Exosomes in Cancer Research

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Abstract

Next generation sequencing has provided the ability to screen for novel microRNA (miRNA) biomarkers in biofluids of patients with cancer. Extravesicular vesicles in the peripheral blood, known as exosomes, provide a reliable source of miRNAs for disease biomarker detection. The molecular content of exosomes, readily available in body fluids such as blood, urine and saliva, is highly specific and a powerful biomedical tool. Exosomes generated during tumorigenesis and derived from cancer cells provide cancer fingerprints, detectable in peripheral blood. In addition, since cancer exosomes are messengers for signaling and alteration of the tumor microenvironment, it is no surprise that cancer features such as angiogenesis, chemoresistance and metastasis are associated with them, and their ability to facilitate the formation of a pre-metastatic niche as a primer for implantation of circulating tumor cells. The aim of this paper is to provide a review of the state-of-the-art of exosomes in cancer research, their role in cancer niche development with clinical correlation as biomarkers for cancer diagnosis and prognosis, as well as their future use in exo-therapy in the era of precision oncology medicine.

Keywords: Exosomes, Extravesicular vesicles, Cancer, miRNA

Introduction

Newer and less toxic treatments for cancer are desperately needed as the group of cancer survivors teaches us about long-term effects of current standard chemotherapy. In addition, identification of novel minimal invasive biomarkers for early cancer detection may improve treatment outcomes. Cancer biomarkers are informative of the pathological status for diagnosis and prognosis of a disease and have received increasing attention in cancer research for screening, early diagnosis and detection of relapse of solid tumors.

One such biomarkers group is a class of small non-coding RNAs, termed microRNAs (miRNAs). An intriguing new strategy for biomarker detection is analysis of miRNAs content of exosomes, isolated from body fluids of cancer patients. Exosomes are extracellular lipid microvesicles (40-100 nm), secreted by nearly all cells in body fluids (1) such as peripheral blood (2), urine (3), saliva, breast milk (4), cerebrospinal fluid and malignant effusions (5). They contain functional biomolecules including oncogenic proteins, lipids, ssDNA and dsDNA (6) and are perceived to be carriers of unique signaling molecules like miRNAs (7). By membrane invagination of late endosomes, exosomal vesicles are formed that contain cytosolic content of the cell of origin in healthy as well as pathological cells (8). Upon release in the body fluid microenvironment, the exosomes represent the fingerprint of the releasing cell type that can be transferred to target cells. The vesicle-associated RNA is called exosomal shuttle RNA (esRNA) that includes miRNAs as well as mRNA and are functional in target cells where they effectively silence genes (9, 10).

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Twelve years after the first major review (8), exosome research is about to explode especially in the field of cancer pathophysiology (11, 12). As vehicles of intercellular communication, exosomes play important roles in cancer tumorigenesis and metastatic disease (13). Tumor-specific exosomes are mediators of oncogenesis as they exhibit tissue-specific addresses and the ability to disturb the exquisite precise regulatory mechanism of secretion and adhesion might contribute to cancer pathogenesis (i.e. mail delivered to the wrong address). The spectrum of current scientific interest in exosomes ranges from studying functions and pathways of exosomes to utilizing them in diagnostics and development of new therapeutics (14, 15), some of which are already in Phase I clinical trial for melanoma (16).

**Figure 1: Exosomes Biogenesis in Cancer**

(A) **Budding**: Exosome biogenesis in the cell for origin (normal and cancer cell) is a two-step process with formation of intraluminal vesicles by (a) endocytic budding of the cell membrane with trapping of extracellular material intraluminally with formation of endosomes. Through a second inward budding of the endosomes with trapping of a portion of the cell’s cytoplasm, (b) multivesicular bodies containing exosomes are formed. (B) **Fusion**: After fusion with the cell membrane of the target cell (c), exosomes release their cargo (d). ESCRT: Endosomal Sorting Complex Required for Transport; TSAP6: Tumor Activated Pathway-1 (p53 target gene); RabGTPases: exocytic pathway family members. Blue vesicles indicate endosomes in normal cells; Yellow vesicles indicate exosomes as regulators of normal homeostasis; Orange vesicles indicate exosomes as regulators of tumorigenesis.
Exosomes biogenesis in cancer

