Review

Cancer Research

The Biology of Cancer Exosomes: Insights and New Perspectives

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Abstract

Exosomes are a subclass of extracellular vesicles involved in intercellular communication that are released by all cell types, including cancer cells. Cancer exosomes carry malignant information in the form of proteins, lipids, and nucleic acids that can reprogram recipient cells. Exosomes have emerged as putative biological mediators in cancer contributing to major steps of disease progression. A leading role exists for cancer exosomes in specific aspects of tumor progression: modulation of immune response, tumor microenvironment reprogramming, and metastasis. This review will address the functions attributed to cancer exosomes in these three aspects of cancer biology, highlighting recent advances and potential limitations. Finally, we explore alternative strategies to develop better models to study cancer exosomes biology. *Cancer Res; 77(23); 6480–8. ©2017 AACR.*

Introduction

Exosomes are a class of extracellular vesicles defined as 40–150 nm diameter membrane nanovesicles of endocytic origin that contain proteins, nucleic acids, and lipids (1, 2). Since they were first discovered, the unveiling of their potential biological significance has prompt major advances in the field. Exosomes have evolved from simple cell "dumpsters" to key players of numerous biological processes in both pathologic and nonpathological contexts (3). Nonetheless, exosomes' normal physiologic functions remain largely unexplored. Until now, the only insights on their role in a healthy background include normal synaptic physiology (4, 5), and modulation of immune response due to their ability of antigen presentation and T-cell activation when derived from competent antigen-presenting cells (APC), such as dendritic cells (DC; ref. 6). However, it was in the field of cancer that exosomes' biological significance was more scrutinized. The intricate exosome-mediated network of communication established between tumor and nontumor cells seems to be involved in every step of cancer progression, from tumor growth to cell dissemination (7). Cancer cells developed exosome-mediated mechanisms to promote a favorable microenvironment that supports tumor growth through enhanced cell proliferation and escape to apoptosis (7). In addition to these features, cancer exosomes also have the ability to induce formation of new vessels, which ensures access to nutrients, oxygen, and waste removal, and contribute to the metabolic reprogramming of cancer cells providing means for their sustained proliferation (8, 9). The capacity of tumors to become invasive and disseminate is highly boosted by cancer exosomes, which carry information that contributes to extracellular matrix (ECM) remodeling, cancer cell migration, and invasion (10, 11). Finally, cancer exosomes portray a crucial role in the escape of the tumor to immune surveillance (12–14). Concerning all the processes in which cancer exosomes involvement has been described, the three main research areas that clearly show the role that exosomes play in specific aspects of tumor progression are: the ability of exosomes to modulate the host immune response towards immune escape; cross-talk of exosomes with the tumor microenvironment to promote tumor growth and progression; and involvement of exosomes in metastasis. This review will explore these hallmarks and how they significantly contributed to our understanding of the role exosomes play in cancer (Fig. 1). We will further address potential approaches that can overcome current limitations on cancer exosomes studies, which can improve our knowledge on cancer exosomes' biological significance.

Exosomes: Biogenesis and Characterization

In the early 1980s, two research groups studying reticulocyte maturation observed a curious form of extracellular vesicles (EV) secretion (15). They showed that a smaller class of vesicles secreted by cells were formed by inward budding of the membrane of an early endosome, leading to the accumulation of intraluminal vesicles (ILV) in their lumen, in a process mediated by the endosomal sorting complex required for the transport (ESCRT) complex family (15, 16). Because of their vesicular appearance, these mature endosomes are also known as multivesicular bodies (MVB). MVBs can then fuse with lysosomes for content degradation or fuse with the plasma membrane (PM) releasing their vesicles to the extracellular space (16). Late stages of exosome production are promoted by the Rab GTPases 27A and 27B, which mediate the fusion and docking of MVBs to the PM

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doi: 10.1158/0008-5472.CAN-17-0994

