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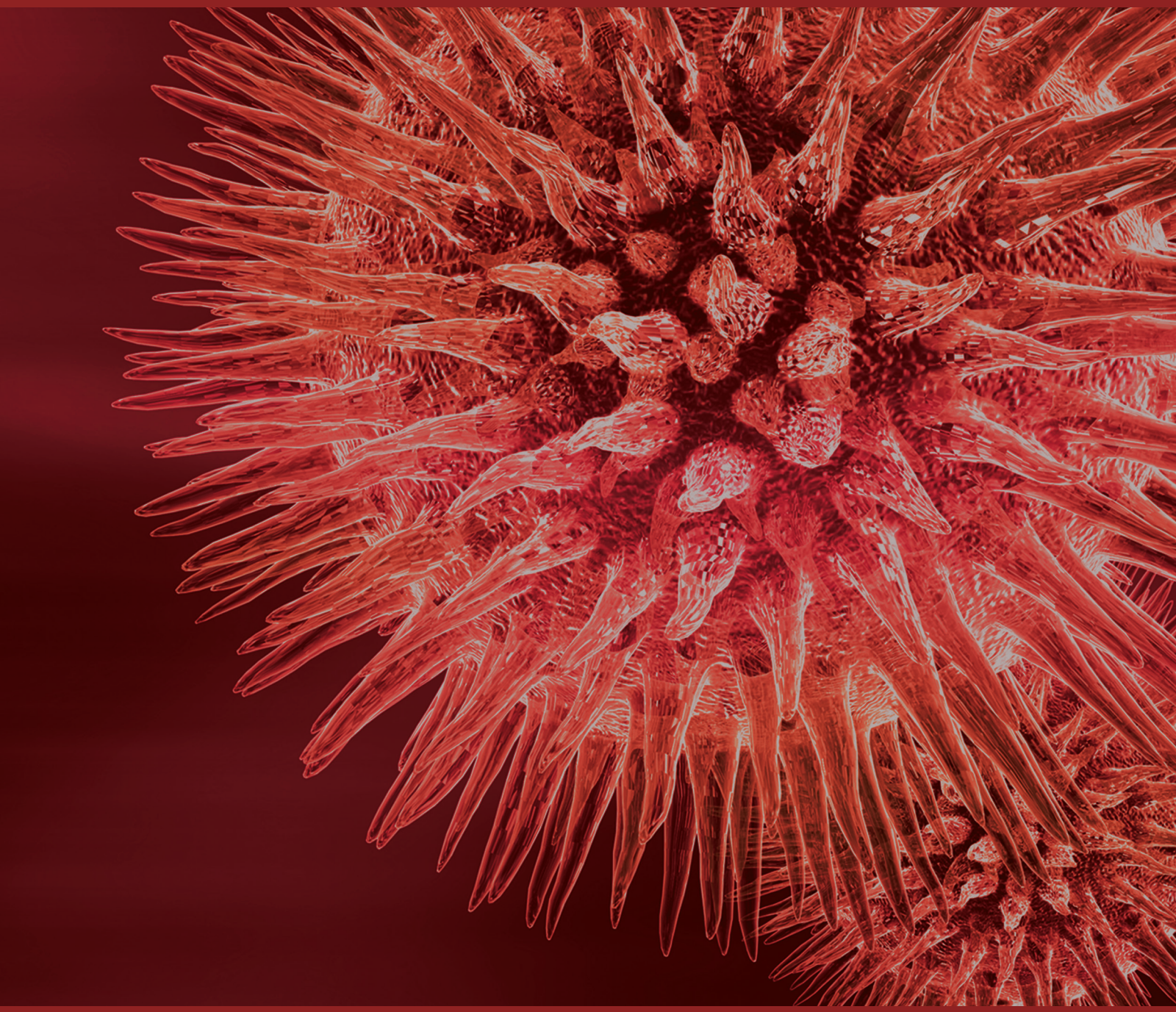
# Transmission of Information in Neoplasia by Extracellular Vesicles

Guest Editors: Aurelio Lorico, Denis Corbeil, John M. Pawelek, and Riccardo Alessandro



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*Editorial*

# Transmission of Information in Neoplasia by Extracellular Vesicles

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Paracrine interactions among neoplastic and nonneoplastic cells in the immediate tumor microenvironment are important for tumor growth and metastatic spreading. Most of the studies in the past decade addressing these cellular interactions have focused on tumor cell-derived soluble molecules. Recently, these studies and interest have shifted to nanosized extracellular vesicles (EVs) and especially ectosome and exosome-associated molecules [1]. They contain not only proteins, but also lipids, mRNA, and microRNA [1], which can regulate gene expression in their target cells in a much more pleiotropic manner [1]. While exosomes originate by a sequential process of inward budding of late endosomes, producing multivesicular bodies (MVBs), followed by release of internal microvesicles into the microenvironment by fusion of the MVBs with the plasma membrane [1, 2], ectosomes bud from plasma membrane, particularly from plasma membrane protrusions (e.g., microvilli) [1]. However, difficulties in obtaining homogeneous exosomal and ectosomal preparations result in incomplete understanding of their formation, composition, and functions [3]. Trafficking of biological materials across cellular membranes is part of any normal cell homeostasis, to maintain proper compartmentalization of important molecules. The physiological functions of EVs are extremely diverse (e.g., cell-cell communication, cellular differentiation, immunity, and inflammation) [4–8]. However, in pathological states, such as cancer, aberrant activity of the export machinery results in expulsion of a number of key proteins and microRNA that modify the tumor microenvironment, in turn stimulating the release of EVs from stromal and immune-competent cells. In cancers, such vehicles might play a role in carcinogenesis and disease progression and promote formation of the premetastatic niche [9–11]. In the present special issue on transmission of information in neoplasia by extracellular vesicles, review articles and research paper illustrate the relevance of EVs to cancer growth and metastasis.

C. Soekmadji and C. C. Nelson discuss the current available literature about the role of EV-mediated drug resistance in cancers, with a particular focus on advanced prostate cancer. Presence of multidrug resistance proteins on the EV membrane, enrichment of ceramide, and sequestration of anticancer drugs are all potential EV-mediated mechanisms to limit the bioavailability or efficacy of anticancer agents.

T. M. Green et al. review the effects of EVs released by breast cancer cells through transfer of mRNA, microRNA, and proteins to different recipient cells within the tumor microenvironment, both in an autocrine and in a paracrine manner, which have a significant impact on signaling pathways, mRNA transcription, and protein expression. The authors point to proteins and microRNA identified within breast cancer-released EVs that give insight as to the nature and severity of the disease and could serve as possible diagnostic markers. Emphasis is also placed on multiple mechanisms by which breast cancer cells avoid immune system

recognition through EVs, such as secretion of immunosuppressive proteins, inhibition of NK cell proliferation, or a decrease in T cell cytotoxicity. Finally, potential benefits deriving from the use of EVs as a vehicle for delivery of anti-breast cancer drugs are discussed.

T. Sun et al. point to exosome-derived noncoding RNA and in particular microRNA as important molecules in lung cancer biology, facilitating lung cancer growth and metastasis. Both the EV field and the microRNA fields being in their infancy, it is conceivable that many biological mechanisms crucial for lung carcinogenesis and progression have yet to be discovered.

Z. Qian et al. review the current literature in the field of EV-mediated changes in the epigenetics of cancer microenvironment. Some of the proteins and nucleic acids transmitted through EVs to recipient cells participate in DNA methylation, histone modification, and posttranscriptional regulation of RNA. They may be responsible for altering expression of oncogenes and tumor suppressor genes in recipient cells.

S. Raimondo et al. examine the role of EVs released from cells of hematological malignancies during cancer progression, mainly focusing on the specific microenvironment of hematological diseases, that is, the bone marrow. Blast cells from acute myeloid leukemia and chronic myeloid leukemia are able to initiate an exosome-mediated cell-cell communication with bone marrow stromal cells, thus affecting angiogenesis and microenvironmental niche. Furthermore, examples of the acquisition of EV-mediated chemotherapeutic resistance are illustrated for multiple myeloma and B-cell lymphoma.

In their review article, G. Schiera et al. summarize the possible physiological roles of EVs in the nervous system, where cell-cell communication is determinant in neuron and glia differentiation as well as in the formation of the blood-brain barrier. They further discuss the involvement of EVs in the horizontal transfer of information for brain pathologies such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

In the article entitled "High LIN28A Expressing Ovarian Cancer Cells Secrete Exosomes That Induce Invasion and Migration in HEK293 Cells," the authors present the results of a study on IGROV1 ovarian cancer cells expressing high levels of LIN28, a stem cell marker and emergent oncogenic driver. Although LIN28 was not present in exosomes released by cultures of IGROV1 cells, addition of exosomes to target cells resulted in changes in expression of genes associated with epithelial-mesenchymal transition and in a more aggressive cell behavior.

Overall, the current issue of this journal highlights the contribution of EVs to tumorigenesis and formation of metastases and points to several potential strategies to effectively utilize EVs for breast cancer diagnosis, prognosis, and therapy.

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*Riccardo Alessandro*

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*Review Article*

# Breast Cancer-Derived Extracellular Vesicles: Characterization and Contribution to the Metastatic Phenotype

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The study of extracellular vesicles (EVs) in cancer progression is a complex and rapidly evolving field. Whole categories of cellular interactions in cancer which were originally presumed to be due solely to soluble secreted molecules have now evolved to include membrane-enclosed extracellular vesicles (EVs), which include both exosomes and shed microvesicles (MVs), and can contain many of the same molecules as those secreted in soluble form but many different molecules as well. EVs released by cancer cells can transfer mRNA, miRNA, and proteins to different recipient cells within the tumor microenvironment, in both an autocrine and paracrine manner, causing a significant impact on signaling pathways, mRNA transcription, and protein expression. The transfer of EVs to target cells, in turn, supports cancer growth, immunosuppression, and metastasis formation. This review focuses exclusively on breast cancer EVs with an emphasis on breast cancer-derived exosomes, keeping in mind that breast cancer-derived EVs share some common physical properties with EVs of other cancers.

## 1. Introduction

Breast cancer is the most prevalent type of cancer in women [1]. Although a multitude of treatment options are available [2–4], approximately one-third of women worldwide diagnosed with breast cancer still die from the disease, largely from metastasis, especially brain metastasis [5–8]. EVs have been hypothesized to have significant roles in breast cancer growth and metastasis and thus have been evaluated as potential avenues of new therapeutic intervention. EVs, including exosomes and MVs, are secreted in large quantities by cancer cells into the local microenvironment and premetastatic "niche" [9]. While both exosomes and MVs are small (usually $< 1\,\mu$m in diameter), these bilipid membrane-enclosed vesicular structures [10–13] have a distinct biogenesis: exosomes are generated through inward budding of an endosome resulting in a multivesicular body (MVB) which is released by subsequent fusion of the MVB with the plasma membrane [14–18], whereas MVs are released directly by budding from the cellular plasma membrane [15, 18, 19]. However, EVs including both MVs and exosomes have been proposed to enter target cells through multiple mechanisms, including ligand-receptor-mediated [20, 21], or lipid raft-mediated entry [22, 23], with EV fusion and uptake greatly influenced by surrounding pH levels [24]. Exosomes are typically smaller than MVs, being 100 nm or less in diameter, while the latter are > 100 nm in size. Some size overlap does exist, however [25–29]. Apoptotic bodies, another type of EV, are poorly characterized and not generally included within the general categories of exosomes or MVs and therefore will not be discussed in this review. This review will discuss breast cancer EV purification, composition, and effect on target cells within the tumor microenvironment to promote cancer growth and metastasis, as well as their possible use as drug delivery vehicles of anticancer therapeutic drugs.

## 2. Breast Cancer EV Characterization and Proteomic Profiling

*2.1. Methods of Isolation.* There are several variations to the method of isolating MVs and exosomes from cell culture

supernatants, but most involve differential centrifugation at various speeds to separate particles based on size and density. For EV isolation, cells are grown in media supplemented with exosome-depleted fetal bovine serum (FBS). The first step in purification involves a low-speed centrifugation at 300 ×g–500 ×g for 5–10 min to remove live cells [30–32]. The supernatant is then centrifuged at 1200 ×g–2000 ×g for 10–30 min to remove dead cells and apoptotic blebs [30, 33–35]. This is followed by centrifugation at 10,000 ×g for 30 min to remove cellular debris [30, 36]. To isolate the exosome and MV fractions, the supernatant then undergoes ultracentrifugation at 100,000–200,000 ×g for 60–120 min [30, 32, 36–38]. The supernatant at this point is discarded and the pellet, containing exosomes and MVs, is washed in phosphate buffered saline (PBS) and ultracentrifuged one final time [30, 36, 39, 40]. The PBS is gently decanted and the exosome/MV pellet is resuspended in a small volume of buffer [35, 41]. If human or mouse serum is used as starting material, an intermediate step using a 0.22 $\mu$m filter can be employed to supplement the centrifugation steps, resulting in an enrichment in exosomes and small MVs [32, 38, 42]. These methods have been used to isolate MVs and exosomes from both cancer and noncancerous cell lines, including LNCaP prostate cancer, vascular smooth muscle cells (VSMCs), BV-2, HCMEC/D3, and several breast cancer cells (MDA-MB-231, MCF-7, and MDA-MB-435) [30, 31, 34, 43]. Exosomes purified using this methodology are suitable for liquid chromatography-mass spectrometry (LC-MS) analysis [30, 36]. However, in order to obtain a density-based purer fraction devoid of additional nonspecific proteins and protein aggregates, the exosomal/MV supernatant can be layered on a sucrose gradient prior to high-speed ultracentrifugation [33, 44]. It is really not possible to separate exosomes from MVs by strictly physical means.

*2.2. Size Variation.* Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to determine the physical properties of EVs from many sources, including cancer and noncancerous cell lines, as well as from patients with a myriad of diseases. In general, exosomes are 30–100 nm in size and have a characteristic round or cup-shape, while microvesicles are larger, approximately 100 nm–1 $\mu$m, and are composed of a round-shaped, heterogeneous population; however, as previously mentioned, a certain degree of size overlap occurs [11, 35, 40, 42, 44–51]. It is important to note that the cup-shape appearance of exosomes visualized by electron microscopy may be an artifact of the fixation process, and their true shape mirrors that of round microvesicles [17]. In addition, there is also some variation in size of EVs based on the method of visualization. For example, exosomes derived from metastatic MDA-MB-231 breast cancer cells were determined to be 40–120 nm in size by SEM and contained the typical exosomal marker CD63 [52]. EVs shed from MDA-MB-231 cells, on the other hand, were larger in size and determined to be 57–440 nm in size with a mean diameter of 121 ± 54 nm by TEM [53]. As another example, tissue factor (TF) antigen has also been identified in breast cancer-derived EVs [53, 54]. 10% of MDA-MB-231-derived MVs were found to express TF, and although EVs up to 220 nm contained active TF, most EVs containing active TF were 100 nm or smaller [53]. In an independent study, TF was instead found in breast cancer MVs 200–350 nm in size. [54]. The term "exosome-like vesicles" (ELV) has been used in some studies to describe vesicles with relatively small size and similar densities, physical characteristics, and protein expression patterns as exosomes [55]. For example, in one study, ELVs from MDA-MB-231 cells were 20–180 nm in size, with the majority being 40–100 nm in size, and contained particles that were both round and cup-shaped [56]. By TEM, ELVs from MCF-7 and MDA-MB-231 cells were reported by Kruger et al. [57] to be 80–200 nm in size and round in shape. For the purpose of this review, ELVs will be referred to as exosomes. Nanoparticle tracking analysis (NTA) is another technique frequently used to determine the concentration and size of EVs [35, 37, 58]. For example, Zheng et al. used NTA to examine Rab27-dependent exosome secretion from MDA-MB-231 cells [37]. NTA analysis determined peak exosome size to be 129 nm, while an average of 79 nm was determined by TEM.

*2.3. EV Proteins as Cancer Markers.* Cancer cell lines are known to secrete significantly more EVs than noncancerous cell lines [59–63]. In line with this, serum from breast carcinoma (BrCa) patients contained significantly higher levels of exosomes than healthy donors [41, 64, 65]. Several EV proteins are differentially expressed in certain stages and types of breast cancer and may be used as diagnostic markers of cancer progression or as a diagnostic marker, that is, "liquid biopsy" for general cancer diagnosis. For example, the oncogenic cancer marker CD24 [66] was uniquely expressed in serum-derived exosomes from BrCa patients [65]. In an independent study, FAK and EGFR, which are overexpressed in cancer [67, 68], were significantly higher in MVs from BrCa patients compared to healthy donors and were found to be differentially expressed depending on the stage of cancer [41]. Survivin-2B, a splice variant of the native protein, which is an apoptosis inhibitor and associated with decreased survivability [69], has also been identified in EVs from BrCa patient serum, and the protein expression is lost with the progression of the disease [70]. In addition to CD24, FAK, EGFR, and Survivin, BrCa serum EVs have been found to contain the disintegrin and metalloprotease ADAM10, as well as the tetraspanin CD9 [41, 65, 70], with the latter being a known functional integrin binding partner in breast cancer cells [71, 72] and a molecular marker of EVs. Similarly to EVs from serum, exosomes from BrCa pleural effusions contained ADAM10, CD9, and CD24 but also contained the epithelial cell adhesion molecule (EpCAM) [65], a highly expressed protein on cancer cells [73]. It should be considered, however, that CD9 is present on EVs of both neoplastic and nonneoplastic origin [74]. HSP70 and Annexin-1, the latter of which regulates apoptosis and inflammation [75], were also identified from BrCa pleural effusion-derived exosomes [65]. In addition to BrCa patient samples, several EV proteins have been identified from immortalized cancer cell lines. In one particular study, the tumor marker extracellular matrix metalloproteinase inducer EMMPRIN [76, 77] was identified on SKBR3 and MCF-7 breast cancer-derived MVs, but not

on exosomes [78]. On the other hand, the tetraspanin CD63, a binding partner of integrins and tumor marker whose expression inversely correlates with cancer metastasis [71, 79–84], and tumor susceptibility gene 101 (TSG101), which is a subunit of the endosomal sorting complex required for transport-1 (ESCRT-1) [85], were present almost exclusively on exosomes [78]. Others have reported CD63, ALG-2-interacting protein X (Alix), which is involved in exosome biogenesis and endosomal sorting [86, 87], and TSG101, or a combination of these proteins, from MDA-MB-231, MCF-7, T47D:A18, and Hs-578T breast cancer-derived exosomes [37, 45, 49, 50]. Lysosome-associated membrane glycoprotein 1 (LAMP1) and heat shock cognate 70 (HSC70) were also both found to be in exosomes from MDA-MB-231 cells [56]. Therefore, specific proteins can be used to distinguish breast cancer exosomes from shed MVs.

*2.4. Proteomic Profiling.* There has been extensive proteomic characterization performed on exosomes from breast cancer cells by liquid chromatography-mass spectrometry (LC-MS). This proteomic characterization demonstrated that exosomal content is diverse in nature and varies depending on the cell line from which they originate. They also contain cytosolic as well as membrane proteins involved in signaling pathways and maintenance of cellular structure. In a study performed by Palazzolo et al., LC-MS identified 32 proteins more abundant in the MDA-MB-231 exosomes compared with the cell lysates [56]. These included cytoskeletal proteins such as cytokeratin 9 (K1C9), tropomyosin 4 (TPM4), and transgelin 2 (TAGL2), proteins regulating cell death, including peroxiredoxin 2 (PRDX2), Annexin A5, and heat shock protein 71 (HSP71), and signal proteins such as integrin $\alpha$6 and integrin $\alpha$3 [56]. The latter two proteins are involved in cell migration and therefore play a role in the growth and angiogenesis of tumors [88–93]. Exosomes were also enriched in the beta chain of MHC Class I molecules (B2MG), supporting the involvement of exosomes in altering immune system recognition to promote cancer growth [56]. Consistent with this result, proteomics performed on exosomes from a pleural effusion of a breast cancer patient identified MHC Class I molecules, in addition to B-cell translocation gene 1 (BTG1) and pigment epithelium-derived factor (PEDF) [44]. Since the decrease in tumor surface expression of BTG1 and PEDF has been associated with increased cancer growth and metastasis [94–96], tumor cell exosomal shedding of these proteins obviously decreases the cell surface content of these molecules, which benefits the tumor. Secretome profiling of BT-474 and SKBR3 breast cancer cell lines also showed that exosomes were enriched in proteins associated with antigen processing/presentation, such as heat shock 70 kDa protein 5 (HSPA5), calreticulin (CALR), and proteasome activator complex subunit 2 (PSME2), and proteins associated with glycolysis/gluconeogenesis, such as triosephosphate isomerase 1 (TPI1) and phosphoglycerate mutase 1 (PGAM1) [97]. Proteomics of MCF-7 exosomes showed they contained lipid raft proteins (G protein) and raft-associated proteins (profilin II, HSP27) [36], giving insight into the composition of their exosomal membranes. Others have also documented flotillin-1 and cofilin, proteins associated with lipid rafts in MCF-7 exosomes [45]. Villarreal et al. demonstrated exosomes composed a portion of the MCF-7 and MDA-MB-231 secretomes, and the former included CD63, along with intracellular proteins such as Annexins A2 and A4, and a variety of histones and chaperones [98]. Immunoblotting also showed BT-549 breast cancer exosomes contained Annexins A2 and A6 [99]. A separate study showed BT-549 exosomes were taken up through lipid raft domains, which possibly involves Annexin A2 in the target cell, while Annexin A6 may play a role in the movement of exosomes to the late endosomal compartment [22]. Recently, LC-MS/MS was used to compare proteins of exosomes from both MDA-MB-231 and MCF-7 cells [57]. MDA-MB-231-derived exosomes contained significantly more extracellular matrix proteins than those from MCF-7 cells [57]. MDA-MB-231-derived exosomes also contained more proteins with catalytic activity, while MCF-7 exosomes contained more nucleic acid binding and transport proteins [57]. Annexin A1 was exclusive to MCF-7 exosomes while EpCAM was exclusive to MDA-MB-231-derived exosomes and both contained Annexin A2 and $\alpha$-enolase [57], the latter of which has been shown to promote cancer cell growth, migration, and metastasis [100]. MDA-MB-231- and MCF-7-derived exosomes also differentially expressed certain miRNAs. For example, MDA-MB-231-derived exosomes had higher levels of tumorigenic mir-130a and mir-328, while those from MCF-7 cells had higher levels of mir-301a [57], the expression of which indicates a negative prognosis of patients with invasive ductal or triple negative breast cancer [101, 102]. Therefore, exosomes from both breast cancer cell lines contain proteins and miRNA that are oncogenic in nature, yet they each have their own distinctive identifying markers that may relate to the nature and aggressiveness of the cancer type. Both breast cancer-derived exosomes and MVs contain significant, yet distinctive proteins related to breast cancer progression and metastasis (Table 1). Additional breast cancer-derived exosomal proteins have functional roles in immune evasion and miRNA biogenesis and serve as surrogate metastatic markers (Table 2).

## 3. Visualization of Breast Cancer EVs

Direct visualization of breast cancer EV release and uptake in both live and fixed cells by microscopy has been facilitated by fluorescently tagged EV proteins, fluorescent antibodies or peptides, or lipid fluorescent dyes. In particular, several groups have utilized breast cancer cell lines overexpressing the exosomal marker CD63 with a green fluorescent protein (GFP) fusion tag [22, 46, 103]. A striking example is shown by Suetsugu et al., who engineered MDA-MB-231 and mouse mammary tumor (MMT) cells stably expressing CD63-GFP [103]. From these cell lines, the transfer of CD63-GFP exosomes to nonfluorescent autologous breast cancer cells was visualized *in vitro* and in xenograft models. Upon injection into immunodeficient mice, breast cancer cells expressing CD63-GFP formed tumors that metastasized to the lungs, secreting fluorescent exosomes into both the primary tumor and metastatic microenvironment. Various studies have also utilized PKH dyes, which intercalate with lipids, [32, 52, 104,

Table 1: Comparison of breast cancer-derived MV and exosomal proteins.

| Role | Microvesicles | Exosomes |
|---|---|---|
| Extracellular matrix degradation | EMPRINN [42, 78]<br>ARF1 [144] | ADAM10 [51, 65] |
| Cancer invasion/metastasis | RhoA/RhoC GTPase [144]<br>FAK [41]<br>EGFR [41]<br>TF [53, 54, 147] | Annexins A2, A5, and A6 [55, 56, 97, 98]<br>EpCAM [57, 65, 120]<br>H2A [46]<br>CD24 [65]<br>HSP70 [36, 65]<br>$\alpha$-enolase [57]<br>Integrins $\alpha$3, $\alpha$6 [56] |
| Cell survival | IDO [128]<br>tTG [107] | PRDX2 [56]<br>HSC70 [56] |

Table 2: Additional breast cancer-derived exosomal proteins and their functional roles.

| Role | Exosomes |
|---|---|
| Immune evasion | CALR [97]<br>MHC Class I molecules [33, 37, 44, 56]<br>PSME2 [97] |
| miRNA biogenesis | Dicer [64]<br>Ago2 [64]<br>TRBP [64] |
| Integrin binding/signaling partner | CD63 [45, 49, 50, 52, 64, 78, 98, 139]<br>CD9 [45, 51, 64, 65, 133, 138, 139] |

105], or fluorescent antibody or peptide markers [43, 106, 107] to stain MVs and exosomes, demonstrating that breast cancer EVs can transfer nucleic acids and proteins to autologous and heterologous cells within the tumor microenvironment, possibly resulting in the acquisition of the cancer phenotypes, favoring tumor progression, immune evasion, and metastasis.

## 4. Horizontal Transmission of miRNA and Proteins

EVs purified from breast cancer cells typically carry specific mRNAs and miRNAs in addition to proteins and can transfer both transcripts and intact proteins to surrounding cancer cells to promote tumor development. In fact, miRNA is enriched in exosomes derived from the breast cancer cell lines 4T.1, MDA-MB-231, and MCF-7 compared to exosomes from normal breast cells MCF10A and NMuMG [64]. Exosomes from metastatic cell lines (MDA-MB-231 and 4T.1) were also enriched in miRNA compared to exosomes from nonmetastatic cells (MCF-7) [64]. miRNAs were found to be secreted into subpopulations of MVs from MDA-MB-231 cells, with different miRNAs packaged into different types of vesicles [48]. Addition of MVs from MDA-MB-231 cells caused an increase in total RNA in human submandibular gland (HSG) cells [108]. In turn, HSG MVs isolated from HSG cells that were treated with MDA-MB-231-derived MVs contained multiple new mRNAs and an increase in protein levels [108]. Cancer cell-derived exosomes are capable of miRNA processing and biogenesis in addition to transfer of miRNA to target cells [64]. This is evidenced by the detection of proteins involved in miRNA biogenesis, including the RISC loading complex (RLC) proteins Dicer, Ago2, and TRBP, in exosomes from breast cancer cell lines and patient samples but not from normal breast cell lines [64].

## 5. Induction of Drug Resistance

Several mechanisms have been described for breast cancer EV-mediated transfer of drug resistance to promote tumor growth and progression. One such mechanism involves the transfer of P-glyoprotein (P-gp), a protein known to be involved in drug resistance [109, 110], through MVs produced from doxorubicin- or docetaxel-resistant breast cancer cells into target endothelial or drug-sensitive cancer cells [111, 112]. These MVs also transferred TrpC5, which caused activation of the NFATc3 transcription factor to stimulate transcription of P-gp mRNA [111]. In addition to the transfer of proteins, the transfer of miRNAs from drug-resistant breast cancer-derived exosomes conferred drug-resistant properties to target cells [47, 104]. Specifically, exosomes from docetaxel-resistant MCF-7 cells contain miRNAs which downregulate mRNA encoding chemosensitive properties when transferred to nonresistant MCF-7 cells [47, 104]. Exosomes from doxorubicin-resistant MCF-7 cells also induced chemoresistance in nonresistant MCF-7 cells through transfer of miRNAs [47]. In another study, exosomes produced from tamoxifen-resistant MCF-7 cells were taken up by MCF-7 wild type cells and released miR-221/222 [32]. miR-221/222 subsequently caused a decrease in P27 and ERa (targets of miR-221/222) protein levels, which caused an increase in tamoxifen resistance in MCF-7 target cells. Besides conferring drug resistance, cancer exosomes can decrease the effectiveness of the therapeutic drug trastuzumab, an antibody that binds HER2 [40]. The presence of HER2 on cancer cells is linked to increased cancer metastasis and tumor proliferation [113, 114]. Exosomes from SKBR3 and BT-474, both of which are HER2-overexpressing breast cancer cell lines, contain active HER2 and, along with exosomes from HER2-positive breast cancer patient serum, bound to trastuzumab to decrease the drugs' effectiveness at inhibiting SKBR3 cell proliferation [40]. Treatment of BT474 cells with epidermal growth factor (EGF) and heregulin (HRG), which are growth factors secreted by cancer cells [115–117] and activators of HER2 [118, 119], caused a significant increase in exosome production [40], leading to the possibility that enhanced secretion of exosomes may be a way for the tumor microenvironment to support cancer growth in the presence of therapeutic agents. Consistent with these results, $HER2^+$ exosomes from BT-474 and SKBR3 cells decreased the trastuzumab-induced cytotoxic effects of peripheral blood mononuclear cells (PBMC) against BT-474

cells [120]. Besides the transfer of proteins and miRNA to cancer cells, breast cancer EVs can also directly secrete the anticancer drug doxorubicin [121]. The number of secreted MCF-7-derived EVs positively correlated with increasing drug concentration, which suggested EV release is a direct mechanism for imparting chemoresistance within a tumor [121].

## 6. Therapeutic Implications of EVs

It is possible to take advantage of the drug delivery capabilities of exosomes for breast cancer therapy, as shown by Tian et al. [122]. Doxorubicin, incorporated into exosomes from immature dendritic cells (imDC) and taken up by both MDA-MB-231 and MCF-7 cells, caused an inhibition of cell proliferation. When injected into nude mice implanted with MDA-MB-231 tumors, it caused a reduction in tumor growth [122]. Specific targeting to tumors was achieved by incorporating an RGD integrin target sequence in the exosomes which bound to MDA-MB-231 cells, which naturally express high levels of integrin $\alpha$v [123]. Exosomes from HEK293 cells stably expressing EGF or EGFR peptide have also been studied as therapeutic agents by targeting breast cancer cells expressing high levels of EGFR (such as the HCC70 breast carcinoma cell line) [39]. The tumor suppressor let-7a miRNA was transfected into HEK293 cells expressing EGFR peptide, and exosomes produced from these cells significantly decreased HCC70 tumor growth in mice [39]. Additionally, treatment of 4T.1 murine breast cancer cells with epigallocatechin gallate (EGCG), a chemical with antitumor effects, caused an increase in the levels of the tumor suppressor miR-16 within exosomes [124]. Exosomes from EGCG-treated breast cancer cells subsequently decreased NF$\kappa\beta$ activity and M2 polarization in tumor-associated macrophages (TAM) through miR-16 [124]. M2 macrophages, which inhibit inflammation during the end stages of inflammatory processes such as wound healing [125], are known to have tumor-promoting phenotypes [126, 127]. Consequently, therapeutic agents can induce transfer of tumor suppressors to surrounding macrophages through exosomes to decrease their cancer-associated properties.

## 7. EVs as Immunomodulators

Many groups have studied the immunosuppressive effects of tumor-derived EVs and their relation to cancer progression within the tumor microenvironment (Figure 1). In one study, MVs from 36% of breast cancer patients contained indoleamine 2,3-dioxygenase (IDO), an enzyme that has a role in immunosuppression and tumor survival [128]. Another example of exosomal-mediated immune modulation was described in a study involving TS/A murine mammary tumor-derived exosomes, which were found to act as immune system suppressors to promote tumor progression by inhibiting the differentiation of myeloid precursors in the bone marrow into dendritic cells, partially through induction of IL-6 mRNA [129]. Myeloid-derived suppressor cells (MDSCs) are involved in tumor growth, partially through the inhibition of T-cell activation via Toll-like receptor (TLR) adaptor protein MyD88 [130]. Murine melanoma-derived exosomes were found to exacerbate the effects of MDSCs, which included the induction of the proinflammatory cytokine CCL2 through MyD88 [131]. This same study also discovered that 4T.1-derived exosomes caused an increase in lung metastases through CCL2 [131]. Similarly, breast cancer TS/A-derived exosomes injected into a mouse model caused an increase of MDSCs within both the primary tumor and the spleen [132]. Breast tumor-derived exosomes have also been found to reduce the immune response by inhibiting NK cytotoxicity. In one case, TS/A and 4T.1 breast tumor-derived exosomes inhibited activated NK cell proliferation and prevented them from killing TS/A and 4T.1 tumor cells [133].

