

Review

Exosomes as divine messengers: are they the Hermes of modern molecular oncology?

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Exosomes are cell-derived vesicles that convey key elements with the potential to modulate intercellular communication. They are known to be secreted from all types of cells, and are crucial messengers that can regulate cellular processes by ‘trafficking’ molecules from cells of one tissue to another. The exosomal content has been shown to be broad, composed of different types of cytokines, growth factors, proteins, or nucleic acids. Besides messenger RNA (mRNA) they can also contain noncoding transcripts such as microRNAs (miRNAs), which are small endogenous cellular regulators of protein expression. In diseases such as cancer, exosomes can facilitate tumor progression by altering their vesicular content and supplying the tumor niche with molecules that favor the progression of oncogenic processes such as proliferation, invasion and metastasis, or even drug resistance. The packaging of their molecular content is known to be tissue specific, a fact that makes them interesting tools in clinical diagnostics and ideal candidates for biomarkers. In the current report, we describe the main properties of exosomes and explain their involvement in processes such as cell differentiation and cell death. Furthermore, we emphasize the need of developing patient-targeted treatments by applying the conceptualization of exosomal-derived miRNA-based therapeutics.

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Facts

- Exosomes are key elements that facilitate intercellular communication; depending on their vesicular content (‘cargo’), they can modulate tumor cells by influencing major cellular pathways such as apoptosis, cell differentiation, angiogenesis and metastasis.
- This communication can involve the exchange of molecules such as small noncoding RNAs (e.g. miRNAs) between malignant, nontransformed and stromal cells (in all directions).
- Exosomal miRNAs represent ideal candidates for biomarkers, with multiple applications in the management of an array of pathologies such as cancer.
- Manipulating exosomal miRNAs suggests new alternatives for patient-tailored individualized therapies.

Open Questions

- What are the mechanisms through which exosomal contents (e.g., miRNA) are selected to be further secreted

from tumor cells? Are these mechanisms similar/different when the secretion is from nontransformed or stromal cells? Are the miRNAs conveyed in exosomes a reflection of the cellular miRNA composition?

- How are the molecules sequestered in exosomes influencing the cancer hallmarks (e.g., mediating immune evasion or establishing metastatic niches)?

In ancient Greek mythology, Hermes was the wing-shod messenger of the Olympians, the beloved son of Zeus and of the nymph, Maia. He was committed to numerous responsibilities given by Zeus, and the most important one was to serve as a link between two worlds, taking messages from the gods to mankind.¹ By applying the wisdom of ancient philosophy to modern biomedical research, there is clear resemblance between the way the two worlds – mankind and gods – co-evolved, with the ways ontogenesis and oncogenesis are thought to develop: by communicating through messengers that for years were unknown to scientists.

The release of membrane-bound vesicles is a highly conserved biological event in prokaryotes and eukaryotes, a

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Abbreviations: CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; ESCC, esophageal squamous cell carcinoma; miRNA, microRNA; mRNA, messenger RNA; ncRNA, noncoding RNA; MBL, monoclonal B-cell lymphocytosis

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fact that attributes these vesicles an important role in regulating physiological cellular processes.² Interestingly, recent studies have discovered that transformed-tumor cells can take advantage of these endogenous 'trafficking systems' by transferring molecules that activate cancer-related pathways such as anti-apoptotic, proliferative or other tumorigenic ones. Initially, malignant tumor cells develop and proliferate in their local niche through the activation of endogenous oncogenic proteins and pathways. However, after some time, these cells recruit endogenous systems such as vesicle secretion, to broaden communication within the local tumor microenvironment and beyond. For example, at the vascular interface they orchestrate the enrollment of endothelial, perivascular or inflammatory cells, as well as platelets and clotting factors to supply tumor requirements. Actions such as these lead to the disruption of the local vascular homeostasis and also to the alteration of vital pathways that can favor the development of a tumor microenvironment with metastatic potential.^{3,4} Through their 'trafficking', membrane-bound vesicles transport 'molecular machinery' with the potential of causing physiological effects that can very well favor tumorigenesis. Several key elements have been shown to be sequestered and transported through these vesicles: cytokines, growth factors, proteins, lipids, messenger RNAs (mRNAs) or noncoding transcripts, including microRNAs (miRNAs).²⁻⁵

MiRNAs are short single-stranded (19–25 nucleotides in length) nonprotein-coding RNA transcripts (ncRNA) that are initially produced in the nucleus and then transported into the cytoplasm, where they undergo a series of steps to acquire maturation. Mature miRNAs regulate gene expression by binding (through Watsonian complementarity) to the sequence of a target mRNA. This interaction results in translational repression and/or mRNA cleavage, which consequently decreases the levels of the mRNA coding protein.^{6,7} MiRNAs have been found to be aberrantly expressed in many diseases.⁸⁻¹² For example, in cancer, the tumor microenvironment contains deregulated miRNA levels, and a reason for their altered levels is because they are being actively secreted as membrane-bound vesicular content.

Cells can secrete 'molecular machinery' through several types of vesicular carriers that are composed of both membrane and cytosolic constituents. These carriers have been shown to contain nuclear/cytoplasmic proteins, but also nucleic acids¹³ as well as other molecules. The biogenesis and main characteristics of these vesicular systems are presented in Table 1 and Figure 1a. Vesicular carriers can be classified based on their tissue of origin, their size and targeted function. In this manner, they can be categorized as either exosomes, microvesicles or apoptotic bodies.¹³⁻¹⁵ Out of the three, exosomes are currently studied the most. They are depicted as vesicular carriers involved in mediating both cell-to-cell communication¹⁶ and the trafficking of several molecules that are regarded as insoluble or classified as endogenous cellular components. Exosomes are usually transported through biological fluids such as plasma or serum. Thus, body fluids comprise a complex variety of extracellular vesicles that are being secreted from different tissues and therefore different cell types. The mechanisms regarding exosomal formation from their tissue of origin, their

traveling through body fluids and their evolutionary significance has been studied in the past.^{7,8} It is known that exosomes display a set of proteins that reflect their tissue of origin and more specifically their target cells.¹⁷ Furthermore, their trafficking is cell specific,^{18,19} and the exosomal content of the vesicles is highly regulated by a group of proteins that together form the endosomal sorting complex required for transport (ESCRT).^{2,3} This complex determines the relative abundance of proteins, mRNA,²⁰ miRNAs (...and so on), that will be segregated into a vesicle to be further transported elsewhere.

