes carrying these molecules can be used for cancer treatment. However, related research is lacking. Although the use of exosomes as a delivery system has its drawback (for example, quickly eliminating by the reticuloendothelial system, lack of efficient encapsulation methods, and potential immune responses), exosomes targeted at tumors may allow systemic administration of miRNA as cancer treatment and are thus worthy of attention.

Advantages of Exosomes for Drug Delivery Systems

Nanotechnology has been developed for drug delivery, but intrinsic immunogenicity and residual cytotoxicity have hindered its application. During recent decades, researchers turned to delivery systems based on natural and synthetic polymers and lipids because such liposomes possess valuable qualities, such as the incorporation of hydrophilic and hydrophobic drugs, and membrane penetration (Farooqi et al., 2018). However, disadvantages, such as lower circulation stability, rapid clearance by phagocytosis, and increased toxicity, challenge the application of liposomes (Ha et al., 2016). In this respect, exosomes display better tolerance and lower toxicity due to their ubiquitous presence and similarity in structure and composition to biological membranes (Bang and Thum, 2012). Exosomes can penetrate through tissues, deliver contents directly into cellular compartments, and evade the immune system. They are also able to target specific organs and tissues (Hood et al., 2011). The application of engineered cell strains with special plasmid vectors that encode fusion proteins helps to develop targeted exosome-based delivery systems by achieving amenable membrane modifications and desirable attributes especially when targeting a specific cell type (Farooqi et al., 2018).

Application of Exosomes Providing a Novel View for Ferroptosis-Based Cancer Treatment

Modified exosomes can be selectively used to deliver drugs to specific cells and present advantages, such as high effectiveness

and reduced toxicity. Exosomes generated by genetically engineered cell stains present designed proteins on the surface, which can selectively drive exosomes and their contents to targeted cells. For example, engineered immature dendritic cells (imDCs) in mice express a well-characterized exosomal membrane protein (Lamp2b) fused to αv integrin-specific iRGD peptide (CRGDKGPDC). Doxorubicin (Dox), produced by engineering imDCs, can be loaded to exosomes through electroporation. *In vivo* and *in vitro* experiments have shown that the Dox-exosomes process possesses high efficacy in Dox delivery and targeting to breast cancer, effective cancer suppression, and low toxicity (Tian et al., 2014). One study showed that exosomes derived from brain cells that expressed brain specific surface proteins can cross the blood–brain barrier and deliver drugs to the other side (Yang et al., 2015). For ferroptosis, therapeutic drugs, such as erastin and newly recognized natural ferroptosis-inducing compounds, can be loaded onto tumor targeting exosomes. This may provide new avenues for cancer treatment. Attempts have been made and the results are positive. Nevertheless, challenging issues remain to be solved, such as poor encapsulation efficiency and the interference form exosomal endogenous nucleic acids and proteins.

CONCLUSION AND FUTURE PROSPECT

Apoptosis, necroptosis, pyroptosis, and ferroptosis are the most widely studied types of programmed cell death. These types of programmed cell death are all involved in cancer progression and therapy. In our lab, we focus on regulating the crosstalk among different types of programmed cell death to broaden the application of anti-tumor drugs (Liu S. et al., 2017; Huang et al., 2018, 2021a,b; Xiang et al., 2019; Li T. et al., 2020). Inducing a certain type of programmed cell death specifically can have profound significance for cancer treatment. Ferroptosis is an iron-dependent form of programmed cell death triggered by unrestricted lipid peroxidation and subsequent plasma membrane rupture. It is well known that cancer development and treatment can be affected by genetic factors, epigenetic modifiers, CSCs, and the TME (Xiang et al., 2019). As mentioned above, ferroptosis could be induced to exert anti-tumor functions *via* signaling pathway modulation, non-coding RNA expression, DNA methylation, histone modification, CSCs, microenvironment remodeling, and so on. However, it is not clear how to utilize and manipulate ferroptosis in cancer treatment, specifically. On the one hand, studies need to deeply exploit the molecular and cellular mechanisms underlying ferroptosis; on the other hand, combining ferroptosis with biological materials is a promising alternative strategy. As the smallest extracellular vesicles and endogenous source of nanocarriers, exosomes show great potential for cargo delivery, including RNA, protein, drugs, and ions. Most importantly, exosomes have been shown to transport iron out of the cell to regulate ferroptosis (Brown et al., 2019). In addition, gene engineered exosomes exhibit promising characteristics in cancer treatment (Cheng et al., 2021). Therefore, adjusting the cargo of exosomes and/or engineering their spreading pathways could target cancer cells (especially CSCs) or the TME in order to induce ferroptosis, thus achieving a positive therapeutic outcome.

AUTHOR CONTRIBUTIONS

SW and YH wrote the manuscript. TL and WL reviewed the manuscript. All authors approved the manuscript.

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MINI REVIEW
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Pre-metastatic Niche Formation in Different Organs Induced by Tumor Extracellular Vesicles

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Primary tumors selectively modify the microenvironment of distant organs such as the lung, liver, brain, bone marrow, and lymph nodes to facilitate metastasis. This supportive metastatic microenvironment in distant organs was termed the pre-metastatic niche (PMN) that is characterized by increased vascular permeability, extracellular matrix remodeling, bone marrow-derived cells recruitment, angiogenesis, and immunosuppression. Extracellular vesicles (EVs) are a group of cell-derived membranous structures that carry various functional molecules. EVs play a critical role in PMN formation by delivering their cargos to recipient cells in target organs. We provide an overview of the characteristics of the PMN in different organs promoted by cancer EVs and the underlying mechanisms in this review.

Keywords: pre-metastatic niche (PMN), extracellular vesicles (EVs), vascular permeability, extracellular matrix (ECM), bone marrow-derived cells (BMDCs), immunosuppression

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INTRODUCTION

Primary tumors selectively modify the microenvironment of distant organs before metastasis (Hiratsuka et al., 2002; Kaplan et al., 2005). This supportive metastatic microenvironment in distant organs was first termed the pre-metastatic niche (PMN) by Kaplan et al. (2005). In the last decade, PMN induced by various cancers has been identified in the lung (Hiratsuka et al., 2002; Hoshino et al., 2015; Liu et al., 2016; Tyagi et al., 2021), liver (Hoshino et al., 2015; Zhang and Wang, 2015; Houg and Bijlsma, 2018; Sun et al., 2021), brain (You et al., 2020), bone (Kaplan et al., 2007; Xu et al., 2017), and other organs. The PMN is characterized by increased vascular permeability (Gupta et al., 2007; Huang et al., 2009; Zhou et al., 2014), a modified extracellular matrix (ECM) (Aguado et al., 2016; Kim et al., 2019; Mohan et al., 2020), recruited bone marrow-derived cells (BMDCs) (Kitamura et al., 2015; Wang et al., 2019), and immunosuppression (Chen et al., 2011; Tacke et al., 2012; Giles et al., 2016) in the future metastatic organs.

Extracellular vesicles (EVs) are a heterogeneous group of nano-sized membranous structures that are released by almost all cells into extracellular spaces and have many different physiological and pathophysiological functions (Raposo and Stoorvogel, 2013; Colombo et al., 2014). Different EV types, including exosomes, microvesicles, apoptotic bodies, oncosomes, and megasomes have been characterized on the basis of their biogenesis pathways and sizes. Among them, exosomes and microvesicles are the most intensively studied types. Exosomes have an endocytic origin and form by the fusion between multivesicular bodies and the plasma membrane, whereas microvesicles are generated by plasma membrane shedding (van Niel et al., 2018). However, it is difficult

to classify EVs according to their biogenic origin once they are secreted into the extracellular space. Therefore, size-based or density-based nomenclature is recommended by the International Society of Extracellular Vesicles (Thery et al., 2018). EVs contain various functional molecules (proteins, mRNAs, miRNAs, long non-coding RNAs, and double stranded DNA, etc.) that can be trafficked between cells as a means of intercellular communication at both paracrine and systemic levels (Valadi et al., 2007; Balaj et al., 2011; Choi et al., 2013; Thakur et al., 2014). EV cargos are protected by the membrane during the delivery process, which is critical for the communication between primary tumors and distant organs. EVs can be internalized by recipient cells via different mechanisms, including phagocytosis, macropinocytosis, endocytosis, and direct membrane fusion (van Niel et al., 2018). In addition, ligands on EV membrane can interact with receptors on the recipient cell surface and elicit biological responses directly.

In this mini-review, we provide an overview of the characteristics of the PMN in different organs promoted by cancer EVs. The terms, exosomes, microvesicles, or EVs were used to describe their roles in PMN formation to ensure consistency with the original articles.

CHARACTERISTICS OF THE PRE-METASTATIC NICHE

The characteristics of the PMN formed in various organs by EVs are summarized in **Figure 1** and **Table 1**. The lung is the most commonly involved organ, followed by the liver, bone, brain, and lymph nodes (LNs). As shown in **Figure 1**, EVs produced by tumor and stromal cells enter the circulation and arrive at distant organs, where they trigger a sequence of local changes including increased vascular permeability, ECM remodeling, BMDC recruitment, angiogenesis, and immunosuppression.

Increased Vascular Permeability

Increased vascular permeability is an early event in PMN formation (Huang et al., 2009; Araldi et al., 2012). Vascular destabilization in the PMN promotes the extravasation of tumor cells and facilitates metastasis. Both EV-associated miRNAs and proteins contribute to vascular destabilization by destroying adhesion molecules between endothelial cells. Exosomal miR-25-3p derived from human colorectal cancer cells promotes vascular permeability in mouse models by regulating the expression of the tight junction proteins zonula occludens-1

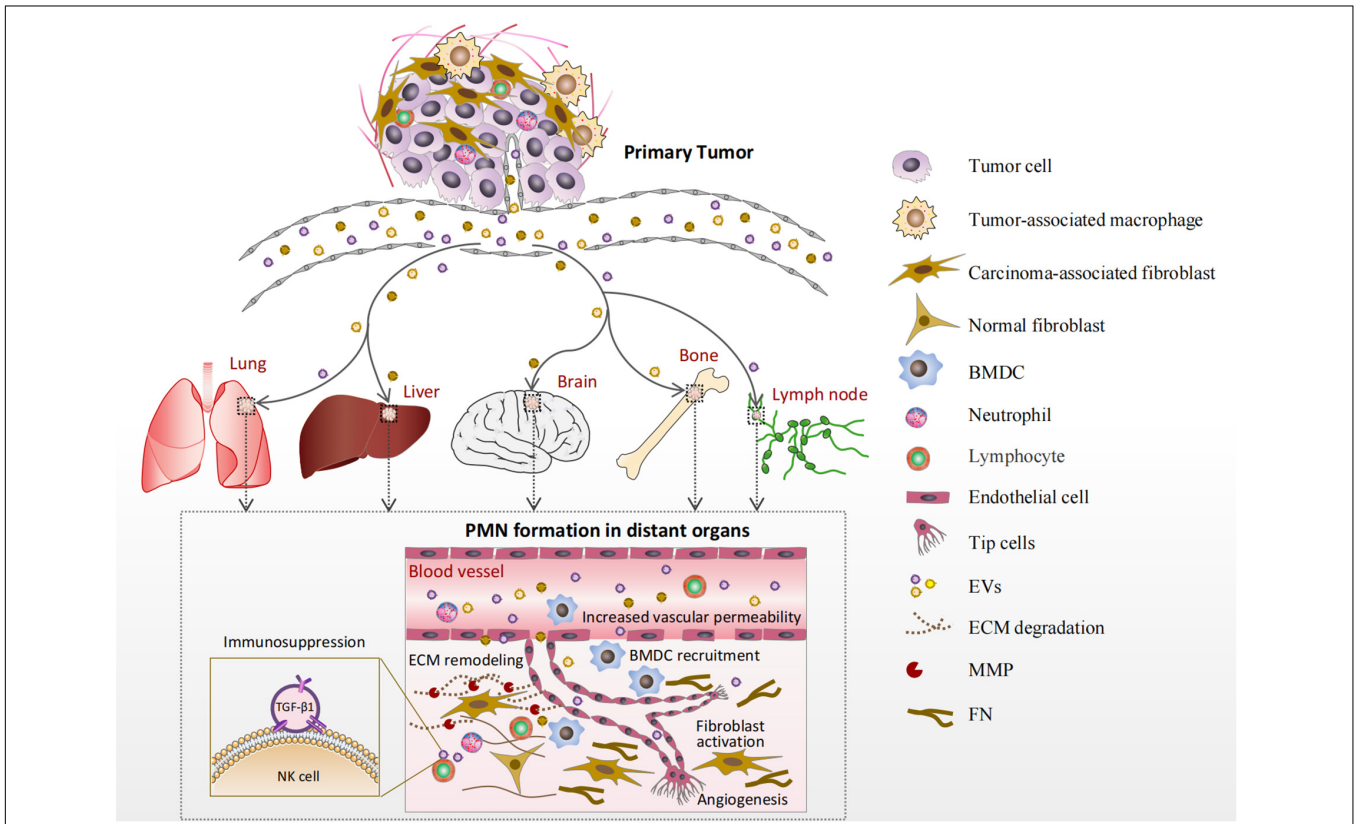

FIGURE 1 | Formation of the pre-metastatic niche (PMN) in various organs. Extracellular vesicles (EVs) produced by tumor and stromal cells enter the circulation and arrive at distant organs such as the lung, liver, brain, bone marrow, and lymph nodes (LNs). Increased vascular permeability is an early event in PMN formation. Then, EVs cause extracellular matrix (ECM) remodeling principally by activating resident normal fibroblasts. The activated fibroblasts deposit new ECM components, such as FN, and produce matrix metalloproteinases (MMPs). Bone marrow-derived cells (BMDCs) are recruited into the target organs by EVs and involved in angiogenesis and/or immune responses. Furthermore, EVs can deliver their cargos to endothelial cells or immunocytes directly in the PMN to promote angiogenesis or create an immunosuppressive microenvironment.

TABLE 1 | Pre-metastatic niche (PMN) formation promoted by extracellular vesicles (EVs).

Organs	Tumor	PMN-promoting EVs	PMN characteristics	References
Lung	Breast cancer	Exosomes from EO771 cells	BMDC recruitment and immunosuppression	Wen et al., 2016
Lung	Breast cancer	EV miR-122	Reprograms glucose metabolism	Fong et al., 2015
Lung	Breast cancer	miR-105-rich EVs	Vascular permeability	Zhou et al., 2014
Lung	Breast Cancer	Exosome-associated Annexin II	Angiogenesis	Maji et al., 2017
Lung	Melanoma	Pigment epithelium-derived factor-positive exosomes	Immune cells recruitment	Plebanek et al., 2017
Lung	Melanoma	Melanoma-derived exosomes	BMDC recruitment; Vascular permeability	Peinado et al., 2012
Lung	Melanoma	EVs from insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1)-overexpressed/knockdown melanoma cells	ECM remodeling (fibronectin deposition); Recruit CD45 + cells in the lung	Ghoshal et al., 2019
Lung	Hepatocellular carcinoma	Nidogen 1 (NID1) in metastatic HCC cell-derived EVs	Angiogenesis	Mao et al., 2020
Lung	High-metastatic hepatocellular carcinoma	miR-1247-3p positive exosomes	Inflammatory microenvironment	Fang et al., 2018
Lung	Colorectal cancer	Exosomal miR-25-3p	Vascular permeability; Angiogenesis	Zeng et al., 2018
Lung	Colorectal cancer	Integrin beta-like 1-rich-EVs	Inflammatory microenvironment	Ji et al., 2020
Lung	Pancreatic ductal adenocarcinomas	Exosomal Podocalyxin	ECM remodeling	Novo et al., 2018
Lung	Osteosarcoma	Osteosarcoma (143-B) cells-derived EVs-associated TGFβ1	Fibroblast activation	Mazumdar et al., 2020b
Lung	Osteosarcoma	Highly metastatic 143-B osteosarcoma cell-derived EVs	CD11b + myeloid cells recruitment	Mazumdar et al., 2020a
Lung	Prostate cancer	Exosomes from human prostate cancer (PCa) PC3 cells under hypoxic conditions	ECM remodeling; BMDC recruitment	Deep et al., 2020
Lung	Nasopharyngeal carcinoma	EV packaged latent membrane protein 1	Fibroblast activation	Wu et al., 2020
Lung	SACC	Epiregulin-positive exosomes	Vascular permeability; Macrophage recruitment	Yang et al., 2017
Lung	Human renal cell carcinoma	MVs derived from CD105-positive cancer stem cells	Angiogenesis	Grange et al., 2011
Lung	Non-small cell lung carcinoma (NSCLC)	Exosomal RNAs (small nuclear RNAs snRNAs)	Immunosuppression	Liu et al., 2016
Lung	Metastatic rat adenocarcinoma	Exosomes from the metastatic rat adenocarcinoma BSp73ASML (ASML)	ECM remodeling; Immunosuppression	Rana et al., 2013
Liver	Breast Cancer	Breast cancer derived-EVs-associated nucleoside diphosphate kinase A and B(NDPK)	Vascular permeability	Duan et al., 2021
Liver	Pancreatic cancer	Exosomes from the highly metastatic pancreatic cancer cell line (Panc02-H7 EXO)	BMDC recruitment	Yu et al., 2017
Liver	Pancreatic Cancer	EV-associated TGF-β1	Immunosuppression	Zhao et al., 2019
Liver	Pancreatic ductal adenocarcinomas	PDAC-derived exosomes-associated macrophage migration inhibitory factor (MIF)	ECM remodeling; BMDC recruitment	Costa-Silva et al., 2015
Liver	Gastric cancer	EGFR-containing EVs	ECM remodeling	Zhang et al., 2017
Liver	Colorectal cancer	Exosomal miR-25-3p	Vascular permeability; Angiogenesis	Zeng et al., 2018
Liver	Colorectal cancer	Integrin beta-like 1-rich-EVs	Inflammatory microenvironment	Ji et al., 2020
Bone	Lung cancer	miR-192-enriched- exosome-like vesicles (ELV)	Angiogenesis	Valencia et al., 2014
Bone	Prostate cancer	Phospholipase D (PLD) isoforms PLD2-riched exosomes	Shift the bone balance in favor of osteoblasts	Borel et al., 2020
Bone	Prostate cancer	Enzalutamide resistant (EnzR) CWR-R1 cells derived EVs (EnzR EVs)	BMDC recruitment	Henrich et al., 2020
Brain	Breast cancer and melanoma	EVs derived from brain metastases cancer cells (Br-EVs)	Blood–brain barrier (BBB) permeability	Busatto et al., 2020
Brain	Glioblastoma multiforme	EV-associated VEGF-A	Angiogenesis	Treps et al., 2017
LNs	Metastatic rat adenocarcinoma	Exosomes from the metastatic rat adenocarcinoma BSp73ASML (ASML)	ECM remodeling; Immunosuppression	Rana et al., 2013
LNs	Melanoma	Melanoma-derived exosomes	ECM remodeling; Angiogenesis	Hood et al., 2011

(ZO-1), occludin, and claudin-5 in endothelial cells, thereby promoting cancer metastasis in the liver and lungs of mice (Zeng et al., 2018). In breast cancer, metastatic cancer cells secrete miR-105-rich exosomes that regulate vascular permeability and promote tumor metastasis by downregulating ZO-1 expression (Zhou et al., 2014). Melanoma-derived exosomes upregulate tumor necrosis factor-α expression in the lung, disrupting endothelial cell–cell junctions and increasing vascular permeability (Peinado et al., 2012). Vascular endothelial growth factor-A (VEGF-A) is carried by EVs derived from glioblastoma stem-like cells, increasing vascular permeability *in vivo* and the angiogenic potential of human brain endothelial cells (Treps et al., 2017). In addition, nucleoside diphosphate kinase B enriched in EVs derived from triple negative breast cancer cells (MDA-MB-231) enhances pulmonary blood vessel leakage and experimental lung metastasis (Duan et al., 2021).

Extracellular Matrix Remodeling

Extracellular matrix remodeling, a key event in PMN formation, is characterized by the deposition of new ECM components and the expression of enzymes related to ECM modification. The remodeled ECM provides substrates for incoming cancer cells and increases matrix stiffness, which affects the properties of cancer cells (Erler et al., 2009; Cox et al., 2013; Wu et al., 2021). Several ECM components are involved in PMN formation, including fibronectin (Murgai et al., 2017), tenascin-C (Urooj et al., 2020), periostin (Malanchi et al., 2011; Fukuda et al., 2015; Wang et al., 2016), and versican (Kim et al., 2009; Gao et al., 2012). Fibronectin is reported to be upregulated in the livers of mice treated with exosomes from highly metastatic pancreatic cancer cells (Yu et al., 2017). Macrophage migration inhibitory factor (MIF) is highly expressed in pancreatic cancer cell-derived exosomes. Uptake of exosomal MIF by liver Kupffer cells causes transforming growth factor-β (TGF-β) secretion, leading to the activation of hepatic stellate cells. Fibronectin production by the activated hepatic stellate cells promotes the infiltration of bone marrow-derived macrophages and neutrophils in the liver, leading to the formation of the PMN (Costa-Silva et al., 2015). Insulin-like growth factor 2 mRNA-binding protein 1 is rich in melanoma cell EVs and promotes PMN formation in the lungs through the deposition of fibronectin and accumulation of $CD45^+$ cells (Ghoshal et al., 2019). Exosomes from mutant p53-expressing pancreatic ductal adenocarcinoma cells affect the deposition and remodeling of the ECM by fibroblasts to generate a microenvironment highly supportive of tumor cell migration and invasion (Novo et al., 2018). Proteolytic enzymes play a critical role in ECM remodeling during PMN formation. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that target ECM proteins, and MMP induction is one of the hallmarks of PMN formation. Deep et al. (2020) found that exosomes secreted by prostate cancer cells under hypoxia increase MMP activity and upregulate the expression of MMP-2, MMP-9, fibronectin, and collagen IV in the lung, liver, kidney, and spleen. A growing number of EV-associated miRNA have been involved in the regulation of ECM in regional sites. EV-associated miR-494 and miR-542-3p in metastatic rat adenocarcinoma cells regulate tumor-draining LNs and lung tissue by upregulating MMPs to form the PMN (Rana et al., 2013).

Stromal ECM proteins are mainly produced by fibroblasts. ECM remodeling by activated fibroblasts in the PMN has been reported by several groups. Wu et al. (2020) demonstrated that EVs from nasopharyngeal carcinoma package latent membrane protein 1 and activate the conversion of normal fibroblasts into carcinoma-associated fibroblasts (CAFs), thus, increasing the levels of typical PMN biomarkers, including fibronectin, S100A8, and VEGFR1 in lung and liver tissues. Ji et al. (2020) reported that colorectal cancer cells release integrin beta-like 1 (ITGBL1)-rich EVs that activate fibroblasts through the EVs-ITGBL1-CAFs-TNFAIP3-NF-κB axis in the liver and lung; the activated fibroblasts promote PMN formation by secreting pro-inflammatory factors. Fang et al. (2018) found that the highly metastatic hepatocellular carcinoma cell-derived exosomal miR-1247-3p induces the activation of normal fibroblasts into CAFs, which secrete inflammatory cytokines such as IL-6 and IL-8 to promote PMN formation in the lung, thus, promoting lung metastasis of liver cancer. Mazumdar et al. (2020b) provided strong evidence that osteosarcoma cell-derived EVs can activate fibroblasts into CAF phenotypes in the lung PMN through EV-associated TGF-β1 and SMAD2 pathway activation.

Bone Marrow-Derived Cell Recruitment

One mechanism by which tumor factors promote PMN formation is by mobilizing BMDCs to establish a suitable environment in specific secondary organs. The proto-oncoprotein MET is a receptor tyrosine kinase involved in cancer cell growth and invasion. Exosomes from highly metastatic melanomas reprogram BMDCs toward a pro-vasculogenic phenotype by transferring EV-associated MET, and thus, increasing the metastatic behavior of primary tumors (Peinado et al., 2012). Osteosarcoma-derived EVs can increase $CD11b^+$ myeloid cell infiltration in the lungs (Mazumdar et al., 2020a). In pancreatic cancer, highly metastatic pancreatic cancer cell-derived exosomes recruit $CD11b^+$ and $CD45^+$ hematopoietic progenitor cells at the PMN (Yu et al., 2017). An opposite example of tumor EVs promoting PMN formation is the inhibition of lung metastasis in melanoma cells with low metastatic potential (Plebanek et al., 2017). These low-metastatic tumor cell-derived exosomes can amplify $Ly6C^{low}$ patrolling monocytes in the bone marrow and trigger a wide range of innate immune responses. The pigment epithelial-derived factor on the external surface of exosomes plays a critical role in the process.

Angiogenesis

Angiogenesis is a prominent characteristic of the PMN. It was demonstrated that treating immunodeficient mice with epiregulin-enriched exosomes derived from salivary adenoid cystic carcinoma greatly enhanced tumor metastasis to the lung. Epiregulin-enriched exosomes upregulate the expression of VEGF, FGF-2, IL-8, and VEGFR1 in lung vascular endothelial cells, thus, contributing to angiogenesis (Yang et al., 2017).

Human renal cancer stem cells promote angiogenesis and the formation of a PMN in the lung; the process involves the induction of pro-angiogenic mRNAs and miRNAs by $CD105^+$ microvesicles in the whole organ (Grange et al., 2011). Nidogen 1 in metastatic hepatocellular carcinoma cell-derived EVs is reported to promote PMN formation in the lung by enhancing angiogenesis and pulmonary endothelial permeability to facilitate colonization of tumor cells. EV-associated nidogen 1 activates fibroblasts to secrete tumor necrosis factor receptor 1, thereby facilitating lung colonization of tumor cells (Mao et al., 2020). Annexin II is one of the most highly expressed proteins in exosomes. Maji et al. (2017) showed that exosomal Annexin II generates a PMN to facilitate breast cancer metastasis in distant organs by promoting tissue plasminogen activator-dependent angiogenesis.

Immunosuppression

The PMN is an immunosuppressive microenvironment comprising T cells, natural killer (NK) cells, neutrophils, monocytes, and macrophages (Seubert et al., 2015; Patel et al., 2018). Wen et al. (2016) reported that exosomes derived from highly metastatic murine breast cancer cells are distributed predominantly to the lungs of mice, where they suppress T-cell proliferation and inhibit NK cell cytotoxicity, likely suppressing the anticancer immune response in premetastatic organs. EVs isolated from pancreatic cancer induce a dysfunctional phenotype in NK cells, which contributes to an immunosuppressive microenvironment in the liver, and ultimately results in PMN formation. The study provided evidence that pancreatic cancer-derived EVs induce Smad2/3 phosphorylation and downregulate NKG2D in NK cells by delivering TGF-β1 to NK cells, which contributes to PMN formation in liver (Zhao et al., 2019). It is demonstrated that lung alveolar epithelial cells are stimulated by tumor exosomal RNAs via Toll-like receptor 3, which triggers neutrophil recruitment and lung metastatic niche formation (Liu et al., 2016). EVs secreted by brain metastases cells cause low-density lipoprotein aggregation, which accelerates EVs uptake by monocytes and macrophages. These monocytes and macrophages secrete immunosuppressive factors such as interleukin 10, chemokine ligand 2, and TGF-β, which contribute to PMN formation (Busatto et al., 2020). Maus et al. (2019) investigated lymphatic EVs of melanoma patients and showed that EVs traffic from the primary tumor microenvironment to the sentinel LNs and regulate the immune microenvironment in LNs, thereby promoting PMN formation.

Others

Altered glucose metabolism, a hallmark of cancer, is characterized by increased glycolysis and glucose uptake. Fong et al. (2015) demonstrated that EV-associated microRNA-122 secreted by breast cancer cells can be transferred to normal cells (lung fibroblasts, brain astrocytes, and neurons) in the PMN, leading to reduced glucose uptake in these cells. Thereby, the niche accommodates a massive energy for cancer cell metastatic growth by suppressing the nutrient utilization in other cell types.

PRE-METASTATIC NICHE FORMATION IN DIFFERENT ORGANS

Lung

Our understanding of PMN biology is mostly based on studies of lung metastasis. Tumor EVs can target lung endothelial cells and cause vascular leakage and angiogenesis at pre-metastatic sites (Grange et al., 2011; Peinado et al., 2012; Zhou et al., 2014; Maji et al., 2017; Yang et al., 2017; Zeng et al., 2018; Mao et al., 2020). Lung fibroblasts are another common target of tumor EVs at PMN sites. Tumor EVs induce lung fibroblast reprogramming, by which fibroblasts are activated and differentiate into myofibroblast/CAF phenotypes, resulting in ECM remodeling, angiogenesis, secretion of pro-inflammatory cytokines, and immunosuppression (Fang et al., 2018; Novo et al., 2018; Ji et al., 2020; Mazumdar et al., 2020b; Wu et al., 2020). Alveolar epithelial cells released from tumor EVs secrete chemokines and neutrophils in the lungs to promote the formation of the microenvironment before lung metastasis (Liu et al., 2016). In addition, BMDCs ($CD45^+$) from tumor exosomes in the lung inhibit the proliferation of T cells and the cytotoxicity of NK cells to form an immunosuppressive microenvironment (Wen et al., 2016). Overall, EVs target lung endothelial cells, fibroblasts, and alveolar epithelial cells to construct an inflammatory and immunosuppressive niche for the colonization of circulating tumor cells.

Liver

Tumor EVs can be internalized by Kupffer cells (F4/80 positive) and hepatic stellate cells (alpha smooth muscle actin and desmin positive) in the liver and activate downstream signaling pathways. Costa-Silva et al. showed that macrophage MIF is highly expressed in pancreatic cancer-derived exosomes. Uptake of these exosomes by liver Kupffer cells causes TGF-β secretion, leading to activation of hepatic stellate cells. Fibronectin production by activated hepatic stellate cells promotes the infiltration of bone marrow-derived macrophages and neutrophils in the liver, leading to the formation of the PMN (Costa-Silva et al., 2015). In gastric cancer cells, epidermal growth factor receptor-containing exosomes target Kupffer cells and hepatic stellate cells to favor the development of a liver-like microenvironment, thereby promoting liver-specific metastasis (Zhang et al., 2017). In addition, cancer-derived EVs may target NK cells in the liver. Zhao et al. (2019) demonstrated that pancreatic cancer-derived EVs induce a dysfunctional phenotype of NK cells, which contributes to an immunosuppressive microenvironment in the liver, and ultimately results in PMN formation. These studies indicate that EVs establish a fibrotic and immunosuppressive liver PMN mainly through Kupffer cells and hepatic stellate cells, but not hepatocytes. It suggests that targeting circulation EVs and/or the fibrotic niche may provide an early prevention and therapeutic intervention for liver metastasis.

Brain

Disruption of the blood-brain barrier is related to metastatic initiation and progression. Treps et al. (2017) reported

that VEGF-A is carried by EVs derived from glioblastoma stem-like cells, and VEGF-A-enriched EVs promote PMN formation in the brain by targeting endothelial cells and increasing vascular permeability. In the same study, they demonstrated that EV-associated VEGF-A exerts pro-angiogenic activity on brain endothelial cells to stimulate angiogenesis. In addition, EVs may target non-endothelial cells in the brain to promote cancer brain metastasis. Busatto et al. revealed that brain metastasis cell-derived EVs interact with low-density lipoprotein and accelerate EV uptake by monocytes. These monocytes are key components in the brain niche and secrete immunosuppressive factors, such as interleukin 10, chemokine ligand 2, and TGF-β, which contribute to PMN formation (Busatto et al., 2020).

Bone Marrow

Bone marrow myeloid cells and osteoblasts may contribute to PMN formation in the bone marrow. Henrich et al. characterized EV-mediated communication between prostate cancer cells and bone marrow myeloid cells, and demonstrated that cholesterol homeostasis in bone marrow myeloid cells regulates pro-metastatic EV signaling and metastasis. The phospholipase D (PLD) isoforms PLD1/2 regulate tumor progression and metastasis by catalyzing the hydrolysis of phosphatidylcholine to yield phosphatidic acid (Henrich et al., 2020). Borel et al. (2020) demonstrated that PLD2 is present in EVs of prostate cancer cells and activates proliferation and differentiation of osteoblasts by stimulating ERK 1/2 phosphorylation, leading to the formation of a microenvironment before bone metastasis. By contrast, Valencia et al. reported that lung cancer cells release miR-192-enriched EVs, which target endothelial cells and show antimetastatic activity *in vivo*. EV-associated miR-192 is internalized by endothelial cells and inhibits the expression of proangiogenic factors including IL-8, ICAM, and CXCL1 to reduce metastatic colonization (Valencia et al., 2014). Bone marrow is a complex microenvironment and contains multiple cell types such as osteoblasts, osteoclasts, myeloid cells, fibroblasts, macrophages, adipocytes, and endothelial cells. More studies are required to elucidate how these cells cooperate in PMN formation in the bone marrow.

Lymph Nodes

Metastasis to LNs is common in various cancers. Melanoma-derived exosomes home to sentinel LNs and can convert a remote LN into a PMN before tumor cell colonization by inducing the expression of factors responsible for cell recruitment, matrix remodeling, and angiogenesis (Hood et al., 2011). Maus et al. (2019) investigated lymphatic EVs of melanoma patients and showed that EVs traffic from the primary tumor microenvironment to the sentinel LNs, where they regulate the immune microenvironment, thereby initiating PMN formation. However, these studies did not identify the definite target cells of tumor EVs in LNs.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Studies show that various cancers can promote PMN formation in the lung, liver, brain, bone, and LNs by targeting endothelial cells, fibroblasts, alveolar epithelial cells, Kupffer cells, hepatic stellate cells, monocytes, and macrophages in these organs. Tumor EVs play a critical role in the communication between the primary tumor and distant organs by precisely delivering tumor products to target cells. Thus, tumor EVs in circulation should be a potential target of liquid biopsy to predict metastasis. Blocking the delivery of tumor EVs to target cells may prevent cancer metastasis. However, the biomarkers of tumor EVs that promote PMN formation have not been fully identified, and the mechanisms underlying the uptake of tumor EVs by target cells remain unclear. Except tumor cell-derived EVs, our group demonstrated that EVs secreted by CAFs can promote PMN formation in the lung by activating lung fibroblasts (Kong et al., 2019). Further study is necessary to determine whether EVs from other stromal cells in the primary tumor can induce PMN formation in more distant organs. Potential future studies in the field may focus on the following: (1) the different roles of EVs secreted by tumor and stromal cells in PMN formation; (2) the biomarkers of tumor or stromal EVs that promote PMN formation in various cancers; (3) the mechanisms by which recipient cells take up tumor or stromal EVs. The identification of PMN characteristics and a better understanding of the roles of EVs in PMN formation in various organs may help prevent cancer metastasis at an early stage.

AUTHOR CONTRIBUTIONS

QD, XL, KC, JS, and JK collected the literatures. TL and QD prepared the manuscript. All authors contributed to the article and approved the submitted version.

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miR-371b-5p-Engineered Exosomes Enhances Tumor Inhibitory Effect

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Background: Exosomes are well-known natural nanovesicles, that represent one of the recently discovered modes of intercellular communication due to their ability to transmit cellular components. Exosomes have been reported to have potential as natural vectors for carrying functional small RNAs and delivering chemotherapeutic agents to diseased cells. In this study, we aimed to investigate the role of exosomes in carrying miRNA for targeting tumor cells.

Methods: We present a novel method for engineering exosomes with functional miR-317b-5b to target tumor cells. MiR-317b-5b exerts its anti-tumor function *via* its expression in tumors. RT-qPCR was performed to assess the levels of miR-371b-5p, FUT-4. Western blot was performed to measure the levels of CD9, CD81, and FUT-4 proteins. Confocal microscopy was used to observe the internalization of miR-317b-5b in tumor cells. CCK-8, EdU, flow cytometry, wound-healing migration and transwell assays were performed to evaluate cell viability, proliferation, migration, and invasion, respectively.

Results: Our findings illustrated that miR-317b-5b-loaded engineered exosomes were internalized by tumor cells. MiR-317b-5b was overexpressed in tumor cells treated with miR-317b-5b-loaded engineered exosomes. The internalization of miR-317b-5b in tumor cells was accompanied by changes of cell viability, proliferation, apoptosis, and migratory and invasive capability. We found that miR-317b-5b-loaded engineered exosomes were presence in tumor tissue sections and miR-317b-5b was overexpressed in tumor tissues of osteosarcoma tumor-bearing mice infected with miR-317b-5b-loaded engineered exosomes. MiR-317b-5b-loaded engineered exosomes had the anti-tumor efficiency *in vivo*.

Conclusion: Our findings show that miR-317b-5b-loaded engineered exosomes can be used as nanocarriers to deliver drug molecules such as miR-317b-5b both *in vitro* and *in vivo* to exert its anti-tumor functions.

Keywords: engineered exosomes, miR-317b-5p, tumor cells, proliferation, migration, xenograft

INTRODUCTION

Extracellular vesicles (EVs), that contain exosomes, microbubbles and apoptotic bodies are produced by various cell types including mesenchymal stem cells, endothelial, epithelial, and tumor cells (Sutaria et al., 2017). Exosomes contain various macromolecular substances with the size of approximately 30–150 nm, and these macromolecules mediate local or systemic intercellular communications (El Baradie et al., 2020).

In recent years, studies on exosomes have developed into many practical applications, including biomarkers of diseases and therapeutic responses, and drug carriers (Marcus and Leonard, 2013; Hong et al., 2014; Batrakova and Kim, 2015). Different from synthetic liposomes or nanoparticles, exosomes composed of natural ingredients that could avoid clearance in blood circulation (Lundqvist et al., 2008; Kopac, 2021). The exosomes transport their cargos to recipient cells by adhesion proteins or direct fusion with the plasma membrane. At present, exosomes are explored to be used as drug carriers for siRNA, miRNA simulators, miRNA inhibitors, mRNAs, and proteins expressed by plasmid DNAs (Mulcahy et al., 2014).

The main challenge of gene therapy is to develop non-toxic molecular transporters, that can effectively deliver functional copies of therapeutic genes to target cells (Niidome and Huang, 2002). Exosomes can transport mRNA, miRNAs, and proteins to remote target cells *via* the specific interaction between proteins on exosome membrane and receptor molecules on target cells (Fuhrmann et al., 2015). Hence, the construction of engineered exosomes has become one of the research hotspots of gene therapy. Previous studies have shown that the molecular cargo delivered by engineered exosomes to target cells can affect the pathophysiological processes of target cells or tissues, including cell biological activity, tumor growth, and tissue repair (Gidlöf et al., 2013; Tian T. et al., 2014; Qiu et al., 2018). In addition, the presence of specific genetic information in exosomes derived from tumor cells provides an opportunity for cancer detection or monitoring the effectiveness of cancer therapy (Ghai and Wang, 2016).

Studies have shown that most miRNAs are differentially expressed during tumorigenesis (Calin and Croce, 2006). MiRNAs are promising therapeutic targets because they could regulate an entire signaling pathway rather than a single protein. If they are overexpressed in tumors, they are easily inhibited by anti-miRNAs. If they are under expressed, they may be supplemented by miRNA mimics (Henry et al., 2010). Furthermore, MiRNAs can be used as molecular therapy for a variety of diseases, particularly cancers (O'Connor et al., 2016). Most therapeutic applications of miRNA require packaging nucleic acids in vectors or nanocarriers. At present, the packaging of miRNAs into exosomes has been widely studied (Rincon et al., 2015; Nie et al., 2020). It was known that MiR-317b-5p is involved in tumor cell proliferation, migration, and invasion and is differentially expressed in various cancers, including non-small cell lung cancer, chondrosarcoma, and bladder cancer (Mutlu et al., 2015; Li et al., 2020; Luo et al., 2020).

In this study, miR-317b-5b-loaded engineered exosomes were constructed to explore the effect of miR-371b-5p in tumor pathological behaviors. We developed an active delivery method that uses the interaction of HIV-1 TAR RNA-TAT peptides to exchange pre-miR-317b-5p rings with TAR RNA rings. The pre-miR-317b-5p, modified with LAMP2A fusion protein, and identifies TAT peptides in electric vesicles. Using this TAT-TAR interaction, the load of miR-317b-5p to the outer cut can be enhanced (Sutaria et al., 2017; Liang et al., 2018).

MATERIALS AND METHODS

Mice

Female BALB/c mice, 4 to 8 weeks old, purchased from Shanghai Sippr-BK Experimental Animals Co., Ltd., (Shanghai, China). The mice were kept in cages in airy rooms with free access to food and water. The animal experiments are conducted in accordance with national-specific ethical standards for biomedical research and approved by the animal ethics committees of Nantong University.

Cell Culture

The 143B, HeLa, and A549 tumor cells were provided by Procell Life Science and Technology Co., Ltd., (Wuhan, China). The cells were cultured in Minimum Essential Medium (MEM, Roche, Basel, Switzerland) containing 1% penicillin Streptomycin Solution and 10% FBS (Solarbio, Beijing, China). All cells were cultured in a 37°C humidified incubator containing 5% CO_2.

Construction of Plasmids

Supplementary Figure 1 summarizes the process by which miR-317b-5p is incorporated into the HEK293T exosome. The pEGFP-1 carrier expressing Lamp2b is provided by Procell Life Technology Co., Ltd., (Wuhan, China). The LAMP2A was cloned into the carrier skeleton using *Nhe*I and *Bam*HI enzyme inching points. Four peptide sequences were introduced into the LAMP2A sequence: 3X Flag peptides for exosome purification, PC94 peptides for liver cancer targeting, TAT peptides combined with modified TAR, and His tag for verifying the protein is translated within the framework. Because HEK293T cells are resistant to neomycin, a methromycin box is introduced downstream of the SV40 initiator. Human miR-317b-5p was inserted into the artificial intron of LAMP2A gene. The miR-317b-5p sequence was cloned into the exon binding site of LAMP2A gene.

Preparation of Exosome

According to the previous research, the engineering exosomes was separated by overspeed centrifugal method (Théry et al., 2006). About 360 mL 293T cell culture (approximately 4×10^8 cells) was centrifuged at 300 *g* for 10 min, moved into a clean test tube and centrifuged again at 2,000 *g* for 20 min. The cell fragments were removed. Subsequently, the fluid was transferred into a clean test tube, centrifuged at 10000 *g* for 30 min, and filtered in a vacuum with a 0.22 M filter (EMD, Billica, MA, United States). Furthermore, the supernatant was transferred into a high-speed centrifuge tube (Beckman Coulter, Braille, CA, United States) and centrifuged at 11,000 *g* for 4 h in the

overspeed centrifuge (Beckman Coulter Optima TM Xe, Billica, MA, United States). The sediment was rinsed once with sterile phosphate buffer (PBS, pH 7.4) and then suspended in a 0.3 to 0.6 mL PBS containing 1% DMSO. The outer cut^{TM} was prepared with APILES. The protein contents of exosomes were measured using the Pierce^{TM} BCA protein kit.

Characterization of Exosomes

The images of exosomes were taken with a transmission electron microscope (TEM). Briefly, the exosome sediment was washed with PBS three times, 10 min each time, and then fixed in PBS containing 2.5% dialdehyde at 4°C. Subsequently, exosomes were dehydrated in increasing concentration of alcohol. The samples were observed under 80kV TEM (JEM-2100; JEOL, Tokyo, Japan). The particle size of exosomes was measured using Zetasizer Nano S (Malvern Instrument, Malvern, United Kingdom). The surface charge was evaluated by measuring the Zeta level in the PBS buffer.

Exosome Labeling and Loading

The exosomes were labeled with the fluorescent probe PKH26 (Invitrogen, Carlsbad, California, United States). The purified exosomes were incubated with 5 μL PKH26 at 37°C for 15 min, and the free probe was removed by centrifugation at 120,000 *g* for 90 min. After two washing and centrifugation cycles, the labeled exosomes were suspended in PBS and used for cell studies.

The exosomes with a total protein concentration of 10 μg/ml were mixed with the 400 nm Cy5 labeled miR-317b-5p in a 1 mL phosphate buffer. The mixture was electrophoresis at 400V, 50 sf, 30 ms pulse/2 s, and suspended for three cycles. After loading miR-317b-5p, it was diluted 10 times with PBS and centrifuged at 110,000× *g* for 70 min. The free miR-317b-5p was removed. RT-PCR was used to detect miR-317b-5p in the exosomes.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA was extracted with TRIZOL reagent (Takara, Kusatsu, Japan), and the concentration of RNA was measured at 260 nm. M-MLV reverse transcriptase (RNase H) kit (Takara, Kusatsu, Japan) was used to synthesize cDNA. RT-qPCR was performed by using SYBR Green PCR Master Mix (Takara, Kusatsu, Japan). GAPDH was used as a negative control for mRNA detection, and U6 was used as a negative control for miRNA detection. The reaction was conducted under the following condition: template denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 60°C for 15 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression. The primer applied to this study were shown in **Table 1**.

Exosome Uptake

HeLa, 143B, and A549 cells (3×10^5) were seeded in a 3.5 cm glass-bottom culture dish and cultured to 70% confluence. Subsequently, cells were washed with PBS and incubated with cell culture medium containing 10^8 particles/mL of exosomes labeled with PKH26 Laser scanning confocal microscope (CLSM) was used to scan fluorescence images to record the fluorescent signals of PKH26 and to process images using the Zen software (CLSM; Zeiss lsm710, Oberkochen, Germany).

TABLE 1 | Primer sequences.

Primer name	(5′-3′)Primer sequences
F-miR-371b-5p	5′-GTGGCACTCAAACTGT-3′
R-miR-371b-5p	5′-CATCTTTTGAGTGTTAC−3′
F-FUT-4	5′-CAGTGGCCCGCTACAAGTTC-3′
R-FUT-4	5′-GCCAGAGCTTCTCGGTGATATAA−3′
F-U6	5′-CTCGCTTCGGCAGCACA−3′
R-U6	5′- AACGCTTCACGAATTTGCGT−3′
F-GAPDH	5′-GAGTCAACGGATTTGGTCGT−3′
R-GAPDH	5′-TTGATTTTGGAGGGATCTCG−3′

Western Blot

Total proteins were separated with cell lysis buffer (Beyotime, Nanjing). The protein was analyzed with a 10% decanyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a TBST sealed polyfluoroethylene (PVDF) film in 5% skimmed milk powder, which was 1 h warmed at room temperature. Anti-FUT-4 antibody (Ab188610), anti-calcitonin antibody (Ab76011), anti-CD81 antibody (Ab79559), and anti-CD9 antibody (Ab92726) were incubated at 4°C overnight. β-actin (AB8226) was used as the internal control. Subsequently, membranes were incubated 2 h at room temperature with a secondary antibody (1:2,000). All antibodies were purchased from Abcam (Cambridge, United Kingdom, 1:1000). The optical density of protein band was determined by ImageJ software, Inc.

CCK-8 Assay

Cell Counting Kit-8 (Beyotime, Nanjing, China) was used to assess the viability of cells. Briefly, the cells were inoculated to 96 well plates (3650, Corning, NY, United States) for 2 h. Subsequently, 10 μL of CCK-8 solution was added to the cell wells, incubated at 37°C for 2 h, and finally, the fluorescent microplate was used to detect the light absorbance reflecting the cell viability at 450 nm.

EdU (5-Ethynyl-2'-deoxyuridine) Assay

After inoculation of 143B cells treated with engineered exosomes on a 24-well plate for 24 h, the EdU media was added. After incubation for 2 h, the culture medium was removed, the cells were digested with trypsin, and then washed twice with 1 × PBS. Cells were fixed with formaldehyde 30 min, decolored with glycine, and washed in PBS for two times. Subsequently, cells were soaked in 0.5% Triton X-100 for 10 min, and then washed twice with 1 × PBS. Finally, the staining was performed using the Cell Light^{TM} EdU Cell Proliferation Assay (Sigma, St. Louis, MO, United States) according to a previously published report (Xiao et al., 2019).

Wound-Healing Migration Assay

The effect of miR-317b-5b engineered exosomes on the migration of 143B cells was quantitatively tested *in vitro*: 3×10^5 143B cells

were inoculated into six well plates and incubated to reach 70% confluence (about 24 h). These cells were treated with engineered exosomes loaded with miR-317b-5b and incubated at 37°C for 4 h. A scratch wound was created using a sterile pipette tip. The cells were incubated in fresh culture medium at 37°C, and images of scraped monolayer cells were recorded at 0 and 48 h.

