

Roles of Extracellular Vesicles in Metastatic Breast Cancer

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ABSTRACT: Cells can secrete extracellular vesicles (EVs) to communicate with neighboring or distant cells by EVs which are composed of a lipid bilayer containing transmembrane proteins and enclosing cytosolic proteins, lipids, and nucleic acids. Breast Cancer is the most frequently diagnosed malignancy with more than 1 million new cases each year and ranks the leading cause of cancer mortality in women worldwide. In this review, we will discuss recent progresses of the roles and mechanisms of cancer-derived EVs in metastatic breast cancer, with a special attention on tumor microenvironment construction, progression, and chemo/radiotherapy responses. This review also covers EV roles as biomarker and therapeutic target in clinical application.

KEYWORDS: Extracellular vesicles, breast cancer

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Introduction

Cells can secrete extracellular vesicles (EVs) to communicate with neighboring or distant cells. Extracellular vesicles are composed of a lipid bilayer containing transmembrane proteins and enclosing cytosolic proteins and RNA. Based on their cellular origins, EVs can be classified into 2 groups. The first group of EVs is formed and released by budding from the cells' plasma membranes, generally known as microvesicles, ectosomes, or microparticles. These sizes of the EVs range from 100 to 1000 nm in diameter. The second type of EVs, referred to the exosomes, is generated inside multivesicular endosomes or multivesicular bodies and released when these compartments fuse with the plasma membrane. Exosomes are usually smaller than 150 nm in diameter. Surface molecules of EVs can be recognized by recipient cells and trigger EVs' internalization. Thus, recipient cells' physiological state can be modified by cytosolic proteins and RNAs carried by EVs to achieve cell-cell communication.

In this review, we will discuss recent progresses in our understanding toward the roles and mechanisms of cancer-derived EVs, with a special attention on metastatic breast cancers. Because most current purification protocols (differential ultracentrifugation, 220 nm filtration, commercial kits) cannot distinguish the subtypes of EVs, we will provide a broad view of all types of EVs in general.

EVs and the Primary Tumor Microenvironment

Tumors are composed of malignant cancer cells embedded in vasculature and surrounded by tumor stroma consisting of various nonmalignant cells, such as fibroblasts and myeloid cells. The

tumor microenvironment plays a critical role in tumorigenesis. Communications between tumor-tumor cells and tumor stromal cells are involved in primary tumor formation and progression.

In the primary tumor, exosomes can be exploited to share oncogenic molecules among tumor cells and thus can directly modify tumor cells' signaling and metabolic state. Proteins and microRNAs (miRNAs) regulating apoptosis, cytoskeleton remodeling, cell mobility, cell cycle, tumor invasion, and metastasis are identified in EVs isolated from breast cancer cell lines (MCF-7, MDA-MB-231).^{1,2} Exosomes expressing CD63-GFP have been directly observed transferring between tumor cells both in vitro and in xenograft murine models.³ Aggressive subclone cell line Hs578Ts(i)₈-derived EVs can promote cell proliferation, migration, and invasion of recipient cancer cells.⁴ Uptake of EVs of 4T1 cells can notably stimulate proliferation and suppress apoptosis of CD133⁺ breast cancer cells in vitro.⁵ Cell adhesion has been shown to have indispensable effects on tumor growth and metastasis through the interaction of tumor endothelial cells.⁶ BT-549-released exosomes can promote focal adhesion, attachment, and spreading through the association of fetuin-A with histone H2A.⁷ In addition to proteins and messenger RNAs, miRNA and other noncoding RNAs are also possible active EV cargos. Recent report has shown that MDA-MB-231 EV-mediated secretion of miR-10b and miR-21 in tumor microenvironment is responsible for elevated cancer cell viability, proliferation, and colony-forming capacity.⁸ Overall, breast cancer cell-derived EVs can transport plenty of miRNAs and proteins to facilitate neoplastic formation and development.^{9,10}

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In addition, breast cancer cell-derived EVs can alter the cellular signaling and metabolic state of surrounding nontumor cells. Exosomes derived from tumor cells tagged with CD63-GFP can be incorporated into tumor stromal cells as well as circulate in the blood with metastases.³ Mesenchymal stem cells (MSCs) have potential to regenerate and differentiate into multiple types of cells, which can further contribute to tumor stroma and provide an applicable microenvironment for tumor progression. Breast cancer cell-derived EVs can induce a tumor-associated myofibroblastic phenotype of adipose tissue-derived MSCs, with increased expression of α -SMA, promoting expression of stromal cell-derived factor 1 (SDF1), transforming growth factor β (TGF- β), vascular endothelial growth factor (VEGF), and C-C motif chemokine ligand 5 (CCL5) via the SMAD-mediated signaling pathway.¹¹ In addition to tumor-derived EVs, EVs from cancer-associated stromal cells can stimulate invasiveness of recipient breast cancer cells, in this case, by activating Wnt-planar cell polarity-dependent signaling process.¹² Collectively, EVs function as critical mediators of tumor-tumor cells and tumor stromal cells' interaction and their adaptive responses.

