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Extracellular vesicles as carriers of microRNA, proteins and lipids in tumor microenvironment

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Abstract

In recent years, the knowledge about the control of tumor microenvironment has increased and emerged as an important player in tumorigenesis. The role of normal stromal cells in the tumor initiation and progression has brought our vision in to the forefront of cell-to-cell communication. In this review, we focus on the mechanism of communication between stromal and tumor cells, which is based on the exchange of extracellular vesicles (EVs). We describe several, evergrowing, pieces of evidence that EVs transfer messages through their miRNA, lipid, protein and nucleic acid contents. A better understanding of this sophisticated method of communication between normal cancer cells may lead to developing novel approaches for personalized diagnostics and therapeutics.

Keywords

extracellular vesicles; exosomes; mesenchymal stem cells; microRNA; MSCs; Lipids; proteins; RNA; tumor microenvironment

One form of intercellular communication is through the exchange of secreted cell membrane fragments known as extracellular vesicles (EVs) into the extracellular space.[1] The interest in understanding the role of EVs in cancer started in the late 1970s, when studies showed the secretion of EVs in both normal and cancer cells[2–5]. A correlation between elevated blood EVs levels in cancer patients[5–7] and other studies implicated EVs as potential diagnosis markers for cancer.[7–9] This has shifted recent research to focus on whether EVs play a supportive role in cancer pathology, including effects associated with cancer initiation, progression, angiogenesis and metastasis.

An important factor in the support of the tumor microenvironment is the cell–cell communication between stromal cells and transformed cancer cells. The role of gap junctions in transport of cellular communicators and juxtacrine regulation based on direct communication is well documented.[1] Recently, Tsuyada *et al.* demonstrated the cancer-stroma signaling circuit, in which breast cancer cells stimulate the expression of chemokine

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CCL2 in normal fibroblasts that become cancer activated fibroblasts. In turn, this stimulates the stemness of breast cancer cells constituting the cancer-stroma-cancer signaling circuit.[9]

Recent studies have demonstrated that EVs exchange cargo of small RNA, mRNA, proteins, lipids and other regulatory molecules, between breast cancer cells and stromal cells.[9] They are recognized as being involved in regulating a variety of extracellular signals and paracrine signaling,[9] including breast cancer invasiveness.[9] A dynamic interaction between stromal cells, cancer cells and the tumor microenvironment facilitates tumor progression (Figs 1 and 2)

Tumor Microenvironment

Stromal cells in the tumor microenvironment play a very important role in tumor progression. This microenvironment includes untransformed cells surrounding the tumor, which is composed of extracellular matrix (ECM) and numerous stromal cell types, including endothelial and inflammatory immune cells, fibroblasts, adipocytes and tumor-associated vasculature. Tumor malignancy is highly dependent on interactions between tumor cells and the tumor microenvironment. Studies on comprehensive gene expression and genomic profile study of epithelial, myoepithelial and stromal cells have revealed diverse microenvironments between normal breast tissue and breast carcinomas. Stromal elements secrete chemokines that act as paracrine factors that could induce ECM remodeling, enhance tumor cell proliferation and invasion.[9] As an example of paracrine signaling in the tumor microenvironment, overexpression of the chemokines CXCL14 and CXCL12 in myoepithelial cells and myofibroblasts can enhance the proliferation, migration and invasion of breast cancer epithelial cells. Tumor stroma is also associated with therapeutic resistance and relapse—a main reason for breast cancer treatment failure.[9]

Mesenchymal Stem/Stromal Cells in Cancer

Mesenchymal stem/stromal cells (MSCs) are multipotent cells of nonhematopoietic origin and constitute a minor population (0.01%) of nucleated cells in bone marrow.[–] MSCs are subsets of stromal cells and are known for their active mobilization from bone marrow and migration to sites of injury.[–] Various reports suggested that bone marrowderived MSCs are preferentially recruited to the tumor surrounding stroma when compared to normal stroma, mainly by the inflammatory factors in the tumor microenvironment. These reports increased interest in understanding the potential role of MSCs in tumor progression. The MSCs recruited to the tumor microenvironment by various cytokines[–] act as precursors for pericytes and cancer associated fibroblasts.[–] MSCs promote tumor cell proliferation through their immunosuppressive properties and direct cell supportive properties.[9] Earlier studies suggest that under nutrient-deprived conditions the MSCs associated with tumor stroma undergo autophagy, thereby facilitating tumor support through an anti-apoptotic secretome made of cytokines, growth factors and secreted vesicles such as EVs.