Transwell Assay

Cell migration was evaluated in transwell chambers with a membrane pore size of 8 μm. 143B cells treated with engineered exosomes were inoculated in the top well of Millicell suspension culture plate (PIEP12R48) in triplicate with a density of 1×10^5. The chamber insert was placed in a 24-well transplate containing DMEM and 30% fetal bovine serum as a chemical inducer. After 24 h incubation, the cells in the upper chamber were taken out with a cotton swab, and the cells on the lower surface of the membrane were fixed with 100% methanol for 15 min and stained with 0.05% crystal violet. Cells were counted at five random sites in each chamber and the mean number of cells was determined.

Flow Cytometry Assay

Fluorescence Activated Cell Sorter (FACs) were performed to evaluate cell apoptosis. In brief, cells were inoculated to 96-well plates (3650; Corning, NY, United States) for 2 h. Heterocyanate fluorin (FITC) and propylene iodide (PI) were added to A549, HeLa, and 143B cells (5 μL/well) and incubated at 37°C for 2 h. The number of apoptotic cells was counted by flow cytometry. Apoptosis is defined as FITC (+) and PI (+).

In vivo Visualization of Intravenously Injected Exosomes

In order to evaluate the biological distribution of miR-317b-5b containing exosomes, female BALB/c mice between 6 and 8 weeks of age were received subcutaneous flank injections of 1×10^6 143B cells tumor cells. Mice with tumors were monitored daily. When the tumor volume reaches 400 mm^3, the 30 mg PKH26 labeled exosomes were injected into the nude mouse and the tumor-free mouse was used as a control. The 200 μL PBS + 5% glucose was injected into the abdominal cavity of the lotus mouse as a background control. Tumor bearing mice were anesthetized with 2.5% isoflurane at 3, 12, 24, and 48 h after administration, and small animal imaging system (Kodak, Rochester, NY, United States) was used. The fluorescent signals of each tissue sample were quantified in the target area drawn with the free hand.

In vivo Anti-tumor Analyses

The 143B cells were injected subcutaneously into the ventral side of female BALB/c mice, and 1×10^6 cells in 100 μL PBS were used. On the 12th day after inoculation, when the tumor was touchable, the mice were divided into four different treatment groups. The treatment group includes PBS, Unload Exo, NC Exo, and miR-317b-5b-Exo (30 μg, iv each). The tumor growth rate was measured with a caliper every 4 days. The survival of mice was monitored for 40 days. The probability of survival in mice was assessed by Kaplan–Meier method, and the survival ratio of each group was compared with the number of rank tests. The mice were sacrificed by cervical dislocation and the tumor was removed for further study.

Tissue Dissection and Fluorescent Microscopy

The tumor tissue and the important organs (lung, liver, kidney, and spleen) were removed, immersed in 5, 10, and 14% sucrose, frozen with an OCT culture base at −80°C, and sliced with a Leica cm1800 cryostat. Frozen tissue slices (8 m thick) were fixed with acetone and the nuclei were dyed with DAPI. Fluorescently labeled exosomes were displayed using the Circle-3 Cell Imaging Multi-Mode Reader (Bio-Tek Instruments, Winooski, VT, United States).

Statistical Analysis

Data were presented as mean ± standard deviation (SD) from three independent experiments. GraphPad Prism 5.0 Software (GraphPad Software, Inc.) was used for statistical analysis. *T*-test or one-way analysis of variance was used for comparison between two or more groups, and Tukey post-test was used for comparison within multiple groups. When $P < 0.05$, the difference was statistically significant.

RESULTS

Isolation and Identification of Engineered Exosomes

Transmission electron microscopy (TEM) analysis showed the disk-like appearance of the engineered exosomes with the average size between 30 and 150 nm (**Figure 1A**). Subsequently, the purified exosomes were characterized by dynamic light scattering (DLS). DLS analysis showed that the average size of the engineered exosomes was 103 nm (**Figure 1B**), indicating that the secreted exosomes were successfully isolated. Furthermore, Western blot analysis showed that CD81, CD9, and other important extracellular markers were expressed in the exosomes ($P < 0.05$; **Figure 1C**). These findings revealed that the engineered exosomes were successfully isolated.

Uptake of Engineered Exosomes by Tumor Cells

Compared with other vectors, one of the unique characteristics of exosomes is that they are simply incorporated into the target cells by co-culture and penetrate the cells by endocytosis or membrane fusion (Liang et al., 2018). To investigate the specificity and efficiency of uptake of engineered exosomes by tumor cells, human osteosarcoma cell 143B, Hela cell and human lung cancer cell A549, were treated with 200 μg/mL engineered exosomes at 37°C for 24 h. The engineered exosomes were labeled with fluorescent probe PKH26. The fluorescence intensity of PKH26-labeled exosomes in tumor cells was monitored by confocal laser scanning microscope (CLSM). CLSM showed that the engineered exosomes labeled with PKH26 successfully entered 143B, Hela, and A549 cells following 24 h of coculture (**Figure 2A**). Flow

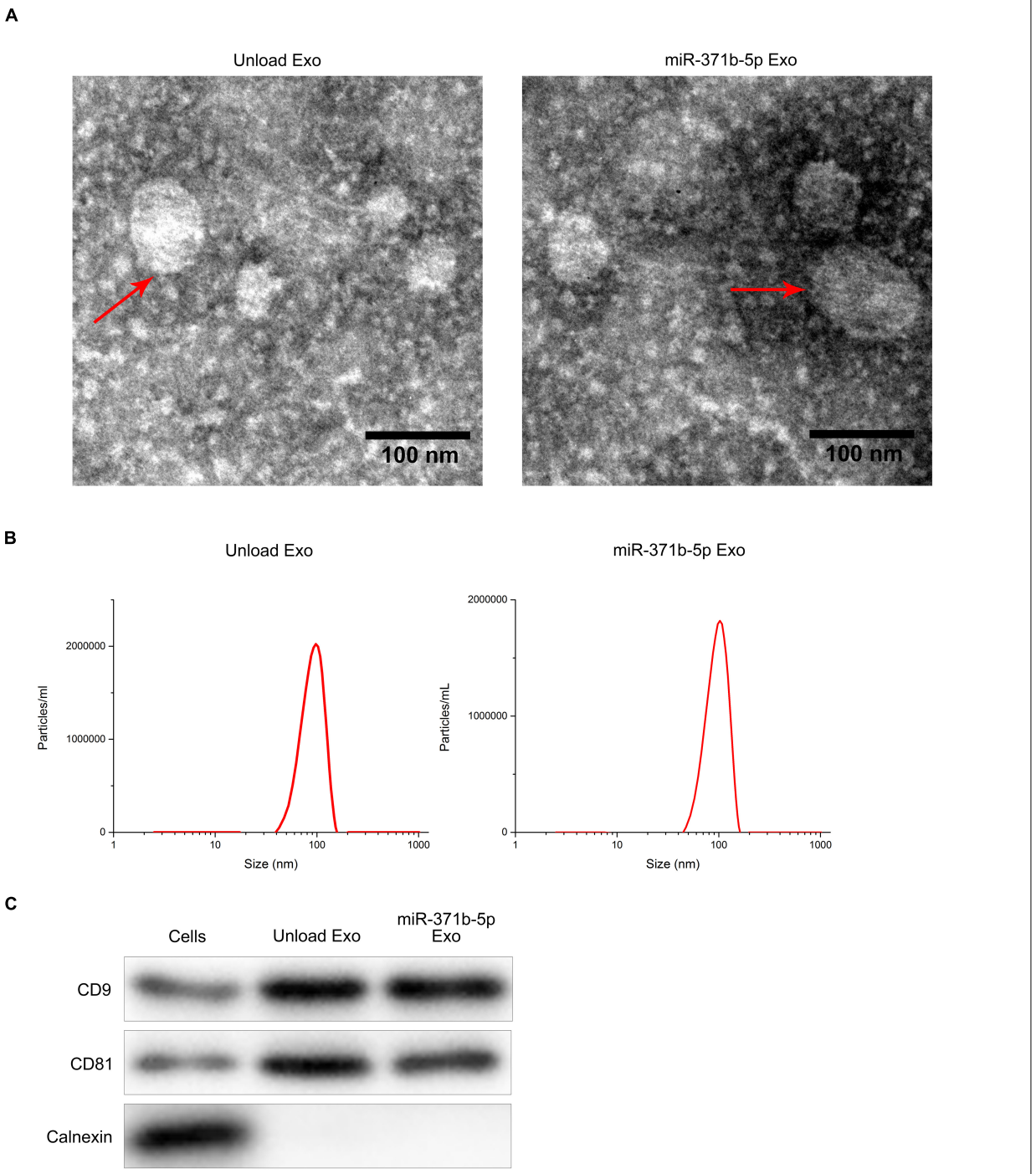

FIGURE 1 | Isolation and identification of engineered exosomes. **(A)** TEM micrographs of engineered exosomes before and after loading with miR-371b-5p. **(B)** DLS assay analyzed particle size of engineered exosomes. **(C)** Western blotting assessed the levels of CD9, CD81, and Calnexin proteins that are the markers of endoplasmic reticulum.

cytometry (FCM) analysis showed that engineered exosomes marked by CD63 were taken into tumor cells, including 143B, Hela, and A549 cells ($P < 0.05$; **Figure 2B**). The uptake rate of PKH-67-labeled engineered exosomes was higher in 143B cells than in other cells. Thus, 143B cells were selected for subsequent functional experiments. To explore the expression of miR-317b-5b in tumor cells, miR-317b-5b level in 143B cells incubated with miR-317b-5b-engineered exosomes was detected by RT-qPCR and Western blot. The results showed that compared with the control, miR-317b-5b level was significantly

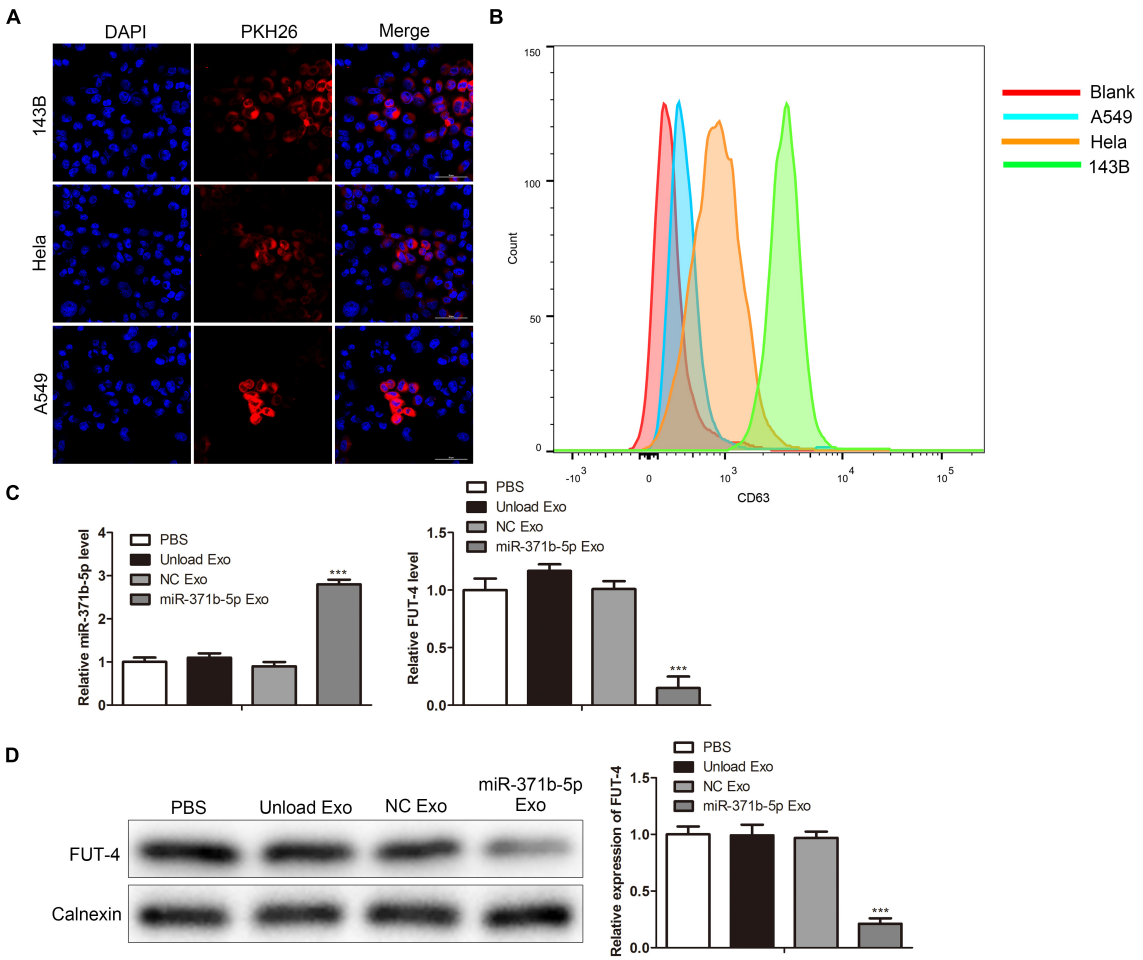

FIGURE 2 | Engineered exosomes internalized by tumor cells. **(A)** Cellular uptake of PKH26-labeled engineered exosomes by 143B, Hela, and A549 cells. **(B)** FCM assay performed to evaluate the ability of tumor cells to uptake engineered exosomes. 143B cells incubated for 6 h with each of the following: PBS, unload exosomes (unload Exo), NC exosomes (NC Exo), and miR-317b-5b-loaded engineered exosomes (miR-317b-5b Exo). **(C)** RT-qPCR assessed the levels of miR-317b-5b and FUT4 in 143B cells incubated with miR-317b-5b-loaded engineered exosomes. **(D)** Western blot measured the level of FUT4 protein in 143B cells incubated with miR-317b-5b-loaded engineered exosomes. ***$P < 0.001$ vs. NC Exo. Error bars represented SD. Data represented three independent experiments.

up-regulated after miR-317b-5b-loaded engineered exosomes were incubated with 143B cells. It has been reported that, FUT-4 is a target of miR-371b-5p (Li et al., 2020). In order to prove the functionality of the engineered exosomes rich in miR-371b-5p FUT-4 expression level was detected. As shown in **Figures 2C,D**, FUT4 level in 143B cells incubated with miR-317b-5b-loaded engineered exosomes was lower than that in the control group. These findings indicated that miR-317b-5b-loaded engineered exosomes could be internalized by 143B, HeLa, and A549 cells. In the 143B cells co-incubated with miR-317b-5b-loaded engineered exosomes, miR-317b-5b level was high.

The Effects of miR-371b-5p Delivered by Engineered Exosomes on Tumor Cell Proliferation and Apoptosis

To further investigate the effects of miR-317b-5b-loaded engineered exosomes on the proliferation and apoptosis of 143B, Hela, and A549 cells, CCK-8 assay was performed on these cells following the incubation with miR-317b-5b-loaded engineered exosomes over 4 days. Findings showed that cell viability of 143B, HeLa, and A549 cells treated with miR-317b-5b-loaded engineered exosomes was significantly lower than that of untreated cells ($P < 0.05$; **Figure 3A**). The reduction of cell viability in 143B cells was more significant than the other cells. Thus, 143B cells were selected for subsequent functional experiments. EdU assay was carried out to assess the cell proliferation of 143B cells following incubation with miR-317b-5b-loaded engineered exosomes over 4 days. It was found that the rate of cell proliferation was significantly down-regulated in miR-317b-5b-loaded engineered exosomes-treated 143B cells compared to untreated cells ($P < 0.05$; **Figure 3B**). On the contrary, FCM analysis indicated that the rate of apoptosis was significantly up-regulated in miR-317b-5b-loaded engineered exosomes-treated 143B cells compared with the control ($P < 0.05$; **Figure 3C**). These findings indicated that the internalization of

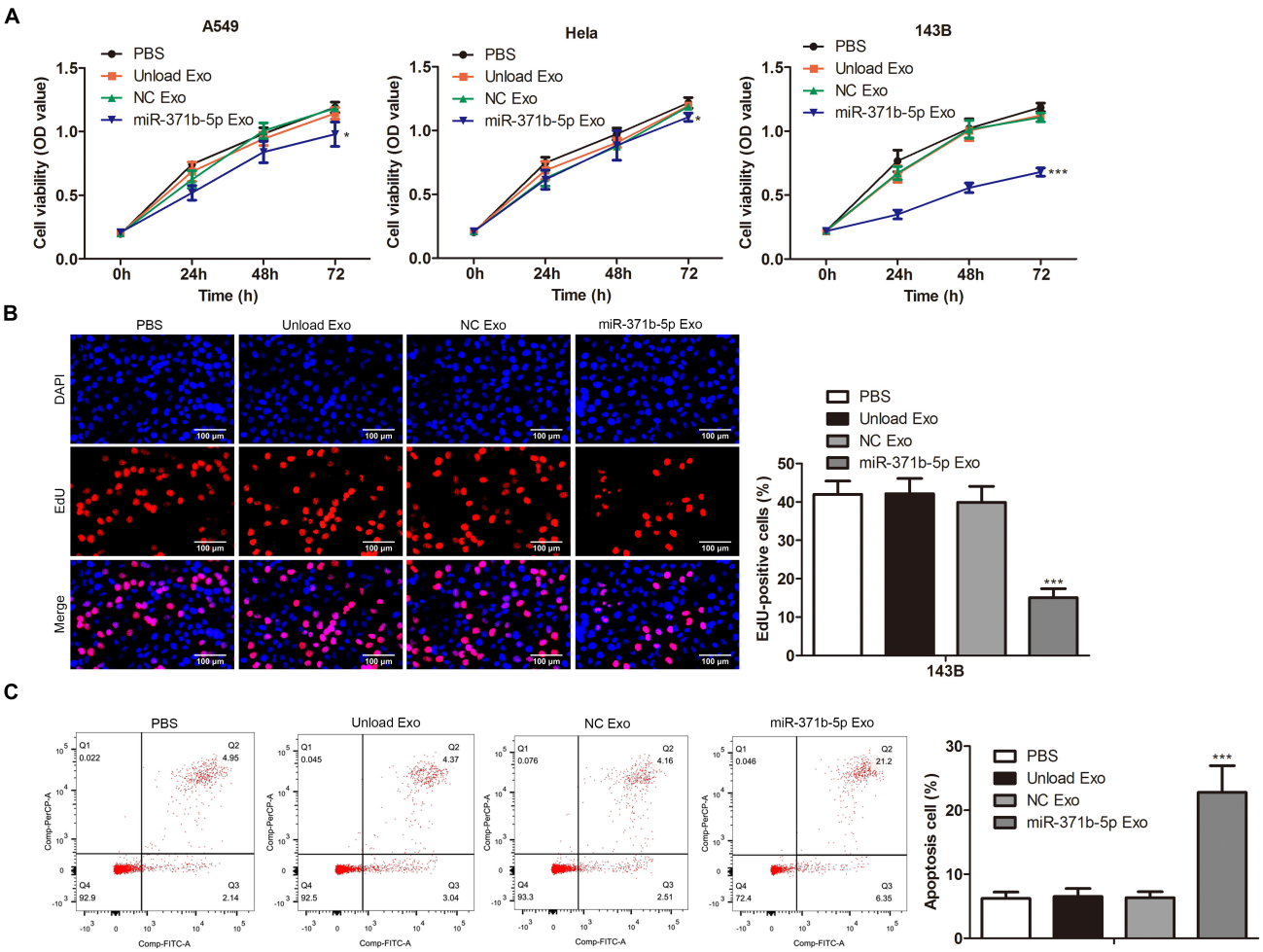

FIGURE 3 | The effects of miR-371b-5p delivered by engineered exosomes on tumor cell proliferation and apoptosis. 143B, Hela, and A549 cells incubated with each of the following for 6 h: PBS, Unload Exo, NC Exo, and miR-317b-5b Exo. **(A)** CCK-8 assessed cell viability. **(B)** EdU assay on cell proliferation. **(C)** FCM evaluated cell apoptosis. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. NC Exo. Error bars represented SD. Data represented three independent experiments.

miR-317b-5b is accompanied by specific changes in cell biological behaviors including cell viability, proliferation, and apoptosis in 143B, HeLa, and A549 cells.

The Effects of miR-371b-5p Delivered by Engineered Exosomes on Tumor Cell Invasion and Migration

Next, we investigated the effects of miR-317b-5b-loaded engineered exosomes by 143B cells on cell wound-healing response, invasion, and migration. Findings showed that the wound-like gaps in 143B cells of PBS, unload Exo and NC Exo groups had almost healed completely. However, compared with the control, the wound closure of 143B cells treated with miR-317b-5b-loaded engineered exosomes was significantly delayed ($P < 0.05$; **Figure 4A**). Similarly, transwell analysis indicated that there were significant reduction of cell invasion and migration in miR-317b-5b-loaded engineered exosomes-treated 143B cells compared to the control cells ($P < 0.05$; **Figure 4B**). These results suggested that the internalization of miR-317b-5b in 143B cells resulted in changes in cell invasion and migration.

Visualization of Fluorescently Labeled Engineered Exosomes in Tumor-Bearing Mice *in vivo*

Furthermore, we evaluated the biodistribution and tumor penetration of miR-317b-5b-loaded engineered exosomes in mice with osteosarcoma. To observe the presence of miR-317b-5b engineered exosomes in tumors and tissues such as heart, liver, spleen, lung, and kidney, 10^6 143B cells were injected into female BALB/c mice aged 6 to 8 weeks. Mice with osteosarcoma were monitored daily. When the tumor volume reached 400mm^3, the tumor-bearing mice were intravenously injected with 30 μg engineered exosome or 200 μL PBS containing 5% glucose as the negative control. Fluorescence intensity of engineered exosomes labeled with PKH26 was monitored from 3 to 48 h after injection. Findings indicated that

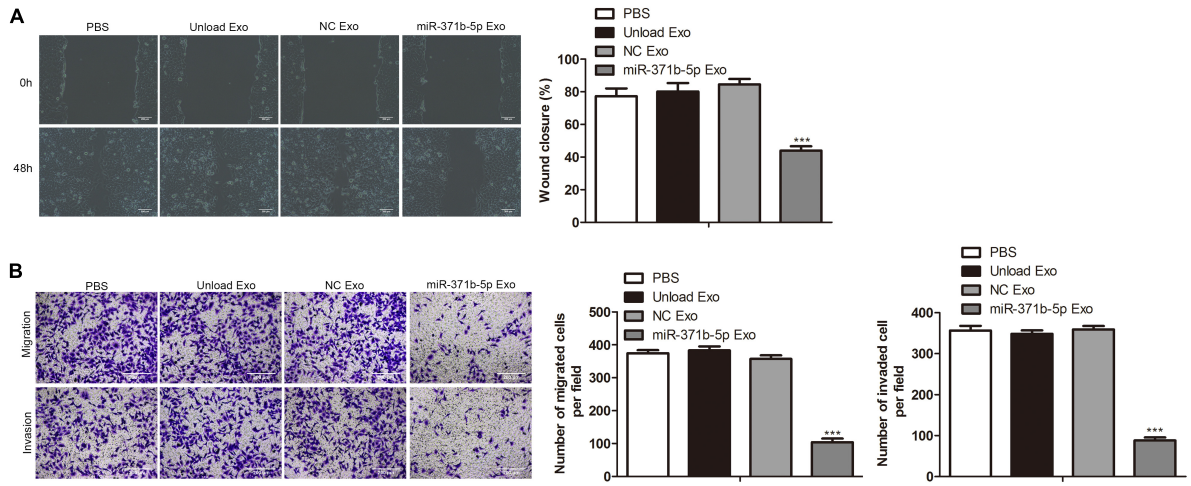

FIGURE 4 | The effects of miR-371b-5p delivered by engineered exosomes on tumor cell invasion and migration. 143B cells were incubated for 6 h with each of the following: PBS, Unload Exo, NC Exo, and miR-317b-5b Exo. **(A)** Wound-healing assay assessed the effect of miR-317b-5b-loaded engineered exosomes on 143B cells migration. **(B)** Transwell assay on the effect of miR-317b-5b-loaded engineered exosomes on 143B cells migration and invasion. ***$P < 0.001$ vs. NC Exo. Error bars represented SD. Data represented three independent experiments.

compared with the control, PKH26-labeled and miR-317b-5b-loaded engineered exosomes were easily observed in different tissues and tumor sites of tumor bearing mice (**Figure 5A**). Subsequently, all mice, including control mice, were sacrificed at 0, 3, 12, 24, and 48 h after intravenous administration of labeled engineered exosomes or PBS, and fluorescence signals were immediately observed from fresh dissected tissues using the Interactive Video Information System (IVIS). Results showed that the fluorescent signal from PKH26-labeled and miR-317b-5b-loaded engineered exosomes were easily observed in tumor tissues and most tissues, including liver, spleen, and kidney. The fluorescent signal from PKH26-labeled and miR-317b-5b-loaded engineered exosomes in tumor tissues was the strongest ($P < 0.05$; **Figure 5B**). These results demonstrated that miR-317b-5b-loaded engineered exosomes were preferentially accumulated in tumor tissues.

Anti-tumor Efficiency of Engineered Exosomes *in vivo*

The antitumor effect of miR-317b-5b delivered by engineered exosomes was evaluated in the osteosarcoma mouse model. Tumor growth analysis of osteosarcoma showed that miR-317b-5b-loaded engineered exosomes inhibited tumor growth. The unload Exo and NC Exo had the same effect on tumor growth rate. Compared with PBS treated mice, tumor volume was significantly reduced in miR-317b-5b-loaded engineered exosomes-treated mice ($P < 0.05$; **Figure 6A**). Furthermore, survival analysis showed that the mean survival time of osteosarcoma-bearing mice treated with miR-317b-5b-loaded engineered exosomes was significantly higher than that of mice treated with PBS or Exo-unloaded and NC Exo ($P < 0.05$; **Figure 6B**). These findings revealed that miR-317b-5b-loaded engineered exosomes had the anti-tumor efficiency *in vivo*.

Quantification of miR-317b-5b *in vivo*

To explore the levels of miR-317b-5p in osteosarcoma tumor-bearing mice following injection with miR-317b-5b-loaded engineered exosomes, RT-qPCR and Western blot were performed to quantify the levels of miR-317b-5b and FUT4 in tumor tissues. The results showed that miR-317b-5b expression was significantly up-regulated in tumor tissues that had been injected with miR-317b-5b-loaded engineered exosomes compared with the control. On the contrary, FUT4 expression in tumor tissues that had been infected with miR-317b-5b-loaded engineered exosomes was lower than that in control group ($P < 0.05$; **Figures 7A,B**). These findings revealed that miR-317b-5b was overexpressed in tumor tissues of osteosarcoma tumor-bearing mice injected with miR-317b-5b-loaded engineered exosomes.

DISCUSSION

Exosomes are natural vehicles for the exchange of macromolecular goods and information among cells in the human body (Tkach and Théry, 2016). Proteins on exosomes may bind to target receptors on target cells, where they are internalized through receptor-mediated endocytosis (Malhotra et al., 2016). Compared with artificial nanoparticles, exosomes circulate in the body for much longer time, increasing the chance of encountering distant target cells. These favorable properties promote researchers to explore engineering exosomes to deliver exogenous nucleic acids and therapeutic drugs to diseased cells, particularly tumor cells (Tian Y. et al., 2014; Choi et al., 2016). In this study, we designed exosomes as *in vivo* vectors for tumor-targeted delivery and uptake of miRNAs therapeutic agents to tumor cells. Findings showed that miR-317b-5p was efficiently delivered to target tumor cells, leading to specific changes in

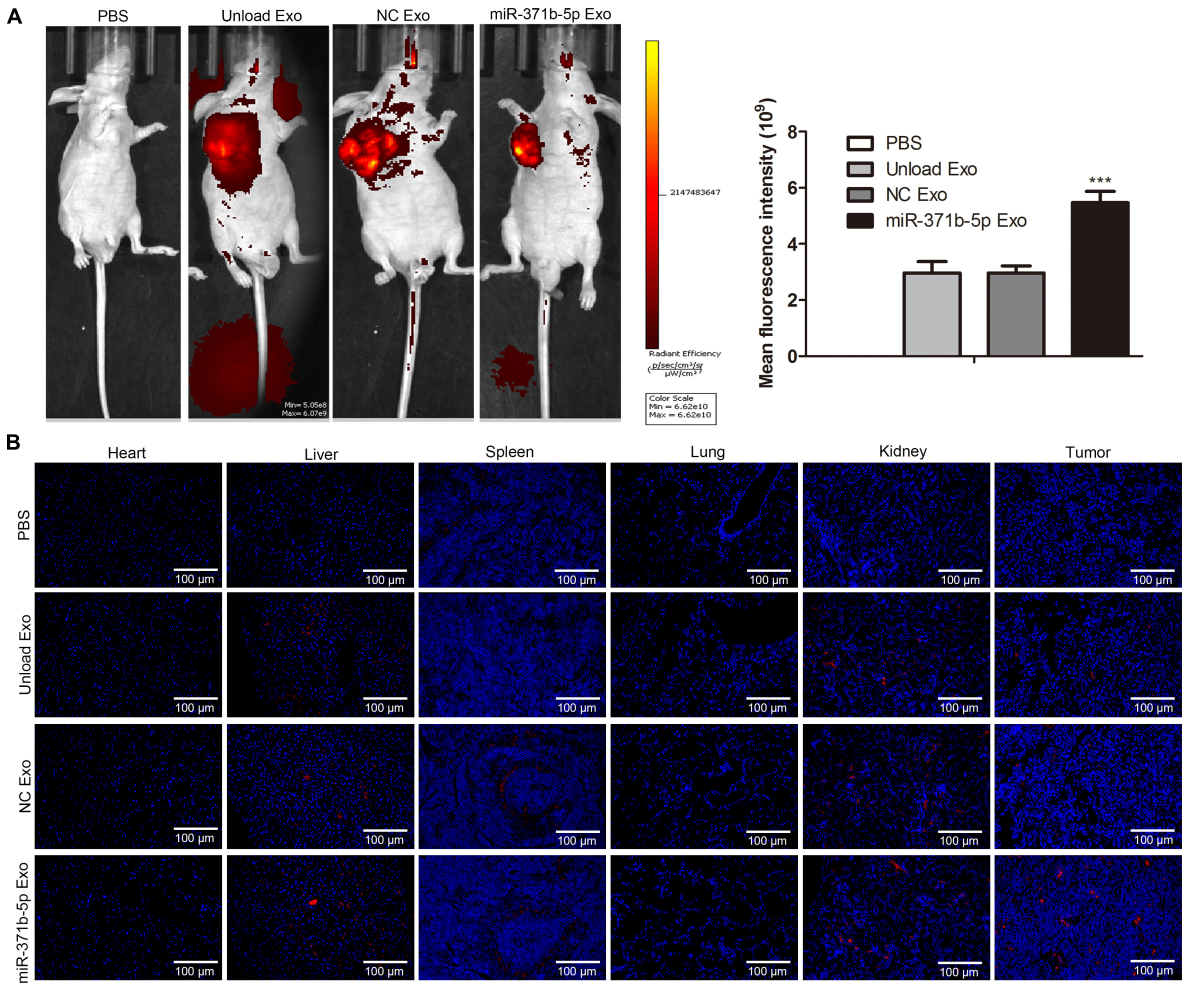

FIGURE 5 | Visualization of fluorescently labeled engineered exosomes in tumor-bearing mice *in vivo*. BALB/c tumor bearing mice injected with each of the following: PBS (200 μL), unload exosomes (unload Exo; 30 μg), NC exosomes (NC Exo; 30 μg), and miR-317b-5b-loaded engineered exosomes (miR-317b-5b Exo; 30 μg). **(A)** *In vivo* miR-317b-5b-loaded engineered exosomes biodistribution in tumor-bearing mice by animal imaging. **(B)** miR-317b-5b-loaded engineered exosomes labeled with PKH26 were intravenously injected into mice bearing osteosarcoma tumors. Lung, liver, spleen, kidney, and tumor tissues were harvested after 3, 12, 24, and 48 h post injection for *ex vivo* imaging. The fluorescent signal of PKH26-labeled miR-317b-5b-loaded engineered exosomes detected using IVIS. ***$P < 0.001$ vs. NC Exo. Error bars represented SD. Data represented three independent experiments.

tumor cell bioactivities. We further developed a simple method to design human cell-derived exosomes to deliver specific and functional therapeutic miRNA to tumor cells.

Exosomes have been recognized as carriers for a variety of nucleic acid therapeutic drugs, including siRNA, plasmid DNA, miRNA mimics, miRNA inhibitors, and mRNAs (Tian T. et al., 2014). In this study, cargo RNA was endogenously loaded into exosomes. After the RNA vector was loaded into the exosomes, the miR-317b-5p precursor sequence was modified. The introduction of TAR RNA/TAT peptide interaction between the LAMP2A fusion protein and the modified pre-miR-317b-5p loop significantly improved the delivery of pre-miR-317b-5p. Namely, we successfully constructed miR-317b-5b-loaded engineered exosomes.

It was reported that engineered exosomes delivering miRNAs play an important role in cancer diagnosis and treatment (Malla et al., 2017; Nie et al., 2020). Our findings showed that miR-317b-5b-loaded engineered exosomes were internalized by tumor cells, including A549, Hela, and 143B cells. The internalization of miR-317b-5b in tumor cells was accompanied by specific changes in cell viability, proliferation, apoptosis, migration, and invasion. Furthermore, the antitumor effect of miR-317b-5b-loaded engineered exosomes was evaluated in osteosarcoma bearing mice. According to the *in vivo* antitumor study, the lowest tumor volume and the longest survival time were observed in mice treated with miR-317b-5b-loaded engineered exosomes, which showed significant antitumor efficiency compared with the unloaded exosomes and NC exosomes. In addition, *in vivo* studies using tumor bearing mice showed that following iv injection, miR-317b-5b-loaded engineered exosomes mediated the effective delivery of miR-317b-5b to tumor tissues.

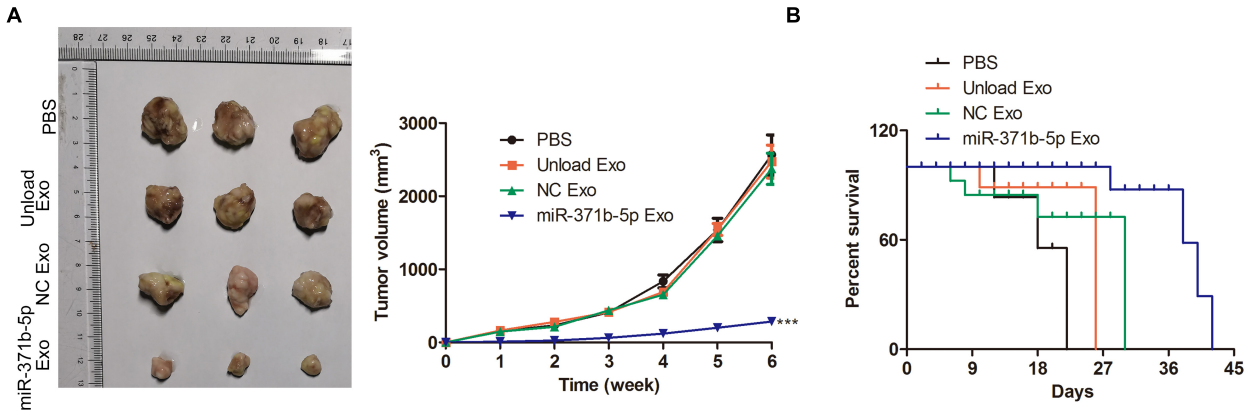

FIGURE 6 | Anti-tumor efficiency of engineered exosomes *in vivo*. BALB/c tumor bearing mice were injected with each of the following: PBS (200 μL), unload exosomes (unload Exo; 30 μg), NC exosomes (NC Exo; 30 μg), and miR-317b-5b-loaded engineered exosomes (miR-317b-5b Exo; 30 μg). **(A)** Average osteosarcoma tumor volume, 16 days following infection. **(B)** Survival analyses of osteosarcoma tumor-bearing mice, 60 days following infection. ***$P < 0.001$ vs. NC Exo. Error bars represented SD. Data represented three independent experiments.

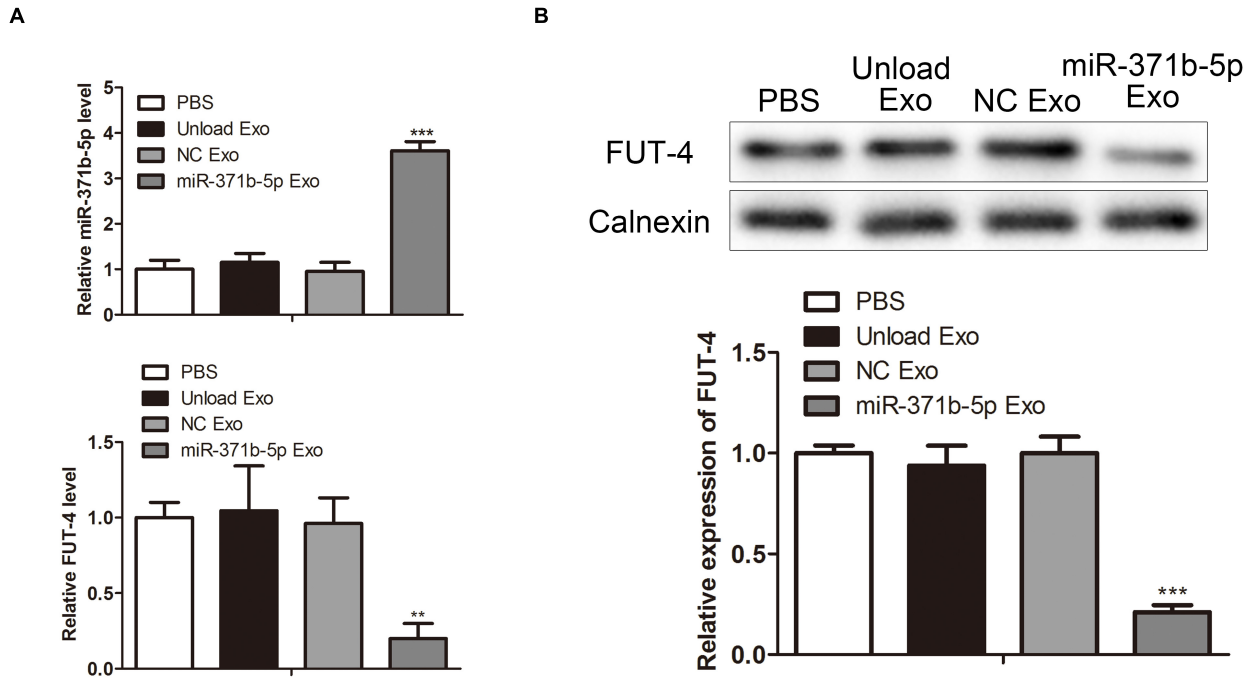

FIGURE 7 | Quantification of exosome-loaded miR-317b-5b loading *in vivo*. BALB/c tumor bearing mice were injected with each of the following: PBS (200 μL), unload exosomes (unload Exo; 30 μg), NC exosomes (NC Exo; 30 μg), and miR-317b-5b-loaded engineered exosomes (miR-317b-5b Exo; 30 μg). **(A)** RT-qPCR assessed the levels of miR-317b-5b and FUT4 in tumor tissues of osteosarcoma tumor-bearing mice infected with miR-317b-5b-loaded engineered exosomes. **(B)** Western blot measured the level of FUT4 protein in tumor tissues of osteosarcoma tumor-bearing mice infected with miR-317b-5b-loaded engineered exosomes. **$P < 0.01$, ***$P < 0.001$ vs. NC Exo. Error bars represented SD. Data represented three independent experiments.

Although our research has shown that miR-317b-5b-loaded engineered exosomes is a promising treatment. However, it is important to emphasize that the clinical therapeutic potential of engineered exosomes is limited by the difficulty of their manufacture and the need for a comprehensive molecular, and functional characterization of each formulation prior to treatment, both of them are expensive and labor-intensive.

In summary, our results suggest that miR-317b-5b-loaded engineered exosomes can be used as viable nanocarriers to deliver drug molecules such as miR-317b-5b both *in vitro* and *in vivo* and exert their anti-tumor effects. The modified exosomes will eventually be made into injectable forms as therapeutic drugs, including miRNAs and protein-based therapeutic agents in the future.

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committees of Nantong University.

AUTHOR CONTRIBUTIONS

XL and WZ designed the study. QX and YY performed the major work about mice, cell, molecular, and biochemistry experiments. LY, XY, ZS, and JL performed the molecular and biochemistry experiments. JX, XL, and WZ collected and analyzed the data. XL and WZ prepared the draft. All authors reviewed and agreed the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.750171/full#supplementary-material

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REVIEW
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Extracellular Vesicles in Acute Leukemia: A Mesmerizing Journey With a Focus on Transferred microRNAs

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Despite their small size, the membrane-bound particles named extracellular vesicles (EVs) seem to play an enormous role in the pathogenesis of acute leukemia. From oncogenic hematopoietic stem cells (HSCs) to become leukemic cells to alter the architecture of bone marrow (BM) microenvironment, EVs are critical components of leukemia development. As a carrier of essential molecules, especially a group of small non-coding RNAs known as miRNA, recently, EVs have attracted tremendous attention as a prognostic factor. Given the importance of miRNAs in the early stages of leukemogenesis and also their critical parts in the development of drug-resistant phenotype, it seems that the importance of EVs in the development of leukemia is more than what is expected. To be familiar with the clinical value of leukemia-derived EVs, this review aimed to briefly shed light on the biology of EVs and to discuss the role of EV-derived miRNAs in the development of acute myeloid leukemia and acute lymphoblastic leukemia. By elaborating the advances and challenges concerning the isolation of EVs, we discuss whether EVs could have a prognostic value in the clinical setting for leukemia.

Keywords: acute myeloblastic leukemia, acute lymphoblastic leukemia, extracellular vesicles, prognosis factor, disease pathogenesis, miRNAs, non-coding RNAs

INTRODUCTION

As the most common type of leukemia, the incidence of acute leukemia, either acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL), is increased in the last few years (Hunger and Mullighan, 2015; Park et al., 2015; Terwilliger and Abdul-Hay, 2017; Bosshard et al., 2018; Wiese and Daver, 2018). The aggressive and heterogeneous behavior of acute leukemia is originated from not only the diversity of genetic abnormalities but also the occurrence of extensive epigenetic changes. The list of involved genes and molecules in the pathogenesis of these malignancies is endless and new candidates are continuously emerging. This heterogenic characteristic of acute

leukemia brings obstacles in the way of precise treatment of the disease and somehow ends the era of the conventional chemotherapeutic approaches (Bassan and Hoelzer, 2011; Selim and Moore, 2018). Despite the efficacy of the common chemotherapy regimen consist of cytarabine and anthracycline, which thus far is the most effective treatment strategy for leukemia patients, a considerable proportion of patients succumb to the disease due to the therapy failure (Bassan and Hoelzer, 2011; Luger, 2017).

Recently, a group of extracellular vesicles (EVs) derived from the cancer cells that carry the property of their parental cells ranging from signaling proteins to nucleic acids such as DNAs was successfully identified (Voso et al., 2019). It became evident that cancer cell-derived EVs are auxiliary tools that support cancer growth by carrying the factors that could enhance angiogenesis (Skog et al., 2008), provide the metabolic needs of tumor cells and promote their proliferative capacity (Fong et al., 2015). In hematologic malignancies, for example in leukemia, EVs were showed to participate in the primary tumor growth as well as inducing multi-drug resistance through recruiting microenvironment resident cells such as endothelial cells (ECs) or leukocytes (Litwińska et al., 2019). In ALL, it has been proposed that the communication between leukemic cells and the bone marrow residential cells could increase the survival of leukemic cells through exporting exo-RNAs. In pre-B ALL, for example, some evidence revealed that the delivered exo-miRNAs could change the architecture of the BM environment in the manner that it reinforces the proliferation of leukemic cells and suppresses the activity of immune system (Xu et al., 2018). MicroRNAs are one of the main non-coding RNAs that play a critical role in the development of leukemia. There are multiple evidence reporting that miRNA transferred by EVs into the leukemic cells could be fundamental in the pathogenesis of leukemia. For example, the transferred miR-155 to AML cells could suppress the expression of C/EBPA to reinforce the development of leukemogenesis (Alemdehy et al., 2016). Or, the upregulation of long non-coding RNA SNHG1 expression has also been suggested to elevate the growth of the AML cells through targeting miR-489-3p (Li et al., 2021). The transfer of miR-181 family to ALL cells has also been suggested to be associated with CNS involvement (Egyed et al., 2020). Moreover, it should be noted that not all the transfer of miRNAs are participated in the early stages of leukemogenesis and sometimes the transferred miRNAs are responsible of protecting the leukemic cells from the devastating effects of the anti-cancer agents. In this vein, it became evident that exosomes carrying miR-155 could confer drug-resistance against tyrosine kinase inhibitors in AML cells (Viola et al., 2016). The transfer of miR-19b and miR-20a has also been reported to be associated with induction of drug-resistance in APL cells through increasing the expression of multiple drug resistant proteins (MDPs) (Bouvy et al., 2017).

In addition to their key roles in the pathogenesis of acute leukemia, EVs are also a valid repertoire of genetic and epigenetic abnormalities of the parental cells. The next-generation sequencing (NGS) and GeneScan-based fragment-length analysis on the identified double-stranded DNA (dsDNA) in AML-derived EVs revealed that the majority of dsDNA mirror the mutations found in the genomic DNA obtained from primary leukemia cells (Kontopoulou et al., 2020; Bernardi et al., 2021). It has been suggested that there is a similar pattern between the expression of miRNAs in the EVs and pre-B ALL-derived leukemic cells (Xu et al., 2016). These findings together with the fact that these membrane bilayer-enclosed structures could be reachable in the body fluids suggest EVs as a valid tool for early detection, and for predicting patient outcome of both AML and ALL. The disease diagnosis/prognosis is not the only area that leukemia-derived EVs could be employed, as these groups of microvesicles could also integrate into the risk stratification of patients and even in the therapeutic strategies. To understand the clinical value of leukemia-derived EVs, this review aims to summarize the biology of EVs and discuss how EV-derived miRNAs could play a part in the development of AML and ALL. By combining the advances and challenges concerning the isolation of EVs, we discuss whether EVs could have a prognostic value in the clinical setting for leukemia patients.

THE BIOLOGY AND FUNCTION OF EXTRACELLULAR VESICLES

The Origin of Extracellular Vesicles

The evidence of the existence of EVs dated back to almost 60 years ago when the 20–50 nm-sized vesicles carrying clotting factors were identified in human platelet-free plasma (Wolf, 1967). In an article published by Wolf (1967), these lipid-rich vesicles were described as minute particulate material referred to as platelet dust because they were released from platelet (PLTs) during activation. The results of electronic microscopy revealed that these particles carry phospholipids and platelet factor 3 (tissue factor) to facilitate the coagulation process (Wolf, 1967). Since then, the definition and the perspective of this particle which is now named as EVs has changed. Now, EVs are envisioned as a group of lipid bilayer-enclosed structures that are released into the extracellular space (Doyle and Wang, 2019; Cocozza et al., 2020; Kalluri and LeBleu, 2020) by most cells to either discard their unwanted products or communicate with other neighboring or distant cells and even with extracellular matrix (ECM) (Doyle and Wang, 2019; Cocozza et al., 2020). This means that the content of EVs could be varied from proteins, lipids, and nucleic acids that could change the behavior of the recipient cells (Doyle and Wang, 2019; Cocozza et al., 2020). Thus far, several types of EVs have been identified in biological fluids such as urine, blood, ascites, and cerebrospinal fluid (Ibrahim and Marbán, 2016), and according to their size, these vesicles are classified into three groups: exosomes with the size range from 30 to150 nm, apoptotic bodies that have the size of 50–5,000 nm, and microvesicles (also known as ectosomes, shedding vesicles, or microparticles) with 100–1,000 nm in diameter (Kim et al., 2018; Liu et al., 2021). Apart from the size, the mechanism of release of EV differs from each other. While exosomes require multivesicular bodies (MVBs) for transportation, apoptotic bodies and microvesicles are released through interacting with the plasma membrane (Hessvik and Llorente, 2018). Among

the mentioned EVs, it should be noted that exosomes have the most participation in intercellular communication, neuronal communication, antigen presentation, immune responses, organ development, and reproductive performance. This means that exosomes may have the most fundamental roles in pathophysiologic conditions, as well (Crenshaw et al., 2018; He et al., 2018). However, it should be noted that the size classification could not properly distinct microvesicles from exosomes, as not only both vesicles may overlap in the size but also both could be found in extracellular fluids and may exert similar intracellular effects (Zaborowski et al., 2015). Given these, in most cases, both microvesicles and exosomes are referred to as circulating vesicles (Raposo and Stoorvogel, 2013).