In this review, we aim to emphasize the roles that exosomal miRNAs have as essential communicators between the cancer cells and all other cell types. We also describe the consequences of their 'molecular trafficking' in cancer development, progression and metastasis.

Exosomes, Their Synthesis and Internal Content

Exosomes are naturally produced, membrane vesicle-like structures that range in size between 40 and 100 nm.¹⁵ They have a very unique cup-shaped morphology when they are observed through transmission electron microscopy.^{16,21} After being secreted, these structures have been described to have a buoyant density of 1.10 to 1.21 g/ml in sucrose. Aside from lipids such as cholesterol, ceramide or sphingolipids, their composition includes proteins like Rab GTPases, annexins or Tsg101, as well as integrins or tetraspanins (such as CD63, CD9 or CD82)^{8,15,22,23} (Figure 1b).

Several experimental procedures have served as assessment tools to detect the presence, size, purity and content of exosomal structures. In general, exosomal content can be assessed by a wide range of approaches such as western blot, mass spectrometry, fluorescence-activated cell sorting (FACS) or immunoelectronic microscopy. These techniques have individually proven that exosomes contain a variety of lipids, hundreds of proteins, thousands of RNA species²¹ including over 500 different miRNAs.^{24,25}

Immediately after their synthesis,¹⁵ exosomes are released and can remain in the extracellular space near the cell they originated from. Alternatively, they can also travel through body fluids such as blood, urine, amniotic fluid, saliva, lung surfactant, malignant effusions or breast milk. The end result of this dynamic process is a variety of regulative molecules being transported to different tissues in different places, and influencing cellular processes. Exosomes have been shown to carry proteins,²⁶ many of which have the potential to influence multiple regulatory mechanisms. For example, exosomes can transport annexins that have the ability of altering the dynamics of the cytoskeleton. They can also transport Rab GTPases that promote a wide range of cellular events regarding membrane fusions or endosomal sorting complexes desired for transport.²⁶⁻²⁸ In hematopoietic tissues, exosomes express high levels of MHC class I or II molecules on their surfaces as well as other types of adhesion molecules such as CD146, CD9, EGFRvIII, CD18, CD11a or LFA-3/CD58.²⁹⁻³¹ Specific surface markers such as these allow exosomes to be uptaken even by distant cells with a high level of specificity. This mechanism facilitates the transfer of proteins, mRNAs, miRNAs, lipids or other types of molecules to different parts of the body, and thus

Table 1 Extracellular vehicles and their characteristics

Extracellular vehicles	Size	Biogenesis	Characteristics	Content	Role	References
Apoptotic bodies	50–5000 nm	Broad plasma membrane blobbing and breach of cell fragments	Heterogeneous group of vesicles; contains surface markers for the recognition by phagocytic cells markers: Annexin-V	Fragmented nuclei as well as cytoplasmic organelles, histone, DNA fragments	Activated as response to a cellular stress or injury	5,22,23,30
Microvesicles	50–1000 nm	Bud directly from the plasma membrane through unique cellular mechanisms	No characteristic markers; lack of transferrin receptors; secreted during normal cellular processes	Cellular proteins, lipids and RNA, including miRNAs	Intercellular communication; transfer of proteins and genetic material	5,22,23,30
Exosomes	30–100 nm	Originate from the endosomal network named multivesicular bodies (MVBs) and released upon MVB fusion with the plasma membrane	More homogenous in size than other vesicles; highly enriched in transferrin receptors; secreted during normal cellular processes; markers: CD9, CD63, Alix, flotillin-1 and Tsg101	Cellular proteins, lipids and RNA, including miRNAs	Intercellular communication; transfer of proteins and genetic material	5,22,30

in this way ‘exosome-trafficking’ becomes a type of particle-based endocrine system.

Exosomes as Key Determinants of Intercellular Communication

The release of cellular invaginations from the membrane into the extracellular space has been known and described by Black³² over three decades ago. Initially, exosomes were described as key players with important roles in cell-to-cell communication as well as key physiological and pathological processes.³³ However, it was recently proven that vesicles released by cells could be indicative of the cell type and/or its function.^{2,14} With the progression of exosomal-related studies, these vesicles have now become a useful tool with diagnostic utility, and a novel strategy to develop highly specific drug delivery systems.

Exosomes can contain a variety of differentiation factors that could be released into body fluids affecting key processes of cellular homeostasis and differentiation.¹⁸ The disruption of this homeostasis has important repercussions that may result in very different outcomes such as programmed cell death or, the contrary, chaotic cell proliferation and growth.³⁴ A deregulated homeostatic environment can eventually lead to the development of several pathological conditions, including malignancies such as cancer. Thus, the contribution of exosomes in the altering of normal intercellular as well as intracellular signaling has been proven to be as significant, or even more so, as lysosomes or proteasomes.¹³

Adult tissues, more specifically the neoplastic ones, are heterogeneous complex systems composed of a variety of cell types that continuously interact with one another. Both normal and tumor cells typically secrete proteins, and thus different subtypes of exosomes can be responsible for carrying these proteins from cell to cell, resulting in a type of ‘message delivery’. In cancer, the ‘messages’ are highly dysregulated and therefore intracellular circuits are either disrupted or overwhelmed, resulting in tumor initiation and development. Exosomes are the link between disruption of

homeostasis in normal tissues and the development of oncogenesis (because they influence crucial modulators of cell cycle, proliferation, apoptosis, etc).^{35–39}

