Cancer Extracellular Vesicles, Tumoroid Models, and Tumor Microenvironment

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Abstract

Cancer extracellular vesicles (EVs), or exosomes, promote tumor progression through enhancing tumor growth, initiating epithelial-to-mesenchymal transition, remodeling the tumor microenvironment, and preparing metastatic niches. Three-dimensionally (3D) cultured tumoroids / spheroids aim to reproduce some aspects of tumor behavior in vitro and show increased cancer stem cell properties. These properties are transferred to their EVs that promote tumor growth. Moreover, recent tumoroid models can be furnished with aspects of the tumor microenvironment, such as vasculature, hypoxia, and extracellular matrix. This review summarizes tumor tissue culture and engineering platforms compatible with EV research. For example, the combination experiments of 3D-tumoroids and EVs have revealed multifunctional proteins loaded in EVs, such as metalloproteinases and heat shock proteins. EVs or exosomes are able to transfer their cargo molecules to recipient cells, whose fates are often largely altered. In addition, the review summarizes approaches to EV labeling technology using fluorescence and luciferase, useful for studies on EV-mediated intercellular communication, biodistribution, and metastatic niche formation.

Keywords: Extracellular vesicles; Exosome; 3D Tumoroid Models; Cancer Stem Cells; Tumor Microenvironment; Metastatic Niche

Contents

1. Introduction

Poor prognosis in cancer patients is associated with rapid tumor progression, a tumor-stroma interactive microenvironment, the dissemination of tumor cells to the blood circulation and distant organs where metastatic secondary tumors are formed [1, 2]. Recent studies have shown that "exosomes" or small extracellular vesicles (sEV: 50–150 nm diameter) released from the cells can transfer bioactive molecules to the neighboring cells and also deliver them to distant organs through body fluids such as the bloodstream. The term "exosome" is so designated as it is originated from combining the terms endosomes and secreted via exocytosis. However, it is experimentally difficult to distinguish exosomes from other EV types, such as ectosome (100–500 nm) released by membrane shedding, and few papers have concentrated on such differences. For this reason, most experts recently have recommended calling exosomes sEV, which is clearly stated in the position paper [3]. Therefore, we use mainly the term sEV but sometimes exosome in this review. (More information is in the section of "Diversity and Heterogeneity of Extracellular Vesicles")

sEV-mediated molecular transfer is essential for several events in tumor progression, including epithelial-mesenchymal transition (EMT), tumor-stroma interaction in the cancer microenvironment, and metastasis [4-7]. Tumor-derived sEV / exosomes can promote the dissemination of cancer cells after transit through body fluids, the formation of the pre-/pro-metastatic niche in target organs [8], and through bone marrow education of myeloid cells [9]. Tumor sEV can contain oncogenic or pro-tumorigenic proteins such as RAS and mutant KRAS [10], epidermal growth factor (EGFR) family members, including EGFR [11-13], mutant EGFR [14], EGFR variant III (EGFRvIII) [15], and ErbB1 [16], metalloproteinases [5, 17], CD326/EpCAM (epithelial cell adhesion molecule) [4, 18-21], heat shock protein (HSP) family members, including HSP90 [4, 22-24], HSP70 [24], HSP60 [25], TRAP1 [24], Gp96/Grp94/Endoplasmin [22], and programmed cell death-ligand 1 (PD-L1) [26, 27]. It has been shown that the molecular transfer of oncogenic proteins can trigger the transformation of recipient cells, mediating such processes as EMT [4, 11, 22] and initiating tumorigenic potential [28], prompting us to use the conceptual term “oncosome” for the oncogenic vesicles.

Two-dimensional (2D) cell cultures are simple and easy to use for cancer research and drug screening. In conventional 2D cultures, cells are grown as monolayers on flat surfaces, allowing the cells access to the same amount of growth factors and nutrients present in the medium, resulting in homogenous growth and proliferation. However, the strong physical interaction between cells and 2D materials alters tumor cell behaviors that differ from those growing *in vivo*. Thus, the 2D culture model fails to correctly mimic the proper tissue architecture and complex

microenvironment of tissues encountered *in vivo*. To overcome limitations of the 2D culture system, 3D cell culture models (known as spheroids, organoids, tumoroids, and micro-fluidic systems) have been developed to better mimic *in vivo* cell microenvironments. The 3D culture model maintains the interactions between cells and their ECM and creates gradient access of oxygen, nutrient, metabolic wastes buildup, and a combination of tissue-specific scaffolding cells as in tumors in vivo [29-31]. Proliferating, quiescent, and dying cells coexist in normoxic, hypoxic, or necrotic zones within tumoroids (or spheroids) as in human cancers [18, 28, 32, 33]. In addition, the population of cancer cells recovers cancer stem cell (CSC)-like properties in 3D cultured tumoroids [18, 34, 35]. Thus, the 3D cancer models reflect more closely some of the behaviors of *in vivo* human tumors. EV functional assays in 3D culture systems recently became available, and 3D-cultured tumoroid-derived sEV were shown to be rich in CSC markers [4, 18]. Also, 3D-cultured tumoroids release small EVs (sEV) and large EVs (L-EV), which are pro-tumorigenic in vitro and could penetrate deep inside the tumoroids and their cell nuclei [28].

However, classical spheroid models fail to reproduce some of the features of tumors such as *de novo* angiogenesis, infiltration of normal cells such as fibroblasts, exposure to host immune cells and the acute acidity of many large tumors due to build-up of CO_2 and lactate [36]. We review here some of the recent progress in cancer EV research using 3D culture systems. We will first discuss the diversity and heterogeneity of EVs (section 2) and then review EV-mediated molecular transfer and signaling (section 3). Multi-functional proteins, such as HSPs and metalloproteinases, loaded in sEV are featured (section 4). We describe EVs released from 3D tumoroids with CSC properties (section 5) and then review tumor tissue culture and engineering platforms compatible with EV research (section 6). We then review EV labeling methods using fluorescence and luciferase (section 7). Some tumoroid models have been generated with aspects of tumor microenvironments, such as ECM, vasculature, and hypoxia (section 8). Therefore, the 3D culture tumoroid models are useful among the tools for research on the tumor microenvironment (section 9), tumor EV-driven premetastatic niche formation (section 10), and circulating tumor cell (CTC) cluster and metastasis (section 11).

2. Diversity and Heterogeneity of Extracellular Vesicles

Extracellular vesicles (EV) are small structures surrounded by lipid membranes that are released by cells of diverse organisms, including eukaryotes and prokaryotic cells. In pluricellular organisms, EVs exist in body fluids such as blood, saliva, urine, breast milk, cerebral spinal fluid, synovial fluid, bile, ascites, amniotic fluid, and pleural ascites [37]. EVs contain molecular cargo types, including proteins, RNA, DNA, lipids, minerals, and metabolites [38]. The diversity and

heterogeneity of EVs and extracellular non-vesicular particles are currently crucial in biology and medicine. Earlier studies classified EVs into the following three types: (1) exosomes (50–150nm) originating from the endosome; (2) ectosomes, also called microvesicles or microparticles (100–500 nm), generated by the budding and shedding of the plasma membrane of cells; and (3) apoptotic bodies (1–5 μm), also known as apoptosomes, originating through blebbing of the plasma membrane [3]. Moreover, according to EV functions or origin, recent studies reported additionally defined vesicle types, such as oncosomes [5, 39-45], stressomes [22], matrix vesicles [46, 47], migrasomes [48] (50 nm–3 μm), exopheres (~4 μm), and bacterial outer membrane vesicles (OMV) [49, 50]. Recently, EVs have been re-defined by their size as small EVs (sEV), medium EVs (mEV), and large EVs (L-EV), depending on the context of studies [3]. The stressome is a secretory phenotype released upon cell stress with large EVs (100–500 nm), membrane-damaged EVs, and HSPs [22]. DNA damage regulates senescence-associated extracellular vesicle release via the ceramide pathway to prevent excessive inflammatory responses [51]. Additionally, non-membranous nanoparticles termed exomeres (~35 nm) have been found and involve metabolic enzymes, microtubule, hypoxia, coagulation proteins, and glycosylation [52].

Oral fluids are rich in large and small EVs derived from saliva, gingival crevicular fluid, and cellular components [53, 54]. Three-dimensional (3D) tumoroids of metastatic colorectal cancer cells released large and small EVs [28]. Exosomes or sEV often have phosphatidyl serine (PS) on their membrane surface and a PS-TIM4 affinity-based EV purification method is frequently used [55]. Since PS is an "eat me" signal, PS+ EVs are phagocytosed by macrophages. The negative charge of PS in exosomal membranes is involved in the recognition and clearance of intravenously injected exosomes by macrophages [56]. The heterogeneous population of EVs in a given tissue or body fluid is thus a function of both of the varieties of EVs generated by single cells and their origin in multiple cell types, close or distant, each with its own repertoire of vesicles.

It is not easy to determine exactly the origin of EVs present in body fluids in animal tissues in vivo. However, if a cell type-specific marker is present on the EV surface, it can be a clue to infer its origin. Tissue-exudative extracellular vesicles (Te-EVs) are defined as EVs exuded from tissues extracted from animal bodies [57]. These are obtained when tissue is excised from a living body and immersed in a medium to collect EVs exuded from such tissue. Te-EVs are assumed to be equivalent to the EV produced by the original in vivo tissue and may also have applications in cancer research [58].

