Cancer Cell International

REVIEW Open Access

Exosomes and breast cancer angiogenesis; Highlights in intercellular communication

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Abstract

Breast cancer (BC) is a prevalent and highly lethal cancer in females. Like other cancer types, the intricate cellular and molecular heterogeneity leads to the variation of therapeutic outcomes. The development and progression of blood vessels increase the tumor cell expansion and metastasis to remote sites. Based on several pieces of scientific data, different mediators and cells are involved in the promotion of angiogenesis into the tumor parenchyma. Recent data have indicated the critical role of extracellular vesicles, especially exosomes (Exos), in the transfer of angiogenesis molecules between the BC cells. Due to unique physicochemical properties, and the transfer of certain signaling molecules, Exos are at the center of attention in terms of biomarkers and therapeutic bullets in cancer patients. Along with these statements, understanding the modulatory role of Exos in BC angiogenesis seems critical in the clinical setting. Here, the mechanisms by which BC cells can orchestrate the angiogenesis phenomenon via Exos are discussed in detail. The present study can help us to understand the pro-/anti-angiogenesis role of Exos in BC and to design better oncostatic strategies.

Keywords Breast cancer, Angiogenesis, Paracrine communication, Extracellular vesicles, Exosomes

Introduction

Breast cancer (BC) is the most common prevalent lethally neoplastic condition in females [1]. In most circumstances, the rate of cellular and molecular heterogeneity is high in BC because of the distinct genomic and proteomic signatures of tumor cells. These features lead to the emergence of different cell clones with specific membrane-bound receptors [2]. This heterogeneity can predetermine the interaction of cell-to-cell and cell-to-surrounding extracellular matrix (ECM) and lead to the lack of effective treatment responses [3]. Within the tumor niche, cells can communicate with each other via providing physical contact (juxtacrine) and the release of soluble factors (paracrine) [4]. The close physical contact acts as a cue to dictate specific tumor cell morphology and motility. Along with the paracrine activity, the juxtacrine capacity is integral to prognosis in patients with cancers [5].

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In the clinical setting, tissue biopsy using invasive surgical procedures and subsequent histological analysis are the main valid available modalities for the detection of BC [6]. This approach does not give data to monitor the progress of tumor growth and/or atresia in the next steps. Besides, sampling is not applicable in all BC patients and some cases can predispose the possibility of metastasis [7]. Commensurate with these comments, attempts have been focused on liquid biopsy for the detection of BC patients. Both circulating tumor cells (CTCs), genetic elements (ctDNA), tumor cell-extracellular vesicles (EVs), etc. can be monitored in biofluids [8]. Compared to direct tissue biopsy, liquid biopsy is and could exhibit the relatively whole genetic traits of tumors [9]. The release of biomarkers to biofluids such as blood, etc., can be monitored using different modalities that are useful in BC detection, prognosis, therapeutic protocol efficiency, and decision-making for personalized medicine [10]. Unfortunately, the quantity of biomarkers in biofluids is not enough and detectable during early-stage tumor formation. Even, the existence of natural barriers can limit biomarker distribution in biofluids [11, 12].

Emerging data have indicated that EVs can harbor different signaling molecules between the cells under physiological and pathological conditions [13]. Three types of EVs, including exosomes (Exos), microvesicles (MVs), and apoptotic bodies have been diagnosed in paracrine activity between the cells [14]. During recent years, Exos have been novel frontiers in monitoring the dynamic growth of tumor cells, cancer metastasis, and therapy [15]. Exos can harbor BC-associated biomarkers such as carcinoembryonic antigen (CEA) 19−9, and carbohydrate antigen 125 (CA125) with valid theranostic properties [16–18]. Monitoring biofluid Exos can help clinicians to diagnose the BC cases in the early stages [19]. Recent years have witnessed the application of Exos as direct therapeutics, or bioshuttles for the delivery of pharmaceutics into the tumor niche. However, due to bulk distribution and off-target effects, sophisticated approaches, such as engineered Exos have been used to improve the therapeutic efficiency [20, 21]. Overall, the biomedical use of Exos is diverse and continually expanding [22]. Exos can reflect the in-time metabolic status of parent cancer cells indicated by specific cargo such as carcinogenic proteins, and genetic elements [23]. Despite the advantage of Exo application in terms of BC patients, several aspects remain to be addressed. Here, we aimed to highlight the critical role of Exos in the modulation of molecular mechanisms related to BC resistance/treatment.

Exo biogenesis and abscission

As above-mentioned, EVs are the most important bioshuttles that mediate inter-cellular communication by transferring several signaling molecules [24]. Based on size, and origin, EVs encompass three main types including endosomal Exos, plasma membrane- MVs (also known as ectosomes), and apoptotic bodies [25]. MVs possess a mean diameter from 50 to 1000 nm and are produced by direct budding of the plasma membrane. In contrast, Exos with an average size of 50–150 nm, originate from the endosomal system via the invagination of endosome membrane [24]. It is postulated that the phenomenon of Exo production is specific and tightly regulated in comparison with the MV synthesis [26]. In short, the process of Exo production includes cargo sorting, endosome formation, transport, and fusion with the cell membrane. Several molecular mechanisms may be engaged in the sorting of cargo into the intraluminal vesicles (ILVs). Compared to normal cells, tumor cells can use various strategies to control the Exo biogenesis. Thus, it is logical to hypothesize that the heterogeneity of Exo cargo is high in tumor cells with various functions [27]. The internalized Exos are enclosed inside the early endosomes whereas *de novo* Exos are generated via the invagination of the endosome membrane. Two main molecular mechanisms are involved in membrane invagination and ILV formation as follows; endosomal sorting complex required for transport (ESCRT)-dependent and -independent pathways (Fig. 1) [22]. ESCRT complex encompasses four subunits, -0, -I, -II, and -III [28]. The ESCRT-0 composed of Hrs and STAM uses a ubiquitin-binding domain to target ubiquitylated proteins. Using the Hrs domain, ESCRT-0 is recruited into the clathrin-coated microdomain. Along with these changes, ESCRT-I, and -II are added to ESCRT-0 to develop a complex that can per se recall ESCRT-III [29]. The addition of ESCRT-III coincides with the polymerization of long filaments at the microdomain neck, leading to the detachment of generating ILVs into the endosome lumen [30]. Of note, during cargo sorting, ubiquitylated proteins are deubiquitinated but this step is not required for all cargo contents. Certain factors such as Alix, and HD-PTP can help the ESCRT system in the sequestration of cargo into ILVs [31]. In an ESCRT-independent pathway, lipid rafts, ceramides, and phospholipids can collaborate to force endosomal membrane curvature, and protein sorting into ILVs without the ESCRT complex [32, 33]. The early endosomes can mature into late endosomes and multivesicular bodies (MVBs) where numerous intraluminal vesicles (ILVs) are generated and accumulated in the endosome and MVB lumen (Fig. 1) [15, 34]. In the next steps, MVBs can interconnect with other organelles such as the trans-Golgi network (TGN) apparatus, endoplasmic reticulum network, mitochondria, or phagosomes

