Extracellular vesicle-mediated transport: Reprogramming a tumor microenvironment conducive with breast cancer progression and metastasis

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A B S T R A C T

Breast cancer metastatic progression to critical secondary sites is the second leading cause of cancer-related mortality in women. While existing therapies are highly effective in combating primary tumors, metastatic disease is generally deemed incurable with a median survival of only 2, 3 years. Extensive efforts have focused on identifying metastatic contributory targets for therapeutic antagonism and prevention to improve patient survival. Excessive breast cancer release of extracellular vesicles (EVs), whose contents stimulate a metastatic phenotype, represents a promising target. Complex breast cancer intercellular communication networks are based on EV transport and transference of molecular information is in bulk resulting in complete reprogramming events within recipient cells. Other breast cancer cells can acquire aggressive phenotypes, endothelial cells can be induced to undergo tubule formation, and immune cells can be neutralized. Recent advancements continue to implicate the critical role EVs play in cultivating a tumor microenvironment tailored to cancer proliferation, metastasis, immune evasion, and conference of drug resistance. This literature review serves to frame the role of EV transport in breast cancer progression and metastasis. The following five sections will be addressed: (1) Intercellular communication in developing a tumor microenvironment & pre-metastatic niche. (2) Induction of the epithelial-to-mesenchymal transition (EMT). (3) Immune suppression & evasion. (4) Transmission of drug resistance mechanisms. (5) Precision medicine: clinical applications of EVs.

Introduction

In the United States alone, breast cancer metastatic progression accounts for 42,000 deaths each year [1–3]. There is immense inter-tumor and intra-tumor heterogeneity in breast cancer [4]. However, amongst the vast variety of subtypes, once distant metastases have occurred patients have a low probability for long-term survival [1,4,5]. It is rare for metastatic breast cancer to be effectively treated, rather the general therapeutic approach is management through palliative care [6]. Critical organs such as the lung, liver, brain, and bone are common sites for breast cancer spread and directly relate to poor prognosis [7–10]. According to the American Cancer Society, the 5-year relative survival rate is 26% for patients diagnosed at stage IV compared to nearly 100% for patients diagnosed at stage I [10]. Of the three major breast cancer molecular subtypes (hormone receptor-positive/ERBB2 negative-70% of patients, ERBB2 positive-15% and triple-negative-15%), the median overall survival for metastatic triple-negative breast cancer is estimated only at 1 year and 5 years for the other two [5]. Consequently, there is a

Abbreviations: BCRP, breast cancer resistance protein; CAFs, cancer-associated fibroblasts; DAMP, damage-associated molecular pattern; DISK, death signaling inducing complex; ECM, extracellular matrix; EGFRvIII, epidermal growth factor receptor variant III; EMMPRIN, extracellular matrix metalloprotease inducer; EMT, epithelial-to-mesenchymal transition; ERBB2/HER2, erythroblastic oncogene B/human epidermal growth factor receptor; ERK, extracellular signal-regulated kinases; ETA, endothelin receptor A; EVs, extracellular vesicles; FGFR, fibroblast growth factor receptor; HIF-1α, hypoxia-inducible factor-1α; HISLA, hypoxia-inducible factor-1α stabilizing long non-coding RNA; HMECs, human mammary epithelial cells; HMGA2, high mobility group A2; IDO, indoleamine-2,3-dioxygenase; LncRNAs, long non-coding RNAs; MAC, membrane attack complex; MAPK, mitogen-activated protein kinase; MDSC, myeloid-derived immune suppressor cells; MET, mesenchymal-to-epithelial-transition; MHCII, major histocompatibility complex; MMP, matrix metalloproteinase; MRP1, multidrug-resistant protein 1; MVE, multivesicular endosome; NFs, normal fibroblasts; P-gp, p-glycoprotein; PI3K, phosphoinositide-3-kinase; SMR, secretion modification region; STAT3, transforming growth factor-β; TLR-4, toll-like receptor-4; TNBC, triple-negative breast cancer; TRAIL, TNF-related apoptosis-inducing ligand; TrpC5, transient receptor potential channel 5.

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pressing clinical need for translational research detailing metastatic mechanisms to facilitate the identification of novel therapeutic targets [11].

Breast cancer cells are known to have significantly heightened EV secretion with differential content release based on staging [12]. Recent studies have focused on investigating EV-mediated intercellular interactions that generate a pro-metastatic tumor microenvironment [13–16]. EV is a broad term, encompassing different types of membrane-encased molecular transit such as microvesicles or exosomes that are created via budding of the plasma membrane and fusion of multivesicular bodies, respectively [17]. In contrast to cellular signaling reliant on receptor binding, EV transport is in bulk with a more extensive scope of potential recipient cells [18, 19]. Acting as local and systemic messengers, EVs contain a myriad of bioactive molecules such as oncogenic proteins, lipids, miRNA, RNA, and even in rare findings genomic and mitochondrial DNA [20, 21]. These bioactive molecules are capable of altering numerous distinct pathways involved in signaling and gene regulation [18, 19]. Frequently EV uptake is referred to as a “cancer-induced reprogramming event”, as recipient cells are recruited into highly coordinated efforts to reinforce cancer progression in areas such as proliferation and angiogenesis [13, 14, 22–24]. The specific cargo within EVs gaining the most attention are oncogenic proteins and microRNAs [24, 25]. They are consistently associated with stable transmission and pro-metastatic phenotype reprogramming in recipient breast cancer cells [24, 25].