3. Exosome- or sEV-mediated Molecular Transfer and Signaling

sEV or exosomes are characterized as vesicles carrying, propagating, and delivering the loaded molecules to receiving cells [59, 60]. sEV mediate several types of molecular transfer and signaling (**Figure 1**). Intra-EV molecules, such as RNA and proteins, can be transferred into EV-receiving cells and exert their functions on these target cells. For example, sEV mediate the protein transfer of multifunctional proteins, such as MMP3 [5, 28] and HSPs [61], into recipient cells. Notably, MMP3 loaded in colorectal cancer EVs is transferred into recipient cell nuclei, and thus MMP3 plays *trans*-activating roles on the cellular communication network factor 2 (CCN2) gene, known as a tumor-promoting factor [5]. EV transmembrane proteins, such as EGFR, can be transferred to the cell membrane of the EV receiving cell and become involved in intercellular communication and signaling [45]. Cytokines, chemokines, and growth factors can also be decorated on the sEV surface by binding with proteoglycans. CCL2 is thus decorated on exosomes by binding with proteoglycan and is bound with its receptor CCR2 on the surface of receiving cells, determining uptake and biodistribution [62]. Integrins are often found on cancer cell-derived exosomes and determine organotropic pre-metastatic niche formation [63]. Ligands for integrins include fibronectin, vitronectin, collagen, and laminin. Fibronectin on the surface of sEV mediates EV-cell interactions [64, 65].

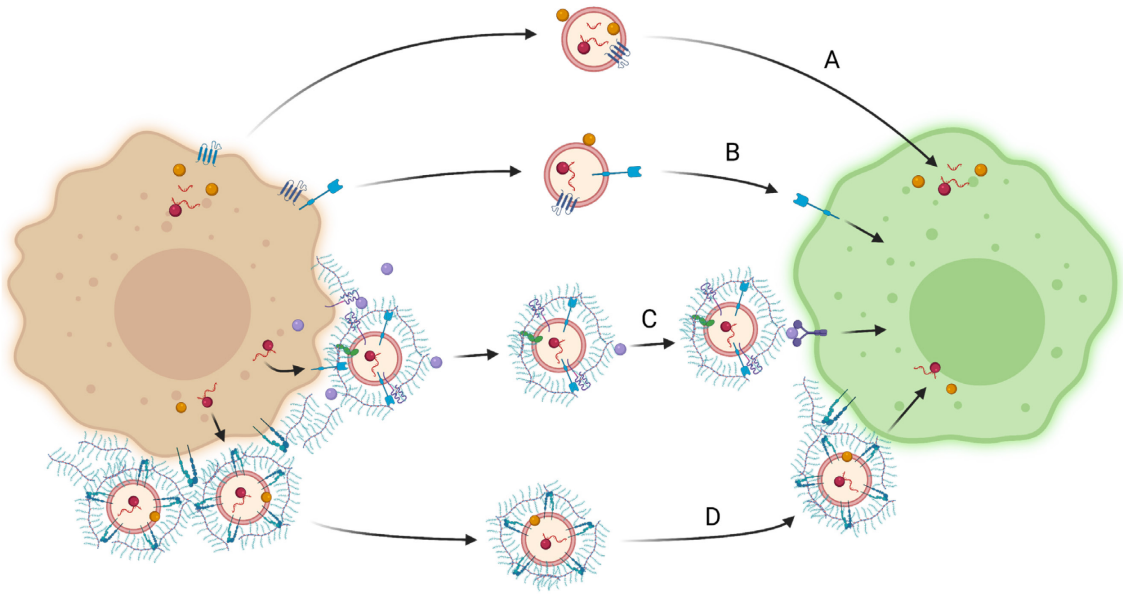


Figure 1. Exosomes or sEV-mediated molecular transfer and signaling. (A) Intra-EV molecules, such as RNA and proteins, can be transferred into sEV-receiving cells and exert their functions on target cells (red hexagram and arrow) [61]. MMP3 [5, 28] and HSPs [61] loaded in sEV are transferred into recipient

cells. Protein palmitoylation may be required for this event [66]. (B) EV transmembrane proteins, such as EGFR, can be transferred to the cell membrane of the EV receiving cell and become involved in intercellular communication and signaling (blue rectangle and arrow) [11, 45]. (C) Cytokines, chemokines, and growth factors can be decorated on sEV by binding with surface proteoglycans. CCL2 (purple ball) is decorated on sEV by binding with proteoglycan and bound with its receptor CCR2 (purple box) on the surface of receiving cells, determining uptake and biodistribution [62]. (D) Integrins are often found on cancer cell-derived exosomes and determine organotropic pre-metastatic niche formation [63]. Ligands for integrins include fibronectin, vitronectin, collagen, and laminin. Fibronectin on the surface of exosomes mediates EV-cell interactions [64, 65].

Protein palmitoylation is the covalent attachment of fatty acids, such as palmitic acid, to cysteine (S-palmitoylation) and less frequently to serine and threonine (O-palmitoylation) residues of proteins [67] and plays a signaling role in the association with the lipid membranes of cells and vesicles. Palmitoylation enhances the hydrophobicity of proteins and is thus vital to their membrane association in cells and vesicles. Palmitoylation also plays a key role in protein-protein interactions between cells and protein transport across the cell membrane. Notably, in contrast to prenylation and myristoylation, palmitoylation is usually reversible.

Tetraspanins (TSPAN) are often palmitoylated and found in sEV or exosomes. They are transmembrane proteins belonging to the Transmembrane 4 superfamily (TS4SF) and comprise 33 members from TSPAN1 to TSPAN33. Tetraspanins form molecular complexes on membranes with integrins, growth factor receptors, and other molecules, known as a tetraspanin-enriched microdomain (TEM) [68]. The formation of TEM is enhanced by palmitoylation and is important for exosome molecular loading and transfer [69]. CD9, CD63, and CD81 are tetraspanins often used as exosome markers [3]. Fluorescent proteins such as GFP and tdTomato are usually not secreted by the cells, but after adding, a palmitoylation signal can become palmitoylated and attached to the membrane of EVs. Palmitoylated GFP (palmGFP) and palm-tdTomato can be loaded on EVs [70]. We have generated palmGFP-expressing macrophages and fractionated their large, medium, and small EVs by the SEC method [66]. Palmitoylated GFP was co-loaded in CD9-loaded exosomes (large exosomes) and CD63 / HSP90-loaded particles and then very efficiently transferred from the macrophage-derived exosomes to receiving cancer cells [66]. By contrast, the other EV types did not carry GFP, CD9, CD63, and HSP90. Thus, it is conceivable that protein palmitoylation is essential for molecular loading on exosomes and subsequent transfer to cells.

4. Multi-Functional Proteins Loaded in EVs – HSPs and Metalloproteinases

EVs are a treasure trove of multifunctional molecules. EV research is beginning to explain the functional principles of multifunctional proteins that have not been explained before. We introduce heat shock proteins and matrix metalloproteinases, multifunctional proteins installed in EVs [17]. Heat shock protein (HSP) family members are often found in extracellular space and EVs or exosomes, including molecular chaperones such as HSP90 [4, 22-24], HSP70 [24], HSP40 [22], TRAP1 [24], Gp96/Grp94/Endoplasmin [22], Grp78/BiP [22, 24], Hsp105/HspH1 [24], HspB paralogs (such as alpha-crystallin and Hsp27) [22] and chaperonin such as HSP60 and CCT family members. HSP90 is essential in intracellular protein folding, wound healing, and cancer progression [71-73]. However, more recent studies, including ours, have revealed the secretion of HSP90 that leads to novel functions in cell-cell communication. More recently, HSP90 has also been found as a major cargo contained in sEV [22, 23, 74, 75]. The HSP90 family consists of four members, including the proteotoxic stress-inducible HSP90α encoded by *HSP90AA1*, the constitutively expressed HSP90β encoded by *HSP90AB1*, the mitochondrially localized TRAP1, and an ER-resident paralog Gp96 (aka Grp94 or endoplasmin) and elevated expression of these HSP90 paralogues are often found in cancer [74, 75]. Notably, HSP90α is often overexpressed in cancer cells and can be secreted to the extracellular space as a soluble protein or a cargo protein of sEV or exosomes [22, 24, 76, 77]. Recently, the EV-proteome analysis of cancer cells has revealed abundant secretion of sEV enriched with HSP90 from high-metastatic oral cancer cells [24] and castration-resistant prostate cancer (CRCP) cells [22]. HSP90-rich sEV initiated epithelial cell transformation to a mesenchymal phenotype, known as EMT [4, 22]. HSP90-rich sEV also promoted migration and invasion of oral cancer cells [4]. Notably, HSP90-rich sEV promoted the spheroid formation of oral cancer cells in a 3D culture system [4]. These pro-tumorigenic potentials of HSP90-rich sEV were inhibited by the depletion of HSP90 from the sEV [4]. Thus, HSP90-sEV plays oncogenic and pro-tumorigenic roles in cancer. Pioneering studies first showed the inclusion of Hsp60 in extracellular vesicles that may have profound physiological effects [78, 79].