Exosomal miRNAs in Cell Death and Differentiation

Exosomes are important for various aspects of tumor biology, such as cell de-differentiation and programmed cell death. Yang *et al.*⁴⁰ have proven that purified exosomes from the supernatant of T24 bladder cancer cells demonstrated to have overexpressed levels of Bcl-2 and cyclin D1, but reduced levels of Bax and caspase-3 proteins. This same research group also proved that there is a direct, proportional link between the amount of secreted exosomes and the expression of phosphorylated Akt and extracellular signal-regulated protein kinases (ERKs), both of which are crucial elements regulating apoptosis and cell differentiation processes. ‘Auto-crine’ or ‘paracrine’ intercellular trafficking of proteins/genetic material via exosomes has proven to be extremely efficient for intercellular communication (specifically in tumors). For example, the release of exosomes may protect tumors from cell death, and surge the transmission of signals that may lead to an increase in cell proliferation.⁴¹ The fact that exosomes can tightly regulate cellular processes such as these, most certainly implies that these vesicles could be targeted therapeutically to induce apoptosis and cell differentiation or to inhibit proliferation.⁴

The physiological role of exosomes is still a controversial topic, although to this date increasing amounts of experimental data have implicated them in various key processes.²⁸ Nevertheless, some studies are still considered speculative. For example, Turchinovich *et al.*⁴² suggested that circulating miRNAs are components or remaining parts of exosomes from dying cells that persist in the extracellular environment. This group proposed that those same miRNAs could act in a paracrine manner because exosomes supply a stable intravesicular environment for them as well as for other proteins that they require for their biogenesis and maturation (e.g. Ago2).⁴² Similarly, additional studies have demonstrated

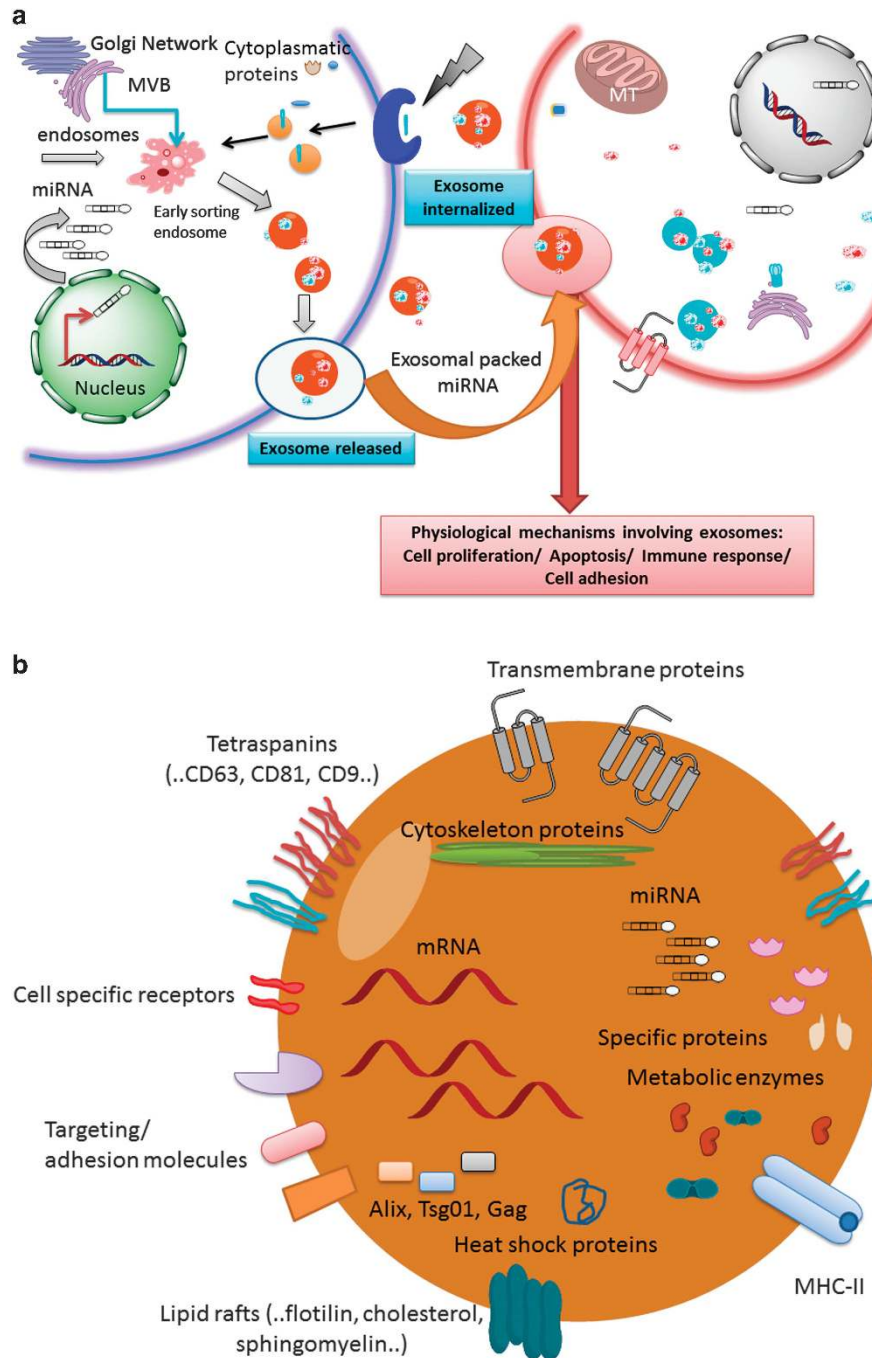


Figure 1 Exosome structure and function. (a) Exosome biogenesis and function: once released, exosomes can travel through biological fluids (e.g. serum, lymph) and furthermore, they can be internalized by cells from nearby tissues, or more so, reach body sites very far from their tissue of origin. (b) Main classes of molecules included in the exosomal cargo. MT, mitochondria; MVB, multivesicular bodies

that molecules, but specifically miRNAs, embedded in exosomal vesicles are protected from RNAases in the blood because they are surrounded by a lipidic membrane.⁴³ Thus, by increasing their stabilization and protecting them from degradation, exosomes enable molecules such as miRNAs to evade immunosurveillance mechanisms and thereby prevent events such as apoptosis. In this manner, exosomes allow an increase in pathological cancer-related processes such as

angiogenesis, and even more, they confer tumors the ability of resisting chemo- and radiotherapy.⁴³