Roles of EVs in Tumor Progression

During tumor progression, cells within them develop the ability to invade into surrounding normal tissues and through tissue boundaries to form new growths (metastases) at sites distinct from the primary tumor. Cell-cell and cell-matrix adhesion, degradation of extracellular matrix (ECM), initiation, and maintenance of early growth at the new site are generally accepted to be critical in tumor invasion. Tumor-derived EVs are believed to influence tumor invasion by increasing tumor cell motility and ECM degradation. Extracellular vesicles can directly contribute to ECM degradation by spreading matrix metalloproteinases present on EVs. Intravital imaging demonstrates persistent and efficient *in vivo* movement of cancer cells which relies on secretion of exosomes bearing ECM.¹³ In the work by Hendrix et al,¹⁴ rab27b-mediated exocytic release of HSP90-positive exosomes from metastatic breast cancer cells can promote directional cancer cell invasion ability through degradation of ECM components and release of growth factors by MMP2 activation. Another view by Wang group also demonstrates that EVs shed by hypoxic breast cancer cells promote focal adhesion formation and invasion.¹⁵ In addition, recipient cells treated with exosomes from CXCR4-breast cancer cells showed increased proliferation, migration, and invasion capacities.¹⁶ Furthermore, MSC-derived exosomes accelerate migration of the breast cancer cell line MCF7.¹⁷ However, more intensive studies *in vivo* are required to clarify definitive roles of EVs in tumor invasion.

Epithelial-mesenchymal transition (EMT) is a biological process by which epithelial cells are transdifferentiated to a mesenchymal state and has been implicated in the progression toward an advanced cancer phenotype.^{18,19} Extracellular vesicles have been shown to participate in EMT, and some groups

have described how tumor-derived EVs are involved in this process. Release of MDA-MB-231 EVs, stimulated with linoleic acid, induces a transient decrease in E-cadherin expression, accompanied by increase in Snail 1/2, Twist 1/2, Sip1, Vimentin, and N-cadherin expression. Extracellular vesicles also promote MMP-2 and MMP-9 secretion, nuclear factor κ B (NF- κ B)-DNA binding activity, migration, and invasion of MCF10A cells.¹⁸

Recent report showed that tumor-derived exosomes influence the survival and proliferation of metastatic tumor cells at distant sites.²⁰⁻²² MDA-MB-231-, T47DA18-, and MCF-7-derived exosomes can be taken up by human primary mammary epithelial cells (HMECs), resulting in an increase in reactive oxygen species, autophagy, and secretion of tumor factors from human primary mammary epithelial cells (HMECs). This permissive microenvironment supports survival and proliferation of incoming metastatic tumor cells.²³ A novel mechanism employed by breast cancers to induce pro-inflammatory activity has been highlighted that circulating tumor-derived EVs can promote NF- κ B activation and secretion of pro-inflammatory cytokines such as IL-6 (interleukin 6), TNF- α (tumor necrosis factor α), GCSF (granulocyte-colony stimulating factor), and CCL2 of distant macrophages.²⁴

Glucose-enriched niche is generated by transfer of miR-122-bearing tumor EVs to stromal cells, which prevents glucose uptake of stromal cells via miR-122-mediated inhibition of pyruvate kinase.²⁵ Overall, the data above indicate the functional implications proposed for EVs of supporting metastatic tumor cells' survival and proliferation at distant site. However, this working model of circulating tumor EVs has not been demonstrated in a fully physiological *in vivo* context.