HSP90 is essential for intercellular communication via sEV. The siRNA-based triple knockdown of HSP90α, HSP90β and CDC37 lowered levels of HSP90α /β and tetraspanins (CD9 and CD63) in both oral carcinoma cells and their sEV [4]. The reduction of HSP90, CD9, and CD63 in sEV resulted in the loss of molecular transmission from the tongue cancer sEV to macrophages [4]. Also, EV-mediated molecular transfer from macrophage-sEV to cancer cells can depend on CD9+ exosomes and CD63+ HSP90+ protein / EXO-S fraction [66]. In patient-derived oral carcinoma cases, HSP90α is significantly more expressed in cancer cells in metastatic cases (stage IV) than in non-metastatic stage I cases and stromal cells. HSP90β is expressed in

cancer cells in stage I non-metastatic cases and significantly reduced in stage IV metastatic cancer cells, while HSP90β is significantly found in stromal cells, including tumor-associated macrophages (TAMs) in the metastatic stage IV cases [4]. Therefore, it is conceivable that EV-mediated HSP90β transfer from cancer cells into macrophages is involved in the M2 polarization of TAMs. It was reported that intercellular chaperone transmission via exosomes contributes to maintaining protein homeostasis at the organismal level [61].

HSP70 loaded in/on sEV plays immuno-stimulatory roles in cancer. Firstly, it was shown that extracellular HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine [80]. It was then reported that HSP70 surface-positive tumor exosomes / sEV stimulate the migratory and cytolytic activity of natural killer (NK) cells [81]. Active secretion of Hsp70 from peripheral blood mononuclear cells (PBMC), including lymphocytes and monocytes, is exosome-dependent [82]. Tumor-derived exosomes educate dendritic cells to promote tumor metastasis via HSP72/HSP105-TLR2/TLR4 pathway [83].

Chaperonins such as HSP60 (aka. Cpn60) and CCT family members are also found in EVs [24, 78, 79]. The key difference between chaperons and chaperonins is that the chaperones perform a wide array of functions, including folding and degradation of the protein, aiding in protein assembly, etc., whereas the key function of chaperonins is to assist in the folding of large protein molecules. Human Hsp60, the product of the *HSPD1* gene, is a Group I mitochondrial chaperonin, phylogenetically related to bacterial GroEL [84]. The presence of Hsp60 outside the mitochondria and outside the cell, e.g., in circulating blood, has been reported [78]. Hsp60 is actively secreted with exosomes to extracellular space from many tumor cell lines but not non-tumor cells [85, 86]. Hsp60 is found in tumor tissues and circulating exosomes in human large bowel carcinoma [25], brain tumors such as gliomas [87, 88], lung carcinoma [89], and other tumors. Protein acetylation may be involved in the release of Hsp60 because the HDAC inhibitor SAHA inhibited their release [89].

Metalloproteinases consist of matrix metalloproteinases (MMP) family [27], membrane-type MMPs (MT-MMPs), a disintegrin, and metalloproteinases (ADAM) family [90], and ADAM with thrombospondin motifs (ADAM-TS) [91]. These metalloproteinases are zinc/calcium-dependent endopeptidases and main players in extracellular matrix (ECM) remodeling due to their ability to degrade numerous components of ECM and non-ECM proteins, such as adhesion molecules, cytokines, protease inhibitors, and membrane receptors. Later studies have shown that moonlighting / matrix metalloproteinase 3 (MMP3) was a bifunctional protein that acts as a proteolytic enzyme and a transcriptional factor, playing crucial roles in tumor progression [33–

35]. Recent studies showed that metalloproteinases are loaded in cancer exosomes or sEV [43, 92-96]. As expected, MMPs on the sEV surface play pro-tumorigenic proteolytic roles in cancer [97-99]. Notably, MMP3 loaded in colorectal cancer sEV is transferred into recipient cell nuclei, in which MMP3 plays trans-activating roles on cellular communication network factor 2 (CCN2) gene, known as a tumor-promoting factor [5]. MMP3-EVs promote tumorigenesis through the bloodstream in mice in vivo [5] and tumoroid formation in a 3D culture system in vitro [28]. MMP3 was found in the border area between tumor and stroma [6], indicating that MMP3-sEV may play key roles in EV-driven tumor-stroma interaction in the tumor microenvironment. MMP3 loaded in EVs can penetrate deep inside 3D tumoroids and promote in-vitro tumor growth with increases in Ki-67+ proliferating cells [28]. The pro-tumorigenic role of MMP3-EVs was canceled by the depletion of MMP3 from EVs using the CRISPR/Cas9 genome editing system [5, 28].

Knockout of MMP3 also resulted in the loss of CD9 ad CD63 tetraspanins in tumoroid-sEV, while MMP3-rich EVs increased CD9 levels in tumoroids [28], indicating that MMP3 is involved in CD9-exosome / sEV biogenesis and release in carcinoma. Interestingly, 3D tumoroids formed by LuM1 cells produced sEV (50–200 nm) and aggregated EVs (300–1000 nm), which appeared upon MMP3 knockout [28]. The MMP3-high LuM1 cells possess increased stem cell properties [34] and show lung-tropic metastasis [6, 32, 35, 100]. The LuM1-derived MMP3-rich sEV showed lung-tropic accumulation [4]. At the same time, siRNA-based knockdown of MMP3 and MMP9 significantly decreased primary tumor growth and lung metastasis in the mouse LuM1 tumor allograft model [6]. Thus, MMP3 / MMP9-loaded sEV may play key roles in lung-tropic metastasis.

5. Tumoroids Releasing EVs with Cancer Stem Cell Properties

Two-dimensional (2D) cell cultures are commonly used for cancer research and drug screening [31]. In these conventional 2D cultures, cells are grown as monolayers on flat surfaces, allowing access to similar amounts of growth factors, nutrients, and oxygen in the medium, thus resulting in homogenous growth and proliferation [18, 101]. However, the strong physical interaction between cells and 2D substrates results in alterations in tumor cell behaviors that differ from those of tumors growing *in vivo* [18, 31]. Thus, the 2D culture model fails to correctly mimic the tissue architecture and complex microenvironment of tissues encountered *in vivo* [18]. To overcome these limitations of the 2D culture system, novel 3D cell culture models, including spheroid, organoid, and tumoroid, have been developed to better mimic *in vivo* tumor microenvironments [18, 33, 102]. The 3D culture model maintains the interactions between cells

and their extracellular matrix (ECM) and creates gradient access of oxygen and nutrients, variation in pH, metabolic waste buildup, and multiple cell types in the tumor microenvironment [29, 103-105]. As with human cancers, proliferating, quiescent, and dying cells coexist in normoxic, hypoxic, or necrotic zones within tumor organoids [18, 28, 32, 106]. Thus, the 3D cancer models reflect many properties of in vivo tumors more closely, which prompted us to define the term tumoroids. (Tumoroid models with tumor microenvironment were reviewed in section 7.)

Several groups reported that 3D culture promotes the secretion of in vivo-like exosomes or sEV more than was seen in 2D culture [5, 18, 32, 107, 108]. We showed that significantly distinctive sEV and L-EV are produced by 3D tumoroids of LuM1 metastatic colon cancer cells [28]. Cell aggregates of LuM1 colorectal cancer cells with high metastatic properties expressed CSC markers such as CD326/EpCAM (also found in exosomes) [4, 18, 22, 24], MMPs [28, 34, 35, 100] (also found in EVs / exosomes), DLL1 (delta-like 1, a Notch ligand) [18, 32], ATP-binding cassette transporters G1 and G2 (ABC-G1 and ABC-G2) [32, 34], phosphorylated STAT3 [35] and other stem cell markers [18, 34]. We showed that the gel-free tumoroids with stem cell properties acquired resistance to cisplatin in metastatic colorectal cancer [34]. Therefore, the use of 3D cultured tumoroids is highly recommended for future cancer research, pharmacological research, and EV research.

It has gradually become clear that many tumors harbor CSC, also known as cancer-initiating cells (CIC), in dedicated niches [109]. Normal stem cells are often derived from undifferentiated pluripotent/multipotent populations, such as MSCs and hematopoietic stem cells (HSCs), and primitive cells that have the potential to differentiate or provide a variety of specific cell types. The crosstalk between stem cells and the surrounding microenvironment, also called "niche" is an important interaction and a material basis for tissue homeostasis, and stem cells can thus keep an accurate balance between self-renewal and the direction of differentiation [110, 111]. Recent studies have reported MSC-derived sEV or exosomes to contain stemness-specific molecular information capable of promoting stem cells assertively within the niche [112, 113]. However, upon certain circumstances of abnormal cellular communication, a series of morphological, physiological, and phenotypic changes can create a microenvironment favoring the transformation of normal stem cells into CSCs [114-116]. Also, EVs secreted in response to cell stresses, such as chemotherapy [117] or hypoxia [94], can promote stemness in surrounding cancer cells. Although CSCs present many similar characteristics to other stem cells, such as MSC, embryonic stem cell (ESC), and induced pluripotent stem cells (iPSC), in terms of their capacity for self-renewal, the expression of ESC transcription factors, and similar regulation of several

signaling pathways, they are unique in contributing to carcinogenesis, tumor growth, metastasis, and cancer recurrence [34, 118, 119] **(Figure 2)**.

Some drugs have been shown to inhibit CSC-like tumoroid growth. Anti-malarial artesunate is a pleiotropic drug that inhibits CSC-like tumoroid growth [35, 120, 121]. Benztropine also inhibits CSC-like tumoroid growth by inhibiting the expression and activities of STAT3, NF-κB, β-catenin, and MMP9 [35]. As a potent inhibitor of autophagy and cytotoxic agent, Salinomycin effectively inhibited mammosphere formation of CSC-like cells [122]. Tumor acidosis enhanced cytotoxic effects and autophagy inhibition by salinomycin on cancer cell lines and cancer stem cells.