Exosomes were first identified in 1985 as small vesicles floating in supernatant of in vitro cultured adherent cell layers that were associated with plasma membrane enzymatic activity of the parent cells (17, 18). Historically however in the late 70s, extracellular vesicles have been isolated from physiological media such as seminal plasma and prostatic fluid, hence called “prostasomes” after the organ of origin i.e. prostate (19, 20). Prostasomes have been studied for their dual role in normal reproduction as well as malignant prostate growth (21, 22). Exosome biogenesis is a two-step process with formation of endosomes by invagination of the cell membrane with trapping of extracellular material intraluminally. Consequently, a second inward budding with trapping of a portion of the cell’s cytoplasm on multiple loci of the endosomal membrane, gives rise to intraluminal vesicles (forming the multivesicular body, MVB) that, after fusion between the membrane surrounding the MVB and the cell surface plasma membrane, are released as exosomes extracellularly. Exosomes are shed in the extracellular environment allowing for re-uptake by target cells to deliver their cargo (8) (Fig. 1). The 2013 Nobel Prize in Medicine was awarded for the discovery of the molecular principles that govern how this cargo is delivered to the right place at the right time (23-25). By repetitive ‘budding’ (the vesicle pinches off from a ‘donor’ membrane) and ‘fusion’ (the membrane of the vesicle merges with the target membrane) of exosomes, a beneficial vesicle transfer process is created with an important role in intracellular and extracellular physiology (23). Exosome concentrations are increased in body fluids of cancer patients compared to healthy controls, implicating their role in tumor growth and metastatic spread (26, 27). Two mechanisms have been proposed: first, cancer cells might increase their exosome biogenesis by upregulation of tumor activated pathway-1 (TSAP6 - a direct p53 transcriptional target gene) (12, 28, 29); Second, exosome secretion by tumors is induced via the exocytic pathway by Rab-GTPases family members, more precisely the Rab27B protein (Fig.1) (30, 31).

Molecular markers of exosomes are the surface tetraspanins CD9, CD63, CD81, CD82 and CD151 in addition to major histocompatibility complex (MHC) class I and MHC class II proteins (32). Several detailed protocols are readily available that describe exosome isolation methods using different techniques: ultracentrifugation, OptiPrep density gradient centrifugation, ExoQuick and total exosome isolation precipitation (1, 33). Exosomal equipment such as lipids, proteins, miRNA and messenger RNA (mRNA) are concealed by the cancer source cell in exosomes and, once absorbed by target cells of different lineage, are capable of inducing pathways involved in cancer initiation, support and progression (12). The proteomic content of exosomes is very specific, combining common plasma membrane (cell surface receptors) and cytosolic proteins as well as distinct cell-type specific proteins (34, 35). Heat shock proteins (HSP-70), which are known to have immunological properties and the ability to induce dendritic cells, have been identified as part of the cytosolic proteins. Therefore, exosome-based vaccines may be a promising new strategy as immunotherapy in cancer (36, 37).

MicroRNAs are small, non-coding RNA molecules implicated in post-transcriptional gene expression regulation. Since exosomal miRNA (exo-miRNA) can be transferred to target cells and translated into functional proteins, they have appropriately been called “exosomal shuttle RNA” (esRNA) (7). Moreover, exo-miRNAs are protected from degradation by RNase enzymes in the circulation (38)(Fig.1). Because of the complexity of tissue/cell type specific proteins present in exosomes a compendium called ExoCarta ([http://exocarta.ludwig.edu.au](http://exocarta.ludwig.edu.au)) of the most commonly found exosome-related proteins (n=4,563), lipids (n=194), mRNAs (n=1,639) and miRNAs (n=764) was established as a resource for exosomal research (39, 40). In addition, two new recent initiatives are free available online as reference databases for exosomes investigators: Vesiclepedia (a compendium for extracellular vesicles with continuous community annotation) (41) and EVpedia ([http://evpedia.info](http://evpedia.info)) (an integrated database of high-throughput data for systemic analysis of extravesicular vesicles) (42, 43).

