

## The Role of Exosomes in Breast Cancer

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**BACKGROUND:** Although it has been long realized that eukaryotic cells release complex vesicular structures into their environment, only in recent years has it been established that these entities are not merely junk or debris, but that they are tailor-made specialized minimaps of their cell of origin and of both physiological and pathological relevance. These exosomes and microvesicles (ectosomes), collectively termed extracellular vesicles (EVs), are often defined and subgrouped first and foremost according to size and proposed origin (exosomes approximately 30–120 nm, endosomal origin; microvesicles 120–1000 nm, from the cell membrane). There is growing interest in elucidating the relevance and roles of EVs in cancer.

**CONTENT:** Much of the pioneering work on EVs in cancer has focused on breast cancer, possibly because breast cancer is a leading cause of cancer-related deaths worldwide. This review provides an in-depth summary of such studies, supporting key roles for exosomes and other EVs in breast cancer cell invasion and metastasis, stem cell stimulation, apoptosis, immune system modulation, and anti-cancer drug resistance. Exosomes as diagnostic, prognostic, and/or predictive biomarkers and their potential use in the development of therapeutics are discussed.

**SUMMARY:** Although not fully elucidated, the involvement of exosomes in breast cancer development, progression, and resistance is becoming increasingly apparent from preclinical and clinical studies, with mounting interest in the potential exploitation of these vesicles for breast cancer biomarkers, as drug delivery systems, and in the development of future novel breast cancer therapies.

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According to the WHO, cancer is a leading cause of death worldwide, with breast cancer being the fifth most

common cancer (1). In the US it was estimated that approximately 232 670 new cases of breast cancer would be present in 2014. This staggering figure was predicted to account for 29% of all new cancer cases (2). Of note, breast cancer is not restricted to the female population, with approximately 1% of these cancers occurring in males. Primary breast tumors typically do not kill; this occurs as a result of cancer spread/metastasis to secondary sites in the body. In fact, the 5-year survival rates are 99% for localized breast cancer, 84% for regional stage (nearby lymph nodes), and 23% for metastases (distant organs and lymph nodes) (3).

Breast cancer is a highly heterogeneous disease and, although this review is not focused on describing these subtypes but more so on the relevance of exosomes, the following information may help to add context for those who have not previously studied breast cancer. In 2000, Perou et al. (4) made a fundamental contribution to defining breast cancer subclassifications, using DNA microarrays. From this development, hierarchical clustering analysis revealed various subtypes based on gene expression patterns, i.e., basal-like [mostly classified as triple-negative breast cancer (TNBC),<sup>3</sup> although basal-like and TNBC are not synonymous (5)], human epidermal growth factor receptor 2 (HER2)/neu-overexpressing, normal-like, and luminal epithelial/ER<sup>+</sup> (estrogen receptor positive). Subsequent to this study, the luminal subclass was further subdivided into luminal A and luminal B. Without treatment being considered, the poorest survival rates have been associated with basal-like and HER2-overexpressing tumors (6, 7). Because of the heterogeneous nature of breast cancer and different stages of diagnosis from individual to individual, treatment typically involves multimodality approaches with surgery, chemotherapy, radiation therapy, hormone therapy, and other newer targeted treatments, including monoclonal antibodies and small molecules.

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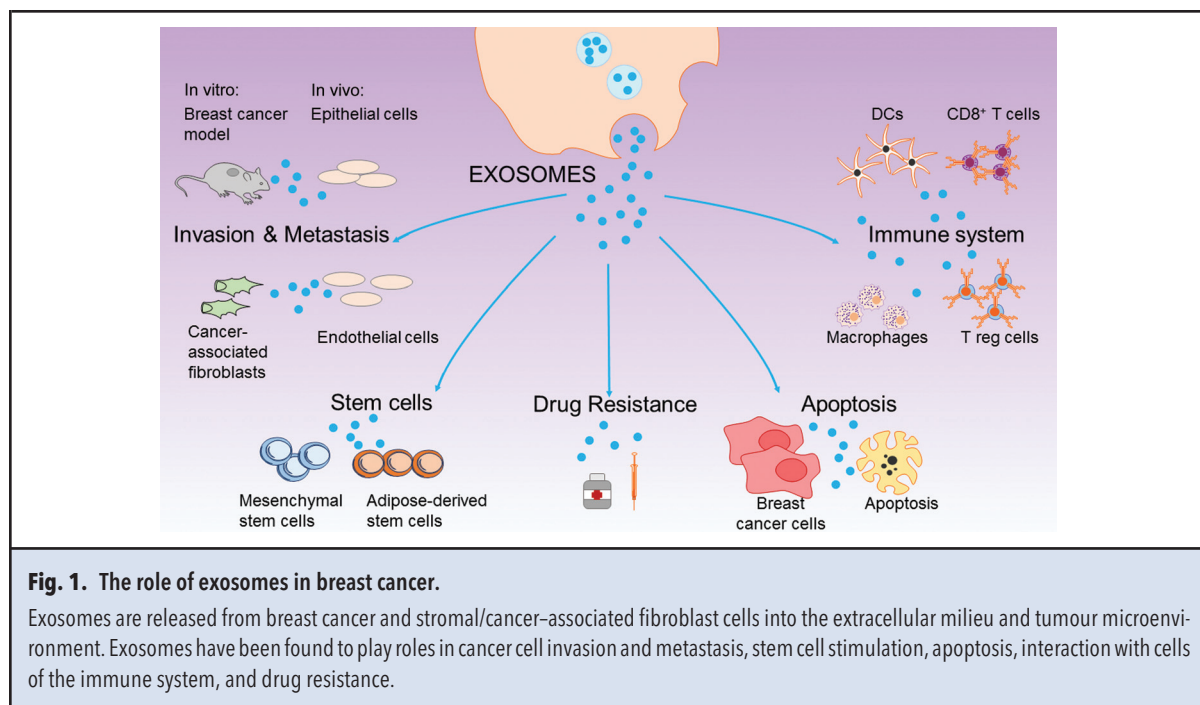
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Received March 11, 2015; accepted August 14, 2015.