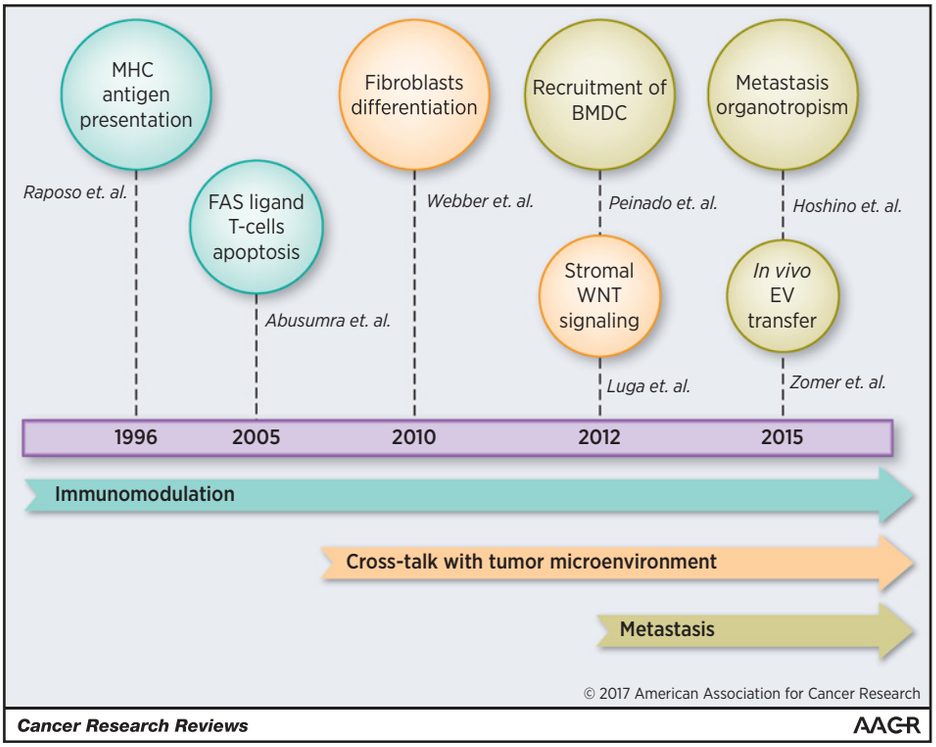

Figure 1.
Chronological highlights of studies on cancer exosomes. Chronological hallmarks of the main findings related with the involvement of cancer exosomes in immunomodulation, tumor microenvironment reprogramming, and metastasis.

(17). Exosomes are characterized by the expression of a common subset of proteins, due to their endosomal origin. These proteins are involved in membrane transport and fusion processes such as Rab GTPases, annexins, and flotillin, components of the ESCRT complex, integrins and tetraspanins, including CD9, CD63, and CD81 (18). On the other hand, exosomes' cargo is dependent on the cell of origin and comprises proteins, nucleic acids including several types of RNAs (e.g., mRNA and noncoding RNAs) that were found to modulate recipient cells in most instances favoring tumor development (19). Exosomes were also found to carry out cell-autonomous processes such as the processing of pre-miRNAs (20). Besides RNA and proteins, the presence of other molecules including mitochondrial DNA (21), transposable elements (22), and more recently double stranded DNA (23) has been reported inside exosomes.

Despite the reports describing a plethora of roles for exosomes in cancer, their classification is still under debate. A brief search of the literature can easily identify a variety of nomenclatures used: exosomes, oncosomes, dexosomes, exosome-like particles, membrane blebs etc., are just a few examples. However, it is evident that many authors prefer the general term EVs, independently of the vesicles characterization, subcellular origin, and isolation methods. The current criteria used for naming EVs is mostly based on their physical characteristics such as size and morphology, as well as the presence of known markers and isolation techniques. In addition, the EV–TRACK consortium created an open-access knowledgebase that stores methodologies used for isolation and characterization of EVs with the aim of facilitating and homogenizing the complexity associated with EVs studies (24). In this review, we have adopted the nomenclature according to the original article and/or the most enriched EVs expected by isolation method used and subsequent vesicles' characterization.

Exosomes Modulate the Immune Response

The demonstration that exosomes derived from human Epstein-Barr Virus-transformed B-lymphocytes contain the major histocompatibility complex (MHC) class I and II at their surface, was the first evidence that exosomes could potentially be involved in the modulation of the immune response (25). The MHC-I and -II complexes are responsible for presenting antigens to $CD4^+$ and $CD8^+$ T-cells, through which they regulate the antigen specific immune response (26). These exosomes bearing MHC-II are capable of presenting antigens and activate $CD4^+$T cells, a process that was only attributed to APCs (27). Following this report, human DC-derived exosomes were also found to express both MHC-I and -II classes (12). Because DCs have been associated with antitumor immune responses and anticancer vaccination (28), the possibility that DC-derived exosomes could present antigens to T-cells was further explored. With this purpose, murine bone marrow–derived DCs were incubated with tumor peptides along with MHC-stimulatory production molecules to generate exosomes expressing tumor antigens (12). The isolated exosomes were then used *in vivo* as an immunization treatment that primed an immune response against tumors. Interestingly, DC-derived exosomes significantly reduced tumor burden in an aggressive mouse model of mastocytoma as well as in a model that spontaneously develops breast carcinoma. This antitumor response triggered by DC-derived exosomes was abrogated in *in vivo* models that lack functional T cells (12). Overall, these findings shed light into APC-derived exosome's role in a healthy physiologic setting, as they participate in the immune response against cancer. In addition, it was hypothesized that cancer-derived exosomes could also express MHC complexes and trigger an immune response. Indeed, exosomes of murine cancer cell lines have MHC-I complex and are capable of indirect