In another study, injection of TS/A tumor-derived exosomes into the general circulation of mice decreased the cytotoxicity of NK cells by reducing the percentage and activity of NK cells [105]. TS/A-, 4T.1-, and MDA-MB-231-derived exosomes also decreased stimulated NK cell proliferation, and mice implanted with TS/A or 4T.1 tumors and treated with TS/A or 4T.1 breast tumor-derived exosomes had more rapid tumor growth and metastases compared to controls [105]. The human MHC Class I chain-related genes MICA and MICB are ligands of the NKG2D receptor and stimulate the immune response by activating T-cells [134–137]. To evade detection by the immune system, MDA-MB-231-derived exosomes lack MICA or MICB and therefore there is no immune stimulation [138]. Furthermore, ADAM17 in MDA-MB-231 breast cancer cells cleaves MICA/MICB from the cell surface of the cancer cell to produce a soluble form, sMICA/sMICB, which may result in a type of immune decoy [138]. Exosomes from T47D breast carcinoma cells decreased the amount of NKG2D-positive lymphocytes within peripheral blood lymphocytes (PBL), which was modulated through NKG2D ligands such as MICB, CD81, and MHC Class I on exosomes [33]. These T47D-derived exosomes also led to inhibition of the cytotoxic function of $CD8^+$ T-cells, which may be related to the downregulation of NKG2D [33]. This could be yet another mechanism for cancer to evade and/or inhibit the host immune system. A study by Deng et al. showed exosomes isolated from the 4T.1 breast cancer cell line that had interacted with immunosuppressive leukocytes from the tumor microenvironment conferred properties that enhanced tumor metastasis through production of MMP-9 and proinflammatory cytokines [139]. One interesting study suggested how the structural interactions of MDA-MB-231- and MCF-7-derived MVs with human beta-defensin 6 (hBD6), a class of proteins with antimicrobial activity [140], were linked to metastatic potential [141]. There were more nuclear magnetic resonance (NMR) chemical shifts and larger resonance intensity ratios associated with MCF-7-derived MVs compared to MDA-MB-231-derived MVs upon association with hBD6, which indicated stronger binding to the MCF-7-derived MVs [141]. This group proposed the weaker interaction of MDA-MB-231-derived MVs with hBD6 could suggest a greater capability of evading immune detection and one explanation for the increased metastatic properties of this cell line [141].

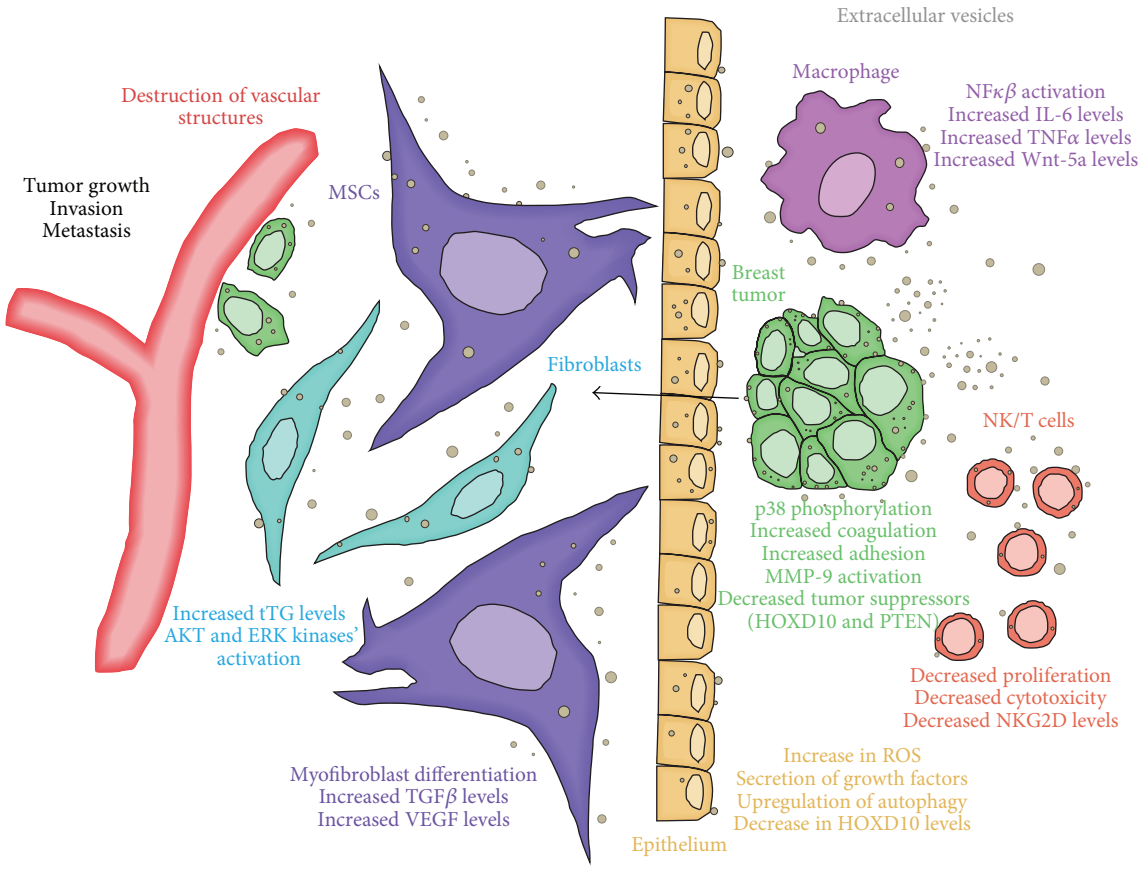


Figure 1: Schematic of the numerous interactions between breast tumor-derived EVs and stromal, epithelial, and immune cells within the tumor niche and the resulting changes that enhance cancer growth and metastasis. Breast cancer cells avoid detection from the immune system through EV-mediated decrease in the cytotoxicity of NK and T-cells and secretion of proinflammatory cytokines from macrophages. Tumor EVs enter fibroblasts and stimulate proangiogenic ERK and AKT kinase activation. Concurrently, EVs cause the secretion of oncogenic cytokines TGF$\beta$ and VEGF in mesenchymal stem cells. Additionally, tumor EVs enter surrounding cancer cells to upregulate signaling pathways that promote growth and metastasis, including increases in coagulation, adhesion, and a decrease in levels of tumor suppressors. To promote invasion, EVs induce epithelial cells to secrete growth factors and cause destruction of endothelial vascular structures, thus enhancing tumor growth.

## 8. EV Contribution to Breast Tumor Growth and Metastasis

*8.1. EV Transfer of Cancerous Phenotypes.* As mentioned above, cancer cell-derived EVs contain a number of oncogenic proteins and miRNAs, which can be transported to surrounding cancer cells to elicit phenotypic changes within target cells. Several groups have investigated the mechanisms through which breast tumor-derived EVs upregulate prometastatic pathways after entering nearby cancer cells. One scenario involves breast cancer MVs increasing cancer cell invasion through extracellular matrix metalloproteinase inducer- (EMPRINN-) dependent phosphorylation of p38 [42]. MDA-MB-231-derived MVs also contain RhoA and RhoC GTPases, which are upregulated in cancer and involved in invasion and metastasis [142, 143], as well as ADP-ribosylation factor 1 (ARF1), which is associated with MV release and MMP-9 activity within MVs [144]. MMP-9 is involved in the degradation of extracellular matrix, a critical step in cancer cell invasion [145, 146]. MDA-MB-231-derived MVs have also been reported to transfer TF to MCF-7 cells to cause increase in procoagulant activity [147], which has been correlated with increased metastatic capabilities [148]. Cellular adhesion is also known to be important in the growth and metastatic progression of cancer cells [149, 150]. To this end, the uptake of exosomes derived from BT-549 cells into target cancer cells caused increased cellular adhesion and spreading through histone H2A binding to heparin sulfate proteoglycans [46]. Another study by Melo et al. [64] highlighted the importance of exosomal miRNA processing in tumor growth. This group showed that breast cancer exosomes have all the components required for cell-independent processing of pre-miRNAs into mature miRNAs. By treating normal breast cells with exosomes from metastatic breast cancer cells and measuring miRNA changes within the target cell, they showed that exosomal miRNAs have the ability to alter the transcriptome of target cells. Similarly, treatment of MCF10A cells with MDA-MB-231-derived exosomes led to upregulation of miRNAs involved in cancer metastasis, resulting in downregulation of proteins including PTEN

and HOXD10, as well as increases in MCF-710A viability, proliferation, and colony formation, all processes in which the capability of miRNA biogenesis was essential. MDA-MB-231-derived exosomes injected with MCF10A cells also caused tumor growth in nude mice, which was decreased when miRNA biogenesis within exosomes was inhibited [64]. Therefore, cancer growth is influenced by miRNA biogenesis in exosomes. In addition to miRNA, exosomes can also transfer cancerous properties to heterologous cancer cells to promote tumor aggressiveness and metastasis. O'Brien et al. demonstrated that exosomes isolated from an invasive variant of triple negative breast cancer (TNBC) cells ($Hs578Ts_{(i)8}$) caused an increase in the growth rate, migration, and invasive capacity of SKBR3, MDA-MB-231, and HCC1954 breast cancer cell lines, as well as causing an increase in endothelial cell angiogenesis [50]. Galindo-Hernandez et al. reported that an epithelial-mesenchymal transition-like process was induced in MCF10A cells by EVs isolated from plasma of women with breast carcinoma [151]. Exosomes from the serum of patients with TNBC also produced significantly greater invasion of breast cancer cell lines compared to exosomes from healthy donors [50]. Recently, using a TNBC xenograft model of inflammatory breast cancer (MARY-X) [152], a model which forms tight tumor cell aggregates (termed spheroids) *in vitro*, which represent the *in vivo* equivalent of lymphovascular tumor emboli, complete exosomal biogenesis could be observed by TEM (Figure 2). In this model, large numbers of secreted exosomes observed within the intercellular space between cancer cells suggest the possibility that important exosomal-mediated autocrine signaling occurs in actual tumor emboli which may give them a survival advantage (unpublished observations). Since most breast cancers are adenocarcinomas, which are present in both metastatic sites and the circulation as circulating tumor cells (CTCs), this exosomal-mediated autocrine signaling may have wide therapeutic implications.

*8.2. Hypoxic Effects on EV-Influenced Metastasis.* Cancer cells have a unique ability to survive and grow under hypoxic conditions [153]. Exosome secretion plays a role in the ability of tumors to metastasize under limited oxygen conditions. Hypoxic conditions are known to increase MV and exosome release from a variety of breast cancer cell lines, including MCF-7, MDA-MB-231, and SK-BR3 [43, 45], which requires hypoxia-inducible factor- (HIF-) dependent RAB22A expression [43]. Hypoxic breast cancer-derived MVs caused increases in tumor metastasis and invasion, which were dependent on the expression of RAB22A within the MVs [43]. A low pH often accompanies hypoxia in cancerous phenotypes [154], and it has been previously reported that melanoma-derived exosome secretion and uptake increases with decreased pH levels [24]. Additional studies showed that the MCF-7 breast cancer cell line became more resistant to the chemotherapeutic drug cisplatin at lower pH levels [155], and treatment of metastatic breast cancer patients with proton-pump inhibitors (PPI) led to enhanced efficacy of chemotherapy [156]. From these results, it can be proposed that treatment of breast carcinomas with PPIs could lead to decreased exosome number within the tumor microenvironment, leading to enhanced chemosensitivity. Breast cancer-derived exosomes produced under hypoxic conditions can also promote inflammatory cross talk within the tumor niche to promote growth, which is decreased by activation of nuclear receptors within the cancer cell [157]. This leads to the possibility of nuclear receptors as new therapeutic targets, whose efficacy can be increased by taking advantage of the exosome-mediated delivery system between cancer cells.

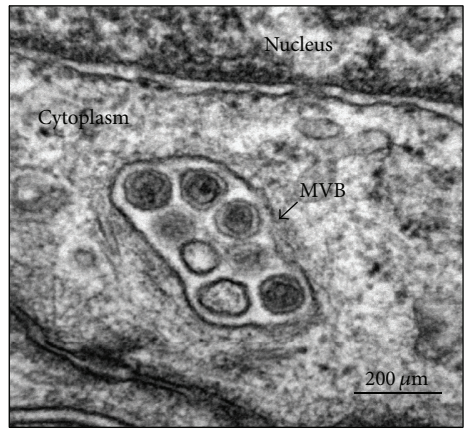


Figure 2: Early biogenesis of exosomes present within a multivesicular body (MBV) of the MARY-X spheroids is depicted. The MBV will subsequently fuse with the plasma membrane and exosomes will be released from the cell.

*8.3. Effects on the Tumor/Metastatic Niche.* Breast tumor exosomes have the ability to prime the premetastatic niche, which involves the transfer of proteins and activation of signaling pathways within epithelial cells, stromal cells, macrophages, and fibroblasts that promote tumor progression (Figure 1). Several studies have demonstrated that exosomes from cancer cells have this effect on the surrounding microenvironment. MVs derived from MDA-MB-231 have been shown to confer cancerous phenotypes to NIH 3T3 fibroblasts as well as noncancerous MCF10A human breast epithelial cells, including increased survival and cellular transformation, partly through the transfer of tissue transglutaminase (tTG) [107]. MDA-MB-231-derived MVs caused NIH 3T3 tumor growth in nude mice through activation of AKT and ERK kinases in NIH 3T3 fibroblast cells, requiring tTG [107]. In another example, MDA-MB-231-, T47DA18-, and MCF-7-derived exosomes were taken up by human primary mammary epithelial cells (HMEC), causing an increase in reactive oxygen species (ROS) and secretion of tumor growth factors from HMECs [49]. Increases in ROS by breast cancer-derived exosomes led to upregulation of autophagy through an increase in the phosphorylation of DNA damage response proteins (DDR) (ATM, H2AX, and Chk1) and p53 stabilization [49]. Autophagy, which is the internalization of damaged material within a cell, is an important survival mechanism for cancer cells to acquire energy to keep up with the demands of tumor growth [158]. In addition, cancer exosomes caused an increase in HMEC

autophagy in part by transferring LC3 proteins, involved in autophagosome cargo sorting [159], to HMEC cells [49]. A study done by Cho et al. indicates that breast tumor-derived exosomes can differentiate mesenchymal stem cells (MSC) into tumor-associated myofibroblasts [160], which are known to contribute to tumor growth, progression, and metastasis within the tumor microenvironment [161]. In particular, MDA-MB-231- and MCF-7-derived exosomes increased the expression of $\alpha$-SMA, which is an indicator of myofibroblasts, through the activation of SMAD2-dependent pathways, and cytokines such as TGF$\beta$ and VEGF, which support tumor growth, when added to adipose-derived mesenchymal stem cells (ADSC) [160]. Metastatic enhancement of breast cancer cells is regulated through exosomal miR-105, which can target tight junctions in primary human microvascular endothelial cells (HMVEC), resulting in the increased permeability of cell monolayers, the destruction of vascular structures, and induced HMVEC migration [162]. Additionally, treatment of HMVECs with MDA-MB-231-derived exosomes increased transendothelial migration of MDA-MB-231 cells through HMVEC monolayer, and mice pretreated with MDA-MB-231-derived exosomes and then injected with MDA-MB-231 cells had increased lung and brain metastases [162]. MDA-MB-231-derived exosomes can also transfer miR-10b, which is more highly expressed in exosomes from metastatic as opposed to nonmetastatic cell lines, to immortalized human mammary epithelial (HMLE) cells to reduce levels of the tumor suppressor HOXD10 [163] protein and subsequently increase cell invasion [52]. Inflammation from macrophages promotes tumor growth and metastasis to distant sites [164, 165]. Concurrently, macrophages treated with breast cancer-derived exosomes caused significant increases in NF-$\kappa\beta$ activation and production of inflammatory cytokines (including IL-6 and TNF$\alpha$) compared to exosomes from normal breast cells through exosomal binding to the Toll-like receptor 2 (TLR2) [106]. Additionally, MDA-MB-231 cells transplanted into immunodeficient mice released exosomes that entered distant macrophages in lymph nodes, causing increases in IL-6 expression [106]. This showed breast cancer exosomes can alter macrophage phenotypes to cause an increase in metastasis. In another study, MCF-7-derived EVs transferred Wnt 5a mRNA, which plays an important role in tumor invasion, to macrophages, where it was translated into Wnt 5a protein [78]. This Wnt 5a protein in macrophages was then packaged into EVs and secreted from cells. The macrophage EVs containing Wnt 5a subsequently caused an increase in MCF-7 cell invasion [78]. Therefore, breast cancer EVs can transfer mRNA to macrophages, which can, in turn, translate and package cancerous proteins into vesicles, which are then subsequently secreted into the tumor microenvironment to enhance the invasive capacity of surrounding cancer cells in a paracrine manner.

## 9. Conclusions

There have been many proteins identified within breast cancer EVs that give insight into the nature and severity of the disease. These could serve as possible diagnostic markers to be used in conjunction with current analytical techniques. Both exosomes and MVs are key players in the progression of cancer and elicit a multitude of cellular responses within their target cells, at both the mRNA and protein levels. There are multiple mechanisms by which cancer cells avoid immune system recognition through EVs, such as secretion of immunosuppressive proteins, inhibition of NK cell proliferation, or a decrease in $CD8^+$ T-cell cytotoxicity. Some targeted breast cancer therapies have been investigated employing exosomes as a vehicle for drug delivery, but due to the complex nature of EV-cell interactions, additional studies need to be performed to achieve a therapeutic strategy with a favorable therapeutic index using an exosome approach. Tumors have the unique ability to support their progression by using MVs and exosomes to deliver procancerous transcripts and proteins, to both other cancer cells and nontransformed cells. EVs are key players within the cancer niche and are able to thrive in the acidic, hypoxic environments common to tumors in order to confer prometastatic phenotypes such as inflammation, migration, and invasion. While there have been many advances in recent years, future investigations involving EVs and their contribution to tumor growth are crucial to the continued understanding of cancer development.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# The Emerging Role of Extracellular Vesicle-Mediated Drug Resistance in Cancers: Implications in Advanced Prostate Cancer

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Emerging evidence has shown that the extracellular vesicles (EVs) regulate various biological processes and can control cell proliferation and survival, as well as being involved in normal cell development and diseases such as cancers. In cancer treatment, development of acquired drug resistance phenotype is a serious issue. Recently it has been shown that the presence of multidrug resistance proteins such as Pgp-1 and enrichment of the lipid ceramide in EVs could have a role in mediating drug resistance. EVs could also mediate multidrug resistance through uptake of drugs in vesicles and thus limit the bioavailability of drugs to treat cancer cells. In this review, we discussed the emerging evidence of the role EVs play in mediating drug resistance in cancers and in particular the role of EVs mediating drug resistance in advanced prostate cancer. The role of EV-associated multidrug resistance proteins, miRNA, mRNA, and lipid as well as the potential interaction(s) among these factors was probed. Lastly, we provide an overview of the current available treatments for advanced prostate cancer, considering where EVs may mediate the development of resistance against these drugs.

## 1. Introduction

Extracellular vesicles (EVs) are vesicles secreted by cells [1, 2], they are involved in mediating communication between cells by transferring signaling molecules, initiating a variety of cellular processes [2, 3]. The role of EVs in normal cellular growth and development has been reported as well as in the context of disease progression and cancer metastasis [4–6].

Exosomes and ectosomes are subclasses of secreted EVs; they have both been extensively characterized and shown to be functionally active in a number of studies [7–9]. The exosomes are nanosized vesicles, formed through intracellular budding at the multivesicular bodies (MVB). The biogenesis of exosomes is mediated by the action of Endosomal Sorting Complex Required for Transport (ESCRT) or by the lipid ceramide ([1, 2, 10], Figure 1). The MVB is an organelle involved in trafficking of vesicles from late endosomes to the plasma membrane, to mediate secretion [2, 11]. As such, exosomes contain MVB-associated proteins as well as RNAs encapsulated in a lipid bilayer with a specific composition of lipids, rich in sphingomyelin, cholesterol, and glycophospholipid [3, 12–14]. The ectosomes or microvesicles are vesicles bud from plasma membrane with a diameter up to 1 $\mu$m. The ectosomes also have a specific lipid composition as they are enriched in phosphatidylserine. The exposure of phosphatidylserine on the cell surface is a characteristic of ectosome secretion [15]. Ectosome biogenesis is triggered by plasma membrane activation including intracellular calcium influx, mediated by ARF-6 and interactions between cytoskeletal resident proteins actin and myosin [1, 9, 16]. Others such as oncosomes, prostasomes, exosome-like vesicles, and viruslike vesicles are also members of EVs [2, 17]. The oncosomes are newly characterised EVs and are large vesicles (~0.8 $\mu$m) which bud at the plasma membrane by "amoeboid" migratory cancer cells [18].

Understanding EV biogenesis has helped to differentiate exosomes and ectosomes; however classical ultracentrifugation and density gradient methodologies could not purely

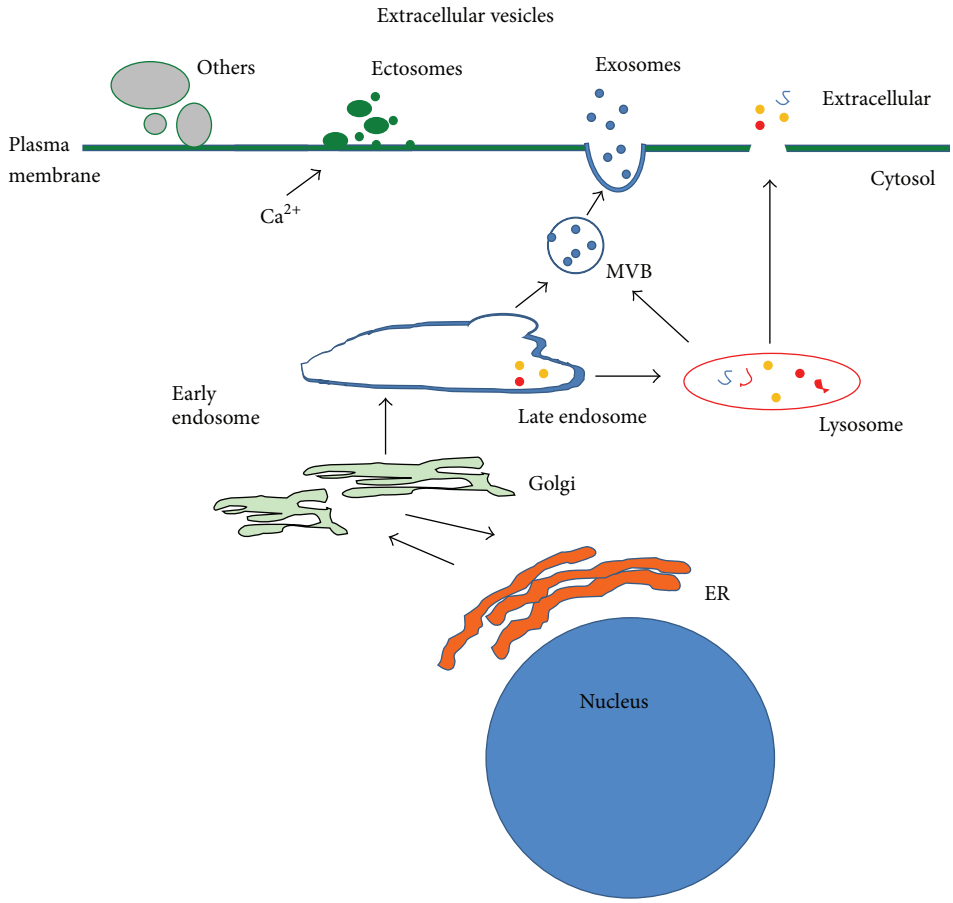


Figure 1: Schematic representative of ectosome and exosome secretion. Ectosomes or microvesicles are defined as extracellular vesicles which form through budding from plasma membrane. The exosomes are formed intracellularly, as proteins destined for exosome secretion are sorted in endosomes and packaged for exosome secretion through MVB pathways. Cross talk between MVB and endosomes has been reported as lysosomal markers such as LAMPs are found to be coisolated in secreted exosomes. Cells also secrete other types of vesicles such as oncosomes, prostasomes, and viral-like particles.

separate the subclasses of EVs, in particular to separate exosomes from other members of the EVs [2, 19]. Emerging evidence has shown the diversity of EVs; in particular, the previously characterized exosomes are not a homogenous population of vesicles [8]. As such, it was proposed to use the term EVs to include all secreted vesicles, even though classification based on biogenesis is still used in various publications [1, 2, 20].

Studies have explored EVs as potential biomarkers for various diseases, as the protein and RNA content of EVs has been shown to resemble the cell of origin [21, 22]. EV-derived protein glypican-1 has been shown to specifically indicate the presence of pancreatic cancer, indicating the real potential of EVs as a cancer biomarker [23]. Recently, it is becoming more accepted that EV secretion is not always constitutive as originally thought but is likely to be a cellular response toward changes in their environment. Exposure of cells to an effector can alter the EV content and their secretion process. For example, changes in cellular pH levels and exposure of cells to hypoxia growth conditions can alter the EV secretion [24, 25].

EVs have also been looked at as natural delivery agents for several treatments. By exploiting the "natural" path for vesicle endocytosis, EVs can bypass the immune system with no or low cytotoxic effects [26, 27]. This has been tested in melanoma cancer, as treatment of cancer with EVs containing the MAGE3 peptide has shown promising results, with no grade II toxicity [28]. EVs have been reported to internalize into cells through clathrin mediated endocytosis and micropinocytosis in PC12 cells [29] and in KRas expressing MiaPaCa-2 cells [30]. The cytoskeleton seems to play a role in this process [31]. However, due to the heterogenous nature of EVs, other mechanisms such as caveolin-dependent and caveolin-independent endocytosis and lipid raft mediated endocytosis were also observed [32–34]. As a shuttle mechanism between cells, EVs have been demonstrated to transfer prion and tau proteins as well as prion RNA [35, 36]. The transmission of prion protein (PrP) shows the role EVs play in the mechanism of transmissible neurodegenerative disorders [37].

In this review, we will discuss and assess the recent developments in the reported role of EVs in mediating drug

resistance in cancer. We then discuss the potential role of how EVs could be involved in mediating the progression of prostate cancer to a more advanced, castrate resistant form of the disease. It is beyond the scope of this review to discuss in detail the many mechanisms of drug resistance; in-depth reviews on this topic have been published and are referred to in this paper.

## 2. The Emerging Role of EVs in Mediating Drug Resistance in Cancers

Acquired drug resistance is a major obstacle in cancer therapy. Drugs can be transported into target cells by diffusion, transport (by the action of transporters), or endocytosis (such as immunotoxins) [38]. Drug resistance accounts for cases of treatment failure by altering the absorption, metabolism, and/or efflux of drugs from the target cells. Drugs can also induce mutations of the targets and inhibit pathways for cellular apoptosis, leading to resistance. The development of new drugs with increased sensitivity and reducing patients' side effects toward treatment, while effective, can unfortunately induce the development of new pathways involved in drug resistance.

Cancer cells are capable of developing multidrug resistance (MDR). The acquired resistance toward a particular drug could lead to resistance toward other drugs with different structures and targets. Cancer cells do not respond equally towards drugs due to their distinctive genetic make-up and the expression of various oncogenes or tumor suppressor genes. Alteration of the cell cycle and checkpoints as well as expression of drug efflux pumps can contribute toward development of MDR [38, 39]. Changes in genetic or epigenetic factors and alteration in the interaction between host and tumor microenvironment also contribute toward drugs resistance [38, 39]. Tumor microenvironment can create a physical barrier, slowing the absorption of drugs by target cells and reducing the impact of oxygen radicals [40], thus reducing the effect of drugs in the body. In prostate cancer, chemotherapy drugs can prompt the secretion of WNT16B by the surrounding fibroblast cells, which then activates cellular survival Wnt pathways in prostate cancer cells, PC3 [41]. Furthermore, increasing evidence has shown that while drugs can cause apoptosis in the majority of a cell population, a small percentage of cells can survive [42, 43]. These cells, known as Cancer Stem Cells (CSC), express cell survival pathways BCL2/NF-$\kappa$B; they are able to self-renew and have pluripotent capacity [44–46]. The ability of CSC to self-renew is thought to drive its malignant phenotype and could be responsible for progression of cancer into a more advanced form of the disease.

Apart from their specific cell surface markers, CSC also express drug efflux pumps, the ABC drug transporters (ATP-binding cassette or multidrug resistance (MDR) protein) [47–49]. The characteristic expression level of ABC transporters in tumors has proposed the use of these proteins as biomarkers [50]. The ABC transporter protein superfamily is generally found at the plasma membrane and is recently shown to be present in EVs [51, 52]. Forty-eight members of ABC superfamily are found in humans [53, 54] including P-gp and multidrug resistance-associated protein (MRP) protein family. The ABC transporters maintain low intracellular drug concentrations and allow CSC to survive under therapy. It is hypothesized that MDR cell lines entrap drug molecules from their cytosol into acidic vesicles [55].

The multidrug resistance-1 (MDR1 or ABCB1) is the most characterized member of the ABC superfamily, also sometimes referred to as P-gp (P-glycoprotein). P-gp is able to transport a range of molecules, including amphipathic drug compounds, chemotherapy agents (such as doxorubicin), lipid, steroids, and peptides. P-gp activity is highly sensitive to its lipid environment [56]. The expressions of P-gp in cancers vary. For example, colon and kidney cancers have been shown to express high levels of P-gp, while ovary, breast, melanomas, lymphomas, lung, and multiple myelomas cancers express low levels of P-gp, even though P-gp expression elevates upon acquired drug resistance. Various strategies to overcome MDR by inhibiting the ABC transporters have been tested in phase III clinical trials with minimal success [57]; this may indicate that additional pathways are involved in the development of acquired MDR.

The members of ABC transporter family play a role in maintaining cellular homeostasis, by maintaining cellular lipid bilayers and transporting fatty acids and sterols in the body [58]. Chen et al. have reported that ABC transporters play a role in sequestration of drugs into intracellular organelles, as shown by accumulation of cisplatin in melanosomes [59], limiting the efficacy of drugs. It has been shown that MDR can also be mediated by changes in the membrane lipid ceramide [60]. The presence of P-gp has allowed the conversion of ceramide to sphingolipids, a major constituent of phospholipid membrane [61]. P-gp may be involved in the encapsulation of drugs into vesicles, which would then be exocytosed by cells [62]. P-gp has recently been shown to function through its association with F-actin and p-ERM [63].

Another study in pancreatic cancer has strengthened the benefit of targeting exosome pathways. In a pancreatic cancer cell model, GAIP interacting protein C terminus (GIPC, a regulator of G protein signaling) has been shown to modulate the exosome secretion and content. Interestingly, in the GIPC-depleted cells, the secreted exosomes contain high expressions of ABCG2, a member of the ABC transporter protein superfamily [64].

The presence of ABC transporters in exosomes has also been shown in breast and prostate cancers, where chemotherapy drug, docetaxel, can increase the presence of ABC transporters in secreted exosomes [51, 52, 65]. Docetaxel, a microtubule-targeting drug, decreases glucosylceramide synthase and sphingosine kinase-1 and increases ceramide synthase genes [66], confirming the potential to combine therapies by targeting ceramide and ABC transporters in a clinical setting. Cotargeting EV pathways would then be a promising avenue to overcome MDR in cancers, even though the pathways in which EV-derived ABC transporters can mediate MDR are relatively unexplored. Other molecules shown to mediate MDR, such as DeltaNp73 in colon cancer [67], have also been investigated for their role in mediating MDR through EVs such as exosomes.

The EV member ectosomes have also been shown as regulators for MDR. A study using ectosomes secreted by the adriamycin-resistant breast cancer cell line, MCF-7, can transfer $Ca^{2+}$-permeable channels, TrpC5, to endothelial cells. The TrpC5 then stimulates the expression of P-gp through activation of the transcription factor NFATc3 (nuclear factor of activated T cells isoform c3), mediating the development of MDR in endothelial HMEC cells [68].

Apart from the presence of MDR-associated proteins, EVs can be involved in mediating resistance for drugs by sequestration of drugs in vesicles. It was reported in cisplatin-resistant melanoma cells that cisplatin is found in secreted exosomes [69]. Cisplatin is an alkylating agent that binds to DNA bases causing cross-links and breaks in DNA strands, interfering with DNA replication [70]. Encapsulation of cisplatin in exosomes is shown to be pH dependent. Accumulation of cisplatin has been shown in EVs when cells are grown in acidic extracellular pH rather than in neutral pH [69]. Exosome secretion and uptake are also enhanced in low pH [71], highlighting the importance of the extracellular environment in regulating the exosome secretion process.