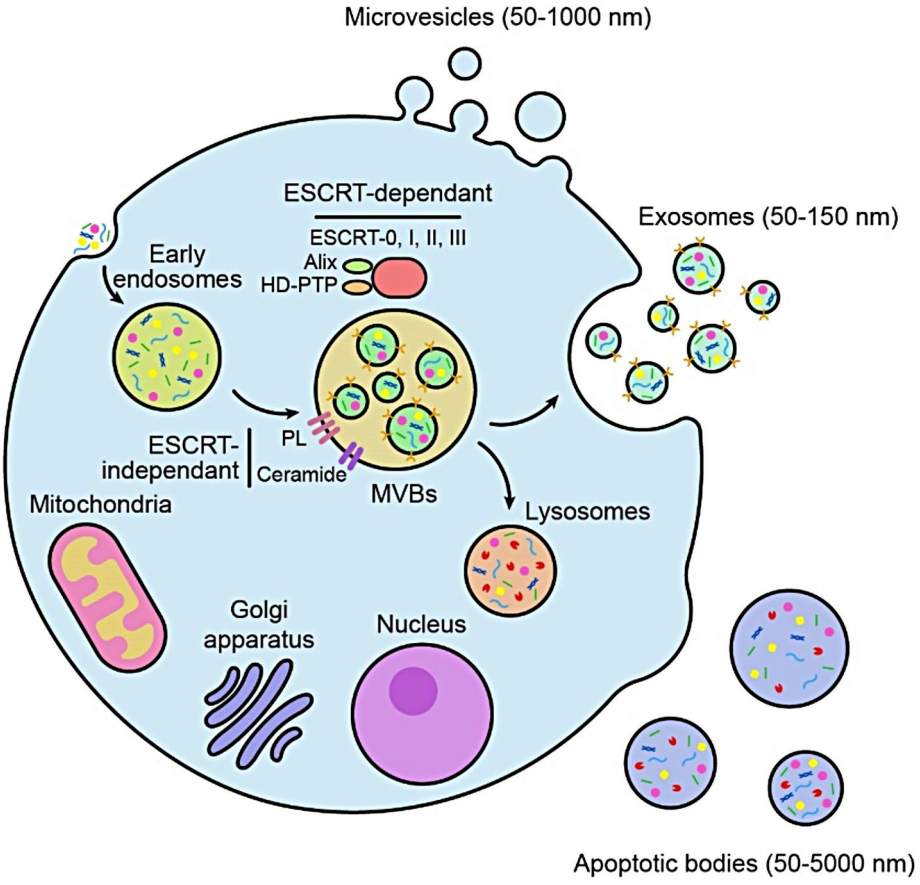

Fig. 1 Schematic illustration of Exo biogenesis. Exos are produced inside the donor cells via the endosomal system. The internalized Exos are sequestrated inside the early endosomes. In the next step, these endosomes mature into late endosomes and MVBs. MVBs can guide toward lysosomal digestion and/or fusion with the cell membrane to release their contents. The endosomal system comprises molecular machinery that orchestrate the formation of ILVs via the invagination of the endosome membrane. Different ESCRT-dependent and independent mediators along with other factors are recruited to accelerate the formation, and abscission into the ECM

[35]. It is thought that MVBs can fuse with lysosomes or cell membranes to release ILVs which are known as Exos [36].

Exos and BC cell resistance

Exos significantly influence BC cell resistance via engaging numerous mechanisms [37]. These particles can transfer specific proteins and non-coding RNAs with the potential to accelerate drug efflux [38], stimulate pro-survival signaling, and control epithelial-mesenchymal transition (EMT) [39]. Besides, Exos can influence the hormonal resistance in BC cells via the expression and modulation of certain receptors such as estrogen receptor alpha and downstream effectors, leading to immunotherapy resistance [40].

Emerging data have indicated that Exos use three main mechanisms to increase drug efflux as follows; Exos can directly transfer intracellular components and internalized drugs out of the tumor cells [41]. For instance, BC cells can escape from the cytotoxic effects of chemotherapeutics such as doxorubicin (DOX) using this strategy [42]. Inside the cancer cells, Exos are valid biological platforms to transfer membrane-embedded efflux pumps such as ATP-binding cassette (ABC) transporters such as P-gp among cancer cells horizontally [43, 44]. Interestingly, Exos efficiently transfer P-gp from resistant cancer cells to susceptible cells within the tumor parenchyma in in vitro and in vivo settings [45, 46]. Based on the previously published data, the transfer of P-gp from docetaxel-resistant BC cells to docetaxel-sensitive counterparts led to the acquisition and activation of resistance mechanisms [47]. Besides these mechanisms, the genetic makeup and Exo profile are also involved in the production and synthesis of P-gp. It was suggested that transient receptor potential channels (TRPCs) in BC cells can up-regulate the expression of P-gp [48]. The uptake of TRPC5-loaded Exos can simultaneously increase Ca^{2+} influx P-gp35 overexpression in adriamycin-sensitive MCF-7 cells [49]. Other factors such as exosomal UCH-L1 can stimulate the expression of P-gp via the regulation of MAPK signaling pathway. In support of this notion, it confirmed that the higher levels of P-gp and UCH-L1

adriamycin-resistant MCF-7 cells in which the selective inhibition of UCH-L1 using LDN-57,444 in MCF-7 cells make them sensitive to chemotherapeutic protocols [50]. Of course, it should not be neglected that Exos carry specific enzymes and components with the ability to metabolize the drugs. For instance, exosomal glutathione S-transferase P1 can neutralize several drugs [51]. Even though, the level of this factor is high in adriamycin-resistant tumor cells [52]. As above-mentioned, the internalization of certain cytokines and growth factors via Exos can stimulate distinct pro-survival signaling pathways, and effectors such as survivin in various cancer cells [53, 54]. In response to Paclitaxel treatment, MDA-MB-231 cells produce survivin-enriched Exos with the putative role of accelerating the viability of fibroblasts and SK-BR-3 cells [55]. Additionally, the existence of certain genetic factors like non-coding RNAs inside Exos helps BC cells circumvent the therapeutic regimes. For instance, exosomal levels of miR-423-5p increase by MDA-MB-231 cells after being treated with cisplatin. The uptake of miR-423-5p-bearing Exos can blunt the apoptotic response and concomitantly stimulate the tumor cell proliferation and metastasis [56]. In an experiment conducted by Wang et al., they found that exosomal lncRNA-H19 can reduce the sensitivity of BC cells to DOX [57]. The hormonal resistance is another critical issue in the context of BC progression and development. For example, exosomal lncRNA urothelial cancer associated-1 can and miR-222 heighten resistance mechanisms in estrogen receptor$^+$ BC cells in the presence of tamoxifen via the modulation of mTOR signaling pathway [58–60]. This effect would be related to the up-regulation of p27, and estrogen receptor [58]. Notably, the potency of Exos in the transfer of mitochondrial DNA from resistant and progenitor BC cells can promote the acquisition of stemness, dormancy, and thereby hormonal resistance in other cells [60].

Exos have putative roles in the elimination of antibody drugs trastuzumab-resistant BC cells. In support of this claim, it has been shown that SK-BR-3 and BT-474 BC cells release HER2-enriched Exos with the potential to directly neutralize trastuzumab before attachment to the membrane-bond receptors [61]. The exosomal TGF-β1 and PD-L1 are also other signaling biomolecules that participate in HER2$^+$ BC cell dynamic growth and progression [62].

The phenotype shifting between the epithelial and mesenchymal states (EMT) can be also regulated via Exos in BC cells [63, 64]. The increase of exosomal miR-155 in CSCs can promote the stemness feature in the presence of paclitaxel and DOX [65, 66]. Other factors such as exosomal TGF-β2 can induce stemness features in cancer and normal cells via the remodeling of cytoskeletal proteins, loss of cell-to-cell physical connection (E-cadherin↓), and expression of certain factors such as alpha-smooth muscle actin and vimentin [67].

Exos in BC angiogenesis and vasculogenesis

It has been indicated that tumor cell expansion and metastasis are impacted by blood perfusion. The vascular units can provide sufficient micro and macronutrients into the tumor parenchyma. Compared to natural vascular units, the blood vessels within the tumor masses exhibit a disorganized pattern and uncontrolled leakage [68]. In the context of blood perfusion, two distinct terms include angiogenesis and vasculogenesis exit. Angiogenesis is the process of new blood vessel formation from the vascular trunk in response to hypoxic/ischemic conditions. The hypoxic cancer cells release diverse proangiogenesis factors to switch on the angiogenesis behavior of endothelial cells (ECs). Compared to angiogenesis, vasculogenesis is the formation of vascular units by the recruited progenitor cells, especially endothelial progenitor cells (EPCs) [69, 70]. The newly generated blood units can provide O_2 and essential nutrients to different cells within the tumor parenchyma and drain the waste byproducts [71]. In general, the balance between the pro- and anti-angiogenesis factors can predetermine the fate of angiogenesis inside the tumor mass. The hypoxic conditions, inflammatory response, and genetic mutations of cells within the tumor mass can foster the formation of angiogenesis [70]. It has been touted that the inhibition of blood perfusion into the tumor parenchyma can diminish the expansion of non-resistant cancer cells and metastasis to remote sites. On the other hand, this strategy can activate resistance mechanisms in resistant tumor cells [70]. Even though, in some circumstances, the activation of vasculogenesis can compensate for vascularization into the tumor mass when angiogenesis is prohibited. Under hypoxic conditions, the release of hypoxia-inducible factor 1 alpha (HIF-1α) and stromal cell-derived factor 1 alpha (SDF-1α) can promote the recruitment of progenitors into the tumor parenchyma and thus vasculogenesis [72]. For example, it has been indicated that the suppression of HIF-1α, and SDF-1α using different strategies such as irradiation can blunt the recruitment of CD11b$^+$ myeloid cells and EPCs and reduce vascular unit formation [72]. Recent data have shown that tumor-derived Exos are potent bioshuttles for the transfer of factors to modulate angiogenesis/vasculogenesis. Exos can harbor signaling molecules like matrix metalloproteinases (MMPs), microRNAs (miRNAs), vascular endothelial growth factor (VEGF), peptides, lipids, etc. with the potential to alter behavior ECs [68, 73–75]. Tumor-derived Exos (TDEs) from different BC cell lines, i.e., MDA-MB-231 and MCF-7 cells, are valid nanoparticles in the promotion of cancer angiogenesis. Therefore, monitoring the TDEs provides a valuable avenue

to conceive intricate molecular mechanisms related to angiogenesis [76, 77]. The hypoxic TDEs stimulate the tumor cell migration, and metastasis, tubulogenesis activity of ECs in different animal models compared to normoxic TDEs. The exposure of cancer cells to hypoxia leads to profound changes in the metabolic profile of cancer cells and the production of Exos with angiogenesis potential [78–81].