presentation to T-cells (6). Treating an e*x vivo* culture of DCs derived from the bone marrow with tumor-derived exosomes potentiates T-cell activation, supporting the role of cancer exosomes in modulating the immune response (6). Tumor-derived exosomes can activate an immune response through cross-presentation of tumor antigens. Exosomes isolated from patient's ascites malignant effusions have MHC-I and tumor antigens for Her2/Neu, Mart1, TRP, and gp100, and were used to cross-present antigens to patient-isolated lymphocytes via DCs (29). The sensitized DCs promoted the expansion of specific T-cell clones, which were then cocultured with autologous tumor cells causing cell death. Altogether, these studies show that MHC^+ exosomes from both tumor and immune cells could constitute an alternative mechanism of antigen presentation. Nonetheless, different studies show that exosomes that do not express MHC, when loaded with antigens of ovalbumin, can still generate T-cell antigen-specific responses. However, these MHC-independent mechanisms of exosomes in the modulation of the immune response remain largely unexplored (30, 31). Considering the described properties of exosomes, they have been explored as anticancer immunization or vaccination systems in phase I clinical trials with melanoma, lung, and colorectal cancer, using exosomes derived from autologous DCs or ascites-derived exosomes (32–34). These studies concluded that it is possible to obtain a sufficient amount of autologous exosomes for several treatments isolated from *ex vivo* expanded cells and that administration of exosomes is well tolerated by the patients. However, the immune response triggered with these treatments was not efficient and there were no clear signs of activated T-cell responses against specific antigens (35).

In opposition to the described ability to trigger a cancer immune response, tumor-derived exosomes are well known by escaping immune surveillance. In fact, prostate cancer–derived exosomes decrease T-cell proliferation in a dose-dependent manner due to the expression of Fas ligand (13). In a similar fashion, murine mammary carcinoma cells' interaction with natural killer (NK) cells induced a reduction in NK cells' cytotoxic activity and proliferation (36). Moreover, by treating mice with cancer exosomes previous to tumor inoculation, it was demonstrated that the immunosuppressive effect on NK cells hampered any immune protective effect of DCs exposed to tumor-derived exosomes. Cancer exosomes also drive the impairment of DC maturation *in vivo*, contributing to an immunosuppressive context. Breast cancer exosomes are internalized by bone marrow myeloid precursors, which impair the differentiation into DCs by promoting overexpression of IL6 and phosphorylation of Stat3 (37). Further validation was obtained by using bone marrow precursor cells isolated from an IL6 knockout (KO) model, that are able to differentiate into DCs, upon treatment with cancer exosomes. Collectively, these findings support an immunosuppressive role for cancer exosomes through NK and DC modulation. However, the majority of the described studies fail to identify the molecular effectors in exosomes responsible for the observed modulation of immune cells.

In addition, cancer exosomes seem to be involved in the activation of key pathways related to macrophage biology, which ultimately can support tumor development. Tumor-associated macrophages correlate with an oncogenic, proinflammatory, and tumor-permissive niche by the secretion of growth factors and promotion of fibrosis (38). Exosomes derived from a breast cancer cell line induced NFκB activation in macrophages, contrarily to exosomes from nonmalignant cell lines (39). NFκB pathway is responsible for the inflammatory response of macrophages, including induced secretion of several cytokines such as IL6, CCL2, and TNFα (39). Moreover, using an *in vivo* breast cancer xenograft model, Chow and colleagues demonstrated that cancer exosomes were responsible for the activation of NFκB via Toll-like receptor (TLR)-2 (39).

As illustrated by these studies, cancer exosomes are able to interact with practically every type of immune cells. The complexity of these interactions shows that exosomes can both potentiate the immune response against the tumor or act as immunosuppressive agents. How is this dual response occurring within the same tumor and how it may vary according to the immunogenicity and cancer heterogeneity is still largely unexplored. *In vivo* models to trace cancer exosomes would allow a better understanding of their interaction with different cells of the immune system, and therefore would be a valuable tool to help understand how these complex communication mechanisms are modulating tumor progression.