Biogenesis of Circulating Vesicles

Like their intracellular functions, the biogenesis of circulating vesicles is complex and numerous internal and external factors are involved in this process. Different membrane receptors, lipid raft complexes, and endosomal sorting complexes regulate the biogenesis of EVs; however, this process could not be accomplished without the presence of Ras superfamily GTPase (Kim et al., 2018; Jadli et al., 2020).

Biogenesis of Exosomes

The biogenesis of exosomes includes three main steps; early endosomes (EEs) and late endosomes (LEs) or MVB formation, intraluminal vesicles (ILVs) formation, and the secretion of ILV into the extracellular space.

Early endosomes (EEs) form as a result of membrane budding inward the cell. The generated EEs subsequently fuse with endocytic vesicles to incorporate their cargos. After the evacuation of recycling endosomes from EEs, they undergo several stages of maturation to produce late endosomes (LEs), also known as multivesicular bodies (MVBs). There are two destinies for MVBs; either merge with the lysosome which leads to their destruction, or package their contents as 30–100 nm vesicles named intraluminal vesicles (ILVs) to integrate with the plasma membrane and release into the extracellular space (Akers et al., 2013). In this process, the companionship of several factors including cytoskeleton, motors proteins, and Rab family of GTPases may decide whether MVBs move toward lysosome or plasma membrane. For example, while ubiquitylated 7 guides MVBs toward lysosomal degradation, the members of the Rab family of GTPases such as Rab27A, Rab11, and Rab35 regulate the fusion of MVBs into the membrane through generating ILVs (Ostrowski et al., 2010).

The formation of ILVs consists of two main steps. At the primary stage, the endosome membrane should be organized by specific transmembrane proteins known as Tetraspanins (Pols and Klumperman, 2009). The presence of Tetraspanins is critical for the formation of ILVs, as these proteins construct Tetraspanin enriched microdomains (TEMs) domains within the membrane which could later cluster the essential proteins for ILVs generation (Hemler, 2005). Thus far, CD9 and CD63 are the most common as well as important Tetraspanins on the surface of ILVs that are also used as an identifier to isolate EVs in the body fluids (Jansen et al., 2009; Kosaka et al., 2010). The second step of ILVs formation is allocated to the addition of a group of multi-protein complexes named endosomal sorting complex required for transport (ESCRTs). The results of the in-depth molecular investigations showed that the presence of 4 types of ESCRT called ESCRT 0, I, II, and III is essential for membrane budding. Among them, the presence of ESCRT III is necessary for complete membrane budding. Upon EE formation and cargo mono-ubiquitination (Crenshaw et al., 2018; Mashouri et al., 2019), ESCRT-0 in the complex with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and STAM1/2 recruit ESCRT I/II to bind to the phosphatidylinositol 3-phosphate (PIP3) located on the membrane. This event curves the membrane inward the lumen and also recruits the complex of ESCRT III-Alix- TSG101 that is essential for complete secretion of the vesicle from the membrane (Crenshaw et al., 2018; Juan and Furthauer, 2018; Mashouri et al., 2019; Jadli et al., 2020). It should be noted that if the cargo does not undergo mono-ubiquitination, ALG-2 interacting protein-X (ALIX) forms a team with Syntenin-Syndecan complex (Juan and Furthauer, 2018) or PAR1 (Kim et al., 2018; Skryabin et al., 2020) to mediate ESCRT-dependent secretion of exosomes (Kim et al., 2018; Mashouri et al., 2019).

Previous studies showed that even in the absence of HRS, Alix, and TSG101, the biogenesis of exosomes is continued. It has been suggested that this process could be mediated independently of ESCRT complex and through raft-dependent mechanisms. Sphingomyelin is a key player in this process (Skryabin et al., 2020), as its cleavage *via* sphingomyelinase 2 produces ceramide, a waxy lipid molecule which facilitates the formation of ILVs through regulating membrane budding (Juan and Furthauer, 2018). Moreover, ceramide could be catalyzed into sphingosine 1-phosphate (S1P) (Kajimoto et al., 2013), a signaling molecule which attracts cargos such as CD63, CD81, and flotillin into ILVs *via* interaction with inhibitory G protein (Gi)-coupled receptor. No matter through which mechanism ILVs may be produced within the MVBs. NSF attachment protein receptors (SNAREs) provide a platform for their secretion into the extracellular space. Through binding of Ca^{2+} to synaptotagmin VII, SNAREs complex could be activated, resulted in secretion of EVs (van Niel et al., 2018; Jadli et al., 2020). When the exosomes were released by the parental cells, VPS4 separates the remained components to recycle them for further uses.

Biogenesis of Micro Vesicles and Apoptotic Bodies

Since micro vesicles (MV) differ from the size from the exosomes, their biogenesis might have some differences with EVs. In the biogenesis of MV, actin rearrangement play a fundamental role (Gurunathan et al., 2021). In GTPase-dependent pathway, an enzyme named LIM kinase (LIMK) adds a phosphoryl group cofilin, which is a to actin depolymerizing enzyme, and thereby by inactivating this enzyme facilitate MV budding (Li B. et al., 2012). Another mechanism that could lead to MV biogenesis could be mediated through Small GTPase, ADP-ribosylation factor 6 (ARF6) signaling. Once ARF6 is activated, it could recruit ERK signaling pathway to activate myosin light-chain kinase (MLCK), which in turn activates myosin light-chain (MLC) at the necks of MVs. Activated MLCK interact with

actin filaments, a process leads to MV production (Muralidharan-Chari et al., 2009). The biogenesis of MV could be triggered by phospholipid redistribution or cytoskeleton reorganization (Akers et al., 2013; D'Souza-Schorey and Schorey, 2018). The best example of a biological process that could activate MV biogenesis is the translocation of phosphatidylserine (PS) to the out membrane, a process that occur in the apoptosis (Akers et al., 2013; D'Souza-Schorey and Schorey, 2018). In apoptosis, cellular contents are packed in the form of small membrane-bound vesicles known as apoptotic bodies (ApoBDs), which contains externalized phosphatidylserine, calreticulin, and calnexin (Nunez et al., 2010). When actin-myosin located at the membrane starts to concentrate, a mechanism leads to membrane blebbing, the formation of ApoBDs begins (Xu et al., 2019). In addition to membrane blebbing, the reduction in the volume-to-surface ratio of cells also is another factor that provoke ApoBDs formation (Nunez et al., 2010).

Extracellular Vesicles as a Vehicle to Deliver Essential Components to Target Cells

Based on the type of the parental cells and the mission that EV may have, the cargo of these circulating vesicles varies. However, proteins, lipids, metabolites, RNAs, and cDNAs are the common components of EVs (Crenshaw et al., 2018; He et al., 2018; Huang and Deng, 2019). **Table 1** listed the most common molecules that could be detected in EVs, irrespective of their origin and cellular function.

Proteins

Apart from structural proteins such as tetraspanins (CD81, CD82, CD37, and CD63), TSG101, Alix, and Rab family, heat shock protein, clathrin, protein kinase G (PKG), ATPase, syntenin, and RNA binding proteins (RBPs) are the most common types of proteins that are detected in EVs (Crenshaw et al., 2018; Huang and Deng, 2019; Jeppesen et al., 2019). It should be noted that the protein content of EVs depends on the original cell (He et al., 2018). MHC class II, ICAM-1, integrin, CD20, PD-L1, EGFR, IGF-1R, and cytokine receptors are among the most important proteins that are isolated from EVs (Pegtel and Gould, 2019).

Nucleic Acids

One of the most important contents of EVs is nucleic acids that could be either mitochondrial DNA (mtDNA), double strands DNA (dsDNA), single strands DNA (ssDNA) (Kalluri and LeBleu, 2020), or different types of RNAs ranging from coding RNAs such as mRNAs to non-coding species (Chu et al., 2020; Kalluri and LeBleu, 2020; Skryabin et al., 2020). Among different types of nucleic acids, it seems that microRNAs (miRNAs) are the most important cargos of EVs. Carolina Villarroya-Beltri and colleagues have successfully reveal that sumoylated hnRNPA2B1 and Ceramide are critical for loading miRNAs into EVs (Kosaka et al., 2010; Villarroya-Beltri et al., 2013). Upon integrating with mRNAs in the recipient cells, miRNAs could conveniently change their cellular behaviors. This process is well-defined in cancer cells, where the exosome miRNAs provide a platform for cancer cells to grow, invade into distant organs, and resist chemotherapeutic drugs (Ingenito et al., 2019; Rahbarghazi et al., 2019). Recently, it has been indicated that the resident-long non-coding RNAs in EVs could epigenetically alter the behavior of target cells (Wang et al., 2019; Da et al., 2021). The list of the most common miRNAs and lncRNA in EVs can be found in **Table 1**.

MicroRNAs

miRNAs are the best tools for regulating gene expression, either transcriptionally or post-transcriptionally (Huntzinger and Izaurralde, 2011; O'Brien et al., 2018). In complex with other proteins, which is called as MiRISC [miRNA and Argonaute 2(AGO-2)], the 5′-proximal region (nucleotide 2–8) of the miRNA binds to the 3′ UTR of the targeted mRNA and thereby inhibit or stimulate their expression. It should be noted that not all miRNAs bind to 3′ UTR of the mRNA and in some cases, miRNAs such as miR-10a could bind to 5′ UTR of the targeted mRNA (Ørom et al., 2008; Valinezhad Orang et al., 2014). Once miRNA interact to its targeted mRNA and if miRNA and miRNA response elements (MRE) are entirely complementary,

TABLE 1 | The content of circulating EVs.

Types of cargo	Common contents
Proteins	
Structural proteins	Tetraspanin proteins (CD81, CD82, CD37, and CD63), Syntenin, Alix, tumor susceptibility gene 101 protein (TSG101), Syndecans (SDC1–4), Intercellular adhesion molecule-1 (ICAM-1), Integrins, and Chaperons.
Outer membrane lipid-anchored proteins	CD39, CD73, CD55, CD59, Glypican-cellular prion protein (PrPC), and Amyloidogenic conformer.
Inner membrane lipid-anchored proteins	Small GTPases superfamily, and Protein kinases (Src).
Cell signaling proteins	Epidermal growth factor receptor (EGFR), Vascular endothelial growth factor receptor type-2 (VEGFR2), Insulin-like growth factor I receptor (IGF-1R), Notch receptors, Cytokine receptors, G protein-coupled receptors (GPCRs), Wnt proteins, Bone morphogenetic proteins (BMPs), Transforming growth factor β (TGF-β), tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand, first apoptosis signal (FAS) ligand, and extracellular matrix (ECM) proteins.
Enzymes	Phosphatases, Pyrophosphatases, Calcium-binding annexins, and phosphate transporters, RNA editing enzymes, Lipases, Proteases, Glycosyltransferases, Glycosidases, and Metabolic enzymes.
LncRNAs	lincRNA-p21, ANRASSF1, lncRNA SYSIL, lncRNA ROCK1, and lncRNA Paupar.
miRNAs	miR-150, -221, -1246, -140-3p, -16-5p, -20a-5p, -15a-5p, -17-5p, -18a, let-7b.

the endonuclease activity of AGO-2 be provoked to cleave the mRNA (O'Brien et al., 2018). There are some piece of evidence suggesting that miRNAs could also enhance the expression of some genes (Huntzinger and Izaurralde, 2011). For example, when AGO makes a team with another protein associated to the miRNA-protein complex (microRNPs) named Fragile-x-mental, this complex could bind to AU-rich elements (AREs) at the 3′ UTR of the targeted mRNAs to enhance its expression. The best example of this regulatory process could be seen in Let-7 which by recruiting this mechanism could enhance the expression of the genes leading to cell cycle arrest (Forman et al., 2008). miR-24-1 is another miRNA that seems to could enhance the transcription of the target genes *via* inducing chromatin remodeling at enhancer site (Xiao et al., 2017). Given the importance of miRNAs in gene regulation, intense attention has been attracted to the biogenesis of these small non-coding RNAs.

Biogenesis of MicroRNAs

The biogenesis of miRNAs could be mediated through two main pathways; canonical and non-canonical mechanism (O'Brien et al., 2018). In canonical pathway, the primary miRNA (pri-miRNA) produced by RNA polymerase 2 converts to precursor-miRNA (pre-miRNA) by DROSHA/DiGeorge Syndrome Critical Region 8 (DGCR8) complex. The produced pre-miRNA, then, exported to the cytoplasm *via* exportin5, where it loses its pri-terminal miRNA's loop *via* RNase III endonuclease Dicer. Helicase, then, come to play to convert the mature double stranded miRNA to single stranded miRNA (Han et al., 2004; Okada et al., 2009). In non-canonical pathway; however, the biogenesis of miRNAs could be mediated through Drosha/DGCR8 and Dicer-independent manner (O'Brien et al., 2018). In this manner, the non-cleaved pre-miRNA recruit exportin 1 to transport into the cytoplasm, where it makes a team with Argonaute-2 (AGO-2) to become mature (O'Brien et al., 2018).

Transfer of MicroRNAs to Extracellular Vesicles

Through binding to either RNA-Binding Proteins (RBP) or membrane proteins, miRNAs could be packaged into EVs (Groot and Lee, 2020). For example, RNA-Binding Proteins (RBP) such as heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) binds miRNA to transfer them into the EVs (Villarroya-Beltri et al., 2013). AGO-2, apart from its role in miRNA maturation, not only could aid miRNAs to transfer into EVs by recruiting the KRAS-MEK-ERK signaling pathway (Li L. et al., 2012; McKenzie et al., 2016) but also could protect them from degradation (Groot and Lee, 2020). Synaptotagmin-binding cytoplasmic RNA-interaction protein (SYNCRIP) is another protein that could join miRNA to transfer them into EVs. Through binding to extra-seed sequence (hEXO) motif of miRNAs, SYNCRIP aid miRNAs to gather into the exosomes (Santangelo et al., 2016).

Extracellular Vesicles Transfer to Target Cells

As a carrier of information to the recipient cells, EVs should be delivered properly as well as safely to the target cells. Indeed, the vesicular structure of EVs protects their contents from enzymatic degradation and guarantees that the cargo be delivered to the target cells in its original state. One striking point about EV secretion is that under a stressful condition, cells are more prone to produce EVs (King et al., 2012). This phenomenon could be explained in two ways; first, this is an attempt of the cells to discard the damaging and harmful factors, or second, this is a communicational tool to aware the neighboring cells of the ongoing event. Valid evidence exists for both of these hypotheses. It has been reported that in response to the DNA damage and as the result of p53 activation, the pace of EVs release is exacerbated (Yu et al., 2006). Also, during hypoxia, the tendency of the cells to produce EVs is much higher than normoxic conditions (Park et al., 2010). No matter what is the purpose of EV secretion, when these circulating vesicles are released, they should be delivered to the recipient cells. The membrane-bound activating or inhibitory molecules in EVs transmit a signal to the recipient cells to allow EVs entrance. Like other circulating vesicles, EVs also integrates with the membrane of the responder cells *via* either endocytosis or membrane fusion. Once EVs enter the cells, they evacuate their valuable biologically active molecules repertoire to alter the cellular behavior. For example, the delivered miRNAs or lncRNAs could epigenetically change the expression of a wide range of molecules, activate/suppress different signaling pathways, and change the chromosomal structure. Also, the transferred mRNA could be translated into proteins that previously did not exist in the responder cells (Parolini et al., 2009).

EXTRACELLULAR VESICLES-DERIVED MICRORNAs, A TROJAN HORSE FOR CANCER DEVELOPMENT

Although these seem to be striking methods to alter the characteristics of cells, production and delivery of EVs by cancer cells can not only transform the neighboring cells into the malignant counterpart but also engage the properties of the surrounding cells for their favor. The first evidence of the involvement of EVs in tumorigenesis was reported by Skog et al. (2008) who indicated that secreted EVs from glioblastoma cells enforce angiogenesis in brain endothelial cells. Very soon, the other pieces of evidence were found in other types of human cancers such as squamous cell carcinoma (Park et al., 2010), breast cancer (O'Brien et al., 2013; Zomer et al., 2015), and colorectal cancer (Tian et al., 2018), all suggesting that EVs act as Trojan horses to alter the microenvironment according to the needs of the tumor cells. Tumor-derived EVs could also induce immune exhaustion in the tumor microenvironment through upregulating the expression of inhibitory molecules of lymphocytes such as NK cells and CD8 positive T-lymphocytes or enhancing the differentiation of myeloid-derived suppressor cells (Lane et al., 2018). EVs also play a fundamental role in the pathogenesis of hematologic malignancies, as having a precise cross-talk with other residential cells within the BM niche is vital for the survival of hematologic malignant cells. The delivered EVs induce a "homing and nurturing" microenvironment in BM and protect the leukemic/neoplastic

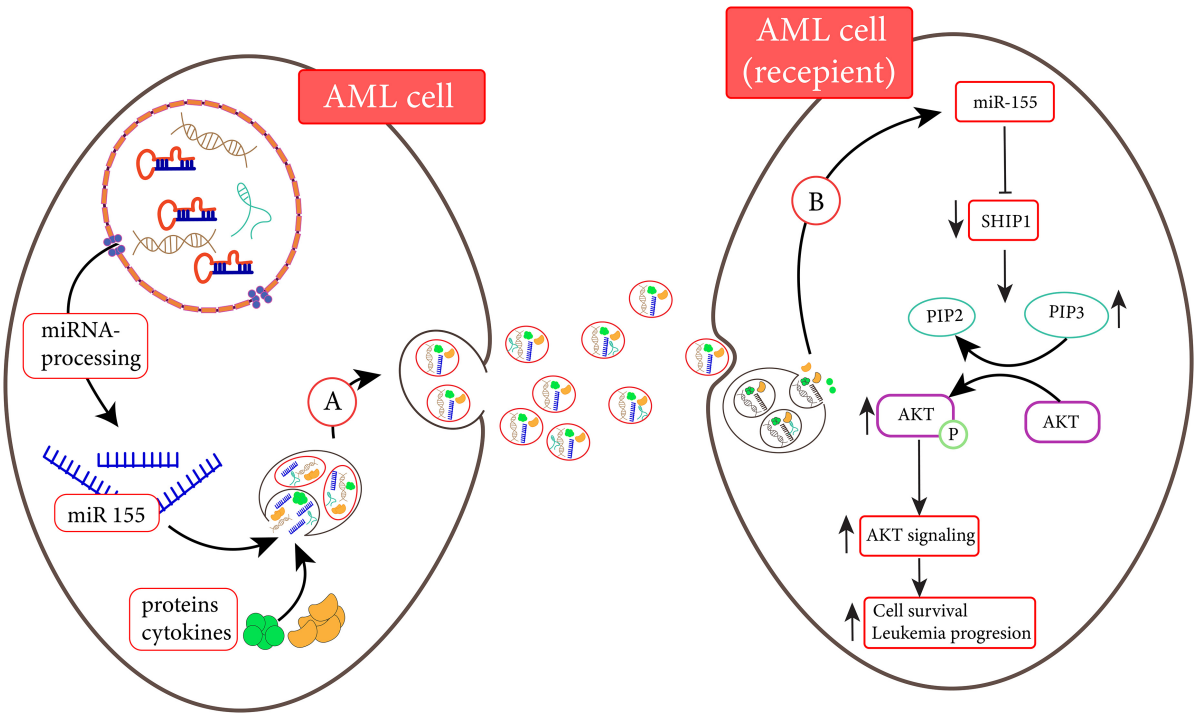

FIGURE 1 | AML cells are able to transfer miR-155 to another AML cell (Hornick et al., 2015). (A) MiR-155 and other elements are transferred to recipient cells *via* exosomes. (B) MiR-155 derived from exosomes is released into the recipient cell and inhibits the activated SH2 domain-containing inositol 5′-phosphatase1 (SHIP1) expression. Following the inhibition of SHIP1, the enhancement of AKT signaling promotes cell survival (Xue et al., 2014).

cells from the devastating effects of chemotherapeutic drugs. For example, it has been reported that chronic myeloid leukemia (CML)-derived EVs enforce BM stromal cells to produce IL-8, a cytokine which prolongs the survival of CML cells (Corrado et al., 2014). In multiple myeloma, it has been reported that the secreted EV from the BM mesenchymal stromal cells enhanced the proliferative capacity of multiple myeloma cells through transferring miR-15a (Roccaro et al., 2013). For acute type of leukemia (AML and ALL), there are multiple lines of evidence shedding light on the role of EVs in the pathogenesis of this common type of hematologic malignancy. In the following part of this article, we discuss the roles of EVs in the pathogenesis of acute leukemia.

EXTRACELLULAR VESICLES AS A COMMUNICATION TOOL IN ACUTE LEUKEMIA

Extracellular Vesicles Participate in the Pathogenesis of Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia

In acute leukemia, EVs serve as a bridge to provide a dynamic cross-talk between leukemic cells and the stromal cells that reside in the BM niche. In another word, EVs are responsible for turning BM microenvironment into a leukemia-permissive space. There are multiple evidence suggest that EVs might have a key role in the early stages of leukemogenesis. It has been revealed that the transfer of EVs from the leukemic cells to either HSCs or myeloid progenitor cells could abrogate the proper differentiation and thereby lead to the development of leukemia. Moreover, the transferred EVs protect leukemic cells from apoptotic stimuli such as chemotherapeutic drugs (Kumar et al., 2018). In the following part of the paper, more details will be discussed about the participation of EVs in the pathogenesis of leukemia.

The Role of Extracellular Vesicles Derived MicroRNAs in the Regulation of Leukemogenesis

One of the main mechanisms through which EVs promote the progression of leukemia is mediated through delivering the essential oncogenic RNAs into HSCs to change its characterization for developing into leukemic cells. In this process, the delivery of miRNAs plays fundamental roles. In ALL, for instance, it has been claimed that the leukemic cells released EVs containing miR-43a-5p to the BM microenvironment. After internalizing to the BMSCs, this miRNA targets Wnt signaling axis and thereby inhibits osteogenesis in the BM. The malignant HSCs transform to leukemic cells as osteogenesis is suppressed (Yuan et al., 2021). In addition, it has been found that the secreted EVs from BMSCs consisting of miR-21 are delivered into HSCs and consequently enhance the development of B-ALL cells. On the other hand, the exo-miR-21 could interact with TGF-β

and shut down the anti-tumor immune responses in the BM microenvironment (Lv et al., 2021). The same mechanism was also observed in AML. A previous study showed that the serum level of EVs carrying miR-10b is elevated in AML patients as compared to the healthy counterparts (Fang et al., 2020). miR-10b is notorious for its role in halting the granulocytic/monocytic differentiation in HSCs and enhancing the proliferative capacity of immature myeloid progenitors, leading to AML development (Bi et al., 2018). The large amount of EVs containing miR-10b in newly diagnosed AML patients suggested the significant roles of this delivering system in inducing AML (Fang et al., 2020). MiR-4532 is another delivered miRNA that could be transferred to HSCs through AML-derived EVs to suppress the expression of LDOC1. LDOC1 is an inhibitor of the STAT-3 signaling pathway and thereby its downregulation leads to STAT-3 activation. When AML-derived EVs deliver such cargo to the HSCs, they manipulate the proliferative capacity of these cells by stimulating the STAT-3 signaling pathway (Zhao et al., 2019). The results of the previous studies also indicated that some of the leukemic-derived EVs could induce early leukemogenesis in myeloid progenitors through transferring miR-155 (**Figure 1**). Through binding to 3/UTR of c-Myb, miR-155 inhibits the expression of this differentiating transcription factor in myeloid cells and thereby induces differentiation arrest, a critical step in AML development (Hornick et al., 2016). Apart from miRNAs, some evidence suggest the exosomal transfer of oncogenic mRNAs such as those encoding NPM1 and FLT3-ITD to the myeloid progenitors from leukemic cells could also lead to leukemia development (Huan et al., 2013). By silencing the expression of hematopoiesis-related growth factors such as IGF-1, CXCL12, KIT ligand, and IL-7, AML-derived EVs could enforce neoplastic HSCs committed to the myeloid progenitor, enhancing the production of leukemic cells (Kumar et al., 2018). The transfer of the anti-apoptotic proteins such as MCL-1, BCL-2, and BCL-XL to the immature myeloid blasts could also guarantee their survival in the BM microenvironment (Wojtuszkiewicz et al., 2016). In ALL, it has been found that leukemia-derived EVs could induce metabolic switch in BMSCs. Johnson et al. (2016) have proposed that the leukemia-derived EV recipient stromal cells have minimal mitochondrial aspiration and use an aerobic glycolysis instead of oxidative phosphorylation, which in turn could provide the desired energy for ALL development in the BM microenvironment. It seems that during leukemogenesis, EVs act as a Trojan horse by delivering either miRNAs or onco-mRNAs. These tiny vesicles could alter the characteristic of BMSCs in a way that they increase the possibility of AML or ALL development.

The Role of Extracellular Vesicles Derived MicroRNA in Regulating Survival of Leukemic Cells

As mentioned earlier, one of the main purposes of EVs delivering in acute leukemia is to evolve a leukemia-permissive space in BM, where leukemic cells could have an opportunity to survive, proliferate and grow. The number of studies that cover this mechanism in the progression of both AML and ALL is skyrocketed over the last decades and thus far, numerous molecules have been identified to be involved in this process. EVs could potentiate the survival and proliferative potential of ALL. In a study conducted by Patel et al. (2016), it has been reported that when non-proliferating ALL cells were cultured with ph^+ ALL-derived exosomes, their proliferative capacity were vigorously reinforced, suggesting that the contents of these EVs might have proliferative factors. But what components could be involved in this process? Haque and Vaiselbuh (2020) came up with the answer when they successfully isolated miR-181 from the EVs in the serum of pediatric cases of ALL. By conducting further analysis, they proposed that the delivered miR-181, on one hand could enhance the expression of anti-apoptotic proteins such as MCL-1 and BCL-2 in leukemic cells, and on the other hand, could elevate the expression of proliferation-related genes, including PCNA and Ki-67. Moreover, the authors also claimed that the up-regulation of miR-181 in EVs-derived from ALL patients suppressed the expression of pro-apoptotic genes. As a straightforward interpretation of these results, it was proposed that ALL cells might have longer survival and more potent proliferative capacity by uptaking these EVs (Haque and Vaiselbuh, 2020).

For AML, multiple studies declared the importance of EVs in increasing the survival of leukemic cells. Through delivering DKK1 to the BM stromal cells (BMSCs), for example, AML-derived EVs could halt the progression of hematopoiesis and osteoblast differentiation in the BM niche and promote the uncontrolled proliferation of leukemic cells (Kumar et al., 2018). BMP-2 is another cargo that could be transmitted between AML cells and the mesenchymal stem cells (MSCs) in the BM microenvironment to guarantee the survival of leukemic cells. The excessive amount of BMP-2 in leukemic cells transfer in the form of EVs to MSCs, where this transcription factor could reinforce osteogenic differentiation. As a result, through secretion of connective tissue growth factor (CTGF) from MSCs, AML cells could find a chance to grow (Battula et al., 2017). So far, many exosome-delivered miRNAs have been identified for endowing the AML cells the survival advantages. MiR-125b, for instance, is one of these delivered miRNAs that target the expression of pro-apoptotic proteins such as BAK and Bmf, and P53 in AML cells (**Figure 2**). This miRNA could not only halt the induction of apoptosis in AML cells but also induce cell proliferation through promoting cell cycle (Bousquet et al., 2010; Zhang et al., 2011; Vargas Romero et al., 2015). Ji et al. (2021) have also suggested that BMSC-derived EVs could enhance AML development through delivering miR-26a-5p to leukemic cells. By activating the Wnt/B-catenin signaling pathway, the delivered miR-26a-5p promotes AML cell proliferation, migration, and invasion (Ji et al., 2021). Not all the alterations should be delivered by exo-miRNAs and in some cases exo-lncRNAs might also have a role in the regulation of the BM microenvironment. Exo-circ-0009910 has been claimed to block the expression of miR-5195-3p and thereby enhance the progression of cell cycle in AML cells through up-regulating the growth factor receptor-bound protein 10 (GRB10) (Wang et al., 2021). Another exo-lncRNA that has been detected in the sera of AML patients is Circ-0004136, which is a sponge for miR-570-3p, a tumor suppressor miRNA that reduced the expression of TSPAN3 in AML cells. When EVs containing Circ-0004136 is delivered into AML cells, not only the

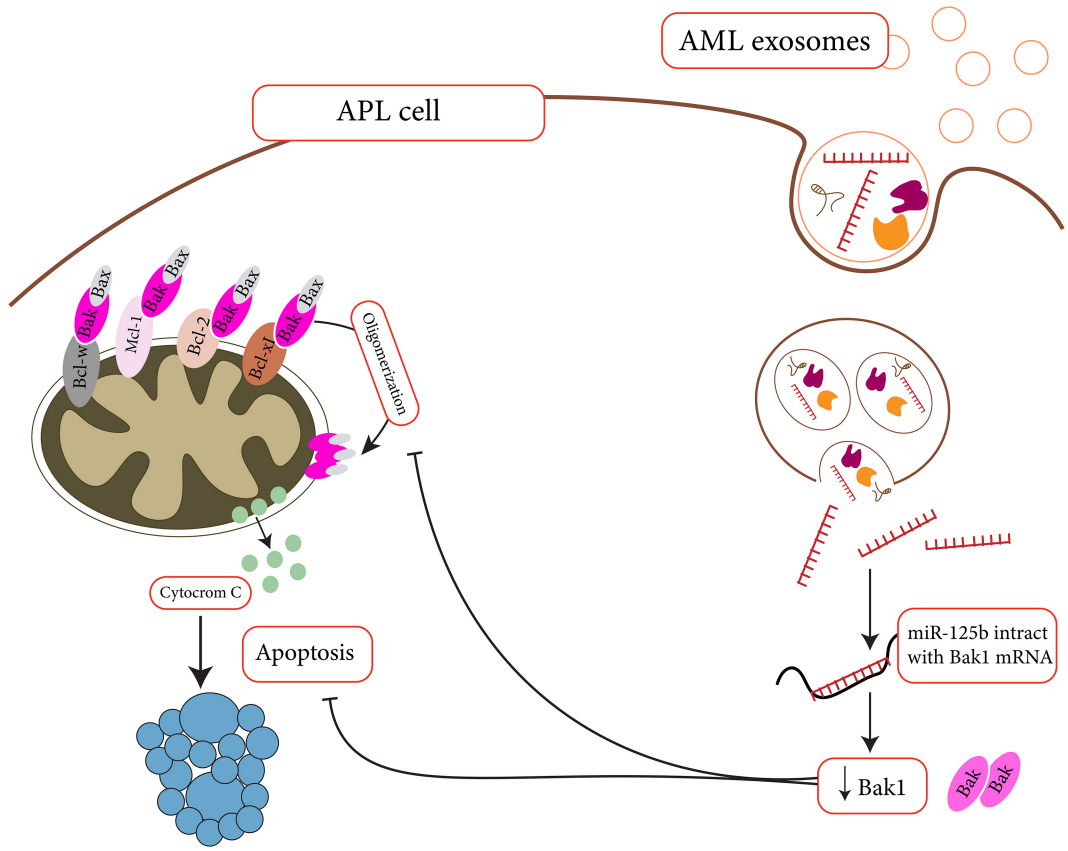

FIGURE 2 | Apoptosis suppression by miR-125b might help APL cells survive in cytotoxic situation (Zhang et al., 2011). MiR-125b is a microRNA that targets Bak1. As miR-125 levels rise, Bak levels fall, and apoptosis decreases.

viability of the cells be sustained but also these cells proliferate more autonomously (Bi et al., 2021).

The Role of Extracellular Vesicles in Regulating Angiogenesis

Since the density of BM microvessels is one of the main criteria that could protect the survival of leukemic cells in the BM, it comes as no surprise that EVs might participate in the regulation of the density of BM microvesicles. Through delivering angiogenic factors/proteins and miRNAs, EVs change the characteristics of endothelial cells and promote angiogenesis (Huan et al., 2013). The angiogenic contents of EVs increase the proliferative capacity of endothelial cells, enforce their invasion and subsequently enhance the expression of proangiogenic factors such as IL6 and VEGF. Fang et al. (2016) reported that the transferred EVs could deliver IL-8 and VEGF to endothelial cells to change their angiogenic signature and thereby prolong the survival of NB4 cells. Among the different angiogenic cargos, perhaps the miR-17-192 family is the most important non-coding RNAs that could stimulate angiogenesis in acute leukemia (Doebele et al., 2010). Through suppressing integrin A5 in human umbilical vein endothelial cells (HUVECs), it has been proposed that miR-92a, one of the members of the miR-17-92 family, could increase the density of microvesicles in the BM microenvironment (Xin et al., 2017). Apart from direct delivery of angiogenic factors, leukemic cells could also induce hypoxia in endothelial cells through EVs carrying HIF-1α. In response to hypoxia, endothelial cells activate their angiogenic properties, so that the new vessels might bring enough supplies of oxygen to the cells (Park et al., 2010). In ALL cases, the delivered exo-miR-181 could also be transferred into endothelial cells and enforce these cells to produce VEGF to enhance angiogenesis (Patel et al., 2016). Overall, these findings shed light on the importance of EVs in the progression and dissemination of myeloid leukemia cells through regulating the angiogenic process.

The Role of Extracellular Vesicles in Regulating Drug-Resistance

Constructing a leukemia-friendly environment, EVs also protect leukemic cells from anti-cancer agents. Viola et al. (2016) was the first group who have reported that the transferred TGF-β and miR-155 from AML cells to MSCs provide a chemotherapy-protective environment for AML cells. Wojtuszkiewicz et al. (2016) realized that the chemo-sensitive AML cells could acquire the resistant phenotype through receiving Bcl-2 containing EVs from the chemo-resistant AML cells. They proposed that EVs are communicational tools for inducing drug resistance (Wojtuszkiewicz et al., 2016). The same results were also reported

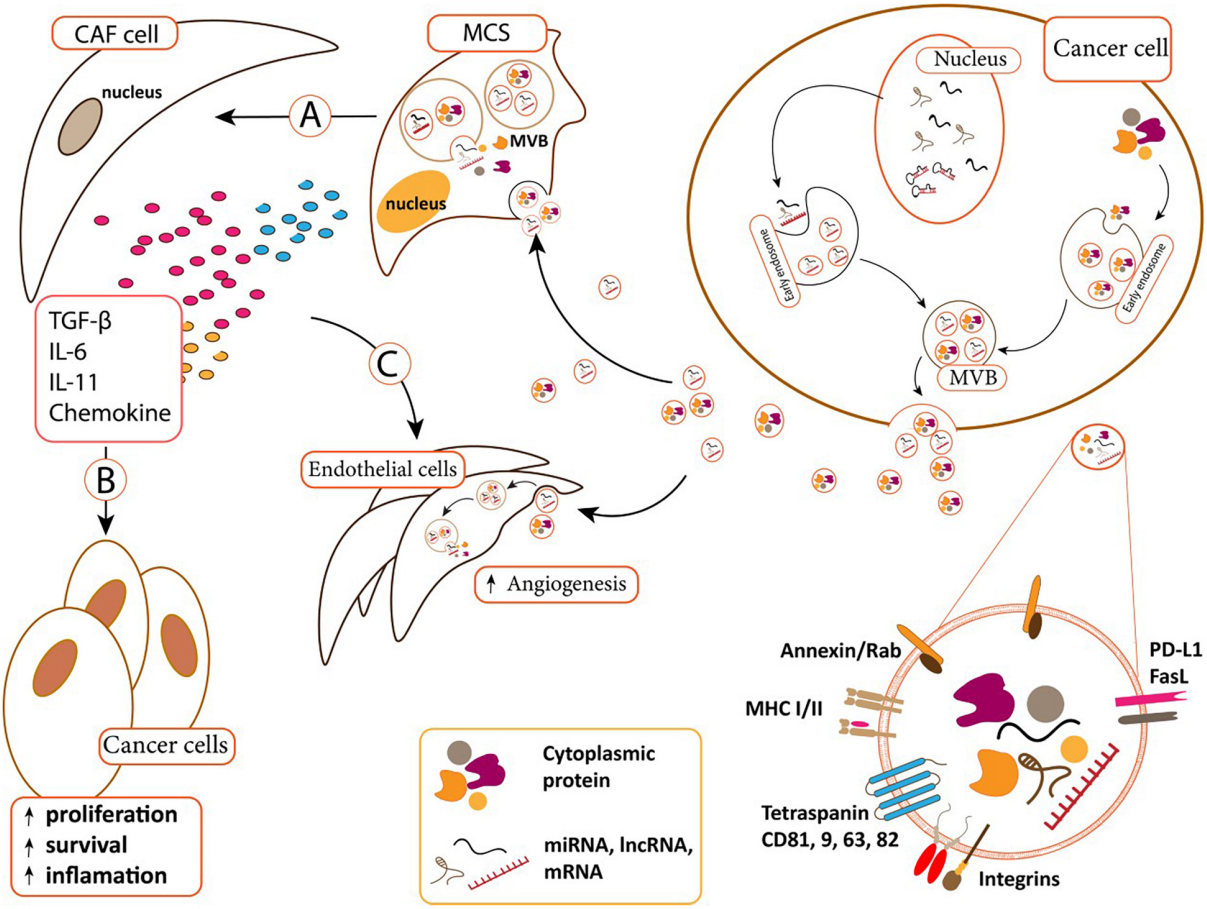

FIGURE 3 | (A) Cancer-derived exosomes can transfer to MSCs and change MSCs. MSCs differentiated to cancer-associated fibroblast (CAF) in B.M microenvironment. (B) CAFs supports cancer cells *via* secreted content such as TGF-β, IL-11, IL-6, and chemokine. CAF secreted contents affect cancer cells and increase cancer cell proliferation, survival. (C) Cancer derived exosomes can also uptake by endothelial cells and induce angiogenesis. CAFs secreted content can also induce angiogenesis in endothelial cells (Padua and Massagué, 2009; Mineo et al., 2012; Pickup et al., 2013; Costanza et al., 2017).

by Bouvy et al. (2017) who indicated that daunorubicin-resistant AML cells could induce drug resistance in other leukemic cells through delivering drug efflux pump multidrug resistance protein 1 (MRP-1). Aberrant delivery of the anti-apoptotic molecules/proteins to AML cells is well-studied in numerous studies and has been suggested as a mechanism to increase the survival of leukemic cells against anti-cancer agents. For example, miR-155 and miR-375 enriched EVs could be transferred from BMSCs to confer drug resistant phenotype in AML cells against tyrosine kinase inhibitors (Viola et al., 2016). MiR-19b and miR-20 could induce chemo-resistance through activating TGF-β and PI3K/Akt signaling axis (Tazzari et al., 2007). In the induction of resistance against immune-therapies, the footprint of EVs is also observed. Hong et al. (2017) suggested that AML-derived EVs could attenuate the efficacy of adoptive natural killer (NK) cell therapy by delivering inhibitory ligands that counteract the activity of NKG2D receptor on NK-92 cells. The transferred TGF-β from AML cells into NK-92 cells reduced the expression of NKG2D in these cells through recruiting TGFβRI/II pathway (Hong et al., 2017).

The number of studies demonstrated the role of exo-miRNAs/proteins in conferring drug resistant phenotype is rare in ALL cases. It has been reported that when BMSCs absorb leukemia-derived EVs, they would be transformed into cancer associated fibroblasts (CAFs) that could evolve a protective niche against chemotherapeutic drugs (**Figure 3**). On the reciprocal manner, BMSCs-derived EVs could deliver galectin-3 into ALL cells to increase their drug resistance through activating NF-κB signaling axis (Fei et al., 2015).

Extracellular Vesicles and Acute Leukemia Prognosis

Given the importance of EVs in the pathogenesis of acute leukemia, many studies have come to a consensus that analyzing the contents of these circulating vesicles could provide a valuable perspective about the outcome of patient with leukemia. **Table 2** summarized the results of several studies evaluating the prognostic value of EVs in leukemia patients.

TABLE 2 | The correlation between leukemia-derived EVs and the outcome of patients.

Study	Results	References
AML		
Bernardi et al.	AML patients with higher exosome levels of miR-10b had shorter survival as compared to those with lower levels of miR-10b.	Bernardi and Farina, 2021
Bernardi et al.	The higher expression of exosome miR-532 in AML patients is associated with lower survival, suggesting that miR-532 can act as an independent prognostic marker in AML.	Bernardi and Farina, 2021
Fang et al.	The higher expression of miR-10b in exosomes harvested from the sera of AML patients is associated with the aggressive clinical characteristics of the disease and poorer outcomes in the patients.	Fang et al., 2020
Jiang et al.	The identification of miR-125b in EVs of AML patients is suggestive of elevated risk of disease relapse and shorter 2-years overall survival, introducing miR-125b as an independent prognostic marker.	Jiang et al., 2018
Kontopoulou et al.	Genetic analysis of EVs harvested from pediatric AML patients could be used as a tool to evaluate MRD in the patients.	Kontopoulou et al., 2020
Chen et al.	EVs encapsulating miR-1246 could increase the survival of leukemic stem cells (LSCs) in AML patients through targeting LRIG1 and STAT-3 signaling pathway and thereby induce poor outcomes in the patients.	Chen et al., 2021
Bouvy et al.	MRP-1 proteins could be delivered from chemo-resistance HL-60 cells to chemo-sensitive leukemic cells. Circulating EVs containing miR-19b and miR-20a are responsible for the induction of chemo-resistance in AML patients and thereby reduce their overall survival.	Bouvy et al., 2017
Barzegar et al.	AML-derived EVs containing MRD proteins could induce chemo-resistance against idarubicin, suggestive of the participation of EVs in the induction of poor prognosis in AML patients.	Barzegar et al., 2021
Viola et al.	BMSC-derived EVs that contain miR-155 could confer drug resistance against tyrosine kinase inhibitors in AML patients, reduce the opportunity of complete remission in patients.	Viola et al., 2016
Lin et al.	Elevated plasma exosome-derived miR-532 is associated with favorable outcomes in AML patients.	Lin et al., 2020
Jiang et al.	An increased in the expression of exosome miR-125b could increase the risk of relapse in AML patients and is associated with the reduced overall survival.	Jiang et al., 2018
Hong et al.	The reduction in the levels of plasma EV-TGFβ1 protein in AML patients who received chemotherapy is indicative of the favorable response to treatment and induction of long-term complete remission. Changes in EV-TGFβ1 levels in AML patients could be considered a prognostic and risk stratifying factor.	Hong et al., 2017
Hornick et al.	Elevated serum EV levels containing let-7a, miR-99b, -146a, and -191, is associated with poor prognosis in AML patients.	Hornick et al., 2015
Caviano et al.	EV-derived miR-155 is a well-known independent prognostic factor for AML patients. The level of this miRNA correlates with the number of WBCs and complex karyotypes in patients.	Caivano et al., 2017
ALL		
Egyed et al.	The elevation in EV containing miR-181a could be an indicator for CNS involvement for the pediatric patients with ALL.	Egyed et al., 2020
Labib et al.	The upregulation of extracellular miR-22 in pediatric ALL is associated with poor prognosis and shorter overall survival.	Labib et al., 2017
Rzepiel et al.	Detetction of miR-128-3p and miR-222-3p in blood of ALL patients could be indicator of MRD and thus far these circulating miRNAs could be considered a prognostic maker for ALL patients.	Rzepiel et al., 2019

CHALLENGES OF EXTRACELLULAR VESICLES IN THE CLINICAL APPLICATION FOR ACUTE LEUKEMIA

Given the importance of EVs in the pathogenesis of leukemia and based on the number of reports suggesting the benefits of evaluating EVs contents for early diagnosis or predicting the outcome of patients, it could be known that EVs are promising circulating biomarkers. However, the story is not as simple as it looks. The study of EVs as biomarkers in clinical approaches is still a new field, and no standard methods have been established yet for the proper enrichment and isolation of these circulating vesicles. The diversity in the protocols used for EVs isolation, enrichment, and measurement leads to the fact that in many cases, the results of studies are not comparable with each other, and this may have a negative effect on the validation of the results (Lane et al., 2018). For example, for EVs isolation, based on the available equipment and materials, methods such as differential ultracentrifugation, density gradient ultracentrifugation, polymer-facilitated precipitation, immune-affinity isolation and, size exclusion chromatography (SEC) are used. Among them, differential ultracentrifugation is considered a gold standard method for EV isolation (Théry et al., 2006). Despite the great popularity, ultracentrifugation could lead to vesicle aggregation and contamination of the protein contents of EVs (Baranyai et al., 2015; Nordin et al., 2015). Moreover, the variability in the methods in evaluating pelleting efficiency has led to the discrepancy in results obtained from different studies. To tackle the contamination problem, density gradient ultracentrifugation was developed (Kalra et al., 2013; Choi and Gho, 2015). However, the difficulty in the procedure of this method and the risk of loss of samples have muted the enthusiasm in employing density gradient ultracentrifugation (Muller et al., 2014). For the polymer-facilitated precipitation and the immune-affinity isolation, the poor purity of isolated EVs and the lack of proper antibodies have been claimed as the main factors that

could restrict the efficacy of the methods (Van Deun et al., 2014; Lane et al., 2018).

The diversity in the isolation techniques could not only produce variability in the results but also influence the precise molecular characterization of EVs. The high purity of EVs and ability to distinct the EV-derived proteins/nucleic acids from non-EV sources are essential for the proper characterization of EVs. Apart from this, thus far, several techniques, including transmission electron microscopy (TEM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), flow cytometry, and tunable resistive pulse sensing (TRPS) are used for the physical characterization of EVs. Although effective, each of these techniques also have some limitations and complications. Problems such as vesicle shrinkage and the limited number of vesicles that could be analyzed by TEM have made flow cytometry superior to TEM which is currently a gold standard method (Van der Pol et al., 2014; Erdbrügger and Lannigan, 2016). However, the application of flow cytometry is not without practical limitation, as the small size of EVs and their low refractive index result in improper scattering of the vesicles by flow cytometry (Van Der Pol et al., 2012; Nolan, 2015). The labeling of EVs with protein-dye was not successful in tackling this problem, as EVs possess a restricted amount of target molecules (Welsh et al., 2017). A similar problem could be seen in studies using DLS and NTA for the molecular characterization of EVs. Although these techniques are rapid, i.e., NTA could measure the contents of thousands of single EVs in less than a few minutes (Bi et al., 2021) and could analyze bulk samples, only larger particles with the ability to scatter the greater amount of light signal could be characterized by these methods (Anderson et al., 2013; Lane et al., 2018). The nature of TRPS is different from other methods and this non-optical measurement technique uses the electrical impedance for analyzing the physical characterization of EVs. However, the necessity of an expert user to operate this technique has made it difficult to use TRPS for the characterization of EVs (Lane et al., 2018). It should be noted that both qRT-PCR and Western blotting analysis for evaluating the miRNA/RNA and protein content of EVs could also be affected by the methods that are used for the isolation of EVs. Taken together, all mentioned limitations suggest the necessity of an appropriate protocol for EV handling and measurement. The more accurate the methods, the faster EVs could be employed in clinical settings for risk stratifying patients.

CAN EXTRACELLULAR VESICLES MEET THE CLINICAL CHALLENGES FOR RISK STRATIFICATION IN PATIENTS WITH ACUTE LEUKEMIA?

The application of EVs in risk stratification for patients with acute leukemia is beneficial, though it has a long way to be achievable. First, as mentioned earlier, due to the limitation of all mentioned isolation techniques in distinguishing exosomes from micro-vesicles, both of these cellular components referred to as circulating vesicles, which limits our understanding of the property of these circular vesicles. The less we know about the contents of circulating EVs, the longer it might take for receiving approval EVs as a diagnostic tool in clinical application (Witwer et al., 2013). Moreover, some studies revealed that the level of circulating EVs could be affected by numerous factors, and thereby, EVs varied based on the time of sample collection (Pickup et al., 2013). This finding threatens the value of EVs in the clinical application and prioritizes the importance of an optimized protocol for the collection, isolation, and storage of EVs. Additionally, many studies have thus far evaluated the efficacy and the value of EVs in *in vitro* analysis. So, experiments conducting on patient's samples are required to achieve better results and provide a wider perspective about the application of EVs as a prognostic factor in patients with acute leukemia.