Hananan and Weinberg conceptualized the hallmarks of cancer transformation (growth, apoptosis, migration and angiogenesis) as manifestations of a somatic mutational theory (44). However, Sonnenschein refuted this concept and promotes cancer as a tissue-based disease whereby carcinogenesis is due to alteration of the reciprocal interaction between cells and their microenvironment, which includes endothelial cells, immune cells, extracellular matrix, fibroblasts and mesenchymal cells, mediated by signaling molecules such as RNA and miRNA (45). End-stage disease in cancer patients seems to support the latter, where multiorgan failure may originate from widespread dissemination of tumor cells derived from a single primary tumor. Since cancer-derived exosomes carry
both genomic and proteomic material, they may interact as propagators of the cancer niche, with pathways supported by both theories (Fig. 2).

**Exosomes: the language of the stem cell niche?**

One of the proposed disparities between a normal stem cell and a cancer stem cell is the difference in reliance on the stem cell niche, a specialized habitat to ensure their survival (46). miRNA regulates stem cell maintenance as well as oncogenesis within the niche, by promoting or inhibiting proliferative signaling. Homing and recruitment molecular mechanisms from the normal stem cell can be seized by cancer stem cells, as tools for invasion and metastasis (47, 48). Cancer-cell derived exo-miRNAs are cast within exosomes into the circulation and body fluids. Bearers of the cancer signature to surrounding and long-distance cells, exosomes communicate signals between stem cells and the microenvironment as translators of the language of the cancer cell niche. This language is a two-way communication, whereby according to the “seed and soil” theory (49), miRNA from the ‘seed’ can alter the ‘soil’ and vice versa (48, 50).

**Figure 2: Exosomes and the Cancer Niche**

(A) Exosomes are secreted in the cancer niche by normal supportive cells of the microenvironment (endothelial cells, mesenchymal stem cells, fibroblasts, bone marrow stem cells) as well as by the cancer cells. (B) During the homeostatic process of self-renewal, exosomes may play a role in stem cell maintenance as well as terminal differentiation. (C) Exosomes as propagators of oncogenesis (a) at the primary tumor site, (b) at the pre-metastatic niche, and (c) in metastatic disease. Cancer niche: Blue cancer cells in cancer niche (A) indicate dormancy; Red cancer cells in primary tumor environment (a) indicate activation.
The secretory Rab GTPases Rab3D, Rab27A and Rab27B are main regulators of exocytosis (51). Rab GTPases constitutive exocytic trafficking has been involved in regulation of exosomal matrix metalloproteases (MMP), which modulate the matrix in the target organ necessary for cancer cell invasion (52). Since Rab27B exo-miRNA levels are significantly higher in patients with invasive breast cancer (estrogen receptor-positive with nodal involvement), they might serve as a biomarker of ER-positive breast cancer with poor prognosis (30).

In hematological malignancies, exosomes are shed by normal blood cells (platelets, endothelial cells, leukocytes and monocytes) as well as by leukemia cells (53). B-cell chronic lymphatic leukemia (CLL) is a clonal B-cell disorder driven by constitutional expression of the receptor tyrosine kinase AXL (RTK-AXL). Circulating exosomes in plasma of CLL patients are at higher levels than in healthy controls and carry phosphorylated RTK-AXL, which are able to activate the AKT-target rapamycin signaling pathway in CLL bone marrow stroma cells (27). Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease caused by Bcr-Abl oncoprotein-driven tyrosine kinase activity (Philadelphia chromosome). Exosomal crosstalk between CML cells and their bone marrow niche induces angiogenesis via exosomal release of interleukin-8 in vitro (54). Moreover, transferred Bcr-Abl DNA from the K562-CML cell line induced a CML-phenotype in mice, emphasizing the pathophysiological role of exosomal tumor genes transfer in leukemogenesis (55).

In acute myeloid leukemia (AML), exosome trafficking alters the microenvironmental niche by transferred mRNA, reprogramming the bone marrow during invasion of AML (56). Exosomes also play a role in the immune surveillance by natural killer (NK) cells, building a protective shield for human leukemia niche-invaders. NK cell surveillance is an important physiological tool of cancer restraint that is curbed in patients with AML because blast-derived exosomes suppress NK-cell activity using the TGF-β1 pathway (57). Moreover, changes in exosomal TGF-β1 content may have clinical predictive value for the outcome of chemotherapy (58).

