

Trends in Cancer

Volume 2 Number 2
February 2016
ISSN 2405-8033



Cancer Exosomes The Communication Bubbles

CellPress

February 2016
Volume 2, Issue 2

Review

Signaling by Extracellular Vesicles Advances Cancer Hallmarks

Masamitsu Kanada,¹ Michael H. Bachmann,¹ and Christopher H. Contag^{1,2,3,*}

Mammalian cells secrete various extracellular vesicles (EVs; exosomes, microvesicles, and apoptotic bodies) that differ in biogenesis, composition, and function. Each vesicle type can originate from normal or cancerous cells, transfer molecular cargo to both neighboring and distant cells, and modulate cellular behaviors involved in eubiology and pathology, such as tumor development. Here, we review evidence for the role of EVs in the establishment and maintenance of cancer hallmarks, including sustaining proliferative signaling, evading growth suppression, resisting cell death, reprogramming energy metabolism, acquiring genomic instability, and remodeling the tumor microenvironment. We also discuss how EVs are implicated in the induction of angiogenesis, control of cellular invasion, initiation of premetastatic niches, maintenance of inflammation, and evasion of immune surveillance. The deeper understanding of the biology of EVs and their contribution to the development and progression of tumors is leading to new opportunities in the diagnosis and treatment of cancer.

EVs Are Emerging as Essential Components of Physiological and Pathological Biology

Over the past decade, it has become apparent that cells can communicate via small membrane-bound vesicles called EVs in a paracrine and/or autocrine manner [1,2]. A causal role for EVs has been suggested in a multiplicity of physiological and pathological processes. For example, EVs in plasma are associated with inflammatory and autoimmune diseases, cardiovascular disorders, and metabolic syndrome [3]. EVs from normal and cancerous cells have a role in important biological processes that range from surface-membrane trafficking to horizontal transfer of proteins, DNAs, mRNAs, and noncoding RNAs among neighboring or distant cells [1,2,4]. Molecular profiling of various EV types has been performed to determine whether EVs derived from normal and cancerous cells exhibit unique molecular signatures that differentially modulate cellular functions and may be used for prognosis, diagnosis, and, possibly, directed therapy [2,5,6].

Forty years ago, it was suggested that glycolipid-based EVs contribute to normal cell signaling [7,8]. However, until recently, the exact nature, function, and biogenesis of EVs remained enigmatic. As first described in reticulocyte differentiation, multivesicular endosomes release EVs into the extracellular space by fusing with the plasma membrane [9,10] (reviewed in [11]). At present, at least three main types of EV are recognized according to an evolving consensus nomenclature: exosomes, microvesicles (MVs; also called ectosomes or microparticles), and apoptotic bodies (ABs) [4]. Given the recent and thorough review of EV biogenesis [11], our discussion of biogenesis is limited here to where it is relevant to the hallmarks of cancer.

Trends

Classification: Extracellular vesicles (EVs) are heterogeneous with at least three main classes of EV being distinguished: exosomes, formed by inward budding of the endosomal membrane; microvesicles (MVs), formed by direct budding from the plasma membrane; and apoptotic bodies (ABs), formed in cells undergoing programmed cell death. EV subpopulations continue to be redefined by their size, composition and function.

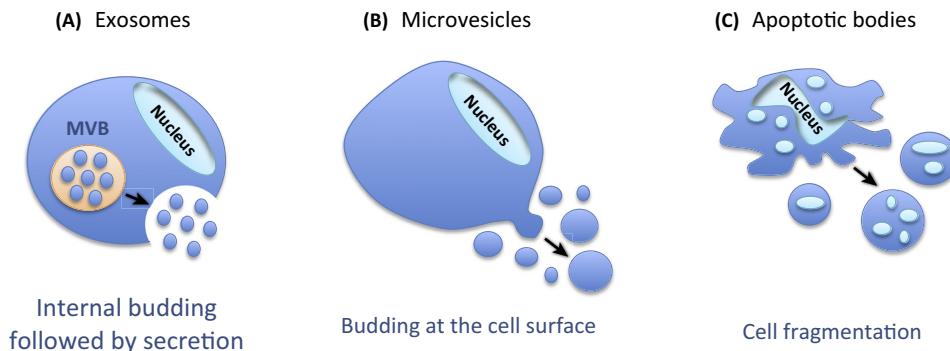
Molecular signatures: EVs derived from normal cells and those from diseased and malignant cells exhibit unique ‘molecular signatures’ that differentially modulate cellular functions in recipient cells. These signatures are identified to aid in cancer diagnosis and prognosis.