A frequent observation in patients with cancer is thrombocytosis. One possible explanation is coagulation and platelet accumulation at cancer sites can evade immune surveillance and promote cell migration.²⁶ Extracellular vesicles are reported to be involved in coagulation by carrying tissue factor (TF) and other coagulation-promoting factors.^{27,28} In addition to tumor-derived EVs, EVs from platelets and cancer-associated inflammatory cells participate in coagulation.²⁹ The EVs bearing TF derived from breast cancer cells can exchange between tumor cells with different aggressiveness potentials, which may contribute to the propagation of a TF-related aggressive phenotype among heterogeneous subsets of breast cancer cells.³⁰ However, more *in vivo* data are required to elucidate the roles of TF-bearing EVs in promoting coagulation in breast cancer.

After dissemination, cancer cells intravasate to the circulation. Tumor-derived EVs can alter the cellular signaling and metastatic state of recipient endothelial cells and trigger vascular permeability and recruitment of bone marrow progenitor cells.³¹ Some groups have described how EVs are involved in this process. Recent report has shown that vascular leakiness in lung is triggered by breast cancer-derived EVs, which upregulate a subset of S100 proteins and activate Src kinase signaling.³² Another report by Zhou et al³³ also shows that breast

cancer cell-derived EVs bearing miR-105 can induce tight junction protein ZO1 destruction in recipient endothelial cells, resulting in increasing vascular permeability.

Tumor growth and progression depend on exploitation of preexisting vessels and development of new vessels to obtain necessary nutrient and oxygen especially under hypoxic conditions called vascularization. Recent reports have shown that breast cancer cell-derived EVs have potential roles in promoting angiogenesis. One group discovered that EVs bearing bioactive form of VEGF are released from tumor cells under acidic condition.³⁴ Treatment of adipose stem cells (ASCs) enriched in mammary microenvironment with breast cancer cell-derived EVs leads to VEGF secretion from ASCs and angiogenic sprouting of human umbilical vein endothelial cells (HUVECs).³⁵ Moreover, another group displayed that breast cancer cell-derived EVs contain a unique oligomeric species of VEGF called VEGF_{90k}. After cross-linking with VEGF₁₆₅, VEGF_{90k} will be catalyzed by the enzyme tissue transglutaminase and associated with EVs through the interaction with Hsp90. Both in vitro and in vivo studies indicate that VEGF_{90k}-EVs can activate endothelial cells to migrate toward angioreactors and stimulate HUVECs to undergo tubulogenesis.³⁶ These observations indicate that EVs isolated from tumor cells may exert important effects on tumor angiogenesis. However, more experiments should be performed to draw a solid conclusion.

Whether tumor cells can evade immune surveillance becomes a crucial step in tumor metastasis and several ways are employed by tumor cells: deleting immune cells via death ligands, suppressing immune reaction by regulatory T cells, and inducing tolerization by cytokines or cross-presentation related to dendritic cells (DCs) and macrophages.³⁷ NKG2D, a homodimeric C-type lectin receptor, is widely expressed in various immune cells. On binding of ligands, NKG2D can directly trigger NK cytotoxic capacity and activate costimulatory signaling pathway in T cells in addition to T cell receptor-dependent process. Breast cancer cell-derived exosomes can inhibit immunologic functions by repressing expression of the NKG2D receptors on lymphocytes, resulting in decreased CD8+ T-cell cytotoxicity.³⁸ Dendritic cells, originating from hematopoietic stem cells, act as antigen-presenting cells to stimulate T-cell activation and induce the host antitumor immune response. Previous report showed that bone marrow-derived CD11b+ myeloid precursor cells can take up tumor exosomes in vivo, which further blocks DC differentiation and maturation via the induced IL-6 production in vitro. Consistent with the observation in murine model, coculture of exosomes isolated from MDA-MB-231 breast tumor cells with CD14+ monocytes results in decreased DC differentiation.³⁹ Tumor-associated macrophages (TAMs) play vital roles in the tumor microenvironment and are associated with poor diagnosis due to the tumor-promoting inflammatory M2 phenotype, which is the main existing form of TAM. Breast cancer cell-derived

exosomes can stimulate NF- κ B activation in TAMs, resulting in secretion of pro-inflammatory cytokines such as IL-6, TNF- α , GCSF, and CCL2 both in vitro and in vivo.²⁴ Collectively, breast cancer cell-derived EVs can function as critical mediators of tumor cells to evade immune surveillance.