Extracellular Vesicles—Definition

The word “extracellular vesicle” is actually a generic term that refers to a series of membrane-bound organelles, which are commonly distinguished by their size range. More specific nomenclature for EVs include exosomes (40–100 nm diameter), ectosomes (50–1,000 nm), argosomes and apoptotic bodies (50–5000 nm). There are problems in establishing a standard terminology in this field of research that have led to uncommon words such as “microparticles,” and even organ-specific classifications such as “prostasomes” used in literature. Any discrepancies between the characteristics of specific types of EVs is largely subject to debate, mainly due to the way these organelles are isolated (*e.g.*, ultracentrifugation, use of a sucrose gradient, by biological markers), the precise context of study, or vesicle-specific properties. For the purposes of this review, EVs will be used for all organelles in this general category between 40 and 1,000 nm in diameter unless explicitly noted.

EVs are evolutionarily conserved, which suggests that they carry out important biological functions. Cells are known to secrete EVs due to factors such as environmental stress, cellular activation or apoptosis.[7] The composition of EVs varies between cell types and environmental conditions, and a formal classification based on vesicles components is still being actively debated.[7] As an example, exosomes can be characterized by membrane markers (CD63, CD81, CD9, TSG101 and Alix) though these markers are not exclusive to this type of EVs.[7] Review article by van der Pol *et al.* elegantly describes other commonly used techniques to characterize exosomes, which include size distribution assays using optical microscopy and nonoptical microscopy based assays.[7]

Extracellular Vesicles—Genesis

At least three mechanisms for EVs generation have been proposed: *(i)* decay of dying cells into apoptotic bodies, *(ii)* cellular cell membrane blebbing ectosomes and *(iii)* inward budding of endosomal limiting membrane followed by emission of cell membrane into EVs.[7–9] The result is outward budding and fission of vesicles from the tumor cell surface (Fig. 3). Some observations have also described a direct formation and release of EVs from cytoplasmic membrane budding of immune cells.[7] The genesis of EVs that fall in the size of exosomes have been shown to occur both through inward budding of the endosomal limiting membrane to form multivesicular bodies (MVBs), which can fuse with the cell membrane and/or by budding off plasma membrane. The early stages of EVs synthesis starts with inward budding of the endosome membrane; such enriched endosomes are referred to as MVBs. A fraction of such MVBs can fuse with the plasma membrane releasing EVs into the extracellular milieu as exosomes or alternatively the exosomes can be directly secreted into extracellular fluid.

Extracellular Vesicles—Isolation

Several methods of isolation have been described ranging from ultracentrifugation, density gradient centrifugation, immunoaffinity capture using magnetic beads and commercially available precipitation methods. Comparative studies using these techniques demonstrated

that the purity and the quality of preparations is dependent on the source of exosomes.[–] Reports of EVs isolation, size, density and morphology should be interpreted with caution. Due to their small size and heterogeneity, conventional methods of classification for this type of biomolecule have proven to be difficult.[,] EVs are hard to detect with basic light microscopy and flow cytometry because they are generally less than 200 nm in size. Several methods have been in use for isolation and purification of EVs, ranging from centrifugation techniques to antibody precipitation.[,] Most commonly used is a differential ultracentrifugation including a sucrose density gradient.[,,] A recent study demonstrated that the *g* force and time of centrifugation significantly affect the quality of preparation. In addition, techniques such as these have been shown to change the size and morphology of EVs. For instance, while exosomes are frequently described as cupshaped in literature,[,–] Thery *et al.* demonstrated that this morphology was actually an artifact caused by the fixation process for transmission electron microscopy. In another study by Connor *et al.*, repeated freeze-thaw cycles of plasma rich in platelets caused a considerable increase of annexin-V in EVs. The EVs count in a sample can vary with storage time, temperature, buffer composition and agitation. Moreover, the presence of fetal bovine serum (FBS) in culture media has many limitations with issues of contamination from EVs of foreign species. Such variability warrants a systematic and detailed methods section be supplemented with every publication.