CONCLUSION

From the first description of EVs in the samples of patients with leukemia, there is no doubt that these tiny circulating vesicles play fundamental roles in leukemogenesis, cell proliferation, survival, and also angiogenesis. The components of EVs conveniently alter the structure of BM in the way that it protects leukemic cells from either the adaptive arm of the immune system or anti-cancer agents. Thus far, many studies are focusing on the importance and value of EVs in determining the outcome of patients with leukemia; however, a long way should be passed to reach the best results. As it was mentioned earlier, according to the size of EVs, three classes of these circulating vesicles have been identified in body fluids and for sure, each of them may participate in some specific biological processes. However, due to the size overlap and the disability of the current isolation and characterization technologies, it is impossible to distinguish these vesicles from each other and analyze their cargos individually. Moreover, many of the results published in this area are conflicting and incomparable, as each research group might use different techniques for the collection, processing, and storage of EVs. Given these limitations, it seems that more issues should be addressed before EVs could enter into the clinical application for acute leukemia. Nevertheless, the journey of EVs in leukemia is still mesmerizing.

AUTHOR CONTRIBUTIONS

MI: conceptualization, literature survey, figure designing, review structure, writing review and editing, and references collection. ZH: conceptualization, literature survey, review structure, writing review and editing, and references collection. FJ: literature survey, writing review, and references collection. AH and AG: critical review and editing. Y-DL: formating and reference collection.

LJ: conceptualization, literature survey, writing review and editing, references collection, tables preparation, supervision, and correspondence. Z-SC: critical review and editing, correspondence, and supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Biology and Function of Extracellular Vesicles in Cancer Development

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Extracellular vesicles (EVs) exert their biological functions by delivering proteins, metabolites, and nucleic acids to recipient cells. EVs play important roles in cancer development. The anti-tumor effect of EVs is by their cargos carrying proteins, metabolites, and nucleic acids to affect cell-to-cell communication. The characteristics of cell-to-cell communication can potentially be applied for the therapy of cancers, such as gastric cancer. In addition, EVs can be used as an effective cargos to deliver ncRNAs, peptides, and drugs, to target tumor tissues. In addition, EVs have the ability to regulate cell apoptosis, autophagy, proliferation, and migration of cancer cells. The ncRNA and peptides that were engaged with EVs were associated with cell signaling pathways in cancer development. This review focuses on the composition, cargo, function, mechanism, and application of EVs in cancers.

Keywords: EVS, Cancer, ncRNA, drug loading, target

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INTRODUCTION

EVs are 40–100 nm extracellular vesicles that are released by cells (Kahlert and Kalluri, 2013). EVs were initially observed in sheep reticulocytes in the 1980s (Raposo and Stoorvogel, 2013). Recently, studies have focused on the source of their endocytosis and on distinguishing them from micro-vesicles (Théry et al., 2002). EVs have anti-tumor functions associated with the development of a variety of cancers, such as breast, stomach, liver, and lung cancers (**Table 1**).

The Biogenesis and Composition of EVs

Mammalian cell, EVs are highly heterogeneous. They contain lipid membranes, proteins, RNAs, and DNAs (Kowal et al., 2016). The lipid membrane of EVs carries the ligands and receptors from the source cells and has a role in cell-to-cell communication (Valadi et al., 2007; Kahlert et al., 2014). Due to the specificity of the lipid membrane, EVs can invade target cells through biogenesis (Balaj et al., 2011). The components on the membrane also play a key role in cell-to-cell communication (Wu et al., 2021). EVs use lipid membranes to enter recipient cells to release cargo and affect recipient cells. These characteristics indicate that EVs have potential applications in regulating cancer development.

The Formation of EVs

Many EVs formed from normal and pathological cells. In contrast to micro-vesicles, EVs are mainly derived from multivesicular bodies (MVBs) that are formed by intracellular lysosomal particles. EVs are released into the extracellular matrix through the fusion of the outer membrane of the MVBs with

TABLE 1 | The function of EVs in cancers.

Name	Fatality rate (%)	Function of EVs	References
Lung cancer	89	Diagnosis	Kahlert and Kalluri, (2013)
Liver cancer	60–70	Inhibited cell growth	Raposo and Stoorvogel, (2013)
gastric cancer	12.4	Induce cell apoptosis	Théry et al. (2002)
Colon cancer	12	Inhibited EMT	Kowal et al. (2016)
Breast cancer	6.6	Plasma biomarkers	Kahlert et al. (2014)

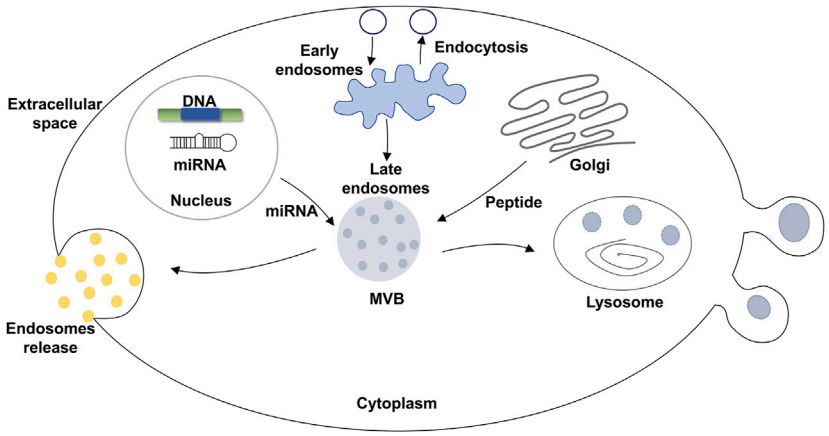

FIGURE 1 | Formation of EVs.

the membrane of source cells (**Figure 1**). Specifically, EVs are formed through the endosomal pathway. First, the endosome is formed by the invasion of the plasma membrane during cell maturation process (Harding et al., 1983). The endosome is a membrane-encapsulated vesicular structure and includes both early and late endosomes. Early endosomes are usually located outside of the cytoplasm. In contrast, late endosomes are located inside of the cytoplasm, near the nucleus. Endosomes are acidic vesicles without lysosomal enzymes (Bainton and Farquhar, 1968). The invasion of endosomes produces MVBs which contain 40–150 nm vesicles. The inner membrane forms intraluminal vesicles (ILV). Finally, the late lysosome melts or fuses with the plasma membrane of the source cell and degrades MVBS to release EVs (Harding et al., 1983). This process is known as EV biogenesis and is different from apoptotic bodies (Taylor and Gercel-Taylor, 2008). EVs are widely observed in tumor cells, mesenchymal stem cells, fibroblasts, neurons, endothelial cells (ECs), and epithelial cells (Kalluri, 2016). Previous reports have suggested that the tumor cells can specifically absorb their own secreted EVs (Kahlert and Kalluri, 2013). This implies that during the formation of EVs, specific biomarkers are formed on the surface of the EVs. These biomarkers are the cues that render EVs to be absorbed by specific cells.

EVs Cargo

Nucleic acids such as DNAs or RNAs, proteins, or drugs can be carried in EVs as cargo to be delivered for cell-to-cell communication (**Figure 2**). In the past decades, miRNAs and mRNAs have been found to be major components of EVs. The improvement of EV detection techniques has allowed more RNA species, including transfer RNAs (tRNAs), long non-coding RNAs (lncRNAs), and viral RNAs, to be observed (Valadi et al., 2007; Su et al., 2021). An increasing amount of data suggests that these RNAs, such as lncRNA, have crucial functions that affect the development of cancer cells (Gusachenko et al., 2013). Moreover, numerous studies have demonstrated that the abnormal expressions of miRNAs, lncRNAs, and mRNAs are associated with cancer development (Chan and Tay, 2018; Huang et al., 2020). Hence, these RNAs, that are contained within EVs, can either preserve or degrade their target genes.

Cancers develop because of the expression and interaction of numerous genes or proteins. EVs can express proteins through genetic engineering (Silva et al., 2021). The EVs were obtained from the source cells that were transfected with the target gene plasmids. These EVs contain the synthesized proteins or peptides through cell culture (Perin et al., 2011). There is evidence that fusing the exosomally-enriched membrane protein (Lamp 2b) with the ischemic myocardium-targeting peptide (IMTP) can be used to inhibit cancer development by molecular cloning lentiviral packaging protocols (Fernández et al., 2002). EVs secreted by tumor cells can be taken up by the same tumor cell with specificity. Some molecules (such as Let-7a) can be easily introduced to donor cells through EVs, and tumor targeting EVs carrying these molecules can be used for cancer treatment (Wu

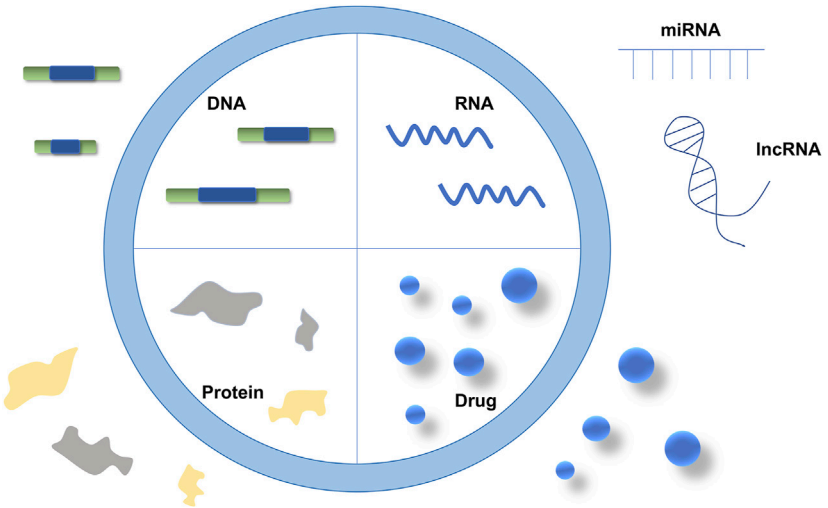

FIGURE 2 | The contents of EVs.

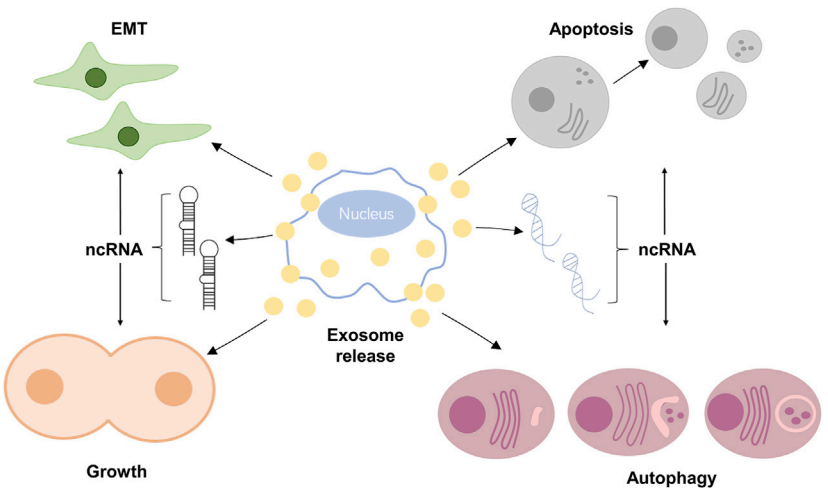

FIGURE 3 | EVs decide cell fate.

et al., 2021). In addition, EVs can carry various chemotherapeutic drugs and materials for targeted treatment of cancers (Wang et al., 2019a).

EVs can Decide Cell Fate

The function of EVs depends on the source cells, such as tumor cells or stem cells (Draganov et al., 2019; Dzobo et al., 2020). The EVs released from these source cells can affect the apoptosis, growth, cell cycle, migration, invasion, and differentiation of recipient cells. Previous studies have indicated that tumor-released EVs could deliver genetic information to the recipient cells for cell-to-cell communication (Valadi et al., 2007). This process promotes cell growth, invasion, and active angiogenesis in a tumor microenvironment (**Figure 3**).

Initially, EVs were considered to be "garbage bags" that could not affect other cells (Kalluri, 2016). However, it was found that EVs could be absorbed by target cells and their cargos could be released to affect cell signaling transduction, therefore determining the fate of the recipient cells (Pan et al., 1985). Additional evidence suggested that tumor cells released EVs that promoted tumor growth and invasion *in vivo* (Ramírez-Ricardo et al., 2020). EVs that carried tumor suppressors, such as let-7a, could inhibited tumor growth (Melo et al., 2014).

The Function of EVs in Cell Proliferation

Indefinite proliferation is a key feature of tumor cells. The abnormal cell cycle of tumor cells is associated with uncontrolled cell growth. Previous reports confirmed that miRNA-122 was involved in the cell cycle as well as the proliferation of hepatocellular carcinoma (HCC) cells (Fernández et al., 2002; Xu et al., 2011). A recent report showed that the EVs carrying circRNA plays a role in the

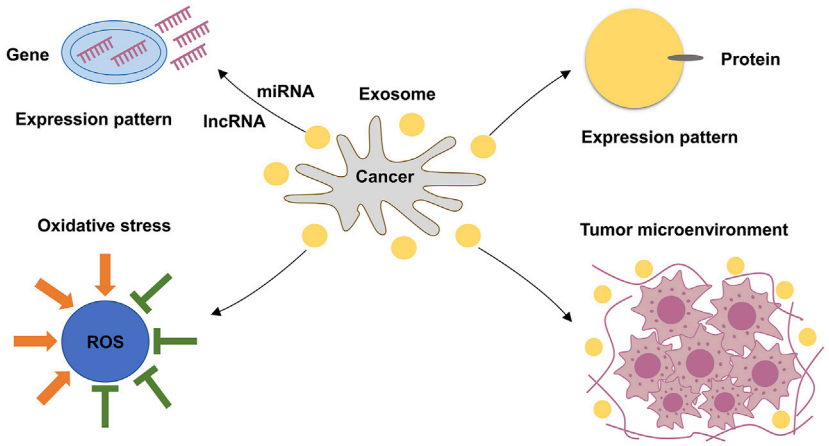

FIGURE 4 | The function of EVs.

proliferation of HCC cells (Xue et al., 2017). In addition, arsenite could increase the expression of circRNA_100284 carried by EVs, altering the cell cycle and their proliferation by acting on miR-217 (Lu et al., 2015). The expression of the cell proliferation biomarkers E2H2 and cyclin D1 were regulated by the circRNA_100284 contained within EVs, and the expression of circRASSF2 was increased in laryngeal squamous cell carcinoma (LSCC) tissue compared to paracancerous tissue. The circRASSF2 carried by EVs promoted LSCC cell growth via the miR-302B-3p/IGF-1R axis (Tian et al., 2019). Thus, EVs have the ability to regulate cell proliferation through their cargos.

The Function of EVs in Epithelial-Mesenchymal Transition

The cell-to-cell communication in tumors might promote EMT of cancers. Previous data has shown that the EV-released circRNA PED8A was associated with increased lymphatic invasion, TNM staging, and low survival rate of patients. Furthermore, the circRNA PED8A from EVs promoted tumor cell growth by activating MET, which is a tyrosine kinase receptor (Luna et al., 2019). In addition, the release of circRNA PED8A contained within EVs into the blood circulation promotes invasion and metastasis through the MACC-MET-ERK or AKT pathway. More evidence indicated that EV-released circRNA NRIP1 promoted proliferation, migration, and metastasis through AKT1/mTOR signaling pathway in gastric cancer. The involvement of this pathway has also been confirmed in breast cancer cells in patients (Wang et al., 2019b; Zhang et al., 2019). The circPTGR1 carried in EVs was found to contribute to the metastasis of hepatocellular carcinoma (Wang et al., 2019c). Interestingly, knock out of circPTGR1 in the source cells, their EVs inhibited invasion and migration of cancer cells. The increased expression of EV-released circ-IARS is related to the EMT of pancreatic cancer (Li et al., 2018). Therefore, EVs can act as messenger vehicles for cell-to-cell communication, releasing ncRNAs that contribute to the EMT in cancers.

The Function of EVs in Apoptosis and Autophagy

Cell apoptosis and autophagy are programmed cell death, both of them are abnormal in cancers. Previous reports have indicated that EVs containing anti-tumor drugs can induce cell apoptosis in HCCs (Slomka et al., 2020). Furthermore, EVs containing miRNA mimics such as let-7a have been found to induce cell apoptosis in breast cancer (Ahmed et al., 2021). In addition, EVs have the ability to regulate autophagy. There is evidence that EVs can enhance autophagy in glioblastoma (GBM) (Pavlyukov et al., 2018). These findings suggest that EVs play a role in cell apoptosis and autophagy.

EVs Stimulate Oxidative Stress

Studies have shown that low levels of reactive oxygen species (ROS) were observed in the stem cells of liver cancer and breast cancer (Shi et al., 2012). The EVs of SV-HUC-1 cells were found to mediate the P38/NF-kB signaling pathway, enhancing the levels of OS (Xi et al., 2020). This suggests that EVs were involved in OS, that may contribute to the development of cancers (**Figure 4**).

EVs Regulate the Expression of lncRNA

LncRNA usually acts as a regulator of nuclear transcription factors (Wu et al., 2021). An increasing amount of data has shown that long non-coding RNAs (lncRNAs) are associated with the development of cancers (Huang et al., 2021a). EVs containing lncRNA-APC1 inhibited tumor growth in colorectal cancer (CRC). lncRNA-APC1 is an important mediator of APC development through the APC1/RAB5B axis (Wang et al., 2021). The increased expression of lncRNA H19, which is normally regulated by DNA methylation, was observed in numerous cancers (Yang et al., 2021). Previous studies have suggested that EV-contained H19 promotes cell migration and invasion in CRC (Ren et al., 2018). The abnormal expression of *XIST*, a key factor in the X chromosome inactive (XCI) process, was observed in gastric cancer (Chen et al., 2016; Huang et al.,

TABLE 2 | The miRNA of EVs in cancers.

EVs source	miRNA	Mimics/Inhibitor	Function	Cancer	References
LIM1863 cells	miR-106b-3p	Mimics	Inhibits cell growth	CRC	Valadi et al. (2007)
LIM1863 cells	miR-126–3p	Inhibitor	Inhibits metastasis	Breast cancer	Balaj et al. (2011)
LIM1863 cells	miR-126–5p	Mimics	Inhibits EMT	Prostate cancer	Wu et al. (2021)
LIM1863 cells	miR-355–3p	Mimics	Inhibits cell growth	CRC	Harding et al. (1983)
Urine	FOLH1	Mimics	Diagnostic	Prostate cancer	Bainton and Farquhar, (1968)
Urine	HPN	Mimics	Diagnostic	Prostate cancer	Bainton and Farquhar, (1968)
Urine	ITSN1	Mimics	Diagnostic	Prostate cancer	Bainton and Farquhar, (1968)
Urine	CFD miR-21	Inhibitor	Diagnostic	Prostate cancer	Bainton and Farquhar, (1968)
PDAC cell lines	miR-195	Mimics	Diagnostic	PDAC	Taylor and Gercel-Taylor, (2008)
PDAC cell lines		Mimics	Diagnostic	PDAC	Taylor and Gercel-Taylor, (2008)

2021a; Huang et al., 2021b). EV-contained *XIST* was found to stimulate cell growth in breast cancer (Xing et al., 2018).

To investigate the role of EVs that contained lncRNAs in cancers, appropriate EVs were collected. The EVs were mostly obtained from the cells that were enriched in expressed lncRNA, such as the A549 cell line which exhibited increased H19 expression (Hao et al., 2017). In addition, the EVs were cultured in an environment that encouraged the increased expression of lncRNAs (Born et al., 2020).

EVs Regulate the Expression of miRNA

In contrast to lncRNAs, miRNAs are 20–22 nucleotides long. Both miRNAs and lncRNAs are single-stranded, endogenous RNAs, and play roles in the development of cancers. Some miRNAs, such as let-7a and the miR29 family, are involved in EMT, metastasis, migration, invasion, cell cycle, proliferation, and apoptosis of numerous cancers (Rostas et al., 2014; Song et al., 2020). A few miRNAs have been confirmed to be post-transcriptional regulators for target mRNAs. They can be used as the potential biomarkers for classification, prognosis, chemotherapy, and radiotherapy resistance in triple-negative breast cancer (TNBC) (Ding et al., 2019). Results show that miRNA of EVs have a curing effect on breast cancer (Ohno et al., 2013). MiRNAs can be coated by EVs and delivered to target cells, affect the H19/MAPK/ERK pathways (Ding et al., 2018; Wu et al., 2021).

A database indicated that EVs are enriched in miRNAs, lncRNAs, and proteins (Berardocco et al., 2017). In contrast to transfected mimics or miRNAs inhibitors, EVs that obtained from source cells can specifically and accurately deliver these miRNAs endogenously (**Table 2**). Considering the characteristics of EVs, therapies using EVs could be a potential approach for cancer treatment.

EVs Regulate Gene Expression by siRNA

SiRNAs are produced by short, exogenous double-stranded RNAs (dsRNAs) as an RNA interference (RNAi) tool (Kim et al., 2018; Dharamdasani et al., 2020; Feng et al., 2020). SiRNA can be used to effectively silence target genes. A recent study showed that the use of siRNA, such as siRNA-027 can inhibit cell growth and induce apoptosis in numerous cancers (Chen et al., 2020). Hence, siRNA can be used to potentially analyze the development of cancers. A barrier to the RNAi-based therapy of cancers is the low specificity of siRNA delivery. EVs are nano-scale vesicles that can be used to deliver siRNAs as cargos to the target cells by cell-to-cell communication. Previous reports have suggested that the EVs of human plasma cells can deliver siRNA to monocytes and lymphocytes that can silence the expression of mitogen-activated protein kinase 1 (Wahlgren et al., 2012). This suggests that EVs can be used as gene delivery vehicles (GDV) to transport exogenous siRNA in cancer research. Consequently, EVs combined with siRNA are more effective and demonstrate higher specificities than traditionally siRNA delivery in cancer treatment.

EVs Regulate the Expression of Protein

The mitochondrial proteins contained in EVs can promote tumorigenesis by cell-to-cell communication (Al-Nedawi et al., 2008; Demory Beckler et al., 2013). The expression of MET (also known as hepatocyte growth factor receptors) associated with circulating EVs and phosphorylated MET (Tyr1349) was increased in patients with stage 3 and stage 4 melanoma compare to control (Peinado et al., 2012). This finding indicates that EVs can be used to detect the development of cancer (Costa-Silva et al., 2015). This assumption was confirmed when the expression of MIF and GPC-1 proteins in EVs was detected in cancer patients, allowing them to analyze the prognosis of cancer (Melo et al., 2015). Furthermore, phospholipid-binding proteins-carrying EVs can inhibit cell growth and induced apoptosis in numerous cancers (Dhondt et al., 2020). Thus, the proteins contained in EVs were useful for the detection and prognosis of cancers.

The Function of EVs in the Tumor Micro-environment

EVs are a key component of the tumor microenvironment. Tumor heterogeneity includes genomic heterogeneity in both tumor cells and non-cancerous microenvironments. Moreover, the tumor nanoenvironment (TNE) is a special nano-scale tumor microenvironment that possesses complex structures and unique components (Eguchi et al., 2018). The TNE includes EVs and apoptotic bodies. EVs released by tumor cells were absorbed by other cells in the tumor microenvironment, influencing the development of cancer through tumor heterogeneity (Tredan et al., 2007). EVs thus contribute to the formation of the tumor microenvironment in the form of cell-to-cell communication.

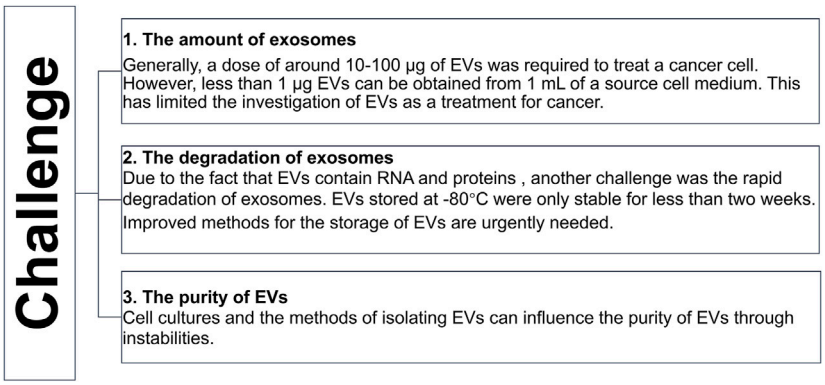

FIGURE 5 | The challenge of EVs.

DISCUSSION

Considering that EVs can carry any cargos, including nucleic acids and proteins, EVs can thus be used as clinical diagnostic biomarkers. For example, the detection of tumor-specific RNAs in EVs can be used as biomarkers for cancer diagnosis (Gurunathan et al., 2019). Furthermore, proteins contained within EVs such as TSG101, RAS-related protein RAB-11B (RAB11B), CD63, and CD81 can be used as biomarkers for diagnosis of HCCs and other cancers (Möbius et al., 2003; Valadi et al., 2007). In contrast to traditional diagnostic methods such as peripheral blood or histopathology, the accuracy and specificity of EVs were more closely associated with the development of cancers.

EVs can be combined with engineered materials to specifically affect cancer cells. Gold nanoparticles (AuNPs) can mediate photothermal therapy (PPT) to inhibit cell growth and induce cell death (Hu et al., 2020). However, most AuNPs have low specificity. EVs combined with AuNPs can increase their specificity and accelerate the release of their cargos, enhancing the anti-tumor effect of PTT (Nasseri et al., 2020). This could be an important form of therapy for the treatment of cancers in the future. Due to the endogenous nature of EVs, their cargos can escape the immune system and accurately and effectively target tumor cells. In addition, as nano-vesicles, EVs can bypass the blood-brain barrier (Yin et al., 2012). The EVs of immature dendritic cells have been engineered to contain proteins that can target tumors originated from the neuroendothelial and nerve cells in the brain (Federici et al., 2014). Therefore, EVs as nano-vesicles can be used to cross the blood-brain barrier in cancer treatment.

EVs containing anti-cancer drugs, such as therapeutic agents, can be used in the treatment of cancers. In contrast to liposomes, EVs injected *in vivo* can be absorbed without the interference of the immune system (Ferguson and Nguyen, 2016; Kalluri, 2016; Barile and Vassalli, 2017; Fitts et al., 2019; Liao et al., 2019). Furthermore, EVs are safe and are tolerable *in vivo*. Recent studies have demonstrated that repeatedly injected mesenchymal cells (MHC) or the IPCs of EVs do not induce toxicity (Zhu et al., 2017; Mendt et al., 2018).

The EVs that carry chemotherapeutics can decide the cell fate by cell-to-cell communication. For example, αv integrin-specific EVs have been shown to have a therapeutic effect on breast cancer (Tian et al., 2014). Another report suggested that paclitaxel surrounding the EVs of macrophages inhibited lung cancer growth in mice (Kim et al., 2016). These reports indicated that chemotherapeutic agent encapsulating EVs have an anti-tumor effect. Recently, studies have shown that the bioavailability of EVs-engineered doxorubicin was improved compared to the free doxorubicin (Tian et al., 2014; Kojima et al., 2018). These studies suggested that as a vesicle, EVs can enhance the efficacy of drugs. Despite the advancements in the understanding of EVs, there are still some challenges that need to be solved (**Figure 5**).

CONCLUSION

EVs are derived from multivesicular bodies formed by intracellular lysosomal particles that are released into the extracellular matrix. The source cells determine the specificity of their EVs. EVs contained RNAs, proteins, and drugs that can play important roles in the development of cancers. EVs have the ability to decide the fate of cells by cell-to-cell communication. EVs have potential applications in anti-cancer treatments in the future.

AUTHOR CONTRIBUTIONS

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

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Early Detection and Investigation of Extracellular Vesicles Biomarkers in Breast Cancer

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Breast cancer (BC) is the most commonly diagnosed malignant tumor in women worldwide, and the leading cause of cancer death in the female population. The percentage of patients experiencing poor prognosis along with the risk of developing metastasis remains high, also affecting the resistance to current main therapies. Cancer progression and metastatic development are no longer due entirely to their intrinsic characteristics, but also regulated by signals derived from cells of the tumor microenvironment. Extracellular vesicles (EVs) packed with DNA, RNA, and proteins, are the most attractive targets for both diagnostic and therapeutic applications, and represent a decisive challenge as liquid biopsy-based markers. Here we performed a study based on a multiplexed phenotyping flow cytometric approach to characterize BC-derived EVs from BC patients and cell lines, through the detection of multiple antigens. Our data reveal the expression of EVs-related biomarkers derived from BC patient plasma and cell line supernatants, suggesting that EVs could be exploited for characterizing and monitoring disease progression.

Keywords: breast cancer, extracellular vesicles, plasma, liquid biopsy, biomarkers

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed malignant tumor in women worldwide, and the leading cause of cancer death in the female population. Although it has been calculated that in Europe, between 2014 and 2019, cancer mortality rate has declined steadily by about 8.7% (Malvezzi et al., 2019), an incidence of 2,261,419 cases and 684,996 deaths were reported

in 2020, remaining an alarming concern for public health (Sung et al., 2021). In fact, despite improved clinical management resulting in better prognosis, up to 30% of node-negative BC patients and a larger part of patients with node-positive carcinoma, develop distant metastases after several years from the time of primary tumor detection and surgical resection (Barone et al., 2020). This provides only a relatively poor chance for successful treatments and survival, and identification of new molecular markers for diagnosis and prognosis, especially in a metastatic setting, and for development of innovative therapeutic molecules, are necessary. Furthermore, BC is characterized by a considerable tissue heterogeneity, showing distinct clinical and biological features, which make tumors respond differently to treatments and adverse in their management. In the last years, molecular profiles have been largely explored, providing a well-established classification of BCs into four well-settled subtypes: Luminal A, Luminal B, Basal-like, and human epidermal growth factor receptor 2 (Her2)-enriched. In addition, BC staging also provides useful information about appropriate treatment options, due to its ability to estimate prognosis at each tumor stage. In particular, the Tumor-Node-Metastasis (TNM) system represents an attempt to classify cancer based on the major morphological attributes of malignant tumors that were thought to influence disease prognosis: size of the primary tumor (T), presence and extent of regional lymph node involvement (N), and presence of distant metastases (M) (Singletary and Connolly, 2006; Bandini and Fanini, 2019).

Neovascularization has become a pivotal aspect of tumor and metastasis growth, involving endothelial cell (EC) proliferation, migration, and vascular formation (Chen et al., 2019). In the last years, research has narrowed its attention to the study of the tumor microenvironment (TME) as a target for cancer therapy. In fact, chemoresistance of tumor cells and the development of metastases are no longer due entirely to their intrinsic characteristics, but are also regulated by signals derived from cells of TME. Secreted factors from cancerous cells enable the recruitment of several types of cells required to form the TME, contributing to the formation of a premetastatic niche and to development of chemoresistance (Madden et al., 2020). Tumor stromal cells, including fibroblasts, immunoinflammatory cells, vascular EC and other components of TME, as well as the extracellular matrix, not only play a crucial role in cancer response to therapies, but also orchestrate cancer proliferation, invasion, and metastasis. In particular, ECs are the building pillars of vessels and as such are key players in sprouting angiogenesis (Draoui et al., 2017). Recently, several models and analysis tools have been developed to investigate the crosstalk between mammary cells and neighboring vascular ECs, in order to explore their potential applications in basic research and drugs development. In fact, it could be useful to establish new approaches to develop anti-angiogenic strategies, which represent the few available therapies against the most aggressive BCs (Devadas et al., 2019; Kourti et al., 2020).

Since most of the current methods used for diagnosis and prognosis of cancer are expensive, invasive, and time consuming, new diagnostic panels need to be investigated to make the process less invasive, more cost-effective, and rapid. Among the most promising potential diagnostic targets, extracellular vesicles (EVs) are nanometer-sized, lipid membrane-enclosed vesicles released by many types of cells and classified by different size, components, and functions (Xue et al., 2021). EVs are normally distinguished into three main classes: microvesicles produced through outward budding and fission of the plasma membrane, exosomes derived from endosomes and fusion of multivesicular bodies with the plasma membrane, and apoptotic bodies released as blebs from apoptosis undergoing cells (Wu et al., 2019). Importantly, EVs are the most attractive targets for both therapeutic and diagnostic applications, especially because they are enriched in a large batch of body fluids such as breast milk (Shah et al., 2021), blood plasma (Hu H. et al., 2021), saliva (Hoshino, 2021), urine, serum (Salvi et al., 2021), and cerebrospinal fluid (López-Pérez et al., 2021), becoming an excellent source of potential biomarkers. All cell types are expected to secrete EVs, but their main functions remain to be fully understood. In particular, exosomes are membrane-bound vesicles, 50–200 nm in size, secreted from cells *via* a multivesicular-body endocytic process. This vesicles population has been proposed to perform main functions, among which they are counted to support processes to eliminate DNA, RNA, or protein content that could be detrimental to cell viability, to maintain a cell-to-cell communication system by delivering cargo to a recipient cell, or even to develop a mechanism for surveying cell content for viral infections (Sempere et al., 2017; Jayaseelan, 2020). EVs are enriched in proteins involved in the vesicles' trafficking, cell surface receptors such as tumor susceptibility gene 101 (TSG101), integrins and a number of tetraspanins such as CD9, CD53, CD63, CD81, and CD82 (Burgio et al., 2020). The study of exosomes is relatively difficult and, as referred by the International Society of Extracellular Vesicles (ISEV), the assignment of a specific biogenesis pathway to EVs remains not easy to establish as it could be validated only through a live imaging assay of EV release. Accordingly, due to current technical limitations, almost all studies are unable to isolate and investigate a pure population of exosomes (Romano et al., 2021). Despite being a validated source of biomarkers, liquid biopsy (LB) has not yet succeeded in becoming part of the standard clinical practice in BC patients (Chan et al., 2021). The deepening of isolation and analysis of EVs is essential for understanding their biological roles and for investigating their potential clinical use. Several methods have been developed thus far, but with some limitations (Jong et al., 2017).

The present work aimed at identifying new BC tumor biomarkers through an easy and fast approach, based on a

multiplexed phenotyping of EVs released from 30 plasma samples of 10 BC patients. Second, results obtained from patients revealed adhesion molecules markers usually present on the surface of circulating endothelial cells, prompting us to investigate also cell models. Through the analysis of supernatant of BC cell lines, cultured alone or with ECs, we aspired to find clues regarding: 1) EV origin subtypes comparing biomarkers found in plasma and in BC cell cultures and 2) cancer-normal cell interplay, detecting potential marker expression changes in co-culture conditions. EVs were isolated by size exclusion chromatography (SEC) and characterized by a bead-based cytofluorimetric method able to simultaneously detect 37 surface exosomal-related proteins.

MATERIALS AND METHODS

Cell Cultures

The human breast carcinoma cell lines, MDA-MB 453 (Her2-enriched subtype) and MCF-7 (Luminal A molecular subtype) were purchased from ATCC (ATCC; Manassas, Virginia, United States), and cell lines T-47D (Luminal A molecular subtype) and HUVEC (Normal Primary Human Umbilical Vein Endothelial Cells) were purchased from zooprophylactic Institute of Genova (Italy). MDA-MB 453 were maintained in Leibovitz's L-15 medium (ATCC 30-2008, United States). MCF-7 were maintained in EMEM medium (ATCC 30-2003). T47D were maintained in DMEM High Glucose (Euroclone, Italy). HUVEC were maintained in M199 medium (Sigma Aldrich, Merck, Germany). Each medium was supplemented with FBS exosome-depleted (Gibco, Thermo Fisher Scientific, United States) to a final concentration of 10%, according to the information sheet of the manufacturer. Penicillin-streptomycin (PAA, Carlo Erba Reagents, Italy) to a final concentration of 1% and MycoZap Prophylactic (Lonza Group Ltd., Switzerland) to a final concentration of 0.002% were added to all media. The cultures were maintained in an incubator Heraeus, in an 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 the sub-cultivation of cell lines by using Trypsin-EDTA (Life Technologies, United States). Cell lines were tested every 2 months with MycoAlert™ *Mycoplasma* Detection Kit (Lonza Group Ltd., Switzerland) to check a possible contamination by *mycoplasma*.

Patient Sample Collection

The study was conducted on 21 individuals: 10 BC patients diagnosed with early-stage BC enrolled between 2013 and 2014, as well as 11 healthy donors. The samples were enrolled at the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRST. Peripheral whole blood was collected at three time points: 1 day before surgery (A), 1 month after surgery (B), and after adjuvant therapy/6 months after surgery (C). None of the patients underwent neoadjuvant therapy or had detectable metastasis at diagnosis. Histological and clinical characteristics are listed in **Table 1**. Written informed consent was obtained from all subjects before sample analyses. The study was approved by the Ethical Committee of our Institute, Romagna Ethics Committee (CEROM) of Meldola (IRSTB008) and conducted in accordance with the Declaration of Helsinki. Healthy donors were enrolled at the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRST and were matched to BC patients for age classes (all female with an average age of 55).

Plasma and Supernatant Collection

Approximately 5 mL of whole blood were collected in EDTA tubes and centrifuged at 1,000 × g for 15 min, followed by a second centrifugation at 1,500 × g 10 min for obtaining plasma. The blood was collected from all individuals before any surgical intervention, 1 month after surgery and after adjuvant therapy/6 months after surgery. Plasma samples were conserved at −80°C until use. Approximately 30 ml of supernatant of BC controls and co-cultured cells were collected 48 h post co-culture and immediately processed to isolate EVs.

Co-Culture Experiments

Transwell Permeable Supports (Corning, United States) with a 0.45 μm polycarbonate membrane were used in the co-culture model system to separate BC and HUVEC cells

TABLE 1 | Clinical pathological characteristics of patients. Tumor stage was reported based on the tumor (T), lymph node (N), and metastasis (M) system.

Patient	Age	Subtype	Histology	T	N (positive/asported)	M	Grade	Vascular invasion
1	57	TNBC	Ductal infiltrant	2	0 (1)	0	3	Yes
2	75	LumA	Ductal infiltrant	1b	0 (2)	0	2	No
3	43	TNBC	Ductal infiltrant	1c	1a (1/35)	0	3	No
4	58	TNBC	Ductal infiltrant	2	1a (2/28)	0	3	Yes
5	59	LumA	Ductal infiltrant	1b	0(1)	X	3	Yes
6	43	TNBC	Ductal infiltrant	1c	0 (1)	X	3	No
7	53	LumA	Ductal infiltrant	2	1a (1/15)	X	2	Yes
8	57	LumA	Ductal infiltrant	1b	0 (1)	X	1	No
9	59	LumA	Lobular	1c	0 (2)	0	2	No
10	54	LumA	Ductal infiltrant	1c	0 (1)	X	2	No

into different compartments. HUVEC cells were seeded into a six well plate (lower chamber) and an equivalent number of BC cells (ratio 1:1) were seeded into the transwell insert, which was then placed directly over the 6-well plate containing the HUVECs. BC and HUVEC controls were seeded separately into a six well plate. All cells were maintained in FBS exosome-depleted medium. Two independent experiments were performed and all the 3 cell lines were used (control groups, $n = 3$ and co-cultured groups, $n = 3$). Cells were incubated for 48 h and then washed in PBS 1X and harvested for further analysis.

Isolation of EVs From Cell Culture Medium and Plasma of BC Patients

30–40 mL of supernatants from BC and HUVEC cells containing exosome-depleted FBS (Gibco, Thermo Fisher Scientific, United States) were collected after 48 h co-culture, centrifuged at 300 × g for 10 min, filtered by 0.22 μm syringe to exclude cell debris and further purified by centrifugation for 15 min at 1,000 × *g* and for 15 min at 2,000 × *g*. Subsequently supernatants were concentrated through Centricon Plus-70 centrifugal filter devices (Merck Millipore, Darmstadt, Germany). For BC patients, 500 μL of plasma were used. For EVs isolation, qEV10 Size Exclusion Columns (70 nm, Izon Science) were used. After rinsing the columns with PBS 1X, 300–500 μL of concentrated culture medium were applied on the top of a qEV column and 0.5 mL fractions were collected. Four vesicles-enriched fractions (7–10) were firstly analyzed, then EVs content analysis was performed on fraction 8 after nanoparticle tracking analysis (NTA) evaluation.

Nanoparticle Tracking Analysis

NTA was used to determine particles size and estimate number/ml of isolated EVs from subjects and cell lines. EVs were characterized by NTA with a NanoSight NS300 (Malvern Instruments, United Kingdom), equipped with NTA 2.3 analytical software laser. Five 30 s videos were recorded per sample in light scatter mode with a camera level of 14 and from these the software calculated the mean and the mode diameter (nm) and EV concentrations. Software settings for analysis were kept constant for all measurements. All samples were diluted in 0.1 μm filtered PBS to an appropriate concentration before analysis. Based on the data obtained at NTA, which highlighted the fraction eight to be more concentrated and homogenous, we proceeded with downstream analyses with fraction eight for all the samples. Data were analyzed with the NTA version 2.3.

Extraction of Proteins

Proteins were concentrated from the fraction eight of EVs obtained from plasma of patients and from supernatant of cell lines. Then 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 manufacturer's protocol. The lysates were centrifuged at 4°C at 13,000 × g for 30 min. Then, the supernatant was transferred to another tube. Proteins were subsequently quantified following the protocol of the BCA Protein Assay (Pierce, Thermo Fisher Scientific, United States) and using a Multiscan EX microplate reader (Thermo Fisher Scientific, United States), with a wavelength filter of 490 nm.

Protein Expression Analyses

Western blotting was used to evaluate the expression of the exosome markers CD9, Alix, CD81, TSG-101, and Calnexin. Twenty μg of proteins were denatured and separated by electrophoresis using 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. The electrophoretic run was performed at a constant voltage of 180 V in a TRIS/*Glycine*/SDS 1X buffer (Bio-Rad Laboratories, CA, United States). Proteins were then transferred onto a PVDF membrane (Trans-Blot Transfer Turbo midi-format 0.2 μm; Bio-Rad Laboratories) 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 Laboratories, CA, United States) at 0.1% and 1X Dulbecco's Phosphate Buffered Saline (Invitrogen, Thermo Fisher Scientific, United States) supplemented with 5% milk powder (Blotting Grade Blocker Non-Fat Dry Milk; Bio-Rad) in order to facilitate the saturation of non-specific binding sites. Primary antibodies and dilutions used are the following: CD9 (D8O1A, Cell Signaling, United States) 1:1,000, Alix (3A9, Cell Signaling, United States) 1:1,000, CD81 (D4, Santa Cruz, United States) 1:1,000, TSG-101 (T5701, Sigma-Aldrich, Merck, Germany), 1:1,000 and Calnexin (2,433, Cell Signaling, United States) 1:1,000. Secondary antibodies and dilutions used are the following: Goat anti-rabbit IgG-HRP and Goat anti-mouse IgG-HRP (Santa Cruz, United States) 1:5,000, Precision Plus Protein Western C StrepTactin-HRP Conjugate (Bio-Rad Laboratories, CA, United States) 1:10,000. Blocking and immunological reactions were performed in accordance with the protocol Western Immunoblotting of Cell Signaling. Images were developed through the SuperSignal West Femto (Pierce, Thermo Fisher Scientific, United States) or Clarity West-ern ECL Substrate

(Bio-Rad Laboratories, CA, United States) and acquired through Chemidoc (Bio-Rad Laboratories, CA, United States).

Bead-Based Multiplex Exosome Flow Cytometry Assay

Samples were subjected to bead-based multiplex EV analysis by flow cytometry (MACSPlex Exosome Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany). The MACSPlex Exosome Kit (Miltenyi Biotec, Germany) allows the detection of 37 exosomal surface epitopes (CD3, CD4, CD19, CD8, HLA-DR, CD56, CD105, CD2, CD1c, CD25, CD49e, ROR1, CD209, CD9, SSEA4, HLA-BC, CD63, CD40, CD62 P, CD11c, CD81, MCSP1, CD146, CD41b, CD42a, CD24, CD86, CD44, CD326, CD133/1, CD29, CD69, CD142, CD45, CD31, CD20, and CD14) plus two isotype controls (REA and IgG1). The MACSPlex Exosome Detection Reagents for CD9, CD81, and CD63 were used to label the captured EVs. EV-containing samples were processed as follows: vesicles were diluted with MACSPlex buffer (MPB) to a final volume of 120 µL, then 15 µL of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) were added to each sample. One negative/blank control (MACSPlex Buffer only) was used in each run experiment to determine non-specific signals. For counterstaining of particles bound by capture beads with detection antibodies, 5 µL of each APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibody were added to each sample, then they were incubated on an orbital shaker at 450 rpm protected from light for 1 h at room temperature. Next, samples were washed with MPB and incubated on an orbital shaker at 450 rpm protected from light for 15 min. Subsequently, a further MPB washing was performed and flow cytometric analysis was carried out through a BD FACSCanto equipped with two lasers, 488 nm and 630 nm (Becton Dickinson, San Diego, CA, USA), recording a minimum of 50 events for each population of specific beads. The detection of FITC, PE, and APC fluorophores were measured for each sample. For each sample, the 39 bead populations (37 exosomal surface epitopes + 2 isotype controls) were distinguished by different fluorescence intensities detected in the FITC, PE, and APC channels. Final analysis was performed through the corresponding software (BD FACSDiva): from the raw median fluorescence intensity (MFI) of each marker was subtracted the MFI of the negative control used in the same run experiment.

Transmission Electron Microscopy

EVs isolated from the cell lines supernatants were adsorbed to form VAR carbon coated 200 mesh grids (Agar Scientific Ltd., Stansted, United Kingdom) for 2 min, and briefly rinsed in filtered PBS 1X. Vesicles on grids were immediately fixed with 2.5% glutaraldehyde for 1 min and then negatively stained with 2% (wt/vol) Na-phosphotungstate for 1 min. The observations were carried out by means of a Philips CM10 transmission electron microscope at 80 kV.

Data Analysis

The images of the Western blot were acquired through Chemidoc (Bio-Rad Laboratories) and BD FACSDiva software was used to perform Flow Cytometry analysis.

Statistical Analysis

The aim of the study was to evaluate the technical feasibility of a new workflow to investigate the potential role of EVs in early diagnosis of BC. Descriptive statistics were reported as proportions and median values (range). Non-parametric ranking test (Median test) was used to compare continuous data. MACSPlex results were analyzed by *t* test or analysis of variance (ANOVA) for repeated measures. The associations between continuous variables were determined using Spearman correlation analysis. To generate heatmaps of data, data were exported to comma separated files, which were subsequently imported into R Software for further analysis and data visualization. Mann Whitney *U* test (non-parametric ranking test) was used to compare EVs mean diameter and EVs mode diameter between healthy volunteers (n = 11) and patients (n = 10). Robust rank-based ANOVA (ATS) was used to detect a time effect on EVs mean diameter and EVs mode diameter in patients, and multiple comparisons (again ATS) were performed to determine which time points differed. Due to the explorative nature of the study, no formal sample size calculations were performed and no multiple test corrections were made. All p values were based on two-sided testing, and p-values < 0.05 were considered statistically significant. Statistical analysis was carried out using SAS software, version 9.4 (SAS Institute, Cary, NC, United States) and R statistical package version v 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria) with nparLD package version 2.1.