Mechanisms of exo-mediated angiogenesis

The release of several factors can prepare the tumor parenchyma to change the behavior of ECs and blood vessel formation. Along with these changes, TDEs can transfer the angiogenesis factors between the cells, especially ECs, within the tumor niche [82]. It has been shown that TDEs are enriched with certain angiogenesis factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), VEGF, transforming growth factor beta (TGF-β), MMPs, miRNAs, etc. After being internalization, Exos can release these factors inside the ECs [68, 77, 83, 84]. By the digestion of basal membrane (BM) and ECM via exosomal MMP-2, -9, and −13, ECs are released and participate in the angiogenesis process in a cytokine gradient manner in several tumor types like glioblastoma, melanoma, myeloma, and nasopharyngeal carcinoma [68]. The exosomal MMP14 can cleave endothelial receptor VEGF receptor 1 [VEGF receptor 1: VEGFR1], resulting in the migration and proliferation of ECs in the presence of VEGFA [85]. Genetic materials exit inside the Exo lumen such as non-coding RNAs (ncRNAs), and miRNAs can alter the expression of genes at the post-transcriptional step. For instance, exosomal miR-181b-5p, -182-5p, -222-3p, -1246, -19a, -340-5p, -93-5p, -25-3p, -105, -363-5p, -146a, -129, -188-5p, and let-7a-5p can modulate the expression of angiogenesis-related genes in the acceptor cells [80, 86, 87]. In the presence of TDEs, the level of inhibitory HIF-1α (IHIF-1α) is reduced and exerts an eminent impact on angiogenesis response [68]. It was suggested that BC cells produce Exos with miR-10b, -101, -105, -122, -145, -210, and −373, influencing the angiogenesis response, and thus tumor invasion and metastasis [88]. Some miRNAs like miR-9 and −210 can prohibit the anti-angiogenesis factors, leading to the promotion of vascular unit formation. In an experiment, it was shown that exosomal miRNA-145 affects the expression of N-Ras and VEGF in triple-negative BC (TNBC) in a mouse model [76, 87]. Based on previous data, BC Exo SNHG1 can stimulate miR-216b-5p/JAK2 to trigger tumor metastasis and vascular unit formation via the regulation of small interfering RNAs (siRNAs). Of note, the inhibition of miRNA216b-5p in HUVECs coincides with the rapid elevation of exosomal SNHG1 under hypoxic conditions, leading to the activation of JAK2 and angiogenesis potential [76]. TDE CircHIPK3 can act as a potent inhibitor of miR-124-3p. The suppression of miR-124-3p elevates an intracellular concentration of MTDH in ECs and increases tubulogenic activity (Fig. 2) [73, 74].

Along with the existence of cytokines and peptides and genetic materials inside the TDEs, ExoCarta data confirmed the existence of other components such as lipids (1116 types), and proteins (9796) with the potential to maintain the inter-cellular communication, and angiogenesis potential [68]. Besides exosomal factors, exosomal surface factors like CD147 can act as pro-angiogenesis factors. Upon the attachment of Exo surface proteins to ECs, the angiogenesis behavior of ECs is stimulated [20]. Interestingly, BC Exos in blood and pleural effusion exhibit high levels of CD9, Annexin-1, and HSP70 [89]. Exosomal factors like annexin II and WNT4 are directly involved in the promotion of cancer angiogenesis [20]. For instance, annexin II functions as a tissue plasminogen activator (tPA) [90]. tPA can foster tumor angiogenesis via several mechanisms. It has been indicated that tPA activates MMP-9 to release the membrane-bound c-kit and SDF-1α in bone marrow, the attachment of this factor promotes EPCs to the site of tumors. Besides, the production of F fragments leads to VEGF and EC activation [91]. TDE angiopoietin-2 (ANGPT2) and VEGF can permeabilize the tumor vascular system to release ECs for subsequent angiogenesis behavior. However, sophisticated approaches targeting the ANGPT2 signaling pathway can be used as antiangiogenic therapy in the cancer niche [86]. The exposure of tumor cells to hypoxic conditions accelerates the production of Exos with higher Wnt4a levels. The uptake of Wnt4a-contained Exos stimulates the β-catenin signaling pathway, and angiogenesis in ECs [86]. Glioblastoma cells can produce Exos with VEGF, FGF, interleukin (IL)-6, -8, TIMP-1, and −2 [92]. Similar to this statement, the existence of VEGF and IL-6 was also indicated in melanoma-derived Exos [68]. In response to TDEs, mesenchymal stem cells (MSCs) can commit to myofibroblasts and/or fibroblasts to promote angiogenesis [77]. It is also possible that the interaction of TDEs with immunosuppressive factors such as PD-L1 can foster angiogenesis [93]. VEGFRs are also activated via MV-associated VEGF90K, a specific form of VEGF. The production of VEGF90K is stimulated by crosslinking of tissue transglutaminase (tTG) with VEGF165 [94]. The uptake of TDE miR-105 by ECs leads to the weakening of ZO-1 and enhanced vascular permeability. These features increase the possibility of pre-metastatic niche formation, and tumor cell metastasis to remote sites [81]. It was suggested that tumor microenvironment (TME) encompasses several cell lineages with different functions. Exos can modify TME via the alteration of parenchymal cells such as immune cells, fibroblasts, and ECs on behalf of the pro-angiogenic milieu [78]. TDEs containing CD39/CD73 and adenosine

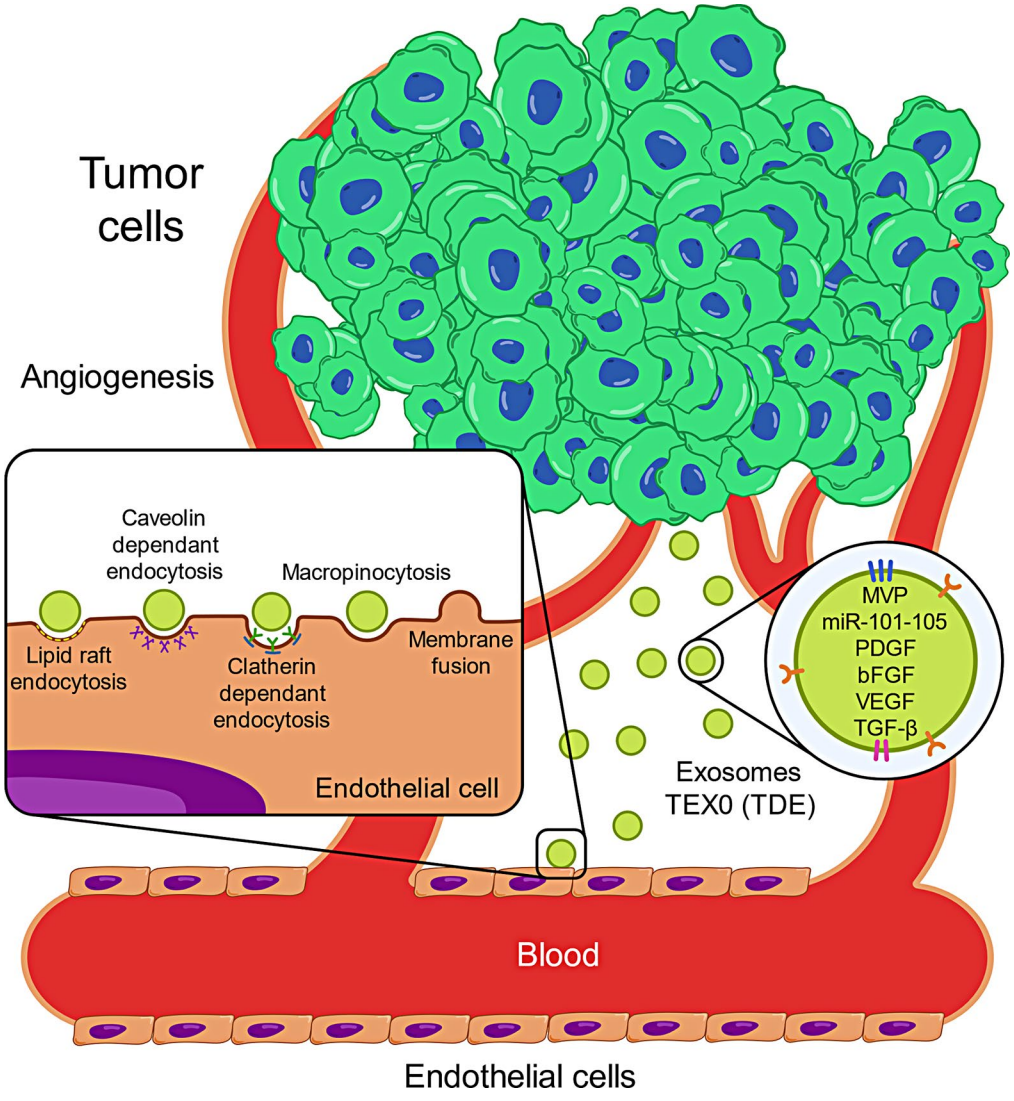

Fig. 2 Angiogenesis behavior of BC tumor Exos. Tumor-derived Exos contain several genetic elements with certain cytokines and growth factors that manage the bioactivity and dynamic growth of ECs. These features lead to the formation of *de novo* vascular networks and support blood into the tumor parenchyma