Cancer Exosomes and Tumor Microenvironment

The tumor microenvironment corresponds to the biological components that surround cancer cells. The components that are generally more abundant are: mesenchymal cells, such as fibroblasts, endothelial cells, hematopoietic cells, both from lymphoid and myeloid origin, and the ECM that provides physical and biochemical support (40, 41). The bidirectional network of communication between cancer and stromal cells is based on the release of soluble compounds like growth factors or on the release of exosomes. Cancer exosomes were found to modulate the surrounding cells to support tumor growth and dissemination. Indeed, exosomes derived from prostate cancer cells were shown to drive fibroblast differentiation into activated-fibroblasts or myofibroblasts in a TGFβ-dependent manner (42). $TGF\beta^+$ exosomes induce a different myofibroblast phenotype from the one induced using serum TGFβ, highlighting distinct roles of both forms of intercellular communication. Activated fibroblasts are commonly found at tumor sites and are well described for their active role in tumor progression by the secretion of growth factors, chemokines, and deposition of ECM constituents (43), facilitating tumor growth and invasion. Breast cancer cell–derived exosomes mediate the conversion of mesenchymal stem cells (MSC) derived from adipose tissue into myofibroblast-like phenotype caused by SMAD2 activation (44). SMAD cascade leads to the production of tumor-promoting factors such as the VEGF and TGFβ. Recently, Baglio and colleagues demonstrated that membrane-associated TGFβ carried by EVs derived from metastatic osteosarcoma cells are involved in tumor microenvironment reprogramming (45). *In vitro* treatments of MSCs with these vesicles resulted in an increased expression of IL6 that promotes inflammatory reaction. Injection of the reprogrammed MSCs *in vivo* promoted tumor growth and metastasis. Nonetheless, EVs/exosomal TGFβ signaling is also found to be associated with fibroblast activation by mRNA transfer upon wound repair in kidney fibrosis (46), which points to the fact that not only cancer exosomes communicate with resident fibroblasts.

Tumor growth depends greatly on cancer cells' access to nutrients, oxygen, and waste removal that is largely provided by the vasculature (47). In this regard, tumors secrete angiogenic factors

such VEGF to promote endothelial cells' migration and proliferation. Moreover, a hypoxic environment promotes exosome secretion by tumor cells, which are then internalized by endothelial cells, promoting the formation of new tubules and contributing to the local development of new blood vessels (9). Exosomes containing hypoxia-regulated mRNAs and proteins [metalloproteinases (MMP), IL8, platelet-derived growth factor, and caveolin-1] mediate hypoxia-dependent intercellular signaling of glioblastoma cells (48). Furthermore, exosomes isolated from cells growing in hypoxic conditions compared with normoxic conditions induce angiogenesis *in vitro* and *ex vivo* by stimulating endothelial cells to secrete specific growth factors and cytokines (48).

Concerning the bidirectionality of this network of communication, stromal cells talk back to cancer cells and promote tumor progression. Exosomes from CAFs induce an autocrine production of Wnt family member 11 (Wnt11)-associated exosomes by breast cancer cells, activating signaling pathways that increase cell motility and invasion (49). Together with the mechanism of juxtacrine signaling, breast cancer CAF exosomes released under Rab27b control have been shown to activate the retinoic inducible gene 1 (RIG-I) antiviral pathway in cancer cells (50). Activation of antiviral response in cancer cells is closely associated with the tumor reaction to chemo and radiotherapy. Classically, RIG-I activation is triggered by specific motifs of pathogen-associated nucleic acids. Boelens and colleagues observed that CAF-derived exosomes contained noncoding RNAs specially enriched in transposable elements that were able to trigger RIG-1 (50). More recently, pancreatic cancer CAFs showed increased production of exosomes when treated with gemcitabine (51). This treatment also affected the exosomes' content by increasing the presence of SNAIL1 and miR-146a. By treating pancreatic cancer cells with gemcitabine-derived CAFs exosomes, cancer cells were able to resist therapy and increase proliferation (51). These findings pinpoint to stromal-derived exosomes ability to enhance prooncogenic features such as migration and therapy resistance.

The role of exosomes in intercellular communication within the tumor microenvironment has been addressed in multiple studies. However, the majority present limited *in vivo* data and lack validation on human samples. This urges for the development of new strategies that efficiently address cancer exosomes interplay with the tumor microenvironment.