Recently, it has been demonstrated that exosomes are uptaken in a selective way by neighbor cells but also by distant ones (including immune cells, through microenvironment cooperation), and that their uptake can lead to the activation of cell proliferation and differentiation.^{43,44} They can contribute to these processes by embedding (as part of their

'molecular cargo') miRNAs that are able to reprogram multiple cellular mechanisms in recipient cells.⁴⁴

Recent reports suggest that exosomes are implicated in facilitating tumors with immune-evasive strategies (a known cancer hallmark).^{45,46} Previous literature has suggested that these processes may occur as an effect of the direct interactions of immune effector cells and cancer exosomes; however, several other molecular intermediates are thought to be contributing as well (although the exact detailed mechanisms are not completely understood).^{4,5,45,46} Through predictions, it has been speculated that a group of exosomal derived-miRNAs (e.g., let-7, miR-17-92 cluster, miR-21, miR-25, miR-106, miR-155 or miR-200 family) participate as mediators that alter immune responses by regulating T cells.⁴⁵⁻⁴⁷ Further research aims to focus on deciphering not only which miRNAs mediate immune evasion, but also the mechanisms through which they do so (Figure 2).

The current direction regarding the study of exosomal miRNAs contributing to immune evasion, is to verify their vesicular content by recovering them from body fluids. With strategies such as reversing immune dysfunction clinicians could obtain an increase in the initial therapeutic response from patients, or alternatively, the reversal of therapeutic resistance, or relapse prevention.⁴⁸ Exosomes or more specifically their vesicular content have already shown to target immunosuppressive pathways. For example, exosomal-derived miR-155 directly influences tissues and their microenvironments by causing an evasion of immune cell response.⁴⁹ Its inhibition actually proved to sensitize CD4 + Th cells for TREG suppression.⁵⁰ Therefore, miR-155 represents a primeval modulator of the immune system with significant clinical implications because it targets receptors/ligands requested for anticancer immunity. Thus, the delivery of anti-miR-155 through artificial exosomes could be considered a potential novel therapy.

Exosomal miRNAs in Cancer

Generally, tumor cells secrete a large amount of exosomes. Exosomal release has been proven to have prognostic relevance, where a higher amount of serum exosomes typically predicts unfavorable outcomes.^{12,44,45} In 2007, Valadi *et al.*⁵¹ made significant progress by proving that genetic materials, mRNAs and miRNAs, are indeed transported through exosomal trafficking of molecules. Further along, other studies confirmed the presence of miRNAs in microvesicles or exosomes.⁵² Several others have been published associating exosomal miRNAs and their influence in cancer. For example, glioblastoma-derived exosomes were proven to be able to transport actively translated mRNA to different target cells. Even more, these exosomes were capable of inducing proliferation and tumor growth.⁵³ Similarly, in metastatic gastric cancer, the let-7 miRNA family (known to have a tumor-suppressive role by targeting oncogenes such as RAS and HMGA2) is selectively released through exosomes and this allows the targeting of oncogenic signals during metastasis development in the recipient tissue.⁵⁴ For a detailed example/model of exosomal content contributing to metastasis in cancer cells, refer to Figure 3. In addition, tumor-derived exosomes may also

transport proteins that inhibit normal cellular processes such as apoptosis. MiRNA transportation from tumor cells into the vascular system promotes a rapid method of exosome trafficking, which can promote processes such as disease spread/metastasis (refer to Figure 4).

Interestingly, when cells are subjected to stressful conditions, miRNAs can promote tumor survival through an exosomal-mediated pathway, increasing the levels of oncogenes involved in survival and evasion of apoptosis.⁴ For example, in a study regarding cervical tumor cells, the secretion of survivin through exosomes was noticed after cells were exposed to radiation treatment. These mechanisms may be considered important for cancer cells to develop strategies that facilitate drug resistance.⁵⁵ On a different study, proteins such as FasL or TRAIL were shown to be expressed on the surfaces of exosomes in melanoma and colorectal carcinomas.³⁷ This has negative consequences on patient therapeutic responses, as they inhibit the ability of T cells to enable apoptosis. In this way exosomes can interfere with cellular pathways not only by trafficking specific molecules, but also by interacting with immune cells through surface-surface interactions.

To some extent, exosomes have been thought to set up an environment that could favor tumor development. Peinado *et al.*⁵⁶ described exosomes as key elements in the assembly of the pre-metastatic niche. This last study presented for the first time the role of exosomes as part of the 'seed and soil' theory. Exosomes isolated from highly metastatic melanomas have been shown to increase the metastatic potential of primary tumors by facilitating (with their vesicular content) bone marrow progenitor cells to acquire a pro-metastatic phenotype via tyrosine kinase receptor activation.⁵⁷ A more recent investigation reported that exosomal miR-105 enhances angiogenesis in a cancer context by targeting ZO-1 (a protein that in humans is encoded by the *TJP1* gene).⁵⁸ In this way exosomes target proteins related to cell junctions and increase epithelial cell permeability, thereby promoting tumor invasion.