Previously published online at DOI: 10.1373/clinchem.2015.240028

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<sup>3</sup> Nonstandard abbreviations: TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor 2; EV, extracellular vesicle; miR, microRNA; EGFR, epidermal growth factor receptor; CAF, cancer-associated fibroblast; TIMP, tissue inhibitor of metalloproteinases; CSC, cancer stem cell; MSC, mesenchymal stem cell; VEGF, vascular endothelial growth factor; ADSC, adipose tissue-derived stem cells; IL-6, interleukin 6; SEB, staphylococcal enterotoxin B; EXO/SEB, exosomes from MDA-MB-231 cells were anchored with SEB; DC, dendritic cell; NK, natural killer; NKT cells, natural killer T cells; NKG2D, NK group 2, member D; TAM, tumor-associated macrophage; NF-κB, nuclear factor-κB; EGCG, epigallocatechin gallate; TLR2, Toll-like receptor 2; hTERT, human telomerase reverse transcriptase; P-gp, P-glycoprotein; Adr, adriamycin; Doc, docetaxel; CTL, cytotoxic T lymphocyte; PTEN-CT, PTEN C-terminus.



## Exosomes and Breast Cancer

Exosomes, cell-derived vesicles, were initially described as vesicles released during the maturation process of reticulocytes (8). Exosomes and microvesicles (also termed ectosomes) are typically differentiated on the basis of size, origin (endosomal or cell membrane), markers, and contents (9). Exosomes are typically described as 30–120 nm, and microvesicles/ectosomes are often described as 120–1000 nm (10). Collectively these vesicles are termed extracellular vesicles (EVs).

Exosomes/EVs have been described as a means of communication between tumor cells and other cell types, including those of the microenvironment and beyond, in breast, as well as other cancer types (11). Dysregulation in this cell-to-cell communication and thus undesirable cellular cross-talk is understood to contribute to cancer development and progression. The multiroles of exosomes/EVs in breast cancer are reviewed here and summarized in Fig. 1.

## Exosomes in Invasion and Metastasis

The most common sites for breast cancer metastasis are to bone, brain, liver, and lung (12). A number of interesting studies, using cell lines, preclinical in vivo studies, and/or clinical samples, have started to unravel the role of exosomes/EVs in such breast cancer invasion and metastasis.

The first published study of exosomes in TNBC included the TNBC cell line Hs578T and its more ag-

gressive isogenic subclone Hs578Ts(i)<sub>8</sub> (13) to investigate the exosomal transfer of phenotypic traits (14). Here we found that exosomes from Hs578Ts(i)<sub>8</sub> cells confer their more aggressive phenotypic traits to all secondary breast cancer cells assessed (including the Hs578T parent cells, SKBR3, MDA-MB-231, and HCC1954). These characteristics included increased proliferation, migration, and invasion, as well as increased angiogenesis, when these exosomes were applied to human endothelial cells (14). Additionally, regarding the TNBC cell line MDA-MB-231 and the noninvasive mammary epithelial cell line HMLE, microRNA (miR)-10b was found to be expressed at significantly higher concentrations in the TNBC cells (15) and, in turn, HMLE cell invasion was promoted upon their exosomal uptake of MDA-MB-231-derived miR-10b. Similarly, MDA-MB-231-derived exosomal miR-105 was successfully transferred to an endothelial cell line, HMVEC, in vitro, resulting in impaired endothelial monolayer tight junctions, reduced vessel sprouting, and increased migration (16). Advancing to in vivo studies, mice were pretreated with these MDA-MB-231-derived, miR-105-loaded exosomes (or PBS as control) by intravenous injection into the tail vein and, following subsequent intracardiac injection of MDA-MB-231 cells, lung and brain metastases were observed in these mice (16). Additionally, MDA-MB-231-derived exosomes have been shown to transfer miRNAs to MCF10A cells, causing miR-10b and miR-21 to be upregulated in the MCF10A cells. This resulted in cell viability, cell proliferation, and colony-forming capacity to be increased in a Dicer-dependent manner. Further-