Exosomes and Metastasis

The role of cancer exosomes in metastasis was extensively addressed over the past years. One of the first events to take place in metastasis is the acquisition of cell motility and invasive capacity (52). Cancer cells have developed exosome-mediated strategies to potentiate numerous biological processes, one of which being the ECM degradation. Invadopodia are subcellular structures found in cancer cells, mainly composed of actin, that degrade ECM. *In vitro* studies showed that cancer exosomes induce invadopodia formation (53). Similarly, invadopodia were identified as crucial docking sites for CD63 and Rab27a MVBs, known as "exosome factories," demonstrating a tight binding between exosomes secretion and cancer invasion processes (53). Further *in vivo* findings highlight exosomes as important effectors of cancer cells' ability to move in a nonstochastic fashion. It was shown that exosomes are involved in a critical autocrine mechanism responsible for directing cell movement and influencing the migration speed of cancer cells (10, 11). These processes are dependent on exosomes ECM cargo, namely fibronectin. Exosome cargo was also found to contribute to ECM degradation both through direct or indirect manners. Proteases in cancer exosomes can degrade collagen, laminin, and fibronectin (10), while exosomal HSP90α activates two extracellular proteases (MMP-2 and plasmin) in cancer cells (54). Therefore, cancer exosomes seem to regulate cell movement and increase cell invasion potential by promoting ECM degradation.

Toward a prometastatic phenotype, cancer cells acquire additional malignant features besides ECM degradation, which includes the ability to cross the endothelial barrier, a process known as intravasation (52). miRNA-105 is involved in the disruption of vascular endothelial tight junctions by interference with a key protein, the tight junction protein 1 (ZO-1), promoting metastasis formation (55). Notably, miRNA-105 was detected in circulating exosomes from breast cancer patients at premetastatic stages (55). Similarly, a recent study demonstrates the ability of gastric cancer (GC) exosomes to promote peritoneal metastasis through the destruction of the mesothelial barrier (56). *In vitro* assessments proposed apoptosis and mesothelial-to-mesenchymal transition (MMT) as two of the mechanisms that could explain mesothelial barrier disruption upon treatment with GC exosomes. Notably, mesothelial cells that had not completely detached of the peritoneum presented lower expression levels of ZO-1 levels compared with control groups of mice with an intact mesothelial barrier (56).

Following cancer cell invasion and intravasation, cancer cells can then colonize a secondary organ (52). It is well described that primary tumors show a tendency to metastasize to specific secondary sites (57). Recently, the relation between metastatic organotropism and cancer exosomes has been explored. *In vivo* studies show pancreatic cancer exosomes are preferably uptaken by lung, liver, and brain cells (58). Interestingly, Hoshino and colleagues found that metastasization could be redirected using exosomes derived from cells known to metastasize to specific sites. In addition, a distinct integrin expression pattern in exosomes that correlates with cancer cells' metastatic organotropism was identified and the exosomes' integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ were associated with lung metastasis, while $\alpha_V\beta_5$ was associated with liver metastasis (58). Blocking these integrins reduces exosomes uptake and their expression in patient-derived exosomes correlates with metastasis, showing these integrins could be a useful tool to predict future metastasis formation (58). The idea that cancer exosomes biodistribution can preclude metastatic dissemination is of great interest and should urge for further studies in view of validation and understanding if blocking cancer exosomes dissemination can be a new avenue to prevent dissemination and/or establishment of cancer cells at secondary organs.

Finally, exosomes' role in the premetastatic niche formation has also been extensively reported. The premetastatic niche describes the ability of hematopoietic precursor cells from the bone marrow to home to specific sites before the arrival of tumor cells, paving the way for metastatic cells through the formation of niches where they can seed and proliferate (59). In this sense, cancer exosomes are the perfect long distant messengers due to all their known biological features. Indeed, melanoma exosomes influence free melanoma cells distribution within a lymph node *in vivo* (60). The number of melanoma cells found preferentially at the periphery of lymph nodes in mice pretreated with exosomes