Several studies have stated the importance of exosomal-derived miRNAs in determining the overall effect of (already known) chemotherapies in cancer patients. For example, in breast cancer, exosomes released by HER-2-positive cancer cells have their expression modulated by various growth factors that include EGF and heregulin, a pleiotropic molecule that binds to the ErbB receptor family. These are some of the most extensively studied HER-2 cross-activating ligands and are secreted in large quantities in the surrounding neoplastic niche. Through the expression of these receptors, exosomes manage to escape/inhibit the activity of trastuzumab, a previously established therapeutic strategy for this type of cancer.⁵⁹ Furthermore, in this subtype of breast cancer, the HER-2 display on the surface of exosomes has been correlated with an increase in the binding of trastuzumab to exosome vesicles and not the original target (which are the cancer cells). This most definitely hinders therapeutic inefficiency.⁶⁰ In addition to exosome-mediated mechanisms, the clinical resistance to herceptin therapy is also related to the cancer cells protecting themselves from NK-cell mediated antibody-dependent cytotoxicity (ADCC), which minimizes the chances of reducing tumor burden.^{61,62} Thus, in this way exosomes

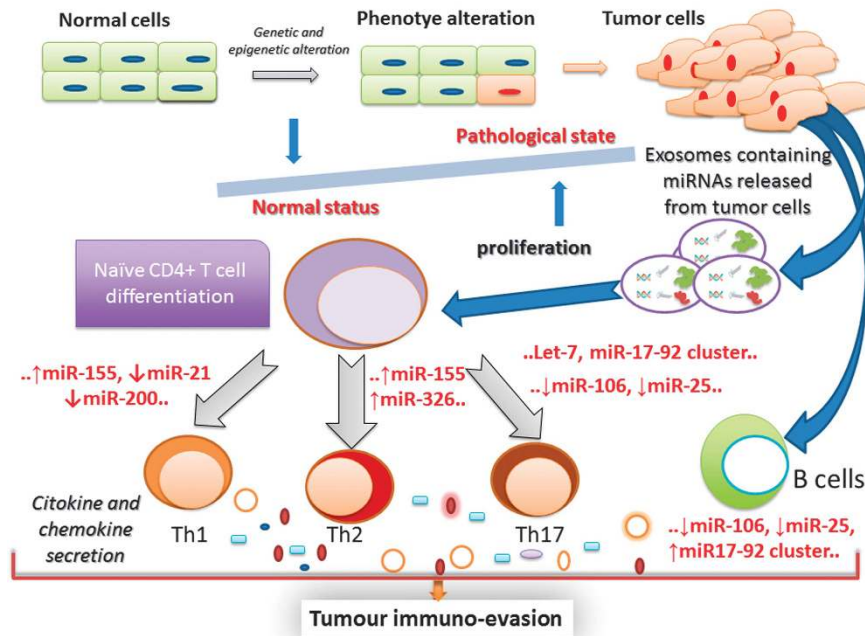


Figure 2 Immune surveillance of T cells and specific miRNAs of exosomes released from tumor cells

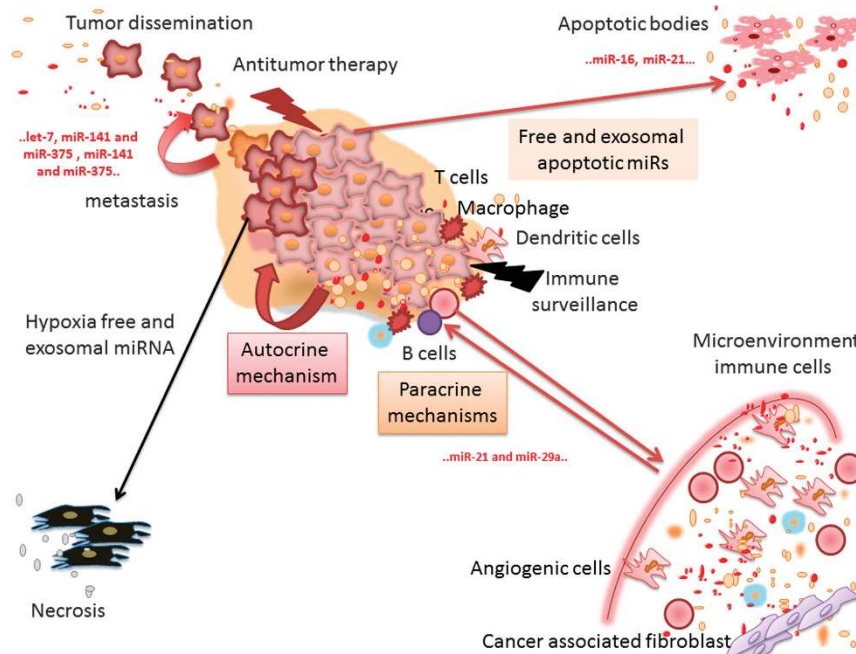


Figure 3 Exosome secretion by tumor cells. The released exosomes are uptaken by neighboring cells and are capable of inducing pathways involved in cancer initiation/progression (e.g. migration, invasion), or alternatively, they can modulate immunogenic responses. Their downstream effect is attributed to their molecular cargo, which can be rich in miRNAs

confer cancer cells additional strategies that can allow them to overcome the effects of pre-existing therapeutics.