can induce macrophage polarization toward the M2 type via the activation of adenosine receptor A2BR [78, 79]. These changes lead to the secretion of different angiogenesis factors such as ANGPT2, IL-8, MMP9, PF4, and TIMP-1 by macrophages [86]. The crosstalk between TDEs and ECs helps these cells to acquire mesenchymal features which is the so-called endothelial-to-mesenchymal transition (EndMT) with enhanced angiogenic and invasive properties [79]. This phenomenon helps the angiogenesis via the sprouting and tip ECs movement to the adjacent tissue [95].

Exo-EC interaction

As described for other cells, Exos can be internalized by ECs via lipid rafts, phagocytosis, and micropinocytosis. However, endocytosis is the main internalization route for Exos in ECs (Fig. 3) [96]. It was suggested that the phenomenon of homotypic endothelial Exos uptake is largely energy-consuming and orchestrated by receptor-mediated, clathrin-dependent pathway [97].

Banizs et al. found that the Exo uptake is closely on environment temperature in which the reduction of temperature from 37 to 4 °C prominently abolishes the internalization rate of DiO^+ Exos, leading to less fluorescence intensity in recipient cells (Fig. 4) [97]. Of note, it should not be forgotten that solid tumors with necrotic cores have less temperature values due to glycolytic activity, and less vasculature system compared to the surrounding normal tissues [98]. Thus, it is logical to hypothesize that the application of Exos for the transfer of certain chemotherapeutics and/or entry into deep layers of the tumor microenvironment is limited due to a lack of sufficient

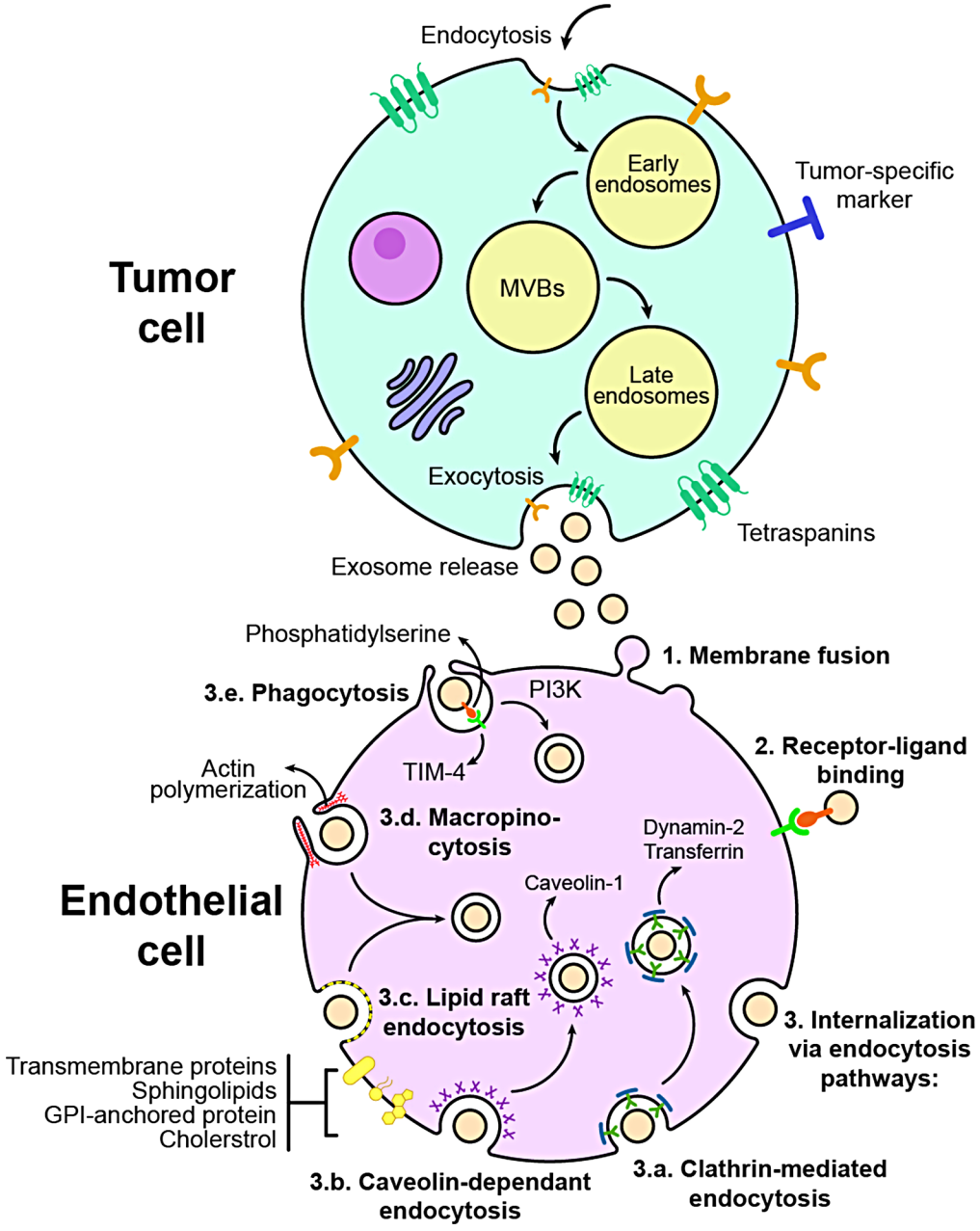

Fig. 3 BC Exos are uptaken by ECs in the proximity or remote sites by using different mechanisms. Exos with certain cargos can be internalized via direct exosomal ligand and recipient cell receptor binding that activates clathrin-, caveolin-, and lipid raft-dependent endocytosis pathways. It is also possible that Exos can enter the target ECs via the activation of micropinocytosis and phagocytosis. The internalized Exos are directed toward specific sites inside the cytosol to exert their effects on various signaling pathways

vascular units and proper environmental temperature. On the other hand, the leakiness, and interstitial pressure within the tumor parenchyma restrict the entry of several compounds from the blood side [99]. Along with these features, the Exos movement to deep layers of tumor mass is also limited as well. The metabolic status of parent cells can contribute to the production and release of specific Exo types with distinct internalization mechanisms [100]. Under severe hypoxia, the activation of Caspase-3 inside the ECs increases the production of apoptotic Exo-like vesicles ($LAMP2^+$, $LG3^+$, and the 20 S $proteasome^+$ particles) from autolysosomes [100]. Brodeur et al. found that incubation of monolayer HUVECs with Cell Tracker Deep Red^+ for apoptotic Exo-like vesicles for 6 h led to internalization and allocation into the cytosol. The internalization rate is time and concentration-dependent and reduced in lower temperatures (4 °C) [100]. One reason for the reduced uptake of apoptotic