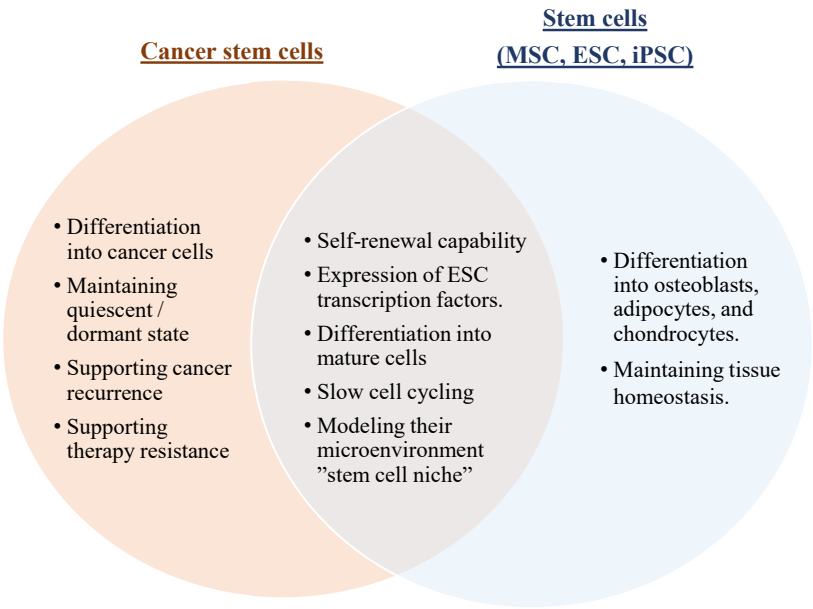


Figure 2. The different and shared characteristics of cancer stem cells (CSCs) and other stem cells (MSC, ESC, iPSC). CSCs and normal stem cells have shared characteristics to the processes that determine stemness [109, 110]. These common features can influence stemness simultaneously, but they can also act independently over time. However, within CSCs, different features can impact cellular stemness properties and shape tumor progression and therapeutic response.

CSCs are also crucial in modulating stromal cells within the tumor microenvironment, promoting cancer progression and recurrence [123]. It is worth noting that tumoroid formation in a 3D culture milieu increased the CSC/CIC properties and secretion of tumor exosomes rich in EpCAM/CD326, a key CSC/CIC marker protein [18]. Consistently, several studies demonstrated the internal contents of exosomes or sEV and their roles in the events of tumorigenesis; a key role was shown in CSC niche formation through modulation of cellular behaviors within the stroma

[94, 124, 125]. Interestingly, despite the different approaches, these studies initially confirmed that treatment with sEV from increasingly aggressive neoplastic cell types, such as thyroid cancer, breast cancer, leukemia, and prostate cancer, mediated the establishment of CSC niches within stromal elements by transporting CSC-related specific molecules and thereby maintaining CSC homeostasis. Another team recently demonstrated that breast cancer cells exposed to chemotherapeutic agents released miR-9-5p, miR-195-5p, and miR-203a-3p-rich exosomes, which promoted CSC-like phenotype within breast cancer cells [117].

6. Tumor Tissue Culture and Engineering Platforms for EV Research

The 3D culture systems can reflect many aspects of in-vivo tumors, increase stem cell properties, and are thus useful in cancer EV research. Various 3D and 4D culture and co-culture systems are currently used, including Transwell systems, bioreactors, spheroids/tumoroids, Te-EV, and cancer-on-a-chip (**Figure 3**). It is difficult to balance high-throughput and pathophysiological relevance in a single method. Therefore, it is recommended to properly combine any of the methods. Among several methodologies of 3D culture models, gel-free 3D culture models are a good fit for EV research. A big advantage of the gel-free tumoroid model is the simplified collectability of EVs from their culture supernatants [18, 28] compared with gel-based 3D cultures such as the matrigel system [126]. There are several gel-free 3D culture systems, including low-adhesion plates [18, 28, 32], hanging drop [127], microfluidic systems [128, 129], 3D bioprinting [130], and bioreactors (such as rotational bioreactor and pinner bioreactor) [131]. Notably, microfluidic systems have also been used for single EV isolation and analysis [132, 133]. NanoCulture Plates (NCP) are a nanopatterned gel-free scaffold-type 3D cell culture system among the low-adhesion plates. The mogul field structure on the NCPs restricts cells from sprawling on the base and enables tumor cells to migrate from a scaffold to another scaffold more actively than cells cultured on 2D plates. The increased migration and lesser attachment of cancer cells on the NCPs enable tumor cells to form cell aggregates [18, 28]. Besides, ultra-low attachment (ULA) plates have also been useful for collecting EVs, including exosomes [28]. Cells do not rapidly migrate on ULA plates compared to NCP. In contrast to tumor spheroids formed by cancer cell lines, cancer organoids are tissue structures containing tumor microenvironmental constituent cells or an in vivo tumor using a patient-derived tumor [134-136]. So far, a matrix or an air-liquid interface is required to form cancer organoids [137].

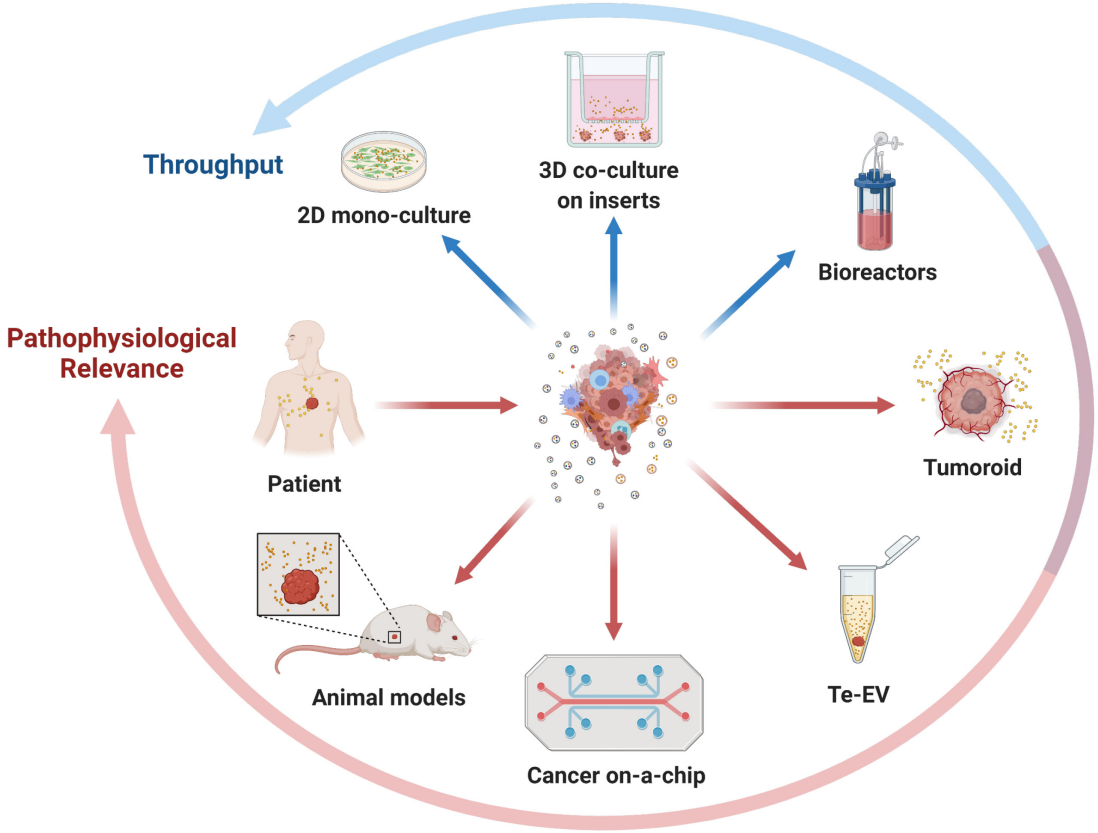


Figure 3. Tumor tissue culture and engineering platforms for EV research. It is difficult to balance high-throughput (far left) and pathophysiological relevance (far right). Therefore, it is recommended to properly combine any of the methods. Co-culture systems, including cancer-on-a-chip, accelerate EV-mediated inter-cellular and inter-organ communication research. Tumoroids produce abundant exosomes, large and small EVs that reflect properties of the tumoroids, such as EpCAM/CD326 [18, 28]. EV uptake and penetration in 3D tumoroids can be monitored in high contents screening system [28, 32]. Modeling and development of tumor microenvironment are attempted within tumoroids and cancer-on-a-chip. Although the origin of EVs in body fluids cannot be identified, Te-EV is a great clue as to what kinds of EVs the tissue (such as carcinoma) produces.

In vitro co-culture systems have been used to mimic the multicellular nature of the in vivo environment. To uncover the biological functions of EVs such as exosomes, two approaches can be used, including either: (i) EVs of the interest are collected then added to the target cells, thereby the effect of the EVs on the second cell population can be investigated [4, 11, 22, 28, 32]. Or (ii) the two cell populations can be co-cultured but are physically separated into two different populations using trans-well inserts and/or microfluidic chips that allow communication only via

secretory factors [66, 138]. The latter approach maintains the continuous communication between the studied cells, with a greater resemblance to the in vivo conditions.