As EVs are not limited to the local tumor microenvironment, they can advance “tumor-nourishing” environments at distant sites to encourage metastatic organotropism [26]. In the context of the “seed and soil” hypothesis, whereby cancer cells of the primary tumor and in circulation (seeds) can only proliferate within “fertile” tumor microenvironments (soil), Chin and Wang described EVs as the “soil conditioner in breast cancer metastasis” [18, 27]. Notably, breast cancer-derived EVs, through miR-122 transmission, were found to inhibit non-tumor cells’ use of glucose locally and systemically, thereby increasing metastatic potential through the enrichment of nutrient availability [28]. Inhibition of miR-122 returned glucose uptake by non-tumor cells and reduced the in vivo incidence of metastasis [28]. Emerging evidence continues to implicate tumor-derived EVs as essential contributors to breast cancer growth, invasion, migration, angiogenesis, immune suppression, and transmission of drug resistance (Fig. 1) [14].

Given the vital role of EVs in breast cancer progression, exploring their mechanisms of action could aid the future design of precision-oriented medicine to positively impact the prognosis of patients with metastatic breast cancer. This literature review aims to merge research findings that define the roles of EVs in the development of cellular environments unopposed by host immunity and entirely dedicated to supporting breast cancer. Consideration will be made for breast cancer-stromal, endothelial, mammary epithelial, immune cells and cancer interactions. As chemoresistance is a significant obstacle and frequent cause for clinical treatment failure, the EV-driven transmission of drug-resistance will be analyzed [29]. Given that EVs contribute to metastasis, the therapeutic potential of inhibiting EV release will be explored. In addition, EVs will be considered as clinical biomarkers and a promising avenue for targeted drug and/or genetic material delivery due to metastatic organotropism [26].

![Fig. 1. Summary of EV-mediated processes that drive breast cancer progression and metastasis.](image-url)

EV-mediated transport plays a critical role in breast cancer progression and metastasis. Degradative enzymes within EVs remove ECM to clear a path for migratory mesenchymal breast cancer cells. Pro-EMT programmes are readily transferred to facilitate migratory capabilities. Chin et al. highlighted EVs as being capable of acting directly as infrastructure for breast cancer cells to seemingly walk along as “stepping-stones.” Both locally and at distant sites, EVs reprogram the microenvironment to enhance breast cancer proliferation, angiogenesis, nutrient availability, and immune suppression. These combined phenomena serve to reinforce breast cancer progression and metastatic organotropism. Drug resistant mechanisms can be transferred throughout the tumor population via EVs and contribute to treatment failure. Created with BioRender.com.
their innate capacity for specialized transportation, minimal immunogenicity, and low toxicity [30,31].

**Intercellular communication: tumor microenvironment and pre-metastatic niche development**

The tumor microenvironment in breast cancer has been likened to an “ecosystem” and is composed of heterogenous populations of various cell types [32,33]. Breast cancer-derived EVs permit a unified cellular community that acts in coordinated mechanisms to reinforce cancer progression [33]. The primary cells subject to EV-mediated reprogramming within the tumor microenvironment and pre-metastatic niches that will be considered in this section are human mammary epithelial cells, breast cancer-associated fibroblasts and adipocytes, endothelial cells, and breast cancer cells (Fig. 2).

**Human mammary epithelial cell**

Dependent on the transcriptome alterations that occur following breast cancer cell-derived EV uptake, human mammary epithelial cells (HMECs) are recruited to reinforce cancer propagation or converted into cancerous cells [34,35]. HMEC production of reactive oxygen species can be EV-elicited to trigger DNA damage, p53 stabilization, and ultimately induction of autophagy [36]. Martinez-Outschoorn et al. describe the tumor autophagic tactic as a “host-parasite relationship” in which the non-tumor cells produce recycled nutrients that are rapidly taken up for anabolic purposes by the tumor cells [37]. Systemic inhibition of autophagy, with antioxidants, is a proposed method for halting the provision of this nutrient reservoir [37]. In alignment with tumor niche development, EVs derived from multiple breast cancer cell lines have been observed to drive HMEC secretion of tumorigenesis promoting factors [36]. Conversely, EVs derived from HMECs exhibit suppression of breast cancer cell EV release and may serve as a protective mechanism in reducing tumor EV induced activities [38]. While this may help hinder tumor progression early on, unfortunately, the dynamic equilibrium in EV influences shifts to favor the breast cancer cells due to their sheer magnitude of EV release [38]. Often HMECs are converted into breast cancer cells via an oncogenic process thought to be attributed to the horizontal transmission of EV-derived oncogenes such as epidermal growth factor receptor variant III (EGFRvIII) [34].

**Fig. 2.** Brief overview of the diversity of the breast tumor microenvironment and EV driven intercellular communication. (1) Breast cancer derived EV delivery of RNA species is the predominate path for transformation of fibroblasts to breast cancer-associated fibroblasts that are supportive of cancer proliferation. (2 & 4) Breast cancer cells utilize EVs to transfer “EMT programmes” and drug resistant tactics to promote invasive phenotypes and chemoresistance throughout the breast tumor. (3) Mammary epithelial cells, following uptake of breast cancer derived EVs, have been observed to undergo autophagy to increase nutrients for the cancer cells as well as other cancer supporting processes. (5) Vascular endothelial cells have been found to undergo angiogenesis and tight junction destruction to support cancer cell proliferation and spread. (6) Breast cancer-associated adipocytes release EVs that induce breast cancer proliferation, migration, invasion, and chemoresistance. (7 and 8) Immune cell reprogramming to trigger apoptosis and reduce cytotoxicity have been strongly linked to breast cancer EVs. This generates a tumor microenvironment unopposed by host cellular immunity. (9) EV-mediated metabolic reprogramming of macrophages has been involved in advancing breast cancer aerobic glycolysis. (10) Breast cancer EVs enter circulation and can act at distant sites to cultivate a tumor nourishing environment that encourages metastasis. Created with BioRender.com.
Correspondingly, mice injected with non-tumorigenic HMECs and EVs derived from MDA-MB-231 were observed to have tumor formation [35], miR-10b containing EVs, that are overexpressed in metastatic breast cancer, increased invasiveness in recipient non-malignant HMECs through targeting of HOXD10 [39]. In another study, HMECs exposed to EVs derived from MDA-MB-231 cells that were stimulated by linoleic acid were driven to undergo epithelial-mesenchymal-like transitions with increased matrix metalloproteinase (MMP) 2 and 9 secretions [40]. These proteolytic enzymes enable migratory behavior, cellular intravasation/extravasation, and greater invasive abilities [41].