more, tumor formation occurred upon implantation of MCF10A cells into the mammary fat pads of the female athymic nu/nu mouse when coinjected with MDA-MB-231–derived exosomes (17). Conversely, coinjection of MCF10A cells with these exosomes, but with Dicer antibodies, resulted in reduced tumor growth. Because exosomal Dicer protein concentrations have been found to be increased in exosomes from the serum of breast cancer patients ( $n = 11$ ) compared to healthy controls ( $n = 8$ ), this result suggests that exosomes may promote cancer development and progression through miRNA biogenesis and through the oncogenic transformation of normal adjacent cells. Further research into exosomal-cell communication and miRNA transport is necessary to greater understand their roles in invasion and metastasis and to their possible exploitation for TNBC diagnostics and/or therapies.

Breast cancer cell–derived exosomes, released under hypoxic conditions, have been associated with increasing the invasive and metastatic potential of breast cancer cells (18). In studies of human breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-435) under hypoxic conditions, expression of RAB22A [an Rab GTPase, which is a membrane-bound protein and functions as a molecular switch, oscillating between its active and inactive states to integrate intracellular signaling and membrane trafficking events (19)] was found to be regulated through hypoxia-inducible factors that, in turn, increased the release of microvesicles. Conversely, shRNA (short hairpin RNA) knockdown of RAB22A in both MDA-MB-231 and MDA-MB-435 cells resulted in a decrease in microvesicle formation and thus a decrease in their *in vivo* invasion and lung colonization (18). RAB22A may thus have potential as a novel therapeutic target for suppressing tumor cell invasion and metastasis. In keeping with these observations, King et al. (20) reported hypoxic conditions to significantly increase the quantities of exosomes released from MDA-MB-231 and T47D breast cancer cell lines. Here, exosomal miRNA concentrations (miR-16, let-7a, miR-21, and miR-210) were investigated under normoxic and hypoxic conditions and exosomal miR-210 was found to be significantly upregulated under hypoxic conditions, suggesting a role for this miRNA in promoting tumor progression in response to hypoxic conditions (20). Advancing on this, after subcutaneous injection of MCC70-let-7a cells into RAG2<sup>-/-</sup> mice, let-7a miRNA was delivered to the cells via epidermal growth factor receptor (EGFR)-targeting exosomes. The successful delivery of let-7a resulted in inhibition of tumor development *in vivo* (21). This study suggests that nucleic acid therapeutics may be transported via exosomes to target EGFR-expressing tumors and may also have antimetastatic potential. The clinical potential relevance of this observation is further supported by the fact that let-7a is reported to be down-

regulated in RNA from highly metastatic breast cancer tumor samples ( $n = 76$ ) compared to normal breast tissue ( $n = 34$ ) (22).

Fibroblast-derived exosomes have been shown to play a role in increasing breast cancer metastasis and motility via the Wnt pathway (23). As reviewed by Zardawi et al. (24), the Wnt signaling pathway plays a role in cell migration, cell adhesion, stem cell maintenance, tissue patterning, and carcinogenesis. CD81<sup>+</sup> exosomes were found to be present in conditioned medium from mouse fibroblast L cells. Inhibition of breast cancer cell (MDA-MB-231) motility was observed following siRNA (small interfering RNA) knockdown of CD81 in L cells, therefore suggesting that the fibroblastic induction of breast cancer cell motility is regulated by CD81. Following coinjection of an orthotopic breast cancer mouse model with MDA-MB-231 cells and CD81 knockdown L cells, MDA-MB-231 cell metastasis was significantly suppressed. Breast cancer cell motility was found to be regulated by L-cell–secreted CD81-positive exosomes. The mechanism of action was found to be dependent on autocrine Wnt–planar cell polarity signaling (23). Because the Wnt signaling pathway has been shown to be activated in TNBC patient tumors ( $n = 130$ ) and Wnt/ $\beta$ -catenin signaling has been associated with a greater risk of lung metastasis (25), it would be important to investigate Wnt in cancer-associated fibroblast (CAF)-derived exosomes that may be causally involved in these events. Evidence to suggest CAF-derived exosomes may promote cancer cell motility is supported in a study in which ADAM10-rich exosomes were found to promote the activation of oncogenic signaling (26). Here, the CAF-like cell state was investigated to determine the role of the tissue inhibitor of metalloproteinases (TIMP) family in the maintenance of the extracellular matrix. TIMP knockout fibroblasts were found to produce ADAM10-rich exosomes which, in turn, increased expression of cancer stem cell (CSC) markers, including aldehyde dehydrogenase as well as integrin  $\alpha 6$ , and also increased cellular motility. Conversely, loss of the metalloproteinase ADAM10 suppressed lung metastasis in the MDA-MB-231 xenograft model. The clinical relevance of this study is supported by the fact that ADAM10 is significantly upregulated in breast tumor stroma ( $n = 51$ ) compared to stroma from normal healthy breast reduction tissue ( $n = 6$ ) (26).