Extracellular Vesicles—Cargo

Recent evidence shows that EVs can act as a unique vehicle for the release of soluble and insoluble molecules, including lipids, proteins and nucleic acids.[,] EVs uptake by target cells may allow the exchange of these molecules from EVproducing cells. Such a mechanism would affect the target cell phenotype. EVs are enriched in lipids like ceramides, cholesterol and sphingomyelin, which promote vesicle release and play important roles in cell communication.[–] More than 300 different proteins have been detected within EVs. The proteins reportedly belong, but are not limited, to families of surface receptors, signaling molecules and cell adhesion molecules.[,] Nucleic acids such as DNA, mRNA and noncoding RNA (long noncoding RNA, tRNA and microRNA) have been reported in EVs.[–] The mechanisms for EVs loading is unclear, however, a few types of proteins have been shown to be associated with MHC-II proteins that may play a role in protein sorting. For example, chaperones such as Hsc70, Hsp90, 14-3-3 epsilon and PKM2.[,] Most proteins detected in exosomes are a class of proteins that lack secretory signal peptides, which are secreted through the ER-golgi pathway.[,]

MicroRNAs (miRNA) are noncoding small RNA (19–22 nucleotides in length) that play a wide spectrum of roles on both pre and post-transcriptional gene expression. miRNA are thought to regulate at least a third of the human genome by targeting mRNAs for degradation by the RISC complex, principally by targeting the 3'UTR coding mRNA[,]. Circulating miRNAs have been detected in various body fluids including serum, plasma, amniotic fluid, saliva, sweat, urine and milk. Data compilation shows that circulating miRNA are found both inside EVs secreted by all kind of cells or circulating miRNAs found bound to proteins. One example is miRNAs directly bind to Argonaute 2 (Ago2) proteins and form a very stable complex. Because of the high stability of the miRNA-Ago2 complex,

it is challenging to trace the original source of these circulating miRNAs since these protein bound miRNA can be co-isolated in exosomal preparation making it difficult to interpret the data from plasma or serum derived exosomes.

Single miRNAs have been identified to regulate the balance between normal and cancer cells. For example, the transfer of secreted miR-143 from normal prostate cells induces the growth inhibition of prostate cancer cells where miR-143 is downregulated. In another study, the EVs secreted by different cancer cell lines contain specific miRNAs (*e.g.*, miR-9) that promote endothelial cell migration. A more recent study showed miR-23 inhibited metastasis and increased dormancy. The presence of miRNA into cancer cell-derived EVs seems to be driven selectively. For example, it has been shown that breast cancer cell lines secrete a variety of EVs containing more abundant and more diverse miRNA species compared to those secreted by normal epithelial cells.[,] A recent study on quantitative analysis of miRNA content in exosomes suggest that most standard preparations of exosomes carry less than one miRNA molecule per exosome of any given miRNA. Therefore, standard preparations may not carry biologically significant numbers of miRNAs. Furthermore, Kalluri lab demonstrated that the exosomes from cancer cells are capable of synthesizing miRNA independent of originating cell.

Horizontal transfer of mRNA and miRNA has been reported in numerous studies between normal cells,[–] between embryonic stem cells,[] from MSCs to cancer cells, between cancer cells,[] from cancer to normal cells and from normal to cancer cells.[] This phenomenon indicates that transferred RNA may play a role in the regulation of gene expression in recipient cells. The ratio of RNA fragments found within EVs varies depending on the cell type from which the EVs originated.

Evidence that the loading of miRNAs into EVs may not in fact be random, but instead controlled by specific proteins involved in the miRNA network, was demonstrated by Gibbings *et al.* in 2009, by demonstrating the presence of Ago2 protein and a noticeable enrichment of GW182 in purified EVs. A recent study also demonstrates that sumoylation of the ribonucleoprotein hnRNPA2B1 controls the sorting of miRNAs to exosomes.