Breast cancer-associated fibroblast

Tumor-derived EVs are known to exert influence on stromal cells and the functionality of the extracellular matrix (ECM) [42]. Cancer cells promptly activate and transform surrounding stromal cells to contribute to cancer progression [43]. Specifically, cancer-associated fibroblasts (CAFs) are of interest as they aberrantly stimulate transforming growth factor-β (TGF-β) signaling, known to be involved in EMT regulation [44, 45]. Within the 4T1 murine model, triple-negative breast cancer (TNBC) with heightened CAFs displayed extensive fibrosis and an increased metastatic tendency [44]. This association is further supported by the successful inhibition of lung metastasis through the use of pirfenidone, a TGF-β antagonist, and doxorubicin [44]. While diverse in origin, the predominant source of CAFs is from breast cancer cell EV-initiated transformation of normal fibroblasts (NFs) [46]. Evidence has suggested that RNA species, such as miRNA-9 and long non-coding RNAs (LncRNAs), within the breast cancer cell-derived EVs act as inducers in converting NFs to the CAFs phenotype [47,48]. Moreover, inhibition of miRNA-9 resulted in diminished motility and extracellular matrix remodeling, thus impinging on invasive and metastatic propensity [47]. EVs derived from CAFs applied to breast cancer cell lines (BT549, MDA-MB-231, and T47D) drove an aggressive phenotype; stimulating proliferation, stemness, and EMT [49]. Breast cancer cells were found to activate NOTCH-MYC in stromal cells to increase unshielded RN7SL1 content in released EVs so that when received, the breast cancer cells would be prompted to grow and invade [43]. RN7SL1 within EVs is thought to function as a cancer-associated damage-associated molecular pattern (DAMP), which through inflammatory instigation has been repeatedly linked to carcinogenesis [43,50].

Yang et al. described EV driven metabolic reprogramming of CAFs within the context of the “Reverse Warburg effect” and “metabolic symbiosis” [32]. CAFs metabolic functioning can be shifted to glycolytic pathways via upregulation of enzymes, such as lactate dehydrogenase and pyruvate kinase, to generate metabolites for cancer cells [32]. Analogously, metabolic reprogramming of macrophages via hypoxia-inducible factor-1α stabilizing long noncoding RNA (HISLA) within breast cancer-derived EVs demonstrated decreased hypoxia-inducible factor (HIF-1α) expression to advance breast cancer aerobic glycolysis [51]. Yan et al. identified an EV specific mechanism in breast cancer cells wherein EV derived miR-105 induced MYC signaling in breast CAFs to confer specific metabolic alterations that prioritized nutrients for adjacent breast cancer cells [24]. These breast CAFs increased glucose and glutamine availability and actively converted waste products to useful metabolites for the breast cancer cells [24].

Cancer cells undergoing EMT via TNBC with induction of miR-105 and miR-122 within pre-metastatic niches of breast cancer patients was associated with increased metastases [24]. Sung et al. connected integrin beta 4 overexpression in EVs derived from TNBC with induction of miR-122, and the knockdown of integrin beta 4 [52].

Breast cancer-associated adipocyte

Adipocytes are one of the predominant cell types surrounding breast cancer cells [53]. Breast cancer-associated adipocytes have paracrine/endocrine functioning and serve as a major energy supply for breast cancer cells [54]. Breast cancer-associated adipocytes secrete free and EV-associated molecules such as leptin, CCL5, CCL2, as well as other inflammation inducing products to enhance breast cancer proliferation, invasion, and metastasis [53,55]. Leptin levels are high in the obese state and has been accepted as fundamentally involved in the risk and prognosis for breast cancer [56]. Giordano et al. defined a novel mechanism in which leptin increased EV biogenesis in MCF-7 and MDA-MB-231 breast cancer cells via induction of tumor susceptibility gene 101 [57]. This induction was suggested to involve chaperoning activity of Hsp90 and be at a post-translational level as the RNA expression levels of tumor susceptibility gene 101 were unaffected by leptin [57]. Evidence continues to implicate EVs role in obesity-related risk for breast cancer and favoring of a more aggressive breast cancer phenotype [58,59]. Breast cancer cells actively take up EVs from breast cancer-associated adipocytes and undergo significant transcriptome alterations [60]. Application of breast cancer cells with EVs derived from breast cancer-associated adipocytes induced EMT related genes and activated hippo signaling with increased proliferation, migration, invasion, and chemoresistance [60,61]. In an in vivo mouse xenograft model, depletion of EVs combated the tumor-promoting effects of breast cancer-associated adipocytes [60].

Breast cancer derived EVs, with miR-144 and miR-126, that are taken up by adipocytes are reprogrammed to differentiate towards an activated beige/brown phenotype with increased catabolic processing [62]. High-energy metabolites such as lactate, pyruvate, fatty acids and ketone bodies can be readily supplied to breast cancer cells that were analogized to “metabolic parasites” [62]. miR-144 elicited beige/brown features through downregulation of MAP3K8/ERK1/2/PARP and miR-126 was responsible for autophagy through HIFα upregulation [62]. Knockdown of miR-144 and miR-126 diminished breast cancer-associated adipocyte induced tumor growth within a mouse model [62].