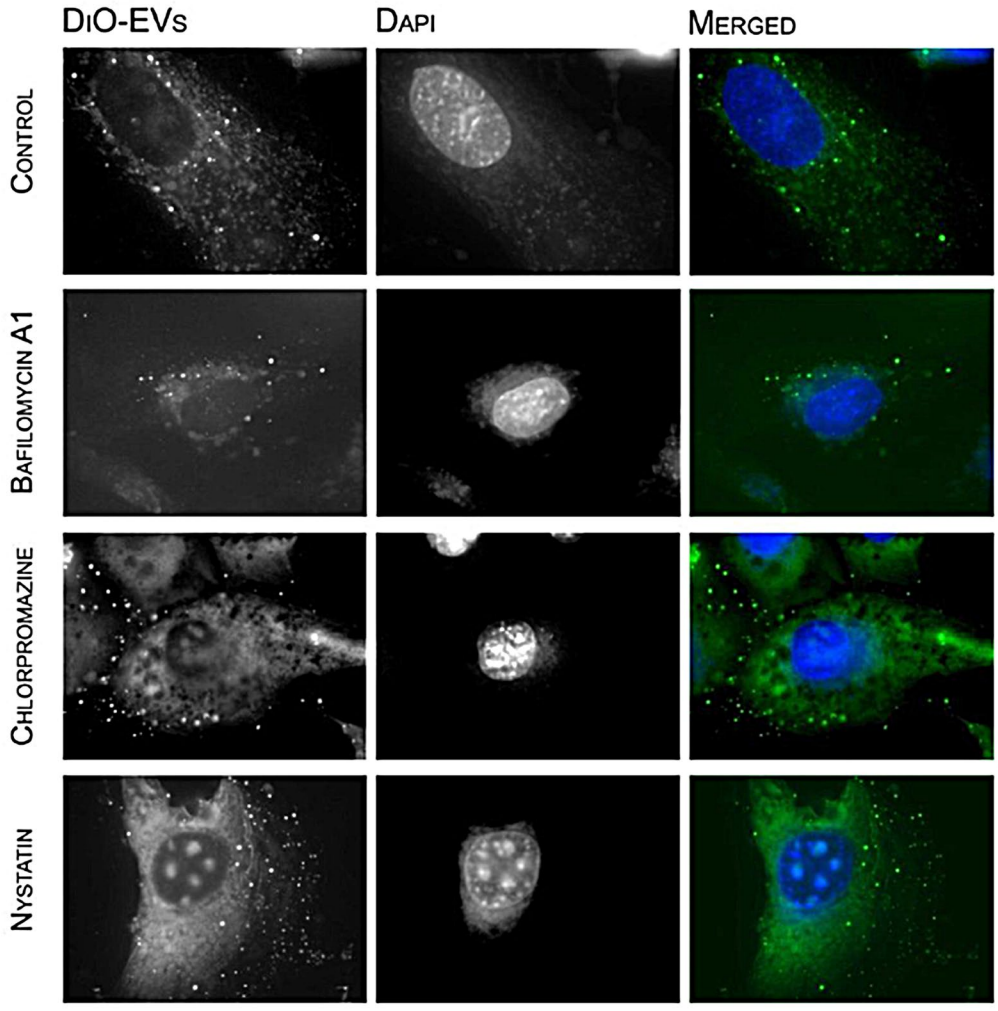

Fig. 4 Immunofluorescence images associated with the uptake of green DiO^+ EVs by mouse aorta ECs pre-treated with 25 µM chlorpromazine (clathrin-dependent endocytosis inhibitor), 25 µM nystatin (caveolin-1 dependent endocytosis inhibitor), and 1 µM Bafilomycin A1 (macropinocytosis inhibitor) for 30 min. In all groups, EVs are in close connection with the cell membrane or internalized into the cytosol. The inhibition of macropinocytosis, clathrin- and caveolin-dependent endocytosis cannot prohibit the entry of EVs in ECs. Blue: DAPI; Magnification: 100X [97]. (Copyright 2018; Molecular Pharmaceutics)

Exo-like vesicles at 4 °C is associated with metabolic activity and cell medium viscosity [100].

Interestingly, the inhibition of endocytosis-mediated internalization, either clathrin or caveolin-based pathway) and non-classical endocytosis (lipid raft) did not alter apoptotic Exo-like vesicle uptake by HUVECs. However, data indicated that macropinocytosis is main the internalization route for apoptotic Exo-like vesicles in which incubation of HUVECs with cytochalasin D, an actin polymerization inhibitor, inhibits the internalization rate [100]. Notably, the released TDEs should be taken by ECs a few hours after production. In support of this notion, it was indicated that PKH26-labeled TDEs are internalized by ECs within 4 h [101]. The phenomenon of endothelial uptake and transfer is a highly dynamic process. For instance, it was shown that Exos are guided to the EC perinuclear zone shortly after internalization [102]. In ECs involved in tubulogenesis (vascular tubes), Exos translocate to the periphery zones and pseudopods. It is also possible that ECs donate exogenous Exos to surrounding ECs or other TME cells [103]. It was suggested that the molecular signature of Exos is very critical in Exo-EC interaction and angiogenesis potential. It has been shown that tetraspanins are potent facilitators in the promotion of adhesion, internalization, and certain factor sequestration [104]. In malignant and metastatic BC tumors, the levels of tetraspanin 8 with an increased cell-to-cell interaction via the increase of E-cadherin. Along with these changes, simultaneous levels of p120-catenin, and E-cadherin are high in tetraspanin 8 bearing EVs [105]. The increase of tetraspanin 8 coincides with the elevation of CD106 and CD49d and can stimulate the Exo uptake by ECs, leading to the induction of angiogenesis behavior (CXCL5↑, vWF↑, CCR1↑, MIF↑, VEGF↑,

and VEGFR2↑) (Fig. 5) [104]. Tetraspanin 8 can directly interact with intersectin-2, involved in clathrin-based endocytosis, for the acceleration of Exo uptake [106]. The close interaction between the tetraspanin 8 and CD49d is required for angiogenesis activity [104]. In support of this statement, Cappellari et al. indicated that blood CD49d^{+} proangiogenic granules from granulocyte sources exhibited higher CXCR4 and VEGFR1 contents with the ability to promote tubulogenesis capacity of HUVECs in vitro [107]. Tetraspanin 8 can also recruit integrin α4β1 for successful interaction between the ECs and Exos [104]. These features indicate that an intricate molecular mechanism with different effector proteins can regulate the entry of Exos into ECs. BC can produce EVs with different integrin proteins like CD47. This protein acts as do not eat me signaling and inhibits the activation of immune cells [108].

Kaur et al. indicated that incubation of HUVECs with MDA-MB-231 cell EVs with higher contents of CD47 led to reduced EC-to-EC connection, loss of cobble-stone shape, and acquisition of elongated spindle shape in in vitro conditions. These features coincide with the alteration of angiogenesis behavior, TNF-α signaling pathway, EndMT transition, etc [108]. Exosomes are very important molecular elements in the organotropism and angiogenesis behavior of numerous tumor cells [109]. For example, a cohort study noted that β4-bearing BC Exos can orient toward pulmonary tissue to form secondary metastatic foci [109]. It seems that the existence of integrin β4 subunit on the Exo surface can increase interaction with ECs and stimulate the angiogenesis behavior. It was suggested that the inhibition of integrin α6, and β4 subunit in colorectal HCT116 and SW620 cancer Exos can blunt the tubulogenic properties of HUVECs [110].