RESULTS

Characterization of EVs Isolated From Plasma

EVs were successfully isolated from plasma of BC patients and healthy donors through SEC, recently established a reliable EVs-isolation method that allows separation of EVs from a considerable portion of lipoproteins and other plasma components (Brahmer et al., 2019). NTA showed that the EVs from the plasma of BC patients had a mean size of 131.1 nm, range 95.3–151.6 nm, and a mode of 97.6. Concentrations ranged between 4.9×10^9 – 5.43×10^{10} particles/ml as shown in **Figure 1**. EVs from plasma of

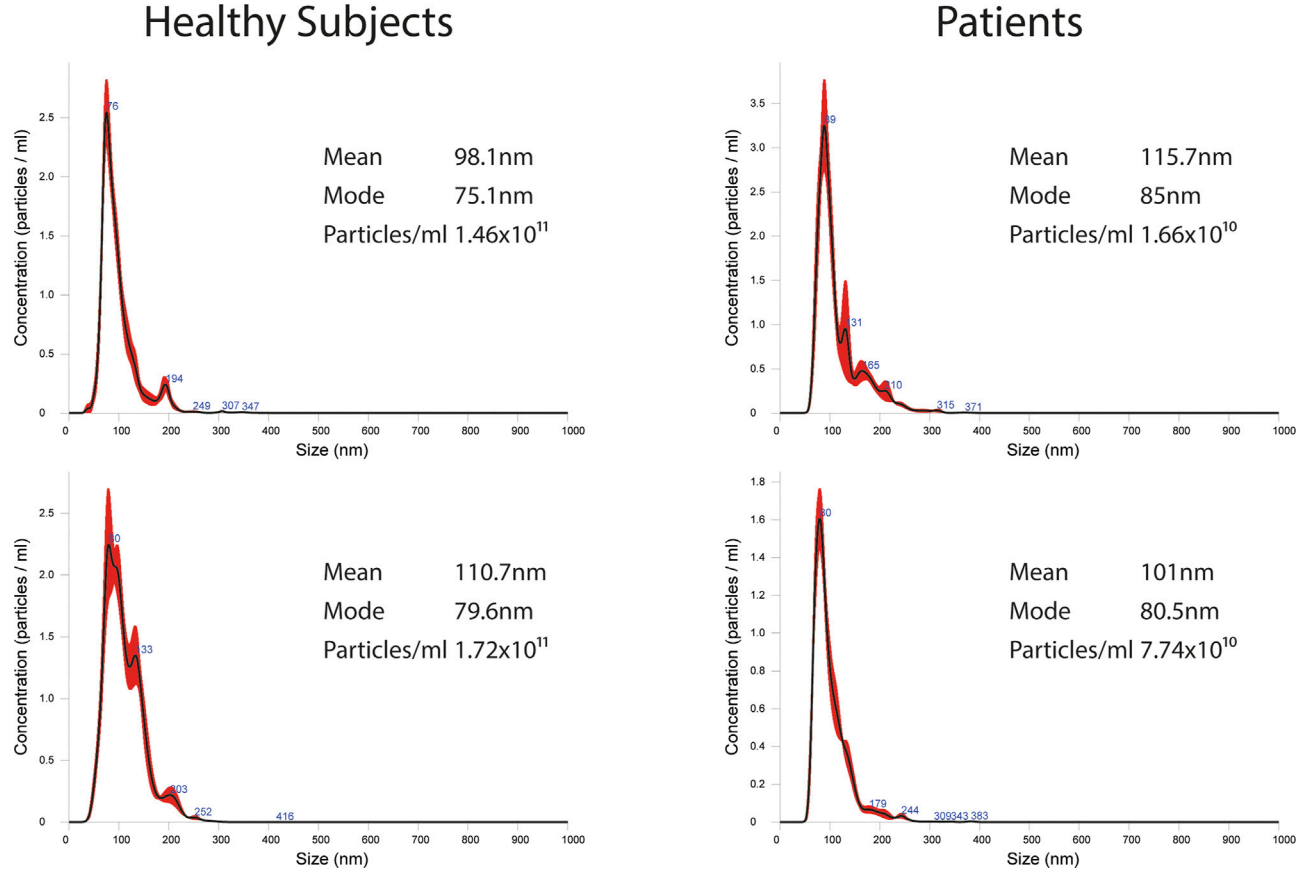

FIGURE 1 | Representative images of NTA profiles analysis of EVs derived from BC plasma samples and healthy donors and corresponding concentration by Nanosight instrument.

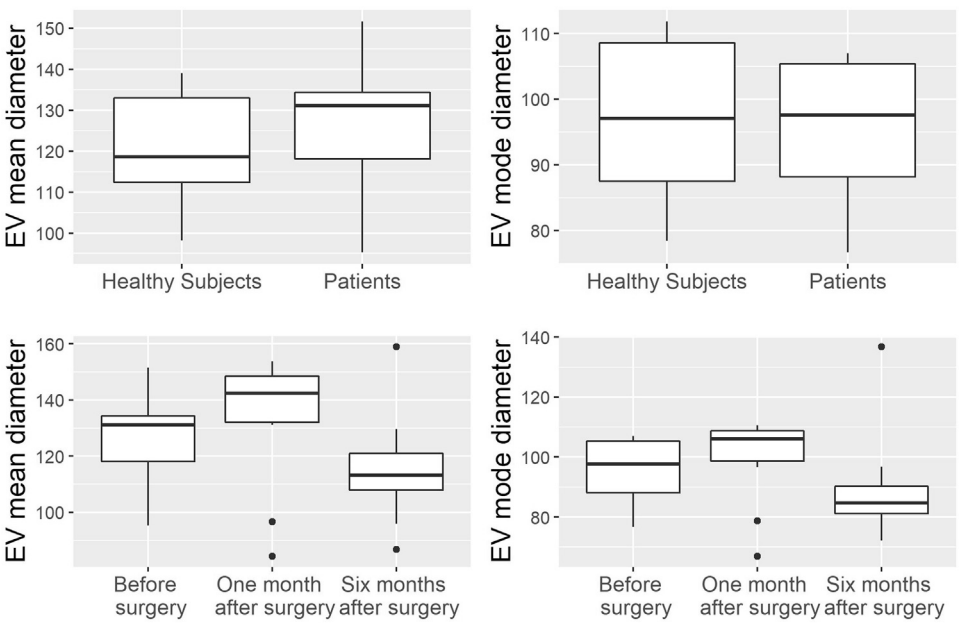

FIGURE 2 | Analysis of EV mean and mode diameters, comparison of healthy subjects vs. patients across three time points. Mann Whitney *U* test was used to compare EVs mean diameter and EVs mode diameter between healthy volunteers ($n = 11$) and patients ($n = 10$). Robust rank-based ANOVA (ATS) was used to detect a time effect on EVs mean diameter and EVs mode diameter.

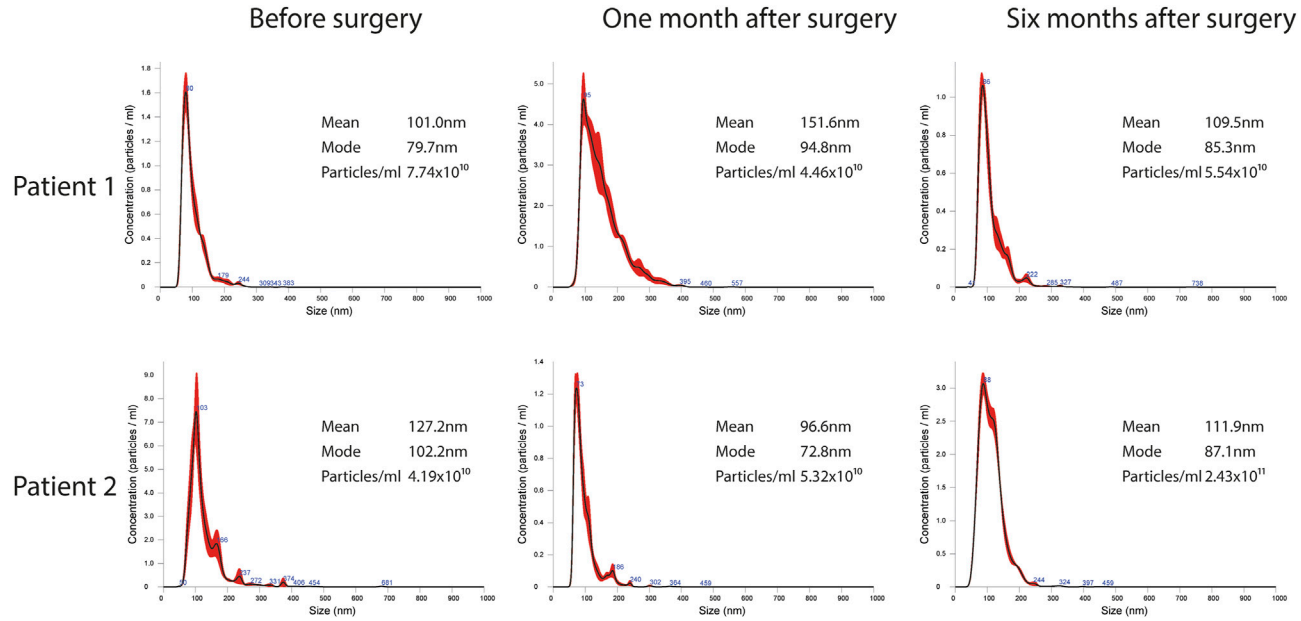

FIGURE 3 | Representative images of NTA profiles analysis of EVs derived from BC plasma samples analyzed at three different time points: pre-surgery, 1 month post-surgery and after adjuvant therapy/6 months post-surgery, and corresponding concentration by Nanosight Instrument.

healthy donors were characterized by a mean size of 118.6 nm, range 98.2–139 nm, and a mode of 97.1. No statistical difference was observed between the EV mean diameters (p = 0.403) and the EV mode diameters (p = 0.802) of healthy subjects vs. patients (**Figure 2**). Concentrations ranged between 3.10×10^9 – 1.72×10^{11} particles/ml. In vesicles derived from BC patients among three time points (**Figure 3**), a significant time effect was detected on EV mean diameters (p = 0.016), in particular for pre-surgery vs. post-surgery (median EV mean diameter 131.1 nm vs. 142.4 nm, p = 0.021) and for 1 month post-surgery vs. 6 months post-surgery (median EV mean diameter 142.4 nm vs. 113.2 nm, p = 0.02). No significant time effect was found on EV mode diameter (p = 0.052) (**Figure 2**). The isolated vesicles were analyzed for the presence of exosomal markers confirmed by western blot, showing positivity at different levels for CD9, Alix, CD81, and TSG-101 while they were negative for the expression of Calnexin (**Figure 4**). Furthermore, they were analyzed through flow cytometry (**Figures 5**, **6**), revealing a variation in the expression of EVs markers between patients and healthy donors and among three different time points of BC patients.

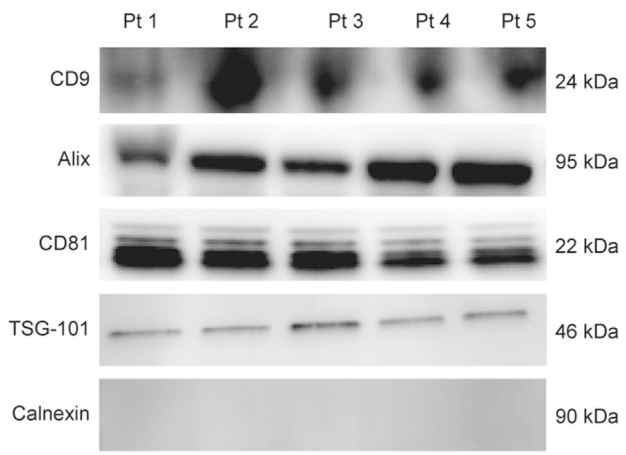

FIGURE 4 | Representative WB analysis of SEC-EVs (fraction 8) derived from plasma of BC patients using EV markers CD9, Alix, CD81, TSG-101, and Calnexin.

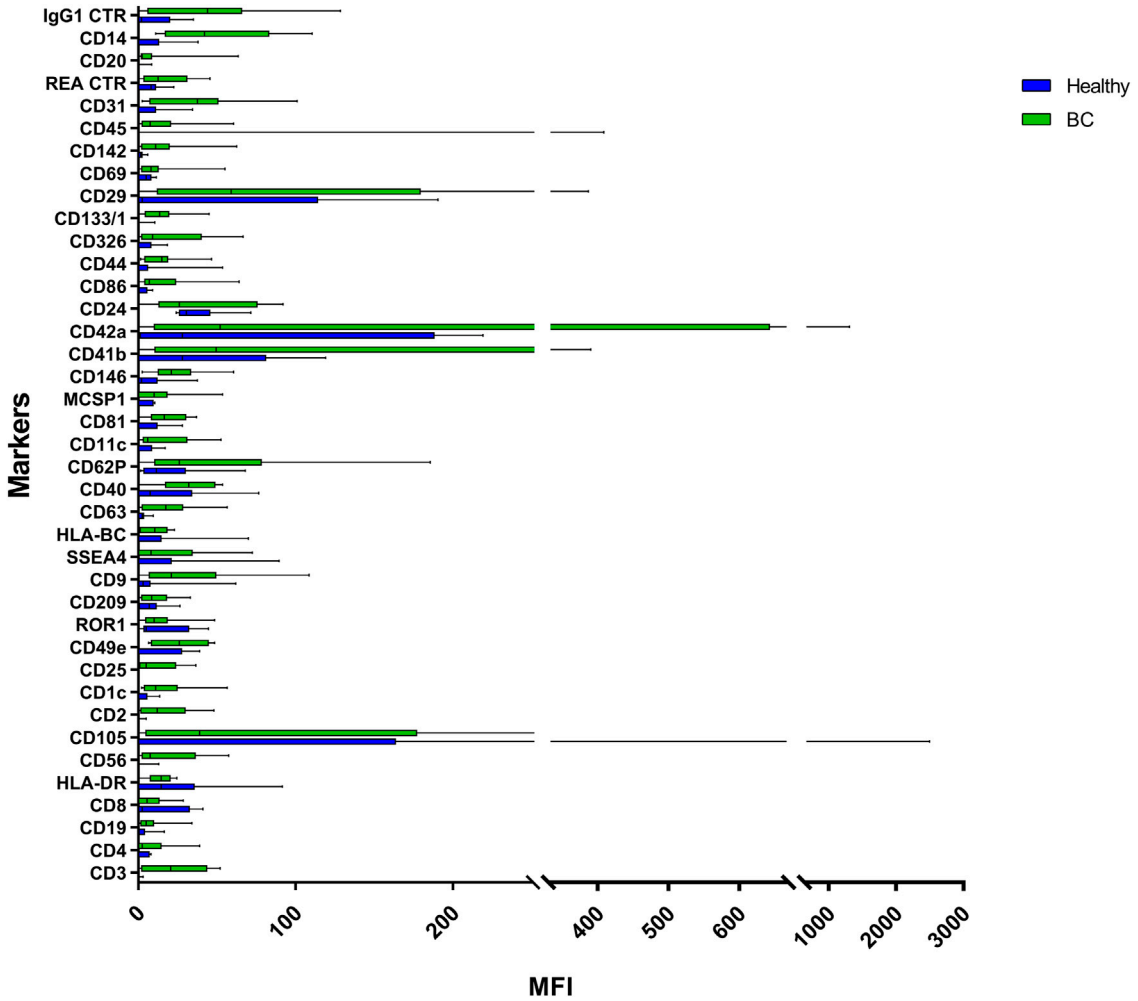

FIGURE 5 | Range from min to max of the Mean Fluorescence Intensity (MFI) for each plasma EVs marker. Plasma from healthy donors in blue; plasma from BC patients in green; values have been normalized to blank control. MFI, mean fluorescence intensity.

EV Markers Differentially Expressed in Plasma of BC Patients

Plasma EVs analysis showed that 11 significant markers were able to significantly discriminate between healthy subjects and patients: CD3, CD56, CD2, CD25, CD9, CD44, CD326, CD133/1, CD142, CD45, and CD14 (**Table 2**). All markers significantly distinguish healthy subjects and BC cases: CD3, CD25, CD56 ($p < 0.001$); CD2, CD9, CD142, and CD14 ($p < 0.01$); CD44, CD326, CD133/1, and CD45 ($p < 0.05$). Statistical results confirmed the trend of tumor samples to have, on average, higher marker values than healthy ones, except for CD45 that decreases its fluorescent intensity in BC cases. Statistical differences were further observed within different time points of BC patients for CD146 ($p = 0.034$) and CD45 ($p = 0.047$) (**Table 3**). More specifically, both markers were found downregulated 1 month after surgery compared to the first access (CD146 $p = 0.042$ and CD45 $p = 0.040$). Data were further evaluated by heatmap analyses, showing however only a weak clustering of CD42a and CD41b that seemed independent from subtype and time points (**Supplementary Figure S1**). The expression of EV markers CD105, CD1C, CD62p, CD41b, CD42a, CD326, and CD29 in BC patients was associated with age of patients (**Table 4**). Among them, CD1c decreased with age while the other antigens increased. In healthy subjects, only CD209 resulted inversely correlated to age, and it decreased along with the increasing of age ($p = 0.026$).

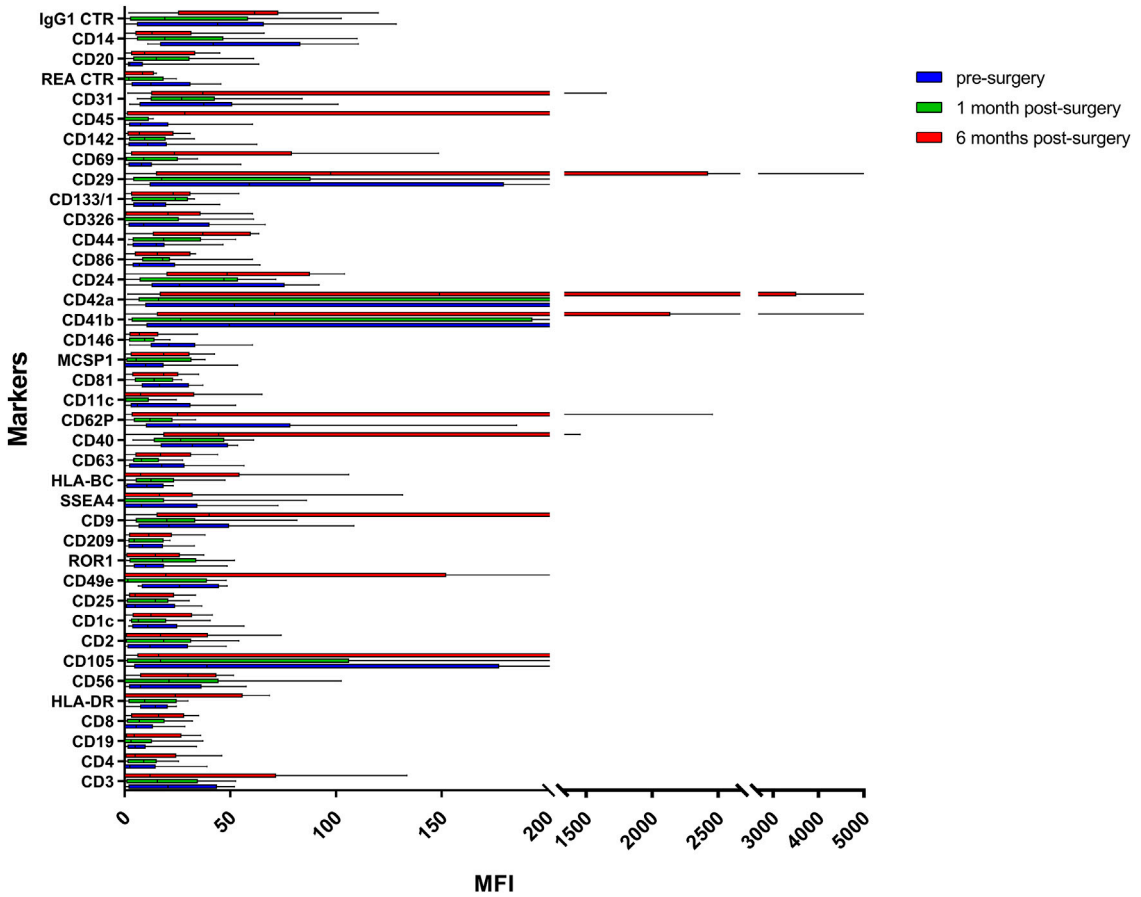

FIGURE 6 | Range from min to max of the MFI for each plasma EVs marker. Plasma from BC patients before surgery in blue; plasma from BC patients 1 month post-surgery in green; plasma from BC patients 6 months post-surgery in red; values have been normalized to blank control. MFI, mean fluorescence intensity.

TABLE 2 | Summary of significant values calculated through analysis of mean values of plasma EVs derived from healthy subjects and BC patients. MFI, mean fluorescence intensity. Unpaired Student's *t* test was used to perform analysis.

Markers	Healthy mean MFI	BCs mean MFI	*p*-value
CD3	0.273	22.444	0.001
CD56	1.182	17.944	0.001
CD2	0.500	16.333	0.006
CD25	0.000	11.444	0.0009
CD9	8.455	33.444	0.006
CD44	9.591	15.056	0.044
CD326	3.864	21.444	0.044
CD133/1	1.045	14.278	0.019
CD142	1.727	15.000	0.006
CD45	40.318	14.167	0.019
CD14	8.773	49.778	0.006

TABLE 4 | Association between baseline markers values and age in BC patients. Spearman correlation was used to perform analysis. If r_s is positive, age and marker expression are directly/positively proportional; if r_s is negative, age, and marker expression are negatively correlated.

	Age	
	r_s	*p*-value
CD105	0.79	0.006
CD1c	–0.82	0.004
CD62p	0.69	0.029
CD41b	0.72	0.019
CD42a	0.68	0.031
CD326	0.75	0.012
CD29	0.68	0.031

TABLE 3 | Summary of significant values calculated through analysis of mean values of plasma EVs derived from BC patients at different time points (before surgical intervention, 1 month after surgical intervention and 6 months after surgical intervention). MFI, mean fluorescence intensity. ANOVA test for repeated measures was used to perform analysis.

Markers	Mean MFI time A	Mean MFI time B	Mean MFI time C	*p*-value
CD146	24.444	8.722	10.111	0.034
CD45	14.167	4.889	104.056	0.047

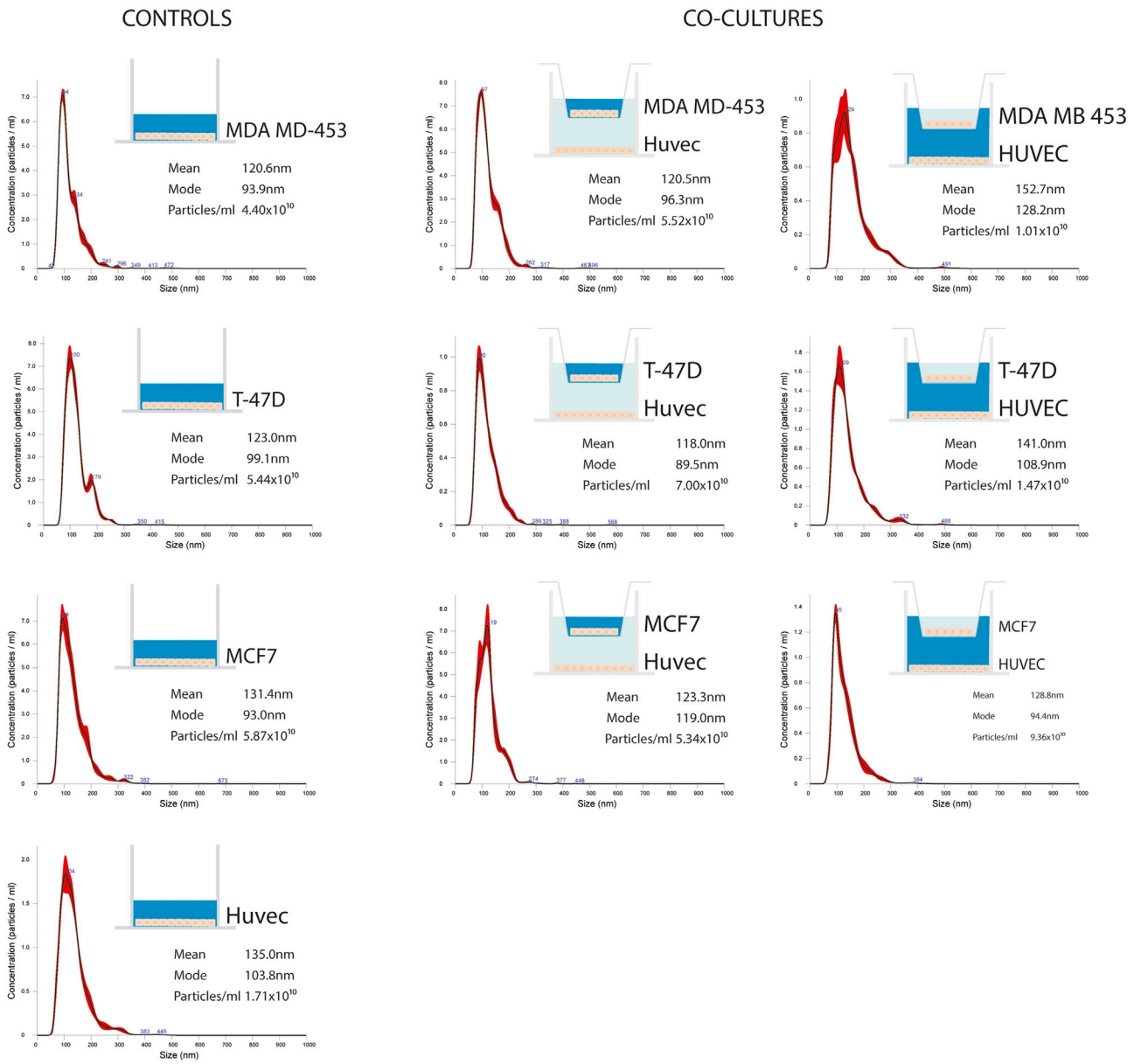

FIGURE 7 | Representative distribution plots of EVs isolated from supernatant of BC cell lines (MDA-MB 453, T-47D, and MCF-7) alone and in co-cultured conditions with endothelial cells HUVEC; each dot plot refers to the corresponding medium analyzed and from which EVs were isolated (in blue). For each plot, there is a corresponding concentration made by Nanosight instrument.

Characterization of EVs Isolated From BC Cell Lines

EVs were successfully isolated from BC and HUVEC cell lines through SEC. NTA showed a similar distribution for EVs from three different BC cells: MDA-MB 453 EVs had a mean size of 120.5 nm in controls and 120.6 in co-cultured samples; T-47D EVs had a mean size of 123.1 in controls and 118 in co-cultured samples; MCF-7 EVs had a mean size of 131.4 in controls and 123.3 in co-cultured samples. All cell lines showed concentrations between 4.4×10^{10} – 7×10^{10} particles/ml as shown in **Figure 7**. Interestingly, we observed a slight trend for vesicles to increase their production or release in co-cultured cells compared to the controls, in particular for MDA-MB 453 (4.4×10^{10} control cells vs. 5.52×10^{10} co-cultured cells) and T-47D (5.5×10^{10} control cells vs. 7×10^{10} co-cultured cells) cell lines. EVs from HUVEC cells were slightly larger compared to those from BC cells, being characterized by a mean size of 134.9 nm in control cells, and by means varying from 118–131.4 nm when co-cultured with BC cell lines, together with lower concentrations between 9.36×10^{9} – 1.7×10^{10} particles/ml. No significant differences were observed between control and

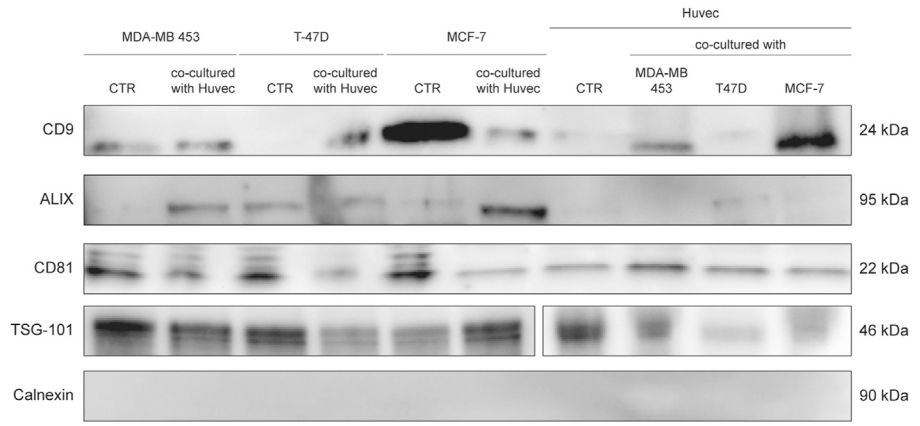

FIGURE 8 | WB analysis of isolated EVs from SEC fraction eight derived from supernatant of BC cells and HUVEC, using EV markers CD9, Alix, CD81, TSG-101, and Calnexin.

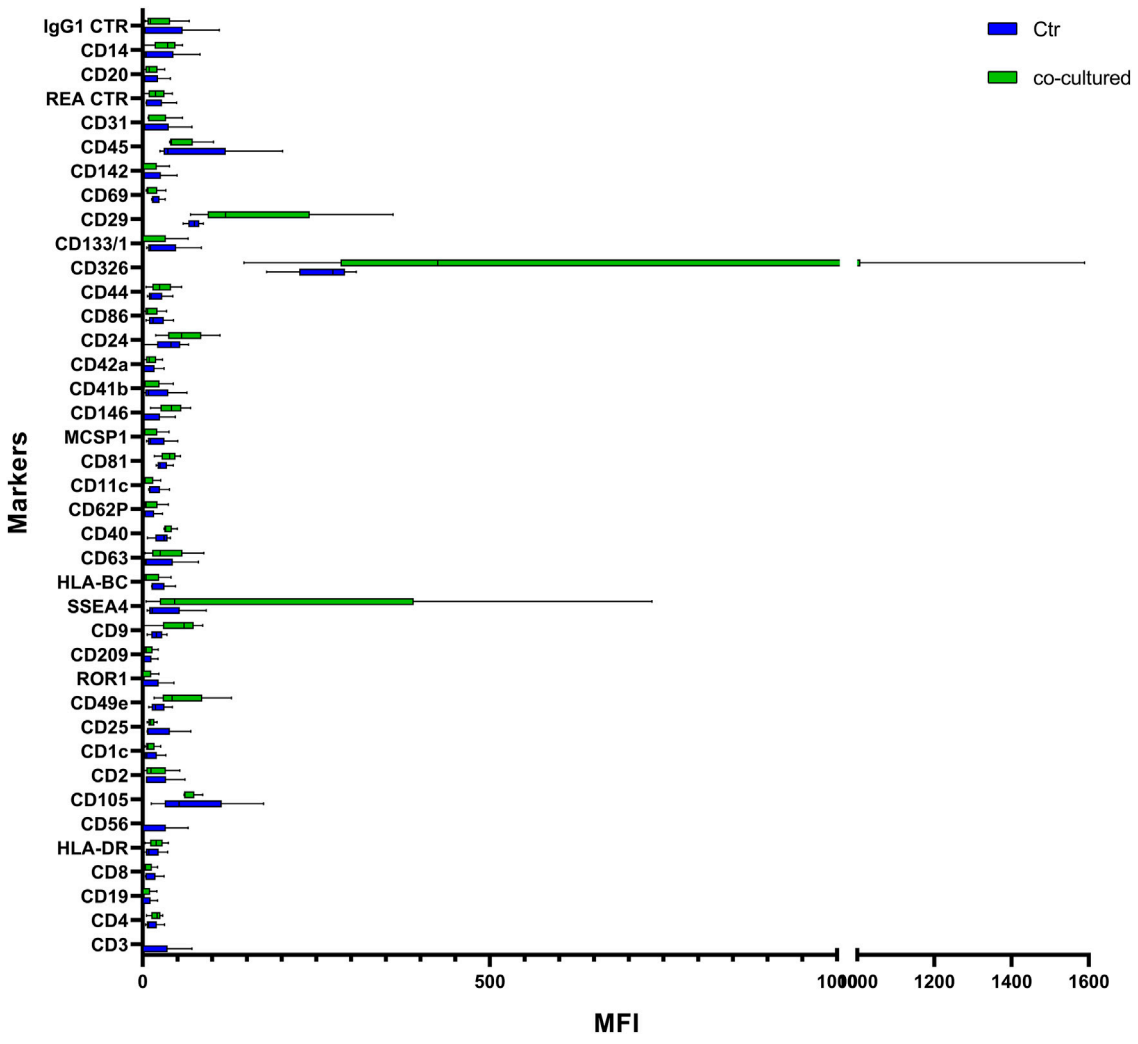

FIGURE 9 | Range from min to max of MFI for each cell EVs marker. Supernatant from cell controls in blue; supernatant from cell co-cultured with HUVEC in green; values have been normalized to blank control. MFI, mean fluorescence intensity.

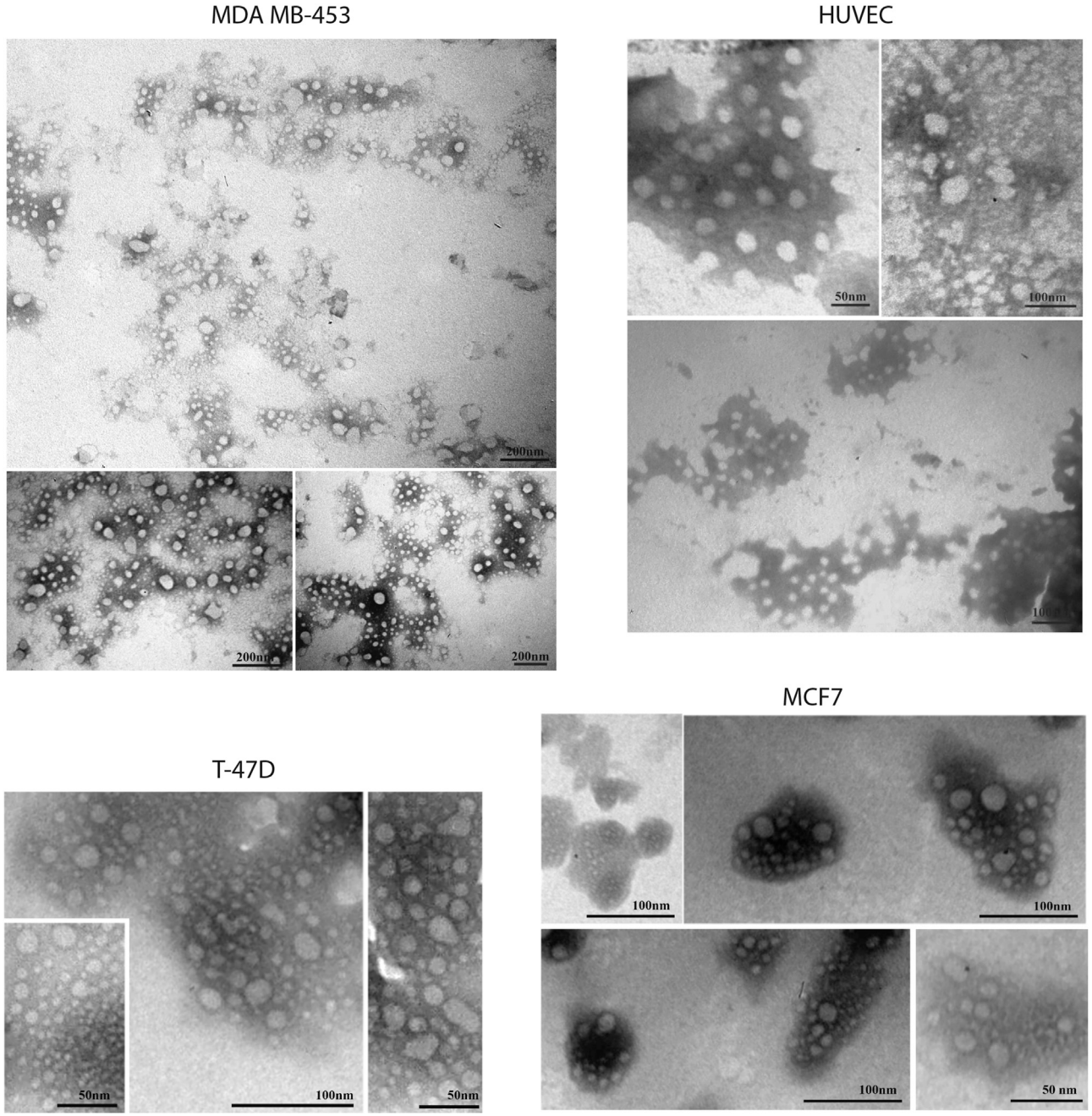

FIGURE 10 | Transmission electron microscopy representative images of SEC-EVs isolated from BC cells and endothelial cells. TEM observations showed numerous EVs between 5 and 100 nm. Scale bar refers to 50–100 nm.

co-cultured cells for vesicle size. Western blot analysis showed that EVs of BC cells and HUVEC were positive, at different levels, for CD9, Alix, CD81, and TSG-101, and negative for the expression of Calnexin (**Figure 8**). Furthermore, vesicles were analyzed both by flow cytometry and TEM. Flow cytometry analysis revealed a variation in the expression of exosomal markers between co-cultured cells and controls (**Figure 9**). The morphology of EVs isolated from BC cells was examined through TEM analysis. MDA-MB 453 were characterized by vesicles with sizes between 20–100 nm and not all of them characterized by well-preserved membranes; T-47D cells were defined by preserved membranes and small vesicles of size between 5–30 nm, some with irregular shape. MCF-7 cells were characterized by preserved membranes and EVs with sizes between 5–25 nm. HUVEC showed less conserved membranes and their vesicles were characterized by a size between 20–60 nm (**Figure 10**).

TABLE 5 | Summary of significant values calculated through analysis of mean values of EVs derived from supernatant of BC cell lines and controls. MFI: mean fluorescent intensity; Ctr: control. Two independent experiments were performed and 3 cell lines were used (control groups, $n = 3$ and co-coltured groups, $n = 3$). Unpaired Student's t test was used to perform the analysis.

Markers	Mean MFI Ctr	Mean MFI co-cultured	*p*-value
CD4	14.833	18.500	0.025
CD105	79.667	69.000	0.025
CD40	26.00	38.000	0.025
CD146	16.333	40.667	0.025
CD44	20.833	28.500	0.025
CD29	73.500	183.000	0.025

EVs Differentially Expressed in Vesicles Derived From BC Cells After Co-culture With Endothelial Cells

Cytofluorimetric EVs analysis showed six significant antigens discriminating between BC cells co-cultured with ECs and BC cells alone with a p-value < 0.05: CD4, CD105, CD40, CD146, CD44, and CD29 (**Table 5**). CD105 ($p = 0.001$) and CD40 ($p = 0.006$) were further confirmed as factors that clearly distinguished EVs derived from BC cells co-cultured with ECs, along with CD81 as correlated to co-cultured phenotype ($p = 0.0005$).

DISCUSSION

In BC, clinicopathological characteristics such as age, grade, stage, and molecular subtypes correlate to different incidences, survival, prognosis, and biology, influencing clinical decisions. In addition to tumor cell biology, an inflammatory microenvironment can be responsible for cancer growth. In fact, to date, it is well known that TME can affect carcinogenesis at various steps, from initiation to progression (Tekpli et al., 2019). Many risk factors have been recognized for BC development including age, family history, genetic mutations, chronic inflammation, obesity, and personal habits. Indeed, cancer development is a complex and progressive process that involves modifications not only in the tumor initiating cells, but also in the surrounding environment constituted by several types of cells and secreted biomolecules (Deshmukh et al., 2019), among which EVs are the key of this interplay.

Equally importantly, the interaction between tumor cells and TME at metastatic sites has been recognized as a key regulator of tumor progression, and a better understanding of the mechanisms through which BC-derived EVs guide secondary metastasis is so crucial (Kim et al., 2020).

TME includes a variety of cell types: fibroblasts, ECs, immune cells, pericytes, adipocytes, and local and bone marrow-derived cells, surrounded by matrix components. Moreover, blood supply plays a pivotal role in cancer progression, allowing access to oxygen and nutrients that support tumor spread and eliminate metabolic waste. In this context, angiogenesis, the process by which new blood vessels arise from pre-existing ones, represents a central step in the progression of tumor growth and metastases dissemination, and the blockade of angiogenesis is a promising challenge for new cancer therapies. Hence, although somewhat partial, studying the possible interaction between ECs and BC cells through *in vitro* studies of EVs could be a fair starting point to solve and more deeply understand the intricate interactions between cancer cells and TME (Bovy et al., 2015). In the future, it should be possible to translate the findings into the clinic after identification of tumor-specific actionable targets and validation of new EV-based markers of prognosis and/or resistance to therapy (Möller and Lobb, 2020).

The release of EVs into the extracellular space means a chance to examine them in body fluids such as blood, urine, liquor, and malignant effusions, making them potential biomarkers for the clinical management of cancer with some notable advantages (Mathew et al., 2020). First of all it is a non-invasive way to recover samples from a number of biologic materials. Secondarily, circulating EVs analysis could represent a "liquid biopsy" with the convenience of not requesting cancerous tissue or the partial or total removal of a tumor to access its molecular information, and the capability to monitor cancer progression due to consecutive repeatable sampling (Lucchetti et al., 2019). However, one of the main issues highlighted by the scientific community concerns the absence of a standardized protocol for enrichment and characterization of EVs (Lane et al., 2018), although numerous methods have been developed in order to investigate their behavior. Generally, EVs can be differentiated by size, density, and protein composition, but it is still demanding to easily fractionate EVs and microvesicles due to the marked similarity of their composition. EVs can be isolated through a variety of techniques, such as centrifugation (high speed, differential, and density-gradient), membrane affinity columns, SEC, filtration, and precipitation. Many of these methods are characterized by poor purity and consistence (Hu T. et al., 2021). Besides the most commonly used approaches to obtain EVs, which have some limitations (Greening et al., 2015; Lobb et al., 2015), several other strategies, including flow cytometry ones, are gaining interest (Maia et al., 2020; Marchisio et al., 2020). Hence, before translating into clinics, methods such as that herein reported (Salvi et al., 2021) need to be tested, and clinical validations need to be performed. In order to investigate blood-related TME and discover new potential disease-related biomarkers through a recent approach, we performed a small case study, highlighting the feasibility of the detection and characterization of BC-derived EVs. We used both plasma samples of BC patients and BC cell lines co-cultured with ECs. Patient samples were taken before surgery, and after 1 and 6 months after surgery, together with adjuvant therapy, to investigate the value of EVs as cancer markers in this clinical setting during the earliest stage of the disease, in order to discover possible biomarkers to monitor patients in the first months after surgery and/or during therapy. We first isolated EVs through SEC, and then characterized EVs through NTA, WB, and a multiplexed phenotyping cytofluorimetric approach able to detect 37 exosomes-related antigens. NTA analysis of EVs derived from BC patients did not show a variation in the dimensions of vesicles compared to that of healthy subjects, but significant results were observed among the three different time points, especially in vesicles analyzed 1 month post-surgery. The MACSPlex-based characterization showed that CD3, CD56, CD2, CD25, CD9, CD44, CD326, CD133/

1, CD142, CD45, and CD14 markers were differentially expressed, with significance, between healthy subjects and patients. Interestingly, CD146 and CD45 were found significantly deregulated at the three different time points. In particular, both CD146 and CD45 expression levels were reduced in EVs 1 month after surgical resection, suggesting that these markers could have a value for monitoring disease after surgical resection, therapy, and progression. In line with these results, CD146 is considered a hallmark of tumor progression and metastasis, especially in TNBC (Li et al., 2021) and CD45 was found deregulated in breast stroma of BC patients at early stages also (Marino et al., 2020). Furthermore, CD146 is a well-known endothelial cell lineage marker. Firstly identified as a melanoma cell adhesion molecule (MCAM), it is overexpressed in many tumors and implicated in vascular and lymphatic metastasis (Wang et al., 2020). CD146 is a molecule known to modulate cell-cell adhesion and to bind to extracellular matrix proteins or other transmembrane proteins, such as VEGFR2, and the secretion of CD146-enriched EVs was reported to be associated with metastatic process, mediating their interaction with specific ligands on endothelial cells of metastatic organs (Ghoroghi et al., 2021). Since we observed a CD146 decrease during the time in which patients did not progress in 5 years, it is tempting to hypothesize that a reduction of this marker could be related to a better prognosis. We further reported deregulated markers whose presence suggests a peculiar asset of EVs in aging cancer patients. Specific age-related EV markers is a field that would be worth being studied, in particular to more deeply understand the well-known association between cancer and aging.

In order to shed some light on the EV epitopes we found in the clinical setting, we set up an experimental plan *in vitro* that could confirm the results obtained for deregulated markers observed. In our cell models, the interaction of BC cells with ECs firstly seems to lead to a slight increase of EV release, compared to the number of EVs produced by cancer or HUVEC cells. A range of markers were identified with increased signal intensity in samples co-cultured with ECs: CD4, CD105, CD40, CD146, CD44, CD29, and CD81, with only CD44 and CD146 variation found common in both patients and cell models. Although results between BC cells and patients were not completely comparable, most probably due to the different nature of samples, the increase of these markers in BC cells-released vesicles may hint some interesting thoughts. The interaction of cancer cells with a simplified normal microenvironment (herein streamlined by HUVEC cells) may trigger the production of EVs exhibiting antigens related to endothelial/neo-angiogenetic and/or aggressiveness features. Indeed, CD146 and CD44 have been related to neo-angiogenesis, cell proliferation, cell survival, cytoskeletal changes, and cellular motility (Chen et al., 2018). This may suggest an involvement of ECs in the acquisition of a more aggressive behavior of cancer cells, as already reported (Hwang et al., 2020). Despite not being significantly different in BC patients vs healthy donors, CD146 expression decreased in our BC patients over time, as previously reported here, suggesting a role in tumor related neo-angiogenetic processes. On the other hand, CD44 was altered both in patients vs healthy subjects and in cell models, suggesting that this could be a cancer-related marker of spreading and prompting a deeper investigation of this antigen in BC patient EVs. Furthermore, a recent study showed that CD44 circulating tumor endothelial cells were associated with poor prognosis in pancreatic ductal adenocarcinoma after radical surgery (Xing et al., 2021). Indeed, the identification of circulating factors that might be responsible for influencing and spreading vessels formation, modulating angiogenesis and aggressiveness, it is of great clinical interest. It is increasingly necessary to support the study of new biomarkers of angiogenesis and metastatic spread, with the final aim of an accurate disease monitoring targeted towards personalized medicine. In agreement, a study reported a high percentage of patients exhibiting high tumor-infiltrating lymphocytes with CD3 positivity exhibited pathological response to neoadjuvant chemotherapy (Gomez-Macias et al., 2020). From this point of view, in the future, the herein feasible detection of CD3-positive EVs, potentially secreted by CD-positive infiltrating lymphocytes, could also be investigated and utilized as a marker of response. In summary, we performed a small preliminary and feasibility study to investigate useful biomarkers possibly exploitable for diagnostic and monitoring intent, as already reported for monitoring therapies in various types of cancer (Stevic et al., 2020). Moreover, although dissimilar from the clinical setting in terms of expression of some markers, the results from *in vitro* analysis of EVs suggested the implication of CD44 and CD146 in biological processes involved in breast tumor and microenvironment interplay. The fact that we observed significant results already in a small but well monitored series of patients recommends continuing in this direction. However, since we are aware of the limitations of this study, principally due to the small size of our patient cohort, future validation studies with a larger set of patients are clearly needed.

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 the Romagna Ethics Committee (CEROM) of Meldola IRCCS IRST (IRSTB008). The patients/participants provided their written informed consents to participate in this study.

AUTHOR CONTRIBUTIONS

EB and TR constructed this study. EB, TR, IV, MM, SSalu, MB, and FF performed the experiments. ES and IA performed the formal data analysis. RM and PS were responsible for clinical resources. GG, SSalv, and WCC performed data curation and interpretation. FF was responsible for the supervision of the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.732900/full#supplementary-material

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New Insights Into the Regulatory Roles of Extracellular Vesicles in Tumor Angiogenesis and Their Clinical Implications

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Angiogenesis is required for tumor growth and development. Extracellular vesicles (EVs) are important signaling entities that mediate communication between diverse types of cells and regulate various cell biological processes, including angiogenesis. Recently, emerging evidence has suggested that tumor-derived EVs play essential roles in tumor progression by regulating angiogenesis. Thousands of molecules are carried by EVs, and the two major types of biomolecules, noncoding RNAs (ncRNAs) and proteins, are transported between cells and regulate physiological and pathological functions in recipient cells. Understanding the regulation of EVs and their cargoes in tumor angiogenesis has become increasingly important. In this review, we summarize the effects of tumor-derived EVs and their cargoes, especially ncRNAs and proteins, on tumor angiogenesis and their mechanisms, and we highlight the clinical implications of EVs in bodily fluids as biomarkers and as diagnostic, prognostic, and therapeutic targets in cancer patients.