**Exosomes in the pre-metastatic niche**

Once tumor cells leave the protective niche of origin, additional conditions must be met for successful sprouting in foreign soil i.e. growth of metastatic disease (59, 60). One of the conditions is the presence of a long-distance signaling system conducive to the creation of an adaptive environment for “traveling” tumor cells. This signaling system needs to be able to alter expression of growth factors (VEGF-1), matrix metalloproteinases (MMP-9) and adhesion molecules (integrin VLA-4) to allow the establishment of a pre-metastatic niche at a distance from the primary tumor location (61). An emerging common theme in solid tumor oncogenesis is the evidence that exosomes drive pre-metastatic niche formation (62, 63). Highly metastatic melanoma exosomes can educate bone marrow progenitors towards a pro-vasculogenic phenotype at a pre-metastatic site via the tyrosine kinase receptor MET. Peinado et al identified an exosome-specific melanoma signature in patients with clinical correlation to advanced disease stage and poor prognosis (64). Modulation of host extracellular matrix degradation by tumor exosomes promotes motility and allows for recruitment and invasiveness of circulating tumor cells to the pre-metastatic niche (65). Breast cancer exosomes are involved in leading the ‘metastatic exodus to the promised niche’ (66). Exosomes produced by breast cancer cells are taken up by stromal fibroblasts and reciprocally cancer-associated fibroblast-derived exosomes stimulate breast cancer cell motility and metastatic behavior via autocrine Wnt-11 signaling (67-69). Tumor-initiating cells that express the mesenchymal cell marker CD105 in human renal cell carcinoma release exosomes that stimulate the formation of the lung metastatic niche in mice (70). Recent studies in cancer cell lines show that ovarian cancer cell invasiveness correlates with excessive exosomal let-7 miRNAs (71) and a metastatic gastric cancer cell line releases let-7 miRs via exosomes in the extracellular environment to promote tumor growth. In normal homeostasis, let-7 controls cell proliferation by negative regulation of Ras GTPases but constitutively activated Ras mutations are common in solid tumors (72). The exact mechanism by which exosomal let-7 regulates cancer growth remains to be elucidated. Proteomic profiling of exosomes from human primary and metastatic colon cancer revealed differential expression of key metastatic factors such as the tyrosine kinase receptor MET, implementing their role in metastatic niche configuration (73).

**Exosomes: novel biomarkers in cancer diagnostics**

In order for biomarkers to be useful in the clinical setting of cancer patients, they must: 1, be easy accessible by minimal invasive procedures for repetitive sampling; 2, represent the signature of the cancer of origin; 3, be distant travelers to deliver their
protected cargo; 4, gain easy and specific entry into target cells; 5, carry the machinery to interfere with intra- and intercellular differentiation signaling; 6, reflect disease-stage and exhibit changes in response to initiated treatment and last but not least 7, be amenable to high-throughput screening in larger patient populations with a reference database available (Table 1).