Breast cancer-associated endothelial cell

Rapidly proliferating breast cancer cells require a continuously increasing supply of oxygen and nutrients present within the circulation [63]. Breast cancer cells are known to elicit neoangiogenesis to meet these nutrient demands through the release of a variety of signaling molecules, however, EVs are becoming revealed as key players within the breast cancer-endothelial crosstalk [63]. Neutral sphingomyelinase 2 in breast cancer regulated EV secretion of miR-210 that subsequently targeted endothelial cells under hypoxic conditions to enrich angiogenesis for sustaining tumor growth [64,65]. Correspondingly, elevated expression of miR-210 in breast cancer tissues was correlated with a worse prognosis [64,65]. In a matrigel plug assay mouse model, breast cancer EV annexin expression was determined to trigger angiogenesis in a t-PA-dependent manner with a 5 to 24 fold increase in hemoglobin content as compared to the negative control [66]. Inhibition of annexin dramatically reduced this angiogenic effect [66]. Ghaffari-Makhmalbaf et al. noted a significant reduction in human umbilical vein endothelial cells expression of VEGF-A, proliferation, and migration when treated with EVs derived from docosahexaenoic acid treated breast cancer cells [67]. Connecting the breast cancer cell profile to secreted EV expression, Aslan et al. found that docosahexaenoic acid inhibition down-regulated pro-angiogenic genes within breast cancer cells that were reflected in the secreted EVs miRNA content [68]. A mechanism behind EV miRNA regulation was identified whereby stromal interaction molecule 1, a calcium sensor, reduced miR-145 in EVs derived from MDA-MB-231 breast cancer cells to elicit angiogenesis [69].

In patients with metastatic breast cancer, lower levels derived from breast cancer-associated endothelial cells correlated with a greater likelihood of survival following chemotherapy [70]. The proangiogenic mediators in EVs derived from breast cancer-associated endothelial cells
may be a promising target for therapeutic inhibition [71]. IL-3R-alpha blockade served to halt the EVs derived from breast cancer-associated endothelial cells from stimulating angiogenesis through targeting of the Wnt/β-Catenin pathway [71]. β-Catenin activates gene expression for proliferative cell cycle functions [71]. C-myc, a target gene of β-Catenin, was upregulated in EVs from breast cancer-associated endothelial cells and downregulated with anti-IL-3R-alpha treated EVs from breast cancer-associated endothelial cells [71]. This indicates deregulation of c-myc as a possible outcome of IL-3R-alpha targeting of the Wnt/β-Catenin pathway [71].

Breast cancer-cancer cell EV transmission

Breast cancer subpopulations with metastatic capability can use EVs to confer malignant abilities throughout the entire tumor populace [72, 73]. Many studies have noted this phenomenon as EVs derived from an aggressive cancer cell line applied to a non-aggressive cancer cell line result in increased malignancy [25,72,73]. In a study comparing triple-negative breast cancer (TNBC) derived EVs from Hs578T vs a highly invasive variant Hs578Ts(i), EVs from Hs578Ts(i) significantly increased proliferation, migration, invasion, and endothelial tubule formation to support angiogenesis within all exposed cell lines SKBR2, MDA-MB-231, and HCC1954 [72]. In another study, EVs in patients with metastatic breast cancer were found to have elevated levels of glycosylated extracellular matrix metalloproteinase inducer (EMMPRIN) whose transmission amongst the tumor cells contributed to increased invasiveness [74]. Strangely, Menck et al. hypothesize a process independent of matrix metalloproteinases in which the increased glycosylation status of EMMPRIN leads to p38 phosphorylation with subsequent proinvasive signaling activation [74]. Kia et al. determined that miRNAs in EVs derived from MDA-MB-231 induced a malignant phenotype in non-metastatic MCF-7 with increased migration and invasion in a dose dependent manner [75]. EV miR-190-190–3p transmission between breast cancer cells enhanced proliferation and migration, as well as protected against apoptosis [76]. Notably, EVs derived from drug-resistant breast cancer cells are capable of eliciting resistance and breast cancer metastatic potential within recipient cells [77]. Gao et al. found that EVs derived from drug-resistant breast cancer cells were enriched with EphA2 that enhanced aggressive phenotypes in recipient breast cancer cells, with increased migration and invasion, through activation of Ephrin A1-dependent reverse pathway [77].

Induction of the epithelial-to-mesenchymal transition and metastasis

Tumor-derived EVs can facilitate the epithelial-to-mesenchymal transition (EMT), a state in which cells gain enhanced migratory abilities, that has been frequently tied to hematogenous dissemination of cancer [20,45,78]. In breast cancer patients with advanced disease, the most pervasive status of circulating cancer cells was the mesenchymal state [45]. The quantity of circulating breast cancer cells in the mesenchymal state decreased with treatment and increased within refractory periods [45]. Thus, mesenchymal biomarkers have been proposed as an indicator for therapeutic resistance [45]. Within normal physiology, EMT is essential to wound healing, regeneration, and other physiological functions [79]. However, within the context of oncogenesis, EMT is a transdifferentiation process where cells shift from an epithelial state, tightly adhered to the primary tumor, towards a mesenchymal state, detached from the primary tumor and able to migrate through the ECM into the bloodstream [78].