EVs and Therapeutic Responses

On the way to successful treatment of breast cancer, drug resistance remains an intractable impediment. Tumor-derived EVs can participate in cancer cell resistance to chemotherapy. P-glycoprotein (P-gp), a membrane transporter, can reduce the accumulation of antitumor drugs in cytoplasm due to its active drug efflux capacity.^{40–43}

Drug resistance can be transferred to sensitive recipient cells by EVs derived from docetaxel-resistant MCF-7 cells, which can promote P-gp expression in dose-dependent pattern.⁴⁴ Different consequences of EV-associated RNA transfer in the breast cancer microenvironment have been recently described. Extracellular vesicles bearing miR-200c can reduce P-gp expression to enhance chemosensitivity to epirubicin,⁴⁵ whereas miR-298 and miR-451 bearing EVs can induce chemoresistance to doxorubicin via the increased P-gp expression.^{46,47} Besides P-gp modulation, suppression of Raf-1 and Bcl2 by miR-195-EVs promotes the chemosensitivity to Adriamycin and radiosensitivity.⁴⁸ In addition, exosomes bearing miRNA cargo derived from stromal cells transfer to breast cancer cells and activate the pattern recognition receptor RIG-1 and stimulate the STAT-1-dependent pathway and NOTCH3, which further collaboratively induce the stroma-mediated resistance process.⁴⁹ Several kinds of miRNAs (miR-17, miR-29, miR-30a, miR-100, miR-221, miR-222, etc) are upregulated in drug-resistant MCF-7 cells and enriched in exosomes. These miRNAs bearing exosomes further transfer to sensitive MCF-7 cells and induce drug resistance.^{50,51} In addition to miRNAs, multifarious proteins in tumor EVs can also regulate P-gp expression. MCF-7 Adriamycin-resistant cell-derived EVs transfer a Ca²⁺-permeable channel TrpC5 to human microvessel endothelial cells, resulting in the elevated expression of P-gp by activation of the transcription factor nuclear factor of activated T cells' isoform c3 (NFATc3).⁵² Recently, a member of ATP (adenosine triphosphate)-binding cassette transporter family called ABCG2 has been reported to play a vital role in multidrug resistance (MDR) induction. Chemotherapeutic drugs have been concentrated in EVs relying on ABCG2, thus reducing drug concentration in cytoplasm.⁵³ It has been reported that PI3K-Akt signaling pathway and Ko143 participate in ABCG2 targeting and biogenesis of EVs.⁵⁴ This mechanism can be employed to overcome MDR. Taking advantages of EVs that can actively concentrate various drugs from cytoplasm, treatment of cells with photosensitive cytotoxic chemicals produces drug-bearing EVs. Reactive oxygen species will be induced in recipient cells, leading to tumor cell lysis to overcome MDR.⁵⁴

Clinical Applications of EVs

Many groups have described that more EVs are secreted from cancer cell lines compared with noncancerous cells, with remarkable highly expressed molecules. This will make circulating tumor-derived EVs as promising biomarkers to evaluate tumor progression and prognosis.^{55–58} In addition, more exosomes can be separated from serum of patients with breast cancer than healthy donors.⁵⁹ Breast cancer–released EVs, regarded as potential indicators at early stage of illness, are worth further investigation. Several proteins, including the oncogenic cancer marker CD24, focal adhesion kinase (FAK), epidermal growth factor receptor (EGFR), apoptosis inhibitor surviving, and its splice variants, cell surface proteoglycan glypican-1 (GPC-1), have been reported to be dramatically overexpressed in EVs derived from serum of patients with breast cancer compared with healthy donors.^{60–64} In addition, researchers found that the exosomes derived from MCF cell line express higher level of 27-hydroxy-cholesterol compared with exosomes derived from MDA-MB-231 and healthy control group.^{65,66} These molecules differentially expressed according to the stage of tumor progression. For example, developmental endothelial locus-1 protein (Del-1) is highly expressed in circulating EVs derived from patients with breast cancer compared with healthy donors. After tumor resection, Del-1 level will decrease to normal level.⁶⁷ Besides, EVs isolated from pleural effusions of patients with breast cancer are enriched in disintegrin and metalloprotease ADAM10, CD9 tetraspanin, and epithelial cell adhesion molecule (EpCAM) compared with healthy donors.^{68–70} Thus, specific proteins can make EVs as candidates to be breast cancer biomarkers. In addition to proteins, circulating exosomal miRNAs can also be employed as a diagnostic marker for cancer progression and prognosis. A tendency in field shows that detection of exosome-bearing miRNAs is more sensitive and reliable than miRNAs directly purified from plasma or serum.⁵¹ MiR-101 and miR-372 are found enriched in exosomes, but not in serum samples. Conversely, significantly higher level of miR-372 is detected in serum other than in exosomes from cancer samples.⁷¹ Compared with healthy control group, miR-21 and miR-1246 are elevated in exosomes derived from plasma of patients with breast cancer and tumor-bearing mice.⁷² In addition, miR-105, a potent regulator of migration via direct interaction with the tight junction protein ZO1, is uniquely expressed and released by metastatic breast cancer cells. Thus, in patients with early-stage breast cancer, high expression level of circulating miR-105 suggests the possibility of tumor metastasis.³³ Overall, these studies suggest that EVs have potential to be employed as biomarkers for diagnosis and prognosis at early stage of disease in patients with breast cancer.