Table 1: Exosomes as Cancer Biomarkers

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<thead>
<tr>
<th>Cancer Biomarker Criteria</th>
<th>Exosomes as Cancer Biomarkers</th>
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<tbody>
<tr>
<td>• Easy accessible by minimal invasive procedures for repetitive sampling</td>
<td>• Body fluids (1, 9, 74)</td>
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<tr>
<td>• Signature of the cancer of origin</td>
<td>• Exo-miRs (75, 76)</td>
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<td>• Distant travelers for safe cargo delivery</td>
<td>• Rab GTPases-regulated vesicle trafficking (51)</td>
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<td></td>
<td>• p53-regulated exosome release (77)</td>
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<td></td>
<td>• Exo-miRs in blood are RNase protected (38, 69)</td>
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<td>• Target cell-specific entry</td>
<td>• Phosphatidylserine exosome uptake signal in NK cells (42)</td>
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<td></td>
<td>• Surface expression of adhesion tetraspanin-integrin complexes on exosomes (66, 78, 79)</td>
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<tr>
<td>• Interference with intra- and intercellular differentiation signaling</td>
<td>• Exosomal shuttle RNA (esRNA) (7)</td>
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<td></td>
<td>• Immunomodulation (80, 81)</td>
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<td></td>
<td>• Cancer dormancy (10)</td>
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<td>• Disease-stage specific and treatment responsive</td>
<td>• Exo-predictive biomarker with chemo responsiveness (82)</td>
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<td></td>
<td>• Exo-transfer of multidrug resistance (83, 84)</td>
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<td>• High-output screening tool</td>
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<td>• Reference database available</td>
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<td></td>
<td>• Vesiclepedia (41)</td>
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<td></td>
<td>• EVpedia (42, 43)</td>
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Exosomes are key-players in intercellular communication, and since their host-cell derived cargo can regulate cellular signaling, they are prime candidates for the role of traveling messengers to deliver that cargo to local or distant target cells (13, 87). The oncogenic cargo of cancer-related exosomes contains bioactive molecules including DNA, mRNA and miRNAs (hence these cancer-related exosomes are often referred to as oncosomes) (88). Noncoding transcripts such as miRs are part of the exosome repertoire by which oncosomes assist in the process of tumor promotion by modulating the microenvironment into receptive metastatic niches. Proof of concept that exosomes represent the signature of the cancer of origin was demonstrated in a xenograft mouse model of human lung cancer cells, labeled with human CD63-
green fluorescent protein (GFP). hCD63-GFP exosomes were identified in blood and saliva of tumor-bearing mice, suggestive of the link between distal tumor progression and biomarker discovery in saliva (76). Entry into target cells seems not to be an at random event either. Human brain tumors (gliomas) express an oncogenic form of the epidermal growth factor receptor, known as EGFvIII. EGFvIII can be ‘shared’ by glioma cells via horizontal transfer by oncosomes with transfer of oncogenic activity and promotion of the cancer phenotype (89). However, identification of the exosomes-target anchorage and internalization components remains greatly elusive, although heparin-glycan proteins and integrins on the exosomal membrane surface have been suggested to play a role (78, 79). The packaging of their molecular load is tissue-specific and strictly regulated by a group of proteins that form the endosomal sorting complex, required for transport (ESCRT) (Fig.1) (90, 91). Because this highly specific packaging process allows exosomes to carry the signature of the cancer cell of origin into the blood stream and supporting stroma, it makes them perfect biomarkers for cancer diagnostics with clinical correlation to disease stage (26, 82). Next-generation deep sequencing (NGS) facilitates high-throughput profiling of miRNAs in biological fluids making this approach a viable screening tool to detect miRNAs biomarkers. NGS has been used to profile miRNAs in exosomes, which appear to provide a consistent source of miRNAs suitable for cancer biomarker detection (86). For example, NGS of exosomal transcripts in breast cancer cell lines revealed their cell of origin, confirming their utility as potential biomarkers in breast cancer (85). In addition, exosomal RNA is protected from degradation by circulating RNases of the blood stream assuring reproducibility and stability (38, 69). However, Argonaute2 (Ago2) complexes carry a population of circulating miRNAs independent of exosomes in human plasma and the circulating Ago2 complexes have been suggested as another mechanism responsible for the stability of plasma miRNAs. This information is important for the development of biomarker approaches based on analysis of circulating miRNAs (92). A consensus needs to be reached on dependable isolation methods for exosome biomarker research (74). Reproducible protocols that obtain the purest exosome fractions for downstream RNA profiling with lack of contaminating Arg2-complexes would meet the standards of clinical care (33, 93, 94). Since exosomes can meet all proposed biomarker criteria, they have been recognized as suitable biomarkers in cancer diagnostics for solid tumors as well as hematological malignancies (Table 2).