### Stem Cells

Stem cells from mesenchyme, from fibroblasts, and from cancer cells themselves—and their associated exosomes—have been implicated in breast cancer.

Mesenchymal stem cells (MSCs) have homing capabilities and can regenerate and differentiate into tissues such as bone, cartilage, muscle, ligament, tendon, fibro-

blasts, and adipose [as reviewed by Chamberlain et al. (27)]. The roles of stem cell–derived exosomes in breast cancer progression and as potential therapeutics are becoming evident. Following treatment with human MSC-derived exosomes, MCF-7 cells underwent migration assessed by transwell assay, via mechanisms that involve the Wnt signaling pathway and are dose dependent on the exosomes added (28). EVs (termed exosomes by the authors) from MSCs have also been shown to suppress angiogenesis in the highly metastatic, invasive, and tumorigenic murine 4T1 mammary carcinoma cell line. Specifically, 4T1 cells were coincubated with murine bone marrow–derived MSC-derived exosomes, and  $\alpha$ -amanitin was co-added to suppress transcriptional activation caused by the addition of exosomes. miR-16, assessed by quantitative reverse transcription–PCR, was shown to be transferred from MSC-derived exosomes to 4T1 cells and subsequently reduced vascular endothelial growth factor (VEGF) expression. BALB/c mice were injected with 4T1 cells alone or 4T1 cells with MSC-derived exosomes, and tumor growth was significantly inhibited upon coinjection of 4T1 cells and MSC-derived exosomes (29). Although substantial work has yet to be done to further investigate this finding, these studies suggest that MSC-derived exosomes may be useful in a therapeutic setting as antiangiogenics or in the transfer of antiangiogenic miRNAs. It is noteworthy, however, that these studies showed somewhat contrasting responses to exosomes from MSCs, i.e., stimulation of breast cancer cell migration (28) but reduced angiogenesis and suppressed tumor progression (29), respectively. Although there may be other contributing factors for these results, we propose that these differing findings may, at least in part, be attributable to the former study being of human breast cancer cells and exosomes from human MSC cells for which the exosomes were isolated using filtration and ultracentrifugation techniques and the latter study having included murine breast cancer cells and extracellular vesicles (isolated using ExoQuick-TC) from murine MSCs. This highlights the relevance of activities by groups such as ME-HaD (European Network on Microvesicles and Exosomes in Health and Disease) that are aimed at standardization of techniques for isolation, characterization, and analysis of EVs, including exosomes, so that true biological differences can be separated from technical differences (30).

Adipose tissue–derived stem cells (ADSCs), obtained from lipoaspirates from individuals undergoing liposuction, were cocultured with exosomes derived from breast cancer cell lines (MCF-7 or MDA-MB-231). Following treatment with exosomes, ADSCs displayed a myofibroblastic phenotypical change with functional characteristics through induced or greatly increased expression of stromal cell–derived factor-1, CCL5 protein, transforming growth factor- $\beta$ , and VEGF. This study

indicates that tumor-derived exosomes may contribute to the conversion of ADSCs into tumor-associated myofibroblasts, thus contributing to the progression of tumor cells in the microenvironment (31).

CSCs are proposed to be involved in breast cancer relapse (32) and, in preclinical studies, have been shown to play a role in spontaneous metastases in an orthotopic breast cancer mouse model (xenotransplantation of patient tumor samples into nonobese diabetic/SCID mice) (33). Following treatment of tumor-associated mammary gland fibroblasts with breast cancer cell (MCF-7)–derived exosomes, mRNA concentrations of CSC activation markers, CD44, interleukin 6 (IL-6), and apolipoprotein E were all found to be significantly upregulated (34). When treated with nuclear receptor agonists (i.e., peroxisome proliferator activated receptor- $\gamma$  and retinoid X receptor), exosomes derived from hypoxic MCF-7 cells reduced mammosphere formation and Notch3 protein expression compared to normoxic conditions. Thus, it was proposed that peroxisome proliferator activated receptor- $\gamma$  and retinoid X receptor agonists may have potential in targeting the CSC niche (34).