Extracellular Vesicles—Transfer

EVs secretion by most of the normal cell types is a regular physiological phenomenon and a mode of intercellular communication for cell growth and activation. The evidence that EVs were involved in cancer was first documented in patients with Hodgkin disease in the late 1970s. Since then, various studies have revealed the active involvement of EVs in different stages of cancer progression. In human breast cancer cell lines, there is a positive correlation between the amount of EVs released and the *in vitro* invasiveness of the cells. Similar results were observed in *in vivo* studies on ovarian cancer fluids. EVs secretion can provide either favorable or unfavorable features to cells depending on the contents of the EVs. Cancer cells can use EVs to evade protective mechanisms of the organism by inducing immune tolerance, expression of pro-apoptotic signals, extracellular matrix remodeling, drug resistance and in other various ways. EVs derived from antigen-presenting cells favor T cell activation. However, EVs secreted by cancer cells induce apoptosis in T cells, thereby promoting tumor

cell survival.[9] Cancer cells dispense caspase-3 through EVs, preventing its accumulation in cells that leads to apoptosis. EVs derived from cancer cells contain proteases and thereby increase the invasiveness of the cancer cells. Furthermore, EVs are shown to play a role in drug resistance in cancer cells through the transportation of multidrug resistant efflux pumps to other cancer cells in the surrounding environment, thus spreading drug resistance among cancer cells.[9] In lung cancer models, an increased secretion of EVs containing VEGF and sphingomyelin under hypoxia conditions facilitates angiogenesis thereby rescuing the cancer cells from nutrient and oxygen deprivation.

Extracellular Vesicles—Stromal Cell-Cancer Cell Crosstalk

Cancer cells actively interact with stromal cells through EVs. One study on invasive prostate cancer cell lines showed that cancer cells could not only activate fibroblasts in tumor stroma by secreting EVs, but also promote EVs release from these activated fibroblasts to advance their own migration and invasion. EVs contribute to the transformation of normal cells into cancer cells, as studies on breast carcinoma and glioma cells showed that EVs transfer tissue transglutaminase from cancer cells to both normal fibroblasts and epithelial cells. Similar to cancer cells, normal cells secrete EVs. Their function depends on the phenotype of the parent cells and the context. For example, EVs secreted by MSCs in breast cancers have been shown to be tumor supportive in primary tumor models and metastasis inhibitory in a metastatic model.

Extracellular Vesicles—Regulatory Packages

Recent studies on miRNA sorting in EVs have indicated that the mature miRNA and its complementary miRNA are regulated. During active miRNA generation, the initial miRNA transcripts are processed by Drosha to produce miRNA hairpin precursors. Once exported from the nucleus by exportin-5, the primary miRNA hairpin precursors are cut by the endonuclease Dicer and released as short double stranded RNA molecules. Based on thermodynamic analysis of Watson-Crick terminal base pairs, one strand has generally been thought of as the active miRNA and the other strand is just a “passenger” (miRNA* also designated miRNA-3p or -5p depending on the context). Typically, the miRNA* strand is degraded, but recent analysis of nucleotide substitutions has implicated some miRNA* strands have a regulatory role. A recent publication demonstrating the presence of miRNA processing enzymes such as Dicer in exosomes further emphasizes the role of regulatory role of exosomes. Taken together, the evidence demonstrates a miRNA mediated regulatory role of EVs. However, other related studies demonstrate that functional properties vary significantly with the method of exosome preparations and the quantity of regulatory molecules loaded in exosomes should be factors of consideration.