## 3. The Emerging Role of EV Lipids in Mediating MDR

Recent reports have highlighted the role of EVs in regulating cellular lipid homeostasis. While exosome biogenesis is generally described as mediated by ESCRT protein complexes, Trajkovic et al. have shown that the lipid ceramide is able to regulate exosome secretion independently from the function of the ESCRT machinery [10]. Interestingly, ceramide, which is enriched in exosomes, is also known to mediate MDR [10]. The presence of ceramide in exosomes initially appears to be a downstream action of ceramide biosynthesis, as inhibition of neutral sphingomyelinases, enzymes which hydrolyse sphingomyelin to ceramide, can inhibit exosome secretion [10]. However, further studies in astrocytes have shown that ceramide in return can alter the exosome secretion, as treatment with extracellular ceramide stimulated exosome secretion in neutral sphingomyelinase 2 (nSMase2) deficient astrocytes cells. These cells do not naturally secrete exosomes; however, addition of extracellular C18 ceramide can stimulate exosome secretion in these cells [72].

Ceramide is a bioactive lipid; it has multiple signaling roles in endothelial cells, macrophages, and fibroblasts [73]. Ceramide accumulation can be mediated by various stimuli such as radiation [74], TNF-alpha [75], chemotherapy drugs [66], and natural anticancer agents (such as curcumin and capsaicin [76, 77]). Ceramide is capable of inducing apoptosis; cotreatment using P-gp antagonist and ceramides increases the rate of cellular death [61]. This process is mediated by alteration of mitochondrial membrane permeability through oligomerization of ceramide channels and reduction of prosurvival Bcl-2 proteins in mitochondria. The disruption of mitochondrial membrane permeability leads to generation of reactive oxygen species (ROS), which lead to apoptosis through caspase activation and mitogen-activated protein kinases (MAPKs)-dependent and -independent pathways [78, 79].

The exogenous ceramide could also induce secretion of ABC transporters in exosomes. For example, in breast cancer, C6 ceramide-induced secretion of breast cancer resistance protein (BCRP/ABCG2-associated exosomes) and inhibition of nSMase2 restored the cellular BCRP. This study suggests that lipid biosynthesis plays a role in recruiting ABC transporters into exosomes. Treatment with nuclear receptor antagonists, farnesoid X receptor antagonist guggulsterone and retinoid X receptor agonist bexarotene, increased the concentration of intracellular ceramide and reduced the BCRP protein in MDA-MB-231 cells [80], suggesting the role of ceramide-enriched exosomes in mediating MDR through ABC transporters.

Interestingly, a recent study has demonstrated that ceramide may not be the only lipid which is important in this process. A study using Synthetic Exosome-Like Nanoparticles, where the ratio of ordered lipids (lipid raft) versus disordered lipids (phospholipids) was equal to 6.0 (SELN6.0), inhibited the Notch-1 pathway in pancreatic cancer MiaPaCa-2 cells. The activation of NF-$\kappa$B leads to expression and the secretion of SDF-1alpha, a chemokine, which then activates the Akt survival pathway [81]. These accumulative findings, however, suggest that maintaining lipid homeostasis is also crucial in overcoming MDR in cancer therapy and that EVs could be important players in this process.

## 4. EV-Derived RNA: An Upstream Regulator of Acquired MDR?

While the exosomal lipids and ABC transporters in EV have emerged as important players in acquired MDR, exosomal RNA has recently been looked at as a player in this process. A recent study has shown that exosomal RNA could mediate MDR through interaction between stroma and cancers. Boelens et al. have reported the role of noncoding transcripts and transposable elements in stroma-derived exosomes in stimulating the activation of receptor RIG-I, which, in turn, activates the transcription factor STAT1-dependent signaling and Notch-3 in breast cancer cells, and accentuates the therapy resistance in tumor-initiating cells [82]. The EV-derived RNA may mediate MDR by regulating expression levels of drug efflux transporters or enzymes involved in lipid homeostasis, which would then disrupt the cellular signaling between the tumor and its microenvironment.

Reports have shown that exosomes-derived miRNAs are involved in MDR. The miR-222 increases the cellular survival in a docetaxel resistant breast cancer cell line MCF-7 [83]. A paper has also reported that secreted miR-221/222 in exosomes could mediate transfer of tamoxifen resistance in MCF-7 cells through downregulation of P27 and ERalpha [84]. In an ovarian cancer model, miR-21-3p has been shown to induce cisplatin resistance in A2780 cells, by targeting NAV3 gene [85].

While these accumulative findings strengthen the role of EV-derived miRNA, a report has also shown association between EV-derived mRNA levels and cancer. The mRNA levels of O(6)-methylguanine DNA methyltransferase and APNG (alkylpurine-DNA-N-glycosylase) are found to be enriched in tumor exosomes obtained from patients' blood

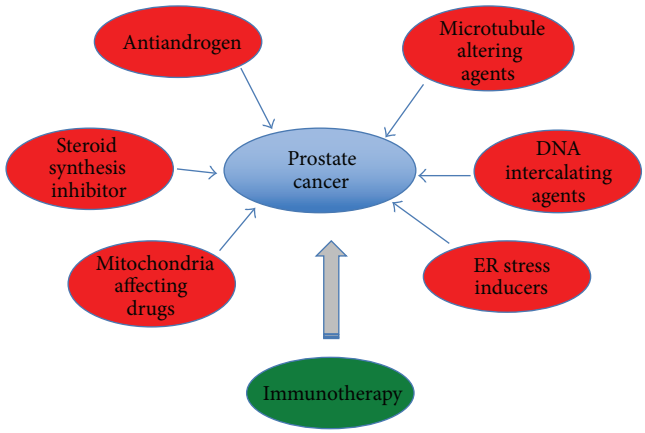


Figure 2: Current treatment options for advanced prostate cancer. Several classes of drugs (red circles) have been tested to target prostate cancer. These drugs inhibit various pathways known to regulate cancer proliferation and survival. A more personalized prostate cancer treatment has been developed through cancer immunotherapy (green circle), by activating patients' own cell mediated immunity against prostate cancer.

and correlate with the level found in tumor cells [86], indicating the potential role on EV-derived mRNA in mediating MDR.

## 5. Treatment Options for Advanced Prostate Cancer

Prostate cancer is the most prevalent cancer in men worldwide and the second highest cause of cancer-related death in men after lung cancer [87]. Male hormones, androgens, are required for normal cell and cancer cell growth and maintenance. The androgens bind to the androgen receptor (AR) and are transported into the nucleus to initiate DNA transcription mechanisms.

Removal of the prostate through surgery followed by radiation therapy and first-line androgen deprivation therapy for men with primary prostate cancer is suboptimal. Within two years, 25–40% of cases develop castrate resistant prostate cancer and continue to progress with metastatic disease [88]. Several classes of drugs/treatments have been developed to interfere with oncogenes/oncoproteins known to be involved in the progression of prostate cancer into a more advanced form of the disease (Figure 2; see also Table 1).

Targeting the AR has remained the main treatment for advanced prostate cancer. The AR is a steroid nuclear receptor; it is transcribed from the AR gene located on chromosome Xq11-12 [89, 90]. AR gene consists of eight exons, which encode four functional motifs: an amino-terminal domain, a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) [91–93]. The amino-terminal domain contains a transactivation domain, AF1, which is the primary transcriptional regulatory region. The LBD contains the secondary transcriptional regulatory region, AF2. The DBD is composed of two zinc fingers that are critical to DNA recognition and binding. The hinge domain of AR contains the nuclear localization signal which regulates the transactivation potential. The hinge domain is involved in intranuclear mobility of the AR and provides a site for binding of various androgen response elements as well as coactivators/corepressors [94].

Antiandrogens are commonly used in therapy to treat advanced prostate cancer. Androgen deprivation therapy (ADT) aims to limit the availability of androgens to bind to and activate AR, inhibiting the prostate cancer growth. ADT involves administering luteinizing-hormone-releasing hormone agonists or antagonists to disrupt the feedback loop within the hypothalamic gonadal axis, suppressing testosterone production by the testes. It has been reported that some prostate cancer cells are able to survive ADT and continue to maintain AR signaling [95–97]. AR antagonists, such as enzalutamide (MDV3100) or bicalutamide (Casodex), are designed to inhibit AR signaling by replacing the natural ligand DHT [98]. Bicalutamide (Casodex), enzalutamide predecessor, has shown some agonistic effect in cells which express high level of AR. Bicalutamide also increases AR recruitment to the enhancer region, thus increasing expression of AR regulated genes, such as PSA. Enzalutamide binds to AR with eightfold greater affinity than bicalutamide and only threefold less affinity than the natural ligand, DHT. MDV3100 also reduces the efficiency of AR translocation to the nucleus and impairs the binding of AR to the androgen response element and other factors that bind to AR [99]. In 2012, a new antiandrogen drug, ARN-509, with no observed agonistic effect was tested to treat castration-resistant prostate cancer (CRPC); it is currently being tested in phase II clinical trials [100, 101].

Docetaxel and its derivative, cabazitaxel, both belong to the taxane group and work by disrupting microtubule dynamics leading to mitotic arrest and apoptosis [103]. Cancer cells are usually rapidly dividing, requiring dynamic microtubule assembly during mitosis. Docetaxel stabilizes microtubules by binding to $\beta$-actin, thus inhibiting microtubule disassembly and mediating G2M arrest [122], and counteracts expression of oncogenes BCL-2 [104]. Prostate cancer is a relatively slow growing disease; thus, docetaxel acts by inhibiting AR nuclear translocation in androgen-dependent prostate cancer rather than stalling the cell cycle progression [105].

While treatment with docetaxel has been beneficial, 30% of CRPC patients who receive docetaxel therapy relapse [123]. Cancer cells can develop resistance to taxanes through mutations of the tubulin gene, expression of alternative tubulin isotypes, or drug efflux through multidrug resistant protein pumps [124]. Treatments combining docetaxel and Bcl-2 family inhibitors were tested in prostate cancer cell lines to overcome docetaxel resistance. ABT-263 and ABT-737 (Bcl-2, Bcl-xL, and Bcl-w inhibitors) enhanced the effect of docetaxel in inhibiting PC3 cell growth [125]. When given with prednisone, treatment with docetaxel every three weeks led to superior survival and improved rates of response in terms of pain, serum PSA level, and quality of life, as compared with mitoxantrone plus prednisone [126]. Cabazitaxel has recently replaced the mitochondria affecting drugs, such as mitoxantrone (discussed below), for prostate cancer patients who progress after docetaxel treatment. Cabazitaxel

Table 1: Classes of drugs used to treat advanced prostate cancer in the clinic.

| Classes | Drugs | Mechanism(s) | References |
|---|---|---|---|
| Antiandrogens | (i) Enzalutamide<br>(ii) Bicalutamide<br>(iii) EPI-506<br>(iv) ARN-509 | Inhibition on the activity of androgen receptor or its splice variant(s) in mediating DNA transcription | [95–102] |
| Microtubule altering agents | (i) Docetaxel<br>(ii) Cabazitaxel | Disruption of microtubule; inhibiting AR translocation to nucleus; counteracting expression of oncogenes BCL-2 | [103–105] |
| DNA intercalating agents | (i) Cisplatin<br>(ii) Satraplatin | Platinum analog drug; creating a DNA adduct to allow DNA translation; overriding mechanism of DNA repair | [106, 107]<br>[108, 109] |
| ER stress inducers | (i) Bortezomib<br>(ii) Estramustine | Inhibition of the 20S proteasome; modulating BCL-2 expression; depolymerizing cytoplasmic microtubules by binding to tubulin and tau protein | [104, 110–113] |
| Mitochondria affecting drugs | Mitoxantrone | Mitochondria affecting drugs; causing mitochondrial stress by depolarization of the mitochondrial membrane; inhibiting topoisomerase II-mediated DNA intercalation | [114–116] |
| Steroid synthesis inhibitor | (i) Abiraterone<br>(ii) Orteronel | Inhibiting *de novo* biosynthesis of androgens by targeting CYP17 in the androgen biosynthesis pathway; suppressing AR signaling in castrate resistant prostate cancer | [117–121] |

binds to different tubulin isotypes, human albumin, and lipoproteins and less efficiently to P-gp [127, 128].

Treatment combining ER stress inducers, bortezomib (Velcade), and docetaxel has been shown to benefit patients. Bortezomib is an inhibitor of the 20S proteasome, involved in ubiquitin-mediated protein degradation [110, 111]. Bortezomib modulates BCL-2 expression [104] but does not inhibit androgen mediated PSA mRNA expression. Bortezomib stimulates p53 translocation to the nucleus and enhances its DNA binding causing accumulation of p53-dependent transcripts in LNCaP pro5 cell line and leading to cell death [129].

Another ER stress inducer, estramustine (Emcyt), is a derivative of estrogen (estradiol) [130]. It has been shown to depolymerise cytoplasmic microtubules by binding to tubulin [112] and tau protein [113]. The microtubule and endoplasmic reticulum (ER) are interdependent, as prolonged disruption of microtubules will cause the ER to retract to the nucleus. This ER extension occurs at the same time as microtubule formation, by attaching to the growing end of microtubules and it is dependent on microtubule motor activity [131, 132].

Estramustine, used in combination with docetaxel, increases prostate cancer patients' median survival by two months in comparison with docetaxel alone [133]. In comparison with a combination of mitoxantrone and prednisone, the docetaxel and estramustine combination improves median survival by nearly two months for men with metastatic, androgen-independent prostate cancer [134]. To improve the synergistic effects, a combination of docetaxel, estramustine, and low-dose hydrocortisone has been trialed; unfortunately, 50% of men with CRPC acquired resistance toward treatments [135].

Satraplatin (JM-216) is an orally bioavailable platinum analog drug that has shown promise for treating advanced prostate cancer by mediating G2 M cell cycle arrest. Satraplatin is an analog of cisplatin and is effective in prostate cancer cells that are resistant to cisplatin [106, 107]. Satraplatin binds to DNA and creates a DNA adduct that is resistant to DNA nucleotide excision repair protein; it also evades DNA mismatch repair protein [108, 109].

Mitochondria affecting drugs, such as mitoxantrone (Novantrone), have also been tested in prostate cancer. Mitoxantrone blocks the cell cycling phase at S and G2 phase and is able to inhibit colony formation by prostate cancer cells *in vitro* [114]. Cells accumulate the drug within or near their mitochondria [115], increasing mitochondrial stress by depolarization of the mitochondrial membrane and causing release of cytochrome c into the cytosol, increased hydrogen peroxide production, activation of caspase cascades, and apoptosis [114]. Treatment with mitoxantrone also causes an increase in the expression of BCL-XI (a member of the BCL-2 family) and delays the onset of the p53 pathway. Both of these pathways are known to be involved in the survival of prostate cancer cells. BCL-2 is involved in mitochondrial membrane integrity and is involved in the Akt apoptosis pathway [136, 137]. Mitoxantrone also inhibits topoisomerase II-mediated DNA intercalation and generates free radicals that kill cancer cells [116].

Treatment by inhibiting *de novo* biosynthesis of androgens in CRPC has recently been investigated by targeting CYP17. CYP17 catalyzes two key steroid reactions involving 17alpha-hydroxylase and C(17,20)-lyase in the androgen biosynthesis pathway [117, 118]. The cytochrome P450 enzymes CYP11A1 and CYP17A1 are involved in intratumoral conversion of the weak adrenal androgens DHEA and androstenedione into the AR ligands testosterone and dihydrotestosterone [138]. CYP17A1 inhibitors, such as abiraterone acetate (Zytiga), are sought as a potential effective

therapy for castrate resistance prostate cancer [117, 119]. However, a study in castration-resistant VCaP tumor xenografts shows development of abiraterone resistance. Abiraterone resistant prostate cancer cells express a T877A mutation in the AR, such as that found in LNCaP cells, which is activated by other steroids through CYP11A1-dependent pregnenolone/progesterone synthesis [138]. In a controlled COU-AA-302 study, abiraterone plus prednisone significantly prolonged median overall survival in chemotherapy-naive men with metastatic CRPC. Another CYP17 inhibitor, orteronel (TAK-700) with higher specificity for 17,20-lyase than for 17$\alpha$-hydroxylase, has been developed to reduce abiraterone-induced toxicity. Orteronel is shown to reduce the expression of PSA (Prostate Specific Antigen), an AR regulated gene and current clinical biomarker for prostate cancer, in metastatic CRPC [120].

Both enzalutamide and abiraterone have been designed to further suppress AR signaling in CRPC. Resistance to enzalutamide and abiraterone is indicated by a return of activated AR signaling. This phenomenon may be mediated by AR splice variants (AR-Vs), in particular, variant 7 (AR-V7) [121]. The AR-V7 lacks the LBD, which is the target of enzalutamide and abiraterone. Interestingly, AR-V7 can remain constitutively active as a transcription factor [121]. Activation of mutant AR by eplerenone can be inhibited by antiandrogens (MDV3100, bicalutamide) or abiraterone. Treatment with abiraterone could overcome resistance caused by activation of AR by residual ligands or coadministered drugs [139]. Recently, a new drug, EPI-506, which targets the N-terminal of AR was announced to be tested in prostate cancer patients [102].

Personalized treatment by implementing cancer immunotherapy is being developed for prostate cancer. Sipuleucel-T (APC8015 or Provenge) is a novel cancer immunotherapy, which utilizes a recombinant fusion protein (PA2024) consisting of granulocyte macrophage colony-stimulating factor and prostatic acid phosphatase to stimulate the patient's T cells response [140]. Sipuleucel-T has shown a positive effect in extending survival by median 4.1 months in men with metastatic CRPC (IMPACT phase III trial data) [141], while combination with docetaxel does not seem to enhance the efficacy of this treatment [142].

Recently, a new phase 1/2 clinical trial has been approved to evaluate the combination of immunotherapy ADXS-PSA and pembrolizumab. The ADXS-PSA (ADXS31-142) is an investigational *Listeria monocytogenes*- (Lm-) LLO immunotherapy to reduce the immunosuppressive activity of myeloid-derived suppressor cells and regulatory T cells in the tumor microenvironment. Pembrolizumab (Keytruda) is a humanized monoclonal antibody that blocks the interaction between programmed death receptor-1 and its ligands. Combinations of both agents will be tested in metastatic CRPC patients [143].

## 6. Can EVs Mediate Drug Resistance in Prostate Cancer?

Drug resistance is a major problem in the treatment of many cancers, including prostate cancer. Various roles of EVs in prostate cancer have been reported; EVs can alter the changes of tumor fibroblasts to myofibroblasts, as well as promoting angiogenesis in the tumor microenvironment [144, 145]. Amoeboid prostate cancer can also secrete oncosomes upon treatment with growth factors, which are involved in cancer cell migration, invasion, and metastasis [17]. EVs have also been looked at as a potential source of biomarkers in prostate cancer [146, 147], while their role in mediating MDR in prostate cancer has just started to be investigated.

Normal prostate cells express BCRP (ABCG2) and MRP (ABCC1) [148, 149] but not P-gp (ABCB1) [149], whereas prostate cancer cells do express P-gp [150]. Corcoran et al. have shown that exosomes can mediate transfer of docetaxel resistance through P-gp transport via EVs in a DU145 cell line [51], even though a mechanism of how exosomal-derived P-gp could mediate drug resistance was not described.

In a non-EVs study, the contribution of P-gp in mediating drug resistance in prostate cancer was previously reported. In a study, prostate cancer cell lines were cotreated with doxorubicin (a gamma topoisomerase poison, which causes DNA damage) and drugs that are known to partially target the MDR in tumors (verapamil, cyclosporine A, and tamoxifen) [151]. Verapamil acts by inhibiting voltage-gated K+ channels, which then inhibit the proliferation of LNCaP cells [152]. The cotreatment resulted in a synergistic effect in PC-3 and DU-145 cells, but not LNCaP cells, indicating the role of P-gp in PC-3 and DU145 resistance. However, the author has also reported that low MDR-reversal rates could suggest that an alternative MDR-independent pathway may be responsible for triggering drug resistance in prostate cancer [151].

Lipid homeostasis seems to play a major role in drug resistance in other cancers as discussed above. Disruption of lipid homeostasis such as treatment with ceramide can also be utilized to target prostate cancer. Ceramide can enhance activation of the intrinsic apoptotic pathways and enhance cell death induced by TNF-alpha in LNCaP cells [153]. Treatment with bioactive ceramide also induces apoptosis in AR expressing LNCaP prostate cancer cells by causing nuclear fragmentation and activation of c-Jun N-terminal kinase (JNK), independent of PKC pathways [79]. In AR negative prostate cancer cells PC3, ceramide activates the protein phosphatase 2A (PP2A) and increased p27(kip1) protein levels via Akt-dependent and Akt-independent pathways [154], suggesting some overlaps in pathways influenced by lipid homeostasis. A natural agent, capsaicin, mediates cell death through sphingomyelin hydrolysis by nSMase, to generate ceramide via the ERK and JNK pathways, resulting in cellular apoptosis in prostate cancer [155]. Alteration of membrane properties could lower the level of resistance [156], an area where EVs could contribute.

In a study on docetaxel resistance in prostate cancer, miR-34a shows clinical relevance. miR-34A could alter cellular response to docetaxel in prostate cancer cells through BCL-2, which then target SNCA and SCL7A5 [157]. Treatment of DU145 cells with fludarabine increase the secretion of exosome-associated miRNA. While the exosome-associated miRNA was not identified, fludarabine treatment is shown to retain the miR-485-3p in parental cells, which could play a role in resistance through regulation of the transcriptional repressor nuclear factor-Y which regulates transcription of

topoisomerase IIalpha, multidrug resistance gene 1, and cyclin B2 prosurvival genes [158].

## 7. Conclusion

Prostate cancer is an extremely heterogeneous disease; the underlying mechanisms for progression to CRPC are complex [138, 159]. Exposure to drugs will prompt tumor cells to secrete proteins to the external environment, where these proteins mediate interaction with stroma, macrophages, dendritic cells, and others. Understanding factors that contribute toward MDR will help patients and clinicians to decide on the most suitable treatment regime for an individual patient, increasing the efficacy of drugs, while reducing unnecessary side effects.

The release of EVs is exacerbated in tumors leading to their increased presence in bodily fluids of cancer patients [160–162]. Tumor-derived EVs express an array of antigens that reflect the originating tumor cells, small RNAs, and specific composition of lipid bilayers. EVs secreted by a tumor can trigger an immune response and cellular differentiation in the tumor microenvironment and they have also been shown to support tumor escape and growth [144, 145, 163]. The presence of P-gp and ceramide in EVs indicates the potential role of EV in mediating MDR, even though the mechanisms by which P-gp and ceramide-derived EVs can mediate MDR in prostate cancer are not clear. The role of EVs in mediating MDR could be limited to drugs that alter the P-gp expression or functions or the lipid ceramide. Understanding the effect on EV pathways of various drugs used to target prostate cancer will help us to understand the role of EVs in mediating MDR in prostate cancer.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Research Article*

# High LIN28A Expressing Ovarian Cancer Cells Secrete Exosomes That Induce Invasion and Migration in HEK293 Cells

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Epithelial ovarian cancer is the most aggressive and deadly form of ovarian cancer and is the most lethal gynecological malignancy worldwide; therefore, efforts to elucidate the molecular factors that lead to epithelial ovarian cancer are essential to better understand this disease. Recent studies reveal that tumor cells release cell-secreted vesicles called exosomes and these exosomes can transfer RNAs and miRNAs to distant sites, leading to cell transformation and tumor development. The RNA-binding protein LIN28 is a known marker of stem cells and when expressed in cancer, it is associated with poor tumor outcome. We hypothesized that high LIN28 expressing ovarian cancer cells secrete exosomes that can be taken up by nontumor cells and cause changes in gene expression and cell behavior associated with tumor development. IGROV1 cells were found to contain high LIN28A and secrete exosomes that were taken up by HEK293 cells. Moreover, exposure to these IGROV1 secreted exosomes led to significant increases in genes involved in Epithelial-to-Mesenchymal Transition (EMT), induced HEK293 cell invasion and migration. These changes were not observed with exosomes secreted by OV420 cells, which contain no detectable amounts of LIN28A or LIN28B. No evidence was found of LIN28A transfer from IGROV1 exosomes to HEK293 cells.

## 1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy worldwide and is often detected in late stages where metastasis has occurred [1]. In ovarian cancer, tumor cells release small cell-secreted vesicles called exosomes [2–4]. Exosomes are endosome-derived vesicles (30–100 nm) that contain bioactive materials and are released by cells into the bloodstream [5], as well as urine [6], saliva [7] plasma [8], epididymal fluid [9], amniotic fluid [10], follicular fluid [11], malignant and pleural effusions of ascites [12], bronchoalveolar lavage fluid [13], synovial fluid [14], and breast milk [15]. Exosomes are also known to affect gene expression as Valadi and colleagues demonstrated RNAs in mast cell exosomes could be delivered to human and mouse mast cells leading to new protein production in recipient cells [2]. Moreover, tumor cell-secreted exosomes can induce increased cell proliferation and invasion in target cells [4, 16–18].

MicroRNAs (miRNAs) are abundantly expressed in human cancers [19, 20]. There are unique miRNA signatures representative of human cancers [21], including ovarian cancer [22] implying miRNAs are key regulators of cellular and molecular function contributing to metastatic disease. miRNAs are nonprotein coding RNAs that function as posttranslational regulators by binding to the 3′UTR of target mRNAs [23]. They are evolutionarily conserved and

approximately 19–22 nucleotides in length. Upon binding to the 3′UTRs of target mRNAs, translational inhibition occurs in the form of mRNA target cleavage or translational repression [24]. The dynamic roles miRNAs have on mRNA target genes can alter signaling pathways associated with the hallmarks of cancer [25]. Furthermore, miRNAs also are present in exosomes and can be delivered from one cell to another [2].

LIN28 is a RNA-binding protein that regulates both mRNA and miRNAs. There are two paralogs of LIN28, LIN28A, and LIN28B, both containing a cold shock domain (CSD) and CCHC-zinc finger RNA-binding domain. They regulate *let-7* miRNA levels by CSD binding to the NGNGAYNNN (N = any base and Y = pyrimidine) sequence on the terminal loop of *let-7* and CCHC-zinc finger binding to the GGAG sequence on the same terminal loop [26]. The linker between the CSD and the CCHC-zinc finger allows for binding of all twelve *let-7* miRNA family members. Studies have focused on elucidating the role of LIN28 and *let-7s* miRNAs in cancer cells [27]; high LIN28A levels are associated with advanced human malignancies [28] and LIN28A is often expressed in ovarian tumors [29, 30]. Considering the positive correlation between LIN28 level and tumor aggressiveness, as well as the observation that tumors are known to secrete exosomes that can induce proliferation, invasion, and/or migration, it is possible that high LIN28 level in cells positively regulates secretion of exosomes with oncogenic potential.

The goal of this study was to test the hypothesis that exosomes from ovarian cancer cells that contain high LIN28 can be taken up by HEK293 cells and lead to changes in gene expression and cell phenotype, whereas exosomes from ovarian cancer cells with low LIN28 levels cannot. To this end we used IGROV1 and OV420 cells; IGROV1 cells can induce peritoneal carcinomatosis in SCID mice, leading to rapid tumor formation and cell growth [31], while OV420 cells do not form tumors in SCID mice [32]. (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/701390).

## 2. Material and Methods

*2.1. Cell Lines and Culture Conditions.* IGROV1 and OV420 cell lines were cultured in Roswell Park Memorial Institute (RPMI 1640) medium with L-glutamine 1X (Cellgro, 10-040-CV), supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, F-0500-D) and 1% antibiotic-antimycotic solution (Cellgro, 30-0004-Cl). HEK293 (human embryonic kidney) cells were kindly provided by Dr. Russell Anthony (Colorado State University) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cellgro, 10-017-CV) supplemented with 10% fetal bovine serum (Atlas Biologicals, F-0500-D) and 1% antibiotic-antimycotic solution (Cellgro, 30-0004-Cl). Cells were cultured in a standard humidified incubator at 37°C in a 5% $CO_2$ atmosphere.

*2.2. Lentiviral Transductions for Exosome Tracking.* IGROV1 cells line were stably transduced with pCT-CD63-GFP Cytotracers (System Biosciences, CYTO120-VA-1) to create an IGROV1-CD63-GFP cell line used for exosome tracking as per manufacturer's instructions. Briefly, $1 \times 10^3$ IGROV1 cells were seeded onto 24-well plates 24 hours before transfection to allow adhesion and were grown to approximately 60–80% confluency. The cells were transduced with pCT-CD63-GFP at a multiplicity of infection (MOI) of 2000 virus particles per cell with addition of Polybrene (Millipore, TR-1003-G) at a final concentration of 2 $\mu$g/mL to increase efficiency of transfection. Cells were incubated for 24 hours and 1-day later fresh medium was added without puromycin for another 24 hours. Infected IGROV1 cells were selected by adding puromycin at a final concentration of 4 $\mu$g/mL.

*2.3. Exosome Isolation.* Complete RPMI 1640 and DMEM medium was ultracentrifuged (Beckman L8-80) at 100,000 g for 16 hours at 4°C to pellet secreted membrane vesicles less than 1000 nm to obtain vesicle-depleted medium. Sterile filtration was performed on vesicle-depleted medium using a 0.2 $\mu$m PES membrane (Thermo Scientific, 565-0020) and stored at 4°C until exosome collection.

For exosome isolation, $1 \times 10^6$ cell were seeded onto four 10 cm cell plates (Celltreat, 229690) and cultured in either RPMI 1640 vesicle-depleted medium or DMEM vesicle-depleted medium for three days. Supernatant was collected and centrifuged at 3,000 g for 15 minutes at 4°C to remove cells and cell debris. Supernatant and ExoQuick-TC Exosome precipitation solution (System Biosciences, EXOTC50A-1) were combined in a 5 : 1 dilution (resp.) and exosomes were collected as per manufacturer's instructions. Briefly, supernatant/ExoQuick-TC biofluid was centrifuged at 1,500 g for 30 minutes at 4°C; biofluid was aspirated and recentrifuged at 1,500 g for 5 minutes at 4°C to remove excess biofluid without disturbance of exosome pellet. Four exosome pellets were combined and either resuspended in 200 $\mu$L of TRIzol LS Reagent (Life Technologies, 10296028) for qPCR or 300 $\mu$L of M-PER (Thermo Scientific, 78501) supplemented with Halt proteinase inhibitor cocktail (Thermo Scientific, 1 : 100, 87786) and phenylmethanesulfonyl fluoride solution (Boston BioProducts, 1 : 100, PI-120). Exosomes were stored at −80°C until RNA or protein was isolated.

*2.4. RNA Isolation.* Total RNA was extracted from confluent cells lysed in 300 $\mu$L of MirVana lysis binding buffer and 30 $\mu$L of miRNA homogenate additive. RNA was isolated per manufacturer's instructions using MirVana miRNA isolation kit (Ambion, AM1561) and resuspended in 30 $\mu$L of RNase/DNase-free water.

Total RNA was isolated from exosome isolates using TRIzol LS Reagent (Life Technologies, 10296-028). RNA isolation was completed per manufacturer's instructions with minor modifications. Briefly, exosomes were lysed in 200 $\mu$L of TRIzol LS Reagent (Life Technologies, 10296-028) and homogenized for 5 minutes. Phase separation was conducted by adding 128 $\mu$L of chloroform to the RNA/DNA/protein phase and homogenization for 5 minutes. Samples were centrifuged for 15 minutes at 4°C to separate the RNA, DNA, and protein phases. The RNA aqueous phase was added to 400 $\mu$L of cold 100% isopropanol and stored at −80°C overnight for RNA precipitation. RNA was pelleted via

centrifugation and was washed twice with cold 75% ethanol and then resuspended in 10 $\mu$L of RNase/DNase-free water.

Once total RNA was isolated from both cells and exosomes, DNase-free DNase Treatment and Removal kit (Ambion, AM1906) was used on all samples to eliminate genomic DNA contamination. RNA quality and concentration were assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Total RNA absorbance of 260/280 was measured and samples with RNA purity between 1.7 and 2.2 were used for experiments. Samples were stored at −80°C until qPCR was performed.

*2.5. Reverse Transcriptase PCR (RT-PCR).* RT-PCR was performed from total RNA (see above) where 1 $\mu$g of RNA was used for cells and 400 ng of RNA was used for exosomes utilizing qScript cDNA Supermix (Quanta Biosciences, 95047-100) as per manufacturer's instructions. Once cDNA was made, the GoTaq DNA Polymerase kit (Promega, M3005) was used with either LIN28A or LIN28B primers. PCR cycling parameters were an initial denaturation step for 5 minutes at 94°C followed by 40 cycles of 15 seconds at 94°C of denaturation, 30 seconds at 60°C for annealing and 15 seconds at 72°C for elongation, and a final elongation step of 3 minutes at 72°C. Amplicons were electrophoresed on a 2% agarose gel at 190 V for 30 minutes and imaged using the ChemiDoc MP System with the Image Lab 4.1 software. Experiments were carried out using three independent biological replicates and the experiments were repeated. The primers used were designed to span introns to ensure no DNA contamination was present. LIN28A forward primer sequence was 5′-GGCATCTGTAAGTGGTTGAACG-3′ and the reverse primer sequence was 5′-CCTTCCATGTGCAGCTTACTCT-3′ (118 bp size) and LIN28B forward primer sequence was 5′-TAGGAAGTGAAAGAAGACCCAA-3′ and the reverse primer sequence was 5′-ATGATGCTCTGACAGTAATGG-3′ (151 bp size).