## Apoptosis

Exosomes have also been implicated in evading apoptosis. For example, exosomes from 4T1 breast cancer cell mouse models were found to increase proliferation and suppress apoptosis of CD133<sup>+</sup> 4T1 cells, supporting the potential role of exosomes in tumor progression through evading apoptosis (35). Other studies, including human cells/clinical samples supporting a role for exosomes in evading apoptosis, are outlined below.

The superantigen staphylococcal enterotoxin B (SEB) induces T-cell activation and proliferation and is involved in Fas-mediated apoptosis (36, 37). When exosomes from MDA-MB-231 cells were anchored with SEB (EXO/SEB) and cocultured with MDA-MB-231 cells or with white blood cells and embryonic kidney cells as normal controls, coculture of EXO/SEB with MDA-MB-231 cells resulted in decreased proliferation of the tumor cells. Conversely, EXO/SEB coculture with both normal cell types resulted in no cytotoxic or antiproliferative effects. This is suggested to be due to EXO directly influencing tumor cells, but not normal cells. In the MDA-MB-231 cells, after 24 h coculture, caspase-3 and caspase-9 were significantly increased, indicating the mechanism involved to be via activation of the mitochondrial apoptotic pathway (38).

The role of S100 family genes, responsible for encoding calcium binding proteins (39), has been investigated in exosomal studies and in the study of breast cancer progression. A number of S100 genes were found to be involved in breast cancer progression, with *S100A11*

(S100 calcium binding protein A11)<sup>4</sup> and *S100A14* (S100 calcium binding protein A14) associated with patient outcome (40). The S100 protein hornerin, which has been detected in exosomes (41), was found to be expressed in breast cancer tissue, including mammary epithelial cells and stromal cells, and found to undergo proteolytic cleavage and differential subcellular compartmentalization. Premalignant, malignant, and metastatic MCF10A cells were developed by transfecting the primary cell line with active H-Ras; cells were then selected according to increased tumor growth from xenograft tumors. Hornerin was found to be decreased in the more aggressive tumor tissue (invasive ductal carcinoma) compared to invasive lobular carcinoma. Furthermore, increased expression and fragmentation of hornerin was observed in breast cancer cells following the induction of apoptosis/necrosis with H<sub>2</sub>O<sub>2</sub>. Hornerin, possibly transported via exosomes, may play a role in promoting apoptosis and suppressing tumor progression (41).

The antiapoptotic protein survivin has been detected within exosomes and found to be released in the extracellular space via exosomes. Initially, exosomes derived from the ovarian cancer cell line HeLa were found to secrete survivin under basal conditions and this was increased following proton irradiation stress, suggesting the involvement of an exosomal pathway in the release of survivin in cancer cells (42). More recently, the use of serum-based exosomal survivin splice variants as early diagnostic biomarkers has shown promise in breast cancer. With the use of acetylcholinesterase activity assays, the amount of cancer exosomes was found to be significantly higher in breast cancer patient serum (n = 40) than in control serum from female patients who had undergone neoadjuvant treatment followed by surgery with no recurrence (n = 10) (43). Considering the roles of survivin and its splice variants in cancer (44), for which survivin is an inhibitor of apoptosis and survivin-ΔEx3 and survivin-2B have apparently retained and lost antiapoptotic potential, respectively, Western blot analysis revealed an increase in antiapoptotic survivin and survivin-ΔEx3 protein in breast cancer patient serum-derived exosomes. Survivin and survivin-ΔEx3 proteins were also found to be increased in breast cancer tissue (n = 23) compared to control samples (female patients who had undergone neoadjuvant treatment followed by surgery with no recurrence) (n = 10). Additionally, differential expression of proapoptotic survivin-2B was evident in patient exosomes and tumor tissue compared to that of healthy controls. Specifically, low expression of survivin-2B was found in the most aggressive breast can-

cer stages and corresponding exosomes. Overall, these studies support a role for exosomal (as well as tissue-based) survivin and its splice variants as diagnostic and prognostic biomarkers with potential for their exploitation in a therapeutic setting (43).

An in vitro study of miR-373-transfected MCF-7 cells showed estrogen receptor expression to be down-regulated and an inhibitory effect on apoptosis was observed. To add a clinical perspective, significantly increased concentrations of exosomal miR-373 have been reported in serum of TNBC patients (n = 168) compared to serum from age-matched healthy controls (n = 28). Although, again, further studies are necessary—ideally including in vitro, preclinical, and clinical studies of each breast cancer subtype to truly determine the potential of exosomal miR-373—this link to increased breast cancer aggressiveness suggests its potential as a diagnostic biomarker with possible benefit for exploitation in a therapeutic approach (45).