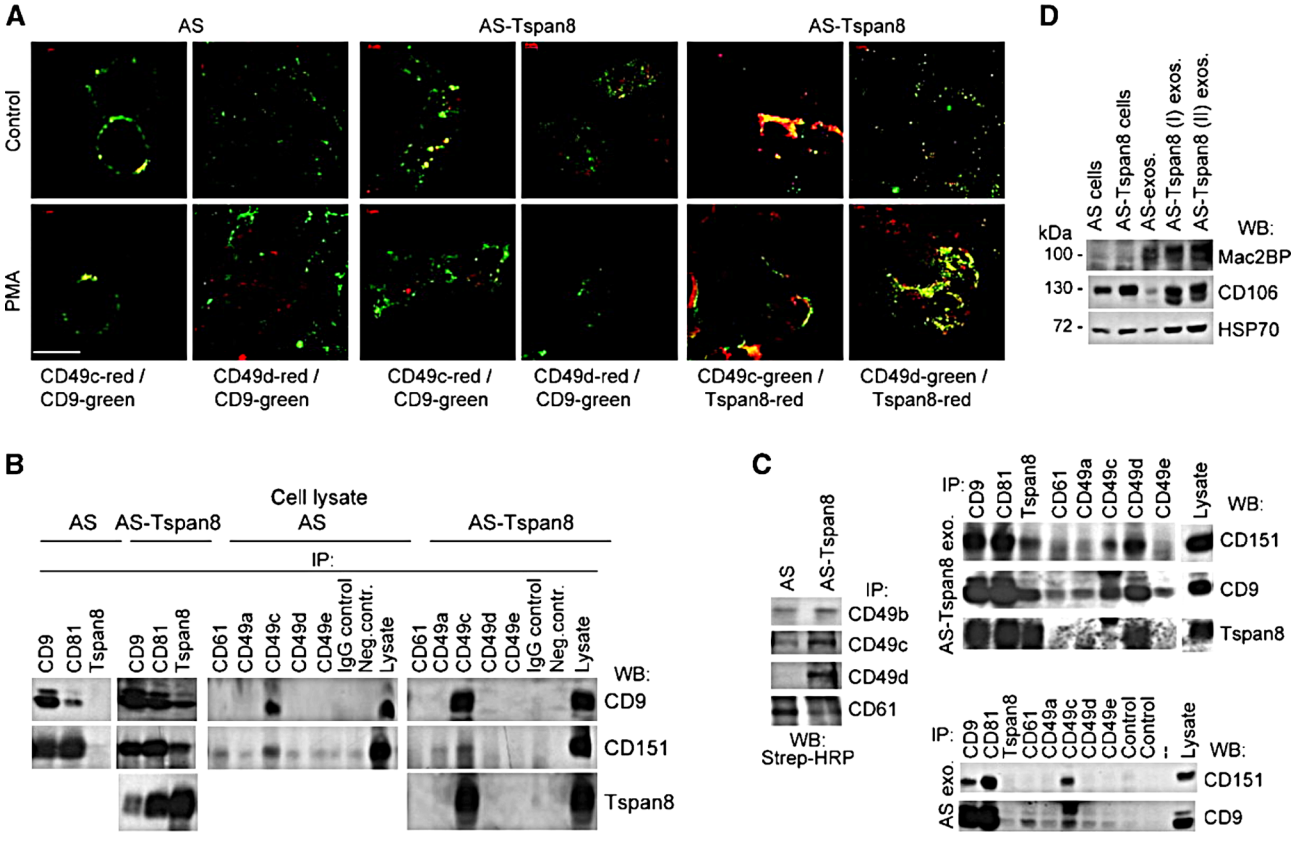

Fig. 5 Monitoring the existence of tetraspanin network in the uptake of rat pancreatic adenocarcinoma BSp73AS cell (AS), adenocarcinoma expressing tetraspanin 8 (AS-Tspan8) cell Exos by ECs. Immunofluorescence images of AS and AS-Tspan8 cells after being treated with phorbol 12-myristate 13-acetate (PMA) to co-localize the tetraspanin and integrin (**A**). Data indicated that CD49c can weakly attach to CD9 with makes strong physical contact with tetraspanin 8 (Tspan8). Incubation with PMA did not alter the co-localization of Tspan8 with CD49d and remained unchanged even after internalization (scale bar: 20 μm). Co-immunoprecipitation of integrins and tetraspanins obtained from AS and AS-Tspan8 cell lysates. It was shown that mediators CD9, 151, and AS-Tspan8 co-precipitate with CD49c (**B**). Immunoprecipitation of Exo integrins. Data indicated that AS and AS-Tspan8 Exos harbor CD49b, c, and CD61 while CD49d can be found in AS-Tspan8 Exos (**C**). Tetraspanins can co-immunoprecipitate in Exos from AS and AS-Tspan8 cells. Of note, CD49c co-immunoprecipitates with CD9 and 151 while CD61 and CD49e co-immunoprecipitates with CD9. It was shown that CD49d has a physical connection with CD9, CD151, and Tspan8 in AS-Tspan8 Exos. In contrast, CD49c can make a physical connection with CD9 and CD151 unless Tspan8 (**C**). Western blotting for detection of galactoside binding protein-3 (Mac2BP), CD106, and HSP70 in the lysates of AS and AS-Tspan8 cells and Exos. The induction of Tspan8 can increase the exosomal content of these factors [104] (Copyright 2010; Cancer Research)

Other integrin subunits such as α4 can form a complex with tetraspanin 8 on the Exo surface and this molecular complex can attach to the endothelial CD54 (ICAM-1) receptor [111]. On the other hand, the production of ICAM-1$^+$ EVs via BC cells can simultaneously activate non-EC internalization. In support of this notion, Duarte-Sanmiguel et al. developed ICAM-decorated mouse embryonic fibroblast EVs loaded with miR-146a and Glut1 using engineering tools. These EVs were prominently taken by immune cells via cognate CD11b receptor, leading to phenotype shifting and recruitment of T lymphocytes to the site of BC in a mouse model [112]. Taken together, there are several mechanisms involved in the internalization of tumor cells, especially BC and Exos by ECs. It is suggested future studies focus on the determination of varied uptake mechanisms in the context of tumors.

Exos as a drug delivery system in BC

Due to unique physicochemical properties, high-rate biocompatibility, low immunogenicity, and target site specificity, Exos are interesting platforms for loading therapeutics. It has been stated that Exo-based cancer therapy can circumvent several issues associated with conventional medication for solid tumors [113, 114]. Compared to naïve Exos, engineered Exos possess higher therapeutics, with less aggregation capacities and improved targeting efficiencies [115]. Because of the bulk distribution rate, Exos can distribute in different parts of the body and sequestrate in non-target organs such as the liver, spleen, pulmonary tissue, etc [116]. Thus, genetic/biological engineering approaches and modification of exosomal surface using via chemical interactions either covalent or non-covalent strategies can increase on-target efficiency into the tumor parenchyma [20, 117]. For non-covalent modification of the exosomal surface, different interactions such as electrostatic interactions, hydrogen bonding, and van der Waals forces can be used to decorate the Exo surface [118]. Otherwise, stable and covalent attachment of target molecules on the Exo surface can be done using click chemistry or azide-alkyne cycloaddition [119]. In the click chemistry approach, the Exo surface is functionalized for the conjugation of specific molecules using simple reactions with high efficiency, and high-rate purification [120]. It is postulated that the decoration of the Exo surface has no profound impact on the size, structural integrity, or uptake capacity. In this scenario, several compounds were conjugated to the Exo surface. For instance, terminal amino acids on the Exo surface can be linked to alkyne groups of polyethylene glycol or vital fluorochromes copper-catalyzed azide-alkyne cycloaddition [121]. During recent years, the Cu^{2+}-free click chemistry method was used to conjugate fluorescent azide for monitoring the on-target efficiency of drug-loaded Exos without detrimental effects on Exo physicochemical properties [122, 123]. In the clinical setting, the administration of chemotherapeutics is limited because of side effects, low retention rate, and poor targeting efficiency [124]. One strategy would be that Exos are loaded with therapeutic (anticancer drugs, photosensitizers) in cancer patients [125].

Several studies have been done for the delivery of chemotherapeutics in BC cancer. For instance, Chen and coworkers developed two distinct Exo-free and Exo-based nano-delivery systems to release mitoxantrone into triple-negative MDA-MB-231 BC cells. Exos were decorated with graphene oxide modified with chitooligosaccharides and γ-polyglutamic acid (EXO-GO-CO-γ-PGA) and loaded with mitoxantrone (EXO-GO-CO-γ-PGA-MIT) [126]. Data indicated an increased apoptotic change (Annexin-V$^+$ cells↑) and reduced survival rate in EXO-GO-CO-γ-PGA-MIT groups compared to cells exposed to GO-CO-γ-PGA-MIT (Fig. 6) [126].

Li et al. used macrophages Exos for the targeted drug delivery in an in vitro setting [127]. For this purpose, poly (lactic-co-glycolic acid) (PLGA)-coated Exos were used for the delivery of doxorubicin for the inhibition of mesenchymal-epithelial transition factor, c-Met in human MDA-MB-231 cells. Incubation of these cells with Exo-PLGA-Dox led to apoptotic changes (TUNEL$^+$ cells). Data also indicated higher targeting efficiency of Cy5.5 labeled Exo-PLGA-Dox to MDA-MB-231 cells in the orthotopic BC mouse model (Fig. 7) [127].