Purified EV is the most reliable for EV function analysis. However, the EV purification step assumes that the original EV structure and function can be lost or modified. Co-culture systems, such as a Trans-well system and Microfluidic chips, are key methods for analyzing inter-cellular communication without EV purification operation. By using a porous membrane of 200-nm, co-culture systems can be constructed, in which sEV such as exosomes pass, but cells and L-EV do not pass. Cancer-on-a-chip (also called tumor-on-a-chip) is a microfluidic chip in which tumors, tumor microenvironment, and multiple organs can be reconstructed as miniaturized.

The transwell co-culture system enables the convenient observation of EV exchanges between multiple cell populations. The cell populations must be separated by a membrane which must have pores with a diameter of 50 nm–500 nm, through which sEV such as exosomes and microvesicles can pass, but large apoptotic bodies (1–5 μm) and cells (5–10 μm) are excluded. However, it should be noted that cytokines, growth factors, etc., smaller than sEV or exosomes, can also pass through the pores together with the sEV. To observe the EV-based molecular transfer, it is thus necessary to delete the molecule in the receiving cells in which the gene expression is not induced. Indeed, we confirmed the transfer of MMP3 proteins from EVs to the nuclei of the receiver tumoroids by allowing MMP3-loaded EVs to act on MMP3-knockout tumor cells [5, 28].

Microfluidic chips have become useful for molecular delivery and functional analysis of sEV or exosomes. It has become possible to produce organ-on-a-chip [139, 140] and tumor-on-a-chip [141] (also called cancer-on-a-chip) using microfluidic chips for modeling EV-based molecular delivery [142], tumor microenvironmental models, and distant metastasis models using a small number of cells.

MMP3 and HSP90 are multifunctional proteins loaded in EVs and play pro-tumorigenic roles in cancers, demonstrated in 3D culture systems. MMP3-EVs promote tumorigenesis through the bloodstream in mice in vivo [5] and tumoroid formation in a 3D culture system in vitro [28]. MMP3 was found in the border area between tumor and stroma [6], indicating that MMP3-EVs may play key roles in EV-driven tumor-stroma interaction in the tumor microenvironment. MMP3 loaded in EVs can penetrate deep inside 3D tumoroids and promote in-vitro tumor growth with increases in Ki-67+ proliferating cells [28]. The pro-tumorigenic role of MMP3-EVs was canceled by the depletion of MMP3 from EVs using the CRISPR/Cas9 genome editing system [5, 28]. Also, HSP90-rich sEV promoted the spheroid formation of oral cancer cells in a 3D culture system [4]. These pro-tumorigenic potentials of HSP90-rich sEV were inhibited by the depletion

of HSP90 from the sEV [4]. Thus, HSP90-sEV or HSP90-exosomes play oncogenic and pro-tumorigenic roles in cancer.

The four-dimensional (4D) culture system includes a time axis with a 3D culture system. EV Uptake, accumulation, and penetration can be monitored using a 4D culture system [28, 32]. The 4D culture system can quantify the degree and speed of uptake and permeation of EV into tumoroids.

7. Tumoroids with Tumor Microenvironment

7.1. ECM, Matrix Vesicles, and Matrix-coated EVs

As a tumor develops, it becomes increasingly important for the cancer cells to sustain their growth and functions by forming the tumor microenvironment; this involves recruiting cellular components and modulating their ECM [123]. Meanwhile, the tumor microenvironment represents the entirety of the tumor components that are not malignant by themselves. The tumor microenvironment consists of the tumor vasculature and various stromal cell types embedded in an altered ECM. Tumor ECM proteins, such as proteoglycans, collagens, and fibronectin, provide essential physical scaffolds to maintain tissue structure and permit migration and various biochemical signals to modulate cellular function [143]. ECM is an important microenvironment or niche for tumors, tumoroid models, and EVs. Cancer cells and the tumor microenvironmental cells such as fibroblasts and endothelial cells produce ECM, structural molecules also functional for cell signaling. Matrix vesicles have been known as EVs embedded in bone and cartilage matrix tissues [46, 144, 145], while the tumor microenvironment is also rich in ECM in which matrix vesicles or other EV types can be embedded. EVs are often kept on the surface of cells and at intercellular space (shown in Figure 1), where they could mediate cell aggregation and organization required for tumor and tumoroid growth [23]. Matrix metalloproteinases and ADAMs can trigger the release of EVs by cleaving ECM surrounding cells [5, 23]. Cancer EVs released by cells are often coated by matrix molecules such as fibronectin, protecting the EVs and mediating EV binding with recipient cells [7, 64, 65].

Nevertheless, metalloproteinase-rich EVs promote tumoroid growth, while MMP3 knockout inhibited tumoroid growth and canceled the pro-tumorigenic activity of cancer EVs [28]. The necrotic area was only 1% in the colon cancer tumoroid but increased up to 15% by MMP3 knockout [28]. The multiple mechanisms of MMP3-driven tumor progression is thought to release growth factors from ECM by metalloproteinase activity and the transmissive and transcriptional activities [5, 146, 147]. Matrigel-free tumoroid models enable us to efficiently collect the released EVs from the culture media. In contrast, matrigel-embedded spheroid, tumoroid and organoid models can store the produced EVs in the matrigel to visualize the released EVs. In the gel-free

tumoroid models, large and small EVs or exosomes are collectible from culture media [28]. Both 3D tumoroid-derived EVs and 2D-cultured cell-derived EVs are pro-tumorigenic for the tumoroid model in vitro and tumor transplantation model in vivo [4, 5, 28], suggesting EVs promote the growth of primary and secondary tumors. There are two types of tumoroids: the sphere-type and the grape-type [18]. Squamous cell carcinoma (SCC) cells often form compact spheroids with strong intercellular adhesion[4, 18]. On the other hand, adenocarcinoma cells form large grape-type tumoroids with ducts, intercellular space between the cells, and increased stem cell properties [18], suggesting that ECM and EVs are deployed in the intercellular space of the adenocarcinoma tumoroids for their growth and active release of EVs.

7.2. Vascularized Tumoroid Models, Hypoxia, and Necrosis

Cancer cells themselves are able to form colonies, spheroids, and tumoroids with increased stem cell properties in vitro [18], while recent studies have evaluated crucial roles of tumor stroma or tumor microenvironment, including vasculature [148], cancer-associated fibroblasts (CAF) [149], immuno-suppressive cells such as myeloid-derived suppressor cells (MDSC) [150] and M2-type macrophages [4]. For modeling in vitro tumors and their microenvironment, co-cultured tumoroid models and tumor-on-a-chip have been recently established. For example, a recent study established a vascularized tumoroid model for human glioblastoma (GBM) angiogenesis [151]. This model comprises 1000-cell spheroids, consisting of HUVEC and GBM cells at a ratio of 3:1, in a 7.5 mg/mL fibrin gel containing 10^6 human dermal fibroblasts (HDF) cells/mL. This tri-culture method triggered robust HUVEC angiogenesis creating lumenized capillaries that sprout radially outwards into the surrounding fibrin gel. NCH82 GBM cells invade into the gel without interacting strongly with the endothelial cells. On the other hand, primary GBM cells integrate more into the vasculature, behaving similarly to the HUVEC [151]. Another study established an engineering vascularized 3D hybrid system to model tumor-stroma interactions in breast cancer [152]. For this model, alginate porous 3D scaffolds are prepared by freeze-drying and particle leaching. In step 1, outgrowth endothelial cells and fibroblasts are co-seeded on the scaffold, and tubular endothelial structures are formed. In step 2, epithelial cells suspended in alginate gel-precursor solution are added to the pre-vascularized scaffold for in situ hydrogel formation. Epithelial organoids are formed within pores in close contact with tubular-like structures, fibroblasts, and their ECM, establishing heterotypic cell-cell and cell-matrix interactions [152].

7.3. Acidity

The existence of an acidic environment as part of the tumor microenvironment has been known since the 1950s, but its involvement in tumor malignant transformation has not been elucidated in detail because it was regarded as a "consequence" of hyperglycemia in a hypoxic environment. However, recent studies have proposed that acidic environments may contribute to malignant transformation through acidic environment-specific cellular responses and metabolic changes in cancer. In tumors, the extracellular pH, which is normally maintained at pH 7.4 in tissues, has been reported to fall to pH 6.8 [153]. This acidic environment is known to be caused by hypoxia and hyperglycemia in proliferating cancer cells. The increased expression of glucose transporter, type 1 (GLUT1), a target of HIF1 in hypoxia, promotes glucose uptake into the cell, and a series of glycolytic enzymes, also target genes of HIF1, metabolize glucose to generate ATP. In the process, lactate and protons (H+) are produced. Both are positively charged, and the intracellular pH tends to decrease. To maintain a constant intracellular pH, Na+/H+ exchanger isoform 1 (NHE1) and membrane proteins such as ATPase and monocarboxylate transporter 1 and 4 (MCT1 and 4) remove lactate and protons from the cell, resulting in a decrease in extracellular pH [154, 155].

The acidic environment has been reported to contribute to the progression of cancer, especially invasion and metastasis [156]. Specifically, it has been reported that the acidic environment induces the expression of angiogenesis-related factors such as VEGF and interleukin (IL)-8 (also called CXCL8), which contribute to tumor angiogenesis, and that the acidic environment induces changes in cell polarity through cytoskeletal remodeling and the degradation of ECM by MMP2 and MMP9. In vivo, cancer cells exposed to an acidic environment in the xenograft model showed increased metastatic potential, suggesting that an acidic environment contributes to the malignant transformation of cancer.