Exosomal contents such as miRNAs typically relate to the ones being expressed in the tissues from which they are secreted. However, in some cases the exosomal miRNA content does not correlate to miRNA profiles of a particular cell type.³³ On occasions, tumor-derived exosomes establish a

pre-metastatic niche which can regulate ‘premetastatic behaviors’ because of the miRNAs that have been transferred from other tissues of origin (Figure 4). For example, in 2008, Taylor and Gercel-Taylor³¹ observed circulating tumor microvesicles in the peripheral blood of patients diagnosed with ovarian cancer, and these vesicles contained specific ‘molecular cargo’ when compared to vesicles from healthy

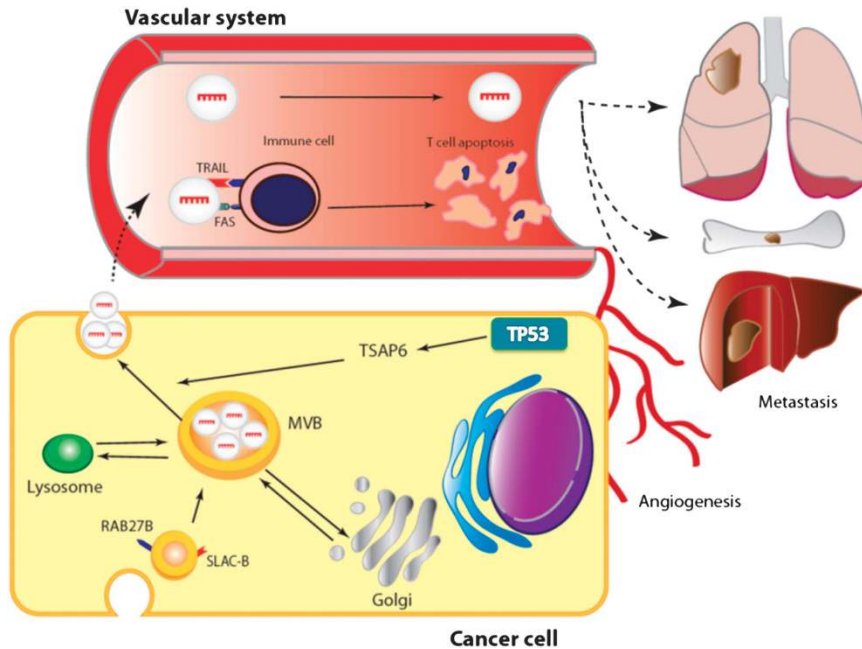


Figure 4 Tumor-derived exosomes are secreted via a constitutive/inducible pathway that can be activated by a p53-mediated response. Through this pathway (and possibly additional ones that remain unknown), exosomes can transport microRNAs into the vascular system and regulate important cellular processes such as immunosurveillance, apoptosis and angiogenesis. For example: (a) TP53 can induce the activity of TSAP6 proteins, thereby modulating key functions associated with tumor-derived exosome trafficking. Exosomes are generated by reverse budding of the membrane of multivesicular bodies (MVBs) that fuse with the plasma membrane, causing the release of these particles outside the cell. (b) From this moment on, exosomes enter the vascular or lymphatic system and circulate freely until they bind to their specific target. (c) The release from the cell is possible after the membrane is stimulated by calcium ionophores or phorbol esters. Inositol 3-kinase inhibitors can inhibit this process

patients. This suggests that vesicular cargo such as miRNAs are different in their content when they are secreted from a tumor microenvironment *versus* when they originate from the exact same tissue, but from a healthy patient. Similarly, studies done in melanoma also described significant differences in mRNA and miRNA signatures when comparing exosomes derived from melanoma with the ones arising from normal melanocytes: the proteins such as HAPLN1, GRP78, syntenin-1 and annexin A1 were present in melanoma-derived exosomes, but not in normal melanocytes.⁶³

Many oncogenes and processes promoting carcinogenesis (as well as other diseases) have been related to specific miRNAs and exosomes, and herein we present some examples. Exosomal-derived let-7 was proven to be involved in the modulation of Toll-like receptor 7.⁶⁴ In addition, exosomal miR-21 and miR-29a were demonstrated to stimulate Toll-like receptor-7 and -8, causing a prometastatic inflammatory response and activating tumor growth and metastasis.⁶⁵ Furthermore, Ferrajoli *et al.*⁶⁶ described an overexpression of miR-155 in free circulating and blood microvesicles for MBL (monoclonal B-cell lymphocytosis) and CLL (chronic lymphocytic leukemia) patients. Separately, in another study, 11 exosomal miRNAs were observed overexpressed in prostate cancer patients and miR-141 and miR-375 were presented as potential markers of metastatic prostate cancer based on exosomal and microvesicle samples.⁶⁷ More so, exosomal miRNA expression profiling proved that AZ-P7a cells (a metastatic gastric cancer cell line) release let-7 miRNAs via exosomes into the extracellular environment to maintain their oncogenesis.⁵⁴ In addition, exosomal miR-223 was shown to be released by macrophages, and to be efficiently transferred

to breast cancer cells, thereby targeting cellular invasion.⁶⁸ Finally, it has already been reported that exosomes are able to transfer phenotypic characteristics from tumor cells to other cell types, conferring aggressive behaviors to receptor cells.⁶⁹

Exosomes as Novel Cancer Biomarkers

Exosome-associated signatures from various malignancies are expected to predict the presence of early disease before a formal diagnosis is done through classic imaging techniques such as MRI, CT or a PET scan.^{70–72} In addition, these signatures might contribute by confirming or discarding the results of biomarkers that are nowadays considered standard of care, such as the prostate-specific antigen (PSA),⁷³ carbohydrate antigen CA-90 or CA-125.⁷⁴

Exosomes are intercellular carriers of many molecules and, thus, they may be considered as novel sources of unknown potential biomarkers.⁷⁵ It would be very useful to develop new mechanisms to monitor the transcriptome expression pattern based on the use of noninvasive biological fluids.^{76–78} As the composition of nucleic acids (specifically RNA) reflect in most cases the nature and origin of the parental cell, exosomes may be considered valuable carriers of cell-specific information because their content provides an insight glance of the proteins being transcribed in the original tissue.^{79–81} In this manner, exosomes may be used in the clinic to achieve early diagnostics and prognosis information, but also to stratify and separate patients into different clinical categories in order to determine specific targeted therapies. With the increase in studies aiming to find selective methods to isolate and purify exosomes, these approaches seem hopeful and