Of course, it should not be forgotten that the method of Exo isolation and drug loading can affect the tumoricidal effects [128]. In a recent study conducted by Ozcelik et al., they isolated Exo from MDA-MB-231 supernatant and human serum using ultracentrifugation and microfluidic systems [128]. Data indicated that Exos isolated by ultracentrifugation lost their typical morphologies compared to the microfluidic system. However, the exosomal surface molecules, such as CD9, 63, and 81 were similar in Exos from both groups. The relatively intact morphological features in Exos isolated using a microfluidic system led to higher lading capacity with paclitaxel and faster internalization into the MDA-MB-231 and MCF-10 A cells [128]. One reason would be that the isolation of Exos using relatively invasive methods with high-speed centrifugation rates can make abnormal morphologies that may affect the close interaction of exosomal ligands with cell surface receptors. However, it is suggested future studies focus on the impact of morphological changes in Exo uptake by several recipient cells. Notably, in some circumstances, the encapsulation of certain anti-tumor agents is mandatory because of low-rate bioavailability and solubility [129]. For example, Thymoquinone, an anti-tumor phytochemical compound, was loaded into adipose MSC Exos using simultaneous incubation,

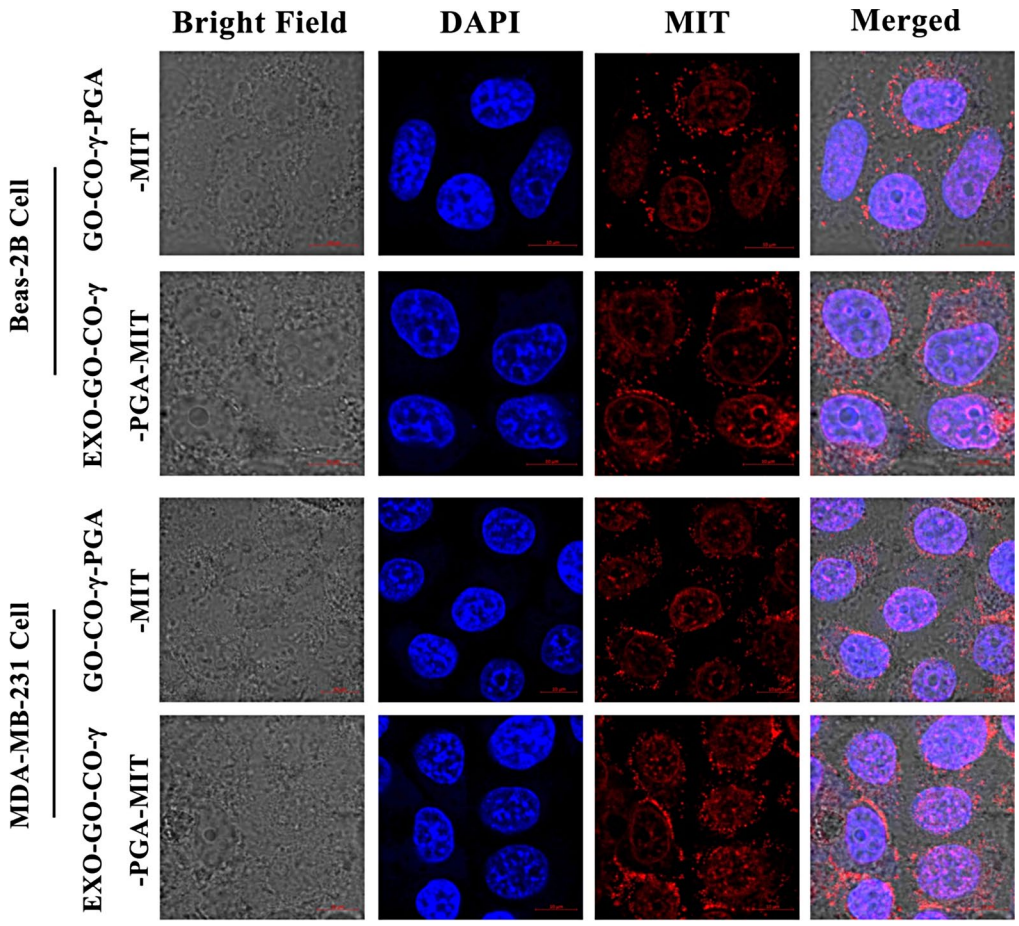

Fig. 6 Distribution of mitoxantrone (MIT) in normal human lung epithelial Beas-2B cells and BC MDA-MB-231 cells using EXO-GO-CO-γ-PGA-MIT after 2 h in in vitro condition. Graphene oxide (GO) was chemically modified using chito oligosaccharides and γ-polyglutamic acid to produce GO-CO-γ-PGA. MDA-MB-231 Exos were decorated with GO-CO-γ-PGA and loaded with MIT. Data indicated that GO-CO-γ-PGA-MIT complex can deliver the drug to cytoplasm while EXO-GO-CO-γ-PGA-MIT had the potential to deliver the MIT into the cell nucleus [126]. (Copyright 2022, Nanotechnology)

freeze-thawing, and surfactant methods [129]. Immunofluorescence data confirmed that FITC-labeled Thymoquinone-loaded Exos were internalized into the MCF-7 BC cells during the 4 h after incubation, resulting in better tumoricidal properties [129]. It has been shown that Thymoquinone can inhibit the vasculogenic behavior of MDA-MB-231 via the reduction of tubulogenesis in a dose-dependent manner. Haiaty and co-workers found that Thymoquinone reduced protein levels of VE-cadherin, MMP-2, and MMP-9 in $CD44^{+}/CD24^{-}$ MDA-MB-231 BC cells. In the presence of Thymoquinone, MDA-MB-231 BC cells lost their stemness features indicated by the increase of cell surface CD24 marker [130]. Likewise, the hydrophobicity of erastin has limited its application in various tumor types. Yu el al. developed Exo-based approach for the delivery of erastin in MDA-MB-231 BC cells. To this end, the incubation of MDA-MB-231 cells with erastin- and folic acid-loaded Exos (erastin@FA-exo) led to ferroptosis (glutathione peroxidase 4↓, cysteine dioxygenase1↓, 7-AAD/Annexin-V^{+} cells↑) [131]. It seems that drug-loaded Exos can be used for different types of BC cells. Both cancer and normal Exos are applicable in the context of drug delivery. For example, Lapatinib was loaded onto normal breast epithelial cell Exos (MCF10A cell line) using an electroporation technique [132]. The incubation of SKBR-3 BC cells with Lapatinib-loaded Exos yielded to lower survival rate (Annexin-V^{+} cells↑, Caspase-3↑, and −9↑) via the inhibition of HER2/EGFR signaling axis (Erbb1↓, Erbb2↓, AKT1↓, and MAPK↓) in lower doses compared to free-Lapatinib treatment groups [132]. Besides the application of drug-loaded Exos in in vitro 2D culture systems, some studies used an exosomal delivery approach in 3D culture models. The incubation of BC tumoroids (MDA-MB-231 cancer cells+HFFF2 fibroblasts and BT-474 cells and HFFF2 fibroblasts) with paclitaxel-loaded MSC Exos yielded a low survival rate (Annexin-V^{+} cells↑) compared to the free drug groups [113]. In an interesting study, nanoamorphous aspirin-loaded Exos were used for the inhibition of dynamic growth in MDA-MB-231