## Immune System

Many tumors develop immune evasion mechanisms to survive. Such mechanisms include secretion of tumor proteins, T-cell evasion, promotion of T regulatory cells, and reduction of the expression of antigen-presenting proteins (46, 47). Studies have shown that breast cancer cell-derived exosomes can influence the immune system through interactions with T cells, dendritic cells (DCs), macrophages, and T regulatory cells.

Natural killer (NK) cells, natural killer T (NKT) cells, CD8<sup>+</sup> αβ T cells, γδ T cells, and macrophages all express the NK group 2, member D (NKG2D) receptor that, upon ligand binding, activates NK-cell cytotoxic killing and provides a costimulatory signal in T cells. Lymphocyte NKG2D receptor expression and CD8<sup>+</sup> T cell cytotoxic ability have been reported to be significantly inhibited following coculture of peripheral blood leukocytes with breast cancer cell (T47D)-derived exosomes (48). Further studies based on these interesting initial results, including clinical samples, could be very useful.

Advancing from these findings, investigators have shown that bone marrow-derived precursor myeloid cells take up tumor exosomes. This was shown by injecting PKH67-labeled TS/A-cell (metastatic murine cell line)-derived exosomes into BALB/c mice for which subsequent FACS (fluorescence-activated cell sorter) analysis revealed the majority (94%) of PKH67-labeled exosomes to be present in myeloid cells. In keeping with this observation, MDA-MB-231 cell line-derived exosomes, cocultured with human CD14<sup>+</sup> monocytes, were found to inhibit DC differentiation through the induction of IL-6 (49). These studies suggest that exosomes

<sup>4</sup> Human genes: *S100A11*, S100 calcium binding protein A11; *S100A14*, S100 calcium binding protein A14; *PTEEN*, phosphatase and tensin homolog.

may therefore be important biomarkers and immunotherapeutic targets in breast cancer.

Exosomal transport of miRNAs has been shown to influence macrophages in the tumor microenvironment. The role of tumor-associated macrophages (TAMs) in metastasis and cancer progression is still uncertain, although they have been shown to be involved in tumor angiogenesis in breast cancer patients (50). TAMs are polarized to an immunosuppressive phenotype, through inhibitor of nuclear factor- $\kappa$ B (NF- $\kappa$ B) kinase subunit  $\beta$  (IKK $\beta$ )-mediated NF- $\kappa$ B activation (51). The green tea component epicatechin gallate (EGCG) is reported to have antitumorogenic properties (52, 53). TAM infiltration and M2 polarization were found to be suppressed in an in vivo and ex vivo EGCG-treated breast cancer study (54). In studies aimed at elucidating the mechanism of events involved, miR-16 was found to be upregulated following EGCG treatment of 4T1 cells in vitro; miR-16 was transported to TAMs (derived from established murine breast cancer) via tumor-derived exosomes, and so inhibited macrophage infiltration and polarization in the tumor microenvironment (54).

By microarray analysis, overexpression of miR-223, miR-565, and miR-660 was evident in human monocyte-derived macrophages compared to breast cancer cell lines (SKBR3 and MDA-MB-231). IL-4-activated monocyte-derived macrophages were found to overexpress miR-223, but this was not seen in the breast cancer cells. miR-223 was thus advanced to further investigate its expression levels in breast cancer cells upon coculture with IL-4-activated monocyte-derived macrophages. For this purpose, breast cancer cells were cocultured with TAM-derived exosomes and miR-223 was found to be shuttled by the TAM exosomes, resulting in increased miR-223 in the cancer cells. miR-223 was subsequently reported to promote the invasive potential of breast cancer cells (55). In agreement with that observation, a lower rate of overall survival was found in TNBC patients whose tumors had high concentrations of infiltrating TAMs (56). Although yet to be investigated, it is possible that exosomal miR-223 from the TAMs may be playing an adverse role here. In a recent study, exosomes from breast cancer cell lines (MDA-MB-231 and MCF-7) were reported to stimulate macrophages through the activation of NF- $\kappa$ B via TLR2 (Toll-like receptor 2) (57). This suggests bidirectional communication (exosomes from macrophages influencing cancer cells; exosomes from cancer cells influencing macrophages), with the possible exosomal alteration of partner cells adding further to the complexity of breast cancer.

Exosomes from the hTERT (human telomerase reverse transcriptase)-immortalized human normal mammary epithelial cells, HMEC B42, inhibited the release of exosomes from its clonal breast cancer subline B42 clone 16 (which was developed by exposing HMEC B42 cells

to  $\gamma$  irradiation) and, conversely, tumor-derived exosomes inhibited the release of exosomes from the normal mammary epithelial cells, HMEC B42. A novel exosomal release regulatory feedback pathway has thus been proposed (58). Exosomes from HER2<sup>+</sup> (human epidermal growth factor receptor 2-positive) breast cancer cell lines (SKBR3 and BT474) and the normal human mammary epithelium 184A1 cell line were isolated to determine secretome profiling using 2-dimensional gel electrophoresis and MALDI-TOF mass spectrometry (59). In silico functional annotation using the Database for Annotation, Visualization and Integrated Discovery predicted exosomes derived from breast cancer cells to be involved in energy metabolism, antigen processing, and antigen presentation (59). Wet laboratory research into the role of exosomes in tumor immunosurveillance and energy metabolism is warranted.