Further studies demonstrating the regulation induced by the uptake of secreted miRNA in the recipient cells have been reported. Rat gliosarcoma cells expressing an miRNA that lacks homology in rat cells were co-cultured with cells expressing a luciferase reporter encoding the target mRNA. The decrease in luciferase reporter activity that was observed was reversed with the addition of carbenoxolone, indicating that gap junction communication regulates intercellular transfer of miRNA. Other studies suggest the same miRNA transfer mechanism

via gap junctions between cardiomyocytes in culture and between bone marrow stromal cells and a breast cancer cell line. Furthermore, transfer of functional miRNA from immune cells involving EVs was found to be a unidirectional and antigen-dependent driven mechanism. Targeting neutral sphingomyelinase-2 inhibited this transfer.' miRNAs are well described to bind to the RISC protein complex but some miRNA have been reported to directly bind to other proteins, acting as a decoy and preventing the miRNA from blocking translation of mRNA. Fabbri *et al*. showed that EVs contain miRNAs that can reach and bind to Toll-like receptors (TLR)-containing endosomes in recipient cells, triggering a TLR-mediated prometastatic inflammatory response that may lead to tumor growth and metastasis. Thus, the role of transferred miRNAs secreted by donor cells can be not limited to post-transcriptional effects in the recipient cells but can also act as a paracrine signal.

Extracellular Vesicles—Metastasis

Metastasis is the leading cause of cancer death, yet it has been an enigma for researchers. It is considered a mechanistically inefficient process because of its dependence on very regulated and controlled systemic fueling. This premetastatic niche is presumed to play a role in dormancy, relapse and development of metastasis. An emerging role of EVs is formulating the premetastatic niche. Ghasemi *et al*. have termed these EVs “metastasomes” and hypothesized that they may aid foundation of the secondary lesions *via* a “malignant trait” spreading system that regulates the interactions between tumor tissue-specific RNA and the cell-type/tissuespecific RNA within the target organ, thus serving as tumororgan matchmakers. Recent studies have shown that these EVs are actually “customized” to the cancers. In studies comparing EVs from cancer cells and normal cells, the selectively exported miRNAs, whose release is increased in malignant cells, are packaged in structures that are different from those that carry neutrally released miRNAs.'

In closing, the recent discoveries on the study of tumor-derived EVs reveal new insights into the cellular basis of tumor stromal support. There is potential to translate this information into developing novel innovative approaches for cancer diagnostics and personalized therapy. The complexity and variety of the EV cargo implicates them in a multipronged approach toward tumor support, and hijacking their functions to engineer tumor-inhibitory EVs seems plausible. Most of the current knowledge is on the molecular profiling of the circulating EVs as biomarkers for cancers, which induces multiple platforms for personalized diagnostics. Recent literature demonstrates several possibilities of EVs as to whether the preparatory methods and studies performed are specific to the model system used. The debate is not easily resolved, but it stresses the importance of requiring in-process data for preparations and developing models to reconcile the differences in the observations related to the role of EVs in intercellular communication.

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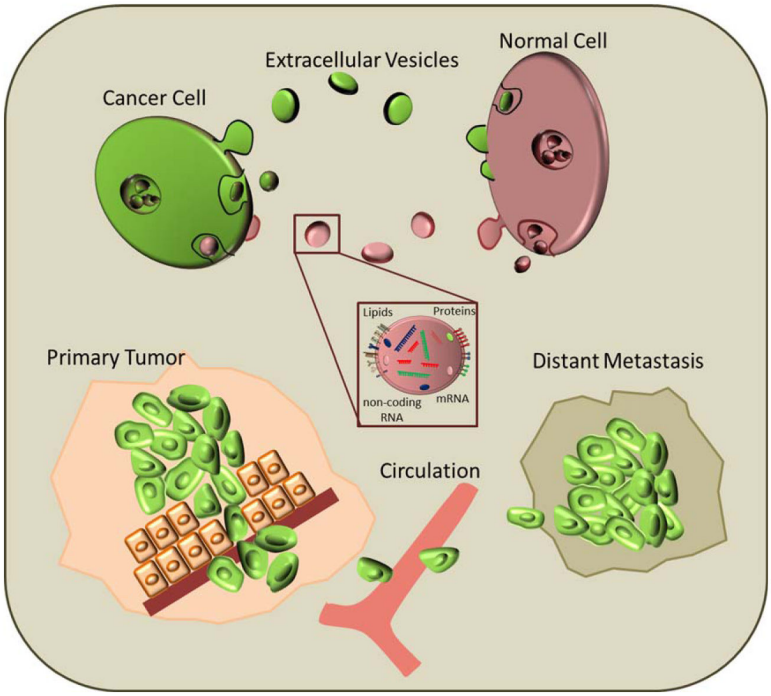

Figure 1.