was significantly higher when compared with treatments with liposomes (60). In addition, sentinel nodes in mice treated with exosomes show upregulation of genes involved in cell recruitment, ECM remodeling, and vascular proliferation factors, contributing to the establishment of a microenvironment that favors melanoma cell recruitment and colonization (60). Additional studies showed that melanoma exosomes home to the subcapsular sinus in lymph nodes (61). Furthermore, the presence of hepatocyte growth factor receptor protein in melanoma-derived exosomes induces vascular leakiness at premetastatic sites, and reprograms bone marrow progenitors toward a provasculogenic phenotype (62). The same holds true for pancreatic cancer exosomes, which create a receptive niche for liver metastasis, through the proinflammatory and fibrotic migration inhibitory factor, and TGFβ production leading to ECM remodeling (63). A recent study also showed GC exosomes' ability to promote metastasis in an EGFR-dependent mechanism (64). Liver cells incorporate EGFR of gastric cancer (GC) exosomes and display the exogenous receptor on their membrane, both *in vitro* and *in vivo*. The exosomal EGFR correlates with the downregulation of miRNA-26a and b that in turn regulate *HGF* mRNA. Therefore, increased levels of HGF in the liver, but not in GC liver metastasis, were observed upon GC exosomes treatment of mice bearing GC. High levels of HGF promoted liver metastasis formation and growth, leading to an activation of MET (HGF receptor) exclusively on GC liver metastases. EGFR was also found in exosomes derived from serum of GC patients but not in exosomes derived from normal human serum (64).

Research on exosomes has come a long way. Intensive efforts have been made to use biological models that can give better insights on exosomes biological significance during the process of metastasis. An ingenious model based on the Cre-loxP system was developed, which allowed EV tracking and, most importantly, showed the transfer of functional cargo *in vivo* (65). In this model, donor cells expressing Cre recombinase are CFP positive (blue), whereas unrecombined Cre-reporter cells are DsRed positive. The cells that internalize vesicles carrying Cre recombinase and suffer recombination, switch from red to green. *In vivo* application of this methodology comprised the first evidence of functional cargo transfer trough EVs *in situ* (65). Moreover, this study further demonstrated the transfer of exosomes among cancer cells and between cancer and noncancer cells, which lead to an enhanced migratory and metastatic potential of less malignant cells upon transfer of exosomes from more malignant cells (66). Nonetheless, alongside *in vitro* to *in vivo* extrapolations, as much as possible all findings should be backed up by human data, which empower the results and reflect their potential biological significance in humans. Indeed, some studies benefit from valuable human data such as the case of using exosomes derived from late-stage human lung cancer serum to treat human bronchial epithelial cells (HBEC), which promoted epithelial-to-mesenchymal transition (EMT) and increase migration and invasive capacities, demonstrating human cancer–derived exosomes' intrinsic ability to promote a prometastatic phenotype (67). Other approaches include validation of specific exosomes effectors and/or targets in human samples. The abovementioned study concerning GC exosomes capability of liver premetastatic niche formation is one good example in which human data supports both *in vitro* and *in vivo* data (64). Collectively, studies addressing exosomes biological functions, namely during metastasis formation, should be developed in biological systems that closely resemble the human disease, moving exosomes studies toward biological significant findings.

Future Perspectives on the Study of the Biology of Cancer Exosomes

Despite current efforts, the tools and the methodologies available to study cancer exosomes do not reflect the great increase of interest in this research area over the past years. Studies that aim to understand exosomes biodistribution and biological functions rely on two main approaches. One is based on the administration of labeled exosomes purified from cell lines into animal's circulation, while the other accounts for the injection of genetically engineered cells that produce labeled exosomes (68). Exosomes labeling techniques are of utmost importance to track their fate whether *in vitro* or *in vivo*. The engineering of an exosomal marker fused to a fluorescent protein has already been put into practice, for instance, by developing a stable cancer cell line expressing CD63-GFP (69). Nonetheless, whether these cells are used to produce labeled exosomes to perform *in vivo* treatments or if they are directly injected into mice, these techniques do not reflect the correct location, concentration, and nature of exosomes released by cancer cells. Moreover, the variations seen when using different exosomes cell sources, the total amount of exosomes injected in mice, their route of administration as well as the fact that most studies are performed in immunodeficient animals, accentuates the difficulty to achieve conclusions regarding their biodistribution and their biological significance (70). These observations highlight the need to develop new strategies to overcome current difficulties in attributing to exosomes a biologically significant role. The previously described approach designed by Zomer and colleagues despite original and very promising present some drawbacks (65). This method is not exosome specific as cells express a fluorescent reporter that can label all sorts of EVs in addition to exosomes, and it depends on the inoculation of cell lines *in vivo*. Nonetheless, it was the first step made toward the development of a more biological significant system to study EVs exchange *in vivo*. Development of genetically engineered mouse models (GEMM) to trace exosomes *in vivo* would be of great value. Indeed, the first CD63-GFP animal model was already created (71). However, the constitutional expression of the fusion protein does not allow the specific tracing of cancer exosomes as every cell type produces labeled exosomes. Ideally, the existing GEMMs available, which reliably recapitulate human cancer development and disease heterogeneity, would also allow the expression of exosomes' marker-reporter specifically by cancer cells (Fig. 2A), hence, enabling the study of the flow of cancer exosomes in a biological background that closely resembles human disease. A method like this involves the overexpression of an exosomes marker, which might have implications on the cell biology and possibly on the exosomes biogenesis itself, influencing normal tumor development (72). The expression of tetraspanins, such as CD63, CD9 and CD82, has already been associated with alterations on cancer cell mobility, which correlate with tumor metastasis (73, 74). Curiously, the effects caused by the downregulation of these surface proteins differ with cancer type. For example, decreased CD63 expression levels are associated with melanoma growth and invasion (75), while in pancreatic and non–small cell lung cancer does not correlate with the disease progression (76). As a result, animal models that follow this strategy need to be