In a preclinical in vivo study of a 4T1 breast cancer metastasis model, primary breast tumor-derived exosomes successfully communicated/interacted with immune cells. Specifically, tumor-infiltrating leukocytes were cocultured with murine breast cancer 4T1 cell-derived exosomes, and fibronectin was found to be successfully absorbed by/recruited into the exosomes (60). When placed in vivo, tumor cell invasion was found to be enhanced through the regulation of CD25<sup>+</sup> T regulatory cells and GR-1<sup>+</sup> myeloid-derived suppressor cells, by the fibronectin that had been recruited into the exosomes (60). The cellular cross-talk evidenced in this study should be further expanded to increase our understanding of the mechanisms of exosomal communication and protein sorting into exosomes.

## Drug Resistance

Drug resistance is a major obstacle in breast cancer treatment and exosomes/EVs are of major interest in drug resistance studies. Stromal cells were found to initiate cross-talk with breast cancer cells (MDA-MB-231) via exosomes. Exosomes were found to be transferred from stromal cells to breast cancer cells, thereby activating antiviral RIG-I (retinoic acid-inducible gene 1 enzyme) signaling and in parallel activating NOTCH3 pathways to regulate the expansion of therapy-resistant tumor-initiating cells. It has been proposed that this mechanism of exosomal transfer is regulated through the stromal cell-induced increase in RAB27B and the activation of RIG-I signaling via the transfer of exosomal 5'triphosphate RNA. In further investigation of these findings, MDA-MB-231 xenograft female nude mice were coinjected with nontransformed MRC5 human diploid fibroblasts (as stromal cells), and in these mice STAT1 (signal transducer and activator of transcription 1) expression was increased, with reduced cell death and increased tumor growth evident in this model (61).

Exosomal transport of P-glycoprotein (P-gp) has been described as a possible mechanism in exosome-mediated drug resistance. Our research group initially showed this in relation to prostate cancer (62) and, more recently, exosomes from docetaxel-resistant MCF-7 cells have been shown to transfer drug resistance to docetaxel-sensitive MCF-7 cells. The suggested mechanism of resistance is via exosomal delivery of P-gp, because P-gp concentrations are higher in exosomes derived from drug-resistant cells than in drug-sensitive cells (63).

Adriamycin (Adr) and docetaxel (Doc) have been shown to have therapeutic efficacy in breast cancer patients, but drug resistance limits their clinical benefits. Drug-sensitive (MCF-7/S) and drug-resistant human breast cancer cell lines (MCF-7/Adr and MCF-7/Doc) were used to investigate possible exosomal transfer of resistance. On coculture of MCF-7/Adr exosomes with MCF-7/S cells, low levels of proliferation and high levels of drug resistance were observed. Similarly, this effect was seen for MCF-7/Doc-derived exosomes. Microarray analysis identified miRNA profiles for both MCF-7/Adr- and MCF-7/Doc-resistant cells in which possible common pathways of resistance were observed, suggesting that the transfer of miRNAs plays a role in this exosomal transfer of resistance (64, 65). In relation to tamoxifen, exosomes from tamoxifen-resistant MCF-7 cells were found to promote proliferation of MCF-7 wild-type cells. Functional assays (cell viability, apoptosis, and colony formation) assessed the involvement of miR-221 and miR-222 in the transfer of this tamoxifen resistance, which was found to be significantly blocked using anti-miR-221/anti-miR-222 (66).

HER2 concentrations are significantly increased in exosomes from breast cancer cell lines, and SKBR3 and BT474 and HER2<sup>+</sup> exosomes have been shown to bind to and interfere with activity of the monoclonal antibody trastuzumab in *in vitro* studies (67). In a study of samples from HER2-overexpressing breast cancer patients (n = 22), higher exosome-trastuzumab-binding capacity was evident in advanced disease-stage serum (n = 11) compared to serum from the early-stage cohort (n = 11). In contrast, exosomes did not interfere with the *in vitro* antiproliferative activity of the small molecule lapatinib that targets EGFR as well as HER2 (67). It is possible that, *in vivo*, HER2<sup>+</sup> exosomes may modulate trastuzumab availability and so adversely affect patient outcome. Although *in vivo* studies and more extensive analysis of relevant patient samples are necessary to further investigate this activity, this study supports a broad range of important roles held by exosomes in breast cancer and highlights their potential in a diagnostic and therapeutic setting.