Table 2 Exosomes application in immune-driven vaccines

Cancer type	Results	Reference
Melanoma	Exosomes from dendritic cells promote the differentiation of melanoma-specific T cells and thus produce IFN- γ lymphocytes	57
Leukemia	Inhibition of tumor growth by lysis of cancer cells	86
Lymphoma	Heat-shocked exosomes against neoplastic cells	83
Leukemia	Induction of spleen cell proliferation and inhibition of cancer cells	87
Lung cancer	Anticancer response of dendritic cell-derived exosomes	94
Pancreatic cancer	Induction of apoptosis via Notch signaling	112
Pancreatic cancer	Induction of apoptosis via PI3K/Akt/GSK-3 β pathway	113
Melanoma	IL-15R α -dependent cell proliferation	81
Lymphoma	Inhibition of tumor growth	88

promising as researchers might actually find relevant biomarkers to diagnose specific diseases or to better define disease stages.^{16,26}

Various functional studies have proven that exosomes display a delimited cell tropism because they target certain cells or tissues by displaying receptors in the membranes (including malignant ones).^{82,83} For example, urine-derived exosomes from prostate cancer patients display (continuously) specific molecular markers such as PCA-3 or TMPRSS2 which are tissue specific.⁸⁴ In addition, exosomes are present in all bodily fluids, minimizing the need of performing invasive or potentially harmful diagnostic procedures (such as continuous rectal examinations for prostate cancer screening). These facts make them strong candidates for biomarkers of disease presence, progression or even therapeutic response.^{85,86}

Recent data promote the use of dendritic cell-derived or even tumor-derived exosomes to understand which therapies are more favorable to use in the clinic.^{87,88} For more details regarding studies using exosomes for anticancer vaccines refer to Table 2. On a separate note, by analyzing the proteomic profile of exosomes isolated from neuroblastoma, Marimpietri *et al.*⁸⁹ discovered that neuroblastoma cells secrete extracellular multivesicular bodies that express the GD2 disialoganglioside, a protein involved in cell immune response, cell differentiation, cell proliferation and progression. Thus, in this manner, proteomic profiles of exosomes could be used to improve prognosis predictions, and diagnostic accuracy but also to predict of potential disease relapse in patients receiving chemotherapy or radiation treatment. Interestingly, in chronic myeloid leukemia (CML), Mineo *et al.*⁹⁰ showed that a co-evolution between endothelial cells and CML cells are essential for leukemia progression and resistance to therapy. This is possible because of the fact that K562 malignant cells secrete growth factors and various miRNAs and transport these 'endothelial inducing factors' via exosomes. As a result, tube formation is stimulated, even when treating with imatinib, a tyrosine kinase inhibitor that targets the Philadelphia chromosome-positive (Ph+) myeloid leukemia cells. In this case, the development of angiogenesis was reported to regulate the progression and dissemination of

this hematological malignancy. In cases such as these, exosomes provide an alternative strategy to target angiogenesis in order to potentially achieve an even better therapeutic response in these patients.

Several studies have already proven successful in identifying exosomes as biomarkers.⁶⁶ For example, increased levels of exosomal miR-141 and miR-375 were capable of predicting metastasis or disease recurrence in prostate cancer.⁹¹ Exosomal miRNAs have shown distinct profiles in ovarian cancer *versus* benign ovarian tumors. Moreover, the lack of exosomal miRNA in normal controls has also been observed and proven useful to distinguish healthy patients from others who have the disease.⁹² Similarly, in a lung cancer study, significant differences in total exosomal RNA levels in patients *versus* controls was found;⁹³ and a similarity between the circulating exosomal miRNA and the tumor-derived miRNA patterns for lung cancer patients was identified.⁹⁴ Finally, miR-1246 was considerably elevated in exosomes from serum samples of esophageal squamous cell carcinoma (ESCC) patients, and this was determined to be a powerful independent risk factor for poor survival.⁹⁵ All together, these data sets suggest that circulating exosomal miRNA would be extremely useful tools to develop novel cancer biomarkers (Table 3).

Exosome-Based Therapy

Exosomes may have significant contribution in drug delivery, based on the facts that they are considered natural carriers of multiple types of molecules, including nucleic acids. They have an ideal prototype to fit the role of 'physiologic liposomes' because of their nano-scaled size.^{96,97} One potential application could be stimulating the body's natural immune system to recognize and eliminate the neoplastic cells through exosomal-mediated recognition. These endogenous delivery systems can overcome the difficulties confronted when trying to use artificially created vesicles.^{20,21} Based on this initial idea, several clinical trials have already started using exosomes with the aim of developing effective anticancer vaccines (Table 4). One of the first steps in developing them has been reducing their immunogenicity⁹⁸ and increasing their half-life in the circulation, thereby assuring protection of their 'therapeutic cargos', particularly in the case of nucleic acids (miRNA or small interfering RNAs (siRNAs)).^{99,100} Thus, exosomes are actively investigated as delivery systems of therapeutic nucleic acids structures, proteins or macromolecular drugs in immunotherapy,^{101,102} cancer and neurological diseases.^{103–106}

Cho *et al.*⁷⁵ published one of the first reports using engineered exosomes to express a specific cancer antigen that could generate an immune antitumor response. In their study, they demonstrated that artificially introduced exosomes have the ability to stimulate and shrink the tumor mass. To this date, liposomes are the predesigned carriers that have been proven to effectively deliver genetic material, drugs, cytokines, adjuvants or antigens into the body. However, nowadays, exosomes seem to be even better carriers because they can be introduced exogenously, but as they are 'naturally endogenous vesicles', they possess a high biosafety profile.⁹⁷