Some reports show that microenvironmental acidity increases the exosome release and cisplatin resistance in tumor cell lines [157-159]. CD81+ PSA+ exosomes were increasingly released by acidity in LnCAP prostate cancer cells and detected at higher levels in plasma samples of prostate cancer patients than healthy donors or benign prostatic hyperplasia (BPH) [159]. The acidic microenvironment induced upregulation of both expression and activity of carbonic anhydrase (CA) IX in cancer cells and their exosomes, together with increasing the number of released exosomes [160]. Tumor microenvironmental acidity involves remodeling the ECM, allowing local invasion, leading to increased metastasis to other organs and bone, where the tumor-generated acidity can be a potent effector of bone pain [161]. Acidic tumors are resistant to radiation and chemotherapy and, in some systems, can induce angiogenesis and lymphangiogenesis. Antitumor effectors such as T and NK cells tend to lose their function and

undergo a state of mostly reversible anergy followed by apoptosis when exposed to a low pH environment [162]. Moreover, the acidity of the interstitial space and the relatively well-maintained intracellular pH influence cancer and stromal cell function, their mutual interplay, and their interactions with the ECM [163].

8. Fluorescence Labeling EVs and EV Exchange Assays

To visualize EV-mediated cell-to-cell communication, fluorescence labeling of EVs is an effective strategy (**Figure 4**). 1) The collected EVs can be labeled with small fluorescence molecules such as tetramethylrhodamine (TRITC) [4, 11, 32] or Cy7 [5, 49] bound to ceramide, a component of sphingomyelin, one of the major lipids in the lipid bilayer. 2) Alternatively, researchers can establish stable cells that produce fluorescent EVs by the cDNA transfection-based expression of palmitoylation (palm) signal-fused fluorescent proteins such as GFP or tdTomato, called palmGFP and palmTomato [28, 66, 164]. 3) EVs or exosomes can also be labeled with fluorescent proteins fused with EV transmembrane proteins such as CD63 [165, 166]. These EV labeling techniques were useful for analyzing EVs' biodistribution in EV transfer assay in vitro and monitoring EVs and exosomes in vivo.

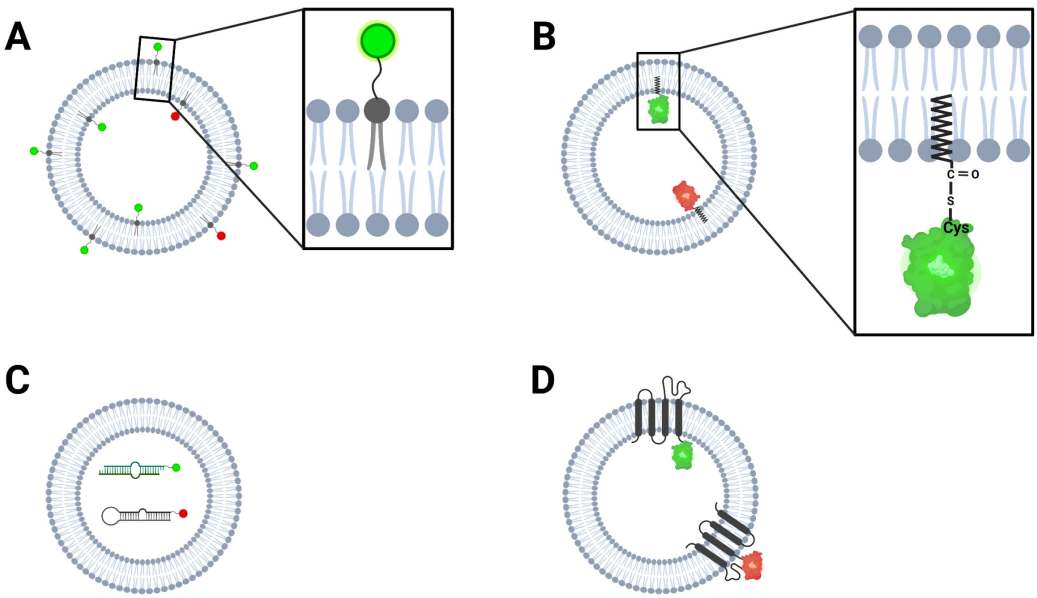


Figure 4. Fluorescence labeling of EVs or exosomes. (A) Fluorescent molecules, such as Cy7 and TRITC, are linked with ceramide, a component of sphingomyelin forming lipid bilayer. (B) PalmGFP and palmTomato are composed of a palmitoylation signal fused with fluorescent proteins. Protein palmitoylation is the covalent attachment of fatty acids, such as palmitic acid, to cysteine, i.e., S-

palmitoylation. Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. Palmitic acid is a major component of the oil from the fruit of oil palms. (C) Labeling miRNA with fluorescent molecules. (D) Tetraspanins fused with GFP or RFP.

Paul Larl Horal (PKH) dyes, such as PKH26 and PKH67, have been used for labeling cellular and vesicular membranes. The PKH dye has a carbon chain that inserts and fixes the fluorescent dye molecule in the lipid region of the cell membrane. The emission spectrum of currently available PKH dyes (such as PKH2, PKH26, and PKH67) are in the limited range of 504 nm to 567 nm. PKH dyes require a dye quenching step by adding serum or BSA protein after dye intercalation in the protocol. If the unbound dye is not quenched, the dye may aggregate, affecting co-culture and causing cells to be aggregate. Relevantly, recent data suggest that labeling with PKH26 causes exosome swelling, which can alter exosome biodistribution and cell uptake, which can confuse the results of studies [167, 168]. ExoSparkler series are useful for staining purified exosomal membrane or protein, which allows imaging of labeled exosomes taken up by cells [169]. The dyes used in ExoSparkler series (Mem Dye-Green, Red, and Deep Red) do not cause aggregation and have little influence on the properties of EVs, allowing a more accurate observation of EV dynamics [169].

Luciferase reporter has also been used for labeling EVs. For example, membrane-bound gaussia luciferase (mbGLuc) consists of a PDGFR transmembrane domain fused with gaussia luciferase [164, 170, 171]. The mbGLuc residues extending from the EVs are cleavable by metalloproteinases and thus as a tool to track the shedding of membrane proteins from the surface of EVs [170]. Gaussia luciferase (gLuc) was also fused with lactadherin, a protein that binds to EV membranes through interaction with phosphatidylserine (PS) [172]. Besides, tetraspanin was fused with nanoLuciferase (nanoLuc) reporters, designated CD9-nanoLuc, CD63-nanoLuc, and CD81-nanoLuc, which are sensitive and rapid quantification of exosomes, or sEV [173].

Several EV inhibitors have been shown to have well-established roles in inhibiting exosome biogenesis or EV release [174]. GW4869 is the best-established exosome biogenesis inhibitor. GW4869 is known as non-competitive neutral sphingomyelinase (N-SMase) inhibitor or phospholipase inhibitor and inhibits biogenesis of multi-vesicular bodies (MVB) of the ESCRT-independent pathway. On the other hand, manumycin A inhibits the ESCRT-dependent pathway by inhibiting RAS [174]. For the inhibition of micro-vesicles (MVs), calpeptin is the most established calpain inhibitor. Calcium-bound calpain activity is essential for MV release, MMP2 release, and integrin-FAK activities. ROCK inhibitor Y27632 inhibits MV release and apoptotic blebbing [174]. Rho-associated protein kinases (ROCK) are serine-threonine kinases involved in cytoskeletal re-organization. Y27632 inhibits ROCK-MLC interaction, essential for MV release

and apoptotic blebbing. In addition, imipramine, a well-known anti-depressant, can promote membrane fluidity by acting on acid sphingomyelinases (aSMase), thus preventing MV generation [174].

9. Cancer EVs Affect the Tumor Microenvironment

Cancer sEV are able to transform normal epithelial cells, which is known as EMT, an initial step of oncogenesis [4, 11]. Cancer sEV can also promote sphere formation of MSC [122]. Multiple different components comprise the tumor microenvironment, including cancer-associated fibroblasts (CAFs), tumor-associated endothelial cells (TECs) forming the tumor neovasculature, adipocytes Immune cells, such as tumor-associated macrophages (TAM), tumor-infiltrating lymphocytes (TILs), including T cells and B cells, and myeloid-derived suppressor cells (MDSCs) are also present. Several studies have demonstrated that CSCs and cells in the tumor microenvironment establish an EV-mediated communication network that fuels the malignant potential and immune privilege of CSCs within the cancerous milieu. It was shown, for instance, that the secretion of miRNA-21-enriched exosomes from glioblastoma stem-like cells could activate the miR-21/VEGF/VEGFR2 signaling axis, promoting permeability and angiogenesis in the glioma microenvironment [175]. It was also shown that exosomes derived from colorectal CSCs promote neutrophil recruitment and survival for tumor infiltration by enhancing the IL-1β / NF-kappaB signaling axis [176]. Similarly, lung CSCs derived exosome-miR-210-3p promote EMT, migration, and invasion of lung cancer cells by downregulating the expression of non-CSCs fibroblast growth factor receptor-like 1 (FGFR-L1) [177].

Several studies showed that CSC-derived sEV or exosomes are immuno-suppressive in cancer. CSCs-derived exosomes actively participate in their dialog with CD8+ T cells, MDSCs, and Tregs leading to immune suppression [178]. In a study on the effects of CSCs-derived exosomes on the immune response of the recipient cells, it was shown that exosomes from glioblastoma stem cells (GSCs) inhibit T-cell activation, proliferation, and Th1-cytokine production [179]. GSCs-derived exosomes can induce the transformation of pro-inflammatory M1 phenotype into immune suppressive M2 phenotype through activation of STAT3 signaling [180]. Furthermore, brain tumor-initiating cells (TICs)-derived exosomes are endowed with their ability to impair T cell functionality by secreting the ECM protein tenascin-C [181].