## Targeted Delivery Systems

Because trastuzumab resistance is a major obstacle in the treatment of HER2-overexpressing breast cancers, there is substantial focus on the development of clinically effective antitumor vaccines, with Hao et al. (68) providing a promising approach. Here the use of ovalbumin-pulsed DC-released exosomes stimulated cytotoxic T lymphocyte (CTL) responses. In this way, a CD4<sup>+</sup> T-cell-based vaccine (OVA-T<sub>EXO</sub>) was found to stimulate long-term CTL memory (69). Further to this study, transgenic HLA-A2/HER2 mice were used to study HER2-specific CD8<sup>+</sup> CTL responses and antitumor activity following administration of the HER2-T<sub>EXO</sub> vaccine (70). The HER2-T<sub>EXO</sub> vaccine was found to be effective at killing trastuzumab-resistant BT474<sub>A2</sub> tumor cells *in vitro* and to eradicate the BT474<sub>A2</sub> tumor *in vivo* (70). This study, therefore, provides early evidence for a novel therapeutic approach involving the use of exosomes for trastuzumab-resistant HER2-overexpressing breast cancer cells.

Reducing immunogenicity and toxicity is essential for the development of new cancer drug delivery systems. To study their potential efficacy as therapeutic delivery systems, exosomes were isolated from mouse immature DCs that were engineered to express the fused protein Lamp2b- $\alpha$ v integrin-specific iRGD peptide. The iRGD exosomes were purified and loaded with doxorubicin. These exosomes were shown to have high affinity for  $\alpha$ v integrin<sup>+</sup> MDA-MB-231 breast cancer cells and significantly inhibited MDA-MB-231 and MCF-7 cell proliferation. Using an MDA-MB-231 tumor-bearing nude mouse model, tumor growth was significantly inhibited using iRGD-exosome-doxorubicin treatment compared to controls (PBS, iRGD exosomes, blank-exosomes-doxorubicin), with no doxorubicin-associated cytotoxic effects reported. This study provides insight into the natural, nanoscale delivery of chemotherapeutic drugs via exosomes. Although substantial work has yet to be done to further investigate this approach, this drug delivery system may prove to be effective in a clinical setting (71).

Mutations in the tumor suppressor gene phosphatase and tensin homolog (*PTEN*) deleted on chromosome 10 are increased in breast cancer patients (72). The PTEN C-terminus (PTEN-CT) stabilizes PTEN. DU145 prostate cancer cell-derived exosomes have been found to secrete PTEN and transfer PTEN to DU145Kd cells *i.e.*, DU145 cells with PTEN knock-down (73). Exosomal delivery of PTEN-CT from HEK293 cells into the murine mammary carcinoma cell line 4T1 caused a reduction in cell viability and impaired colony-forming abilities (74). Although this finding has yet to be fully investigated with breast cancer cells, this exosomal

PTEN-CT delivery system may prove to be beneficial as a targeted therapeutic for *PTEN*-mutated breast cancers.

Although clinical trial studies of exosomes in breast cancer have not yet been reported, there is a precedent for this approach in melanoma, colon, and lung (<https://clinicaltrials.gov> NCT02310451, NCT01294072, and NCT01159288) cancers, giving hope of timely translation of exosomes studies toward clinical utility in breast cancer.

## Conclusion

This review brings together information on the roles of exosomes in breast cancer development, progression, drug resistance, and targeted drug delivery, including specific studies on their role in invasion, metastasis, stimulation of stem cell populations, apoptosis, and modulating cells of the immune system. These studies highlight the growing interest, and growing understanding, of EVs in breast cancer.

Building on these interesting and exciting data, further research is now warranted, both to validate preliminary findings and to expand our knowledge in this area. Although not yet exploited in a clinical setting, the exosome studies reviewed here demonstrate the many roles

of exosomes in breast cancer and their possible exploitation in the development of future therapeutics in the interest of breast cancer patients.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors' Disclosures or Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

**Employment or Leadership:** None declared.

**Consultant or Advisory Role:** None declared.

**Stock Ownership:** None declared.

**Honoraria:** None declared.

**Research Funding:** W.M. Gallagher, the Irish Cancer Society Collaborative Cancer Research Centre BREAST-PREDICT Grant CCRC13GAL; L. O'Driscoll, the Irish Cancer Society Collaborative Cancer Research Centre BREAST-PREDICT Grant CCRC13GAL, H2020 Cooperation in Science and Technology, Microvesicles and Exosomes in Health & Disease [ME-HaD; BM1202], and HEA PRTL Cycle 5 funding of TBSI.

**Expert Testimony:** None declared.

**Patents:** None declared.

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