Cell-to-cell communication: tumor-derived EVs transfer molecular cargo to neighboring stromal and tumor cells and to distant cells, and, thus, affect cellular behaviors involved in tumor development and progression.

Therapeutic tools: EVs whose lipid bilayer membranes protect their cargo from degradation are being explored for their ability to deliver targeted molecules to specific cell types for directed therapy.

¹Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305, USA

²Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA



³Department of Radiology, Stanford University School of Medicine, Stanford, CA 94305, USA

*Correspondence: ccontag@stanford.edu (C.H. Contag).

Trends in Cancer

Figure 1. Main Classes of Extracellular Vesicles (EV). Exosomes are generated by inward budding of endosomal membranes. As these accumulate, they form multivesicular bodies (MVBs) that traffic from the cytosol to the cell surface, where they fuse with the cell membrane and release their exosome content to the extracellular space. By contrast, microvesicles (MVs) are formed by outward budding of the plasma membrane. Apoptotic cells release apoptotic bodies (ABs), which are formed by blebbing of the plasma membrane and contain nuclear fragments.

Exosomes, described first by Johnstone *et al.* in 1987 [12], are small vesicles (40–120 nm in diameter) secreted from endosomal compartments called ‘multivesicular bodies’ (MVBs). Their biogenesis begins with intraluminal vesicle formation by inward budding of the MVB membrane. The endosomal sorting complex required for transport (ESCRT) machinery and other components, such as lipids or tetraspanins, are involved in exosome biogenesis (reviewed in [13]). MVs are larger vesicles (50–1000 nm in diameter) created through direct budding from the plasma membrane. This step requires increased intracellular calcium, changes in membrane asymmetry, and reorganization of cytoskeletal proteins, and leads to phosphatidylserine externalization as MVs are shed [14]. ABs are also larger vesicles (500–2000 nm in diameter) that are released by cells undergoing apoptosis and may contain genomic DNA fragments and histones (Figure 1). In addition, amoeboid cancer cells secrete nonapoptotic large vesicles called ‘oncosomes’ (1–10 µm in diameter) [15]. Given that oncosomes originate from the plasma membrane, some of the pathways implicated in the biogenesis of MVs may also contribute to oncosome biogenesis (reviewed in [16]).

In the tumor microenvironment, populations of genetically and epigenetically highly diverse and often hierarchically stratified cancer cells engage with stromal cells, such as fibroblasts or endothelial and infiltrating immune cells, through reciprocal heterotypic signaling [17,18]. As tumors progress and their intercellular communication becomes increasingly distorted, cancer cells can secrete EVs and affect all of the hallmarks of cancer delineated by Hanahan and Weinberg [18,19]. Since communication in the tumor microenvironment is reciprocal, EVs secreted from stromal cells may in turn modulate the invasive and metastatic potential of cancer cells (Figure 2). Thus, understanding the differences between EVs from normal and cancer cells, the differences in their content, surface characteristics, timing of release, and other parameters of EV biology, will have important implications for cancer biology and medicine.

Below, we summarize the evidence for the involvement of tumor-associated EVs in tumor biology, specifically how EVs participate in and influence each hallmark of cancer. In Box 1, we discuss imaging modalities, tools, probes, and reporter systems that have been used to study EV biology and function. Since accurate characterization and classification of different vesicle types is in its early days, and overlapping subpopulations of vesicles may exist, conflicting data in this emerging field need to be carefully validated before a consensus can be established. Therefore, we use the term ‘EV’ to include all different classes and/or types of extracellular vesicles, when appropriate, to avoid potential misunderstanding and overinterpretation of data.