**Exosomes as cancer therapeutics – ExoDrug**

Ideal cancer therapeutics should exhibit interference with tumor growth and invasiveness as well as circumvent multidrug resistance (MDR) in order to obtain remission and effectiveness in cancer immune surveillance to prevent relapse. Exosomes have been investigated as specific delivery tools of functional molecules to the microenvironment and their uptake by target cells has been confirmed by labeling with fluorescent dyes (GFP, PKH, DiOC18), immunofluorescence and flow cytometry (15). Because exosomes are capable of instigating an immune response (likely through the actions of Hsp70 on the exosomal surface – (95)), an exciting field of exosomal cancer immunotherapy is burgeoning (80, 81, 96). Most studies evolve around the application of dendritic cell-derived exosomes (DEX) that have been pulsed with a tumor antigen (12, 97). DEX harbor functional MHC complexes capable of eliciting T-cell immune (CTL) responses and tumor rejection. This concept was translated in a first Phase I clinical trial in late stage melanoma patients whereby autologous exosomes were pulsed with MAGE3 peptides for vaccination (16). This study highlights the feasibility of ExoDrug based on the properties of: 1/ large scale exosome production, 2/ safety of exosome administration in a clinical setting and 3/ minimal toxicity. Antileukemia immunity was demonstrated in vitro, when exosomes from NB4 cells (a human acute promyelocytic leukemia (APL) cell line) presented leukemia antigens to dendritic cells with the help of ICAM1 and Hsp70, hence inducing effective cytotoxic activity to kill leukemia cells (95). Ascites has been proposed as another rich source of autologous tumor-exosomes (TEX) in ovarian cancer and colorectal cancer patients (81, 98, 99). In colorectal cancer patients, exosomes plus granulocyte monocyte colony stimulating factor (GM-CSF) injections induced beneficial tumor-specific CTL responses, thereby demonstrating that clinical exo-therapy is well tolerated. One of the main causes of disease relapse in cancer patients is phenotypic changes in the tumor that result in multidrug resistance (MRD). Exosome-mediated communication of drug resistance among tumor cells and between tumor cells and microenvironment have been suggested as a mechanism of MRD (83, 84) and exosomal blockages of drug resistance-transfer could mark an improved disease-free survival. Another intriguing approach for ExoDrug
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*clinical trial
application is to outfox the uptake of exosomes by the target cells at the cancer niche (52), hence impeding TEX-initiating angiogenesis (112) in premetastatic niche formation. Exosomal induced-PPARalpha-NF-kappaB-Akt pathways may play a pivotal stimulatory role for neovascularization, which is crucial in tumor growth and maintenance (113). In addition, exosomes can activate angiogenic programming of bone marrow derived endothelial progenitor cells (EPC). Therefore, a potential method for ExoDrug therapy could be exosomal blockage of EPC instead of activation. Since TEX support tumor maintenance via several different mechanisms, mere removal of TEX from the patient circulation by ultrafiltration through affinity binding filters with exosomal ligands and TEX-specific antibodies has been proposed as adjuvant cancer therapy (114).

Nanomedicine explores the use of nano-particles for therapeutic and diagnostic application - termed theranostics- and exosomes are active participants as nano-theranostic delivery agents for gene therapy (115). The use of exosomes as drug delivery systems requires pharmacokinetic studies of circulation time, biodistribution, stability, cellular interaction and cargo loading, lessons that can be learned from the liposome field (74). RNA interference and gene transfer cancer therapy looked promising as methods for effective interruption of the cancer apparatus. However, one of the main problems to be overcome is successful conveyance of nucleic acids across the cell’s plasma membrane. Bioengineered exosomes prepared through expression vector transfection have declared themselves as perfect exo-missiles for targeted gene delivery into cells derived from different lineages (7, 116, 117). Plasma exosomes have been used as gene delivery vectors to transport exogenous small interference RNA (siRNA) that caused selective gene silencing of mitogen-activated protein kinase 1 in human monocytes and lymphocytes (14). The rapid and safe distribution of an exosome-encapsulated anti-inflammatory drug, called curcumin, to the brain via intranasal administration, might open new venues for easy and non-invasive drug delivery in neuro-oncology, bypassing the blood-brain barrier. Fluorescent labeled intranasal delivered exosomes were visible in microglia, suggesting cell uptake in the brain (118, 119).

While the clinical application of exosomes for therapeutic drug delivery in oncology is still immature, issues regarding the understanding of exosomal technology, large-scale production and in vivo toxicity need to be addressed in order to develop lucrative and cost-effective ExoDrug delivery systems.

Future Directions

Exosomes in cancer research is a promising new field in translational medicine. Their use as clinical biomarkers for cancer staging is already in Phase I trial for melanoma. The International Society for Extracellular Vesicles (ISEV) as a global society for researchers around the world with interest in this field addresses best practice in EV isolation and RNA packaging, low-input high throughput sequencing and digital PCR to stress the importance of a unified approach for clinical application. Analysis and modification of cancer derived-exosome content as well as creation of artificial-tailored exosomes for drug delivery may lead to a novel era of cancer therapeutics.

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