Epithelial cell characteristics such as apical-basal polarity and cell-cell adhesions reduce the likelihood of acquiring motility [20]. E-cadherin is highly expressed in epithelial cells and represents the extensive array of tight junctions closely holding the cancer cells to one another [20]. Maintenance of E-cadherin based connectivity prevents the loss of antigungrowth signaling that helps to halt uncontrolled cellular proliferation [80]. By sequestering β-catenin, E-cadherin stops WNT/β-catenin and PI3K activation [81]. Reduced E-cadherin functionality through mutation, degradation, etc., is an essential contributor to metastatic progression in multiple different epithelial based malignancies [81]. In contrast, mesenchymal characteristics such as reduced cell-cell adhesion, expression of matrix-degrading enzymes, front-rear polarity, and a spindle-like morphology promote migratory behavior aligned with metastasis [3,82,83]. Biomarkers for mesenchymal cells include N-cadherin, fibronectin, vimentin, and mortalin [20]. N-cadherin re-inforces fibroblast growth factor receptor signaling (FGFR) to activate MAPK/ERK and PI3K signaling, an opposing mechanism to E-cadherin, to strengthen cellular survival, proliferation, and metastasis of breast cancer [81,84]. N-cadherin is also involved in angiogenesis and antagonists of N-cadherin are being considered for anti-cancer therapy development [85]. As a marker of EMT, fibronectin levels are heightened in metastatic breast cancer compared to normal breast tissue and are associated with elevated mortality risk [86]. Fibronectin contributes to both initiation and stabilization of the mesenchymal state [86]. EMT initiation has been observed to occur via fibronectin elicited activation of STAT3 within metastatic breast cancer MDA-MB-231 cells as well as through cancer cells binding to the fibrillar structure of fibronectin distorting their shape towards a mesenchymal morphology [86,87]. Normally, vimentin expression in HMECs functions to perpetuate a migratory state during wound-healing [88]. Correspondingly, vimentin expression is related to greater migratory and invasive properties and is upregulated in aggressive breast cancer cells lines [89]. Mortalin, a member of the heat shock protein 70 family, is a regulator of the EMT process, upregulating PI3K signaling, vimentin, fibronectin, etc. and downregulating epithelial markers [3,90].

EMT initiates a sequence of events beginning with a singular or collective cellular dissociation from the primary tumor, degradation of the surrounding extracellular matrix, invasion, dissemination, and ultimately colonization to distant organ sites [83]. Note, to “seed” the pre-metastatic niche the cancer cells must undergo the mesenchymal-to-epithelial-transition (MET) [79]. Murine and human metastatic breast cancer EVs with miR-200 enabled lung colonization through MET reprogramming [91]. EMT activation has been shown to increase stemness, enhancing growth potential within neoplastic mammary epithelial tissue [92]. EVs derived from mesenchymal stem cells that contained WNT reinforced EMT expression and promoted breast cancer MCF-7 migration [93,94]. Tumor-derived EVs’ contents have been defined as a “pro-EMT program” including the following contributors: transforming growth factor-beta (TGF-β), cavelin-1, hypoxia-inducible factor-alpha (HIF1α), β-catenin as well as miRNAs such as miRNA-222, miRNA-9 and miRNA-155 [20,78,95]. Breast cancer EV miR-155 may contribute to cachexia by downregulating peroxisome proliferators-activated receptor γ in adipocytes and co-cultivation of breast cancer cells with these reprogramed adipocytes led to EMT induction [96]. Increased expression of miRNA-9 was related to reduced vimentin expression, E-cadherin loss and determined to be a prognostic factor for aggressive breast cancer [95]. TGF-β is a critical inducer in EMT that acts either through SMAD, a potent family of transcription factors, or non-SMAD activation [81,94]. TGF-β/SN-MAD activation leads to p38 MAPK signaling and increased HMGA2 with upregulation of N-cadherin to promote EMT [81]. Conversely, TGF-β/non-SMAD activation increases PI3K signaling [81]. Targeting EV signaling contents to inhibit EMT initiation of the EMT has garnered antitumor therapeutic efforts. For example, Galunisertib (LY2157299 monohydrate), a TGF-β receptor I kinase inhibitor, was found to have antitumor activity within an animal model for breast cancer [97].

EVs are involved in remodeling the extracellular matrix to generate pathways for mesenchymal breast cancer cells [98-100]. EV cargo consists of a variety of degradative enzymes such as MMPs, heparanases, and hyaluronidases, as well as regulators of these enzymes [99]. Increased expression of Rab27B aided EV Heat-shock protein 90α
release, a necessary component for MMP2 activation, resulting in invasive breast tumor growth [99]. EV mircRNA-4443 supported breast cancer cell metastasis to the liver through upregulation of MMPs and downregulation of tissue inhibitors of metalloproteinase 2 [101]. EVs, containing mIRC-105, have been determined to provoke the destruction of tight junctions between vascular endothelial cells to enable cancer cells to begin their migration away from the primary tumor [102]. mIRC-939 transfected MDA-MB-231 breast cancer cells released EVs with mIRC-939 that acted to downregulate VE-cadherin in human umbilical vascular endothelial cells and favored transendothelial migration [103]. In TNBC patients, mIRC-939 expression was heightened and corresponded with reduced disease-free survival [103]. Interestingly, EVs’ role in metastasis includes physically acting as a guiding pathway for the migration of cancer cells towards a pre-metastatic niche [18,104]. Koumangoye et al. considered that detached breast cancer cells’ have a heightened EV secretion with prompt adherence to the extracellular matrix as indicative of the EVs containing various adhesion molecules such as integrins [104]. Chin and Wang proposed a mechanism whereby EVs adhered to the endothelial cell wall via heparan sulfate act as “stepping stones” and express fibronectin that is bound by integrins within EVs on the surface of migrating breast cancer cells [18]. EV secretion may be increased at the leading edge for adhesion and decreased at the lagging edge for release [18]. Thus, breast cancer cells would seemingly “walk” towards the pre-metastatic niche [18]. Reinforcing this premise, inhibition of EV secretion disrupts tumor cell migration both in speed and directionality as the cancer cells were observed to switch directions continuously [105]. Ma et al. discovered and termed these EVs “migrasomes” due to their biogenesis being dependent on migration [106].

**EVs in immune suppression and evasion to promote tumor growth**

Breast cancer derived EVs are closely involved with immune cell reprogramming to evade anticancer host response, reinforcing unopposed tumor growth in primary and secondary sites (Fig. 3) [107,108]. EV-mediated immune suppression creates pockets of immune privilege at distant sites where cancer cells may thrive and is strongly indicated as encouraging metastasis [107]. Long-term exposure to EVs derived from metastatic breast cancer cells impeded host immune defenses by increasing the quantity of myeloid-derived suppressor cells in the lung and liver with corresponding cancer colonization [107]. Contents of EVs, such as indoleamine-2,3-dioxygenase (IDO), are known as direct immunosuppressant molecules and relate to more advanced stages of breast cancer [109]. Multiple cancer types use EV transport to counteract lymphocytes, antigen-presenting cells, and complement to escape host immunity; these processes will be briefly outlined relative to breast cancer [108].