Table 3 The miRNA exosomes in screening and diagnostic

Cancer type	Results	Observation	Role	Reference
MBL and CLL	miR-155 (↑)	Exosomal and whole blood of 224 samples from 2 independent training and validation cohorts	Diagnostic, patient stratification	66
Prostate cancer (PCa)	miR-141(↑), miR-181a-2*(↓), miR-301a(↑), miR-326(↑), miR-331-3p(↑), miR-375(↑), miR-432(↑), miR-574-3p(↑), miR-625*(↑), miR-210(↑)	Plasma and serum microvesicles PCa <i>versus</i> healthy controls, 106 patients for screening; validation on 119 patients for metastatic <i>versus</i> localized PCa (only for miR-141 miR-375)	Diagnostic, prognostic	67,73
Ovarian cancer	miR-21(↑), miR-141(↑), miR-200a(↑), miR-200c(↑), miR-203(↑), miR-205(↑) and miR-214(↑)	Different stages of ovarian cancer (50 cases) <i>versus</i> benign ovarian adenoma (<i>n</i> = 10)	Diagnostic	92
Lung cancer	miR-17-3p(↑), miR-21(↑), miR-106a(↑), miR-146(↑), miR-155(↑), miR-191(↑), miR-192(↑), miR-203(↑), miR-205(↑), miR-210(↑), miR-212(↑), miR-214(↑)	Exosomal and free miRNA in plasma for lung cancer <i>versus</i> control group	Screening and diagnostic	94
Melanoma	miR-31(↑), miR-185(↑), and miR-34b(↑)	Tumor cell-derived exosomes and normal melanocyte-derived exosomes	Biomarkers	63
Esophageal squamous cell carcinoma (ESCC)	miR-1246(↑)	Serum and exosomes	Diagnostic, prognostic	95

The symbol '↑' indicates overexpressed and '↓' indicates downregulated

Table 4 Exosome-based miRNA, small interfering RNAs and protein delivery

Therapeutic agent	Therapeutic target	Reference
<i>Nucleic acids delivery</i>		
<i>miRNA</i>		
let-7a	Targeting EGFR-expressing breast cancer (<i>in vitro/in vivo</i>)	96
miR-29	Tat and morphine-mediated neuronal dysfunction in HIV	99
miR-223 mimics and inhibitor	Targeting β -catenin pathway in breast cancer cells	68
<i>siRNA</i>		
Exosomal BACE siRNA formulation from dendritic cells expressing Lamp2b (assures and reduces immunogenicity)	Knockdown of BACE1, a therapeutic target in Alzheimer's disease, in wild-type mice.	114
<i>Proteins</i>		
MelanA/Mart-1 and gp100 (released by colon carcinoma cells express CEA and HER2)	Stimulate CD4+ and CD8+ T cells and exosomes (obtained <i>in vitro</i>) leading to <i>in vivo</i> activation of T-cell responses and inhibition of tumor growth	98,101, 108,110
Dendritic cell-derived exosomes pulsed with tumor-derived antigens	Antitumor T-cell responses and tumor regression (animal models)	109
Delta-like 4 (a trans-membrane ligand for Notch receptors)	Inhibition of angiogenesis in preclinical models	104
Dexosomes	Melanoma and non-small-cell lung cancer phase I clinical trial	105,106

Emerging progress in nanotechnology has allowed the development of novel exosome-based carriers that can be used in preclinical and clinical trials. In the past, nanoparticles that could mimic the physical characteristics of various viruses have shown to induce a very strong immune response when

testing them as vaccines for similar clinical management.¹⁰⁷

Exosomes are very similar to nanoparticles because they could express/present certain antigens on their surface; however, their toxicity is minimal or none. For example, Lee *et al.*¹⁰⁸ used extracted exosomes from autologous cancers and described them as nano-sized particles enriched with specific antigens. After combining them with dendritic cells *in vitro*, a very strong anticancer effect was noticed in both cell cultures and *in vivo* models of primary and advanced metastatic malignancies.

The highest level of applicable therapeutics expected to be achieved is the use of exosome-based treatments where their content involves miRNAs that can be trafficked to specific tissue types to achieve the silencing of oncogenes (Table 4). In a recent study, Wahlgren *et al.*¹⁰⁹ successfully delivered siRNAs using several types of human exosomes as delivery systems to mononuclear blood cells. Similarly, exosomes expressing let-7a were intravenously injected into a xenograft breast cancer model and the treatment resulted in significant antitumoral activity caused by the targeting EGFR receptors.⁹⁶ Furthermore, some studies have suggested that exosomes themselves can become natural carriers of exogenous siRNA to human cells *in vitro* and *in vivo*.¹¹⁰ An example of this was seen when trying to knock down RAD51, a potential therapeutic target for tumor cells.¹¹¹

Conclusions and Perspectives

In order to achieve early cancer detection, we are in need of discovering and validating better standard-of-care assays that can identify the presence of malignancies through noninvasive techniques. Discovering that exosomes mediate intercellular communication through the delivery of molecules (such as miRNAs and other nucleic acids) has underlined a particular interest for the use of these microvesicles as potential cancer biomarkers. As exosomes express specific markers on their surface, they are ideal candidates that could lead to more efficient management of diseases such as

cancer. Major regulatory pathways such as cell differentiation, proliferation, survival or apoptosis have been demonstrated to be influenced by the transfer of genetic material, such as noncoding RNAs through these secreted vesicles. MiRNAs are an exosomal component that has one of the most significant functions in intercellular signaling and tumor progression. Through the dysregulation protein homeostasis miRNAs facilitate tumorigenesis by reinforcing every single one of the cancer hallmarks. Thus, techniques such as molecular profiling of exosome miRNA signatures will not only add molecular knowledge to our understanding of communication between malignant cells and microenvironment, but will also facilitate the introduction of novel cancer biomarkers and therapeutics.

Conflict of Interest

The authors declare no conflict of interest.

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