*2.6. Quantitative Real-Time PCR (qPCR).* Taqman qPCR was performed on cDNA generated from total RNA (see above). cDNA was diluted to a final concentration of 10 ng/rxn for cells and 20 ng/rxn for exosomes and combined with 2x Ssofast Probe Supermix (Bio-Rad, 172-5230) and 20x Taqman Assay Mix (Applied Biosystems). The 20x Taqman Assay Mix Probes (Applied Biosystems) used for this study were as follows: *LIN28A* (Hs00702808_s1), *LIN28B* (Hs01013729_m1), *GAPDH* (H99999905_m1), *MRPS15* (Hs00229834_m1), and *TBP* (Hs00427620_m1). qPCR was performed using the LightCycler 480 Real-Time PCR System (Roche Applied Science). PCR cycling parameters were an initial denaturation step for 30 seconds at 95°C followed by 45 cycles of repeating denaturing at 15 seconds at 95°C and annealing for 30 seconds at 60°C with a final cooling cycle for 5 minutes at 37°C. Data were normalized using the geometric mean of *GAPDH*, *MRPS15*, and *TBP* and relative levels were calculated using the comparative Cp method to obtain $2^{-\Delta Ct}$ relative expression values. Each sample was run in duplicate with reverse transcriptase negative controls, nontemplate controls, and experiments were repeated. Statistical analysis was determined by analysis of variance (ANOVA) followed by Tukey pairwise comparison (Minitab 17). $p$ values less than 0.05 were considered statistically significant.

Table 1: Human miRNA sequences used for qPCR experiments. These sequences were obtained from miRBase program.

| miRNA | Sequence (5′-3′) |
|---|---|
| *hsa-let-7a* | UGAGGUAGUAGGUUGUAUAGUU |
| *hsa-let-7b* | UGAGGUAGUAGGUUGUGUGGUU |
| *hsa-let-7c* | UGAGGUAGUAGGUUGUAUGGUU |
| *hsa-let-7d* | AGAGGUAGUAGGUUGCAUAGUU |
| *hsa-let-7e* | UGAGGUAGGAGGUUGUAUAGUU |
| *hsa-let-7f* | UGAGGUAGUAGAUUGUAUAGUU |
| *hsa-let-7g* | UGAGGUAGUAGUUUGUACAGUU |
| *hsa-let-7i* | UGAGGUAGUAGUUUGUGCUGUU |
| *hsa-let-7a-3p* | CUAUACAAUCUACUGUCUUUC |
| *hsa-let-7-f1-3p* | CUAUACAAUCUAUUGCCUUCCC |
| *hsa-miR-9* | UCUUUGGUUAUCUAGCUGUAUGA |
| *hsa-miR-17* | CAAAGUGCUUACAGUGCAGGUAG |
| *hsa-miR-18a* | UAAGGUGCAUCUAGUGCAGAUAG |
| *hsa-miR-19a* | UGUGCAAAUCUAUGCAAAACUGA |
| *hsa-miR-19b* | UGUGCAAAUCCAUGCAAAACUGA |
| *hsa-miR-20a* | UAAAGUGCUUAUAGUGCAGGUAG |
| *hsa-miR-22* | AAGCUGCCAGUUGAAGAACUGU |
| *hsa-miR-30a* | UGUAAACAUCCUCGACUGGAAG |
| *hsa-miR-30b* | UGUAAACAUCCUACACUCAGCU |
| *hsa-miR-30c* | UGUAAACAUCCUACACUCUCAGC |
| *hsa-miR-30d* | UGUAAACAUCCCCGACUGGAAG |
| *hsa-miR-30e* | UGUAAACAUCCUUGACUGGAAG |
| *hsa-miR-31* | AGGCAAGAUGCUGGCAUAGCU |
| *hsa-miR-92a* | UAUUGCACUUGUCCCGGCCUGU |
| *hsa-miR-200a* | UAACACUGUCUGGUAACGAUGU |
| *hsa-miR-200b* | UAAUACUGCCUGGUAAUGAUGA |
| *hsa-miR-200c* | UAAUACUGCCGGGUAAUGAUGGA |
| *hsa-miR-125a-3p* | ACAGGUGAGGUUCUUGGGAGCC |
| *hsa-miR-125a-5p* | UCCCUGAGACCCUUUAACCUGUGA |
| *hsa-miR-125b* | UCCCUGAGACCCUAACUUGUGA |
| *Htman t6 snRNA (U6)* | CGCAAGGAUGACACGCAAAUUC |

miRNA qPCR was performed using cDNA diluted to a final concentration of 1.5 ng/rxn and combined with 2x QuantiTect SYBR green PCR master mix, 10x miScript Universal Primer (miScript SYBR Green PCR Kit, Qiagen, 218075). The miRNA PCR primers used for this study are listed in Table 1. qPCR was performed using the LightCycler 480 Real-Time PCR System (Roche Applied Science). The cycling conditions were an initial denaturation step for 15 minutes at 95°C followed by 45 cycles of repeating denaturing at 15 seconds at 95°C, annealing for 30 seconds at 55°C, elongation for 30 seconds at 72°C. Melt curves were generated to ensure single miRNA amplicons using the following cycling parameters: 95°C for 5 seconds and 65°C for 1 minute. Data were normalized using snRNA (U6), and relative levels were calculated using the comparative Cp method. Each sample

was run in duplicate with reverse transcriptase negative controls, nontemplate controls, and experiments were repeated. Statistical analysis was determined by ANOVA followed by Tukey pairwise comparison (Minitab 17). $p$ values less than 0.05 were considered statistically significant. A two-sided unpaired Student's $t$-test was performed on miRNA levels after exosome transfer, with significance at $p < 0.05$.

Human Epithelial-to-Mesenchymal Transition (EMT) $RT^2$ Profiler PCR array (SABiosciences, PAHS-090G-4) was used to examine the relative level of 84 genes related to EMT. Total RNA from 4 biological replicates of confluent HEK293 cells (control) and HEK293 cells exposed to IGROV1 cell-secreted exosomes (treatment) was isolated (see above). cDNA (1 ug) was made, diluted and qPCR was performed per manufacturer's instructions using the LightCycler 480 Real-Time PCR System (Roche Applied Science). The cycling parameters include an initial denaturation step of 10 minutes at 95°C followed by 45 cycles of repeating denaturing at 15 seconds at 95°C and annealing for 1 minute at 60°C with a final cooling step of 5 minutes at 37°C. Biosciences software associated with this Profiling Array was used to determine significant changes in expression level, and EMT-related genes with a fold change of at least 3 were reported.

*2.7. Western Blot Analysis.* Cells and exosomes were lysed in M-PER (Thermo Scientific, 78501) supplemented with Halt proteinase inhibitor cocktail (Thermo Scientific, 1 : 100, 87786) and phenylmethanesulfonyl fluoride solution (Boston BioProducts, 1 : 100, PI-120). Cells were centrifuged at 14,000 g for 5 minutes at 4°C, and protein concentration was determined using the bicinchoninic assay (BCA) method (Pierce BCA Protein Assay Kit, Thermo Scientific, 23225). 30 ug of protein from cell lysates and 40 ug of protein from exosomal lysates were diluted in 6x buffer/DTT loading dye and heated to 95°C for 10 minutes, as described previously [11, 33, 34]. Protein was electrophoresed to 4–20% Ready Gel Tris-HCl Precast Gels (Bio-Rad, 161-1159) at 90 V for 15 minutes followed by 120 V for 1 hour, and transfer onto a nitrocellulose membrane for 1 hour at 100 V on ice. The membrane was washed with 1X TBST for 5 min and blocked at room temperature for 1 hour with 5% dry milk in 1X TBST. The membrane was washed three times for 5 minutes in 1X TBST and incubated with the following primary antibodies: LIN28A (1 : 1000 rabbit polyclonal, ab63740, Abcam), LIN28B (1 : 1000 rabbit polyclonal, 4196S, Cell Signaling Technology), GAPDH (1 : 3000, rabbit polyclonal, ab37168), CYTO C (1 : 100, mouse monoclonal $IgG_{2b}$, sc13156, Santa Cruz Biotechnology), TSG101 (1 : 500, rabbit polyclonal, 14497-1-AP, Proteintech), or EPCAM (1 : 500, rabbit polyclonal, 21050-1-AP, Proteintech). Primary antibodies were resuspended in 5% milk 1X TBST and membranes were incubated overnight at 4°C, except for GAPDH in which case the membranes were incubated 1 hour at room temperature. Membranes were washed three times for 5 minutes each in 1X TBST before the secondary antibodies were applied for 1 hour at room temperature. The secondary antibodies are as follows: goat anti-rabbit IgG-HRP (1 : 5000, sc-2004, Santa Cruz Biotechnology) was for LIN28A, LIN28B, and GAPDH, goat anti-mouse IgG-HRP (1 : 2000, sc-2031, Santa Cruz Biotechnology) was used with CYTO C, and goat anti-rabbit IgG-HRP (1 : 2000, ab6721, Abcam) was used with TSG101 and EPCAM. Membranes were washed with 1X TBST 3 times for 5 minutes and incubated for 5 minutes in ECL Prime Western Blotting Detection Reagent (Amersham, RPN2209) and for 1 second in SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, 34075) for chemiluminescence detection on the ChemiDoc MP System. Image Lab 4.1 software was used to determine densitometry.

Experiments were carried out using at least two independent biological replicates and the experiments were repeated. Densitometry was calculated by dividing the band volume of the gene of interest over the housekeeping gene GAPDH. Statistical analysis was determined by ANOVA followed by Tukey pairwise comparison (Minitab 17). $p$ values less than 0.05 were considered statistically significant.

*2.8. Exosome Transfer.* For exosome transfers, $1 \times 10^6$ IGROV1-CD63-GFP cells were seeded onto 10 cm cell plates (Celltreat, 229690) in complete RPMI 1640 vesicle-depleted medium and grown for 3 days. IGROV1 and OV420 cell-secreted exosomes were isolated from the culture medium using the exosome isolation procedure described above and stained with Vybrant DiD cell-labeling solution (Invitrogen, V22887) per manufacturer's instructions. 24 hours before exosomes were isolated; $5 \times 10^4$ HEK293 cells were grown in complete DMEM vesicle-depleted medium to approximately 60–80% confluency in 4-well plates. Exosomes were resuspended in 500 $\mu$L of complete DMEM vesicle-depleted medium and were added onto $5 \times 10^4$ HEK293 cells, referred to as exosome transfer, for 96 hours. The first control (vehicle) was 500 $\mu$L of complete DMEM vesicle-depleted medium plated onto $5 \times 10^4$ HEK293 cells as a negative control. The second control (supernatant) included 500 $\mu$L of nonprecipitated, supernatant/ExoQuick-TC biofluid plated onto $5 \times 10^4$ HEK293 cells. Cells were imaged on the Olympus FSX100 Bio Imaging Navigator using the FSX100 software or the LSM 510 Meta 405 Confocal Microscope System Zeiss. A series of images were collected at 1 $\mu$m intervals and used to generate a Z-stack. After 96 hours, HEK293 cells were trypsinized and snap frozen in liquid nitrogen and stored in −80°C for RNA/protein analysis or used immediately for migration and invasion assays.

*2.9. Migration and Invasion Assays.* Migration and invasion assays were performed using the 24-well 8.0 $\mu$m BD BioCoat Tumor Invasion System (BD Biosciences, 354166) and the BD Falcon 24-multiwell 8 $\mu$m insert system (BD Biosciences, 351158) as per manufacturer's instructions. Briefly, cell monolayers were pretreated with 10 ug/mL $DiIC_{12}(3)$ in 10% FBS DMEM vesicle-depleted medium for 1 hour at 37°C. Cells were trypsinized in 0.1% FBS DMEM vesicle-depleted serum-free medium. $5 \times 10^4$ cells were seeded onto the apical chambers. Chemoattractant of either 10% FBS DMEM vesicle-depleted medium (positive control) or 0.1% FBS DMEM vesicle-depleted serum-free medium (negative control) was added to the basal chambers. The BD BioCoat Tumor Invasion System and the uncoated BD Falcon FluoroBlok 24-Multiwell System were incubated for 48 hours at 37°C, 5%

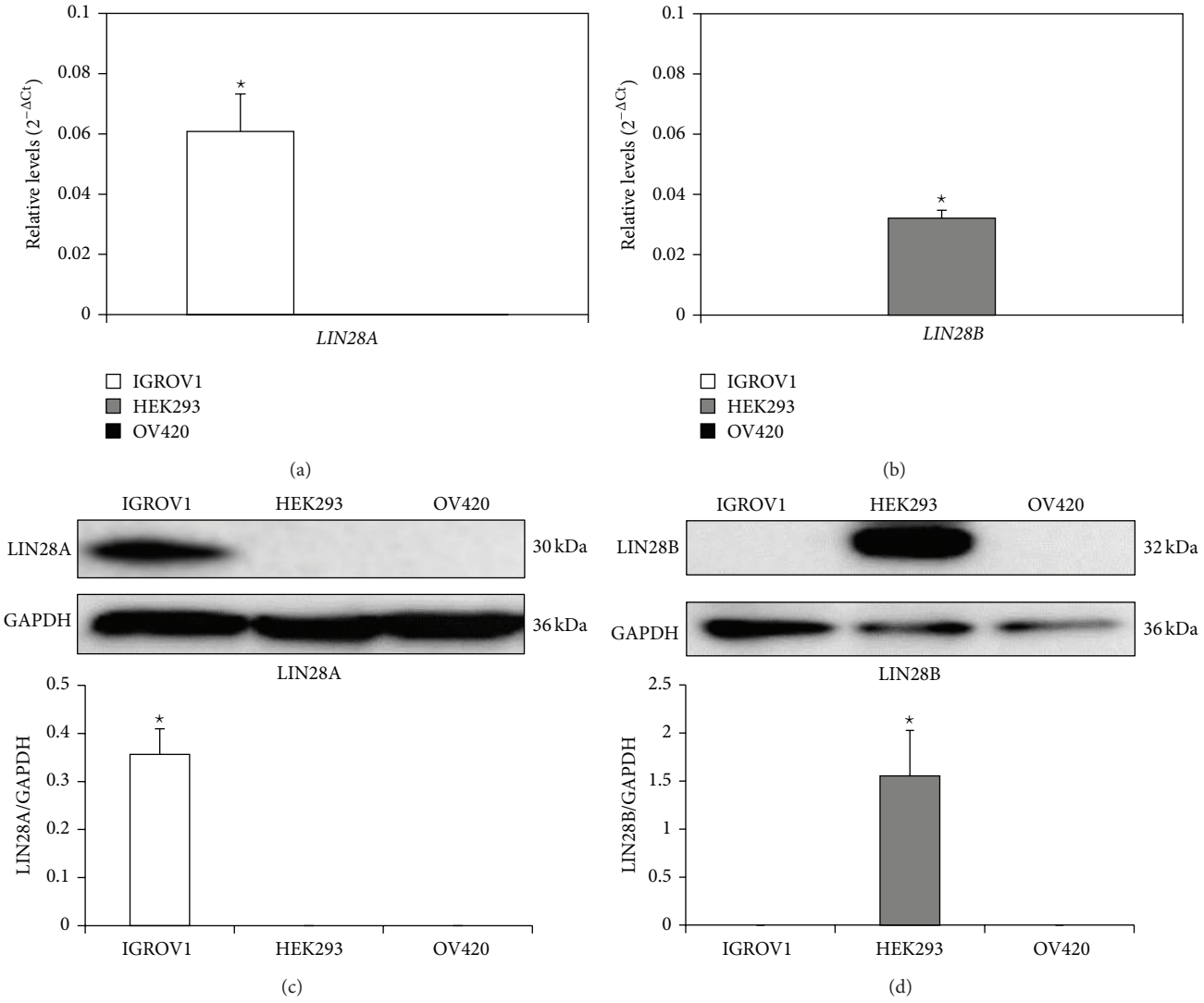


Figure 1: Levels of LIN28A and LIN28B mRNA and protein in IGROV1, HEK293, and OV420 cells. qPCR was performed to obtain *LIN28A* (a) and *LIN28B* (b) mRNA levels. Data were normalized against the geometric mean of *GAPDH*, *MRPS15*, and *TBP*. Western blot analysis was performed to obtain LIN28A (c) and LIN28B (d) protein levels. Densitometry of bands was determined to calculate relative protein amount. Asterisk indicates a $p$ value <0.05.

$CO_2$. Readings were taken every 6 hours, for 48 hours, with Syngene HT and Gen5 program using 530/25-excitation filter and 590/35-emission filter, and sensitivity was set at 52. Fluorescence of invaded/migrated cells was read at wavelengths 549/565 nm (Ex/Em) for detection of $DiI_{12}C$. Background fluorescence was subtracted and data reduction was performed by subtracting values of the negative from the positive control. Migration and invasion relative fluorescence units were plotted separately, and experiments were carried out at least two times with three independent biological replicates.

## 3. Results

*3.1. LIN28A and LIN28B Expression in IGROV1, OV420, and HEK293 Cells.* qPCR analysis revealed significantly higher levels of *LIN28A* in IGROV1 compared to OV420 or HEK293 cells (Figure 1(a)). Alternatively, HEK293 cells were the only cells with detectable amounts of *LIN28B* (Figure 1(b)), whereas OV420 cells did not contain any detectable amounts of *LIN28A* or *LIN28B*. These results were confirmed for LIN28A and LIN28B protein by Western blot analysis (Figures 1(c) and 1(d)).

*3.2. let-7 miRNA Level in IGROV1, OV420, and HEK293 Cells.* LIN28A and LIN28B are known regulators of *let-7* miRNAs by inhibiting their maturation. qPCR analysis revealed that the relative levels of mature *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7g*, and *let-7i* are all significantly higher in OV420 cells compared to both IGROV1 and HEK293 cells (Figure 2).

*3.3. Uptake of IGROV1 and OV420 Secreted Exosomes by HEK293 Cells.* IGROV1 cells were infected with CD63-GFP-cytotracer, which led to GFP-labeling of exosomes by the host cells. In addition, exosomes secreted by OV420 cells were

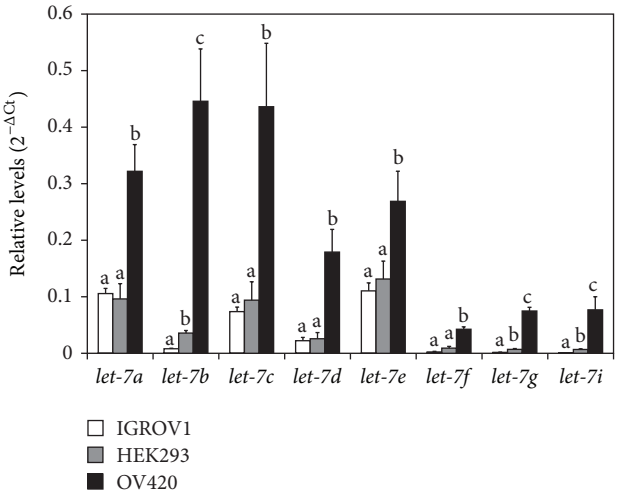


Figure 2: Relative level of *let-7* miRNAs in IGROV1, OV420, and HEK293 cells. qPCR data were normalized against the U6 snRNA. Means without the same superscript are significantly different ($p$ value <0.05).

incubated with the DiD cell-labeling solution, which labels lipids in cell membranes. HEK293 cell incubation with GFP-labeled IGROV1 secreted exosomes or DiD-labeled OV420 secreted exosomes leads to uptake of these exosomes as evident by Z-stack imaging (Figure 3).

*3.4. HEK293 Cell Invasion and Migration following Uptake of IGROV1 and OV420 Cell-Secreted Exosomes.* To determine if uptake of IGROV1 or OV420 cell-secreted exosomes leads to changes in cell phenotype or behavior, HEK293 cells were incubated with exosomes and invasion and migration assays were conducted. Uptake of IGROV1 cell-secreted exosomes leads to significant increase in invasion as well as migration as early as 12 hours in HEK293 cells, compared to HEK293 cells incubated in media with vehicle or supernatant (Figures 4 and 5). Uptake of OV420 cell-secreted exosomes had no effect on HEK293 cell invasion or migration. These results mimic what is seen for IGROV1 and OV420 cells, in that IGROV1 cells are significantly more invasive than OV420 cells (Supplemental Figure).

*3.5. Relative Levels of Epithelial-to-Mesenchymal Transition (EMT) Related Genes in HEK293 Cells following Uptake of IGROV1 Cell-Secreted Exosomes.* HEK293 exposure to and uptake by IGROV1 cell-secreted exosomes leads to invasion and migration; therefore, qPCR analysis was performed using Human Epithelial-to-Mesenchymal Transition RT$^2$ Profiler PCR arrays to determine changes in relative expression of 84 genes known to be involved in EMT. Exposure to exosomes resulted in significant increased levels of 45 EMT-related genes, including *ZEB1*, *NOTCH1*, *WNT5A*, *NODAL*, and *SNAI2* (Table 2).

*3.6. LIN28 and miRNAs in HEK293 Cells following Uptake of IGROV1 Cell-Secreted Exosomes.* In addition to EMT-related genes, relative changes in LIN28A, LIN28B, and selected miRNAs known to be involved in EMT and cancer (oncomirs) were assessed in HEK293 cells following uptake of IGROV1 cell-secreted exosomes. Taqman qPCR assays

Table 2: Epithelial-to-Mesenchymal Transition (EMT) related genes that are significantly upregulated after HEK293 cells exposed to IGROV1 cell-secreted exosomes.

| Fold change | Genes |
|---|---|
| 25.65 | *TIMP1* |
| 10.95 | *FOXC2* |
| 10.55 | *NOTCH1* |
| 9.67 | *PTP4A1* |
| 8.32 | *GNG11* |
| 7.37 | *SNAI1* |
| 7.33 | *FGFBP1* |
| 7.13 | *SOX10* |
| 6.58 | *COL3A1* |
| 6.49 | *JAG1* |
| 6.45 | *WNT5A* |
| 6.41 | *SNAI3* |
| 6.38 | *NODAL* |
| 6.35 | *CDH1* |
| 6.32 | *IL1RN* |
| 6.04 | *TMEM132A* |
| 5.7 | *COL1A2* |
| 5.53 | *GSC* |
| 5.39 | *MMP3* |
| 5.35 | *ERBB3* |
| 5.32 | *SNAI2* |
| 5.24 | *BMP1* |
| 5.2 | *KRT7* |
| 5.15 | *MST1R* |
| 5.13 | *PTK2* |
| 4.82 | *F11R* |
| 4.78 | *AHNAK* |
| 4.49 | *IGFBP4* |
| 4.29 | *FZD7* |
| 4.28 | *TWIST1* |
| 4.19 | *ITGA5* |
| 4.14 | *WNT5B* |
| 4.12 | *STAT3* |
| 4.09 | *BMP7* |
| 4 | *MMP9* |
| 3.95 | *SPP1* |
| 3.86 | *SPARC* |
| 3.82 | *SERPINE1* |
| 3.81 | *ILK* |
| 3.8 | *PLEK2* |
| 3.37 | *WNT11* |
| 3.25 | *TMEFF1* |
| 3.22 | *TGFB1* |
| 3.09 | *TGFB3* |
| 3.03 | *ZEB1* |

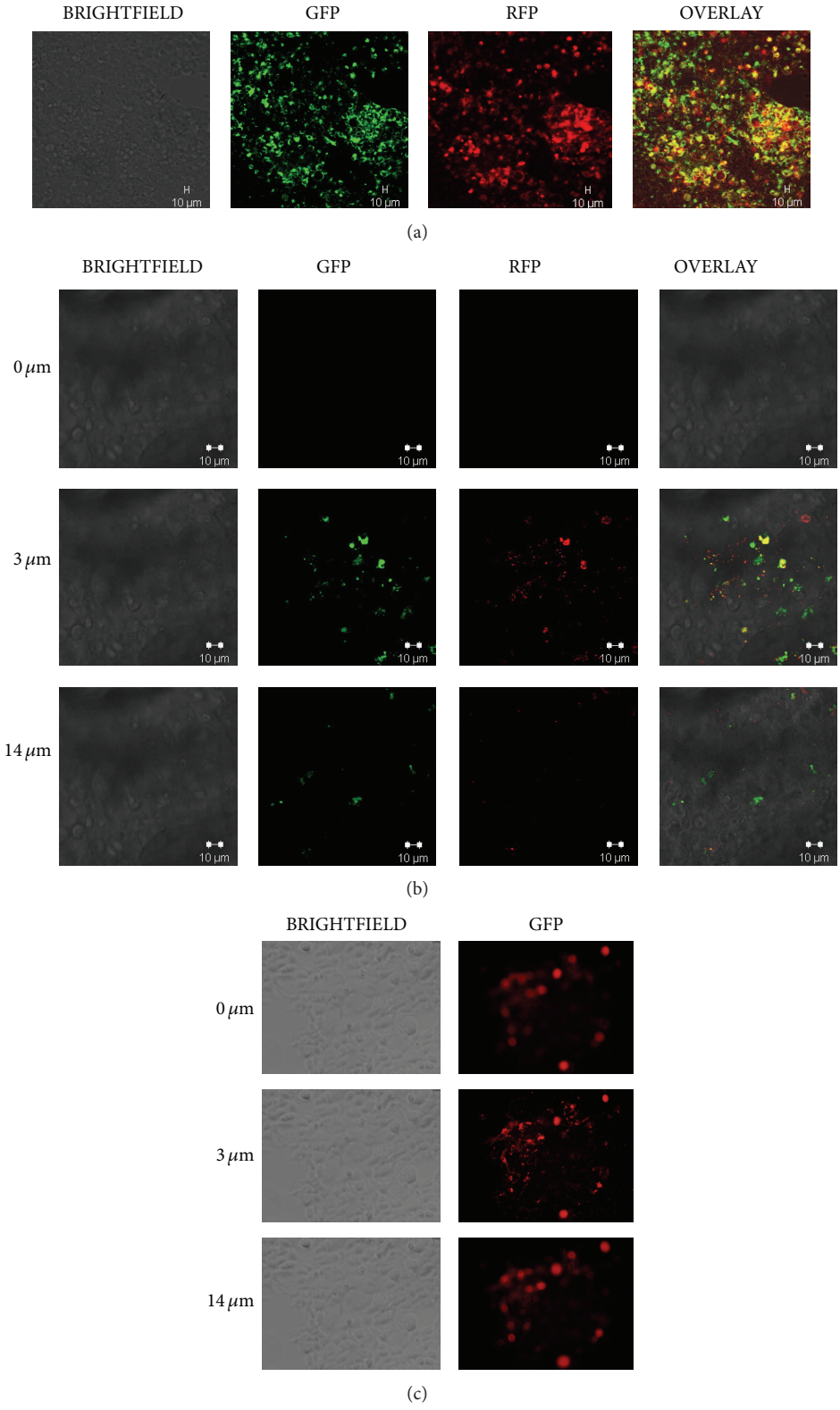


Figure 3: Detection of exosome-uptake by HEK293 cells. (a) HEK293 cells after exposure to CD63-GFP positive (green) and DiD-RFP labeled (red) IGROV1 secreted exosomes. 20x magnification was utilized to image HEK293 cells following exosome exposure using confocal microscopy. (b) Z-stack image of HEK293 cells after exposure to CD63-GFP positive (green) and DiD-RFP labeled (red) IGROV1 secreted exosomes. (c) Z-stack image of HEK293 cells after exposure to OV420 secreted, DiD labeled (red) exosomes. 40x magnification was utilized to image HEK293 cells following exosome exposure using confocal microscopy.

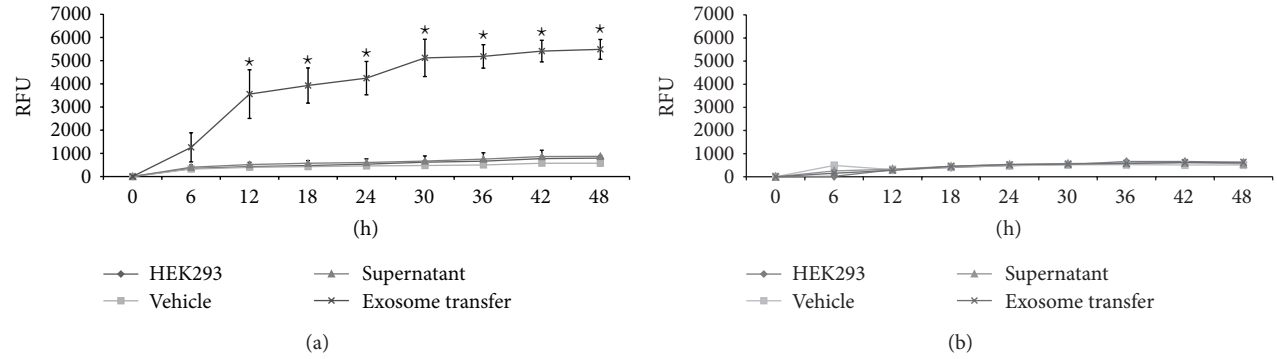


Figure 4: Invasion of HEK293 cells exposed to IGROV1 or OV420 cell-secreted exosomes. (a) HEK293 cells invasion when exposed to IGROV1 cell-secreted exosomes and (b) HEK293 cells invasion when exposed to OV420 cell-secreted exosomes. Asterisk indicates a $p$ value $<0.05$.

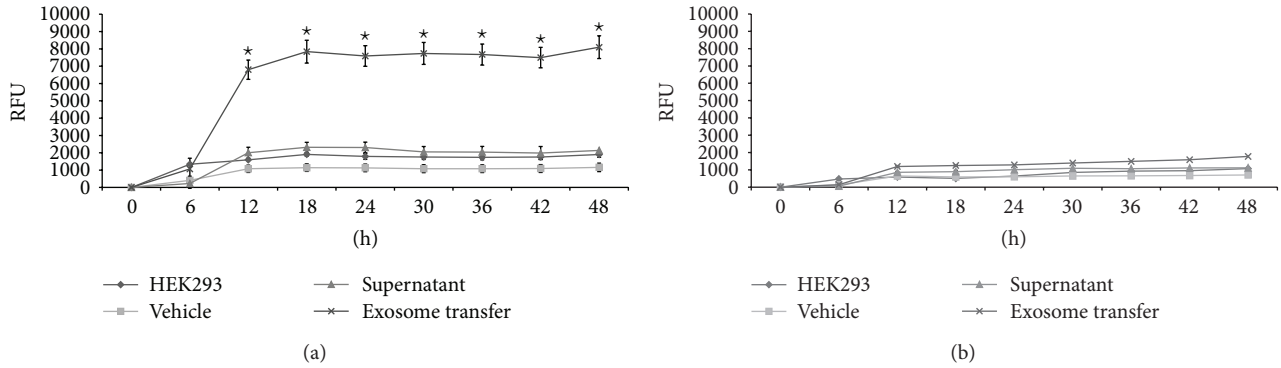


Figure 5: Migration of HEK293 cells exposed to IGROV1 or OV420 cell-secreted exosomes. (a) HEK293 cells migration when exposed to IGROV1 cell-secreted exosomes and (b) HEK293 cells migration when exposed to OV420 cell-secreted exosomes. Asterisk indicates a $p$ value $<0.05$.

revealed that there was a significant, 15-fold increase in *LIN28A* level in HEK293 cells exposed to IGROV1 cell-secreted exosomes compared to IGROV1 cell conditioned media depleted of exosomes (Figure 6(a)), but not *LIN28B* (Figure 6(c)). This change was significant although no change in LIN28A (Figure 6(b)) or LIN28B (Figure 6(d)) protein level was evident in HEK293 cell following exposure to IGROV1 cell-secreted exosomes. No change in *let-7* (*a, b, c, d, ef, g*, and *i*) miRNA was observed in HEK293 cells following treatment with IGROV1 cell-secreted exosomes (Figure 7). Moreover, qPCR analysis of selected other EMT and cancer associated miRNAs, that is, *miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-92, miR-22, miR-200a, miR-9, miR-30b, miR-30d, miR-30e, miR-125a-3p, miR-125-5p*, and *miR-125b*, indicates a small but significant increase in *miR-9* alone in HEK293 cells following treatment with IGROV1 cell-secreted exosomes (Figure 8).