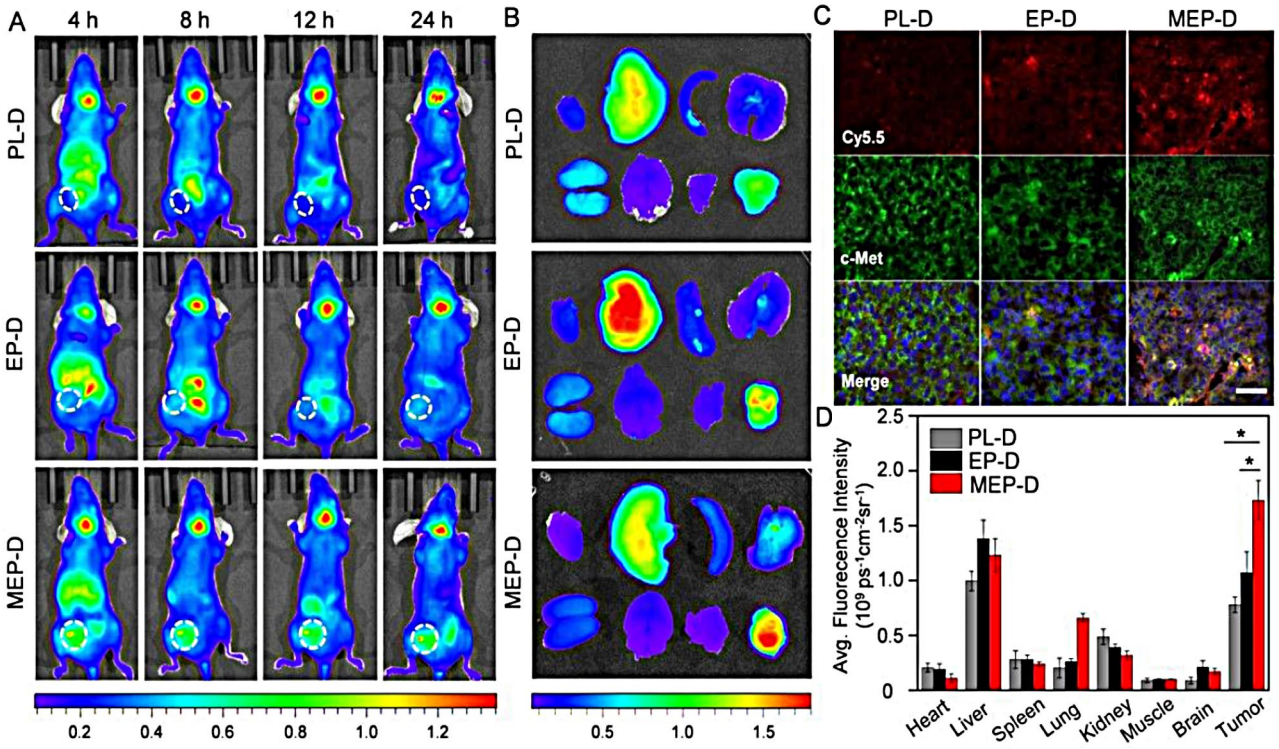

Fig. 7 Targeting potential of macrophage Exos decorated with poly (lactic-co-glycolic acid) (PLGA) nanoparticles and loaded with doxorubicin (DOX) and c-Met peptide (MEP-D). In vivo, images related to on-target efficiency of Cy5.5 labeled MEP-D, PL-D (PLGA + Dox), EP-D (Exo + PLGA + Dox) in nude mice with triple-negative BC (TNBC) 4, 8, 12, and 24 h after systemic injection (**A**). Measuring the fluorescence intensity of normal and cancer tissues 24 h after systemic injection of Cy5.5-labeled PL-D, EP-D, and MEP-D in ex vivo conditions (**B**: top to bottom and left to right are related to cardiac, hepatic, splenic, pulmonary, renal tissues, brain, muscle, and BC). The fluorescence intensity of the tumor section is presented in panel B (**C**). c-Met peptide is shown as a green fluorescent agent (Nuclei: blue DAPI). Measuring fluorescence intensities in tumor and normal tissue presented in panel B (**D**) [127]

BC cells and HT-29 colorectal adenocarcinoma cells. Compared to the free aspirin group, the incorporation of aspirin inside the Exos enhanced uptake rate, apoptosis rate (TUNEL$^+$ cells↑), excessive autophagic response (LC3-II/LC3-I ratio↑), and reduced survival rate [133].

During the last years, studies have expanded to the application of different Exo sources for the delivery of targeted compounds to the tumor sites. Li et al. used milk DSPE-PEG2000-functionalized hyaluronic acid (HA) Exos containing doxorubicin against CD44$^+$ tumor cells [134].

Table 1 Theranostic properties of Exos as in patients with BC

NCT number	Study	Conditions	Interventions/monitoring	Phases
NCT05955521	Prognostic and predictive properties of Exos for detection of patients with an early BC	Triple-negative BC, and HER2$^+$BC	Analysis of Exos and ctDNA	ND
NCT01344109	Prognostic and diagnostic values of BC Exos receiving chemotherapy	Breast Neoplasms	Analysis of exosomal surface protein and luminal RNA	ND
NCT04258735	Genetic Traits and Metastatic Patterns in BC Patients	Metastatic BC	Genomic analysis	ND
NCT04288141	Monitoring her2-her3 dimers in tumor and blood Exos underwent targeted therapies	HER2$^+$BC	Genomic and proteomic analysis and FLIM-FRET monitoring of blood Exos and tissue samples	ND
NCT04653740	Omic technologies to study resistance to Palbociclib in metastatic BC	Advanced BC	Detection of intra-patient variation and Exo molecular profile	ND
NCT03974204	Cerebrospinal fluid Exos analysis in patients with BC metastasis	Metastatic BC	Proteomic and cytological analysis of cerebrospinal fluid and Exos	ND
NCT04298398	Psychological interventions on EVs of patients with different cancers such as BC, colorectal cancer, etc.	Different cancer types	Mindfulness-based cognitive therapy, Emotion-focused therapy group for cancer recovery for monitoring changes in cancer-associated biomarkers such as CA15-3	ND

ND: not determined; BC: breast cancer; Circulating tumor DNA (ctDNA)

The internalization of HA-functionalized doxorubicin-loaded Exos was higher in MDA-MB-231, MCF-7, and A549 cells compared to the HEK293 cells, indicating the close interaction between the exosomal HA with tumor cell CD44 receptor [134]. Along with drugs, Exos can be used for the delivery of other anti-cancers. For instance, fluorochrome sonosensitizer (indocyanine green)-loaded Exos functionalized with folic acid was significantly taken by cancer cells and increased reactive oxygen species (ROS) in MCF-7 BC cells [135]. The rate of Exos uptake was increased in a mouse model of BC after systemic injection. Ultrasonication led to increased ROS production and tumor mass suppression [135].

Clinical application of exos

Recently, Exos have been used in clinical studies of breast cancer (Table 1). Most of these studies have used Exos for early-stage detection, tumor cell metastatic behavior, and changes in Exo profile after the application of specific modalities or therapeutic protocol. It seems that with the progression of Exo isolation from different sources, and engineering techniques in the future we will witness that Exos or other EV types can be used as therapeutics in patients with different tumor types. Currently, naïve and/or engineered Exos are used in animal models with different cancers.

Conclusions

Malignant BC exhibits high morbidity and mortality in humans. The promotion of blood supplements and the formation of vascular units within the tumor niche can help the cancer cells proliferate and metastasize to the remote site. Exos are reciprocal intercellular mediators between the cells, especially in the context of cancer biology. Both BC cells and BC CSCs can produce a large number of EVs mainly Exos with certain cargo that educate the other cells in terms of tumor propagation and development. The intercellular communication between the BC CSCs and ECs should be also considered in terms of angiogenesis for therapeutic purposes. It seems that the inhibition of BC CSC-EC connection via Exos and thus vascularization into tumor parenchyma can lead to the control of tumor cell growth and metastasis to remote sites. By manipulation of Exo cargo and regulation of Exo secretion from BC, especially BC CSCs, the angiogenesis outcome can be regulated. Meanwhile, EVs and mainly Exos can be used as magic bioshuttles for the control of angiogenesis via the delivery of chemotherapeutics. It is recommended that future studies should focus on the determination of underlying angiogenic mechanisms of BC CSCs via Exos in in vitro and in vivo studies. The issues related to the sequestration of angiogenesis factors into the Exo lumen remain to be answered. Despite the recent advantages of the application of Exos in clinical trials, this field faces several bottlenecks and limitations. The isolation, and purification of Exos are laborious, time-consuming, and need high-tech facilities. Of note, the metabolic status of parent cells has a profound impact on the Exo profile. Besides, the lack of GMP-grade preparation protocols can increase batch-to-batch variability [116]. Sterility and the possibility of dormant infections (i.e., viral particles, bacteria, and toxins) before administration and during the preparation steps can affect the regenerative properties of Exos. The rapid elimination from the systemic circulation via splenic tissue or sequestration in non-target organs such as the liver, and pulmonary tract does necessitate several Exo bolus injections. These features increase the likelihood of vascular thrombosis, hemostatic perturbations, and activation of alloreactive immune cells [116].

Abbreviations

ANGPT2	Angiopoietin-2
BM	Basal membrane
bFGF	Basic fibroblast growth factor
BC	Breast cancer
CA125	Carbohydrate antigen 125
CEA	Carcinoembryonic antigen
CTCs	Circulating tumor cells
DOX	Doxorubicin
ESCRT	Endosomal sorting complex required for transport
ECs	Endothelial cells
EPCs	Endothelial progenitor cells
EndMT	Endothelial-to-mesenchymal transition
Exos	Exosomes
ECM	Extracellular matrix
EVs	Extracellular vesicles
HIF-1α	Hypoxia-inducible factor 1 alpha HIF-1α
IL	Interleukin
ILVs	Intraluminal vesicles
MSCs	Mesenchymal stem cells
MMPs	Metalloproteinases
miRNAs	microRNAs
MVs	Microvesicles
MIT	Mitoxantrone
MVBs	Multivesicular bodies
ncRNAs	non-coding RNAs
PDGF	Platelet-derived growth factor
PLGA	Poly (lactic-co-glycolic acid)
ROS	Reactive oxygen species
siRNAs	Small interfering RNAs
SDF-α	Stromal cell-derived factor 1 alpha
tPA	Tissue plasminogen activator
tTG	Tissue transglutaminase
TGN	Trans-Golgi network
TNBC	Triple-negative BC
TME	Tumor microenvironment
TDEs	Tumor-derived Exos
VEGF	Vascular endothelial growth factor
VEGFR1	VEGF receptor 1

Acknowledgements

Authors appreciate the personnel of the Drug Applied Research Center for help and guidance.

Author contributions

P.K., Z.A-M., S.S., and P.H collected data and prepared the draft. S.Z.M. drew the illustrations. R.R., and A.H. supervised the study. R.R. edited the draft. A.H. acquired the funding.

Funding
The present study was supported by a research grant (No: 71007) and the ethical code of IR.TBZMED.VCR.REC.1401.402 from Tabriz University of Medical Sciences.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 14 May 2024 / Accepted: 6 December 2024
Published online: 18 December 2024

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