Schematic representation of cellular cross talk in tumors: EVs are secreted by both cancer and normal cells either by budding directly from the plasma membrane or through invagination of the cellular membrane. This results in formation of EVs that contain cytoplasmic components like proteins, mRNA and other small noncoding RNA. Exchange of information between stem and cancer cells leads to proliferation of tumor at primary site but inhibits metastasis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

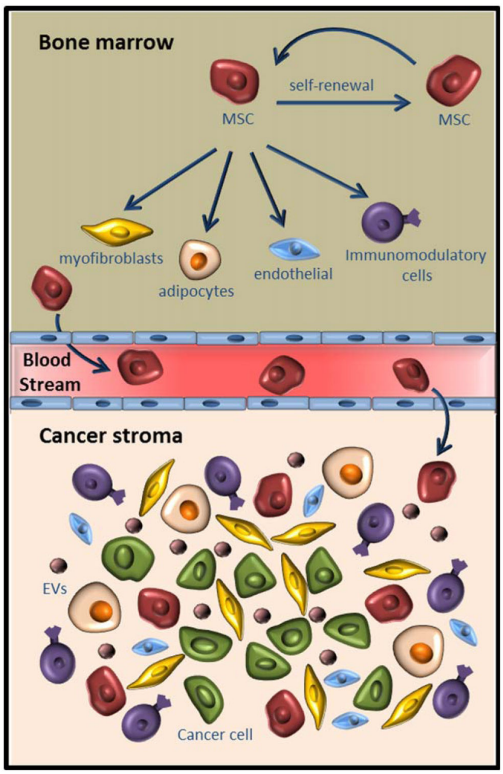

Figure 2.

Schematic representation of interactions between bone marrow niche (top) and cancer stroma (bottom). Bone marrow MSCs self-renew and also differentiate to various cell types that exhibit tumor supportive properties. MSCs are recruited from bone marrow to the tumor site through tumor derived soluble factors. MSCs secrete EVs containing various factors that support tumor progression. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

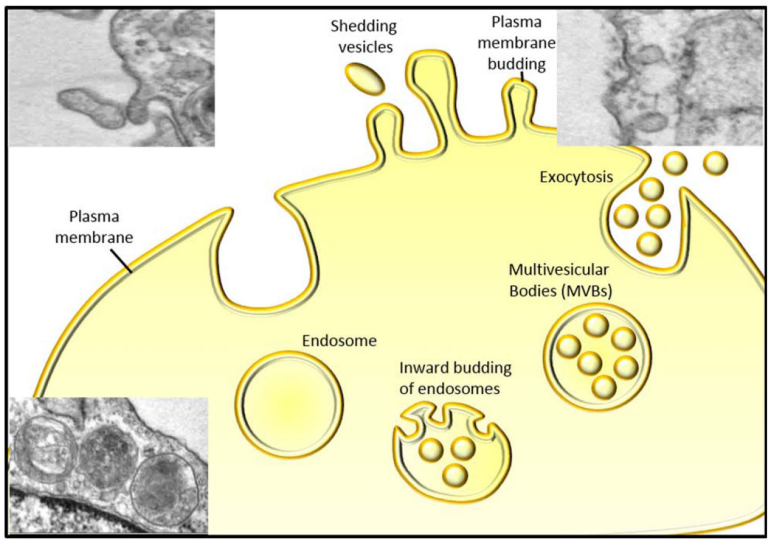

Figure 3.

Schematic representation of formation and release of EVs: in response to cell stimulus EVs are shed from cytoplasm by budding of plasma membrane of the cell. Inset 1 & 2 represents electron microscopy showing shedding and budding EVs, respectively. EVs generated through invagination of plasma membrane accumulated in the MVBs and are released by exocytosis. Inset 3 represent an electron microscopic image showing MVBs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]