*3.7. LIN28 and miRNAs in IGROV1 and OV420 Cell-Secreted Exosomes.* Using Western blot analysis, exosomes secreted by IGROV1, OV420, and HEK293 cells were all positive for exosomal protein markers TSG101 and EPCAM; however, neither LIN28A nor LIN28B was detected in IGROV1, OV420, or HEK293 cell-secreted exosomes (Figure 9). qPCR analysis of miRNAs previously associated with cancer (*miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-92, miR-22, miR-200a, miR-9, miR-30b, miR-30d, miR-30e, miR-125a-3p, miR-125-5p*, and *miR-125b*) in exosomes secreted by IGROV1, OV420, and HEK293 cells indicates that overall miRNA levels were similar with the exception of *miR-200b* and *miR-200c*, which were significantly elevated in OV420 cells, *miR30a*, which was significantly higher in IGROV1 exosomes, *miR-30c*, which was significantly higher in IGROV1 and HEK293 exosomes, and *miR-31*, which was significantly higher in OV420 and HEK293 exosomes (Figure 10). Finally, *let-7*b, *let-7*c, *let-7*g, and *let-7*i miRNAs were significantly higher in OV420 compared to IGROV1 and HEK293 secreted exosomes (Figure 11).

## 4. Discussion

In this study we sought to determine if exosomes from high LIN28A expressing ovarian cancer cells could be taken up by HEK293 cells leading to changes in gene expression and cell phenotype. IGROV1 cells contain high levels of LIN28A mRNA and protein and low levels of *let-7* miRNAs, confirming a previous report [35]. Conversely, OV420 ovarian cancer cells contain no detectable amount of LIN28A or LIN28B, and

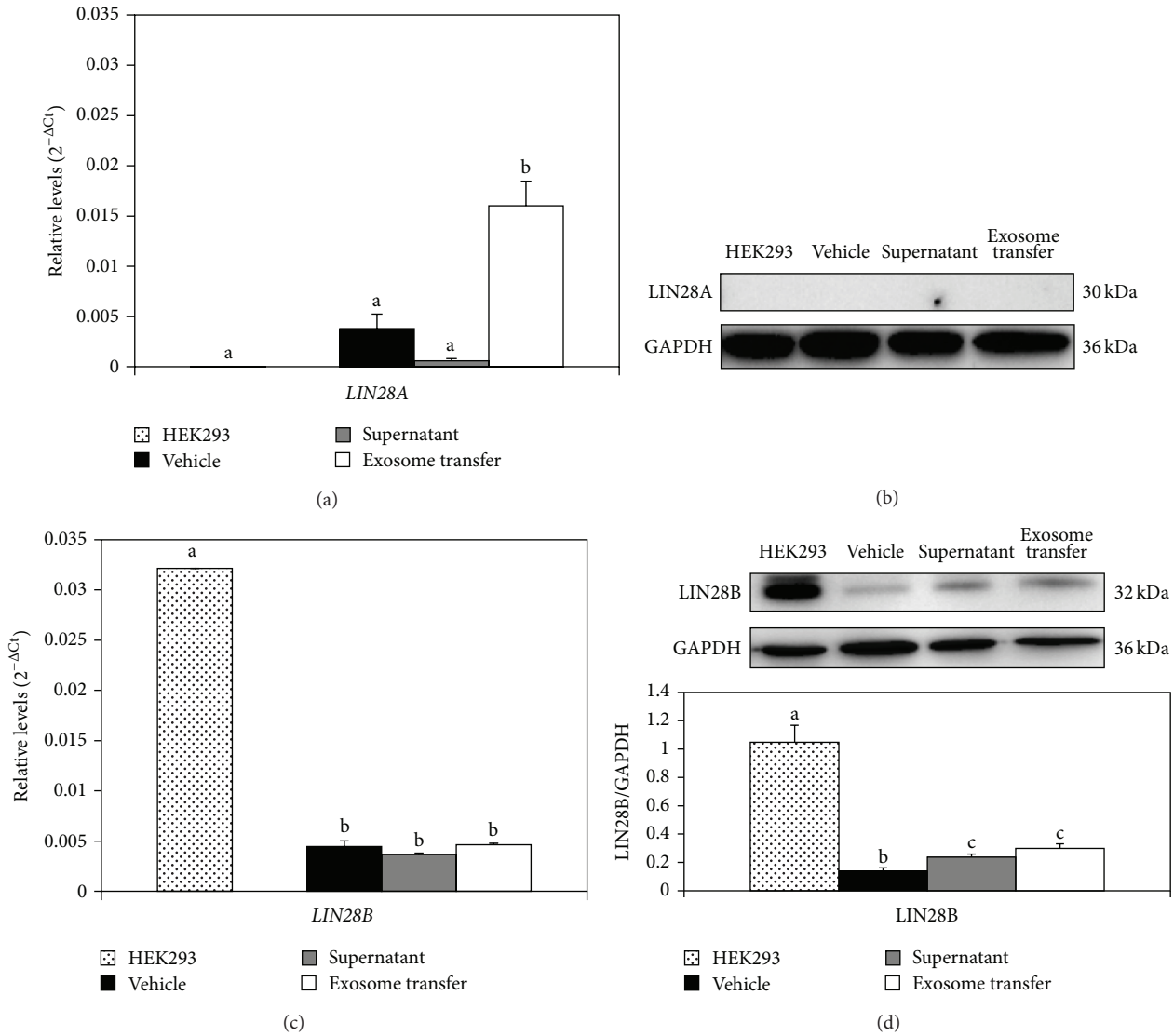


Figure 6: Relative levels of LIN28A and LIN28B mRNA and protein in HEK293 cells exposed to IGROV1 cell-secreted exosomes. HEK293 cells treated with vesicle-deplete IGROV1 conditioned media (vehicle), HEK293 cells treated with supernatant from exosome pellet (supernatant), and HEK293 cells treated with exosomes (exosome transfer). qPCR was performed to assess mRNA levels of *LIN28A* (a) and *LIN28B* (c) after HEK293 cells were exposed to IGROV1 cell-secreted exosomes. Data were normalized against the geometric mean of *GAPDH*, *MRPS15*, and *TBP*. Means without the same superscript are significantly different ($p$ value <0.05). Western blot analysis was performed to determine LIN28A (b) and LIN28B (d) protein levels after HEK293 cells were exposed to IGROV1 cell-secreted exosomes. Densitometry of bands was determined to calculate relative protein amount. Means without the same superscript are significantly different ($p$ value <0.05).

*let-7* miRNAs are significantly higher in these cells compared to IGROV1 cells. HEK293 cells were selected as target cells for IGROV1 and OV420 cell-secreted exosomes, as these cells are readily used and not cancer derived. Interestingly, HEK293 cells were found to contain LIN28B, although this amount was less compared to relative LIN28A levels in IGROV1 cells. Currently it is unclear what the different functions of LIN28A and LIN28B are; both regulate *let-7* miRNA maturation with LIN28A functioning in the cytoplasm, whereas LIN28B appears to sequester *let-7s* to the nucleus [35].

Previous studies demonstrate that tumor secreted microvesicles/exosomes can alter target cell gene expression and cell behavior. For example, glioblastoma tumor cell-secreted exosomes in the brain are enriched with angiogenic proteins and can be taken up by brain microvascular endothelial cells to stimulate tubal formation [4]. More recently, Le and colleagues [36] report that metastatic mouse and human breast cancer cells secrete extracellular vesicles (including exosomes), containing *miR-200s*, *miR-200s*, and can be transferred to nonmetastatic cells. Furthermore, breast cancer cell-secreted exosomes containing *miR-122* induced tumor colonization in brain and lung in xenograft mouse model [37]. Our data reveals that exosomes secreted by high LIN28A expressing IGROV1 cells, but not low

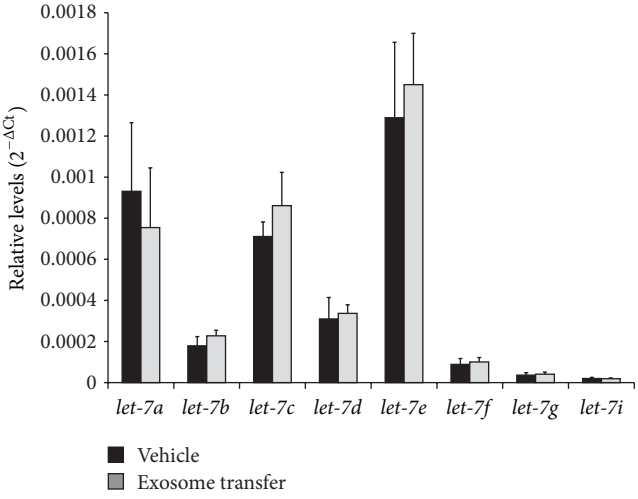


Figure 7: Relative level of *let-7* miRNAs in HEK293 cells following IGROV1 cell-secreted exosome treatment. qPCR was utilized to determine the relative levels and data were normalized against U6 snRNA.

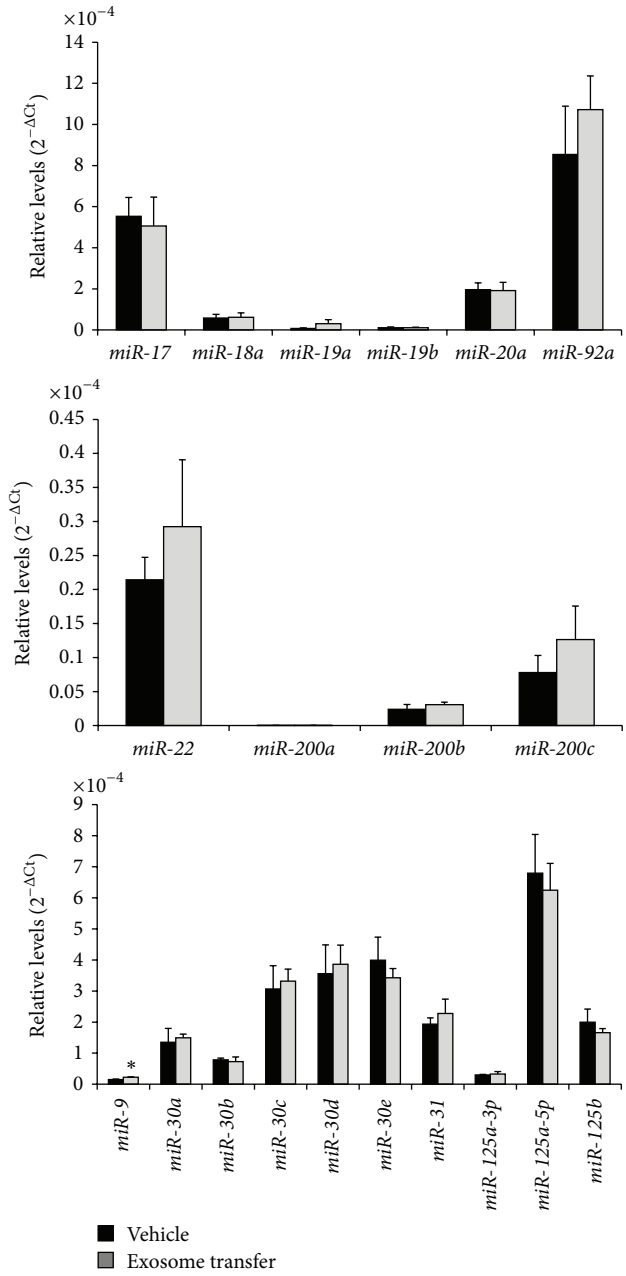


Figure 8: Relative level of selected miRNAs in HEK293 cells following IGROV1 cell-secreted exosome treatment. qPCR was utilized to determine the relative levels and data were normalized against U6 snRNA. Asterisks indicate a $p$ value <0.05.

LIN28 expressing OV420 cells, alter nonovarian HEK293 cell behavior and lead to an increase in invasion and migration. An invasion assay was conducted on IGROV1 and OV420 cells. After 48 hrs IGROV1 cells exhibited significantly higher invasion compared to OV420 cells and support data that exosomes secreted by IGROV1 but not OV420 cells are able to induce invasion in HEK293 cells (Figure S1). Moreover, relative expression levels of several genes related to EMT were upregulated following exposure to IGROV1 cell-secreted exosomes, which could explain the altered behavior of HEK293 cells.

LIN28 is a RNA-binding protein that binds to and regulates both mRNAs and miRNAs. LIN28A and miRNAs can reprogram cells and are known regulators of cell differentiation, and studies have demonstrated deregulation of miRNAs in cancer [38–41]. However, LIN28A protein was not detected in IGROV1 cell-secreted exosomes, and no change in LIN28A was observed in HEK293 cells following exposure to IGROV1 exosomes. Interestingly, we did observe that endogenous LIN28B levels were lower in HEK293 cells when cultured in vesicle-depleted media compared to complete media. The reason for this is unclear at this point.

We also determined that *let-7*b, *let-7*c, *let-7*g, and *let-7*i are significantly lower in IGROV1 exosomes compared to OV420 exosomes, which reflect what was found in the donor cells. Profiling of miRNAs in epithelial ovarian tissues demonstrated *let-7*s were lower in these tissues [42], and lower levels of *let-7*i are found in patients resistant to chemotherapy as well as patients with a poor prognosis in late-stage ovarian cancer [43]. A recent study described exosomal *miR-200* and *let-7* families from SKOV3 cells (highly invasive) and OVCAR-3 cells (low invasiveness), and it was determined that *miR-200* and *let-7* families are sequestered in exosomes of more invasive ovarian cancer cells lines [44]. However, Taylor and Gercel-Taylor [5] report that the expression pattern of *let-7* family is similar between donor cells and exosomes, similar to our findings.

The *miR-17-92, miR-200*, and *miR-30* families are all known regulators of oncogenesis and EMT [45–48]. Therefore, we examined the presence of these miRNAs in exosomes, as well as HEK293 cells following exposure to IGROV1 cell-secreted exosomes. Interestingly, although *miR-200b, miR-200c*, and *miR-31* are lower and *miR-30a* and *miR-30c* are higher in IGROV1 cell-secreted exosomes compared to OV420 exosomes, relative levels of these miRNAs were not

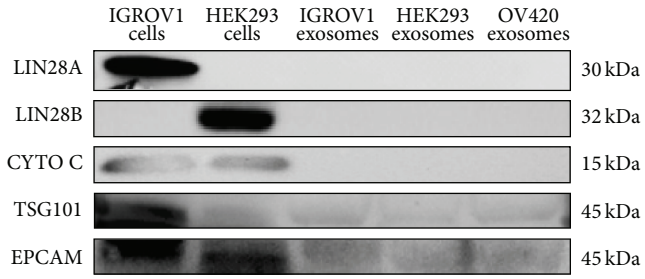


Figure 9: Detection of LIN28A and LIN28B protein in exosomes. Western blot was used to determine the presence of LIN28A protein in exosomes (columns 3–5). The cytoplasmic/mitochondrial protein cytochrome C (CYTO C) was used as a negative control to exclude nonexosomal fractions, and tumor susceptibility gene 101 (TSG101), a component of the endosomal sorting, and EPCAM (epithelial cell adhesion molecule) are used as positive controls for exosomes.

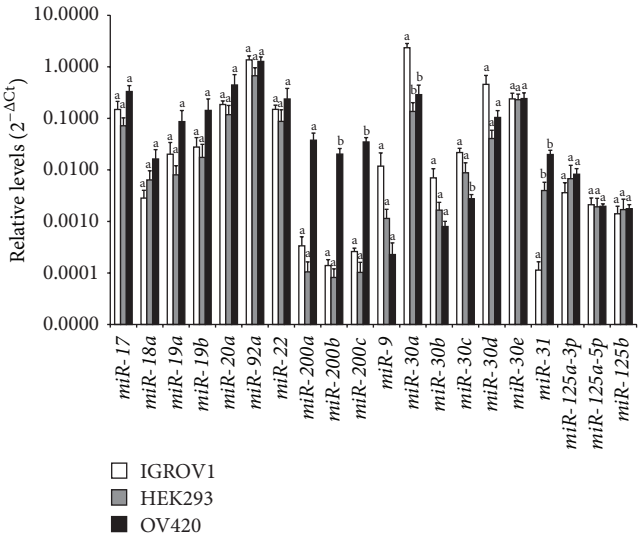


Figure 10: Relative levels of selected miRNAs in ovarian cancer cell-secreted exosomes. qPCR data were normalized against U6 snRNA. Means without the same superscript are significantly different ($p$ value <0.05).

different in HEK293 cells following exposure to IGROV1 cell-secreted exosomes. Our results did reveal that *miR-9* was significantly higher in HEK293 cells after IGROV1 exosome exposure. *miR-9* regulates snail family zinc finger 2 (*SNAI2*) [49], also known as *SLUG*, a necessary factor in type II tumor formation. *SNAI2* was significantly higher in HEK293 cells following exposure to IGROV1 cell-secreted exosomes. It is unclear whether exosomal *miR-9* actually was transferred from IGROV1 to HEK293 cells and whether it directly regulates *SNAI2* transcription and/or translation.

Taken together, data presented here demonstrate that high LIN28A expressing ovarian cancer cells secrete exosomes that, when taken up by nonmetastatic target cells, induce EMT-related gene expression and invasion and migration.

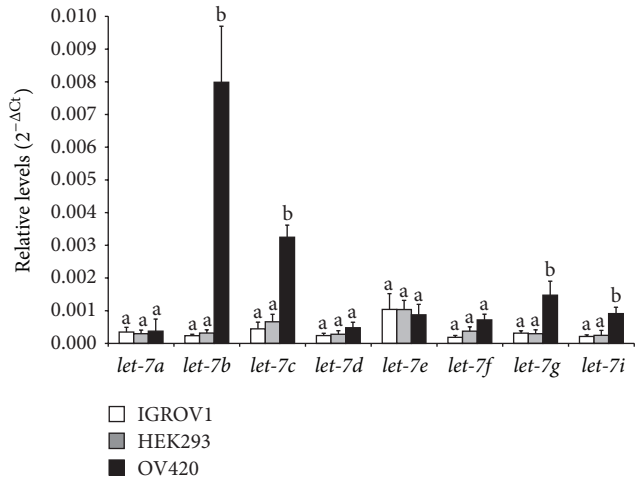


Figure 11: Relative levels of *let-7* miRNAs in ovarian cancer cell-secreted exosomes. qPCR data were normalized against U6 snRNA. Means without the same superscript are significantly different ($p$ value <0.05).

## 5. Conclusions

Our results demonstrate that the more metastatic, high LIN28A expressing IGROV1 cells secrete exosomes that can upregulate genes related to EMT and induce invasion and migration in HEK293 cells. We were unable to demonstrate presence in or transfer of LIN28 itself by exosomes, and it is possible that the observed changes in cell phenotype and gene expression induced by IGROV1 cell-secreted exosomes are not due to LIN28 directly. Future studies using different high LIN28A expressing cancer cells will be important to determine if the observed features of IGROV1 cell-secreted exosomes hold true for other high LIN28A-expressing cells. Although a number of known cancer and EMT-related miRNAs were investigated, only miR-9 was altered in HEK293 cells following IGROV1 cell-secreted exosome treatment. The fact that LIN28 can interact with and bind to many RNAs in addition to miRNAs suggests additional RNAs are potential candidates that can be loaded into exosomes and/or transferred to target cells. Future RNAseq experiments will provide insight into the underlying mechanism of the observed exosome-induced invasion and migration.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# The Role of Extracellular Vesicles: An Epigenetic View of the Cancer Microenvironment

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Exosomes, microvesicles, and other extracellular vesicles are released by many cell types, including cancer cells and cancer-related immune cells. Extracellular vesicles can directly or indirectly facilitate the transfer of bioinformation to recipient cells or to the extracellular environment. In cancer, exosomes have been implicated in tumor initiation, proliferation, and metastasis. Extracellular vesicles can transmit proteins and nucleic acids that participate in DNA methylation, histone modification, and posttranscriptional regulation of RNA. Factors transmitted by extracellular vesicles reflect the donor cell status, and extracellular vesicles derived from tumor cells may be also responsible for altering expression of tumor promoting and tumor suppressing genes in recipient cells. Thus, circulating extracellular vesicles may act as biomarkers of cancer, and detection of these biomarkers may be applied to diagnosis or assessment of prognosis in patients with cancer.

## 1. Introduction

Extracellular vesicles include a variety of nanoscale membranous vesicles [1] released by many cell types into the intercellular microenvironment [2, 3]. Subtle changes in the cellular microenvironment may stimulate malignant transformation of cells, and cellular microenvironment has been implicated in tumor initiation, proliferation, and metastasis. Transformed cancer cells may disseminate bioinformation in autocrine and paracrine manners, to help the cancer proliferate and metastasize. This cell-cell or cell-microenvironment communication may be achieved by direct contact or over longer distances by secreted molecules and secreted membranous vesicles. A growing body of evidence suggests that cancer cells release more extracellular vesicles than healthy cells [4–6], partly due to activation of certain oncogenes, including *ras* [7].

Whether individual extracellular vesicles participate in normal physiological regulation or promotion of pathological processes is dependent on what they contain [8–10]. Exosomes, microvesicles and other extracellular vesicles differ in properties such as size, morphology, buoyant density, and protein composition [11]. Exosomes range in size from 40 to 1000 nm, and microvesicles are >1000 nm. Microvesicles bud directly from the plasma membrane, whereas exosomes are derived from endosomes and are released from cells by fusion of the multivesicular endosome with the plasma membrane [2]. Due to their endosome origin, exosomes contain endosome-associated proteins. Exosomes, microvesicles, and other extracellular vesicles can contain proteins, RNA, DNA, and lipids [12], and thus can deliver these factors to the intercellular environment or recipient cells. Extracellular vesicles are transported in the blood, urine, ascites, and cerebrospinal fluid [13–16] and thus may deliver their contents to either neighboring or distant recipient cells and produce corresponding physiological or pathologic effects. For example, melanoma-derived exosomes can deliver the receptor tyrosine kinase MET oncoprotein to bone marrow progenitor cells, which directs their development toward a prometastatic phenotype [17].

The content of extracellular vesicles, therefore, may be clinically relevant to disease progression, and detection of

extracellular vesicles may be useful in diagnosis of cancer and assessment of prognosis. Circulating microvesicles and exosomes have been detected in the blood samples of patients with glioblastoma [18], colorectal cancer [19], and ovarian cancer [20]. These microvesicles and exosomes contain bioinformation reflecting primary tumor mutations and can act as early indicators of drug efficacy.

Epigenetic regulation involves processes causing functionally relevant changes to the genome which do not alter the nucleotide sequence but do alter gene expression. The genome plays a significant role in the tumor microenvironment, and the microenvironment influences cancer initiation, proliferation, and metastasis [21]. Important mechanisms of epigenetic regulation such as DNA methylation, histone modification and microRNA (miRNA), or long noncoding RNA (lncRNA) regulation are hot topics in cancer research. Recent research has implicated extracellular vesicles in epigenetic regulation of cancer progression [22, 23]. Gene ontology (GO) analysis has indicated that many mRNAs and proteins contained in extracellular vesicles are involved in epigenetic regulation [24], and exosome-mediated transfer of miRNAs is considered to be an important mechanism of genetic exchange between cells [25]. Thus extracellular vesicles regulate epigenetic processes including DNA methylation, histone modification, and miRNA or lncRNA regulation, and the resultant epigenetic modifications are responsible for changes in the expression of tumor promoting genes and tumor suppressing genes. Recent research indicated that the detection of epigenetic biomarkers, such as miRNAs, in extracellular vesicles could be exploited for diagnosis of cancer or assessment of cancer prognosis [26]. In this review we discuss the role of extracellular vesicles in transmission of factors responsible for three forms of epigenetic regulation: DNA, histone, and noncoding RNA modification.

## 2. The Role of Extracellular Vesicles in DNA Methylation and Demethylation in the Cancer Microenvironment

Epigenetic DNA modification of oncogenes or antioncogenes is crucially important for the initiation, proliferation, and metastasis of many tumors [27–29]. Dynamic variation in DNA methylation is one of the most universal factors influencing transcription of oncogenes and antioncogenes. DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3a (DNMT3a), and DNA methyltransferase 3b (DNMT3b) add methyl groups to specific cytosines in the CpG islands of regulatory sequences, thus silencing certain genes [30]. The level of oncogene or antioncogene transcription fluctuates according to promoter methylation status, thus dynamically affecting tumor progression. Enzymes that demethylate DNA, such as activation-induced cytidine deaminase (AICDA) [31] and thymine DNA glycosylase (TDG) [32], protect unmethylated regions of mammalian genomes from *de novo* methylation.

Due to their complex bioactive cargo, extracellular vesicles can cause malignant transformation of normal cells. The protein, DNA, or RNA contained in extracellular vesicles could induce epigenetic changes in recipient cells by affecting the methylation status of their genome. Microvesicles are extracellular vesicles of 100–1000 nm in diameter [2, 33] which bud from the plasma membrane of tumor cells, while exosomes are 30–100 nm in diameter and are derived from the endosome [2, 34]. Microvesicles released from leukemia cells have been demonstrated to increase global DNA methylation levels in recipient cells. Hypermethylation of the promoter regions of tumor-suppressor genes *P53* and *RIZ1* was observed in cells incubated with leukemia-derived microvesicles and attributed to the increased level of DNMT3a and DNMT3b mRNA and protein [34]. Interestingly, protein and mRNA levels of activation-induced cytidine deaminase (AICDA), a deaminase involved in DNA initiative demethylation, was also increased in recipient cells. These results indicate that genomic instability was promoted in recipient cells, which might further induce leukemic transformation [34]. When microvesicles were treated with RNase, the level of DNMT3a, DNMT3b, and AICDA decreased, indicating that leukemia-derived microvesicles influence the methylation status of recipient cells via transmission of microvesicular RNA. Breakpoint cluster region-Abelson leukemia gene human homolog 1 (*BCR-ABL1*) has been reported to be the dominant onco-mRNA in microvesicles released by the K562 leukemia cell line [34]. Extracellular vesicles derived from transformed donor cells may thus transmit enzymes involved in both methylation and demethylation to recipient cells, ultimately inducing alteration in expression of tumor-related genes and accelerating tumor initiation, proliferation, and metastasis.

In addition to inducing malignant transformation of recipient cells, extracellular vesicles may represent useful biomarkers of cancer [35]. Detection of extracellular vesicles, and the molecular markers they contain, in blood or other clinical specimens represents a potentially valuable noninvasive approach for the assessment of tumor initiation, proliferation, and metastasis. Exosomes obtained from the serum of patients with pancreatic cancer were reported to carry genomic double-stranded DNAs (dsDNAs), which contained mutated *KRAS* and *p53* genes [36]. Exosome-associated molecular markers of gastric cancers have been detected in gastric washes and even highly acidic gastric juice [37–39]. Exosomes were purified from the gastric juice of gastric cancer patients, and the content of cancer-related methylated *LINE1* and methylated *SOX17* DNA was analyzed by bisulfite pyrosequencing [37]. The methylation status of *LINE1* and *SOX17* DNA in gastric juice-derived exosomes was found to accurately reflect the methylation status of nuclear DNA in the corresponding tumors, indicating a role in the noninvasive diagnosis of cancer.

## 3. The Role of Extracellular Vesicles in Histone Modification in the Cancer Microenvironment

Chromosomal DNA coils around structural histone proteins to form the basic chromatin structure, which is maintained or altered by histone modification. Relaxation of the chromatin

structure will expose more transcriptional regions of chromosomal DNA, inducing gene expression [40, 41]. Histone modification and, frequently, concomitant methylation have been implicated in pathogenic expression of tumor-related genes, and thus chromatic remodeling represents another potentially detectable biomarker for cancer [42]. Posttranslational modification of specific residues in the N-terminal tails of core histones, including acetylation, methylation, ubiquitination, or phosphorylation influence the chromatin shape, and thus transcription of associated genes [43–45]. Methylation of H3K4, H3K48, and H3K79 is commonly associated with gene activation, whereas methylation of H3K9 and H3K27 is associated with gene inactivation [46, 47]. Histone acetylation is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). The latter removes the positive charge on histones, thereby relaxing the condensed chromatin structure to promote gene transcription [48, 49]. The cancer microenvironment may thus influence epigenetic regulation of cancer-related gene expression by DNA modification and histone modification.

The role of extracellular vesicles in histone modification is currently controversial. Bioinformatic analysis has indicated a striking overlap between genes relevant to transgenerational epigenetic inheritance and the contents of exosomes released by a variety of cells, including cancer cells [24, 50–52]. By examining the *GO* biological processes associated with these overlapping mRNAs and proteins, it appears that the exosome content does not affect cellular activities indiscriminately but is focused on a limited network of processes including several processes related to epigenetic modification. Genes relevant to histone acetylation or deacetylation, histone ubiquitination, and other histone modifications, and even chromatin remodeling, represent a remarkably large proportion of those genes determined to be relevant to transgenerational epigenetic inheritance [24]. These findings indicate that exosomal mRNAs and proteins may directly or indirectly participate in both the response to environmental exposure and epigenetic modification, particularly histone modification. Additional analyses of exosomal miRNAs have indicated a similar association between environmental exposure and histone modification [50].

In the cancer microenvironment, these extracellular vesicle-related processes may participate in cancer initiation, proliferation, and metastasis. One cancer cell line, the G26/24 oligodendroglioma cell line, was reported to release extracellular vesicles containing the differentiation-specific linker histone H1°, which is not released by normal astrocytes [53]. The H1 histone family is the most divergent histone family and each H1 protein subtype or variant is associated with specific functions and distributions [54–57]. H1° is mostly associated with terminal differentiation [58, 59]. Although the precise pathophysiological significance of this phenomenon remains to be understood, enrichment of histone H1° in cancer cell-derived extracellular vesicles represents a promising potential molecular marker for oligodendroglioma diagnosis. Furthermore, extracellular vesicles containing histone H1° may subsequently influence recipient cells in the cancer microenvironment.

## 4. The Role of Extracellular Vesicles in Transmitting Noncoding RNA in the Cancer Microenvironment

Noncoding RNA refers to nonprotein coding transcripts, categorized as lncRNAs and small noncoding RNAs, including miRNAs, according to their length. Circulating tumor-associated miRNAs were first detected in the serum of a patient with diffuse large B-cell lymphoma [60], and the potential for circulating noncoding RNAs to act as noninvasive biomarkers of cancer diagnosis spurred accelerated research in this field. Circulating miRNAs were found to be present in plasma in a stable form that was protected from endogenous RNase activity [61], and recently noncoding RNAs were determined to be packaged in extracellular vesicles secreted by tumor cells, explaining why circulating RNAs are stable and thus detectable in the serum [62]. The capacity of extracellular vesicles containing noncoding RNAs to facilitate cell-to-cell communication and alter the cancer microenvironment remains to be seen; however, the potential for noncoding RNAs in extracellular vesicles to reflect the status of cancer cells or cancer-related immune cells is clinically promising. The lncRNAs and miRNAs contained in extracellular vesicles represent promising molecular markers of cancer diagnosis and prognosis assessment.

*4.1. The Role of Extracellular Vesicles in Transmitting MicroRNAs in the Cancer Microenvironment.* MiRNAs are small noncoding RNA molecules of about 22–25 nucleotides which act to silence RNA through posttranscriptional epigenetic regulation [63, 64]. Probably owing to their relatively small size, miRNAs are the most abundant RNA species in exosomes, making up over 42.32% of all raw reads and 76.20% of all mappable reads in 14 size-selected sequencing libraries [65]. The loading of miRNAs into exosomes may be controlled by specific proteins involved in the miRNA network. GW182 is a protein marker of P-bodies which can bind the Argonaute2 (AGO2) protein. The presence of AGO2 protein and striking enrichment of GW182 in purified monocyte-derived exosome-like vesicles suggests the specific and selective loading of miRNAs into exosomes [66–69]. Furthermore the ceramide-dependent machinery has been reported to regulate release of miRNAs [69].