EV initiated actions against lymphocytes include suppression of proliferation, induction of apoptosis, promotion of terminal differentiation towards a non-functional state, lower cytokine release, and reduction of cytotoxicity (Fig. 3) [107,110,111]. The proliferation of CD8 and CD4 T-cells were reduced via EVs, derived from breast cancer cells, in a dose-dependent fashion [107]. T-cell apoptosis has been identified as a possible primary method for suppression of immune response [107]. The “Fas counter-attack” has been described in breast cancer cells and involves tumor-derived EV FasL delivery to provoke apoptosis within anti-tumor Fas-positive T lymphocytes [110]. FasL expression has been correlated to poorer prognosis in patients with breast cancer [112]. A similar EV-induced cell death approach has been investigated in treatment prospects; EVs armed with TNF-related apoptosis-inducing ligand (TRAIL) have been successfully targeted against tumor cells to control disease progression in vivo [113]. TRAIL is a cytokine that causes apoptosis in tumor cells, while leaving non-tumor cells unharmed [114]. The mechanism of TRAIL involves binding death receptor 4 or 5 with death signaling inducing complex (DISK) formation [114], MEDI3039, a novel TRAIL receptor agonist, inhibited metastatic triple-negative breast cancer growth within an experimental pulmonary metastasis model [115]. EVs derived from aggressive breast cancer cell lines in murine models distributed primarily in the lungs and increased terminal differentiation of T cells towards a non-functional state [107,116]. These, as termed by Jiang et al., “exhausted T cells” had high levels of inhibitory receptor expression and correspondingly poor cancer clearance [116]. TGF-β regulates breast cancer cell secretion of EVs with programmed death-ligand 1 that target cytotoxic T cell resulting in dysfunction [117]. Inhibition of EV release and TGF-β restored “exhausted T cell” function [117].

Natural killer (NK) cell cytotoxicity was reduced upon exposure to tumor-derived EVs containing MICA*008 (MHC class I-related chain A ligand) [118]. Downregulation of NKG2D expression in NK and CD8(+)
T cells occurred in response to excessive tumor-derived EV secretion containing ligands for NKG2D [119]. Thus, tumor-derived EVs with MICA^008 or NKG2D ligands can compromise the ability of lymphocytes to detect cancer cells for destruction. In the murine model, exposure of mammary tumor cell lines to tumor-derived EVs inhibited host natural killer immunity leading to an increased growth rate of the implanted tumors [120]. Dendritic cell expression of toll-like receptor 4 and MHCI has been reduced via tumor-derived EV transferred mRNA resulting in suppression of anti-tumor immunity [108]. A study observed that TS/A murine mammary tumor-derived EVs blocked myeloid precursor differentiation into dendritic cells [121]. Tumor-derived EVs halt dendritic cell production, change the differentiation trajectory within the bone marrow myeloid precursor cells, and trigger a buildup of myeloid-derived immune suppressor cells (MDSCs) in the primary tumor and spleen [122]. An accumulation of myeloid-derived immune suppressor cells was observed in naive mice following conditioning with EVs derived from metastatic murine breast cancer cells [107]. MDSCs reduce T and NK cell functioning and engage in signaling to enhance the breast cancer cells potential for proliferation [123]. EV shedding of the membrane attack complex (MAC) has been noted as a strategy for cancer cell evasion of complement-mediated lysis [125]. MAC is assembled through the combination of C5b-9 and typically results in the insertion of a transmembrane protein channel that disrupts the cell membrane integrity causing cellular damage [126]. However, EV release is significantly increased in cancer cells and often contains MACs [126]. Eliminating MAC from the surface of cancer cells nullifies the membrane disruption and protects against cytotoxicity initiated from the host complement system [126]. Furthermore, inhibition of EV release has been found to restore breast cancer cell sensitivity to complement-mediated lysis reinforcing EVs as the causative agent for complement resistance [127]. Lastly, tumor-derived EVs frequently contain immunogenic factors that may seem contradictory to the goal of immune suppression [128]. However, these EVs act as decoys that actively bind and sequester tumor-reactive antibodies thus allowing tumor cells to evade host antibody-dependent cytotoxicity [128].

EV drug resistance conference throughout tumor

Chemoresistance is a significant obstacle to existing therapeutic strategies and a common cause for treatment failure [129]. An identified contributor to resistance spread is EV-mediated transmission of drug-resistant mechanisms from resistant to sensitive breast cancer cells (Fig. 4) [25]. Horizontal transfer of RNA molecules through EVs has been noted in resistance spread [25]. Breast cancer cell line MCF-7 sensitive to adriamycin and docetaxel acquired resistance through EV miRNA derived from resistant MCF-7 cells [130]. RNase treated EVs were unable to induce resistance in recipient MCF-7 sensitive cells and differential miRNA expression profiles were observed reinforcing the RNA-dependent EV as the trigger for resistance acquisition [130]. Tamoxifen-resistant breast cancer-derived EVs contain miRNA-221/222 that reduce P27 and ERS gene expression in recipient-sensitive MCF-7 breast cancer cells corresponding to increased resistance against tamoxifen [131]. EVs with elevated LncRNA-SNHG14 increased trastuzumab resistance in sensitive breast cancer cells with this effect was abolished through LncRNA-SNHG14 knockdown [132]. Dong et al. attributed the trastuzumab resistance to LncRNA-SNHG14 increasing Bcl-2 and decreasing BAX expression, but noted that further research is required to detail the mechanism [132]. High expression of EV LncRNA actin filament associated antisense RNA 1 corresponded with trastuzumab resistance induction and shortened survival in breast cancer patients [133]. Bioinformatic analysis identified EV miR-567 silencing as contributory to trastuzumab resistance and overexpression of miR-567 results in chemoresistance reversal through targeting of autophagy-related 5 [134]. EMT induction to increase cancer stem cells’ resistance to chemotherapy is linked to miRNA-155 EV transfer amongst breast cancer cells [135]. Revealing of the complexity underlying intercellular communication, EV-mediated breast cancer resistance can also be conferred from stromal cell-derived EVs [136]. RNA EV transmission from stromal to breast cancer cells activates antiviral/NOTCH3 signaling to strengthen therapy resistance [136].