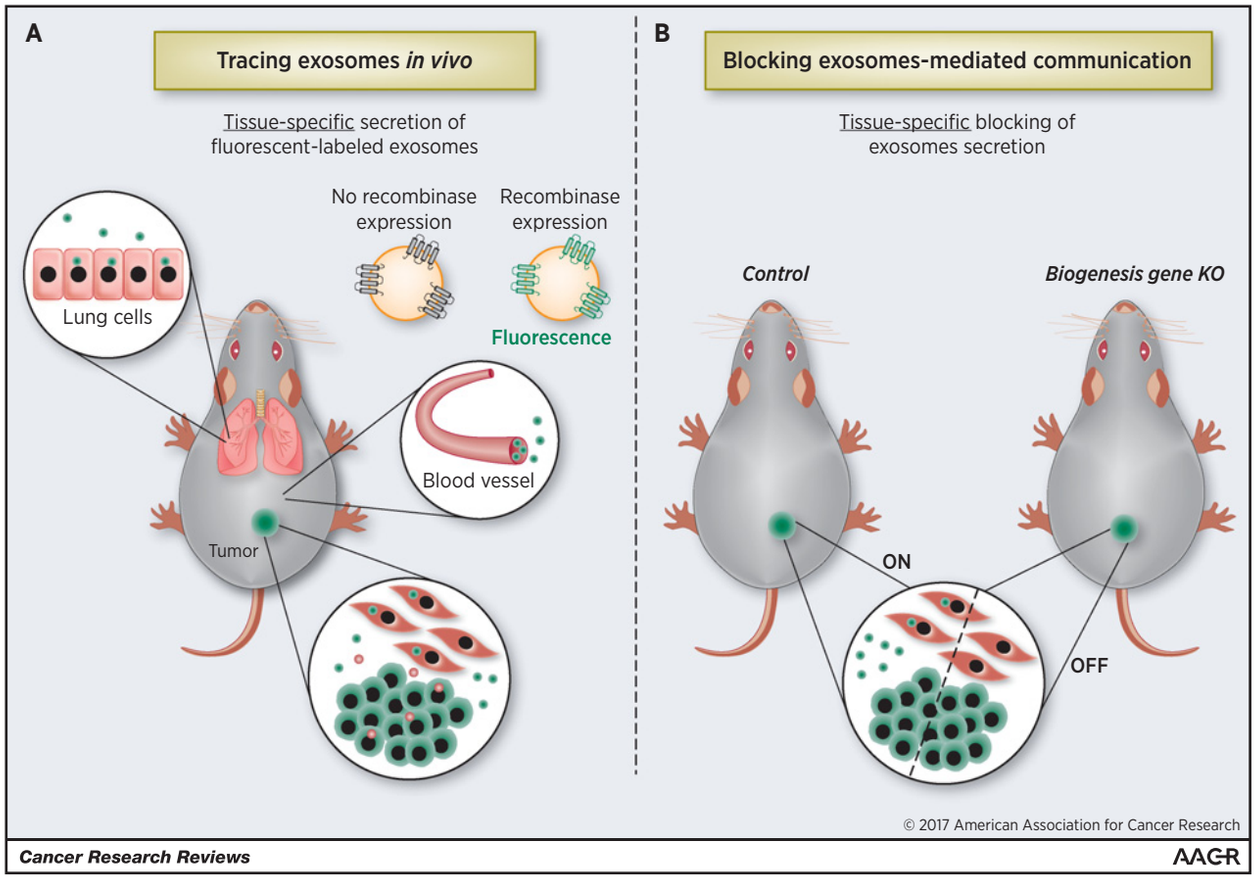

Figure 2.