Metastatic cancer cells shed particular types of miRNAs in extracellular vesicles. Microvesicles released from metastatic melanoma cells contain high levels of prominin-1, which promotes metastatic progression [70–72]. Micro-RNA profiling revealed 49 species of miRNA present at higher concentrations in these metastatic-melanoma derived microvesicles than in donor cells, including 20 species of cancer-related miRNAs. The invasiveness of bone marrow-derived stromal cells was found to be increased following exposure to prominin-1 expressing exosomes [73]. In metastatic gastric cancer, the *let-7* miRNA family is selectively secreted into the extracellular environment via exosomes [74], inducing a prometastatic phenotype in selected host tissues. The exosomes released by the metastatic rat adenocarcinoma BSp73ASML contain higher levels of *miR-494* and *miR-542-3p* than the exosomes of poorly metastatic BSp73ASML

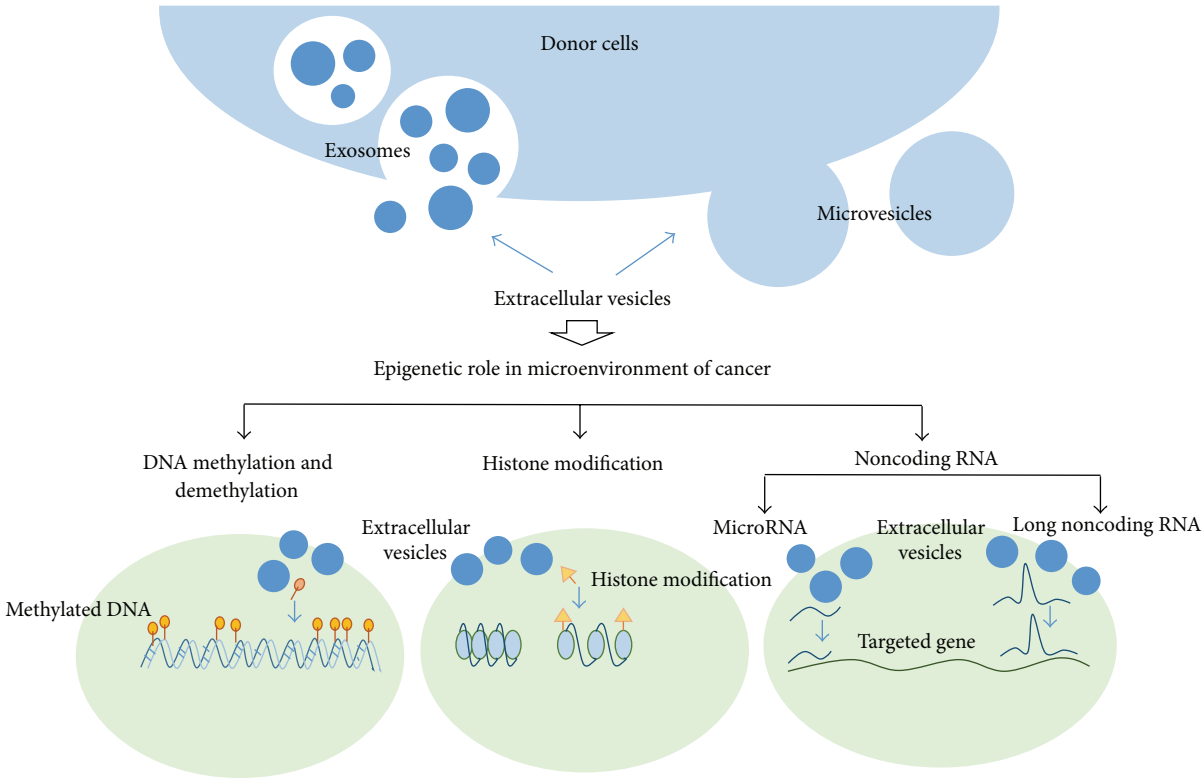


Figure 1: Various extracellular vesicles derived from donor cells play various epigenetic roles in microenvironment of cancer, including DNA modification, histone modification, and noncoding RNA regulations.

CD44v4-v7 knockdown cells [75]. The mRNA and miRNA content of extracellular vesicles derived from cancer stem cells also differ from those derived from differentiated cancer cells. *miR-29a*, *miR-650*, and *miR-151* are associated with tumor invasion and metastases, and *miR-19b*, *miR-29c*, and *miR-151* are upregulated in renal carcinomas and stimulate formation of a lung premetastatic niche [76]. Exosomes derived from chronic myelogenous leukemia cells were shuttled into endothelial cells, causing modulation of their motility and adhesion. This process was associated with exosome content of miR-126, which was concentrated in chronic myelogenous leukemia cell exosomes [77]. Brain metastatic cancer cells released miRNA-181c-containing extracellular vesicles which disrupt the blood-brain barrier [78]. Exosome-mediated transfer of cancer-secreted miR-105 disrupts tight junctions to promote metastasis [79]. Exosomal transfer of miRNA-23b from the bone marrow promotes breast cancer cell dormancy in a metastatic niche [80]. miR-210, released by metastatic cancer cells, could be transported to endothelial cells and regulate cancer cell metastasis [81]. MiRNAs in extracellular vesicles also modulate tumor proliferation [82, 83], and several extracellular vesicle miRNAs have been recommended for cancer diagnosis or assessment of cancer prognosis [10, 26, 37].

*4.2. The Role of Extracellular Vesicles in Transmitting Long Noncoding RNAs in the Cancer Microenvironment.* LncRNAs are nonprotein coding transcripts longer than 200 nucleotides [84] which can participate in epigenetic transcriptional or posttranscriptional regulation. Extracellular vesicle lncRNAs have received less attention than miRNAs, but in the previously described sequence analysis of 14 size-selected sequencing libraries, lncRNAs were found to be the most abundant exosomal RNA species after miRNAs and ribosomal RNA, representing 3.36% of all mappable, countable RNAs [65].

As previously described for miRNAs, the lncRNA content of exosomes differs from that of donor cells, indicating selective secretion of lncRNAs [85]. In two cancer cell lines, HeLa and MCF-7, the difference between the exosome and donor cell content of six lncRNAs (*MALAT1*, *HOTAIR*, *lincRNA-p21*, *GAS5*, *TUG1*, and *CCND1-ncRNA*) was assessed in donor cells under DNA damage stress [86]. Whilst *MALAT1* is prevalent in donor cells, the *MALAT1* level in exosomes turns out to be relatively low; in contrast *lincRNA-p21* is enormously enriched in exosomes. Under cellular stress, the cellular content and selective loading of lncRNAs to extracellular vesicles was altered, indicating the packaging of lncRNAs in extracellular vesicles changes in response to the cancer microenvironment, potentially facilitating adaptation or opposition to the stress environment.

Extracellular vesicle content of lncRNA may reflect tumor growth, metastasis, and response to treatment. LncRNA *TUC339*, found in extracellular vesicles derived from hepatocellular carcinoma cells (HCC), has been implicated in tumor growth, adhesion and cell cycle progression [87, 88].

*Linc-ROR*, another lncRNA enriched in extracellular vesicles from HCC, protects cancer cells from chemotherapy-induced apoptosis and cytotoxicity [89], and *MALAT1*, an lncRNA enriched in extracellular vesicles from cervical carcinoma and breast cancer cells [86], was associated with tumor metastasis and invasion [90, 91]. The potential for *MALAT1* to act as a blood-based biomarker for the diagnosis of nonsmall cell lung cancer is currently being evaluated [92].

## 5. Conclusions

Extracellular vesicles originating from cancer cells contain several types of biomolecules including oncogenes and molecules capable of epigenetic reprogramming. They are shed to the cancer microenvironment and may promote cancer progression [93, 94]. Epigenetic regulation appears to play a major role in this process (Figure 1). Many of the mRNAs and proteins present in extracellular vesicles are ascribed GO biological processes related to epigenetic regulation [24]. Recipient cell methyltransferase and cytidine deaminase can be downregulated by onco-mRNA in microvesicles derived from leukemia cells [34]. LncRNA *TUC339*, found in extracellular vesicles from hepatocellular carcinoma cells, participates in tumor growth, adhesion, and cell cycle progression [87, 88]. Furthermore, detection of the biomarkers present in extracellular vesicles represents a promising, noninvasive method of cancer diagnosis. For instance, methylated *LINE1* and methylated *SOX17* DNA accumulated in gastric juice-derived exosomes [37], and *lincRNA-p21* was detected in extracellular vesicles from cervical carcinoma cells and breast cancer cells [86]. In addition, due to their capacity to mediate intercellular communication, extracellular vesicles may represent targets for therapeutic intervention, and both native extracellular vesicles and artificially engineered vesicles represent promising new tools for drug delivery [22, 23].

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

Zhongrun Qian and Qi Shen contributed equally to this paper.

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*Review Article*

# Role of Extracellular Vesicles in Hematological Malignancies

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In recent years the role of tumor microenvironment in the progression of hematological malignancies has been widely recognized. Recent studies have focused on how cancer cells communicate within the microenvironment. Among several factors (cytokines, growth factors, and ECM molecules), a key role has been attributed to extracellular vesicles (EV), released from different cell types. EV (microvesicles and exosomes) may affect stroma remodeling, host cell functions, and tumor angiogenesis by inducing gene expression modulation in target cells, thus promoting cancer progression and metastasis. Microvesicles and exosomes can be recovered from the blood and other body fluids of cancer patients and contain and deliver genetic and proteomic contents that reflect the cell of origin, thus constituting a source of new predictive biomarkers involved in cancer development and serving as possible targets for therapies. Moreover, due to their specific cell-tropism and bioavailability, EV can be considered natural vehicles suitable for drug delivery. Here we will discuss the recent advances in the field of EV as actors in hematological cancer progression, pointing out the role of these vesicles in the tumor-host interplay and in their use as biomarkers for hematological malignancies.

## 1. Introduction

Cell-to-cell communication is necessary in order to maintain a social and functional order among different cell types within tissues. A number of intercellular communication mechanisms mediated, for example, by soluble factors, extracellular matrix components, ion channels, tunneling nanotubules, and extracellular vesicles (EV) have been described [1].

EV are plasma membrane fragments that include, among several others, microvesicles (MV) and exosomes [2]. MV enclose a heterogeneous population of vesicles with a size greater than 100 nm in diameter and are generated by direct budding off from the plasma membrane; Umezu and colleagues; miR-135b; NF-$\kappa$B [3]. The rate of release of MV is generally low, except for cancer cells, which show an intense surface activity and seem to release them constitutively. Exosomes are vesicles of size ranging from 30 to 100 nm in diameter and are released into the extracellular compartment when the multivesicular bodies (MVB) fuse with the plasma membrane [3]. The secretion of exosomes can be spontaneous or induced, for example, by cell surface receptor activation [2–4]. The mechanisms of assembly and sorting of exosomes are not well defined, but several molecules have been shown to regulate this process, such as RAB11, RAB27, RAB35, and syndecan-syntenin-ALIX [5–8]. Moreover the ESCRT (endosomal sorting complex required for transport) member TSG101 (tumor susceptibility gene 101) and the tetraspanin CD63, which is enriched in specific plasma membrane domains involved in microvesicle budding, have both been described as involved in exosome formation [9]. Recently, the consideration of the tetraspanin CD63 as a specific exosomal marker has been reevaluated, since this protein has also been found in other EV subtypes [10].

The molecular composition of EV varies depending on the type and functional state of the producing cell. During cancer progression, exosomes, released from malignant cells, are enriched in tumor antigens: for example, exosomes isolated from ovarian or breast cancer ascites contain tumor specific antigens HER2/NEU, while MART1 is found in nanovesicles recovered from serum of patients with melanoma [11].

In addition to proteins and specific lipids, coding and noncoding RNAs and DNA may be present in EV [1, 12]. The molecular components found in EV could be transferred from one cell to another by endocytosis or fusion with the recipient cell [13, 14]. Of biological interest, the transferred components are functional in target cells and affect the phenotype of these cells by modulating gene expression [1, 12, 15, 16]. Thus, EV-mediated intercellular communication includes the binding of cell-surface receptors on target cells, followed by induction of cell signaling pathways, transfer and translation of mRNAs, transfer of microRNAs (miRNAs), silencing of mRNA targets, transfer of proteins, and activation of functions. Moreover, after their release into the circulation, EV-dependent signaling can occur not only locally, but also in a paracrine and systemic manner [17, 18]. Even if microvesiculation is a mechanism occurring in all eukaryotic cells, the amount of EV in the blood is increased in several pathological conditions, including Alzheimer's disease, Crohn's, immunological disorders, and cancer [19]. EV have been shown to support tumor invasiveness and metastasis, as well as enhancing angiogenesis. In recent years the scientific community has focused its attention on the specific properties of the tumor microenvironment and how these depend on altered intercellular communication between malignant cells and nonmalignant cells of the host. A number of studies have focused on the role played by cancer-cell-derived EV. For example, the transformation of fibroblasts and epithelial cells upon uptake of glioblastoma- and breast-cancer-cell-derived EV was partly dependent on the transfer of fibronectin and transglutaminase [20]. Cancer cells induce adaptive mechanisms that rely on phenotypic modulation of stromal cells in the neighboring tissue and the recruitment of bone-marrow-derived progenitors from the circulation. This adaptive remodeling of the tumor microenvironment, involving the induction of a large number of effector molecules, ultimately serves to promote the survival and dissemination of malignant cells. EV can modulate the stroma, thus promoting cancer progression and metastasis. Peinado et al. have recently shown that cancer-derived exosomes modulate the crosstalk between malignant cells and the bone marrow (BM) microenvironment. They reported, for the first time, that metastatic melanoma cells release exosomes that are able to "educate" BM progenitors, thereby inducing their mobilization, which supports tumor vasculogenesis, invasion, and metastasis, through the activation of the MET receptor tyrosine kinase. They found that the MET-activated signaling proteins are expressed in highly metastatic melanoma derived exosomes and that the transfer of the exosomal receptor tyrosine kinase MET from tumor-derived exosomes to BM progenitor cells promotes the metastatic process *in vivo*. These results suggest that BM cells retain the educated phenotype after engraftment into a new host [21]. A recent study by Melo et al. presents new and significant data on the role of exosome-shuttled miRNAs in cancer progression. In particular, the authors show that exosomes derived from breast cancer cells, and sera from patients, contain DICER and process pre-miRNAs into mature miRNAs, thus modulating the recipient cell phenotype. This study points out a new role for exosomes, which can be considered not only a shuttle for miRNAs, but also as machinery for miRNA biogenesis [22].

Hypoxia is a common feature of the microenvironment of malignant tumors [23, 24] and is associated with tumor aggression and invasiveness. These effects are mediated, for example, by hypoxic regulation of cytokine release [25] and regulation of tumor suppressors and oncogenes [26]. Several reports have shown a correlation between hypoxia, tumor progression, and exosome release. For example, King et al. found a hypoxic enhancement of exosome release by breast cancer cells [27]. Moreover, exosomes reflect the hypoxic status of tumor cells by mediating microvascular endothelial cell (EC) migration and vasculogenesis [28, 29].

Here we will focus on the role of EV in hematological malignancies and in particular on (i) the role of EV in the crosstalk between cancer cells and the BM microenvironment; (ii) the role of cancer-cell-secreted EV in angiogenesis; and (iii) the role of EV as biomarkers of hematologic malignancies and in mechanisms of drug resistance.

## 2. EV in the Crosstalk with the BM Microenvironment

The importance of tumor microenvironment for cancer progression has, in recent years, been widely recognized. The microenvironment provides crucial signaling to maintain tissue architecture, inhibit cell growth, and modulate differentiated phenotypes. On the other hand, incorrect signals from the microenvironment may lead to destabilization of tissue homeostasis and to the initiation/promotion of normal cells to malignancy. Furthermore, the interaction of cancer cells with their stromal microenvironment overcomes the physiological barrier function of stromal cells and may also modulate the invasive and metastatic phenotype of the cancer cells, as well as angiogenesis [30]. Several studies in the last five years have indicated that EV are important components of the tumor microenvironment and, mediating cell-cell communication, are currently considered one of the contributors to tumor progression and metastasis [31]. Moreover, EV have been found to contribute not only to primary tumor growth, but also to the recruitment of microenvironment resident cells such as ECs or leukocytes (e.g., macrophages, dendritic cells, and T- and B-cells) [17, 32, 33]. Therefore, coevolution of the tumor microenvironment with primary tumor cells is promoted by the crosstalk of different cell types in a tumor EV-dependent manner [34].

BM is the specific microenvironment of hematological disease and is composed of a dynamic network of stromal cells and soluble factors, such as growth factors and cytokines, thus providing a permissive environment for leukemogenesis

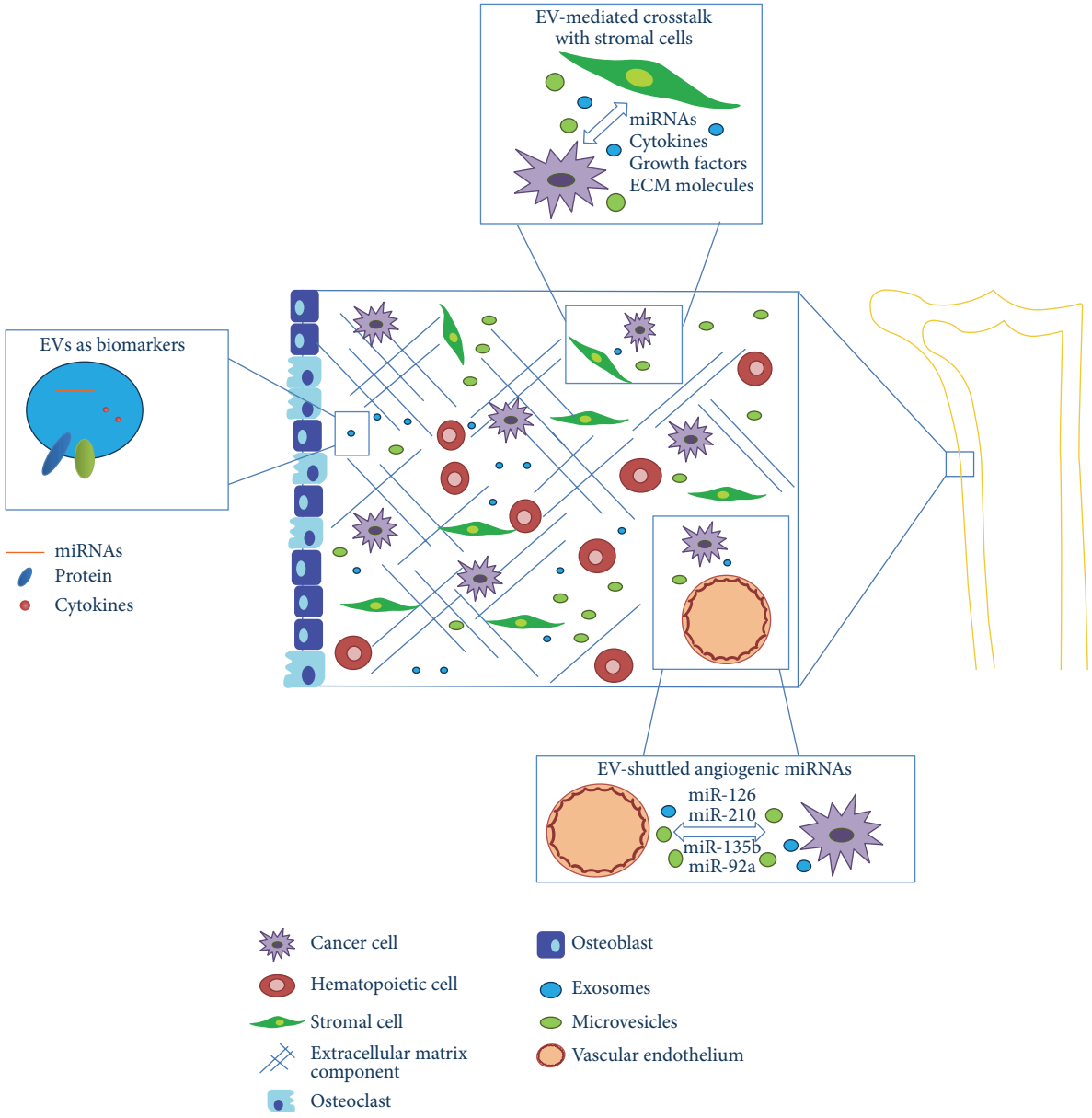


Figure 1: The bone marrow niche and the major constituents relevant to hematological cancer progression: extracellular vesicles (exosomes and microvesicles) are integral part of tumor microenvironment, contributing to the development of a suitable niche for hematological cancer development. Extracellular vesicles released by different cell types allow the bidirectional communication among cancer cells and normal host cells.

and cancer progression (Figure 1). In acute myeloid leukemia (AML), exosomes released from leukemia cells alter the proliferative and migratory responses of cocultured stromal and hematopoietic progenitor cells, helping to explain how the microenvironmental niche becomes reprogrammed during invasion of the BM by AML cells. Specifically, Huan et al. showed the presence of transcripts with prognostic relevance in AML, human CXCR4 and IGF-IR (insulin-like growth factor-I receptor) mRNA, in murine BM stromal cells, thus demonstrating that AML exosomes transfer leukemia-derived mRNAs to BM stromal cells. Moreover, they found that exosomal transfer of IGF-IR can modulate proliferative signaling in bystander cells and can promote expression of VEGF, with the final establishment of conditions that contribute to leukemic spread [35]. We recently provided data showing that chronic-myeloid-leukemia- (CML-) derived exosomes are able to stimulate bone marrow stromal cells (BMSC) to release IL8, which acts as an *in vitro* and *in vivo* prosurvival factor for CML cells. The inhibition of IL8 receptors, using SB225002, was able to abrogate the IL8-driven CML cell survival *in vitro* as well as the growth of CML xenograft *in vivo*, thus indicating a key role of CXCLl8/CXCR1-2 signaling in the growth of CML cells [36]. It is conceivable that IL8 secreted by the BM and ECs

under the stimulation of CML exosomes may modulate both myeloid malignant cells and the BM cellular compartment, thus generating a paracrine loop between hematopoietic malignant cells and resident cells. Our unpublished data further shows that the CML-exosome-mediated release of IL8 by BMSC relies on the activation of the EGF receptor pathway. On the other hand, IL8 is able to activate, in CML cells, an AXL mediated pathway that could be responsible for leukemia cell survival (unpublished data). Ghosh et al. found that EV isolated from plasma of B-cell chronic lymphocytic leukemia (CLL) patients can interact and modulate BMSC, thus providing a "*homing and nurturing*" environment for CLL B cells. In particular, they demonstrated that CLL-EV can maintain the AKT/mTOR/p70S6K/HIF-1$\alpha$ axis in a sustained state of activation and can potentially modulate the AKT/GSK3$\beta$ or AKT/$\beta$-catenin signaling pathways. This leads to the establishment of a tumor microenvironment that favors disease progression [37].

Cell-to-cell communication can also occur between leukemia cells and normal neutrophils, thus providing a mechanism for tumor development. Cai's group found that Bcr/Abl hybrid gene, involved in the pathogenesis of CML, could be transferred through K562 EV to normal neutrophils. Incubation of neutrophils with K562-EV for 24 hours resulted in the expression of the Bcr/Abl hybrid gene in 20% of the cells [38]. Moreover, the same group showed that injection via tail vein of K562 EV into Sprague-Dawley (SD) rats or NOD/SCID mice caused several symptoms of CML in the animals, such as weakness, loss of weight, splenomegaly, and neutrophilia, but reduced neutrophil phagocytic activity. Disease development was accompanied with *de novo* transcription, as well as protein synthesis of BCR/ABL *in vivo*, demonstrating that the transfer of the Bcr/Abl gene from CML-derived EV to neutrophils may promote *in vivo* transformation of normal cells [39]. During tumor development, neoplastic cells actively recruit cells of the immune system, which may provide an immunosuppressive and growth-promoting compartment [40]. Recently, numerous studies have shown that microenvironmental stressors such as low pH, heat, and oxidative stress modulate the molecular composition of EV [41]. For example, leukemia/lymphoma T- and B-cells under a thermal and oxidative stress release exosomes enriched in Natural Killer Group 2, member D (NKG2D) ligands, which abrogate NKG2D-mediated NK-cell cytotoxicity and, thus, may contribute to the immune evasion of leukemia/lymphoma cells [42]. Stromal cells, similarly to cancer cells, can respond to stress-related conditions within the tumor microenvironment by secretion of EV. For example, mesenchymal stem cells stimulated by hypoxia were shown to release MV with angiogenic potential [43]. Roccaro et al. reported that multiple-myeloma-BM-mesenchymal-stromal-cell- (MM-BMSC-) derived exosomes played a role in multiple myeloma (MM) disease progression *in vivo*. In particular, they showed that BMSC transfer exosomes containing miR-15a into MM cells, inducing their proliferation and survival. Importantly, while MM-BM-MSC–derived exosomes promoted MM tumor growth, normal BM-MSC exosomes inhibited the growth of MM cells. These observations suggest the establishment of paracrine growth circuits between BM-MSC and clonal plasma cells and that the BM niche, educated by tumor exosomes, provides an optimal substrate for MM cell localization and growth [44]. The data reported here underscore the importance of the tumor microenvironment for cancer progression. In particular, in the context of hematological malignancies, we found examples of the role of EV released by cells of hematological malignancies in the crosstalk with BM resident cells.

## 3. EV in Angiogenesis

The tumor vasculature is an important component of the tumor microenvironment. Tumors are endowed with angiogenic-inducing capability, and their growth, invasion, and metastasis are angiogenesis-dependent [45]. Over the last few years, many studies have focused their attention on the role of these vesicles in modulating angiogenic processes [46]. EV possess the ability to modulate tumor angiogenesis, depending on their origin and composition, by inducing changes in target cells or by delivering angiogenic proteins or miRNAs that can stimulate EC function. Here we will focus on the role of EV in hematological disorders. Increased angiogenesis has been observed in hematologic disorders, including acute and CML, acute and chronic lymphocytic leukemia, MM, and lymphomas [47–50]. BM microvessel density, a measure of tumor angiogenesis, is greater in patients with advanced myelodysplastic syndromes compared with normal individuals, thus confirming a central role of the process in these disorders [51]. In recent years, we and other groups have focused research on the role of exosomes derived from chronic myelogenous leukemia cells in promoting angiogenesis. Taverna et al. showed that exosomes released from CML cells directly affect ECs by modulating the process of neovascularization, both *in vitro* and *in vivo*, by inducing in ECs the release of proangiogenic cytokines, such as Interleukin-8 [52]. We have also shown that imatinib-resistant CML cells release exosomes with proangiogenic abilities, suggesting that the modulation of their function could be considered for new approaches in CML treatment [53]. Mineo et al. reported that exosomes released by K562 CML cells are internalized by ECs during tubular differentiation on Matrigel and are transferred to neighboring cells through the formation of nanotubular structures connecting the cells. Furthermore, the authors showed that these exosomes stimulate tube formation in ECs through SRC activation [54]. It is now widely known that EV contain miRNAs and that they are delivered in the tumor microenvironment [12, 55]. To date, many angiogenic miRNAs have been identified, and several have been shown to play important roles in exosome-mediated modulation of angiogenesis. Taverna et al. recently found that miR-126, a miRNA involved in angiogenesis, was expressed 6-fold more in LAMA84 exosomes compared with the parental cells. The exosomal transfer of miR-126 to ECs directly targeted the 3′ UTR of Cxcl12 and Vcam1 mRNA, thus modulating adhesive and migratory abilities of CML cells [56]. Other groups have found that exosomes released from K562 cells contain specific miRNAs, which enhanced EC migration and tube formation [57, 58].

Umezu et al. reported that miR-92 derived from K562 cells was transferred to ECs through exosomes, thus promoting endothelial migration and tube formation [57]. It is well established that hypoxia plays a role in boosting tumor angiogenesis. Many studies have shown that the exposure of human cancer cells to hypoxia augments microvesicle shedding in order to modify their microenvironment to facilitate tumor angiogenesis and metastasis [59]. Moreover, microarray and proteomic studies have highlighted the increase of many proangiogenic factors in EV released after hypoxic stimuli [58, 60]. Tadokoro and colleagues reported that the hypoxic human leukemia cell line K562 secretes exosomal miRNAs, which enhances tube formation in human umbilical vein ECs due to inhibition of the receptor tyrosine kinase ligand EPHRIN-A3. In particular, they found that hypoxic exosomes contained higher levels of miR-210, indicating a difference between normoxic and hypoxic exosomes [58]. In AML, the release of EV has been described *in vitro* as well as in the sera of patients affected with the disease. Kurre's group has shown that AML exosome trafficking alters the angiogenic responses of cocultured stromal and hematopoietic progenitor cell lines, thus influencing the invasion of the BM [35]. BM angiogenesis also plays an important role in the pathogenesis and progression of MM. The tumor-host interplay, driven by EV, in MM has been recently established. Liu et al. reported, for the first time, that myeloma RPMI 8226 cells can secrete MV harboring oncogenic CD138, a specific type of angiogenic regulator, and the incorporation of the MM-MVs into ECs leads to the reprogramming of the ECs. Specifically, exosome stimulation promotes EC proliferation and the invasion and the secretion of the proangiogenic factors IL6 and VEGF [61]. Recently, Umezu and colleagues have established an *in vitro* system of hypoxic-resistant multiple myeloma cells (HR-MM), which serves as a model of therapy-resistant MM cells. The authors showed that the amount of exosomes from HR-MM cells was significantly greater than that of parental cells and that specific miRNAs were present. They found that hypoxia-driven accelerated tube formation is attributable to exosomal miR-135b, an oncogenic miRNA shed from HR-MM cells. Specifically, they found that miR-135b directly targets and inhibits FIH-1, which is an asparaginyl hydroxylase enzyme binding to HIF-1$\alpha$, thus inhibiting its transactivation. In this way, exosomal miR-135b accelerated HIF-1 transcriptional activity via inhibition of FIH-1 [60]. Together, these data highlight the crucial role of EV in tumor-host communication, thus contributing to the progression and dissemination of cancer cells through the modulation of angiogenic processes.

## 4. EV as Biomarkers of Hematological Malignancies and Their Role in Mechanisms of Drug Resistance

The identification of new biomarkers differentially expressed in cell populations, as well as in patients, is of primary importance in the detection, diagnosis, and prognosis of cancer. Biologically active exosomes isolated from human plasma can be proposed as new and intriguing biological markers [62]. Because EV (both MV and exosomes) contain genetic and proteomic contents that reflect the cell of origin, it is possible to detect tumor-specific material in EV secreted by cancer cells.

It has been clearly shown that the release of both MV and exosomes is dramatically enhanced during cancer progression. Moreover, EV can be recovered from body fluids and the circulation of cancer patients, thereby constituting a source of new predictive biomarkers involved in cancer development and potentially serving as an information-rich prognostic [63–65]. Working as shuttles, EV can protect their cargo from nucleases and proteases, increasing the biomarker half-life and potentially facilitating sample integrity and downstream analyses [66]. EV-associated cancer biomarkers have been extensively described in a great number of solid cancers, such as prostate cancer, melanoma, and lung cancer [21, 67–70]. Furthermore, in patients affected with melanoma and other solid tumors, total protein levels of exosome fractions isolated from plasma were reported to reflect disease stage, tumor burden, response to therapy, and survival [21, 71, 72]. However, EV derived from hematological cancer cells have been less studied than those derived from solid tumor cells. An interesting study showed that in AML, exosomal protein levels may reflect the extent of disease and correlate with its relapse after therapy. Large differences were noted in exosomal protein levels among patients upon AML diagnosis, and the authors suggested that low exosomal protein levels may be predictive of long-term disease survival. In addition, change in exosomal protein levels may reflect response to chemotherapy, and the exosomal profile analysis may detect residual leukemia cells in patients considered in complete remission. More specifically, TGF-$\beta$1 levels were analyzed and compared among patient cohorts, and the changes in exosome-associated TGF-$\beta$1 levels were evaluated in relation to the therapy given to AML patients. The authors found that the TGF-$\beta$1 levels upon AML diagnosis were higher than those in exosomes of normal controls. Following chemotherapy treatment, TGF-$\beta$1 levels were significantly reduced, while patients in long-term complete remission had low exosomal TGF-$\beta$1 levels. The authors suggested that change in exosomal TGF-$\beta$1 levels may reflect responses to chemotherapy. According to the authors, these data reinforced the relevant role of AML-derived exosomes as potential diagnostic or prognostic biomarkers [71].

EV shuttle diverse RNA species, including mRNAs and miRNAs, to recipient cells, and affect the metabolism of target cells. In addition, several data have shown that miRNA content from their originating cancer cells is similar to that found in circulating exosomes [73]. Exosomes derived from both AML and CML cells were enriched for several coding and noncoding RNAs relevant to both cancer prognosis and treatment, as well as to the leukemic niche function. Still, CML-derived exosomes are characterized by selectively expressed miRNAs and plausibly suggested as possible future biomarkers [35]. A recent study identified miR-155 as a useful biomarker in individuals with monoclonal B-cell lymphocytosis and in patients with B chronic lymphocytic leukemia. The authors found higher miR-155 levels expression in patients who failed to respond to chemotherapy compared

with those who experienced complete response. Furthermore, the authors identified miR-155 in circulating MV from both individuals with monoclonal B-cell lymphocytosis and patients with chronic lymphocytic leukemia [74].

Because the residual disease in patients considered in complete remission is difficult to investigate with conventional methods, the need to elaborate alternative tests for detecting residual disease will become more pressing in future studies. At present, miRNA-based clinical trials involving exosomes have not been initiated because an improved characterization of these carriers and their cargos in normal and disease models is still needed [41].