Keywords: extracellular vesicles, tumor angiogenesis, miRNAs, lncRNAs, CircRNAs, proteins

1 INTRODUCTION

Angiogenesis, defined as the establishment of new blood vessels from pre-existing vascular networks, is triggered by proangiogenic factors and depends on the proliferation and migration of endothelial cells (ECs) (Teleanu et al., 2019; Lugano et al., 2020). In normal healthy tissues, angiogenesis is tightly regulated by a balance that is maintained between proangiogenic and antiangiogenic factors. Solid tumors are generally characterized with aberrant angiogenesis, and tumor angiogenesis is critically required for tumor growth and development (Teleanu et al., 2019; Lugano et al., 2020). Many proangiogenic factors are upregulated in tumor cells and tumor-associated stromal cells, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and delta ligand-like 4 (Dll4). Hypoxia is a key inducer of tumor angiogenesis and promotes the expression of various proangiogenic factors in the tumor microenvironment (Abou Khouzam et al., 2020). Recently, antiangiogenic drugs have been widely applied to the treatment of multiple solid cancers, and cancer patients have gained tremendous survival benefits from antiangiogenic therapy.

Extracellular vesicles (EVs), such as microvesicles and exosomes, are nanosized vesicles with lipid membranes that are secreted by most cells. EVs contain many bioactive molecules, such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), and proteins, and these EV cargoes regulate intercellular communication (Mathieu et al., 2019; Liu

et al., 2021). Donor cell-derived EVs are taken up by recipient cells, and the encapsulated bioactive components are thus delivered to recipient cells, enabling their regulation of recipient cell biological behaviors. An increasing number of studies have demonstrated that EVs play important roles in tumorigenesis, tumor growth, metastasis, immune evasion, drug resistance, and angiogenesis (Todorova et al., 2017; Aslan et al., 2019). Tumor-derived EVs can transfer proangiogenic molecules into ECs to promote their angiogenic activity via various mechanisms such as VEGF/VEGF Receptor (VEGF/VEGFR), Notch, Wingless-type (WNT), and Hypoxia-inducible factor (HIF) signaling pathway (Phng et al., 2009; Horie et al., 2017; Todorova et al., 2017; Aslan et al., 2019). Thus, targeting EVs might be an innovative and promising therapeutic strategy to inhibit tumor angiogenesis.

A wide variety of biomolecules, including ncRNAs and proteins, have been identified as EV cargoes, and these signaling molecules can be transported from donor cells to recipient cells. To date, considerable attention has been directed to the effects of EVs on tumor angiogenesis and the clinical relevance of these effects. A database of exosomes (http://www.exocarta.org/) includes 9,769 proteins, 3,408 mRNAs, and 2,838 miRNAs. The mechanisms triggered by these specific cargos loaded into EVs and delivered from donor cells to acceptor cells are complex (Abels and Breakefield, 2016; Mathieu et al., 2019). This article summarizes the current knowledge on the roles of tumor-derived EVs in angiogenesis, with a particular emphasis on the molecular mechanisms involved. We also discuss the main prospects for their applications in cancer diagnosis, prognosis, and treatment.

2. EXTRACELLULAR VESICLES AND TUMOR ANGIOGENESIS

2.1 EV-Derived ncRNAs and Tumor Angiogenesis

Here, we focus on the effects and mechanisms of EV-derived miRNAs, lncRNAs, and circRNAs on angiogenesis, aiming to elucidate their potential as tumor biomarkers and therapeutic targets for tumor angiogenesis.

2.1.1 miRNAs

Various miRNAs are packaged into tumor-derived EVs and can be transferred into recipient ECs (Muralidharan-Chari et al., 2009). Once internalized by ECs, these miRNAs can initiate an angiogenic switch by modulating EC proliferation and migration and regulating the expression of angiogenesis-related genes (Huang et al., 2020a; Li et al., 2020; Masoumi-Dehghi et al., 2020).

VEGF/VEGFR and HIF signaling pathways are the main targets of miRNAs that regulate angiogenesis. Exosomal miR-130a secreted by gastric cancer (GC) cells targeted c-MYB in ECs and promoted angiogenesis *in vitro* and *in vivo* (Yang et al., 2018). Similarly, GC cell-derived exosomal miR-155 downregulated c-MYB but increased the expression of VEGF in ECs, which enhanced EC tube formation and increased microvessel density in xenografted tumors (Deng et al., 2020). Moreover, inhibition of signal transducer and activator of transcription 3 (STAT3) reduced miR-21 levels in exosomes derived from transformed human bronchial epithelial cells, and these exosomes suppressed angiogenesis by blocking the STAT3/VEGF axis in ECs (Liu et al., 2016). MiR-182-5p in glioblastoma-derived EVs directly targeted Kruppel like factor 2 (KLF2) and KLF4, which resulted in VEGFR accumulation in ECs and thus promoted angiogenesis (Li et al., 2020). In addition, HIF is a critical angiogenesis inducer that regulates the cellular response to hypoxia-induced stress (Shao et al., 2018). Under hypoxic conditions, HIF-1α is stabilized and its expression is increased, which facilitates the expression of various proangiogenic factors (Horie et al., 2017). Tumor cell-derived exosomal miRNAs, such as miR-21-5p, miR-23a, miR-155, miR-181a, miR-182-5p, and miR-619-5p, were also upregulated under hypoxia. Prolyl hydroxylase (PHD) is a negative regulator of HIFs, and inhibition of PHD can induce the accumulation of HIFs in cells. Exosomal miR-23a derived from hypoxic lung cancer cells inhibited the expression of PHD1 and PHD2 and led to the accumulation of HIF-1α in ECs, thereby enhancing angiogenesis (Hsu et al., 2017).

EVs derived from tumor stromal cells, such as cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), can trigger tumor angiogenesis via various mechanisms. Tumor-derived EVs can promote the transformation of fibroblasts into CAFs and induce M2 polarization of macrophages, thereby inducing the proangiogenic macrophage phenotype switch. For example, lung cancer cell-secreted exosomal miR-210 activated the janus kinase 2 (JAK2)/STAT3 pathway by targeting ten-eleven translocation 2 (TET2) in fibroblasts and thus initiated the acquisition of the proangiogenic phenotype in CAFs, as indicated by the upregulation of VEGFA, MMP9, and FGF2 (Fan et al., 2020). TAMs are immune cells that play a significant role in tumor angiogenesis (Zheng et al., 2018) and M2 macrophages express high levels of proangiogenic factors such as VEGF (Corliss et al., 2016). M2 macrophages were associated with increased microvessel density in pancreatic ductal adenocarcinoma (PDAC) tissues, and exosomal miR-155-5p and miR-211-5p derived from M2 macrophages targeted E2F transcription factor 2 (E2F2) and promoted the angiogenic functions of mouse aortic ECs *in vitro* (Yang et al., 2021).

Collectively, miRNA-derived from tumor-secreted EVs regulate angiogenesis primarily by modulating the VEGF/VEGFR and HIF-1α signaling pathways. In addition to those derived from tumor cells, EVs derived from CAFs and TAMs have been shown to regulate tumor angiogenesis via various mechanisms. The effects and mechanisms of other EV-derived miRNAs on tumor angiogenesis are summarized in **Table 1**.

2.1.2 LncRNAs

Tumor-secreted EV-derived lncRNAs can be transmitted to ECs where they promote the expression of proangiogenic genes and initiate angiogenesis by either binding to endogenous miRNAs or interacting with mRNAs and proteins (Ma et al., 2017; De Los Santos et al., 2019; Zhang et al., 2020). For example, lncRNA-H19 functions as an oncogene and is upregulated in multiple types of cancer (Iempridee, 2017). Exosomes derived from $CD90^+$ liver

TABLE 1 | The effects and mechanisms of miRNAs, lncRNAs, and circRNAs derived from tumor EVs on angiogenesis.

Cargoes	Cancer types	Recipient cells	Target genes or signaling pathways	Functions	References
MiRNAs					
miR-9	NPC	HUVECs	MDK, PDK/Akt pathway	Inhibition	Lu et al. (2018)
	Glioma	HUVECs	COL18A1, THBS2, PTCH1, PHD3, HIF-1α, VEGF	Promotion	Chen et al. (2019)
miR-17-5p	NPC	HUVECs	BAMBI	Promotion	Duan et al. (2019)
miR-21	ESCC	HUVECs	SPRY1	Promotion	Zhuang et al. (2020)
miR-21-5p	Hypoxic PTC	HUVECs	TGFBI, COL4A1	Promotion	Wu et al. (2019a)
miR-23a	Hypoxic HCC	HUVECs	SIRT1	Promotion	Sruthi et al. (2018)
	NPC	HUVECs	TSGA10	Promotion	Bao et al. (2018)
	GC	HUVECs	PTEN	Promotion	Du et al. (2020)
miR-25-3p	CRC	HUVECs	KLF2, KLF4, VEGFR2, ZO-1, Occludin, Claudin5	Promotion	Zeng et al. (2018)
miR-26a	Glioma stem cells	HBMECs	PTEN, PI3K/Akt pathway	Promotion	Wang et al. (2019c)
miR-27a	PC	HMVECs	BTG2	Promotion	Shang et al. (2020b)
	ccRCC	HUVECs	SFRP1	Promotion	Hou et al. (2021)
miR-92a-3p	Retinoblastoma	HUVECs	KLF2	Promotion	Chen et al. (2021a)
miR-130a	GC	HUVECs	c-MYB	Promotion	Yang et al. (2018)
miR-130b-3p	OSCC	HUVECs	PTEN	Promotion	Yan et al. (2021)
miR-135b	GC	HUVECs	FOXO1	Promotion	Bai et al. (2019)
miR-135b-5p	CAFs from CRC	HUVECs	TXINP	Promotion	Yin et al. (2021)
miR-141	SCLC	HUVECs	KLF12	Promotion	Mao et al. (2020)
miR-141-3p	EOC	HUVECs	SOCS5, VEGFR2, JAK/STAT3 and NF-κB signaling pathways	Promotion	Masoumi-Dehghi et al. (2020)
miR-148a-3p	Glioma	HUVECs	ERRFI1, EGFR/MAPK signaling pathway	Promotion	Wang et al. (2020b)
miR-155	GC	HUVECs	c-MYB/VEGFA axis	Promotion	Deng et al. (2020)
miR-155	GC	HUVECs	FOXO3a	Promotion	Zhou et al. (2019)
	Melanoma	fibroblasts	SOCS1/JAK2/STAT axis, VEGFA, FGF2, MMP9	Promotion	Zhou et al. (2018)
	Hypoxic HCC	HUVECs	—	Promotion	Matsuura et al. (2019)
miR-155-5p miR-221-5p	M2 macrophages	MAECs	Targets E2F2 in PDAC	Promotion	Yang et al. (2021)
miR-181a	Hypoxic PTC	HUVECs	DACT2, MLL3, YAP/VEGF axis	Promotion	Wang et al. (2021b)
miR-182-5p	Hypoxic GBM	HUVECs	KLF2, KLF4, VEGFR, ZO-1, occludin, claudin-5	Promotion	Li et al. (2020)
miR-183-5p	CRC	HMEC-1	FOXO1	Promotion	Shang et al. (2020a)
miR-205	OC	HUVECs	PTEN/Akt pathway	Promotion	He et al. (2019)
miR-210	LC	CAFs	JAK2/STAT3	Promotion	Fan et al. (2020)
	HCC	HUVECs	SMAD4, STAT6	Promotion	Lin et al. (2018)
miR-210-3p	OSCC	HUVECs	EFNA3, PI3K/Akt pathway	Promotion	Wang et al. (2020a)
miR-221-3p	CSCC	HUVECs	THBS2	Promotion	Wu et al. (2019b)
	CC	MVECs	MAPK10	Promotion	Zhang et al. (2019)
miR-378b	HCC	HUVECs	TGFBR3	Promotion	Chen et al. (2021b)
miR-549a	TKI-resistant ccRCC	HUVECs	HIF-1α, VEGF	Promotion	Xuan et al. (2021)
miR-619-5p	Hypoxic NSCLC	HUVECs	RCAN1.4	Promotion	Kim et al. (2020)
miR-944	Glioma stem cells	HUVECs	VEGFC, Akt, Erk1/2 signaling pathway	Inhibition	Jiang et al. (2021)
miR-1229	CRC	HUVECs	HIPK2, VEGF pathway	Promotion	Hu et al. (2019)
miR-1260b	NSCLC	HUVECs	HIPK2	Promotion	Kim et al. (2021)
miR-1290	HCC	HUVECs	SMEK1	Promotion	Wang et al. (2021a)
miR-3157-3p	NSCLC	HUVECs	TIMP2, KLF2, VEGF, MMP2, MMP9, occludin	Promotion	Ma et al. (2021)
miR-3682-3p	HCC	HUVECs	ANGPT1, RAS-MEK1/2-ERK1/2 signaling pathway	Inhibition	Dong et al. (2021)
LncRNAs					
LncRNA H19	Glioma	HBMVECs	miR-29a, VASH2	Promotion	Jia et al. (2016)
LncRNA H19	$CD90^+$ liver cancer	HUVECs	VEGF, VEGFR, ICAM1	Promotion	Conigliaro et al. (2015)
LncRNA HOTAIR	Glioma	HBMVECs	VEGFA	Promotion	Ma et al. (2017)
LncRNA CCAT2	Glioma	HUVECs	VEGFA, TGFβ	Promotion	Lang et al. (2017b)
LncRNA POU3F3	Glioma	HBMECs	bFGF, FGFR, VEGFA, and ANG	Inhibition	Lang et al. (2017a)
LncRNA MALAT1	EOC	HUVECs	VEGFA, VEGFD, ENA78, PlGF, IL8, ANG, bFGF, Leptin	Promotion	Qiu et al. (2018)
LncRNA GAS5	LC	HUVECs	miR-29-3p, PTEN	Inhibition	Cheng et al. (2019)
LncRNA p21	NSCLC	HUVECs	miR-23a, miR-146b, miR-330, miR-494	Promotion	Castellano et al. (2020)
LncRNA UCA1	PC	HUVECs	miR-96-5p/AMOTL2/ERK1/2 axis	Promotion	Guo et al. (2020)
LncRNA RAMP2-AS1	Chondrosarcoma	HUVECs	miR-2355-5p/VEGFR axis	Promotion	Cheng et al. (2020)
LncRNA APC1	CRC	HUVECs	Rab5b, MAPK	Promotion	Wang et al. (2019a)
LncRNA TUG1	CC	HUVECs	—	Promotion	Lei and Mou, (2020)

(Continued on following page)

TABLE 1 | (*Continued*) The effects and mechanisms of miRNAs, lncRNAs, and circRNAs derived from tumor EVs on angiogenesis.

Cargoes	Cancer types	Recipient cells	Target genes or signaling pathways	Functions	References
LncRNA X26 nt	GC	HUVECs	VE-cadherin	Promotion	Chen et al. (2021c)
LncRNA OIP5-AS1	Osteosarcoma	HUVECs	miR-153, ATG5	Promotion	Li et al. (2021c)
LncRNA AC073352.1	BC	HUVECs	YBX1 stabilization	Promotion	Kong et al. (2021)
LncRNA SNHG16	HCC	HUVECs	miR-4500/GALNT1 axis, PI3K/Akt/mTOR pathway	Promotion	Li et al. (2021b)
LncRNA CCAT1	PC	HUVECs	miR-1138-5p/HMGA1 axis	Promotion	Han et al. (2021)
LncRNA LINC00161	HCC	HUVECs	miR-590-3p/ROCK axis	Promotion	You et al. (2021)
LncRNA SNHG11	PC	HUVECs	miR-324-3p/VEGFA axis	Promotion	Fang et al. (2021)
CicrRNAs					
Circ-100338	HCC	HUVECs	MMP9	Promotion	Huang et al. (2020b)
Circ-SHKBP1	GC	—	miR-582-3p/HUR/VEGF axis	Promotion	Xie et al. (2020b)
Circ-RanGAP1	GC	HUVECs	miR-877-3p/VEGFA axis	Promotion	Lu et al. (2020)
Circ-CCAC1	CCA	HUVECs	SH3GL2, EZH2, ZO-1, Occludin	Promotion	Xu et al. (2021)
Circ-0044366	GC	HUVECs	miR-29a/VEGF axis	Promotion	Li et al. (2021a)
Circ-CMTM3	HCC	HUVECs	miR-3619-5p/SOX9	Promotion	Hu et al. (2021)

Abbreviation: Breast cancer, BC; Cervical cancer, CC, Cervical squamous cell carcinoma; CSCC; Clear cell renal cell carcinoma, ccRCC; Cholangiocarcinoma, CCA; Colorectal cancer, CRC; Epithelial ovarian cancer, EOC; Esophageal squamous cell carcinoma, ESCC; Gastric cancer, Glioblastoma, GBM; GC; Hepatocellular carcinoma, HCC; Lung cancer, LC; Mouse aortic endothelial cells, MAECs; Nasopharyngeal carcinoma, NPC; Non-small cell lung cancer, NSCLC; Ovarian cancer, OC; Oral squamous cell carcinoma, OSCC; Pancreatic cancer, PC; Pancreatic ductal adenocarcinoma, PDAC; Papillary thyroid cancer, PTC; Small cell lung cancer, SCLC., Tyrosine kinase inhibitor, TKI.

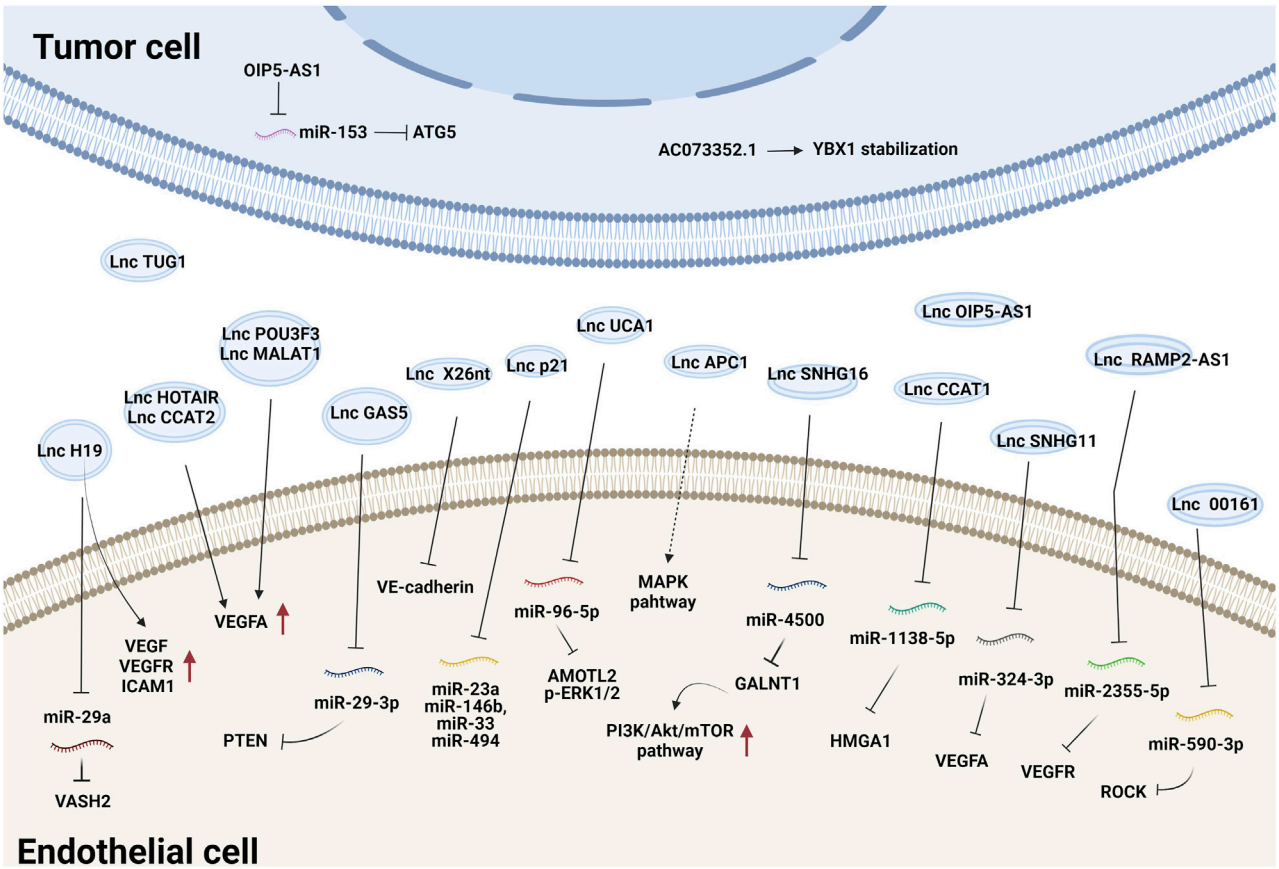

FIGURE 1 | The effects and mechanisms of lncRNAs derived from tumor EVs on angiogenesis.

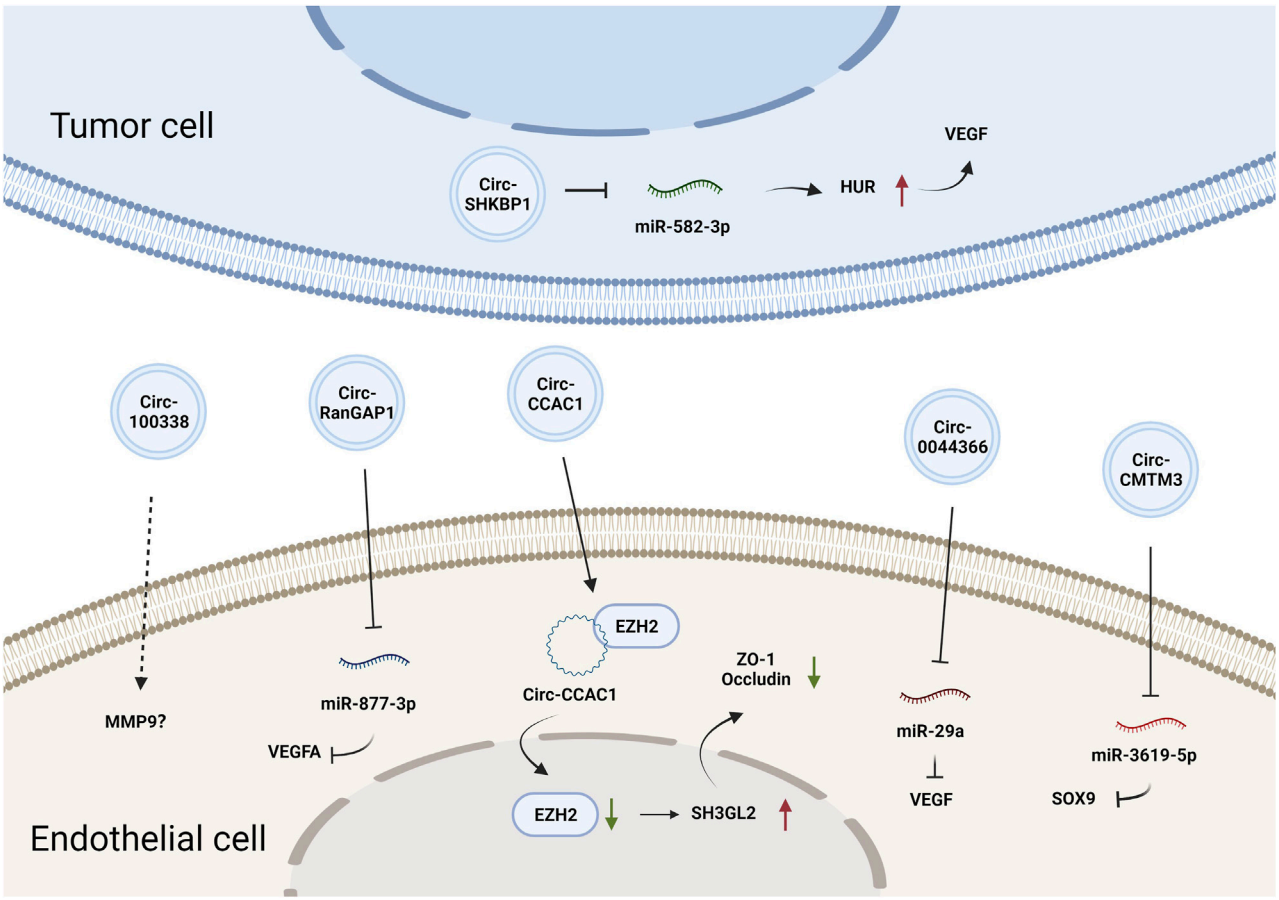

FIGURE 2 | The effects and mechanisms of circRNAs derived from tumor EVs on angiogenesis.

cancer cells were found to be enriched in lncRNA H19 and promoted the angiogenic phenotype of human umbilical vein endothelial cells (HUVECs), probably by regulating VEGF and VEGFR1 expression (Conigliaro et al., 2015). Chondrosarcoma cell-derived exosomes containing lncRNA-RAMP2-AS1 promoted the proliferation, migration and tube formation of ECs by upregulating VEGFR2 by sponging miR-2355-5p (Cheng et al., 2020). LncRNA-UCA1 was highly expressed in exosomes derived from hypoxic pancreatic cancer (PC) cells and promoted angiogenesis and tumor growth by regulating the miR-96-5p/AMOTL2/ERK1/2 axis (Guo et al., 2020). PC-derived exosomal lncRNA SNHG11 promoted the expression of VEGFA by sponging miR-324-3p (Fang et al., 2021). Additionally, glioma-derived exosomal lncRNA-CCAT2 (Lang et al., 2017b) and lncRNA-POU3F3 (Lang et al., 2017a) enhanced angiogenesis by inducing VEGFA expression. LncRNA-APC1, a suppressor of angiogenesis, was significantly downregulated in colorectal cancer cell-derived EVs. It directly bound to and degraded Rab5b mRNA to decrease EV production and block the mitogen-activated protein kinase (MAPK) signaling pathway in HUVECs to suppress angiogenesis (Wang et al., 2019a). Together, these studies demonstrate that tumor exosomal lncRNAs regulate angiogenesis mainly by modulating VEGFA expression and the VEGF/VEGFR and MAPK pathways. The effects and mechanisms of other EV-derived lncRNAs on tumor angiogenesis are summarized in **Figure 1** and **Table 1**.

2.1.3 CircRNAs

CircRNAs constitute a class of endogenous ncRNAs that form a covalently closed loop without a 5′-cap or 3′-poly-A tail (Gan et al., 2021). They are produced by backsplicing protein-coding precursor mRNAs and regarded as variants of competitive endogenous (ceRNAs) that can sponge and thus inhibit the activity of miRNAs (Hansen et al., 2013). Accumulating evidence has demonstrated that circRNAs are involved in various biological processes by regulating gene expression at the transcriptional or posttranscriptional levels (Du et al., 2016). CircRNAs can also be loaded into EVs and mediate cell-cell communication. Circ-SHKBP1 in GC cell-derived exosomes promoted angiogenesis by sponging miR-582-3p and thus increased the expression of hu-antigen R (HUR), which regulated VEGF mRNA stability (Xie et al., 2020b). Circ-RanGAP1 in secreted exosomes derived from the plasma of GC patients and promoted GC progression by targeting the miR-877-3p/VEGFA axis (Lu et al., 2020). Additionally, circ-0044366/circ29, which is highly expressed in GC cell-derived

exosomes, was delivered into ECs and sponged miR-29a to promote angiogenesis by upregulating VEGF (Li et al., 2021a). In summary, tumor EV-derived circRNAs affect tumor angiogenesis primarily by regulating VEGF expression. The effects and mechanisms of other EV-derived circRNAs on tumor angiogenesis are summarized in **Figure 2** and **Table 1**.

2.2 EV-Derived Proteins and Tumor Angiogenesis

In recent years, researchers have identified thousands of proteins from different types of tumor-derived EVs, and some of these proteins were characterized with proangiogenic properties and can stimulate various steps in the angiogenic cascade. For example, EVs derived from colorectal cancer perivascular cells contained growth arrest specific 6 (Gas6) and promoted the recruitment of endothelial progenitor cells (EPCs) to tumors by activating the Axl pathway, thus leading to tumor revascularization after withdrawal of antiangiogenic drugs (Huang et al., 2021). VEGFA was carried in EVs derived from *ex vivo* cultured patient-derived glioblastoma stem-like cells and promoted angiogenesis of human brain ECs (Treps et al., 2017). Breast cancer cell-derived EVs contained $VEGF_{90K}$, which was generated by $VEGF_{165}$ crosslinking and triggered sustained activation of VEGFRs in ECs by interacting with heat shock protein 90 (HSP90) (Feng et al., 2017). Furthermore, EVs secreted by ovarian (ES2), colorectal (HCT116), and renal (786–0) cancer cells, in bodily fluids of tumor-bearing mice, and in ovarian cancer patient ascites could stimulate EC migration and tube formation. These responses were mediated by the 189 amino acid isoform of VEGF ($VEGF_{189}$), which was bound to the surface of these EVs because of its high affinity for heparin (Ko et al., 2019). Collectively, these findings indicate that proangiogenic factors (e.g., Gas6 and VEGFA) and different subtypes of VEGF promote tumor angiogenesis through different mechanisms.

In addition to conventional proangiogenic cytokines, other angiogenesis-related proteins have also been found in EVs. Ephrin type B receptor 2 (EPHB2) in small EVs derived from head and neck squamous cell carcinoma (HNSCC) activated ephrin-B reverse signaling and induced STAT3 phosphorylation in ECs, which promoted angiogenesis both *in vitro* and *in vivo* (Sato et al., 2019). Moreover, soluble E-cadherin, which was localized to the surface of exosomes derived from ovarian cancer (OV) cells, activated the β-catenin and nuclear factor-κB (NF-κB) signaling pathways by interacting with VE-cadherin on ECs, leading to angiogenesis *in vitro* and *in vivo* (Tang et al., 2018). Exosomal Annexin II secreted by breast cancer cells promoted tPA-dependent angiogenesis *in vitro* and *in vivo* (Maji et al., 2017). Wnt5A induced the secretion of exosomes containing proangiogenic proteins (e.g., VEGF and MMP2) and immunomodulatory factors (e.g., IL-8 and IL-6) by melanoma cells (Ekstrom et al., 2014). Additionally, other angiogenic proteins have been found in many cancer cell-secreted EVs, such as yes-associated protein (YAP) (Wang et al., 2019b), angiopoietin 2 (ANGPT2) (Xie et al., 2020a), profilin 2 (PFN2) (Cao et al., 2020), Dll4 (Sheldon et al., 2010), ANG, IL-6, IL-8, tissue inhibitor of metalloproteinases-1 (TIMP-1), TIMP-2, activating transcription factor 2 (ATF2), metastasis associated 1 (MTA1), and Rho associated coiled-coil containing protein kinase 1/2 (ROCK1/2) (Skog et al., 2008; Chan et al., 2015; Yi et al., 2015; Ikeda et al., 2021). More proteins in different types of tumor-derived EVs and their proangiogenic mechanisms are summarized in **Figure 3** and **Table 2**.

3 EXTRACELLULAR VESICLES AND CLINICAL IMPLICATIONS

As ncRNAs or proteins loaded in EVs can be distributed in various biofluids, such as blood, urine, tears, saliva, milk, and ascites (Keller et al., 2011), the ability to analyze their cargoes and levels in bodily fluids makes them promising biomarkers for cancer diagnosis and prognosis (Sun and Liu, 2014). Liquid biopsy is a noninvasive method of detecting precise information about the tumor environment/status, which can provide information prior to treatment (Rekker et al., 2014). Through liquid biopsy, numerous proangiogenic contents in EVs have been identified.

Similar to that on circulating free DNA or cell-free DNA and several oncoproteins, such as prostate-specific antigen (PSA) and alpha-fetoprotein (AFP), emerging evidence has suggested that EV-associated ncRNAs and proteins can serve as biomarkers and diagnostic, prognostic, and therapeutic targets in cancer patients.

The levels of serum miR-210 and serum-derived exosomal miR-210 were much higher in HCC patients than in healthy donors. A high level of miR-210 was associated with higher microvessel density in HCC patients (Lin et al., 2018). Increased expression of exosomal circRNA-100338 in the serum of HCC patients was associated with tumor growth and angiogenesis in primary and metastatic HCC. Exosomal circRNA-100338 can serve as a predictor of poor prognosis and lung metastasis in HCC patients following curative hepatectomy (Huang et al., 2020b). Serum exosomal Annexin II promoted angiogenesis, and a high level of serum exosomal Annexin II was associated with tumor grade, poor overall survival (OS), and poor disease-free survival in African-American women with triple-negative breast cancer (Chaudhary et al., 2020). Increased expression of lnc-UCA1 was positively correlated with microvessel density in PC tissues. Exosomal lnc-UCA1 levels were greatly increased in PC patient serum and were associated with tumor size, lymphatic invasion, late tumor node and metastasis stage, and poor OS (Guo et al., 2020). The elevated expression of metastasis associated lung adenocarcinoma transcript 1 (MALAT1) in exosomes derived from epithelial ovarian cancer (EOC) patient serum was significantly correlated with an advanced and metastatic phenotype and served as an independent predictive factor for the OS of EOC patients (Qiu et al., 2018). NSCLC patients with high levels of lncRNA-p21 in EVs derived from tumor-draining pulmonary veins exhibited shorter relapse-free survival and OS (Castellano et al., 2020). The level of circ-CCAC1 in the EVs in the serum of cholangiocarcinoma patients was significantly

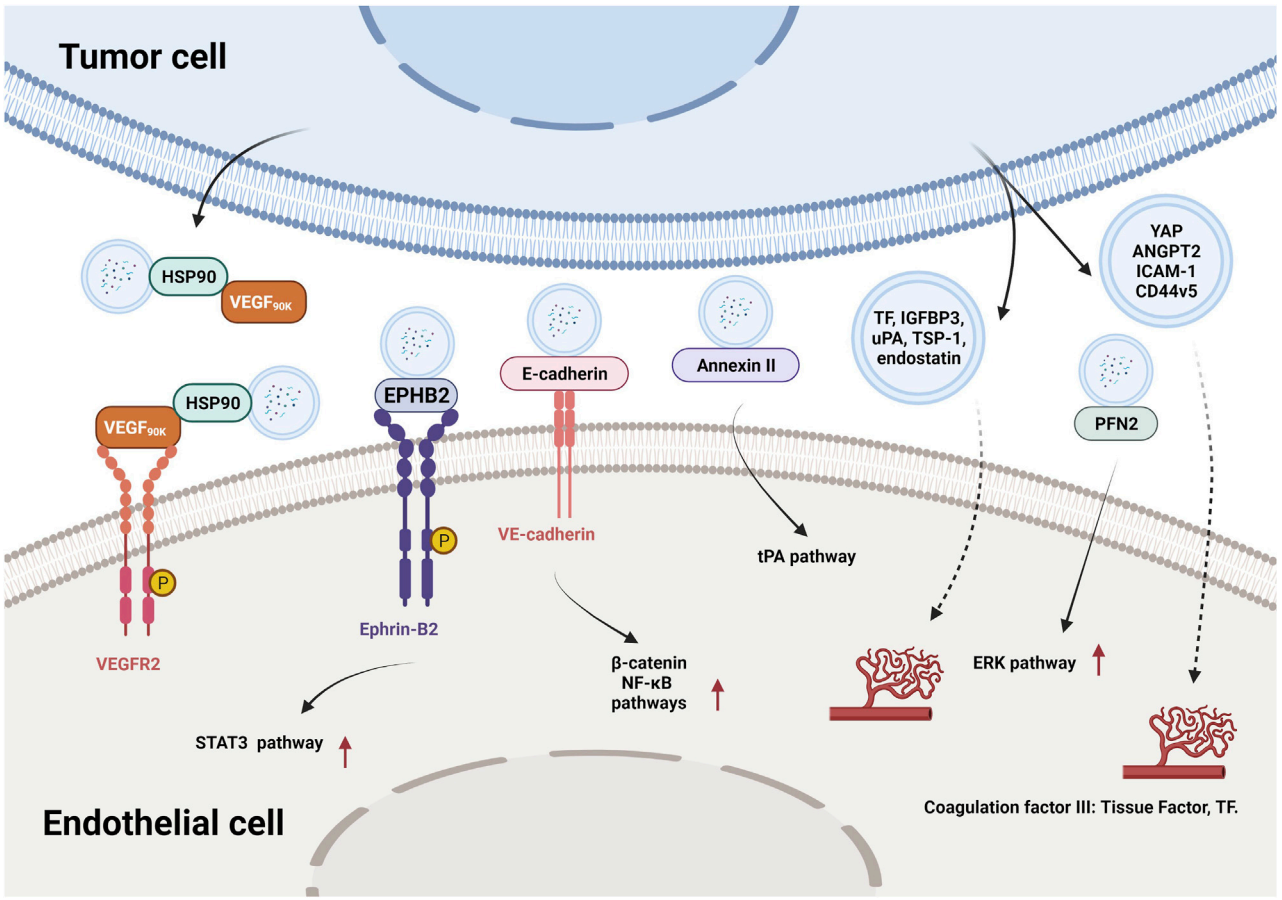

FIGURE 3 | The effects and mechanisms of proteins derived from tumor EVs on angiogenesis.

TABLE 2 | The effects and mechanisms of proteins derived from tumor EVs on angiogenesis.

Cargoes	Tumor types or donor cells	Recipient cells	Signaling pathways	Functions	References
Gas6	Perivascular cells from CRC	EPCs	Activation the Axl pathway	Revascularization	Huang et al. (2021)
$VEGF_{90K}$	BC	HUVECs	$VEGF_{90K}$-HSP90 complex	Proangiogenesis	Feng et al. (2017)
$VEGF_{189}$	OC, CRC, ccRCC, OC patient ascites	HUVECs	Association with the surface of small EVs via heparin-binding	Proangiogenesis	Ko et al. (2019)
EPHB2	HNSCC	HUVECs	Ephrin-B2-STAT3 angiogenic signaling cascade	Proangiogenesis	Sato et al. (2019)
Soluble E-cadherin	OC	HUVECs	Activation of the β-catenin and NF-κB signaling pathways in ECs	Proangiogenesis	Tang et al. (2018)
Annexin II	BC	HUVECs	Activation of the tPA pathway	Proangiogenesis	Maji et al. (2017)
YAP	LC	HUVECs	—	Proangiogenesis	Wang et al. (2019b)
Coagulation factor III, IGFBP3, uPA, TSP-1, endostatin	HNSCC	HUVECs	Functional reprogramming and phenotypic modulation of ECs	Proangiogenesis	Ludwig et al. (2018)
ANGPT2	HCC	HUVECs	—	Proangiogenesis	Xie et al. (2020a)
PFN2	LC	HUVECs	Activation of the Erk pathway	Proangiogenesis	Cao et al. (2020)
ICAM-1, CD44v5	NPC	HUVECs	—	Proangiogenesis	Chan et al. (2015)

Abbreviations: urokinase type plasminogen activator, uPA; tissue plasminogen activator, tPA.

increased compared to that of patients with benign hepatobiliary disease, indicating that circ-CCAC1 in EVs may serve as a biomarker for cholangiocarcinoma (Xu et al., 2021). CRC patients with metastasis showed a higher level of miR-25-3p in exosomes than patients without metastasis (Zeng et al., 2018). The expression of miR-619-5p in exosomes was increased in the serum of NSCLC patients, indicating that miR-619-5p can serve as a diagnostic indicator (Kim et al., 2020). High levels of exosomal miR-1260b were associated with high-grade disease, metastasis, and poor survival in patients with NSCLC (Kim et al., 2021).

Moreover, prostate-specific membrane antigen (PSMA) has emerged as a specific prostate tumor biomarker in prostate tumor-derived exosomes. Ziaei et al. developed a novel biofunctionalized silica nanostructure to capture tumor-derived exosomes through the interaction of PSMA and its ligand TG97, providing a noninvasive approach for prostate cancer diagnosis (Ziaei et al., 2017). The company MiRXES performed a test to analyze the levels of 12 miRNA biomarkers linked to GC and calculated a cancer risk score for each patient (Kapoor et al., 2020). Another study indicated that the level of phosphatidylserine-expressing tumor-derived exosomes in the blood is a reliable biomarker for early-stage cancer diagnosis (Sharma et al., 2017).

4 CONCLUSION AND PERSPECTIVES

Tumor angiogenesis plays a critical role in tumor growth and development, and antiangiogenic therapy has been frequently applied to the clinical treatment of multiple solid tumors. Among the generally known proangiogenic signaling pathways, miRNAs, lncRNAs, circRNAs, and proteins carried by tumor-secreted EVs have recently emerged as important modulators of tumor angiogenesis, acting through a variety of mechanisms, as described in this review.

Antiangiogenic therapy has been widely used for the treatment of various solid tumors and has conferred tremendous survival benefits to cancer patients (Teleanu et al., 2019; Lugano et al., 2020). Antiangiogenic drugs, such as bevacizumab, sorafenib, and regorafenib, inhibit tumor growth by suppressing angiogenesis primarily through blocking the VEGF/VEGFR pathway. However, many patients receive only modest survival benefits and develop acquired resistance to antiangiogenic drugs (Huijbers et al., 2016; Gacche and Assaraf, 2018). Drug resistance is one of the most important obstacles to treatment because it limits the clinical applications of antiangiogenic drugs, and the diseases still progress, which results in poor outcomes and unsatisfactory quality of life (Sennino and McDonald, 2012; van Beijnum et al., 2015). Since exosome-derived ncRNAs and proteins play important roles in tumor angiogenesis, targeting ncRNAs and proangiogenic proteins may be a potential therapeutic strategy to inhibit tumor angiogenesis.

Because a single miRNA, lncRNA, and circRNA species has the potential to regulate angiogenesis by modulating multiple targets, these ncRNAs hold great promise for use in therapeutic approaches to the treatment of tumor angiogenesis. However, in addition to tumors, ncRNAs significantly regulate the biological functions of normal cells, and systemic targeting of ncRNAs might affect physiological angiogenesis in normal tissues. Therefore, it is important to develop more specific therapeutic approaches based on angiogenesis-related ncRNAs. Moreover, EVs have turned out to be possible natural carriers of therapeutic agents with long half-time and non-immunogenic properties (Lakhal and Wood, 2011). These EV-based nanocarriers exhibit several advantages such as a high capacity for overcoming various biological barriers and high stability in the blood (Ha et al., 2016). However, the safety, specificity, and proficiency of this promising approach in clinical trials still remain more mysterious. EVs-based nanocarriers still face many challenges in clinical application.

In summary, this review provides deeper insight into the regulatory role of tumor-derived EVs on angiogenesis. Therefore, revealing the mechanisms of tumor-derived EVs on angiogenesis and seeking their potential as biomarkers and diagnostic, prognostic, and therapeutic targets in cancer patients will be popular research directions in the future.

AUTHOR CONTRIBUTIONS

LD, MiH and MaH designed and revised the manuscript. MaH and YL drafted the manuscript. YZ, CC, and MW participated in the procedures. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Tumor-Derived Extracellular Vesicles Regulate Cancer Progression in the Tumor Microenvironment

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Extracellular vesicles (EVs) are nanosized particles released by numerous kinds of cells, which are now increasingly considered as essential vehicles of cell-to-cell communication and biomarkers in disease diagnosis and treatment. They contain a variety of biomolecular components, including lipids, proteins and nucleic acids. These functional molecules can be transmitted between tumor cells and other stromal cells such as endothelial cells, fibroblasts and immune cells utilizing EVs. As a result, tumor-derived EVs can deliver molecules to remodel the tumor microenvironment, thereby influencing cancer progression. On the one hand, tumor-derived EVs reprogram functions of endothelial cells, promote cancer-associated fibroblasts transformation, induce resistance to therapy and inhibit the immune response to form a pro-tumorigenic environment. On the other hand, tumor-derived EVs stimulate the immune response to create an anti-tumoral environment. This article focuses on presenting a comprehensive and critical overview of the potential role of tumor-derived EVs-mediated communication in the tumor microenvironment.

Keywords: extracellular vesicles, tumor microenvironment, angiogenesis, resistance, immune cells, biomarkers

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INTRODUCTION

Extracellular vesicles (EVs), including exosomes and ectosomes, are nanoscale particles released by nearly all types of cells (Théry et al., 2018). Relying on transferring microRNA (miRNA), long noncoding RNA (lncRNA), messenger RNA (mRNA) and proteins, EVs modulate the functions and phenotypes of target cells (Bayraktar et al., 2017; Choi et al., 2017; Gon et al., 2017). For instance, the delivery of miR-330-3p from plasma cells to ovarian cancer cells by EVs induces a mesenchymal phenotype of ovarian cancers (Yang et al., 2021). In addition, EVs isolated from human vascular endothelial cells contain some cardioprotective proteins, which contribute to promoting human myocardium survival after ischemia-reperfusion injury (Yadid et al., 2020). Vesicular miR-21 derived from tubular epithelial cells stimulates fibroblast and subsequently causes renal fibrosis *in vivo* (Zhao S. et al., 2021). Vesicular lncRNA-SOX2OT from non-small cell lung cancer (NSCLC) cells induces osteoclast differentiation and promotes bone metastasis (Ni et al., 2021).

EVs are closely related to the physical and pathological processes of diseases, especially cancer (Wu et al., 2017; Burnouf et al., 2019; Gamage and Fraser, 2021). Tumor growth requires constant nutrients and oxygen delivered from the vascular network, as they cannot grow above 2 mm^2 with an

inadequate vascular supply (Small et al., 2014). Thereby, angiogenesis, the growth of new blood vessels from the posterior capillary veins and existing capillaries, is vital for tumor progression. EVs mediate communication between tumor cells and endothelial cells, thereby inducing angiogenesis and promoting tumor growth (Wan et al., 2018; Xu et al., 2018). Besides inducing angiogenesis, tumor-derived EVs can also regulate cancer-associated fibroblasts (CAFs) transformation. Since CAFs can remodel the stromal extracellular matrix (ECM) to facilitate tumor cell migration and invasion, CAFs transformation may promote cancer progression. In addition, EVs released from resistant tumor cells have the ability to induce resistance to cancer therapy, which further facilitates tumor progression. Immune cells such as natural killer (NK) cells, macrophages, T cells and B cells can interact with tumor cells via EVs, thereby causing their functions and phenotypes change. Furthermore, crosstalk between tumor-derived EVs and host immune system regulates immune response, thereby influencing cancer progression. Of note, tumor-derived EVs can be isolated from the conditioned medium of cancer cells but also from various body fluids like blood and ascites of cancer patients (Larrea et al., 2016). Due to their cargo diversity and specificity, tumor-derived EVs are promising biomarkers for cancer diagnosis and treatment to reflect the status of parental cancer cells.