It has also been reported that CSC-sEV promotes aggressiveness and distant metastasis in the recipient cells by enhancing EMT [124, 182]. These studies indicated that constructing a pre-metastatic niche is one of the underlining mechanisms for shaping an environment favorable for CICs/CSCs. This concept was supported by a study showing that the interactions between

endothelial cells and CSCs promote the formation of secondary tumor lesions in the lung, enhancing the formation of a pre-metastatic tumor-initiating niche via an exosome-borne tumor RNA signature [183]. In addition, it was demonstrated that exosomes derived from Lewis Lung Carcinoma could reprogram mouse-iPSC into CSCs *in vitro*, endowing them with sphere formation abilities [184]. Moreover, those converted CSCs were granted tumorigenic and metastatic capacities in a nude mice model.

Finally, the CSCs-derived EVs can promote bidirectional crosstalk between the CSCs and their niche, thus promoting the construction of microenvironment supporting tumorigenesis (**Figure 5**).

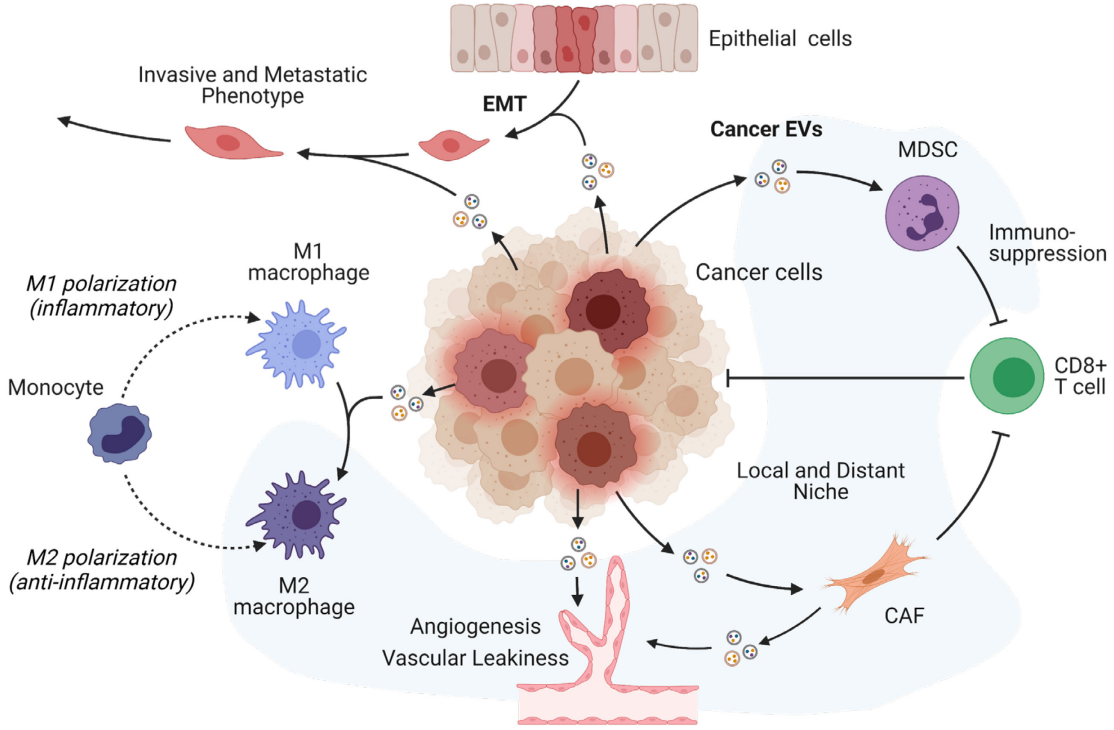


Figure 5. Cancer EVs affect the tumor microenvironment. Monocytes differentiate into inflammatory (anti-tumor) M1 macrophages and anti-inflammatory (tumor-promoting) M2 macrophages (left). Cancer cell-derived sEV or exosomes induce macrophage polarization to M2-type [4, 185]. Cancer sEV has an immunosuppressive effect (right). Inhibition of CD8+ T cells by cancer exosomes is mediated by MDSC [186]. Cancer-derived exosomes trigger endothelial-to-mesenchymal transition (End-MT) followed by the induction of CAFs [129]. Cancer-derived exosomes influence endothelial cells (ECs), which then play multiple roles, such as tumor angiogenesis, endothelial vascular barrier loss by binding to ECs, and the subsequent End-MT, i.e., extracellular matrix remodeling [187].

10. Cancer Exosome-Driven Pre-Metastatic Niche Formation

The process of metastasis relies on the ability of primary tumor cells to invade the surrounding tissues and infiltrate into the bloodstream or lymph vessels, a process known as intravasation. These cells thus turn into circulating tumor cells (CTCs) until they exit from the circulation by extravasation and lodge themselves in new tissue to form a premetastatic niche for subsequent secondary tumor mass generation (known as metastasis formation). sEV from the primary tumor are documented to transfer different molecular effectors to the organs of metastasis. Hence, they trigger signaling cascades which ultimately promote metastasis development.

The metastatic potential of cancer cells depends on their ability to colonize a new environment different from the tissue in which they originated. Hence, before tumor cells arrive at distant organs, primary tumors influence the pre-establishment of a specific permissive microenvironment, so-called premetastatic niche (PMN), to optimize the conditions for colonization of metastatic sites by cancer cells [9, 188]. The stepwise process of PMN generation is initiated with local changes, resulting from the ability of tumor cells to conduct systemic conditioning of distant organs [189], including; (1) remodeling and degrading of stroma and ECM, (2) induction of vascular leakiness by bone marrow-derived cells (BMDCs) and macrophages, followed by (3) neovascularization, angiogenesis, and secondary tumor growth. Interestingly, many of the modifications influenced by secreted sEV could also promote the creation of PMN [190].

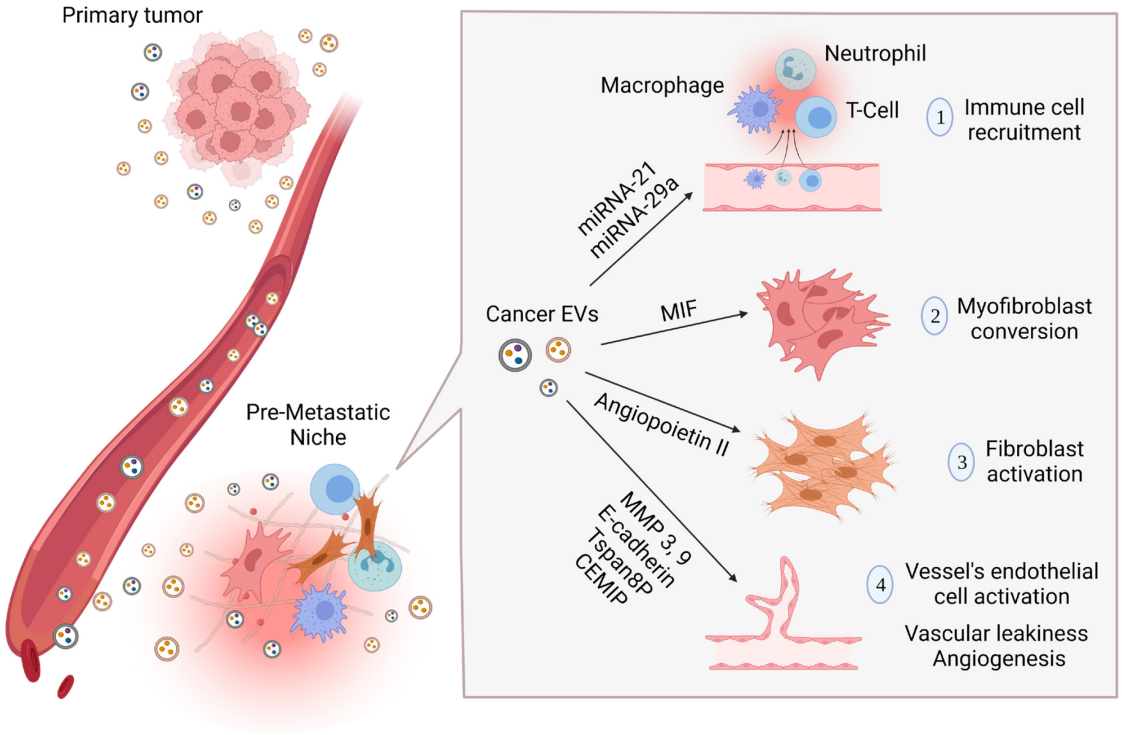
sEV or exosome communication appears to be one of the most important relevant mechanisms promoting vascular leakiness, angiogenesis, and vascular remodeling [187]. Indeed, it was shown that exosomes play key roles in brain vascular remodeling and metastasis [191]. In this study, breast cancer secret sEV expressing the cell migration-inducing and hyaluronan-binding protein (CEMIP) are transferred to brain endothelial and microglial cells. Upon uptake, CEMIP induces upregulation of the pro-inflammatory cytokines Ptgs2, Tnf, and Ccl/Cxcl, which impacted promoting neovascularization in PMN that provide oxygen and nutrients to proliferating cancer cells. Furthermore, tumor-derived sEV carries proteins like Tspan8 [192], soluble E-cadherin [193], or Angiopoietin II that are directly involved in the activation of the endothelium or indirectly through the induction of pro-inflammatory immune cells [194] or pro-vasculogenic phenotypes in the stroma, such as fibroblasts [195].