The development of chemotherapeutic resistance is one of the major factors that contribute to cancer mortality. Though a combination of molecular mechanisms is responsible for drug resistance, the role played by EV in modulating the acquisition of the chemoresistant phenotype is increasingly emerging [41, 75–79]. The effect of BMSC-derived exosomes on the survival and drug resistance of MM cells, using both a murine model and human MM samples, has been investigated in MM. The authors found drug resistance to bortezomib in MM cells treated with BMSC-derived exosomes, as well as the activation of several pathways related to drug resistance and cell survival, such as NOTCH1, STAT3, NF-$\kappa$B, and AKT [80]. In another study, Aung et al. found, both *in vitro* and *in vivo*, a strong exosome production and release from aggressive B-cell lymphoma cells. Such lymphoma-derived exosomes carried the protein CD20, exposed in the membrane, able to intercept rituximab, and thus allowing lymphoma cells to escape from humoral immunotherapy [81]. Because exosomal CD20 is a decoy target for rituximab, the authors suggested that the drug sequestered by circulating exosomes may reduce the efficacy of pharmacological treatment [81].

Cancer cells exposed to chemotherapeutic agents are able to expel drugs in extracellular compartments using specialized transporters of the multidrug resistant ATP binding-cassette transporter (ABC transporter). Recent studies have indicated that leukemia cells express ABCA3 transporters and that their expression is correlated with decreased susceptibility to cytostatic therapy. ABCA3 is localized inside the limiting membranes of multivesicular bodies in which drugs are efficiently sequestered [81–84]. It is now clear that exosomes from different malignant cells carry ABC transporters, and some studies have shown that drugs may also be expelled from the cells through exosomal mechanisms [41, 81, 85, 86]. Finally, immunotherapy studies in aggressive B-cell lymphoma demonstrated for the first time that exosomal evasion of humoral immunotherapy was modulated by ABCA3 [81].

Collectively, these studies provide evidence that EV in hematological malignancies play an important role in cancer drug resistance, thus influencing response to therapy and promoting cancer progression. Advances in the understanding of cancer-derived EV biology, as well as in the development of new sophisticated technologies aimed at isolating and characterizing EV, will be clinically relevant to the identification of new prognostic or predictive biomarkers in hematological malignancies.

## 5. Concluding Remarks

EV have been widely recognized as important actors in cell-cell communication by delivering messages among different cell types. This finding has opened new questions in the field of cancer research, allowing a new interpretation of the tumor microenvironment and of targeted therapies. Here we focused on the role of EV in hematological malignancies, reporting evidence of the importance of these vesicles in modulating cancer properties. Furthermore, EV components can now be considered biomarkers in hematological neoplasia, thus providing important findings for the early diagnosis of these malignancies and the design of alternative approaches to cancer therapy.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Stefania Raimondo and Chiara Corrado contributed equally to this work.

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*Review Article*

# Extracellular Membrane Vesicles as Vehicles for Brain Cell-to-Cell Interactions in Physiological as well as Pathological Conditions

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Extracellular vesicles are involved in a great variety of physiological events occurring in the nervous system, such as cross talk among neurons and glial cells in synapse development and function, integrated neuronal plasticity, neuronal-glial metabolic exchanges, and synthesis and dynamic renewal of myelin. Many of these EV-mediated processes depend on the exchange of proteins, mRNAs, and noncoding RNAs, including miRNAs, which occurs among glial and neuronal cells. In addition, production and exchange of EVs can be modified under pathological conditions, such as brain cancer and neurodegeneration. Like other cancer cells, brain tumours can use EVs to secrete factors, which allow escaping from immune surveillance, and to transfer molecules into the surrounding cells, thus transforming their phenotype. Moreover, EVs can function as a way to discard material dangerous to cancer cells, such as differentiation-inducing proteins, and even drugs. Intriguingly, EVs seem to be also involved in spreading through the brain of aggregated proteins, such as prions and aggregated tau protein. Finally, EVs can carry useful biomarkers for the early diagnosis of diseases. Herein we summarize possible roles of EVs in brain physiological functions and discuss their involvement in the horizontal spreading, from cell to cell, of both cancer and neurodegenerative pathologies.

## 1. Introduction

Extracellular vesicles (EVs) are membrane structures that can be divided into two subgroups: membrane vesicles (MVs), also named ectosomes [1], that derive from plasma membrane exocytosis and have dimensions in the range of 100 nm–1 $\mu$m and exosomes that are smaller vesicles of 50–100 nm in diameter, generated by exocytosis of multivesicular bodies (MVBs) [2]. The two classes of EVs share membrane components, but each of them also contains peculiar proteins, some of which are cell-specific [1]. Lipid composition is also peculiar; MVBs are enriched with specific lipids, probably involved in membrane budding, such as cholesterol, the unconventional phospholipid lysobisphosphatidic acid, sphingomyelins, and ceramide [1, 3–6]. Similarly, cholesterol, sphingomyelin, and ceramide also segregate during ectosomes formation at the plasma membrane [7]. It has been suggested that sorting of proteins to ectosomes is largely based on their interaction with the membrane through their lipid anchors which concentrate them to specific plasma membrane microdomains [8, 9].

Specific enrichment of both proteins and lipids in EVs not only suggests the existence of underlying regulatory mechanisms, which allow selection of cargos, but also points to an important role of EVs in the normal tissue physiology. As a confirmation of such a central role, it is now clear that vesicle release is a universal phenomenon, representing a novel and significant way to shuttle different molecules, such as protein, DNA, and RNA among cells, not only in eukaryotic organisms, but also in prokaryotes [10, 11].

Moreover, although vesicle release was initially discovered in tumor cells [12–16], vesicles are clearly produced by several nontumor cells [17–21] and can reach most biological fluids, such as blood plasma [22], synovial fluids [23, 24], breast milk [25], and saliva [26, 27], where, in addition, their concentration can dramatically change under pathological conditions.

Although EVs have been finally accepted as significant vehicles for cell-to-cell communication and they have been identified in a variety of organisms and conditions, two main problems remain, however, unsolved:

(1) the actual role of these vesicles in the integrated behavior of tissues and organs;

(2) the reason why production of EVs and specific sorting of cargos to them are altered in pathological conditions as different as cancer and neurodegeneration.

Moreover, since most studies have been performed on EV mixtures, it is still unclear whether these different classes of vesicles are equally involved in physiological and pathological processes.

Here we summarize the possible physiological roles of EVs in the nervous system and discuss their involvement in the horizontal transfer of brain pathologies.

## 2. Vesicles in Brain Normal Cells

Cell-to-cell communication is determinant for the right mammalian brain maturation to regulate differentiation of neurons, endothelial and glial cells, as well as allow formation and stabilization of synapses. Cross talk among different classes of brain cells is also essential to generate the blood-brain barrier (BBB), which then maintains brain internal milieu, by controlling trafficking of molecules and ions between the brain and the blood [28]. For example, in a transwell coculture system, containing both rat cortical astrocytes and neurons, brain capillary endothelial cells (BCECs) were found to form over time a functional barrier layer, even in the absence of cell-to-cell contacts [29, 30]. Further analyses demonstrated that both neurons [19] and astrocytes [20] release into the medium, at least in part by EVs, Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor 2 (FGF-2; also known as basic FGF), two growth factors which promote vascularization of developing brain. Both factors are also involved in abnormal neovascularization processes which accompany several pathological conditions [31, 32]. Interestingly, FGF-2 has been known since a long time ago to be secreted in spite of lacking a conventional secretion sequence and independently of the classical cell secretion pathway [33].

One of the main problems in studying metabolism of EVs has been to understand how their content reaches target cells. There is now evidence that their cargos can be at least partially transferred into the recipient cells, through fusion of EVs to the target plasma membranes. For example, after being released via EVs from metabolically labeled astrocytes/neurons, radioactive proteins have been found in unlabeled endothelial cells (Figure 1).

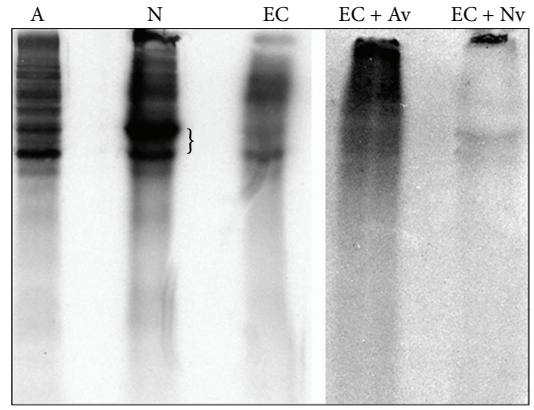


Figure 1: Fluorography of total cell lysates from astrocytes (A), neurons (N), and endothelial cells (EC), metabolically labeled with $^{35}$S-methionine, as well as from unlabeled endothelial cells, incubated for 24 hours with microvesicles shed from labeled astrocytes (EC + Av) or neurons (EC + Nv). The brackets indicate bands present in both labeled neurons and endothelial cells incubated with vesicles shed from neurons.

As mentioned, extracellular vesicles also contain mRNAs and different species of noncoding RNA. Given the importance of RNA-binding proteins (RBPs) in posttranscriptional regulation, mRNAs in vesicles are probably bound to proteins in the form of ribonucleoprotein particles. In agreement with this hypothesis, a microarray analysis, performed on vesicles purified from the cerebrospinal fluid (CSF), recently demonstrated the presence of RNAs containing the recognition sequence for hnRNPA2/B1, an RBP present in the same vesicles, probably in sumoylated form. Moreover, sumoylation has been shown to control hnRNPA2/B1 binding to miRNAs and sorting of these RNAs to exosomes [34]. Both the amount of vesicles and the number of mRNA molecules which bear the hnRNPA2/B1 recognition sequence decrease with development [35].

When we consider the complexity of vesicle cargos, it is not surprising that EVs also contain molecular chaperones and other factors which can regulate protein folding as well as protein-protein and protein-nucleic acid interactions. MVs and exosomes released from astrocytes not only contain, for example, Hsc70/Hsp70 [36, 37] and synapsin I [38], but also matrix metalloproteinases [39], which could be involved in extracellular matrix remodeling. This latter ability is fundamental for tumor cells invasion and migration; however, which function could it have in normal astrocytes or neurons? To answer the question, it could be useful to consider that most brain cells are able to grow branched cellular processes, which can explore the environment, migrate, and contact other cells; thus, an intrinsically high ability to modify the surrounding environment is critical for these cells.

Interestingly, *in vitro* studies demonstrated that release of exosomes from neurons can be modulated by synaptic activity [40]; by functioning as vehicles for both anterograde and retrograde information transfer, exosomes could be then involved in synaptic plasticity and long-term memory [41].

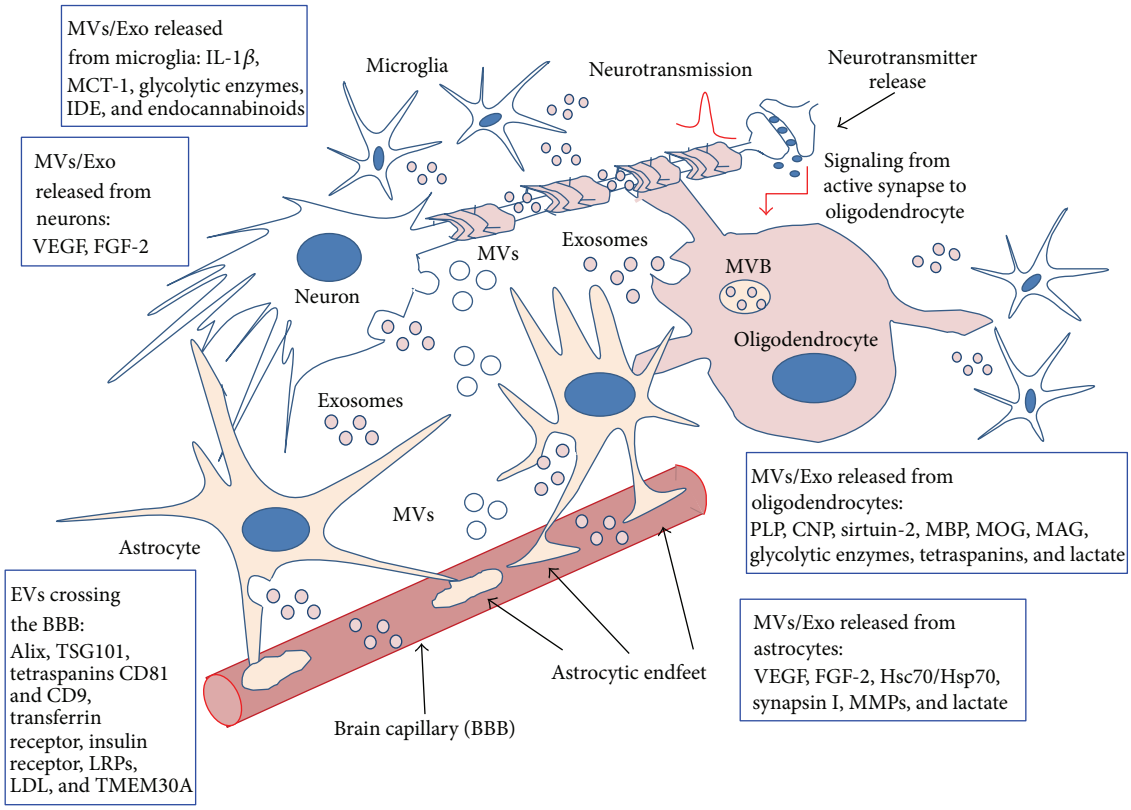


Figure 2: Extracellular membrane vesicles as vehicles for brain cell-to-cell interactions. As shown, all kinds of brain cells can both produce EVs and receive those produced by surrounding cells; this continuous exchange could be a fundamental source of metabolic coupling among neurons and glial cells. Vesicle trafficking from glial cells to neurons has been also suggested to be regulated by neurotransmission, as indicated by the red arrow in the figure. A few examples of molecules present in EVs released from the various brain cell types are given in the boxes. More details and related references are given in the text.

Vesicles are also released from oligodendrocytes, the glial cells responsible in the CNS for producing the myelin sheath which coats the axons, allowing fast impulse conduction; in addition, like astrocytes, oligodendrocytes have a trophic function and provide neurons with energetic substrates, such as lactate [42–44]. The continuous axon-oligodendrocyte cross talk seems to be mostly based on transfer of vesicles [42] which contain myelin proteins, such as proteolipid protein (PLP), 2′3′-cyclic-nucleotide 3′-phosphodiesterase (CNP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), NAD-dependent deacetylase sirtuin-2, glycolytic enzymes, heat-shock proteins, and tetraspanins [45]. It has been also reported that proximal segments of transected sciatic nerves accumulate newly synthesized RNA in axons and that these mRNAs are actually synthesized in Schwann cells and then transferred to neurons through a mechanism that requires actin cytoskeleton and myosin-Va [46].

Most important, vesicle trafficking from glial cells to neurons has been suggested to be regulated by neurotransmission (Figure 2): an increase of cytosolic $Ca^{2+}$ levels in oligodendrocytes, due to activation of glutamate receptors, present on glial cell membrane, induces exosome release [47]. Actually, active neurons should ask oligodendrocytes for metabolites, regulatory proteins, glycolytic enzymes, mRNAs, and miRNAs [48].

Transfer of mRNAs from glial cells to neurons might be of special interest when we consider that localized axonal synthesis may allow remodeling of growing (or regenerating) axons during progression through their extracellular environment. Although translation of localized mRNAs in axons has been debated for a long time [49], periaxoplasmic ribosomal plaques (PARPs) have been only recently described, which contain ribosomes attached to a plaque-like structure, also enriched with $\beta$-actin mRNA, molecular motors, and RNA-binding proteins [50]. Moreover, multiple translation components, including ribosomal subunits and initiation factors, interact with the transmembrane receptor (DCC) for netrin-1, suggesting that their activity can be regulated by extracellular signals [51].

The exciting possibility that ribosomes and mRNAs could be horizontally delivered from surrounding glial cells to axons has been also proposed [52, 53]. For example, Court and colleagues showed that GFP-tagged polyribosomes produced by glial cells can be transferred to axons both *in vitro* [52] and *in vivo* [54].

These findings support the idea that glial cells may contribute to local axonal protein synthesis by supplying protein synthetic machinery and specific mRNAs [55].

Another important class of brain cells is constituted by microglia, the resident macrophages of the brain, which provide the defense during infection and brain injury, and are implicated also in tissue repair. During disease, microglia acquire an activated phenotype, and release soluble mediators, to induce and maintain the inflammatory response. There is also evidence indicating that reactive microglia have the capability to release vesicles of irregular shape and size, characterized by high levels of externalized phosphatidylserine (PS) [56]. These vesicles contain IL-1$\beta$ that may induce and propagate inflammatory reactions in the brain [56, 57]. In addition, microglial MVs, like other glial cell types (see above), are able to modulate synaptic activity and neurotransmission [58]. For example, EVs secreted by microglia have been recently shown to expose on their plasma membrane the active endocannabinoid N-arachidonoylethanolamine (AEA), which binds to and stimulates the type 1 cannabinoid receptors (CB1), thus inhibiting presynaptic transmission in GABAergic neurons [59]. Exosomes released by microglia also contain glycolytic enzymes and the monocarboxylate transporter 1 (MCT1); one role of these exosomes could be delivering to not only target cells energy substrates, but also special enzymes such as the insulin degrading enzyme (IDE), which can degrade the A$\beta$ peptide [60].

Finally, it has been found that BCECs, the endothelial cells which constitute the wall of the brain capillaries and give rise to the blood-brain barrier, are also able to release vesicles. Interestingly, the endothelial cell-derived EVs are able to cross the BBB and are responsible, at least in part, for the brain-specific biomarkers found in blood; accordingly, these vesicles can be useful to analyze the time course of brain diseases. Since their membranes contain many BBB receptors, such as the transferrin and the insulin receptors, these MVs might be also used to deliver drugs across the BBB [61].

Now, if all the brain cell populations are able to release vesicles, an intriguing point is whether EVs produced by a given cell type have promiscuous activities or, on the contrary, have a specific target. Recent analyses suggested that physiological brain vesicles delivery is actually highly specific: exosomes secreted from stimulated glutamatergic cortical neurons were indeed captured only by other neurons; on the other hand, vesicles released from neuroblastoma cells lost this capability and were shown to bind both neurons and glial cells, with an apparent preference for glial cells [62].

Before concluding this brief summary concerning normal brain cells, it should be underlined that, beside their role as carriers of regulatory molecules, EVs can also function as scavengers. Some authors indeed found that exosomes/MVs are necessary for normal cells to eliminate proteins, for example, to discard an excess of glutamate receptor 2 [40].

## 3. Brain Cancer Cells

Variety and complexity of primary tumours of the Central Nervous System (CNS) are probably the highest among human cancers. Their classification is based on both the cell type and/or the brain structure from which they arise and on their grade, from I to IV, according to increasing malignancy of the cancer cells [63, 64].

Most brain primary tumours in the adult originate from glial cells/glial cell precursors and are collectively called gliomas, further divided into astrocytomas, oligodendrogliomas, ependymomas, and mixed oligoastrocytomas [63, 64]. According to the grade of malignancy, oligodendrocytomas and mixed gliomas are grades II and III, while astrocytomas are grouped into low-grade (LGA: pilocytic, grade I; diffuse, grade II) and high-grade astrocytomas (HGA: anaplastic, grade III; glioblastoma multiforme, GBM, grade IV) [65].

The treatment of HGA is mainly done by surgery, also required for definitive histopathologic diagnosis [65, 66], followed by radiation and chemotherapy [67].

Unfortunately, these therapeutic protocols, in spite of undisputed advancements, are not actually effective, and high grade gliomas still remain almost always fatal. In addition, gliomas are difficult to diagnose at an early stage because, at the beginning, the patients may suffer from unspecific symptoms, such as headache and seizures [65]. Difficulties in diagnosing as well as in treating gliomas also depend on the particular location of these tumours, which are protected by the BBB. Many efforts have been, indeed, recently devoted to find out strategies which might improve permeability of BBB to CNS-directed drugs (for review see [68]), although, paradoxically, BBB leakage and the concomitant vasogenic edema (see below) are the main clinical problems in patients suffering from glioblastoma (for review see [69]). For all these reasons, new approaches are urgently needed for an early diagnosis of brain cancer and improvement of therapy.

As shown for many other tumours, brain cancers release much higher amounts of extracellular vesicles than normal cells. EVs are continuously released by cancer cells, but their concentration in the body fluids is somehow proportional to disease grade [70]. Concerning their content, beside proteins more or less present in all the EVs studied up to now, such as different classes of chaperones, they contain tumour-specific antigens, MHC I and II complexes for antigen presentation, apoptosis-inducing (such as FasL and TRAIL) and immune suppressive (such as TGF$\beta$) factors (see below), as well as oncogenic growth factor receptors, such as a truncated form of the epidermal growth factor receptor EGFRvIII [71, 72]. In line with these contents, it has been shown that EVs shed from tumours can facilitate cancer development by suppressing immune responses, stimulating tumour growth, invasion, angiogenesis, and metastasis.

For many decades CNS was considered somehow "invisible" to the immune system because of its protected condition, ensured by BBB. It is now clear, however, that it can be better described as an immune "specialized" site, in which the bidirectional exchange of immune cells with the circulation is fundamental for the maintenance of integrity and functions [73, 74]. In addition, resident microglia can adopt active phagocytic behaviour and release proinflammatory factors upon brain injury [75, 76]. In the context of gliomas, however, glioma-associated microglia and macrophages (GAMs) seem

to loss the ability to induce antitumour immune response and to switch instead to a tumour-promoting, immunosuppressive phenotype. The inducers of this behavioural transition are glioma-derived factors, among which are TGF$\beta$, interleukin- (IL-) 4, IL-6, IL-10, and prostaglandin PGE-2 [76, 77]. At the same time, glioma cells seem to be also able to recruit from the circulation other immature myeloid cells, which also adopt an immune suppressive behaviour, as soon as they start interacting with the cancer site [76]. The cellular and molecular bases for these phenotypic modifications of immune cells, which infiltrate the tumour, are not completely understood but certainly depend on an intense cross talk among glioma and surrounding cells, forced to conform to the signals received and involve at least in part EVs [78].

Among the proteins released not only from glioma cells, but also from GAMs, an important class is represented by the matrix metalloproteases (MMPs) [79–81], especially MMP-2 and MMP-9 [76, 82, 83]. These enzymes can mediate degradation of the extracellular matrix, thus promoting invasion of the surrounding healthy tissues. In line with this idea, microvesicles shed in culture by oligodendroglioma cells were, for instance, shown to contain a TIMP3-sensitive "aggrecanase" activity, which could allow cellular invasion of aggrecan-rich extracellular matrices [23].

Importantly, migration of glioma cells throughout the brain is also guided by the brain vessels, and cancer growth is associated with angiogenesis [69]. Proliferation of BCECs, to produce the new vessels, causes disruption of the tight junctions and generalized fragility of the BBB, which becomes leaky, thus creating the conditions for the vasogenic brain edema, the most serious clinical complication of glioblastoma [69]. Edema and glioma progression have been also correlated with altered expression of different isoforms of aquaporins (AQPs), a family of water channels of the plasma membrane [84], which are upregulated in brain cancer [85–89]. Since in other systems both proteins of the tight junctions [90] and AQPs [91, 92] have been reported to enter exosomes, a new approach to the study of glioma-linked edema could be the search for these proteins in EVs released from brain cancers, with the additional aim of identifying further diagnostic tools. Actually, variations in the number, and possibly in the function, of EVs circulating in peripheral blood have been reported in brain tumors [93].

Importantly, aggressiveness of gliomas has been correlated with hypoxia, which should be the main inducer of both necrosis and BBB alteration. Hypoxia-dependent intercellular signalling, induced by growth factors, such as VEGF, at least in part secreted via EVs [94], stimulates not only BCECs proliferation and angiogenesis, but also activation of the coagulation system, responsible for vascular thrombosis [95]. Microvesicles have been known to be associated with coagulation since the 1940s because they expose PS, a negatively charged phospholipid, which can allow recruitment of calcium ions and active coagulation factors. Actually, tumour-derived vesicles have an even higher coagulation potential because they carry TF (tissue factor), an initiating coagulation factor [78].

An additional serious problem, posed by brain tumours, is the cancer-induced neuronal cell death and neurodegeneration. These events are associated with cytotoxic edema [69, 96]. Again, extracellular vesicles are probably involved. For example, G26/24 oligodendroglioma cells release EVs that, when added to primary cultures of rat cortical neurons, inhibit neurite outgrowth and induce apoptosis in about 75% of the cells [16]. The same amount of EVs induces apoptosis in only 40% of astrocytes [97]. In line with these observations, vesicles released by G26/24 cells were found to contain extracellular proapoptotic ligands, such as FasL and TRAIL, which could cooperate in inducing brain cell death. As already mentioned, these effects depend, at least in part, on the horizontal transfer of proteins, mediated by the vesicles, from tumour to normal brain cells [16, 97].

EVs are probably also involved in expelling from cancer cells regulatory proteins. G26/24 release, for example, vesicles which carry on the differentiation-specific H1° histone variant, thus eliminating a protein otherwise able to counteract proliferation [37].

Perhaps the most intriguing property of EVs is that they contain various classes of nucleic acids and are therefore able to exert an effect on the translational profile of normal cells present in their environment [78, 98, 99]. Transfer of DNA among cells is an evolutionary conserved process: bacterial vesicles contain DNA encoding virulence genes, which can be transferred into other bacteria and then expressed [72]. EVs released from eukaryotic cells carry both DNA and RNA. For example, brain tumours release EVs which contain c-Myc as well as high levels of retrotransposon RNA transcripts, such as those for LINE-1 and Alu elements; these transposable elements are then transferred to normal cells [100]. The presence of noncoding RNAs (ncRNAs) in EVs is actually one of the more fascinating topics in the field, since at least some of these RNAs have been suggested to be involved in epigenetic regulation of gene expression [101] and have thus the potential to induce profound modifications of recipient cells attitudes.

Intriguingly, exosomes released from astrocytes and glioma cells also contain mitochondrial DNA [102].

The collection of glioma-derived mRNAs, transferred into recipient cells via EVs, is actually highly complex and includes a variety of transcripts associated with proliferation, immune repression, and tissue invasion [103]; these transcripts are representative of almost all the transcriptomes of the producing cells; however, some transcripts are clearly enriched in EVs [98], thus suggesting existence of mechanisms, such as the presence of specific "zip code-like" sequences in the untranslated regions of the target passenger mRNAs [104], which allow selective sorting and/or stabilization of given mRNAs in EVs. Interestingly, many transcripts are common among EVs released from different cancer cell types [98].

Among EV-carried RNAs, a special class, which is attracting much attention, is represented by microRNAs [98]. Ten years ago, a microarray analysis allowed, for the first time, identification in glioblastoma, by two different laboratories, of aberrant miRNAs; one of which (miR21) was in particular shown to act as an antiapoptotic factor (for a review see [105]). More recently, a genome-wide miRNA expression profile allowed identification of 55 upregulated

and 29 downregulated miRNAs in malignant gliomas, at the same time suggesting that a group of 23 miRNAs could represent a sort of signature for GBM able to distinguish it even from anaplastic astrocytoma [106]. Although more studies are requested to univocally combine all the results described in the last ten years, a few miRNAs have indeed the potential to contribute to GBM [105, 107].

By performing microRNA PCR array, Camacho et al. also found a difference among brain metastatic (BM) and nonbrain metastatic tumor-derived exosomes; in particular, one upregulated (miR-210) and two downregulated (miR-19a and miR-29c) miRNAs were identified in BM versus non-BM exosomes [108].

The presence of both the proteins and RNAs discussed above in glioma-derived EVs, combined with the fact that these EVs are also present in the circulation of patients with glioma, while they almost disappear after tumor removal [103], suggests that these vesicles could be used as a liquid biopsy of cancer and reflect the disease grade [109–111]. A specific biomarker for glioblastoma is, for example, the already mentioned EGFRvIII, a mutated EGF receptor which lacks the extracellular domain, and triggers a "constitutively on" signal transduction pathway. This protein is found both in tumor cells and in shed vesicles [71, 112].

Recently, the ability of tumor cells to cause damage not only by acting directly on normal cells but also by altering their extracellular environment has been also emerging [113].

Finally, it should be mentioned that exosomes may protect tumor cells from accumulating drugs, thus accounting, at least in part, for drug/multidrug resistance [78, 114]. For example, P-glycoprotein, member of the ATP binding cassette superfamily, and one of the most important drug transporters, has been shown to be exchanged among cells via EVs [115].

## 4. Neurodegenerative Diseases

As in the case of brain tumors, variations in the amount of EVs circulating in peripheral blood have been also reported in several nervous system diseases, such as Alzheimer's disease [116], dementia [117], epilepsy [118], stroke [119], and traumatic brain injury [120]. The association between EV increase and multiple sclerosis is also well documented (as reviewed in [121, 122]), although the exact role of shed vesicles in disease progression is still unclear: it seems that they contain metalloproteinases damaging the blood-brain barrier, as well as factors involved in propagation of neuroinflammation; on the other hand, they seem to promote maturation and migration of oligodendrocyte precursor cells, necessary for repair of the damaged axons [121].

EVs can be implicated in neurodegenerative diseases also because they can deliver toxic proteins such as prions (PrPsc) [123–125], alpha-synuclein [126, 127], amyloid precursor protein (APP) or $\beta$-amyloid peptides [128–133], phosphorylated Tau [134], and SOD1 [135].

Accumulation of aggregates of abnormal proteins has indeed emerged as a common mechanism for most human neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, frontotemporal dementias, and amyotrophic lateral sclerosis. All these diseases should propagate through the brain via prion-like intercellular induction of protein misfolding [136, 137]. Neurons are peculiar cells in that they are postmitotic and cannot self-renew to clear abnormally folded/accumulated proteins; thus they accumulate protein folding errors, especially into aggregation-prone proteins, throughout their life-span [138]. Defects in protein degradation by the proteasome-depending pathway, because of abnormal activity of ubiquitinating/deubiquitinating enzymes, molecular chaperones, and protein hydrolases, can severely affect cell health and survival.

One of the most studied examples of the damaging effects of protein aggregation in neurons is given by the microtubule-associated protein Tau. An increase in Tau concentration, hyperphosphorylation, and aggregation seems to be the principal agent in the transmission and spreading of tauopathies, among which is Alzheimer's disease. Intracellular accumulation of aggregated Tau has been considered over time the main source of its toxicity. However, Tau added from the extracellular side is still toxic to neuronal cells [139]. For a long time, extracellular tau was believed to come out from lysed dead neurons. More recently, however, growing evidence suggested that extracellular Tau in AD brain (and CSF) is very likely due to active secretion [140–143].

A nontransgenic lower vertebrate tauopathy model (the lamprey ABC model) has been used to express full-length wild type and mutant human Tau isoforms in identified neurons, thus allowing localization of toxic Tau sources. Thanks to this model system, Tau was found to be secreted before the onset of neuronal degeneration and to be transferred among neurons, thus spreading in a disease-specific pattern to the brain and playing a major role in pathogenesis [144, 145]. Association of Tau with exosomes suggests that extracellular vesicles is at least one of the routes for active interneuronal transfer of toxic protein [140]. Interestingly, it has been also found that Tau can interact with signaling components localized to the plasma membrane, such as the Src-family of nonreceptor tyrosine kinases [141, 145, 146]. This finding suggests that extracellular signaling might have an effect on Tau sorting to extracellular vesicles and spreading throughout the brain.

Similar to Tau protein, in synucleinopathies, such as Parkinson's disease, SNCA/$\alpha$-synuclein can be released by neural cells into the extracellular space by EV shedding [125, 126]. It has been reported that SNCA/$\alpha$-synuclein is released in two main forms: through exosomes, which contain low-aggregated proteins, and through MVs, which contain high-aggregated proteins. It seems that, while intracellular SNCA aggregation has probably a protective role, released SNCA is toxic, most probably spreading the disease throughout the brain; the toxic effect is highly enhanced when autophagy-lysosomal pathway is inhibited [147].

Involvement of EVs in the pathological spreading of toxic proteins has been confirmed by the presence of vesicles in the CSF. MVs derived from all the major types of neural cells are already detectable in both rodent and human CSF, under normal conditions. In the inflamed brain, in cases of multiple sclerosis in humans and experimental autoimmune

encephalomyelitis (EAE) in mice, however, the amount of MVs increases dramatically depending on disease severity and the extent of microglia activation [148]. Exosomes enriched in prion proteins have been isolated from both ovine CSF [149] and human CSF [150].

In the late 1990s, human Tau was already evidenced in CSF of early stage patients with Alzheimer's disease [151, 152]. More recently, phosphorylated Tau has been found in exosomes, and this is a peculiar feature of AD with respect to both normal aging and other neurodegenerative diseases [141]. The early presence of exosome-associated Tau in CSF is of interest for at least two reasons: (1) it is a further indication of an active secretion of Tau from neurons (discussed in the previous section); (2) it could offer a powerful and noninvasive instrument for early AD diagnosis [138].