The elimination of drugs prior to cytotoxic effects taking hold is a frequent drug resistance strategy seen in breast cancer cells [137]. Tumor-derived EVs have been implicated in the transfer of efflux pump capabilities as well as directly as a disposal site for chemotherapeutic agents [138]. The rate of tumor-derived EV secretion and fluorescent content of doxorubicin was determined to positively correlate with the level of doxorubicin resistance [138]. Multi-drug efflux transporters such as P-glycoprotein (P-gp), multidrug-resistant protein 1 (MRP1), and breast cancer resistance protein (BCRP) have been reported as contents within EVs [129,139]. EVs containing BCRP were observed to sequester mitoxantrone and confer drug resistance 20-fold greater than parental MCF-7 cells; drug sensitivity was reestablished with ABCG2 inhibition through Ko143 treatment [139]. Kong et al. induced EV secretion of BCRP through treatment with guggulsterone and bexarotene resulting in increased intracellular doxorubicin retention and

![Image: Drug resistant mechanisms, established by EVs, conferred throughout the breast tumor populace.](https://www.biorender.com)
subsequent breast cancer cell death 5-fold more than doxorubicin alone [140]. EV-mediated transfer from adriamycin-resistant breast cancer cells of calcium-permeable transient receptor potential channel 5 (TrpC5) enhanced P-gp expression via activation of NFATc3 and fostered drug resistance within sensitive recipient cells [129]. Similarly, adriamycin-resistant breast cancer cells secreted EVs containing UCH-L1 and P-gp proteins that disseminated a chemoresistant phenotype [141].

Breast cancer cell acquisition of mechanisms to reduce drug effectiveness has also been linked to tumor-derived EVs [142]. HER2-overexpressing cancer cell lines, SKBR3 and BT-474, were found to secrete EVs containing full-length active HER2 molecules that bound up Trastuzumab antibody and diminished the drug’s ability to bind cancer cells [142]. Serum analysis of breast cancer patients revealed EVs containing HER2 molecule bound to trastuzumab in greater prevalence within advanced staged patients [142]. The EV compartment has been considered as a “shelter” from small molecule inhibitors [143]. Targeting the epidermal growth factor receptor can be difficult and may be due to EV protection. The epidermal growth factor receptor continues to be transferred and elicit breast cancer progression, but remains hidden from therapeutics [143].

**Precision medicine: clinical applications of EVs**

As EV-mediated transport is vital to breast cancer progression and metastasis, inhibition of EV release is a promising therapeutic avenue that merits further investigation. EV-mediated processes that contribute to pro-metastatic and drug-resistant phenotypes, development of the tumor microenvironment, and evasion of host immunity could potentially be halted with suppression of EV release. EVs have also garnered efforts for tracking purposes in diagnosis, prognosis, tumor evolution, and treatment response [144]. In addition, EVs as drug or genetic material delivery vehicles is attractive as they have natural targeted transport abilities that effectively maintain cargo integrity, low toxicity, and minimal immunogenicity [145].

**Therapeutic promise of agents that antagonize EV secretion**

EV-mediated transport is involved in a broad array of pro-metastatic alterations of the tumor microenvironment including, but not limited to, the advancement of proliferation, angiogenesis, metastasis, immune evasion, and drug resistance. Extracorporeal elimination of tumor-derived EVs has been considered of potential clinical benefit [111]. In turn, inhibitory agents against EV secretion represent a promising avenue for therapeutic targeting [146]. Cannabidiol, Rho kinase inhibitor Y-27,632, PEG-SMR-Clu peptide (SMRwt peptide), Sulfisoxazole, and Ketotifen are EV inhibitory agents that will be described in relation to their therapeutic potential related to breast cancer.

Kosgodage et al. identified a novel use for cannabidiol as a potent inhibitor of EV release in breast cancer cells [147]. The effects of cannabidiol were dose dependent resulting in reduced mitochondrial transport abilities that effectively maintain cargo integrity, low toxicity, and minimal immunogenicity [145].

**EVs as biomarkers**

Repetitive tumor tissue sampling is invasive and impractical for understanding the true heterogeneity of a breast tumor [144] Liquid biopsies that focus on differential cargo protected from degradation within EVs represent a clinically accessible, cost-effective, and precise methodology in indicating the physiological states of the parental breast cancer cells [144,153]. Early detection of breast cancer with EV molecular biomarkers could prove of benefit in enabling treatment prior to metastatic progression [154]. Combined urinary EV miR-21 and MMP-1 expression had a sensitivity of 95% and a specificity of 79% in detecting breast cancer without metastasis and may be beneficial for breast cancer screening [155].

Numerous studies have indicated the potential of using EV cargo as predictive for breast cancer prognosis and treatment response [144,156,157]. Wang et al. determined that the level of circulating EVs with TRPC5 from breast cancer patients was significantly reflective of the TRPC5 expression in the breast cancer tissues and the treatment response of the tumor [156]. High levels of circulating EVs with TRPC5 was predictive for acquiring chemoresistance [156]. Tang et al. found that breast cancer patients had significantly higher levels of serum EVs with HOX transcript antisense RNA as compared to healthy individuals [157]. Increased HOX transcript antisense RNA levels correlated with worse response to tamoxifen and disease-free survival, whereas decreased levels followed surgical intervention [157].