THE BIOGENESIS OF EVS

The term EVs is used to describe almost all types of membrane particles secreted from cells. Based on their size and biogenesis, EV subpopulations can be divided into exosomes and ectosomes (Théry et al., 2018). Exosomes are secreted by inward invagination of the plasma membrane (Wolf, 1967; Johnstone et al., 1987). The first invagination of the plasma membrane leads to the generation of an early-sorting endosome that contains fluids, extracellular components and cell surface proteins. The early-sorting endosome undergoes a series of transformations to mature into the late-sorting endosome. The second invagination of the late-sorting endosome results in the formation of multivesicular bodies (MVBs) that contains intraluminal vesicles (ILVs). The MVBs can fuse with the plasma membrane to release exosomes with a size range of 30–150 nm in diameter. The basic mechanisms responsible for exosomes biogenesis have been reported. The endosomal sorting complex required for transport (ESCRT) machinery, containing four protein complexes (ESCRT-0, -I, -II, and -III) along with associated proteins (VTA-1, Alix and VPS4), is closely related to the biogenesis of MVBs and ILVs (Henne et al., 2011). The specific functional components of ESCRT have also been investigated. While the silence of ESCRT-0 and ESCRT-I (HRS) decreases the biogenesis of exosomes, depletion of other ESCRT components exerts no effects or even increases the biogenesis of exosomes (Colombo et al., 2013). ESCRT proteins also play an essential role in specifying the loading of functional cargoes into exosomes. They mediate the sorting of cargo at endosomal plasma and subsequently induce the late-sorting endosomes to release ILVs (later exosomes) with the sorted cargoes. Exosomes biogenesis can operate in an ESCRT-independent manner in some cancer cells, which has been demonstrated by silencing multiple ESCRTs (Stuffers et al., 2009). In addition, some RAB GTPases (RAB27, RAB11, and RAB31) have been found to drive MVBs transport and ILVs biogenesis (Savina et al., 2002; Ostrowski et al., 2010; Wei et al., 2020). For instance, RAB31 can enhance the formation of ILVs and inhibit the degradation of MVBs in an ESCRT-independent manner (Wei et al., 2020). Mechanically, the high level of RAB31 can drive epidermal growth factor receptor into MVBs to generate ILVs and recruit TBC1D2B to prevent MVBs degradation (Wei et al., 2020). However, the upstream of RAB GTPases is not well clarified. Song et al. (2019) found that KIBRA could inhibit the ubiquitination and degradation of RAB27a, thereby contributing to exosomes biogenesis (Song et al., 2019). Phospholipase D2 and its product phosphatidic acid are involved in ILVs biogenesis and exosomes release (Egea-Jimenez and Zimmermann, 2018). Notably, ESCRT-dependent pathways and ESCRT-independent pathways can also jointly drive exosomes biogenesis. Syndecan-syntenin complexes bind ESCRT-I and ESCRT-III via Alix, leading to enhanced ILVs biogenesis (Baietti et al., 2012). Moreover, tyrosine phosphatase Shp2 has been found to inhibit exosomes biogenesis via dephosphorylating syntenin (Zhang Y. et al., 2021). CD63, belonging to tetraspanin family, is associated with sorting cargoes into exosomes (Theos et al., 2006; van Niel et al., 2011).

Ectosomes with a size range of 50–1,000 nm in diameter are released by shedding or outward budding of the plasma membrane. This process is driven by translocating phosphatidylserine to the outer-membrane leaflet (Zwaal and Schroit, 1997). The mechanisms involved in the biogenesis of ectosomes have been studied. Inhibiting VPS4 is shown to impair ectosomes release, suggesting that ESCRT-III is also required for ectosomes biogenesis (Mathieu et al., 2019). Small GTPase RhoA, an essential regulator of actin cytoskeletal remodeling, is closely related to ectosomes biogenesis in different tumor cells (Li et al., 2012). Moreover, RHO-associated protein kinase (ROCK) has been revealed to mediate the function of RhoA in ectosomes biogenesis. Thus, inhibition of ROCK-1 and ROCK-2 by the small molecule Y-27632 can decrease ectosomes biogenesis (Li et al., 2012). Ectosomes are rich in cholesterol, and knockdown of cholesterol can inhibit ectosomes biogenesis (Del Conde et al., 2005). In addition, nSMAse participates in shedding budding of the plasma membrane, and hence controls ectosomes biogenesis (Menck et al., 2017).

TUMOR MICROENVIRONMENT AND TUMOR-DERIVED EVS

The TME (tumor microenvironment) comprises tumor cells, endothelial cells, fibroblasts, and immune cells as well as extracellular components such as ECM, cytokines and growth factors (Lv et al., 2012; Jin and Jin., 2020) (**Figure 1**). The ECM is a highly dynamic three-dimensional network composed of plenty

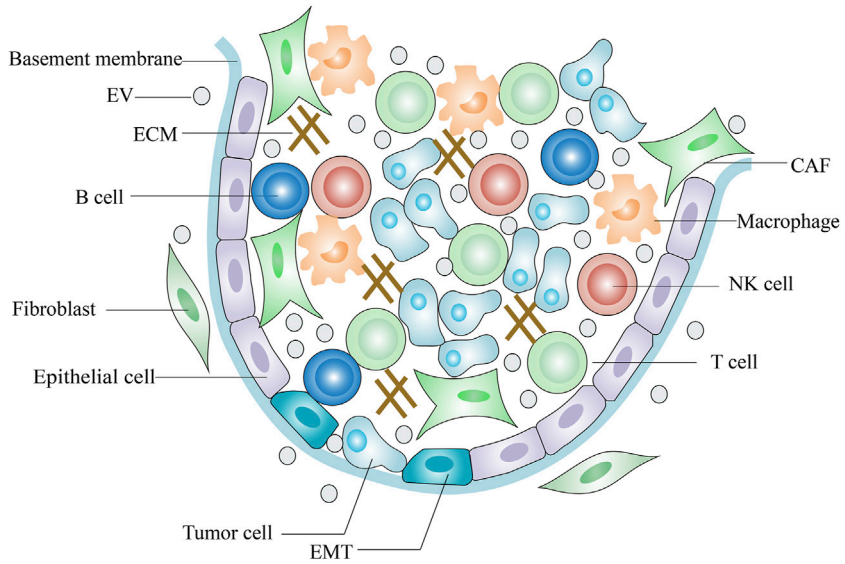

FIGURE 1 | Scheme of tumor microenvironment.

of fibrous proteins and glycoproteins (Mouw et al., 2014). Tumors often exhibit ECM deposition and degradation, and this dysregulation state supports tumorigenesis and metastasis and induces angiogenesis (Sükei et al., 2021). Cytokines are small molecular polypeptides or proteins that serve as immunomodulatory effectors. Overproduction of IL-6 by tumor cells activates STAT-3, a key transcription factor central to immune escape and it is an important regulator in the crosstalk between tumor cells and TME (Lokau et al., 2019). IL-10 is an anti-inflammatory cytokine and its serum levels are negatively related to the tumor prognosis (Pasvenskaite et al., 2021). On the other hand, IL-10 exerts anti-tumor activity by enhancing the immune-stimulatory effect of $CD8^+$ T cell (Naing et al., 2018). Some growth factors in the TME inhibit normal stromal cells proliferation and promote tumor cells metastasis. Transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) form a pro-tumorigenic environment that fosters tumor cell survival, progression and metastasis and directs abnormal vessel growth (Zhou X. et al., 2018; Shang et al., 2020; Zheng et al., 2021). However, TGF-β also shows anti-oncogenic properties in carcinogenesis. TGF-β has been reported to inhibit tumor cell proliferation and induce apoptosis in the early stages of carcinogenesis (Inman, 2011).

As a means of communication between tumor cells and the microenvironment, EVs play an essential role in remodeling the local microenvironment (Milane et al., 2015). Numerous studies have revealed that EVs released by tumor cells contain a variety of biomolecular components, including lipids, proteins and nucleic acids (Taylor et al., 2011; Mathivanan et al., 2012; Agudiez et al., 2020). Especially, those nucleic acids components such as miRNAs and lncRNAs may mediate the formation of a protumoral or an anti-tumoral soil in the microenvironment, thereby influencing tumor progression. Interestingly, hypoxic or metastatic status of tumors appears to a strong force in sorting the loading of composition into EVs, which affects functions of tumor-derived EVs in the TME (Kucharzewska et al., 2013; Yokoi et al., 2017; Chen et al., 2018). Hypoxia is a common feature in most malignant tumors. In hypoxic microenvironment, tumor cells drive glucose mainly into lactate to meet the energy requirements. This phenomenon, called the Warburg effect, is one of the cellular mechanisms by which cancer cells adapt to hypoxic microenvironment and enhance survival (Parks et al., 2017). PKM2, which plays a key role in the Warburg effect, is the enhancer of anaerobic glycolysis. Hypoxic NSCLC cell derived-EVs promote PKM2-dependent glycolysis and subsequently produce metabolites to eliminate ROS, thereby inhibiting tumor apoptosis and promoting tumor growth (Wang et al., 2021). EVs derived from breast cancer enhance the ability of CAFs in response to different metabolic environment by activating MYC signaling pathway in stromal cells resulting in rapid tumor growth (Yan et al., 2018). Moreover, EVs released from Lewis lung carcinoma can induce immunosuppressive macrophages by NF-κB-mediated metabolism reprogramming, leading to tumor metastasis (Morrissey et al., 2021).

REGULATION OF PROTUMORAL FUNCTIONS OF ENDOTHELIAL CELLS BY TUMOR-DERIVED EVS

Tumor-derived EVs are thought to regulate protumoral functions of endothelial cells in numerous types of cancers including hepatocellular carcinoma (HCC) (Lin et al., 2018), colorectal cancer (Huang and Feng, 2017; Zeng et al., 2018; He et al., 2021), cervical cancer (Wu et al., 2019), nasopharyngeal carcinoma (Bao et al., 2018; Tian et al., 2021; Zhang K. et al., 2021), glioma (Ma et al., 2017; Wang Z.-F. et al., 2019), and lung

TABLE 1 | Role of tumor-derived EVs in angiogenesis.

Cargoes	Cancer types	Mechanisms	References
miR-210	HCC	SMAD4 and STAT6↓	Lin et al. (2015), Lin et al. (2018)
miR-21-5P	Colorectal cancer	Krev interaction trapped protein 1↓; β-catenin signaling pathway, VEGFA and Ccnd1↑	He et al. (2021)
miR-221-3p	Cervical cancer	Thrombospondin-2↓	Wu et al. (2019)
miR-144	Nasopharyngeal carcinoma	FBXW7↓; HIF-1α and VEGFA↑	Tian et al. (2021)
HMGB3	Nasopharyngeal carcinoma	Unknown	Zhang et al. (2021a)
miR-26a	Glioma	PTEN↓; PI3k/Akt signaling pathway↑	Wang et al. (2019c)
LncRNA HOTAIR	Glioma	VEGFA↑	Ma et al. (2017)
Wnt4	Colorectal cancer	Wnt/β-catenin signaling pathway↑	Huang and Feng (2017), Yamada (2017)
miR-23a	Lung cancer	Prolyl hydroxylase 1/2↓; HIF-1α↑	Hsu et al. (2017)
miR-23a	Nasopharyngeal carcinoma	Testis-specific gene antigen↓	Bao et al. (2018)
miR-25-3p	Colorectal cancer	Krüppel-like factor 2, Krüppel-like factor 4, occludin, zonula occludens-1 and Claudin5↓; VEGFR2↑	Zeng et al. (2018)

Symbols: ↑, up-regulation; ↓, down-regulation.

cancer (Hsu et al., 2017). As EVs can be internalized by an endocytic-like process, they may deliver regulatory biomolecules into vascular endothelial cells. Thus, tumor cells can participate in the regulation of endothelial cell proliferation, migration, sprouting, branching, as well as tubular-like structure formation by secreting EVs (Zhuang et al., 2012; French et al., 2017).

Some miRNAs, lncRNAs and proteins delivered by EVs have been reported to participate in regulating protumoral functions of endothelial cells (**Table 1**). miR-210 enriched in EVs of malignant tumors may promote tubular-like structure formation of endothelial cells, leading to pro-angiogenic activities and rapid tumor growth. In HCC, abundant miR-210 can be packed into EVs and transferred to endothelial cells (Lin et al., 2015; Lin et al., 2018). After taking up by human umbilical vein endothelial cells (HUVECs), miR-210 stimulates angiogenesis via down-regulating the expression of SMAD4 and STAT6 (Lin et al., 2018). Vesicular miR-21-5p from colorectal cancer decreases Krev interaction trapped protein 1 expression to activate β-catenin signaling pathway and promote the expression of angiogenesis-related factors like VEGFA, thereby stimulating vascular permeability and angiogenesis (He et al., 2021). In addition, cervical cancer-derived vesicular miR-221-3P promotes angiogenesis by inhibiting the expression of thrombospondin-2 in HUVECs, which consequently enhances tumor growth *in vivo* (Wu et al., 2019). miR-144 is a key angiogenesis inducer for neo-angiogenesis in nasopharyngeal carcinoma (Tian et al., 2021). Vesicular miR-144 suppresses FBXW7 and increases hypoxia-inducible factor-1α (HIF-1α) and VEGFA in recipient cells, which consequently promotes endothelial cells migration and invasion (Tian et al., 2021). Moreover, the transfer of high mobility group box 3 (HMGB3) from nasopharyngeal carcinoma cells to endothelial cells via EVs induces angiogenesis (Zhang K. et al., 2021). Interestingly, neo-angiogenesis in nasopharyngeal carcinoma facilitates the formation of pre-metastatic niches, which further causes tumor metastasis (Zhang K. et al., 2021). Vesicular miR-26a from glioma down-regulates phosphatase and tensin homolog (PTEN) expression to stimulate PI3k/AKT signaling, thereby contributing to the proliferation of human brain microvascular endothelial cells (HBMECs) (Wang Z.-F. et al., 2019). Ma et al. (2017) believe that the delivery of lncRNA HOTAIR from glioma cancer cells to HBMECs *via* EVs up-regulates the level of pro-angiogenic factor VEGFA.

It is well-known that disordered vascular distribution and abnormal vascular structure lead to specific hypoxia in many solid tumors. In turn, tumor-derived EVs secreted under hypoxic conditions induce proliferation and migration of endothelial cells, thereby enhancing angiogenesis and tumor growth. For example, EVs isolated from colorectal cancer cells under hypoxia conditions show a more potent pro-angiogenic effect as compared with that from colorectal cancer cells under normoxia conditions (Huang and Feng, 2017). The reason for this phenomenon may be that Wnt4 is highly enriched in hypoxic colorectal cancer-derived EVs, and the increased Wnt4 stimulates the β-catenin signaling pathway in endothelial cells (Yamada, 2017). Similarly, lung cancer-derived vesicular miR-23a under hypoxia conditions enhances the production of HIF-1α in endothelial cells via inhibiting the expression of prolyl hydroxylase 1/2, thereby directly promoting angiogenesis and tumor growth (Hsu et al., 2017).

On the other hand, the emerging evidence has shown that the contents of tumor-derived EVs may be enriched at the metastatic stage during cancer development, and those increased contents can be delivered to endothelial cells to exert biological roles. For example, miR-23a is shown to be significantly higher in nasopharyngeal carcinoma tissues with metastasis than those without metastasis, and its level is associated with angiogenesis (Bao et al., 2018). Furthermore, the molecular mechanism for vesicular miR-23a-mediated angiogenesis may be related to testis-specific gene antigen (Bao et al., 2018). Metastasis-induced vesicular miR-25-3p promotes vascular permeability and angiogenesis, leading to the formation of pre-metastatic niches (Zeng et al., 2018). Mechanically, vesicular miR-25-3p in colorectal cancer can silence Krüppel-like factor 2 and krüppel-like factor 4, thereby enhancing the expression of vascular endothelial growth

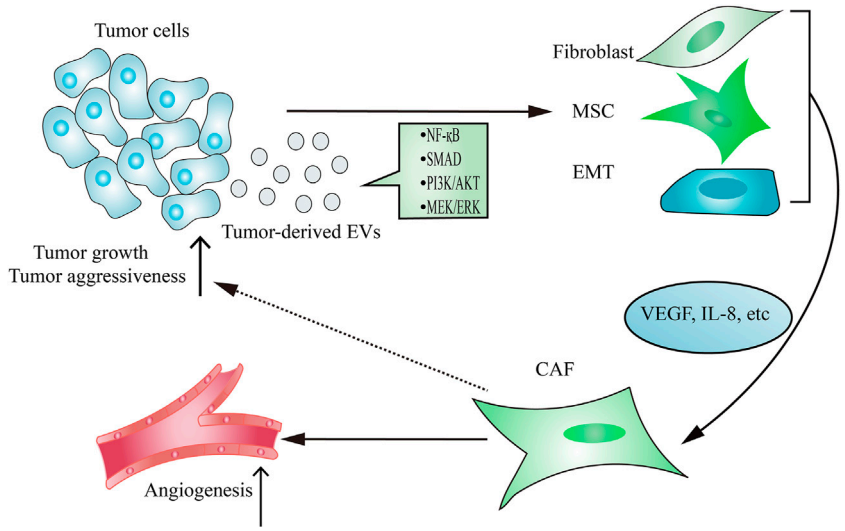

FIGURE 2 | Tumor-derived EVs regulate CAFs transformation. The delivery of functional signaling factors from tumor cells to fibroblasts, MSCs or EMT contributes to CAFs transformation, which consequently promotes tumor growth and aggressiveness and induces angiogenesis.

factor receptor 2 (VEGFR2) and inhibiting the expression of occludin, zonula occludens-1 and Claudin5 (Zeng et al., 2018).

Tumor-derived EVs play an important role in inducing angiogenesis. Similarly, EVs derived from endothelial cell and perivascular cell are also a key player in tumor progression. Anti-angiogenic therapies are thought to improve the prognosis of tumor patients by inhibiting tumor vascularization. However, the outcomes of anti-angiogenic therapies are not ideal for most patients. EVs released from endothelial cells treated with vandetanib enrich VEGF, thus promoting angiogenesis and tumor growth *in vivo* (Zeng et al., 2019). In addition, EVs released from perivascular cell trigger endothelial progenitor cells recruitment after anti-angiogenic therapy cessation, which contributes to blood vessel regrowth and rapid tumor growth (Huang et al., 2021). Mechanically, Gas6-containing perivascular cell-derived EVs activate Axl signaling and subsequently promote tumor revascularization (Huang et al., 2021).

REGULATION OF PROTUMORAL FUNCTIONS OF CAFS BY TUMOR-DERIVED EVS

As the main contributor to remodel tumor stroma, CAFs are often transformed from resident fibroblasts, mesenchymal stem cells (MSCs) and epithelial-to-mesenchymal transition (EMT) cells after taking up tumor-derived EVs (**Figure 2**). The active CAFs may enhance angiogenesis and metastasis, thereby contributing to establishing a tumor-promoting environment. Hodgkin lymphoma-derived EVs transform normal fibroblasts into pathological CAFs utilizing the NF-κB signaling pathway, which leads to the release of neo-angiogenesis factors (Dörsam et al., 2018). Notably, many studies have shown that the delivery of functional biomolecules plays a vital role in regulating CAFs transformation (Paggetti et al., 2015; Fang et al., 2018; Giusti et al., 2018; Yang et al., 2018; Wang J. et al., 2018; Zhou Y. et al., 2018). For instance, EVs released from chronic lymphocytic leukemia cells induce fibroblasts transformed to CAFs by the enrichment of some regulatory proteins and miRNAs from parental cells, which consequently causes rapid tumor growth (Paggetti et al., 2015). In addition to regulating the transformation of fibroblasts into a CAF phenotype, tumor-derived EVs have also been demonstrated to play an important role in inducing the transition of MSCs into CAFs. EVs isolated from breast cancer stimulate SMAD-mediated pathway and subsequently increase CAFs marker expression in MSCs, which consequently enhances angiogenesis and metastasis (Cho et al., 2012). Moreover, tumor cells-derived EVs are capable of regulating the transformation of pericytes into a pathological CAFs phenotype. Relying on releasing EVs, gastric cancer cells promote pericytes proliferation and migration, and induce pericytes transformed into CAFs (Ning et al., 2018). Mechanically, gastric cancer cells-derived EVs stimulate PI3k/AKT and MEK/ERK pathways, leading to the up-regulated expression of CAFs markers (Ning et al., 2018).

Due to the emerging evidence indicates EVs isolated from tumor cells response to hypoxia, many researchers have investigated the potential of tumor-derived EVs under hypoxia conditions in CAFs transformation (Kucharzewska et al., 2013; Wang et al., 2014; Ramteke et al., 2015). It has been reported that EVs secreted from prostate cancer cells under hypoxia conditions promote CAFs transformation and tumor aggressiveness (Ramteke et al., 2015). Interestingly, EVs derived from tumor cells can not only enrich some proteins, but also load some specific proteins that may induce tumor-promoting microenvironment under hypoxia conditions (Ramteke et al., 2015). This finding suggests that unique components loaded in hypoxia tumor-derived EVs may be helpful to CAFs transformation and tumor progression.

TABLE 2 | Role of tumor-derived EVs in therapy resistance.

Cargoes	Cancer types	Functions	Mechanisms	References
miR-155-5p	Gastric cancer	Paclitaxel resistance and EMT↑	GATA3 and TP53INP1↓	Wang et al. (2019b)
miR-423-5p	Breast cancer	Cisplatin resistance, breast cancer cells proliferation and migration↑	P-glycoprotein↑	Wang et al. (2019a)
miR-100-5p	Lung cancer	Cisplatin resistance↑	Mammalian target of rapamycin↑	Qin et al. (2017)
LncRNA H19	NSCLC	Gefitinib resistance↑	Unknown	Lei et al. (2018)
LncRNA-SNHG14	Breast cancer	Trastuzumab resistance↑	Bcl-2/Bax apoptosis signaling pathway↑	Dong et al. (2018)
TrpC5	Breast cancer	Adriamycin resistance↑	P-glycoprotein↑	Ma et al. (2014)
PKM2	NSCLC	Cisplatin resistance and NSCLC cells proliferation↑	CAFs transformation↑	Wang et al. (2021)
Annexin A6	Triple-negative breast cancer	Gemcitabine resistance↑	Epidermal growth factor receptor↓	Li et al. (2021)
Unknown	Squamous head and neck cancer	Radio-resistance and Squamous head and neck cancer cells proliferation↑	Repair of damaged DNA content↑	Mutschelknaus et al. (2016)
miR-301a	Glioma	Radio-resistance↑	TCEAL7↓; Wnt/β-catenin signaling pathway↑	Yue et al. (2019)
Anaplastic lymphoma kinase	NSCLC	Anaplastic lymphoma kinase inhibitors resistance, Ceritinib resistance and tumor growth↑	AKT, STAT3 and ERK signaling pathways↑	Wu et al. (2018)

Symbols: ↑, up-regulation; ↓, down-regulation.

On the other hand, tumor-derived EVs appear to be enriched during fibroblasts reprogramming may be a reaction to the high-metastatic tumor state. Additionally, the functional contents of EVs can also be related to the metastatic status of HCC cells (Fang et al., 2018). The amount of EVs is much higher in high-metastatic cancer cells than that in low-metastatic cancer cells (Fang et al., 2018). This further enhances the regulatory effect of high-metastatic tumor cell-derived EVs on CAFs transformation. Mechanically, elevated miR-1247-3p in EVs isolated from high-metastatic tumor cells that promotes CAFs transformation via inhibiting B4GALT3 expression (Fang et al., 2018).

Tumor-derived EVs play an important role in CAFs transformation, in turn, CAF-derived EVs participate in tumorigenesis. For instance, vesicular miR-92a-3p from CAFs induce EMT, chemoresistance and cancer stemness in colorectal cancer by activating Wnt/β-catenin signaling pathway (Hu et al., 2019). EVs released from CAFs enrich miR-196a by activating heterogeneous nuclear ribonucleoprotein A1, leading to decreased CDKN1B and ING5 in recipient head and neck cancer cancer cells, and ultimately result in enhanced cisplatin resistance and metastasis (Qin et al., 2019). Moreover, ubiquitin-specific protease 7 has been found to inhibit heterogeneous nuclear ribonucleoprotein A1 ubiquitination in CAFs (Zhang et al., 2020). miR-522-bearing EVs released from CAFs inhibit arachidonate lipoxygenase 15 expression and lipid peroxides accumulation, leading to enhanced chemoresistance in gastric cancer (Zhang et al., 2020). Thus, depletion of CAF-derived EVs causes improved chemosensitivity.

REGULATION OF RESISTANT PHENOTYPE OF SENSITIVE CANCER CELLS BY TUMOR-DERIVED EVS

Emerging studies have confirmed that tumor-derived EVs play a vital role in the resistance of tumor cells to cancer therapy, including chemotherapy and radiotherapy (**Table 2**). Some drug-resistant tumor cells have the ability to confer a drug-resistant phenotype upon sensitive cells in an EVs-dependent manner. This may be due to EVs' ability to mediate the transfer of miRNA, lncRNA and proteins associated with drug resistance to recipient cells (Corrado et al., 2013). For instance, paclitaxel-resistant gastric cancer cells have been reported to be rich in miR-155-5p (Wang M. et al., 2019). miR-155-5p can be delivered from resistant cancer cells to sensitive cells by EVs, thereby increasing the expression level of miR-155-5p in recipient cells. The increased miR-155-5p confers paclitaxel resistance and induces EMT in gastric cancer cells via inhibiting the expression of GATA3 and TP53INP1 (Wang M. et al., 2019). Similarly, miR-423-5p-bearing EVs induce the transformation of breast cancer cells from sensitive cells to cisplatin-resistant cells (Wang B. et al., 2019). While the above examples show that drug-resistant tumor-derived EVs can disseminate drug resistance via transferring increased miRNAs to recipient cells, it appears that EVs can also induce drug-resistance by decreased miRNAs. EVs released from cisplatin-resistant lung cancer cells down-regulate a total of 11 miRNAs, in which miR-100-5p is the most significantly down-regulated miRNA (Qin et al., 2017). The down-regulated miR-100-5p modulates the expression of the mammalian target of rapamycin in recipient cells, which induces a chemo-resistant phenotype upon NSCLC cells (Qin et al., 2017).

The expression of lncRNA H19 is up-regulated within EVs from gefitinib-resistant NSCLC cells (Lei et al., 2018). Vesicular lncRNA H19 can be transported to sensitive cells to induce gefitinib resistance (Lei et al., 2018). EVs isolated from trastuzumab-resistant $HER2^+$ breast cancer increase the level of lncRNA-SNHG14, which can induce a chemo-resistant phenotype upon sensitive tumor cells (Dong et al., 2018). Besides mediating the transfer of miRNA or lncRNA to induce drug resistance, EVs have also been shown to deliver proteins to target cells to disseminate resistance. For instance, EVs released from patients with a poor response to chemotherapy up-regulate the expression of transient receptor potential channel 5 (TrpC5) (Ma et al., 2014). Relying on

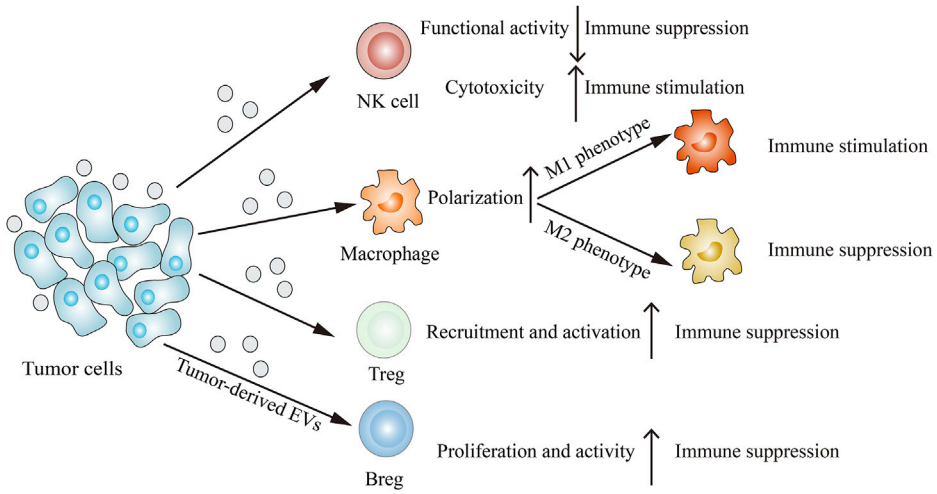

FIGURE 3 | Tumor-derived EVs modulate the immune system. Tumor-derived EVs can suppress NK cells functional activity, induce M2 macrophage polarization, recruit Tregs and proliferate Bregs, thus inhibiting immune response. On the other hand, tumor-derived EVs can also enhance NK cells cytotoxicity and induce M1 macrophage polarization, thereby stimulating immune response.

EVs, the increased TrpC5 can enter sensitive breast cancer cells to disseminate resistance. In addition, PKM2-bearing EVs from cisplatin-resistant tumor cells induce a chemo-resistant phenotype upon NSCLC cells by reprogramming CAFs transformation (Wang et al., 2021). Recently, vesicular transfer of annexin A6 has been found to confer gemcitabine-resistance in sensitive triple-negative breast cancer cells via suppressing and degrading of epidermal growth factor receptor (Li et al., 2021).

Additionally, the involvement of EVs in the resistance of tumor cells to radiotherapy has been reported. Early data demonstrated that the protein composition of tumor-derived EVs might be changed when exposed to radiation (Jelonek et al., 2015). Apart from affecting the composition of EVs, radiation has also been shown to affect the functions of EVs on target cells. EVs isolated from irradiated squamous head and neck cancer cells can confer radio-resistance in recipient cells via repairing damaged DNA content (Mutschelknaus et al., 2016). EVs isolated from hypoxic glioma are rich in miR-301a, which is associated with the resistance of tumor cells to radiotherapy (Yue et al., 2019). Mechanically, miR-301a-bearing EVs directly target TCEAL7 gene to induce radio-resistance in glioma cells and this effect can be reversed by inhibiting the Wnt/β-catenin pathway (Yue et al., 2019). Similarly, EVs released from irradiated cells can also reduce the sensitivity of recipient cells to the drug (Wu et al., 2018). Mechanically, EVs released from NSCLC cells can induce anaplastic lymphoma kinase inhibitors-resistant or Ceritinib-resistant phenotype upon target tumor cells via stimulating AKT, STAT3 and ERK pathways (Wu et al., 2018).

TUMOR-DERIVED EVS MODULATE THE IMMUNE SYSTEM

Immune cells such as NK cells, macrophages, T cells and B cells can interact with tumor cells, resulting in their functions and phenotypes changes. The emerging report reveals that tumor-derived EVs are involved in remodeling the tumor immune microenvironment (**Figure 3**). Through releasing EVs, tumor cells can deliver immune-inhibitory and immune-stimulatory signaling biomolecular components to the tumor immune microenvironment, thus creating a protumoral or an anti-tumoral soil to influence cancer progression (Whiteside, 2016).

NK Cells

NK cells, which play a key role in cancer immunotherapy, are the important subset of innate immune cells. Early research demonstrated that NK cell activity could be inhibited by breast cancer-derived EVs, which resulted in the accelerated growth of xenograft tumors (Liu et al., 2006). Researchers isolated NK cells from the spleens of BALB/c mice that had been pretreated with purified breast cancer-derived EVs and determined NK cell activity by the chromium release assays (Liu et al., 2006). Trials have shown that NK cell cytolytic activity was inhibited in mice by EVs released from TS/A tumor cells (Liu et al., 2006). Further study has demonstrated that pretreated mice with TS/A tumor cells-derived EVs would lead to a significant decrease in the total number and percentages of NK cells (Liu et al., 2006). Given the accumulating evidence for the role of EVs isolated from tumor cells in response to hypoxia, some researchers have investigated the potential of tumor-derived EVs under hypoxia conditions in reprogramming functions of NK cells. For instance, EVs secreted from tumor cells under hypoxia conditions show a more potent ability to impair the cytotoxicity of NK cells as compared with that from tumor cells under normoxia conditions (Berchem et al., 2016). In addition, the expression levels of functional activity markers such as CD107a and IFN-γ in NK cells pretreated with hypoxic tumor-derived EVs are significantly lower than those in NK cells pretreated with normoxic tumor-derived EVs (Berchem et al., 2016). This could be in part explained by the abundance of miR-

23a in hypoxic tumor cells-derived EVs that could function as an additional immunosuppressive activator by directly targeting CD107a in NK cells (Berchem et al., 2016). On the other hand, EVs secreted from pancreatic cancer cells at the high-metastatic state also appear to down-regulate the expression of CD107a and IFN-γ in NK cells (Zhao et al., 2019). Furthermore, EVs isolated from pancreatic cancer patients contain abundant TGF-β1, which can attenuate CD107a and IFN-γ expression in NK cells (Zhao et al., 2019). Mechanically, TGF-β1-bearing EVs activate the TGF β-Smad2/3 pathway in NK cells to impair NK cell-mediated cytotoxicity (Zhao et al., 2019). Apart from inhibiting the cytotoxic activity of NK cells, tumor-derived EVs have also been shown to act as an inducer to stimulate effective NK cell anti-tumor response. For example, EVs isolated from resistant anti-cancer drug-treated HCC cells are able to stimulate the suppressive effects of NK cells on tumor cell proliferation (Lv et al., 2012). The reason for this phenomenon may be that EVs released from HCC cells treated with resistant anti-cancer drugs contain abundant heat shock proteins (HSPs), including HSP60, HSP70 and HSP90 (Lv et al., 2012). Notably, those resistant anti-cancer drugs promote HSP-bearing EVs release, thereby contributing to activating the cytotoxic response of NK cells (Lv et al., 2012).

Macrophages

Macrophages, as one part of innate immune systems, can be affected by many factors to switch their phenotype. Activated macrophages are commonly classified into two phenotypes, classical activation (M1) macrophages and alternative activation (M2) macrophages. M1 macrophages secrete pro-inflammatory cytokines to induce tumoricidal activity, while M2 macrophages secrete anti-inflammatory cytokines to promote tumorigenesis. Tumor-associated macrophages (TAMs) are the main immune cell population in TME, which can be educated by various tumor cells and display an M2-like phenotype to promote the development and progression of tumors. Nowadays, tumor-derived EVs are described as containing a variety of functional components and are now emerging as a key regulator of macrophage polarization. Those components, such as miRNA, lncRNAs and proteins, can be transferred to macrophages via EVs to switch their phenotype.

Epithelial ovarian cancer-derived EVs can induce macrophages to secrete anti-inflammatory cytokine IL-10, leading to enhanced tumor growth and metastasis (Ying et al., 2016). The expression level of vesicular miR-222-3p in epithelial ovarian cancer patients is markedly higher than that in healthy people (Ying et al., 2016). Increased miR-222-3p has the ability to inhibit SOCS3 expression and stimulate the SOCS3/STAT3 pathway in macrophages, thereby inducing TAM-like phenotype macrophages production *in vitro* and *in vivo* (Ying et al., 2016). In addition, hypoxic EVs isolated from tumor cells, including pancreatic cancer and glioma, can generate the M2-like phenotype macrophages (Wang et al., 2018b; Qian et al., 2020). miR-301a-3p is highly enriched in EVs isolated from PANC-1 and BxCP-3 pancreatic cancer cells cultured in hypoxia conditions (Wang et al., 2018b). Mechanically, vesicular miR-301a-3p down-regulates PTEN expression and subsequently activates the PI3Kγ pathway, resulting in increased expression of M2 macrophage marker like CD163 (Wang et al., 2018b). Furthermore, the knockdown of HIF-1a and HIF-2a in pancreatic cancer cells revealed that miR-301a-3p expression level under hypoxia conditions relied on HIF-1a and HIF-2a (Wang et al., 2018b). In glioma, miR-1246 is the most prominently increased content in hypoxic tumor-derived EVs as compared with that in normoxic tumor-derived EVs (Qian et al., 2020). The increased miR-1246 is considered as a key regulator in inducing M2 macrophage polarization, since it can activate the STA3T signaling pathway and suppress the NF-κB signaling pathway (Qian et al., 2020). Notably, the M2 macrophage is a pro-tumor phenotype that promotes tumor cells migration and invasion via facilitating the formation of the immunosuppressive microenvironment. Vesicular lncRNA BCRT1 from breast cancer cells enhances tumor cells migration and invasion (Liang et al., 2020). Injection of breast cancer cells into lncRNA BCRT1-overexpressing mice causes more and larger metastatic lung foci (Liang et al., 2020). In addition, M2 markers (CD206 and MRC-2) expression are shown to up-regulate when macrophages stimulated by EVs derived from lncRNA BCRT1-overexpressing breast cancer cells (Liang et al., 2020). Furthermore, the transfer of Rab22a-NeoF1 fusion protein from osteosarcoma cells to negative tumor cells via EVs contributes to the formation of pre-metastatic niche in osteosarcoma (Zhong et al., 2021). Rab22a-NeoF1 fusion protein recruits bone marrow-derived macrophages and subsequently induces M2 macrophage polarization via its binding partner PYK2 (Zhong et al., 2021). Quantitative real-time PCR (RT-qPCR) analysis showed lncRNA TUC339 was significantly enriched in tumor-derived EVs (Li et al., 2018). Knocking out TUC339 in macrophages resulted in elevated pro-inflammatory cytokines IL-1β and TNF-α (Li et al., 2018). In turn, pro-inflammatory cytokine production decreased in TUC339-overexpressing macrophages (Li et al., 2018). This reveals that the lncRNA TUC339 can serve as a regulator to modulate M2 polarization macrophages.

In addition to mediating transmit miRNA or lncRNA to induce M2 polarization macrophages, EVs have also been shown to deliver miRNA or protein to target cells, thus inducing M1 polarization macrophages. For instance, miR-21 is selectively enriched in EVs isolated from colorectal cancer, which correlates with the increased M1/M2 ratio (Shao et al., 2018). In addition, miR-21 mimic causes increased pro-inflammatory cytokine production in macrophage (Shao et al., 2018). Mechanically, vesicular miR-21 up-regulates IL-6 expression level in macrophages by directly binding to toll-like receptor (TLR) 7, thereby contributing to creating an inflammatory TME (Shao et al., 2018). miR-9 has been found to be markedly enriched in HPV + head and neck squamous cell carcinoma (Tong et al., 2020). In addition, vesicular miR-9 down-regulates PPARδ and subsequently induces M1 polarization macrophages, which consequently leads to enhanced tumor radiosensitivity (Tong et al., 2020). Moreover, EVs isolated from oral squamous cell carcinoma significantly up-regulate the expression levels of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α), while exerting no effect on the expression levels

of anti-inflammatory cytokines (IL-10, MRC1 and CCL18) (Xiao et al., 2018). This suggests THBS1 expression is closely correlated with the expression levels of M1 related cytokines. Mechanically, EVs isolated from oral squamous cell carcinoma induce M1 polarization macrophages via stimulating p38, AKT and SAPK/JNK signaling pathways (Xiao et al., 2018).

T Cells

T cells, including unactivated naive T cells and effector T cells activated by antigen, are the key regulators in the tumor immunity. Helper T cells and cytotoxic T cells are mainly involved in the tumor immunity, while regulatory T cells (Tregs) are mainly involved in tumor immune escape. Nowadays, tumor-derived EVs have been found to act as an immune suppressor to promote recruitment and activation of Tregs in the TME, thereby creating a pro-tumorigenesis environment for tumor progression. For instance, HCC cells-derived EVs have been demonstrated to mediate the delivery of 14-3-3ζ to tumor-infiltrating T cells, which suppresses the anti-tumor effects of T cells (Wang et al., 2018c). Vesicular 14-3-3ζ inhibits the activity and proliferation of peripheral blood T cells, which consequently contributes to deviating the transformation of naive T cells from effector T cells to Tregs (Wang et al., 2018c). In addition, vesicular miR-208b suppresses programmed cell death factor 4 in recipient $CD4^+$ T cells and subsequently promotes Tregs proliferation, which consequently accelerates tumor growth in colorectal cancer (Ning et al., 2021). Nasopharyngeal carcinoma cells selectively up-regulate the transcription of CCL20, which serves as a Treg attractor (Mrizak et al., 2015). In addition, nasopharyngeal carcinoma-derived EVs have the ability to recruit Tregs into the TME and induce the transformation of T cells into Tregs, resulting in an enhanced immunosuppression effect in a dose-dependent manner (Mrizak et al., 2015). In addition to mediating Tregs recruitment in TME, tumor-derived EVs have also been shown to regulate the expression of the immune-related genes in Tregs. For example, mRNA profiles analysis revealed that EVs isolated from head and neck squamous cell carcinoma could up-regulate the expression of CD25, CD39, CD73 and CD26 in activated Tregs (Muller et al., 2016). Heat map analysis further found that tumor-derived EVs co-cultured with Tregs would lead to higher expression levels of adenosine-pathway genes and lower expression levels of immunoregulatory genes (Muller et al., 2016). Previous studies revealed that the adenosine pathway was one of the key mechanisms utilized by Tregs to function as an immunosuppressor (Whiteside et al., 2012; Whiteside and Jackson, 2013). This suggests tumor-derived EVs promote the suppression functions of Tregs *via* regulating the expression of adenosine-pathway genes. $CD73^+$ γδT cells, serve as the main Tregs subset in breast cancer, are able to mediate immunosuppressive effect in an adenosine-dependent manner (Ni et al., 2020). In the context of breast cancer, the release of lncRNA SNHG16 from EVs is shown to regulate CD73 expression on γδT cells (Ni et al., 2020). Mechanically, vesicular lncRNA SNHG16 stimulates the TGF-β/SMAD5 pathway by targeting the SMAD5 gene, which up-regulates CD73 expression on γδT cells (Ni et al., 2020).

B Cells

B cells play a key role in humoral immunity on account of their abilities to produce immunoglobulin and present antigens. The regulatory B cells (Bregs) as a subset of B cells are correlated with immunosuppressive response. Similar to T cells, B cells can be induced into Bregs by tumor-derived EVs. For instance, HCC cells-derived EVs can induce $TIM\text{-}1^+$ Breg with a high expression level of IL-10 (Ye et al., 2018). T cell co-culture with EVs-induced B cell results in decreased TNF-α and IFN-γ production (Ye et al., 2018). Notably, vesicular HMGB1 can activate the TLR-MAPK signaling pathway, which has been found to play a crucial role in inducing the transition of B cells into Bregs (Ye et al., 2018). Further study has demonstrated that blocking TLR or inhibiting MAPK can significantly suppress the Bregs expansion and up-regulate the production of pro-inflammatory cytokines (Ye et al., 2018). In addition, EVs isolated from esophageal squamous cell carcinoma patients significantly enhance the $IL\text{-}10^+$ Breg production (Mao et al., 2019). Correspondingly, flow cytometry analysis showed that the expression levels of IL-10 and PD-1 in B cells were higher when B cells were co-cultured with tumor-derived EVs (Mao et al., 2019). As EVs commonly function via delivering biomolecular components to target cells, researchers further analyzed the mRNAs and lncRNAs composition in EVs. Results revealed that a total of 947 mRNAs and 175 lncRNAs were down-regulated, while a total of 407 mRNAs and 1,331 lncRNAs were up-regulated in EVs released from esophageal squamous cell carcinoma (Mao et al., 2019). Furthermore, EVs derived from head and neck squamous cell carcinoma directly suppress B cell proliferation and activity (Schroeder et al., 2020). Flow cytometry analysis showed that tumor-derived EVs could inhibit the expression of checkpoint receptors (GITR and BTLA) and CD39 on B cells (Schroeder et al., 2020). Notably, as a B cell activation marker, CD39 regulates adenosine production in many immune cells, thus influencing the immunosuppressive effect of B cells (Saze et al., 2013).

Immune Cell-Derived EVs

NK cell-derived EVs significantly enhance the apoptosis of aggressive melanoma (Zhu et al., 2017). Meanwhile, the transfer of miR-186 from NK cell to neuroblastoma cell via EVs inhibits tumor growth and reverses immune escape (Neviani et al., 2018). Most TAMs display an M2-like phenotype, and thus M2 macrophages are the predominant macrophage phenotype in the TME. M2 macrophage derive-EVs (M2-EVs) stimulate PI3k/AKT signaling pathway via enriched apolipoprotein E, leading to cytoskeleton remodeling in gastric cancer cells, and ultimately result in enhanced migration (Zheng et al., 2018). M2-EVs also enrich miR-233 under hypoxia conditions. Vesicular miR-233 affords drug resistance to cDDP in epithelial ovarian cancer cells by activating PTEN-PI3k/AKT signaling pathway (Zhu et al., 2019). Moreover, the transfer of miR-21-5p and miR-155-5p *via* EVs from M2 macrophages to colon cancer cells down-regulates BRG1 and consequently promotes tumor metastasis (Lan et al., 2018). By contrast, M1 macrophage-derived EVs potentiate therapeutic efficacy of gemcitabine by improving

TABLE 3 | Tumor-derived EVs as biomarkers in cancer diagnosis and treatment.

Cargoes	Cancer types	Source of EVs	Applications	References
Let-7a, miR-99b, miR-146a, miR-155, miR-191, miR-1246	Acute myeloid leukemia	Serum	Diagnosis	Hornick et al. (2015)
Circular RNA SETDB1, miR-31-5p	Lung adenocarcinoma	Serum/plasma	Diagnosis	Xu et al. (2021), Yu et al. (2021)
ZFAS1, miR-301a-3p	Gastric cancer	Serum	Diagnosis	Pan et al. (2017), Xia et al. (2020)
GPC1	Pancreatic cancer	Serum	Diagnosis	Melo et al. (2015)
Contactin-1	Melanoma cancer	Plasma	Diagnosis	Pietrowska et al. (2021)
Let-7p-3b, miR-150-3p, miR-145-3p, miR-139-3p	Colon cancer	Plasma	Diagnosis	Min et al. (2019)
miR-92a-3p	HCC	Plasma	Diagnosis	Xia et al. (2020)
LncHILAR	Renal cancer	Plasma	Diagnosis	Hu et al. (2021)
miR-21	Breast cancer	Serum	Diagnosis	Yuan et al. (2021)
HMGB3	Nasopharyngeal carcinoma	Serum	Diagnosis	Zhang et al. (2021a)
TrpC5	Breast cancer	Peripheral blood	Therapy monitoring	Ma et al. (2014)
Annexin A6	Triple-negative breast cancer	Serum	Therapy monitoring	Li et al. (2021)
miR-208b, miR-21-5p	Colorectal cancer	Serum	Therapy monitoring	Ning et al. (2021), He et al. (2021)
S100A4, osteopontin	HCC	Plasma	Prognosis	Sun et al. (2021)
LncRNA-SOX2OT	NSCLC	Peripheral blood	Prognosis	Ni et al. (2021)

chemosensitivity of resistant pancreatic cancer cells (Zhao Y. et al., 2021). T cell-derived EVs containing programmed cell death 1 inhibit tumor cell immune escape by triggering programmed death-ligand1internalization (Qiu et al., 2021). In addition, $CD4^+$ T cell-derived EVs potentiate vaccine-mediated immune responses by enhancing B cell proliferation and antibodies production (Lu et al., 2019). By contrast, B cell-derived EVs compromise chemotherapeutic effect by attenuating $CD8^+$ T cell response (Zhang F. et al., 2019). Thus, inhibition of B cell-derived EVs release contributes to enhanced post-chemotherapeutic T cell responses (Zhang F. et al., 2019).

TUMOR-DERIVED EVS AS BIOMARKERS IN CANCER DIAGNOSIS AND TREATMENT

Due to the lack of ideal biomarkers in the clinic, most cancer patients once diagnosed have been at the advanced stage. Tumor-derived EVs can be released into various body fluids like blood and ascites, which are able to reflect the status of the parental cancer cell. Therefore, tumor-derived EVs are considered as ideal candidates for non-invasive biomarkers in cancer diagnosis. The diversity and specificity of tumor-derived EVs, including miRNA, lncRNA and protein, enable their application in diagnosis (**Table 3**). For instance, EVs released from acute myeloid leukemia cell selectively enrich let-7a, miR-99b, miR-146a, miR-155, miR-191 and miR-1246 (Hornick et al., 2015). Moreover, RT-qPCR analysis showed that the concentrations of those increased miRNAs were 1000-fold above the cellular level, which may better distinguish acute myeloid leukemia from healthy volunteers with high sensitivity and specificity (Hornick et al., 2015). High levels of circular RNA SETDB1 and miR-31-5P are observed in lung adenocarcinoma patients (Xu et al., 2021; Yu et al., 2021). Serum vesicular circular RNA SETDB1 level is closely correlated with the T stage and lymph node metastasis (Xu et al., 2021). In addition, zinc finger antisense 1 (ZFAS1), belonging to competing endogenous lncRNA, is enriched in the serum EVs of gastric cancer patients (Pan et al., 2017). Highly expressed vesicular ZFAS1 may be related to a higher risk of lymphatic metastasis in gastric cancer patients (Pan et al., 2017). Glypican-1 (GPC1) is specifically up-regulated in tumor-derived EVs, thus detection of serum-derived EVs from pancreatic cancer patients distinguishes healthy individuals and patients with a benign pancreatic cancer from patients with early- and late-stage pancreatic cancer in a GPC1-dependent manner with specificity and sensitivity (Melo et al., 2015). In addition, contactin-1 is selectively elevated in plasma EVs of melanoma cancer patients when compared with EVs of normal volunteers (Pietrowska et al., 2021). This indicates that the detection of these differentially expressed proteins of melanoma cancer-derived EVs may play an essential role in the diagnosis and monitoring of tumors. The expression levels of let-7p-3b, miR-150-3p, miR-145-3p and miR-139-3p in plasma-derived EVs from colon cancer patients are much higher than those in plasma-derived EVs from healthy controls (Min et al., 2019). Moreover, EVs derived miRNAs show a more potent diagnosis efficacy than plasma total miRNAs. Recently, Xia et al. (2020) believe that miR-301a-3p is correlated to gastric cancer development and metastasis. In addition, miR-301a-3p is selectively enriched in serum EVs isolated from gastric cancer with peritoneal metastasis (Xia et al., 2020). Similarly, elevated miR-92a-3p expression level in plasma-derived EVs is related to metastasis of HCC patients (Yang et al., 2020). Also, vesicular lncHILAR expression is markedly higher in renal cancer patients with metastasis than those without metastasis (Hu et al., 2021). Yuan et al. (2021) believe that breast cancer patients with high vesicular miR-21 in serum also have bone metastasis. Furthermore, high vesicular HMGB3 level is observed in nasopharyngeal carcinoma patients, especially those with metastasis (Zhang K. et al., 2021).