Breast cancer–derived EVs have been shown to promote tumorigenesis, angiogenesis, invasion, and metastasis, suggesting that interfering EV biogenesis can be a potential way in cancer therapy. Some studies have attempted to do this by inhibiting Ras-related RAB proteins. Rab27a^{-/-} 4T1 cells

exhibit reduced secretion of EVs and lower tumor growth and incidence of pulmonary metastasis.⁷³

All EVs bear surface molecules that allow them to be targeted to recipient cells. Exploiting their cell surface receptors, EVs can also be modified and used as target-specific drug delivery system. Modified exosomes with EGF or EGFR ligand on their surfaces can specifically target EGFR-expressing breast cancer cells and deliver cargos such as miRNAs to them. Loading of tumor suppressor let-7a miRNA in these modified exosomes suppresses xenograft breast cancer growth in murine model, which provides a tool for miRNA replacement therapies in antitumor treatment.⁷⁴ MicroRNA profiling shows miR-134 is the most substantially downregulated miRNA in EVs derived from aggressive breast cancer cells. Delivery of miR-134–enriched EVs to tumor cells leads to distinct reduction in cellular migration and invasion as well as increased apoptosis and drug sensitivity.⁷⁵ Moreover, researchers synthesized a novel structure named exosomes/SEB (staphylococcal enterotoxin B), which bears cytostatic effect on MDA-MB-231 cell. This delicate structure dramatically reduces cell proliferation and induces apoptosis, with increased expression of bax, bak, caspase 3, and caspase 9.⁷⁶ In addition, treatment of breast cancer cells with epigallocatechin gallate (EGCG), a molecule with known antitumor effects, upregulated the expression of tumor suppressor miR-16 in tumor EVs. Ex vivo incubation of exosomes isolated from EGCG-treated breast cancer cells with TAM leads to repressed NF- κ B signaling and M2 polarization, which activates antitumor immune response.⁷⁷ Overall, these discoveries shed light on EVs' capacity as promising candidate vehicles for drug delivery in antitumor therapy.

Future Directions

Recent exosome purification protocols used are based on different protein markers, sizes, and density.⁷⁸ However, few of these purification methods can clearly isolate specific type of EVs. Most studies published so far analyze mixed EV populations. There raise multiple questions about these vesicles themselves: What are the tissues of origin of EVs of different sizes? What is the specific biological function of different types of EVs? How are these EVs interacting with each other (functionally)? Addressing these open questions relies on developing reliable and novel purification method according to deeper understanding of EVs.

In addition, current studies in EVs are limited to in vitro system. More in vivo studies need to be performed, such as transgenic models of breast cancer system, which helps us know the “atlas” of breast cancer cell–derived EVs. By in vivo imaging, we can know the origin of EVs, the releasing rates and numbers of EVs, the recipient cell types, and the relationship between EVs and soluble factors.

As the biology of EVs is continuing to gain more interest, more subtypes of EVs involved in specific biological processes

are discovered and characterized. For instance, Ma et al⁷⁹ identified migrasome, an organelle mediating release of cytoplasmic contents during cell migration. The physiological roles of these EVs in tumor progression remain to be elucidated. In addition, a novel population of EVs named HG-NV was identified recently. HG-NVs derived from 4T1 and MDA-MB-231 contain kinds of RNAs and proteins, which can be potential biomarkers for diagnosis and prediagnosis.⁸⁰ We hope that in the near future, research can provide advanced technical progress and understanding of the multiple roles of each type of EVs, and more efficient therapeutic strategies will be developed by applying these delivery packets in cancer and in many other diseases.

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Author Contributions

JP and WW designed and drafted the manuscript. SR and LL made critical corrections. All authors reviewed the manuscript and approved the final draft. JP takes responsibility for the paper as a whole.

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