Given their potential interest in both diagnosis and basic research, EVs from the CSF have been the subjects of several studies. In particular, many efforts have been devoted to proteomic profiling of CSF exosomes [150, 153]. These analyses demonstrated the presence in CSF vesicles, among other proteins, of Alix, syntenin-1, tetraspanins, heat shock proteins, Rab proteins, transcription factors, MHC antigens, integrin alpha-M, the receptor-type tyrosine-protein phosphatase C, enolase 2, the dihydropyrimidinase-related protein 2, and the vesicle-associated membrane protein 2 (VAMP2) [150].

From all the discussed studies, it is evident that EVs, if taken up from cells different from the producer ones, have the potential to promote deep modifications of properties and behavior of the recipient cells.

## 5. Concluding Remarks

In conclusion, extracellular vesicles seem to play an important role in coordinating intercellular communication in brain. Production of EVs is probably a dynamic process which can undergo both quantitative and qualitative modifications depending on neuronal activity, metabolic state, and perhaps membrane trafficking rate. In pathological conditions, such as cancer as well as neurodegeneration, EV production and release seem to be potentiated and allow secretion into the extracellular environment of proteins, RNAs, and lipids, which can horizontally transfer pathological features to the surrounding cells. The presence of disease-specific proteins and RNAs in EVs, which can also reach the patient CSF, could offer a powerful way for early detection of pathology and is certainly worth of further analyses. Finally, EVs also deserve attention as potential drug carriers, theoretically able to cross the blood-brain barrier.

## Conflict of Interests

The authors declare that there is no conflict of interests.

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*Review Article*

# Role of Exosomal Noncoding RNAs in Lung Carcinogenesis

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Lung cancer is the major cause of cancer death worldwide. Novel, recently discovered classes of noncoding RNAs (ncRNAs) have diverse functional and regulatory activities and increasing evidence suggests crucial roles for deregulated ncRNAs in the onset and progression of cancer, including lung cancer. Exosomes are small extracellular membrane vesicles of endocytic origin that are released by many cells and are found in most body fluids. Tumor-derived exosomes mediate tumorigenesis by facilitating tumor growth and metastasis. MicroRNAs (miRNAs) are a subclass of ncRNAs that are present in exosomes. miRNAs are taken up by neighboring or distant cells and modulate various functions of recipient cells. Here, we review exosome-derived ncRNAs with a focus on miRNAs and their role in lung cancer biology.

## 1. Introduction

Lung cancer is one of the most lethal human cancers worldwide. Nonsmall cell lung cancers (NSCLC) are the majority of lung cancers (80%) while the remaining are small cell lung cancers (SCLC) [1]. Despite recent advances in lung cancer research and the use of novel therapeutic agents for NSCLC, outcomes remain dismal with a 5-year survival rate of about 15% [1]. A deeper understanding of the molecular mechanisms underlying NSCLC development and progression is needed.

ncRNAs are a novel class of RNA molecules that perform many basic regulatory functions in eukaryotes and these include regulating normal cell growth and apoptosis but they also play roles in abnormal cell functions associated with cancer progression and metastasis [2, 3]. However, the functional role of ncRNAs in NSCLC is not well understood. ncRNAs are a large class of molecules that can be divided into two groups based on their transcript lengths: small ncRNAs (<200 bp), such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), small interfering RNAs (siRNA), PIWI-interacting RNAs (piRNA), antisense RNAs, and promoter-associated RNAs (PARs) and long ncRNAs (lncRNAs, >200 bp) [4].

Cell-cell communication can occur by several means, including chemical receptor-mediated events, direct cell-cell contact and cell-cell synapses. Extracellular vesicles (EVs), which include exosomes and microvesicles, are mediators of cell-cell communication and are novel biomarkers for disease. Increasing evidence implicates cell-to-cell communication, which can be mediated by delivery of miRNAs after transfer through exosomes, in physiological and pathological processes. Exosomes are subcellular vesicles (30–120 nm in diameter) present in biological fluids. Different miRNAs including let-7, miR-1, miR-15, miR-16, miR-181, and miR-375, which seem to be specifically packaged exclusively into exosomes [5], have sparked intense investigations into the composition and function of exosomes. miRNAs are small ncRNA gene products discovered in diverse organisms that are thought to regulate target gene expression [6]. A potential role for miRNAs in carcinogenesis comes from their ability to inhibit the translation of tumor suppressor genes and

oncogenes. Many human cancers display unique miRNA expression signatures. miRNAs can be secreted via extracellular vesicles (EVs) and by protein-miRNA complexes. Current studies show that miRNAs are present in extracellular spaces, packaged into various membrane-bound vesicles. The protein-miRNA content of EVs can be transferred to recipient cells and deliver biological information that plays an important role in cancer metastasis and prognosis.

Exosomal miRNAs are also body fluid miRNAs but they differ from free miRNAs circulating in the body fluid because they are encapsulated in exosomes derived from the multivesicular body (MVB) sorting pathway [7]. Exosomes are distributed in various body fluids and are taken up by neighboring or distant cells. In doing so, miRNAs are delivered to the recipient cell and then they alter cell functions. Ratajczak et al. showed RNA contained within exosomes was responsible for the horizontal transfer of genetic information between cells [8]. Ribonuclease pretreatment inhibited the functional effects of exosomes, which provided convincing evidence of RNA involvement. Following translocation from the nucleus to the cytoplasm, RNA molecules bind to membranous organelles or vesicles and are transported to intracellular sites, which may account for RNA populations associating with exosomes. Valadi et al. showed that exosomes contain a subset of both cellular mRNA and miRNA, which could be transferred to recipient cells [5].

Many studies show that miRNAs are differentially enriched in normal exosomes compared with exosomes from pathological conditions such as tumors. Exosome-containing miRNAs are implicated in cancer pathogenesis [9]. For example, microarray analysis revealed selective enrichment of let-7 miRNA family in exosomes derived from highly metastatic gastric cancer cells, when compared with lowly metastatic gastric cells [10]. Members of the let-7 miRNA family have tumour suppressor activity that targets oncogenes such as RAS and HMGA2. Ohshima et al. [10] proposed a mechanism where highly invasive tumour cells release let-7 miRNAs through exosomes into the extracellular milieu, to maintain their tumourigenic phenotype.

Cancer cell communication is an important and complex process, which is achieved through diverse mechanisms and allows tumor cells to mold and influence their environment. Recent evidence shows that cells communicate via the release and delivery of miRNAs packed into tumor-released (TR) exosomes. Some miRNAs are either over- or underexpressed in particular cancer types. Patterns of miRNAs are unique to individual tissue types and differ between cancer and normal samples [11, 12]. Current data support miRNAs as potential biomarkers for NSCLC [13–17]. Measurement of levels of multiple miRNAs can more precisely gauge prognosis and accurately predict survival in lung cancer [18].

## 2. The Release Mechanism of Circulating miRNAs

The biological mechanisms that culminate in the release of circulating miRNAs remain unclear. Possible mechanisms for miRNA release include (1) active secretion of miRNAs as vesicle-containing miRNAs and (2) energy-free passive leakage of cellular miRNAs from disrupted cells. The latter mechanism is only a minor contributor to the process of circulating miRNA generation and occurs predominantly under pathological circumstances such as tissue damage, cell apoptosis, tumor metastatic process, or chronic inflammation [19, 20]. Circulating miRNAs are packaged into various membrane-bound vesicles including exosomes and microvesicles [5], whereas vesicle-free miRNAs are associated with protein or high-density lipoprotein complexes [21]. Only EV-derived miRNAs function in cell-cell communication, and they play roles in various biological processes including immune system regulation, inflammation, and tumor development [22, 23]. EV-derived miRNAs control many aspects of human physiological status and are therefore potentially better disease biomarkers than other circulating miRNAs.

Unfortunately, the release mechanism of circulating miRNAs by tumors is not well established. Rabinowits et al. evaluated the exosomal miRNA circulating levels of patients with lung adenocarcinoma and compared them with those of patients without lung cancer, showing that the miRNA signatures of exosomes parallel those of the miRNA expression profiles of the originating tumor cells. This indicates that the content of tumor cell-derived exosomes is correlated with the miRNA levels in primary tumor [24]. Fabbri et al. provided evidence that, in immune cells, tumor-secreted miR-21 and miR-29a act as ligands that bind receptors of the toll-like receptor (TLR) family, specifically human TLR8, and murine TLR7. Tumor growth and metastasis would be a consequence miRNA binding to TLR that mediates a prometastatic inflammatory response [9].

## 3. Potential Modes for Sorting of miRNAs into Exosomes

Current research describes four possible modes for sorting of the specific loading of miRNA species into exosomes, although the underlying mechanisms are unclear (Figure 1).

The first mode of sorting involves the miRNA-induced silencing complex- (miRISC-) related pathway. Mature miRNAs can interact with assembly proteins to form a complex called miRISC, which includes miRNA, miRNA-repressible mRNA, GW182 protein, and miRNA effector protein argonaute 2 (AGO2). Multivesicular bodies (MVBs, also called late endosomes) are derived from early endosomes. MVBs either release exosomes into the extracellular space upon fusion with plasma membrane or fuse with lysosomes and degrade their content. GW-bodies are distinct foci within the eukaryotic cell cytoplasm that participate in various types of mRNA decay and miRNA-induced mRNA silencing. Gibbings et al. showed GW-bodies appear to contain most of the miRISC complex components (including GW182 protein and AGO2), colocalise with MVBs, and may be sites where miRISCs accumulate and act. They also showed that exosome-like vesicles produced by MVBs were enriched in GW182 protein but not AGO2. Furthermore, blocking the formation of MVB by reducing endosomal sorting complex, required for transport (ESCRT) components,

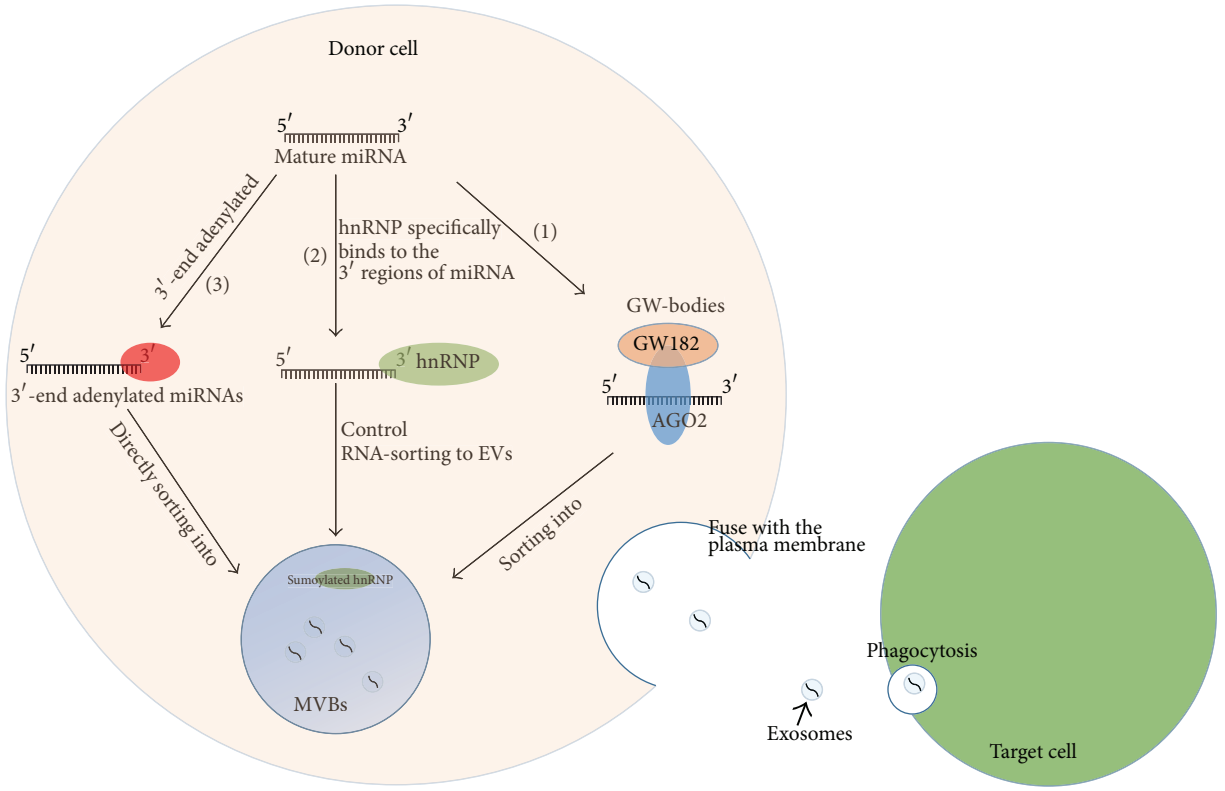


Figure 1: Potential modes for sorting of miRNAs into exosomes: (1) Mature miRNAs can interact with assembly proteins to form a complex called miRISC, which includes target miRNA, miRNA-repressible mRNA, GW182 protein, and miRNA effector protein argonaute 2 (AGO2, which was initially identified as membrane associated). Active miRISCs are recruited into GW-bodies that are physically associated with MVBs and this may represent a method of loading miRNAs into exosomes. (2) The protein hnRNP specifically binds to the 3′ regions of miRNA sequences and controls their loading into exosomes. (3) 3′-end adenylated miRNAs directly sort into EVs. miRNAs release as exosomes and uptake by recipient cell: (a) MVBs are released by exocytosis and fuse with the plasma membrane leading to the release of exosomes. (b) EVs uptake by recipient cells through phagocytosis.

reduced miRNA-mediated gene silencing and resulted in excess accumulation of GW182 protein, while preventing the fusion of MVBs with lysosomes stimulated RISC activity. These data are consistent with the idea that active miRISCs with target mRNAs are recruited into GW-bodies that are physically associated with MVBs and this may represent a method of loading miRNAs into exosomes [25]. The second mode involves a miRNA motif and the sumoylated heterogeneous nuclear ribonucleoproteins- (hnRNPs-) dependent pathway. The protein hnRNPA2B1 specifically binds exosomal miRNAs through the recognition of these motifs in the 3′ regions of miRNA sequences and controls their loading into exosomes. In addition, hnRNPA2B1 in exosomes is preferentially sumoylated, and this sumoylation is important for the loading of exosomal miRNAs into exosomes. The loading of miRNAs into exosomes can be modulated by mutagenesis of the identified motifs or changes in hnRNPA2B1 expression levels [26]. Another two hnRNP family proteins, hnRNPA1 and hnRNPC, could also bind to exosomal miRNAs, suggesting that they might also be candidates for miRNA sorting [27]. The third mode relies on a pathway dependent on the 3′ ends of the miRNA. 3′-end adenylated miRNAs are relatively enriched in cells, whereas 3′-end uridylated isoforms appear overrepresented in exosomes derived from B cells or urine. Collectively, it suggests that posttranscriptional modifications, notably 3′-end adenylation and uridylation, exert opposing effects that may contribute to direct ncRNA sorting into EVs [27]. Therefore, two sorting modes show that the 3′ regions or the 3′ ends of the miRNA sequence contain as yet unidentified critical sorting signal(s). The last mode is mediated by the neural sphingomyelinase 2- (nSMase2-) dependent pathway. Kosaka et al. demonstrate that nSMase2 regulates exosomal miRNA secretion and promotes angiogenesis within the tumor microenvironment as well as metastasis. nSMase2 is the initial molecule associated with miRNA secretion into exosomes. Overexpressed nSMase2 increased the number of exosomal miRNAs whereas inhibition of nSMase2 expression had the converse effect [28].

There are two ways for microvesicles secreting from donor cells: (1) microvesicles are directly shed from the cell membrane; and (2) MVBs which are released by exocytosis fuse with the plasma membrane and release the intraluminal endosomal vesicles into the extracellular space to become exosomes [29]. The miRNAs in cell-released exosomes can circulate with the associated vehicles to reach neighboring

cells and distant cells. EVs, which interact with the functionally active receptors (CCR5, EGFRvIII, or MET) or ligands of target cells, may induce changes in the cell phenotype, eventually leading to triggering intracellular signaling pathways [30]. EVs uptake by recipient cells seems to occur through phagocytosis [31]. After being delivered into target cells, exosomal miRNAs play functional roles (Figure 1).

## 4. The ncRNAs Related to Lung Cancer

Great progress in the last decade has increased our understanding of the role miRNAs play in lung cancer. Several miRNAs that display different patterns in lung cancer compared to normal tissues were identified. Aberrant miRNA levels were detected in lung cancer tumor tissues but also in body fluids and extracellular organelles, such as exosomes. These combined studies gave weight to the notion that miRNAs are promising biomarkers for improved diagnosis and prediction, and they are potential targets for therapeutics to treat lung cancer.

Cazzoli et al. screened 742 miRNAs in circulating exosomes and identified four miRNAs (miR-378a, miR-379, miR-139-5p, and miR-200b-5p) as screening markers to segregate lung adenocarcinoma and carcinoma patients, from healthy former smokers. They also identified six miRNAs (miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p) that segregated lung adenocarcinoma patients and lung granuloma patients [32].

In 2004, Takamizawa et al. [33] identified the first miRNA family, let-7, which was associated with the tumorigenesis of lung cancer. They found that introduction of let-7a and let-7f isoforms into a lung adenocarcinoma cell line with low baseline levels of let-7 expression (A549), inhibited the growth of A549 cells. In a clinical setting, reduced let-7 levels were associated with shorter patient survival after diagnosis. Many targets of let-7 are now known, including the RAS family [34], HMGA2 [34–36], c-Myc [37, 38], CDC25A, CDK6, and Cyclin D2 [39]. These target genes helped to reveal the mechanisms by which let-7 family members exert their function in tumorigenesis. Many miRNAs are oncogenes or tumor suppressor genes and these include miR-17–92 [16, 40], miR-218 [41], miR-21 [42], and miR-34 family members (miR-34a and miR-34b/c) [43–47]. miRNAs play pivotal roles in lung tumorigenesis and are also involved in tumor metastasis. Several miRNAs including miR-17–92 [48–51], the miR-200 family of miRNAs (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) [52], miR-125a-3p/5p [53], miR-21 [54], and the miR-106b-25 cluster (miR-106b and miR-93) [55] are implicated in metastatic lung cancer. Many other miRNAs and their potential mechanism(s) of action are shown in Table 1.

Besides miRNAs, long ncRNAs (lncRNAs) with tumour-promoting and tumour-suppressing functions in lung cancer have also been identified [56]. MALAT1 is lncRNA present at high levels, which is upregulated during metastasis of early-stage NSCLC and is a powerful predictor of metastatic relapse in patients with NSCLC. SCAL1 is lncRNA that shows increased levels as part of the oxidative stress response of airway epithelial cells to cigarette smoke. SCAL1 is also overexpressed in a range of lung cancer cell lines, which may indicate a novel mechanism of smoke-induced malignant transformation. Yang et al. characterized the function of lncRNA PVT1 in NSCLC development and progression [57]. PVT1 levels were increased in NSCLC tissues and lung cancer cell lines when compared with matching nearby normal tissues and the normal human bronchial epithelial cell line 16HBE, respectively. They also found PVT1 levels correlated positively with histological grade and lymph node metastasis, but there was no correlation with age, gender, and tumor size in patients with NSCLC. High levels of PVT1 correlated with lower overall survival rates and were an independent prognostic factor in patients with NSCLC. These results provided evidence of a potentially important role of PVT1 in tumorigenesis and in the progression of NSCLC. Other lncRNAs and their potential mechanism(s) of action are shown in Table 2.

## 5. Potential Mechanism of Some Exosomal miRNAs

Silva et al. showed levels of exosomal let-7f and/or miR-30e-3p in NSCLC patients could distinguish between patients with resectable and nonresectable tumors [58]. Rabinowits et al. assessed patients with and without lung adenocarcinoma for their levels of circulating tumor exosomes and exosomal small RNA as well as specific exosomal miRNAs. Subsequently, these levels were correlated with disease stages as defined by the American Joint Committee on Cancer (AJCC). These studies were used to validate markers for diagnosis and prognosis in patients for lung adenocarcinoma. The levels of 12 exosomal miRNAs (miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, and miR-214) were significantly different between patients and controls [24]. The potential mechanism(s) of those 12 exosomal miRNAs are shown in Table 1.

The analysis of specific miRNAs in exosomes showed enrichment of miRNAs in samples from patients with NSCLC both in plasma and in bronchoalveolar lavage. This finding, together with the higher proportion of exosomes detected in the plasma of NSCLC patients, supports the idea that a proliferative advantage accrues to tumors that release exosomes into plasma [59].

let-7 was the first miRNA shown to be dysregulated in human lung cancer [33]. Decreased levels of let-7 in the sputum of patients with chronic obstructive pulmonary disease (COPD) fueled speculation that chronic airway inflammation, combined with the loss of a major tumour suppressor, may contribute to the increased risk of patients with COPD developing lung cancer. Yu et al. identified a five-miRNA signature that can predict survival in patients with lung cancer, of which hsa-miR-221 and hsa-let-7a were protective, while hsa-miR-137, hsa-miR-372, and hsa-miR-182* were nonprotective [18]. In patients with lung cancer, the total levels of exosomes and their let-7 miRNAs increased compared to controls. Ohshima et al. performed extensive miRNA analysis in three cellular fractions including cells,

Table 1: The potential mechanisms and target genes of miRNAs in lung cancer.

| Onco-miRNA | Potential mechanisms | Target genes | Reference |
|---|---|---|---|
| miR-21 | Overexpression of miR-21 enhances tumorigenesis and inhibits apoptosis through inhibition of negative regulators of the Ras/MEK/ERK pathway and inhibition of apoptosis | PTEN, SPRY1, SPRY2, BTG2, PDCD4, APAF1, FasL, and RHOB | [42] |
| miR-212 | Exert an antiapoptotic effect through direct repression of synaptic acetylcholinesterase expression in NSCLC | AChE | [60] |
| miR-17–92 cluster | Enhances cell proliferation, inhibits apoptosis, and protects against DNA damage | p21, CTGF, Tsp1, PTEN, Bim, and HIF-1$\alpha$ | [16, 61] |
| miR-221-222 | Activates the PI3K/Akt pathway and metallopeptidases | Kit, p27 kip1, PTEN/TIMP3, PUMA, and TRAIL | [62] |
| miR-93, miR-98, and miR-197 | Inhibits tumor suppressor gene FUS1 expression | FUS1 | [63] |
| miR-375 | miR-375 upregulation and correlates with ASCL1 in the cell lines generated from mouse SCLC-like tumors | Not determined | [64] |
| miR-17-5p and miR-20a | miRNA inhibition reduces cell growth<br>miRNA inhibition induces apoptosis and increases the proportions of sub-G1 populations | E2F1 | [65] |
| miR-328 | Associated with cell migration and NSCLC brain metastases by controlling the VEGF/IL1 signaling pathway | PRKCA, VEGF-D, NOTCH1, IL1-$\alpha$, IL1-$\beta$, and PLC-$\gamma$ | [66] |
| miR-106 | Upregulated in lung cancer | RB | [67] |
| miR-155 | Promotes cell proliferation through the repression of SOCS1.<br>Downregulates several tumor suppressors such as PTEN, PDC4, and SHIP1, leading to the activation of the Akt pathway<br>Plays a role in cell invasion by targeting RhoA | CASP3, TP53BP1, SOCS1, PTEN, PDC4, and SHIP1 | [68–70] |
| Tumor suppressor miRNA | Potential mechanisms | Target genes | Reference |
| let-7 family | Suppress cell proliferation by negatively regulating pathways promoting the G1 to S transition | KRAS, CDC25a, CDK6, c-MYC, CCND1, and BCL-2 | [39, 71] |
| miR-126 | Inhibits cell proliferation by arresting the cells in the G1 phase by targeting VEGF | VEGF, CRK, and SLC7A5, | [49, 72] |
| miR-26a | Inhibits cell proliferation, blocks G1/S phase transition, induces apoptosis, and inhibits cell metastasis and invasion *in vitro* | EZH2 | [73] |
| miR-7 | Involved in miR-7-mediated growth suppression and apoptosis<br>Inhibits cancer cell migration | BCL-2 | [74] |
| miR-335 | Reduces cell migration, invasion, proliferation, and metastasis | BCL-W and SP1 | [75] |
| miR-145 | Inhibits cell growth, proliferation, and migration of lung cancer cells and induces cell cycle arrest in G1 by targeting CDK4 | EGFR, NUDT1, CDK4, c-Myc, and OCT4 | [76–78] |
| miR-413 | Inhibits cell proliferation and enhances apoptosis | PKC$\varepsilon$ | [79] |
| miR-192 | Inhibits cell proliferation and induces cell apoptosis in lung cancer cells<br>Arrests cells in G1 phase | RB1 | [80] |
| miR-449 cluster | Induces apoptosis targeting Bcl-2, n-MYC, and HDAC1 and upregulates p53 through the repression of deacetylase gene SIRT1<br>Inhibits cell migration and invasion by suppression of AXL and SNAIL-1 | CDK4, CDK6, c-MYC, CCND1, CCNE2, CDC25A, MET, and E2F | [81–83] |
| miR-206 | Overexpression of miR-206 inhibits migration and invasion of lung cancer cells | Not determined | [84] |
| miR-146a | Inhibits cell growth and induces cell apoptosis<br>Suppresses motility<br>Enhances cell proliferation inhibitory effect of TKIs and cetuximab | EGFR | [85] |
| miR-203 | Inhibits cell proliferation and migration of lung cancer | PKC$\alpha$ | [86] |
| miR-205 | Regulates epithelial to mesenchymal transition by targeting ZEB1 and SIP1 | Not determined | [87] |
| miR-214 | Regulates the acquired resistance to gefitinib via the PTEN/AKT pathway | PTEN | [88] |

Table 2: The potential mechanisms of lncRNAs in lung cancer.

| Onco-lncRNA | Potential mechanism | Reference |
|---|---|---|
| MVIH | Affects expression of MMP-2/MMP-9<br>Proliferation and invasion | [89] |
| BC200 | Upregulated in lung cancer | [90] |
| lncRNA-DQ786227 | Upregulated in lung cancer | [91] |
| MALAT1 | Affects expression of Bcl-2 and metastasis related genes | [92] |
| AK126698 | Decreases NKD and increases the accumulation and nuclear translocation of $\beta$-catenin<br>Antiapoptotic effects and resistance to cisplatin | [93] |
| SCAL1 | Induced by cigarette smoke and NRF2<br>Protective against oxidative stress | [94] |
| HOTAIR | Induced by Col-1 upregulation of cell adhesion-related genes such as ASTN1 and PCDHA1<br>Downregulates genes involved in neuronal growth and signal transduction including NTM and PTK2B<br>Contributes to the cisplatin resistance through the regulation of p21 expression<br>Affects proliferation, migration, and invasion | [95–97] |
| **Tumor suppressor lncRNA** | **Potential mechanism** | **Reference** |
| BANCR | A regulator of EMT during NSCLC<br>Induces apoptosis and inhibits EMT, migration, invasion, and metastasis | [98] |
| MEG3 | Inhibits NSCLC cell proliferation and induces apoptosis by affecting p53 expression | [99] |
| SPRY4-IT1 | Promotes NSCLC cell proliferation and metastasis via effects on the epithelial-mesenchymal transition | [100] |
| TUG1 | Affects cell proliferation in human NSCLC through epigenetically regulating HOXB7 expression | [101] |

exosomes, and medium from cultured cells and they found that let-7 miRNA family is rich in all the fractions from the gastric cancer cell line, AZ-P7a. After comparing the levels of let-7 miRNA family members in exosomes derived from AZ-P7a with exosomes from other cancer cell lines, including the lung cancer cell lines (SBC-3, DMS-35, and NCI-H69), they found members of the let-7 miRNA family were abundant in exosomes derived from AZ-P7a but were less abundant in exosomes derived from other cancer cells. These data were consistent with the notion that exosome-mediated release of let-7 miRNAs into the extracellular milieu resulted in a reduced antitumorigenic effect within the cells and consequently oncogenesis and invasiveness of the cells were sustained [10]. Levels of let-7f, miR-20b, and miR-30e-3p in vesicles from the plasma of NSCLC patients were lower than normal controls [58]. The let-7 family members negatively regulate the oncogene RAS [34], suggesting that let-7 acts biologically as a tumor suppressor.

The let-7 miRNA family members are important regulators that control lung cancer oncogene expression by binding to the 3′ untranslated regions (3′-UTR) of their target mRNAs, and lower levels of let-7 miRNA have been found in NSCLC [34, 102]. let-7 regulates many key cell cycle protooncogenes (KRAS, CDC25a, CDK6, c-MYC, cyclin D, and BCL-2), and in this way let-7 controls cell proliferation by negatively regulating the pathways promoting the G1 to S transition [39]. An interesting feature of the let-7 family is that the 3′-UTR of KRAS (as well as HRAS, NRAS, and various members of the RAS GTPase family) contain multiple let-7 binding sites, and let-7 levels in lung cancer inversely correlate with KRAS expression [103]. Chin et al. described a novel SNP (Single Nucleotide Polymorphism) in the 3′-UTR of the KRAS gene that influences let-7 binding to its target site. This variant, the let-7 complementary site (LCS6) in the KRAS 3′-UTR, is associated with KRAS gene upregulation and let-7 lower levels; however this polymorphism correlates with a modest increase in lung cancer risk [104]. A recent study identified HOXA1 as a new target of the let-7 family. Zhan et al. showed that let-7c represses NSCLC cell proliferation and tumorigenesis by targeting the 3′-UTR of HOXA1 mRNA, which reduced the expression of CCND1, CDC25A, and CDK2 [71]. He et al. showed that let-7a, a member of let-7 family, negatively regulated the expression of NIRF (Np95/ICBP90-like RING finger protein) through NIRF 3′ UTR. Enforced expression of let-7a in A549 lung cancer cells decreased NIRF leading to a coordinated increase in $p21^{WAF1}$ [105]. NIRF binds to methyl-CpGs of the promoter region through a SET and RING finger-associated (SRA) domain. This protein constitutes a complex with HDAC1 (histone deacetylase-1) also via its SRA domain, which binds to methylated promoter regions of various tumor suppressor genes, including $p21^{WAF1}$ in cancer cells [106]. Recently [107], let-7c levels were observed to be inversely correlated to levels of ITGB3 (integrin b3, also known as CD61) and MAP4K3, a member of the MAP4K family in NSCLC tissues.

Gao et al. identified miRNA profiles in NSCLC and showed that deregulated expression of miR-21, miR-143, and miR-181a correlated with patient prognosis, suggesting that miRNAs are involved in the initiation and progression of this disease [108]. miR-21 is the most common miRNA in the human lung but at elevated levels miR-21 contributes to the symptomatic development of asthma, idiopathic pulmonary

fibrosis, and non-small-cell lung cancer (NSCLC) through its effects on inflammatory and cancer suppressing genes. miR-21 is an antiapoptotic miRNA and is regulated by the EGFR pathway and miR-21 levels correlate with phosphorylated EGFR levels [109]. miR-21 stimulates cell growth and NSCLC cell invasion by targeting PTEN (phosphatase and tensin homolog), which enhances the RAS/MEK/ERK pathway by repressing negative regulators of the pathway (i.e., Spry1, Spry2, Btg2, and Pdcd4) and by repressing the expression of proapoptotic proteins (e.g., Apaf1, FasL, RhoB, and Pdcd4). miR-21 is also upregulated by KRAS in NSCLC, both *in vitro* and *in vivo,* through MAPK/AP-1 activation [42, 109–111]. Chiba et al. revealed that exosomes derived from the colorectal cancer cell lines contain miR-21, miR-192, and miR-221 and can be delivered into lung cancer A549 cells [112].

miR-126 is a tumor suppressor, because of its antioncogenic properties. miR-126 inhibits NSCLC cell line proliferation through EGFL7 [113] and targets the Sdf-1a cytokine, which reduces mesenchymal stem cell and inflammatory monocyte recruitment to primary tumors, thereby inhibiting lung metastasis [114]. Rodríguez et al. showed that tumors may eliminate miR-126 by exosome liberation [59].

## 6. Conclusion

Exosomes, despite their nanosize, can act as key communication facilitators between cells. Gradually, we are revealing the roles of exosomes in interactions between distant cells and the complex roles of exosomes in tumorigenesis and cancer progression. Exosomes secrete a variety of biological molecules, including miRNAs, proteins, and their complexes. The study of exosomal miRNAs offers a new and exciting approach to understanding the molecular mechanisms of lung cancer biology. However, the study of exosomes in lung carcinogenesis is still in its infancy. Unresolved issues include whether other noncoding RNAs such as lncRNAs are present in exosomes and whether lncRNAs are involved in target gene regulation in recipient cells. Much work remains to be carried out before we have a complete understanding of the role of exosomal ncRNAs in lung carcinogenesis.

## Conflict of Interests

The authors declare that there is no conflict of interests.

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