In addition to acting as non-invasive biomarkers in cancer diagnosis, EVs have also been shown to serve as "real time" biomarkers during cancer treatment (**Table 3**). For instance, TrpC5 is a regulator of multidrug transporter P-glycoprotein, which promotes the generation of EVs. The expression level of vesicular TrpC5 in breast cancer patients with low drug sensitivity is significantly higher than that in healthy volunteers (Ma et al., 2014). As a result, detection of TrpC5-bearing EVs in peripheral

blood of patients may predict the clinical treatment effect of chemotherapy. Similarly, annexin A6 overexpression relates to poor response to gemcitabine-based chemotherapy (Li et al., 2021). Ning et al. (2021) believe that the elevated miR-208b expression level is associated with oxaliplatin resistance in colorectal cancer patients. In addition, the expression level of vesicular miR-21-5p decreases in colorectal cancer patients after surgical resection (He et al., 2021). Recent studies have found that tumor-derived EVs may be a potential biomarker for cancer prognosis (**Table 3**). For instance, highly expressed vesicular S100A4 and osteopontin are related to short overall survival rates and disease free survival rates in HCC patients (Sun et al., 2021). Similarly, highly expressed vesicular lncRNA-SOX2OT are also related to short overall survival rates in NSCLC patients (Ni et al., 2021).

To better utilize these biomarkers in clinic, progress in EVs detection should be discussed. The conventional EVs detection methods conclude ultracentrifugation pretreatment and downstream western blotting, ELISA or PCR analysis. However, these techniques are time-consuming and insensitive (Lane et al., 2019). To overcome these limitations, plenty of micro and nano-devices have been exploited for detecting EVs. For example, Lewis et al. (2018) developed an Alternating Current Electrokinetic chip, which could capture and quantify vesicular GPC1 and CD63 within 30 min. Zhang P. et al. (2019) designed a fluorescence-based integrated platform called ExoProfile, which could elucidate the differences between the EVs of ovarian cancer patients and healthy people. Pang et al. (2020) used a surface enhanced Raman scattering method to elucidate the expression level of vesicular PD-L1 between NSCLC patients and healthy people. Recently, Marchisio et al. (2021) used a polychromatic flow cytometry technique to perform the detection of EVs captured by the lipophilic cationic dye. Thakur et al. (2021) presented a localized surface plasmon resonance (LSPR) technique to detect tumor-derived EVs using designed TiN-NH-LSPR biosensor and demonstrated that the label-free LSPR technique can be used for glioblastoma monitoring. Park et al. (2021) proposed a high-throughput electrochemical detection platform called HiMEX, which could distinguish colorectal cancer patients from healthy volunteers with high sensitivity and specificity.

CONCLUSION

In recent years, numerous studies of EVs have reported their participation in different stages during cancer progression. The delivery of intercellular information from tumor cells to stromal cells *via* EVs affects the functions and phenotypes of recipient cells, thereby regulating tumor progression. Herein we describe the key findings on how tumor-derived EVs remodel TME to influence tumor progression. In this regard, it is the multiple activators in EVs to create a protumoral or an anti-tumoral soil in the microenvironment. Moreover, such properties also enable their application in cancer diagnosis and treatment as an ideal candidate for non-invasive biomarkers. However, despite significant progress has been made in exploring the role of tumor-derived EVs in TME, many questions remain. Firstly, current studies on tumor-derived EVs use highly heterogeneous cells composed of multiple clones. Therefore, the functions of EVs released from single-cell have yet to be unveiled. Secondly, the enhanced techniques for EVs detection are sensitive and precise, but they also require expensive modification. Thus, highly precise, low-cost and simple techniques for clinical samples detection remain to be exploited. Thirdly, it remains unclear what the main components are at work. This field is in urgent need of more precise characterization of EVs cargo and biology.

AUTHOR CONTRIBUTIONS

LW, YX, and WC provided the direction and guidance throughout the preparation of this manuscript. QB conducted the literature review and drafted the manuscript. QH, YC, RS, and QW edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Exercise-Induced Extracellular Vesicles Delay the Progression of Prostate Cancer

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Increasing evidence suggests that regular physical exercise not only reduces the risk of cancer but also improves functional capacity, treatment efficacy and disease outcome in cancer patients. At least partially, these effects are mediated by the secretome of the tissues responding to exercise. The secreted molecules can be released in a carrier-free form or enclosed into extracellular vesicles (EVs). Several recent studies have shown that EVs are actively released into circulation during physical exercise. Here, we for the first time investigated the effects of exercise-induced EVs on the progression of cancer in an F344 rat model of metastatic prostate cancer. Although we did not observe a consistent increase in the circulating EV levels, RNA sequencing analysis demonstrated substantial changes in the RNA content of EVs collected before and immediately after forced wheel running exercise as well as differences between EVs from runners at resting state and sedentary rats. The major RNA biotype in EVs was mRNA, followed by miRNA and rRNA. Molecular functions of differentially expressed RNAs reflected various physiological processes including protein folding, metabolism and regulation of immune responses triggered by the exercise in the parental cells. Intravenous administration of exercise-induced EVs into F344 rats with orthotopically injected syngeneic prostate cancer cells PLS10, demonstrated reduction of the primary tumor volume by 35% and possibly—attenuation of lung metastases. Hence, our data provide the first evidence that exercise-induced EVs may modulate tumor physiology and delay the progression of cancer.

Keywords: extracellular vesicles, exercise, prostate cancer, RNA cargo, RNA sequencing

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INTRODUCTION

Prostate cancer (PC) is the second most frequently diagnosed cancer in males worldwide affecting more than 1.4 million men per year. In terms of mortality, PC is the fifth leading cause of death from cancer in men (Ferlay et al., 2015). Hence, PC is a global health problem requiring effective primary, secondary, and tertiary prevention measures. Regular physical activity is associated with a lower incidence of many common types of cancer (Moore et al., 2016), whereas the association between physical activity and PC risk remains controversial. Some studies have reported that leisure-time

physical activity is associated with a higher risk of PC, some studies showed no clear relationship, however larger number of studies have found a decrease of PC incidence in physically active men and the effect showed a dose-response relationship with vigorous activity (Shephard, 2017). Furthermore, several studies have shown that exercise reduced fatigue and treatment side effects, improved quality of life, prevented disease recurrence, and improved survival of PC patients (Kenfield et al., 2011; Bourke et al., 2016; Shephard, 2017; Belloni et al., 2021). Moreover, experiments in murine tumor models have demonstrated that exercise leads to a significant reduction in tumor size and incidence, and these effects are associated with remodeling of the immune tumor microenvironment (TME) (Pedersen et al., 2016; Idorn and Thor Straten, 2017). This suggests that apart from the well-documented beneficial effects of exercise on cardiovascular fitness, energy balance and body weight, it may have a direct effect on cancer. Therefore, exercise may not just be preventive but also therapeutic and hence serve as an important tool for tertiary prevention of cancer.

At least partially, the effects of exercise are mediated by various molecules (proteins, lipids, RNAs, metabolites etc.) secreted into the circulation by muscle, bone, brain, liver and other tissues. These molecules can be secreted in a soluble form or packaged into carriers such as extracellular vesicles (EVs). The term "EVs" refers to all kinds of vesicles naturally released from cells that are delimited by a lipid bilayer and cannot replicate (Thery et al., 2018). According to the mode of biogenesis, three main types of EVs have been defined: exosomes, microvesicles and apoptotic bodies (Yanez-Mo et al., 2015). They differ in their molecular content, size, membrane composition, cellular source and specific functions. Although initially considered to be a waste disposal mechanism (Johnstone et al., 1987), it is now clear that EVs generated by both live and apoptotic cells interact with the recipient cells and are important mediators of intercellular communication (Tkach and Thery, 2016; Caruso and Poon, 2018). EVs can be internalized by the recipient cells and trigger various intracellular signal transduction pathways (Yanez-Mo et al., 2015; Zhan et al., 2021) or bind to the cell surface receptors and trigger the respective downstream signaling pathway (Muller et al., 2016; Muller et al., 2017).

Exercise has been shown to induce a rapid release of EVs into circulation (Fruhbeis et al., 2015; Whitham et al., 2018). A recent study demonstrated that exercise induced the secretion of over 300 proteins into EVs, including glycolytic enzymes and myokines, i.e. cytokines secreted by skeletal muscle (Whitham et al., 2018). Myokines have been shown to act as tumor suppressors and may impact several hallmark features of cancer (Hojman et al., 2011; Ruiz-Casado et al., 2017), whereas glycolytic enzymes might induce changes in the metabolic activity of cancer cells (Pedersen et al., 2015). We hypothesized that exercise-induced EVs may directly interact with cancer cells and alter their behavior as well as change the functional phenotype of circulating and tumor-infiltrating immune cells thus affecting the growth rate and metastatic potential of cancer. In this pilot study, we investigated the RNA content of EVs released during forced wheel running and their effects on the progression of cancer in a rat model of metastatic prostate cancer.

MATERIALS AND METHODS

Animal Care and Experimental Design

The experimental procedures in animals were approved by the National animal welfare and ethics committee (permit no. 121/2021) and were performed in compliance with the Directive 2010/63/EU as adopted in the national legislation.

In total, 37 naïve SPF male Fischer 344 rats were obtained from Charles River Laboratories, Germany (immunocompetent inbred strain F344/DuCrl). During the introduction, animals were randomly allocated in cages in pairs or trios; individually ventilated cages GR900, HEPA-ventilated by SmartFlow air handling unit (Tecniplast, Italy) at 75 air changes per hour were used for animal housing. Access to autoclaved water acidified to pH 2.5–3.0 with HCl and standard rodent diet (4RF21 (A), Mucedola) was provided *ad libitum*. Aspen wooden bedding and nesting material (Tapvei, Estonia) together with rat cardboard houses (Velaz, Czech Republic) and aspen gnawing bricks (Tapvei, Estonia) were provided in all cages, and cages were changed every 7 days. Animals were housed in SPF facility under controlled temperature (24 ± 1°C) and relative humidity of 40–60%. Animal health monitoring was performed in line with FELASA recommendations (Mähler et al., 2014).

All animals were subjected to at least 2-weeks acclimatization period with the adaptation to a reverse 12 h light/dark cycle (dark phase set to 10:00 am–10:00 pm; visible light intensity <25 lux), and then used to model either regular physical exercise (i.e. forced wheel running model) or orthotopic PC development [PLS10 rat PC model (Suzuki et al., 2015)]—see **Figure 1** for schematic overview. An individual animal served as an experimental unit in both models. Before starting the procedures, the animals were identified by tattooing their tails using AIMS™ NEO-9 Neonate Tattooing System according to the manufacturer's instructions.

Cell Culture

Rat prostate cancer cell line PLS10 was developed previously using chemically-induced prostate carcinoma in F344 rats (Nakanishi et al., 1996; Suzuki et al., 2015). Cells were maintained in RPMI-1640 medium, supplemented with 10% FBS, 2 mM L-glutamine and 1x Antibiotic-Antimycotic at +37°C in a humidified atmosphere containing 5% CO2.

Cells in the exponential growth phase were trypsinized, counted and resuspended in PBS in aliquots of 5×10^6 cells per 25 μL of PBS. The aliquots were immediately transferred to the animal facility and kept on ice water until injection. Just before injection into the rat prostate, the cell suspension was mixed with an equal volume of ice-cold Matrigel (Corning #356237, USA). Fresh cell suspensions were prepared for every batch of 3-4 animals undergoing laparotomic surgery.

Forced Wheel Running Exercise

In total, 16 7-week-old male F344 rats were subjected to model regular exercise and sedentary lifestyle. The number of animals per group (n = 8) was chosen based on the available data on average EV plasma concentrations and calculations we made to ensure the necessary amount of EVs for the 6-weeks injection

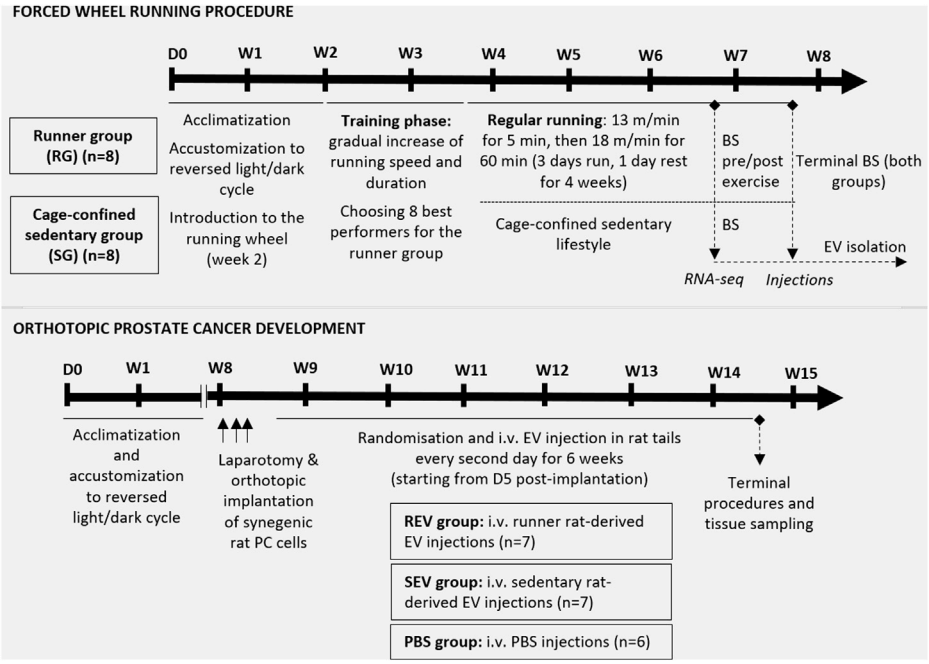

FIGURE 1 | Schematic overview of the *in vivo* study design. The upper panel represents the steps of the forced wheel running procedure—in total, sixteen 7 w/o male F344 rats underwent a 2-weeks acclimatization period. During the second week, rats were introduced to the running wheel, which was followed by a 12-days training phase for all experimental animals. During the training phase, 8 best performers were chosen for the runner group while the remaining 8 rats were assigned to the cage-confined sedentary group. The running exercise phase lasted for 5 weeks in total, including 4 weeks of regular running in a setting described in the figure. After 4 weeks of exercise, blood samples were taken before and shortly after 1 h running from the runner group and a single blood sample from the control animals for further plasma EV analyses. At the endpoint, total blood sample was taken *via* cardiac puncture and used for plasma EV isolation. The lower panel shows the steps of *in vivo* evaluation of the effect of EVs (isolated from the endpoint plasma samples) on orthotopic PC development in syngeneic male F344 rats. Briefly, anesthetized 12 w/o male F344 rats were subjected to a laparotomic incision to enable orthotopic implantation of 5×10^6 PLS10 rat prostate cancer cells into the ventral prostate. After stratified randomization, the animals were divided into 3 groups and, starting from day 5 post-implantation, rats from all 3 groups received i.v. injection of EVs or PBS as shown. After 6 weeks of injections, animals were sacrificed, and tissues of interest were collected. BS, blood sampling; D, day; i.v., intravenous; W, week.

period in the syngeneic PC model animals (**Figure 1**). In the second week after arrival and acclimatization to the reversed light/dark cycle, rats were introduced to the forced running wheel (system for rats, model 80805A, Lafayette Instrument) without a specific running mode. It was followed by a 12-days training phase for all 16 experimental animals (started at 9 weeks of age) by gradually increasing the running speed and duration. During the training phase, 8 best performers were selected for the runner group while the remaining 8 rats were assigned to the sedentary group. After assigning to groups, the animals were kept co-housed with their initial cage mates.

The regular forced running exercise phase for the runner group lasted for 5 weeks in total, including 4 weeks of regular running in a setting described in **Figure 1**. After 4 weeks of regular exercise, rats underwent brief anesthesia with 1.5% isoflurane and 500 µL of blood from the tail vein were taken. This step was repeated for the same animals immediately after 1 h of running. On the same day, a blood sample was collected from the sedentary rats following the same protocol. After 5 weeks of regular exercise, all the animals immediately after 1 h running were subjected to deep surgical anesthesia with 5% isoflurane and underwent terminal blood collection *via* cardiac puncture. Blood was immediately collected into S-Monovette Hematology EDTA K3 tubes (Sarstedt, Germany) or BD Vacutainer™ Blood Collection Tubes with K2EDTA (Fisher Scientific, USA), depending on the volume, and centrifuged at 2000 x g, +4°C for 15 min. Plasma was collected in fresh tubes and immediately frozen for further EV isolation.

Isolation of EVs

EVs were isolated from rat plasma by size exclusion chromatography (SEC) using qEVoriginal/35 nm or qEV10/35 nm columns (IZON, USA) depending on the plasma volume. The SEC fractions were analyzed using ZetaSizer Nano ZS (Malvern Panalytical, UK) and the fractions containing EVs were concentrated using Amicon Ultra-0.5, Ultracel-3 Membrane, 3 kDa centrifugal filter units (Merck Millipore, Germany). EVs that were used for RNA sequencing were treated with proteinase K (Thermo Fisher Scientific, USA) and RNAse A (Thermo Fisher Scientific, USA) to remove all the free proteins and RNAs that are not enclosed in EVs. EVs were visualized by transmission electron microscopy (TEM) and the size distribution profile and concentration of EVs were determined by nanoparticle tracking analysis (NTA) using NanoSight NS500 instrument (Malvern, UK) as described before (Endzelins et al., 2017).

RNA Extraction

RNA was extracted from plasma EVs using miRNeasy micro kit (Qiagen, USA) according to the manufacturer's protocol. The concentration and quality of the obtained RNA were analyzed on Agilent Bioanalyzer with Agilent RNA 6000 Pico chip (Agilent Technologies, Germany).

RNA Sequencing and Data Analysis

RNA libraries were constructed using CleanTag® Small RNA Library Prep Kit (Trilink Biotechnologies, USA), the quality and concentration of obtained libraries were analyzed on Agilent Bioanalyzer using Agilent High Sensitivity DNA chip (Agilent Technologies, Germany). The libraries were cleaned using Blue Pippin DNA Size Selection with 3% gel Blue Pippin Cassette (Sage Science, USA) setting tight target length to 140 bp thus selecting fragments with size in tight range to 140 bp (126–154 bp). The libraries were diluted as required and sequenced on Illumina NextSeq500 instrument using NextSeq 500/550 Mid Output Kit v2.5 (150 cycles) (Illumina, USA).

The obtained raw data in fastq format were analyzed using *ad-hoc* R script pipeline, which included: adapter trimming [cutadapt (Martin, 2011)], read mapping [bowtie2 (Langmead and Salzberg, 2012)] against RGSC rat (*Rattus norvegicus*) genome (version Rnor_6.0), multi-aligned reads reposition [ShortStack (Axtell, 2013)], counting [Rsubread package (Liao et al., 2019)] with RGSC (version Rnor_6.0) and miRbase (Kozomara et al., 2019) annotations. For differentially expressed gene (DEG) analysis, the reads were normalized per sample, the reads mapped to features were counted and analyzed using quasi-likelihood F-tests by edgeR (McCarthy et al., 2012) package. A subset of DEGs (adj. $p < 0.05$) was subjected to GO terms [GOstats (Falcon and Gentleman, 2007)] and enrichment analyses (rentrez (Winter 2017), GO.db (Carlson, 2019a), org.Rn.eg.db (Carlson, 2019b) packages).

Syngeneic Orthotopic Prostate Cancer Model

For modeling an orthotopic PC development, 21 F344 rats were used—the sample size of 7 animals per group (see **Figure 1** for study groups) was calculated by statistical power analyses using G*Power software (Buchner et al., 2021) and taking into account 80% power and $\alpha = 0.05$, the published tumor size variation (Suzuki et al., 2015), expected effect size and the chosen statistical test for the result analyses.

At the age of 12 weeks, each animal underwent laparotomic surgery by using an aseptic technique under 2.5% isoflurane anesthesia, and 5×10^6 syngeneic rat prostate cells PLS10 in 50 µL total volume containing 50% Matrigel were injected into the ventral lobe of the prostate. The incisions were closed with Novosyn 4/0 DS19 mid-term absorbable sutures (B.Braun, Germany) and secured with surgical adhesive. An ophthalmic gel was provided during surgery and 8 ml of warm saline was administered subcutaneously immediately after surgery. Each animal received subcutaneous Meloxicam (1 mg/kg) injection during surgery and for 3 days post-surgery. Animals were single housed in sterile cages for 3 days and then returned to their home cages. Animals were closely monitored for possible postsurgical complications; the wounds were treated with furasol solution. In total, 7 rats divided in 2 sets were implanted with PC cells per day, and animals were allocated to the study groups after stratified randomization by considering this setting (**Figure 1**; one animal did not survive the surgery).

Starting from day 5 post-implantation, rats from all study groups *via* lateral tail vein received i.v. injections of 100 µL EV solution containing 1.5×10^{10} EVs in PBS or PBS every second day as shown in **Figure 1**. During PC development, animals were carefully monitored for possible signs of suffering (following the IACUC Policy #012), and the tumor size was estimated by palpation. After 6 weeks of EV injection, all study animals were humanely euthanized, and terminal blood samples and tissues of interest were collected and fixed in 10% buffered formalin. Primary tumors were measured, and tumor volume was calculated using the following formula: tumor volume = (width)2 x length/2.

Statistical Analysis

A nonparametric one-tailed Mann-Whitney test was used to compare the tumor volume and the number of metastases between groups of animals. Fisher's exact test was used to compare the number of animals with/without metastases between groups of animals. p-value of ≤0.05 was considered to be significant. The statistical analyses were performed with GraphPadPrism 7 (GraphPad, USA).

RESULTS

Effect of Forced Wheel Running Exercise on the Plasma EV Levels

EVs were isolated from rat plasma before (Pre-RUN) and immediately after forced wheel-running exercise (Post-RUN) and the yield, size and purity of EVs were assessed by TEM and NTA. TEM images revealed that the majority of particles were ranging from 30 to 160 nm in diameter and had a cup-shaped morphology that is typically observed for exosomes in TEM. However, smaller particles that possibly represent lipoprotein particles and a small number of large particles of 200–250 nm in diameter were also present in the majority of the samples analyzed and we did not observe any significant differences between Pre-RUN and Post-RUN samples (**Figure 2A,B**). NTA showed that the major fraction of particles was in the size range from 36 to 200 nm and the concentrations of EVs ranged from 6.5×10^9 to 1.9×10^{11} particles per ml of plasma (**Figure 2C**). However, no significant differences neither in size or concentration of EVs between Pre-RUN and Post-RUN samples were observed (**Figure 2D**).

Changes in the EV RNA Content During Exercise

To determine whether exercise affects the EV-enclosed RNA cargo, we performed RNA sequencing analysis of Pre-RUN ($n = 8$) and Post-RUN ($n = 8$) plasma EVs from the runner group and plasma EVs from the sedentary group ($n = 8$). Analysis of the total EV RNA by Bioanalyzer showed the dominant peaks

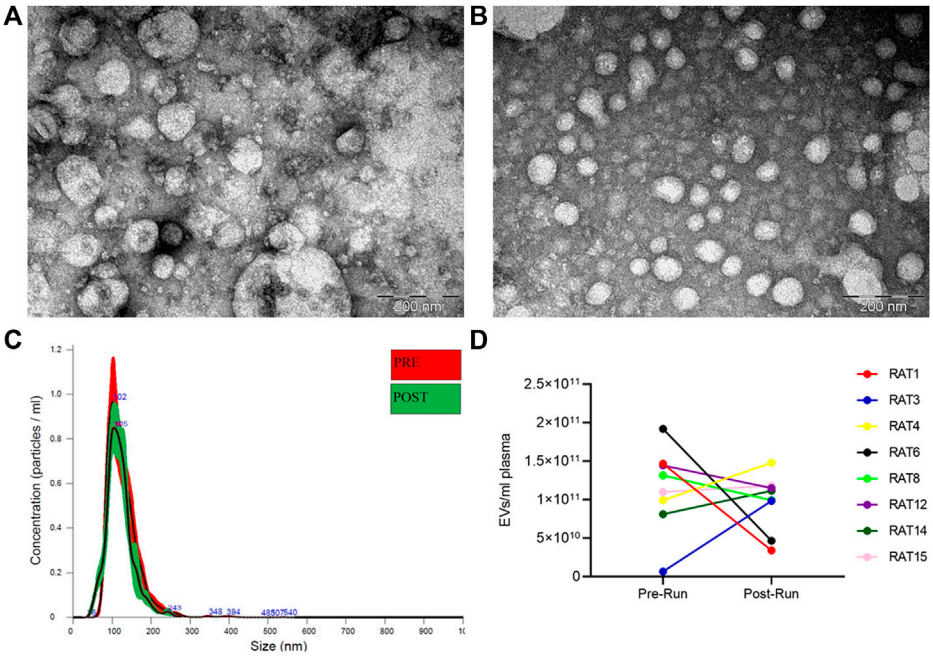

FIGURE 2 | Characteristics of EVs released in plasma during forced wheel running exercise. **(A)** Representative TEM image of Pre-RUN plasma EVs. **(B)** Representative TEM image of Post-RUN plasma EVs. **(C)** Quantity and size distribution of Pre-RUN and Post-RUN plasma EVs assessed by NTA (a representative case). **(D)** Paired dot plots show the number of particles in Pre-RUN and corresponding Post-RUN plasma samples determined by NTA.

in the range from 20 to 150 nt (data not shown). The total EV RNA was used for the RNA-seq library construction without a prior size selection. Importantly, prior to the RNA extraction, EVs were treated with Proteinase K and RNase A to remove RNAs that are attached to the surface of EVs. On average, 5.9 million raw reads were obtained per library, however, on average 2.85 million reads per library remained after the quality control, adapter trimming, and filtering out the reads that were shorter than 16 nt. The average overall alignment rate to the *Rattus norvegicus* genome was 51.5%. The aligned reads were counted using Rsubread package (Liao et al., 2019) and RGSC rat genome and genome annotation (version Rnor_6.0). Results showed that the majority of the reads were mapped to mRNAs (73.8%), followed by miRNAs (13.6%) and rRNAs (6.7%), while the other biotypes—lincRNAs, mitochondrial rRNAs, processed pseudogenes etc. constituted less than 2% each. However, when the reads were counted using miRBase annotation, 0.6% of the mapped reads were counted as mature miRNAs and a total of 194 different miRNAs were identified.

Next, we performed differential expression analysis using edgeR package. RNAs which expression was detected in less than 4 samples were excluded from the analysis. To assess whether the RNA cargo of EVs that are released into the circulation during the forced wheel running exercise is distinct from that in the resting state, Post-RUN EVs were compared against the Pre-RUN EVs. A total of 20 differentially expressed genes (DEGs) (adj. $p < 0.05$)—10 upregulated and 10 downregulated during the exercise were identified (**Figure 3A**; **Supplementary Table S1**). The top 10 DEGs are shown in **Table 1**. All DEGs are protein-coding genes and no differentially expressed RNAs were found in other RNA biotypes. GO term enrichment analysis of DEGs revealed enrichment of genes related to unfolded protein binding (adj. $p = 0.048$).

To assess the long-term effects of exercise on the RNA cargo of circulating EVs, Pre-RUN EVs were compared to the plasma EVs from the sedentary control rats. A total of 52 genes were differentially expressed (adj. $p < 0.05$), including 50 protein-coding genes and 2 miRNAs (**Figure 3B**; **Supplementary Table S2**). Only eleven of these RNAs had higher levels in the Pre-RUN EVs than in sedentary control EVs. The top 10 DEGs are shown in **Table 2**. GO term enrichment analysis showed the enrichment of genes associated with molecular functions "selenium binding" and "oxidoreductase activity, acting on peroxide as acceptor" (both adj, $p = 0.025$).

Effect of Exercise-Induced EVs on the Progression of Prostate Cancer

Next, we investigated the effects of EVs released during the forced wheel running exercise on the progression of *p*C. Briefly, total blood plasma EVs were isolated from F344 rats subjected to forced running wheel exercise or sedentary lifestyle, and the obtained EVs were subsequently administered intravenously in F344 rats with orthotopically injected syngeneic prostate cancer cells PLS10 (established from chemically induced, castration-resistant metastatic PC). The PLS10 PC rat tumor model was adopted based on the protocol originally published by the authors (Suzuki et al., 2015). PC-bearing rats received EV injections

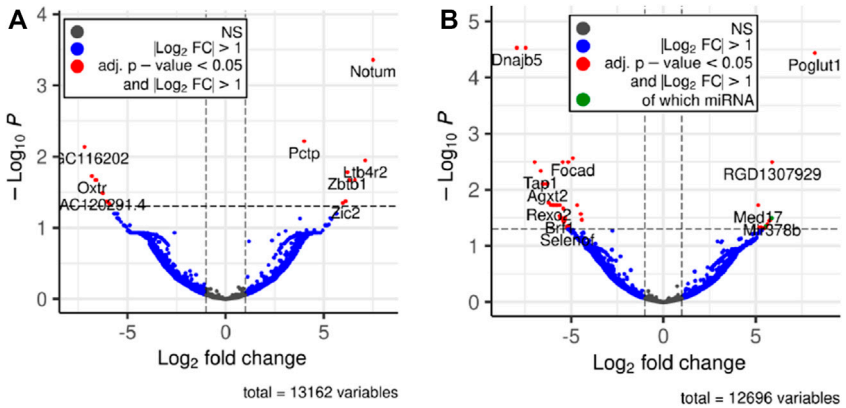

FIGURE 3 | Volcano plots depicting significant changes in the EV-enclosed RNA content. **(A)** Post-RUN *vs.* Pre-RUN EVs from exercised rats. **(B)** Pre-RUN EVs from exercised rats *vs.* EVs from sedentary control group rats.

TABLE 1 | Top 10 differentially expressed genes in Post-RUN *vs.* Pre-RUN EVs.

Gene name	Description	Function Smith et al. (2020)	Expression Yu et al. (2014)	LogFC	Adjusted *p*-value
Notum	NOTUM, palmitoleoyl-protein carboxylesterase	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	Selective expression in Liver (RPKM 96.2), Thymus (RPKM 23.3) and 3 other tissues	7.51	0.0004
Pctp	Phosphatidylcholine transfer protein	Plays a role in intermembrane transfer of phosphatidylcholines	Selective expression in Liver (RPKM 243.0), Testes (RPKM 114.6) and 8 other tissues	4.00	0.0061
MGC116202	LOC688736 uncharacterized protein KIAA0895-like	—	Selective expression in Brain (RPKM 59.9), Testes (RPKM 34.1) and 6 other tissues	–7.19	0.0073
Ltb4r2	Leukotriene B4 receptor 2	Mouse homolog is a chemoattractant for myeloid leukocytes	Selective expression in Lung (RPKM 1.6), Thymus (RPKM 1.1) and 9 other tissues	7.11	0.0113
Zbtb1	Zinc finger and BTB domain containing 1	May bind DNA	Selective expression in Thymus (RPKM 102.4), Spleen (RPKM 62.9) and 9 other tissues	6.21	0.0166
Oxtr	Oxytocin receptor	G-protein coupled receptor for the peptide hormone, oxytocin	Selective expression in Adrenal (RPKM 17.2), Uterus (RPKM 11.7) and 7 other tissues	–6.81	0.0188
Dnajb5	DnaJ (Hsp40) homolog, subfamily B, member 5	Predicted to be involved in chaperone cofactor-dependent protein refolding and response to unfolded protein	Selective expression in Muscle (RPKM 168.1), Brain (RPKM 82.7) and 9 other tissues	6.28	0.0213
Hspa5	Heat shock protein family A (Hsp70) member 5	secreted protein of the endoplasmic reticulum; may be involved in the assembly of secreted and membrane-bound proteins	Selective expression in Liver (RPKM 2693.8), Heart (RPKM 2015.3) and 9 other tissues	–6.64	0.0213
Alox5	Arachidonate 5-lipoxygenase	catalyzes the conversion of arachidonate to leukotriene A4 in leukotriene metabolism	Selective expression in Lung (RPKM 53.5), Heart (RPKM 25.7) and 9 other tissues	–6.57	0.0213
Dxo	Decapping exoribonuclease	Hydrolyzes the nicotinamide adenine dinucleotide (NAD) cap from a subset of RNAs	Selective expression in Adrenal (RPKM 116.7), Kidney (RPKM 46.2) and 9 other tissues	6.59	0.0213

Abbreviations: RPKM, reads per kilobase per million mapped reads (mean values given).

during 6 weeks of PC progression (representing stage 1 to stage 3/4 PC development)—REV group received Post-RUN EVs from runners, SEV group—EVs from sedentary rats, PBS group—vehicle only (**Figure 1**).

Six weeks after implantation, tumor masses were found in the ventral prostate of all rats. Noteworthy, no suffering was observed till the endpoint for the majority of tumor-bearing animals. However, two rats from each group were humanely euthanized before the set endpoint of 6 weeks due to signs of suffering. Postmortem analyses revealed that all the animals had tumor masses developed outside the original injection site leading to the stricture of the urethra, a common complication seen in orthotopic rodent PC models [our

TABLE 2 | Top 10 differentially expressed genes in Pre-RUN *vs*. sedentary control EVs.

Gene name	Description	Function Smith et al. (2020)	Expression Yu et al. (2014)	LogFC	Adjusted *p*-value
Dnajb5	DnaJ heat shock protein family (Hsp40) member B5	Predicted to be involved in chaperone cofactor-dependent protein refolding and response to unfolded protein	Selective expression in Muscle (RPKM 168.1), Brain (RPKM 82.7) and 9 other tissues	−7.95	0.00003
Ripor3	RIPOR family member 3	Orthologous to human RIPOR3, enables protein binding	Selective expression in Kidney (RPKM 21.2), Liver (RPKM 17.8) and 9 other tissues	−7.46	0.00003
Poglut1	Protein O-glucosyltransferase 1	Involved in the pathway protein glycosylation	Selective expression in Thymus (RPKM 128.2), Brain (RPKM 117.2) and 9 other tissues	8.21	0.00004
Focad	Focadhesin	Predicted to be located in focal adhesion. Orthologous to human FOCAD	Selective expression in Brain (RPKM 101.0), Muscle (RPKM 96.0) and 9 other tissues	−4.91	0.0027
Gpx3	Glutathione peroxidase 3	Catalyze the reduction of organic hydroperoxides and hydrogen peroxide (H2O2) by glutathione, and thereby protect cells against oxidative damage	Expression restricted to heart (RPKM 20.0), kidney (RPKM 587.3), lung (RPKM 25.6)	−6.97	0.0032
AC107331.1	—	Predicted to enable G protein-coupled receptor activity and olfactory receptor activity	—	−5.16	0.0032
RGD1307929	Similar to CG14967-PA	Orthologous to human KIAA0100, May be involved in membrane trafficking	Selective expression in Kidney (RPKM 513.2), Adrenal (RPKM 381.1) and 9 other tissues	5.89	0.0032
Lpin2	Lipin 2	Predicted to be involved in several processes, including cellular response to insulin stimulus; fatty acid catabolic process; and triglyceride biosynthetic process	Selective expression in Kidney (RPKM 124.7), Spleen (RPKM 111.6) and 9 other tissues	−5.46	0.0032
Tap1	Transporter 1, ATP binding cassette subfamily B member	May transport antigenic peptides across the endoplasmic reticulum membrane in preparation for MHC class I presentation	Selective expression in Thymus (RPKM 337.5), Spleen (RPKM 282.5) and 9 other tissues	−6.65	0.0046
Agxt2	Alanine-glyoxylate aminotransferase 2	Predicted to enable alanine-glyoxylate transaminase activity and beta-alanine-pyruvate transaminase activity	Expression restricted to liver (RPKM 29.2) and kidney (RPKM 110.3)	−6.28	0.0078

Abbreviations: RPKM, reads per kilobase per million mapped reads (mean values given).

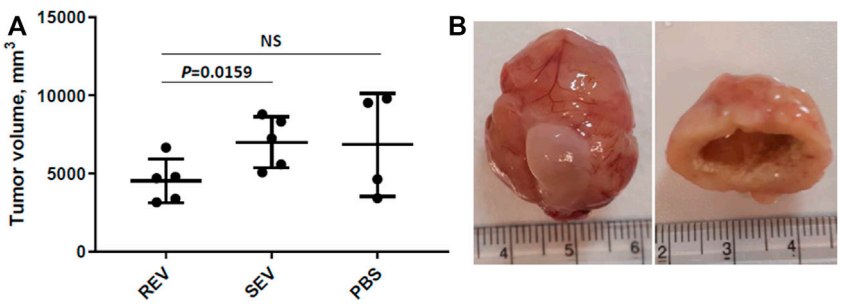

FIGURE 4 | Effects of exercise-induced EVs on the progression of prostate cancer. **(A)** Comparison of primary tumor volumes between groups after 6 weeks of EV or vehicle administration. In the dot plot, mean tumor volumes are shown, whiskers represent standard deviation; dots represent individual animals. Mann-Whitney test was performed to assess the statistical significance of the differences between groups. **(B)** A representative example of an orthotopic PLS10 cell-induced prostate tumor (with bladder) and necrosis found in the center of most tumors.

unpublished data (Suzuki et al., 2015)]. These animals were excluded from the data analyses.

Primary tumor volume was assessed as the primary outcome measure and compared between the groups. The mean primary tumor volumes were 4532 ± 1396 mm^3 in the REV group, 7000 ± 1882 mm^3 in SEV group, and 6841 ± 3291 mm^3 in PBS group (**Figure 4A**). Statistically significant differences were seen only when comparing mean tumor volume of rats that received runner-derived EVs and those of SEV group animals (reduction of the primary tumor volume in REV group by 35%, Mann-Whitney $p = 0.0159$), but the difference was insignificant when compared to the PBS group, due to the small number of animals and large variation in tumor size in this group. All the primary tumors were found to have profound necrosis in the center of the tumor (**Figure 4B**).

Apart from tumor volumes, the location and number of macroscopic metastatic lesions were assessed. The results were highly inconsistent between individual animals and groups—metastases were found in various locations, including

TABLE 3 | Overview of the detected macrometastases in F344 PC model rats.

Group	Total No of metastatic lesions	No of rats with metastases/No of rats per group	Metastatic locations[a]					
			Lung	Peritoneal	Mesenterial	Seminal vesicle	Pancreatic	Intestinal
REV	4	3/5	0/0	1/1	1/1	0/0	0/0	2/2
SEV	8	3/5	2/2	2/1	0/0	0/0	0/0	4/2
PBS	18	3/4	11/2	3/2	1/1	2/1	1/1	0/0

[a]*The number of total metastatic lesions in the given location/the number of animals with metastases in the given location.*

seminal vesicles, mesenteric, iliac and intestinal lymph nodes, and lungs (**Table 3**) and revealed no statistically significant differences between any of the study groups in terms of numbers or locations. However, distal lung metastases were observed only in the animals from control groups, but not rats receiving Post-RUN EVs (REV group) implying for a possible protective role of the EVs in cancer progression.

DISCUSSION

As several previous studies had demonstrated that exercise increases the levels of circulating EVs both in human subjects and laboratory animals (Fruhbeis et al., 2015; Bei et al., 2017; Oliveira et al., 2018; Whitham et al., 2018; Neuberger et al., 2021), we assessed the EV concentration in rat plasma samples collected before and immediately after forced 1-h wheel running exercise. Although the EV levels were increased in some animals, the mean EV concentration and size were not significantly different in the Pre-RUN and Post-RUN plasma samples. These results are in line with several other studies finding no significant changes in the EV concentration after the exercise (Lovett et al., 2018; Hou et al., 2019; Yoon et al., 2021). This controversy suggests that different types, intensity and duration of exercise may affect the kinetics of EV release from various cell types or their clearance from the circulation in different ways. It could be possible that the induction of EV release during wheel running exercise is too slow to observe a substantial increase after 1 h or is compensated by increased clearance rate. Such a version is supported by Fruhbeis et al. showing that small EV levels increased immediately after cycling exercise and declined within 90 min at rest, whereas the increase was moderate but more sustained in response to treadmill running (Fruhbeis et al., 2015). Alternatively, these results may be affected by the use of different EV isolation and quantification methods and the variability of these methods may obscure a moderate increase in EV levels. In this study, we used IZON qEVoriginal/35 nm columns that have an optimum recovery range of 35–350 nm, thus larger microvesicles may be underrepresented in our EV preps, whereas co-isolation of larger lipoprotein particles is possible. Indeed, TEM images revealed small but consistent co-isolation of lipoprotein particles and the presence of some large particles that may represent lipoprotein aggregates, which may affect the NTA measurements. Hence, immunoisolation of EV subpopulations using EV surface markers may give a better insight into the dynamics of EV release and the cell types contributing to the pool of circulating EVs (Brahmer et al., 2019; Rigamonti et al., 2020).

Despite the fact that we did not observe a consistent increase of the circulating EV levels during exercise, RNA sequencing analysis revealed substantial differences in the RNA cargo of Pre-RUN and Post-RUN EVs from exercised rats, thus suggesting that EVs were actively released in the circulation in response to the forced wheel running exercise. Although selective RNA sorting mechanisms have been described that bias the RNA profiles of EVs (O'Brien et al., 2020), at least partially the molecular composition of EVs reflect that of the parental cell and therefore the analysis of RNA cargo may reveal the cell types producing EVs during the exercise. All of the 20 DEGs altered in the Post-RUN EVs were protein-coding genes. According to rat transcriptomic BodyMap (Yu et al., 2014), most of the genes that are upregulated in the Post-RUN EVs have tissue-selective or restricted expression pattern with the highest expression levels in the liver, testis, lung, muscle, brain, thymus and kidney. At least in human blood, the major sources of cell-free RNAs are blood cells, followed by much smaller fractions derived from spleen, liver and other tissues (Larson et al., 2021). Our data suggest that fractions of cell-free RNAs derived from organs involved in exercise are increased during the exercise relatively to the blood cell-derived fraction.

The differentially expressed genes in the Post-RUN EVs reflect the physiological processes that are triggered in various cell types in response to exercise. For example, 3 DEGs—*Notum* (palmitoleoyl-protein carboxylesterase), *Pctp* (phosphatidylcholine transfer protein) and *Cyp4b1* (cytochrome P450, family 4, subfamily b, polypeptide 1), which are induced in Post-RUN EVs, are implicated in various metabolic processes. Two other DEGs—*Dnajb5* (DnaJ heat shock protein family (Hsp40) member B5) and *Hspa5* (heat shock protein family A (Hsp70) member 5) are molecular chaperones that are involved in maturation, re-folding and degradation of proteins and play pivotal roles in cell survival under various stress conditions (Archer et al., 2018). Another set of genes is involved in the regulation of immune responses. *Ltb4r2* (leukotriene B4 receptor 2) and *Alox5* (arachidonate 5-lipoxygenase) are involved in leukotriene signaling that play important role in acute and chronic inflammation (Jo-Watanabe et al., 2019), while *Zbtb1* (zinc finger and BTB domain containing 1) is a transcription factor that is essential for the development, differentiation and effector function of T cells (Cheng et al., 2021). *Fcrlb* (Fc receptor-like B) is an F_C receptor homolog expressed as an intracellular protein in germinal center B cells (Wilson and Colonna, 2005).

We did not find significantly altered miRNAs, when compared the Post-RUN EVs with the Pre-RUN EVs. On the contrary, a recent study by Oliveira et al. found 12 miRNAs that were differentially expressed in rat serum EVs in response to

treadmill running (Oliveira et al., 2018). The levels of eight of these miRNAs (rno-miR-128-3p, 103-3p, 148a-3p, 191a-5p, 10b-5p, 93-5p, 25-3p and 142-5p) were also altered in the Post-RUN EVs in our study, however, the difference did not reach the statistical significance.

Comparison of Pre-RUN EVs from exercised rats with EVs from sedentary control rats revealed 52 DEGs thus showing that exercise has a long-lasting effect on the circulating EV RNA cargo. Among the genes downregulated in the EVs from exercised rats were *Gpx3* (glutathione peroxidase 3), *Pxdn* (peroxidasin) and *Selenof* (selenoprotein F) that are associated with the molecular function "oxidoreductase activity, acting on peroxide as acceptor"; two of them—*Gpx3* and *Selenof* are also associated with "selenium binding". Although several studies have shown that the levels of oxidative stress markers such as glutathione peroxidase increase during the exercise (Khcharem et al., 2021; Mesquita et al., 2021), our results suggest that their baseline levels are lower in exercised than in sedentary animals. Among the DEGs were two miRNAs—miR378b and miR35, that were present in the runner EVs but undetectable in the sedentary rat EVs. Both of them are implicated in the regulation of various processes in the body, including insulin sensitivity (Li et al., 2020) and protection against insulin resistance (Chen et al., 2019).

Several previous studies have investigated the effects of exercise on tumor incidence and progression in animal models. Nilsson et al. studied the effects of early-onset, lifelong voluntary wheel running in a naturally aging mouse model and showed that exercise protected against multiple types of cancer, lowered systemic inflammation and extended the health-span of naturally aged mice (Nilsson et al., 2019). Another study showed that voluntary wheel running reduced tumor incidence and growth across 5 different tumor models, including subcutaneous injection of B16F10 murine melanoma cells, intravenous injection of B16F10 melanoma cells, diethylnitrosamine (DEN)—induced liver cancer, Lewis Lung carcinoma model, and Tg(Grm1)EPv transgenic male mice model. In the subcutaneous B16 models, tumors from running mice showed higher infiltration of NK cells, $CD3^+$ T cells and dendritic cells, while the metastasis model had infiltrated NK cells only (Pedersen et al., 2016), suggesting that the mobilization or increased cytotoxicity of NK cells may be one of the mechanisms of action.

Here, we for the first time studied the effects of exercise-induced EVs on the progression of cancer in a rat model of metastatic prostate cancer. Results showed that regular injections of exercise-induced EVs into tumor-bearing rats reduced the primary tumor growth by ~35% and possibly may have delayed the development of lung metastasis. Pedersen et al. proposed four key mechanisms implicated in exercise-mediated cancer protection: normalization of tumor vasculature and blood flow, boosting immune cell functions, reprogramming metabolic pathways in cancer cells and controlling tumor growth by bioactive molecules secreted by skeletal muscle during contraction (Pedersen et al., 2015). In principle, exercise-induced EVs may contribute to all of these mechanisms, however, what is the scale of their contribution and which of the mechanisms dominate in the exercise-induced EV-mediated cancer protection remains to be established.

A shortcoming of our study, however, is the small sample size that remained after excluding animals showing the signs of suffering and large variation of the obtained data, therefore the results of this exploratory study should be regarded as indicative and should be validated in a larger cohort of animals. Moreover, the biodistribution, uptake and intracellular fate of the exercise-induced EVs and the effects triggered in the recipient cells remains to be investigated.

In summary, we show that the RNA cargo of EVs released into the circulation during exercise is altered as compared to the resting state and provide evidence that exercise-induced EVs may modulate tumor physiology and delay the progression of cancer thus supporting the idea that regular physical exercise should be prescribed to prostate cancer patients as a tertiary prevention measure.

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.ebi.ac.uk/arrayexpress/, E-MTAB-11020.

ETHICS STATEMENT

The animal study was reviewed and approved by National animal welfare and ethics committee (permit no. 121/2021).

AUTHOR CONTRIBUTIONS

AL, ZK and LiS designed research, LiS, JA, LK, NR, and ZK performed research and participated in the analysis and interpretation of the results, PZ performed the RNAseq data analysis and statistical analyses. AL, LiS, JA, LK, and ZK wrote the manuscript. MK and LaS helped with animal surgery and postsurgical care. ST provided the cell line. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.784080/full#supplementary-material

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