MMPs may contribute to PMN formation and extravasation of CTCs. MMP3 and MMP9 are also found in the metastatic lung sites in the LuM1 tumor model [5, 6, 100]. It was also shown that angiopoietin 2, MMP3, and MMP10 are up-regulated in the lung in the premetastatic stage by primary B16/F10 tumor, which leads to the increased permeability of pulmonary vasculatures and extravasation of CTCs [196].

It has also been shown that sEV promotes lymph node metastasis [197]. Melanoma sEV was shown to be selective for homing to sentinel lymph nodes [197], whereas empty liposomes that mimic empty EVs as a control showed nonselective homing. Besides, melanoma exosomes were shown to recruit melanoma cells to sentinel lymph nodes by altering the ECM, promoting melanoma cell trapping within sentinel nodes. Later, it was found that melanoma-derived exosomes can induce pro-inflammatory gene expression in the lungs to favor the generation of PMNs [194].

Activation of macrophages by toll-like receptors (TLR)-7 and -8 could be achieved by binding of EV miRNAs (miR-21, miR-29a), which support matrix remodeling for hosting metastatic cells [198]. Moreover, the generation of PMNs in the liver is promoted by Kupffer cells taking up pancreatic cancer-derived exosomes carrying migration-inhibitory factors (MIF), leading to increased fibronectin production [7]. Fibronectin deposits in the liver promote the arrest and sequestration of bone marrow-derived cells (BMDCs), creating a pre-metastatic niche.

In this way, tumor-derived sEV appeared to act as metastasomes, helping transmit oncogenic signals from primary tumor cells triggering preparation of PMN, a process that induces clonal tumor initiation within the distant target organ (**Figure 6**). Microfluidic applications on



circulating tumor cell isolation and biomimicking of cancer metastasis were recently reported [199].

Figure 6. Roles of cancer EVs in preparation of premetastatic niche. Tumor-derived sEV or exosomes directly transfer different molecules and miRNAs from the primary tumor site into the environment at the site of metastasis, by which the tumor EVs can induce; (1) recruitment of immune cells, such as macrophage, neutrophils, and T cells, which eventually contribute to the generation of pro-inflammatory and immunosuppressive niche [194], (2 and 3) conversion and activation of fibroblasts [195], and (4) activation of vessel's endothelial cells, in response to EV bioactive factors such as MMPs, E-cadherin, Tspan8P, and CEMP leading to vascular leakiness and angiogenesis [192, 193]. Thus, tumor EVs promote the generation of optimal premetastatic niches, a key process in metastasis development.

11. Circulating Tumor Cell Clusters and Metastasis

Cancer cells can disseminate in the bloodstream as anchorage-independent circulating tumor cells (CTCs) [200, 201]. These CTCs can remain loose in circulation and cluster together (from 2 up to >100 cells) as they travel or lodge themselves in new tissues for subsequent metastasis formation [202]. The presence of CTC clusters is advantageous to the malignant cells since clustered cells are protected from various stress conditions, such as the risk of anoikis in the absence of anchorage, shear forces, and immunologic surveillance. Cancer-EVs can indirectly facilitate CTC clustering via the regulation of adhesion proteins and the conversion of fibroblasts into CAFs, which also play a major role in cluster formation [203-206]. Furthermore, in hepatocellular carcinoma (HCC), tumor-derived exosomes were shown to transfer SMAD family member 3 (SMAD3) protein and mRNA to detached HCC cells, promoting their homotypic adhesion, which is associated with an earlier onset of metastasis [207].

Upon reaching the distant organ, CTCs must overcome many barriers to re-colonize their tumor of origin, i.e., primary tumor, in a process termed tumor self-seeding [208]. Since primary tumor-derived sEV stimulates CTCs' survival, migration, and conditioning PMN, it is reasonable to hypothesize that they may also be involved in the seeding process of CTCs. For example, EVs derived from a breast cancer lung metastatic cell line (MDA231-LM2) were found to boost the growth of the primary tumor (MDA-MB-231 xenograft) in an in vivo self-seeding nude mouse model [209]. Notably, MDA231-LM2-exosomes promoted the self-seeding capacity of MDA231-LM2 cells in MDA-MB-231 xenografts. Liu and coworkers found that miR-25-5p-rich exosomes derived from HCC downregulate the expression of leucine-rich repeat-containing 7 (LRRC7) in CTCs, which correlates with increased self-seeding capacity [210]. Besides, the epigenetic silencing of PTEN in CTCs, through transferring PTEN targeting miRNAs from brain astrocyte exosomes, leads to increased CCL2 chemokine secretion and, consequently, myeloid cell recruitment which promotes CTCs expansion through enhanced proliferation and reduced

apoptosis [211]. Overall, the presence of tumor-derived sEV can enhance the metastatic ability of CTCs for long-distance dissemination and metastasis formation (**Figure 7)**. CTCs can be isolated and analyzed in single-cell analysis by on-chip microfluidic technologies, including clinical applications [35, 212].

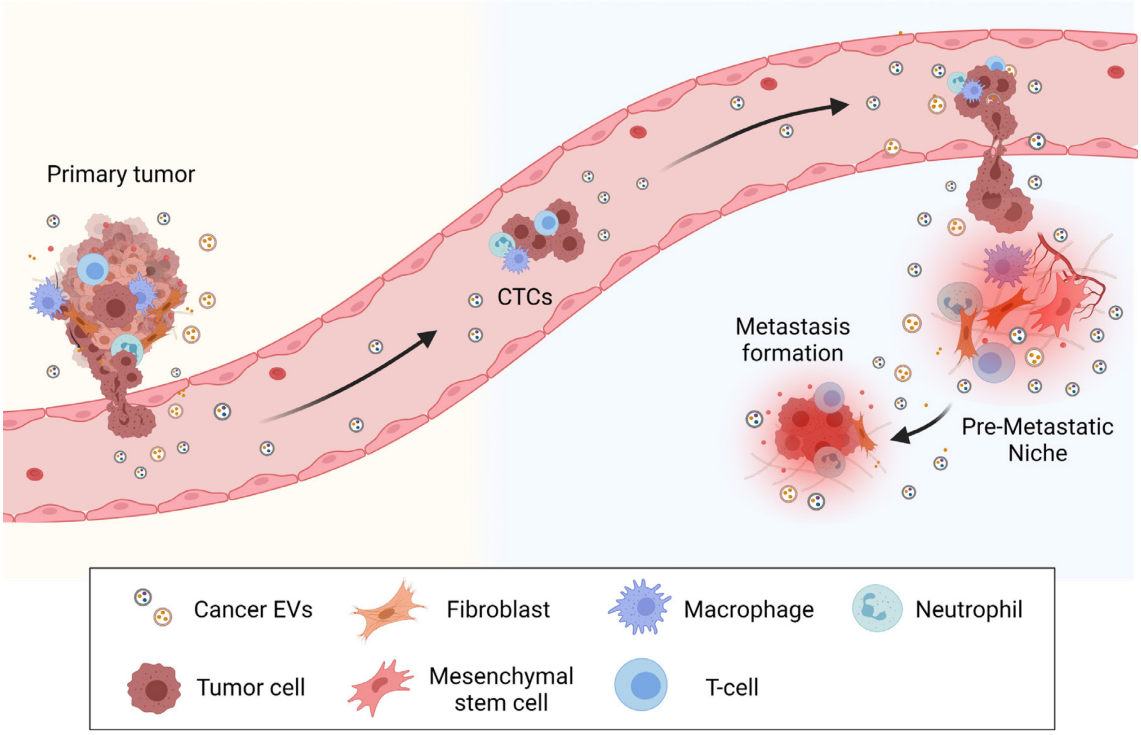


Figure 7. Cancer-derived EVs or exosomes contribute to the metastatic potential of CTCs. EVs or exosomes derived from tumor supports distant tumor spread by favoring tumor cells intravasation (1), CTC cluster and survival in circulation (2) [203-206], and CTC extravasation (3) at the site of distant tissue [207]. Moreover, cancer EVs contribute to the formation of the pre-metastatic niche (4), in which CTCs can be disseminated and proliferate for promoting metastasis formation (5) [190].

12. Conclusions

In recent years, knowledge of the diversity and heterogeneity of EVs has expanded, and 3D culture technology has also diversified. 3D-cultured tumoroids with increased CSC potential significantly release small and large EVs, inheriting CSC properties such as high expression of CD326/EpCAM [4, 18] and promoting tumor growth [28]. MMP3 loaded in metastatic cancer-derived EVs was pro-tumorigenic and transferrable deep inside 3D tumoroids and cellular nuclei, leading to the transcriptional activity [5, 28]. EV-associated HSP90 is a key molecule involved in the intercellular molecular transfer, cell survival, and differentiation in the tumor

microenvironment [4]. By targeting multifunctional proteins such as MMP3 and HSP90, EVs' ability to promote tumoroid formation was suppressed [4, 5, 28]. In addition, EV fluorescent labeling technology [4, 28] and luciferase labeling technology are indispensable for highly sensitive visualization of EVs to analyze EV transfer and exchange in vitro [4, 28, 66] and EV dissemination and distribution in vivo [5, 49, 164]. 3D culture and EV labeling techniques will be important foundations for elucidating and resolving the tumor microenvironment and pre-metastasis niche affected by EVs.

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