New strategies to approach the biological significance of exosomes *in vivo*. **A,** GEMM to trace tissue-specific exosomes. Using a transgene knock-in comprised of an exosomal marker fused with a fluorescent reporter, it is possible to activate its expression through recombinase action regulated by a cell lineage–specific promoter. Once activated, both cell of origin and exosomes will be labeled with fluorescence. **B,** GEMM for blocking exosomes production. Knockout model of genes involved in exosomes biogenesis would allow to downregulate exosomes secretion and demonstrate their role in cancer progression.

comprehensively characterized for biological alterations that might affect tumor features. Also, exosomal markers should be tailored according to each cancer type. In addition, another drawback of such models would be the limitation of looking at only a subpopulation of exosomes, due to the heterogeneous nature of the markers on their surface (18). Nonetheless, we believe this would be a considerable leap toward the establishment of better models to study exosomes biology.

In addition to the described studies that try to identify specific exosomes' molecular effectors implicated in cancer biological processes, others focus on a more general approach by blocking cancer exosomes production. A sphingomyelinase inhibitor drug, the GW4869, is frequently used to impair the formation of ILVs and/or exosomes release by the MVB fusion to the PM (77–79). GW4869 treatments induce a significant reduction on the number of secreted exosomes (80). Other studies showed exosomes impaired secretion through the silencing of different genes involved in exosomes biogenesis, like Rab27a or nSMase2 (17, 62, 81). Further *in vivo* experiments show hampered tumor growth and metastasis burden when exosome-mediated communication is inhibited (62, 81). In addition to the models previously described, it would be interesting to develop GEMMs in which exosomes biogenesis would be affected specifically in cancer cells (Fig. 2B). Such models could be achieved through the knockout of a gene involved in exosome biogenesis. This type of model would contribute to the understanding of exosomes role during tumor initiation and progression, addressing their role in cancer development. Nonetheless, as clearly pointed by Bobrie, these proteins might also be involved in the secretion of nonexosome-related proteins, as is the case of Rab27a, which is also involved in the secretion of the prometastatic MMP-9 (82). These data highlight the need for thorough controls when exosomes biogenesis proteins are being tampered with. Only in this case we can fully understand which phenotypes can be attributed to impaired exosome secretion, rather than secondary effects of other secreted factors that might be altered in these models.

We are aware that the described models are not ideal and could present limitations. However, in light of the current available tools, these approaches would serve as means to study cancer exosomes in a more direct and specific manner and in a biological system that more closely resemble human disease.

Concluding Remarks

Since their first description three decades ago, information on exosomes biology and their role in cancer has led to an exciting period of progress and developments in the field (15). Exosomes intrinsic ability of horizontal cargo transfer at local and distant sites enables them to phenotypically reprogram cells (19). Because of this, there is no doubt that exosomes are important players in major steps of cancer development, from escape to immune surveillance, to tumor microenvironment reprogramming and metastasis establishment. Nonetheless, the bulk of these findings arise from *in vitro* experiments or *in vivo* xenograft models, approaches that face several limitations (68). We believe there is a pressing need for the development of informative animal models that enable exosome study closer to the normal biological system. As those models are still not available, all studies should comprehensively gather *in vitro* data and build careful *in vivo* approaches that address cancer exosomes proposed biological functions. Before this is achieved, the translation potential of data on cancer exosomes must be interpreted with caution.

On the other hand, despite the major advances observed, a void still holds regarding the function of exosomes in a nonpathologic condition. Much like hormones manage processes such as development, growth, reproduction, and behavior, do exosomes have central roles in a nonpathologic context? Further research on this unexplored side of exosomes is important to pursue. Exosomes biology is intriguing, to say the least. Much like platelets, which were first described as "spherules" in 1865 and thought to be "debris" of megakaryocytes (83), exosomes have come a long way and the first insights on their functions in health and disease are only now being unraveled.

Disclosure of Potential Conflicts of Interest

S.A. Melo has ownership interest (including patents) in patents. No potential conflicts of interest were disclosed by the other authors.

Grant Support

The laboratory is supported by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the projects "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274), and "The Role of Exosomes in Tumor Heterogeneity: More than Just Bubbling" (PTDC/BIM-ONC/2754/2014); and by Norte Portugal Regional Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), in the framework of the project NORTE-01-0145-FEDER-000029, and Maratonas da Saúde Prize. C. F. Ruivo is supported by FCT (PTDC/BIM-ONC/2754/2014), B. Adem by NORTE-01-0145-FEDER-000029, M. Silva by FCT (PD/BD/114160/2016), and S.A. Melo is supported by FCT (IF/00543/2013).

Received April 11, 2017; revised July 21, 2017; accepted September 25, 2017; published OnlineFirst November 21, 2017.

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