Comparison of EV cargo at different stages in breast cancer progression could have applications in enhanced clinical differentiation [158]. Martinez et al. explored this in breast cancer patients under neoadjuvant chemotherapy. They found that EV miR-21 and miR-105 was elevated in metastatic breast cancer patients as compared to non-metastatic patients. Still, solely miR-21 was elevated in metastatic breast cancer patients as compared to localized breast cancer patients [158]. EV miR-21 had a direct relationship with tumor size, and elevated levels of miR-21, miR-222, and miR-155 were associated with circulating tumor cells in breast cancer patients [158]. Plasma EV miR-223–3p was significantly elevated in patients with invasive ductal carcinoma compared to ductal carcinoma in situ and may have potential as a biomarker for detecting invasive ductal carcinoma [159]. This evidence suggests that EV cargo can serve as reliable biomarkers that aid in the diagnosis, prognosis, tracking of treatment response, and risk for chemoresistance in future breast cancer patients.

**Potential use of EVs for drug and/or genetic material delivery**

As chemotherapy has the unfortunate combination of low specificity with high toxicity, significant research efforts focus on developing drug delivery vehicles that only target the cancer cells [145]. Currently, natural EVs are intensively being explored as chemotherapeutic delivery agents to maximize their inherent function as extracellular messengers with minimal immunogenicity, and low toxicity [30,31].
Macrophage-derived EVs filled with paclitaxel and doxorubicin effectively targeted and eliminated tumor growth of MDA-MB-231 breast cancer cells within the mouse model [160]. EVs with anti-HER2 mRNA were successfully directed to HER2 receptors of HER2+ve breast cancer tumors within mice leading to reduced tumor growth [161]. Targeting circulating breast cancer cells is essential to directly halting metastasis; Wang et al. developed an EV-like sequential bioactivation prodrug nanoplatform (EMPC) that releases curcuminib A to trigger oxidative stress in recipient circulating breast cancer cells [162]. Drugs that were previously limited by toxicity or pharmacodynamic properties have more significant potential for application within an EV model as targeting is highly specific and solubility barriers can readily be circumvented [163]. For example, Erastin, a chemotherapeutic drug limited by renal toxicity, within an EV had optimized uptake efficiency into MDA-MB-231 cells with an enhanced inhibitory effect; therefore, rationally, lower concentrations could be applied with similar clinical benefit while minimizing toxicity [163]. A review on targeted therapy for metastatic breast cancer noted that EV loaded doxorubicin had significantly lowered cardiac toxicity [164].

Excitingly, EVs are also being explored as delivery vehicles for genetic material for anticancer effect, improvement of chemotherapeutic response, and in minimizing metastatic organotropism in breast cancer [165-167]. Sheykhbasan et al. used EVs derived from mesenchymal stem cells to effectively deliver anticancer miR-145 into breast cancer cells [166]. Gong et al. targeted TNBC cells with EV co-delivery of doxorubicin and miR-159 that exhibited synergy and had successful silencing of TCF-7 gene [168]. Liu and Chen were successful in using CXCR4/TRAIL enriched EVs with carboplatin in the treatment of brain metastasis of breast cancer in a mouse model [165], miRNAs with roles in regulating cancer proliferation, apoptosis, invasion, and migration are being considered for EV transmission in preventing metastasis [166]. EVs can be designed with trajectories towards distant sites that may act as a pre-metastatic niche [167]. Zhao et al. designed cationic bovine serum albumin conjugated with siRNA EV nanoparticles that successfully targeted the lung and significantly suppressed postoperative lung metastasis within a breast cancer mouse model [167]. These favorable findings continue to encourage endeavors into improving EV synthesis and loading to advance the likelihood for clinical application [145].

Conclusion

In conclusion, as breast cancer metastasis is the central drive of mortality, there is an urgent clinical need to find targeted and effective methods to disrupt the metastatic process [3]. While EV-mediated transport was overlooked in the past, current research discoveries have strongly indicated it is a vital player in a multitude of breast cancer processes [12–14]. Essential not only to promoting local proliferation of breast cancer cells, EV-mediated transport also is fundamental to the development of metastatic organotropism. With bulk transference of molecular information, a complete reprogramming event occurs within the recipient cells [13,14]. As any cell with machinery for EV uptake is susceptible to this reprogramming, entire host cellular communities can be altered to solely reinforce cancer growth [13,14,20,21]. Other breast cancer cells can be transformed towards an aggressive phenotype with migratory mesenchymal tendencies and mechanisms that make chemotherapy ineffective [20,25,78,95]. Mammary epithelial cells can either be recruited to become cancerous or to downregulate nutrient use and release growth factors that contribute to cancer growth [34,35]. Immune cells can be reprogrammed to hinder host anticancer response efforts [107,108]. As current research is only beginning to reveal the profound role that EV-mediated tactics play within the breast cancer microenvironment and distant pre-metastatic niches, this literature review serves as a preliminary perspective highlighting the need for further translational research. Perhaps the most beneficial would be research detailing the underlying mechanisms of EV transport as well as defining the roles of specific EV cargo. After these processes begin to be elucidated, it will be possible to identify novel therapeutic targets to halt EV-elicted cancer spread. Host anticancer immune response efforts could be restored. Previously ineffective chemotherapeutic agents may be rendered effective. In addition, EVs hold promise as clinical biomarkers and in being exploited as drug and/or genetic material delivery vehicles [30,31]. Taken altogether, EV transport merits greater research efforts as this could contribute to improving the survivability of future patients with metastatic breast cancer.

Authors’ contributions

Dara Brena, dbrena@msm.edu, and Ming-Bo Huang, mhuang@msm.edu, designed the manuscript. Dara Brena was a major contributor in writing the manuscript and drawing the figures. Ming-Bo Huang mainly revised the manuscript and the figures. Vincent C. Bond, vbond@msm.edu, and Ming-Bo Huang participated in the review design and made some revisions to the study. All the authors read and approved the final version of the review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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