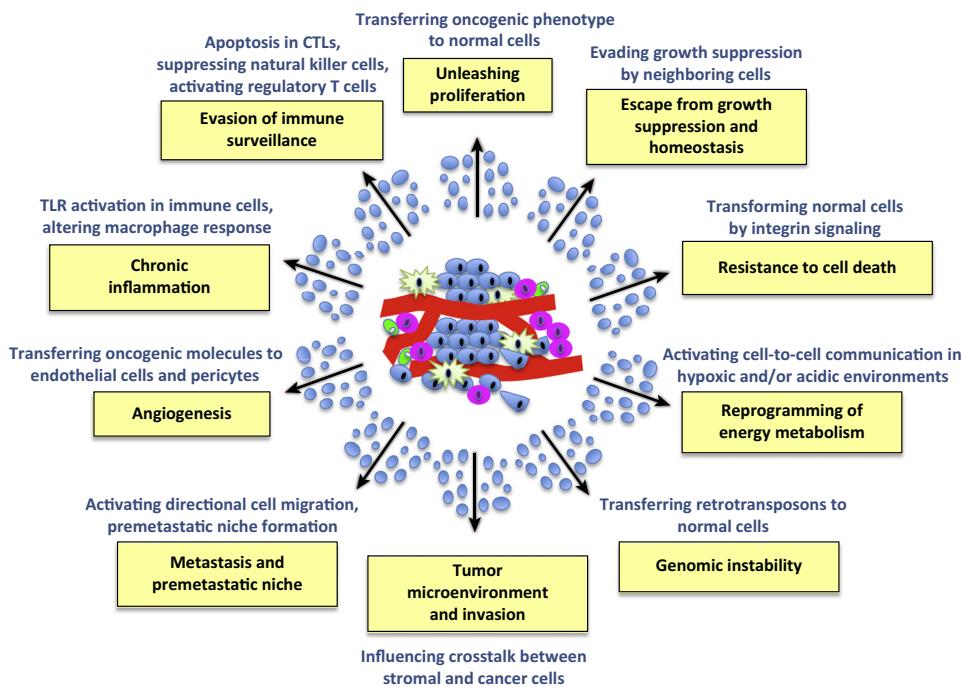


Figure 2. Cancer Hallmarks and Extracellular Vesicles (EVs). The hallmark capabilities that drive tumor progression are reinforced by the communication between cancer cells and tumor microenvironment cells via EVs.

Box 1. Imaging EVs in Cancer Biology

To visualize EVs and understand their biology, most studies have used optical imaging because of the abilities to image over a range of scales (from micro- to macroscopic) and to use the same marker in cell culture and in animal models (reviewed in [83]). Protein-based EV reporters were developed by genetically engineering membrane proteins or adding membrane-anchoring acylation tags [84–87]. With these imaging strategies, EV exchange between cells, loading of mRNA into EVs, and, more importantly, *de novo* translation in the recipient cells upon EV delivery were visualized [84]. In addition, a sensitive EV reporter combining GLuc and metabolic biotinylation allows multimodal *in vivo* imaging of EVs [85].

All these imaging tools are undoubtedly useful for the study of EV biodistribution, a critically important part of EV biology. However, the imaging of EVs does not reveal the complexity of EV-mediated cell-to-cell communication. To fully understand the role of EVs in cancer, their cargo needs to be comprehensively analyzed and their function visualized. The Cre-lox reporter system provides an imaging signal that reflects the transfer of active biomolecules to recipient cells *in vivo* and can lead to a persistent signal [41,88,89]. Although the functional transfer of Cre may not inform on physiological cell-to-cell communication, this imaging strategy enables recipient cell-specific analysis of molecular alterations associated with the cargo transferred by the EVs [88,89]. In addition, the permanence of the resulting signal may allow for smaller amounts of EVs to be used and these levels may be more physiologically relevant. Alternatively, fluorescent proteins can be palmitoylated for fluorescence imaging of EVs and of cell-to-cell communication both *in vitro* and *in vivo* [84]. Development of such imaging tools that reveal functional cell-to-cell communication is needed to fully understand EV-mediated cancer biology.

A noteworthy limit of *in vivo* studies of cancer cell-derived EVs is the experimental design itself. Most such studies have been conducted using a bolus injection of highly concentrated EVs derived from cell culture supernatants. Since EV concentrations in tumors, in the tumor microenvironment, and at the distant premetastatic sites are unknown, such studies may not represent a relevant physiological and/or pathological situation. Furthermore, tumor cells are thought to release EVs continuously and, therefore, these experimental designs, even with repeated bolus injections, may not adequately simulate the conditions that occur naturally in tumors. These important points should be carefully considered when biological effects mediated via EVs are studied and interpreted.

Unleashing Tumor Cell Proliferation

During tumor formation, accumulated genetic and epigenetic alterations constitutively activate the expression of proto-oncogenes and inactivate tumor suppressor genes [18]. Malignant transformation is reported to correlate with an increased number of secreted EVs [20,21] that may contribute to the spread of the transformed phenotype by transferring activated oncogenes between cells [4,20]. As an example, ABs may horizontally transfer tumor DNAs from mutant H-ras^{V12} and human c-myc-transfected rat fibroblasts to wild-type mouse fibroblasts, inducing a tumorigenic phenotype *in vivo* [4]. Similarly, aggressive glioma cells expressing EGFRvIII, a truncated oncogenic form of the epidermal growth factor receptor (EGFR), release EVs containing EGFRvIII that can be taken up by indolent glioma cells lacking this isoform. The acquired EGFRvIII activates growth promoting mitogen-activated protein kinase (MAPK) and Akt signaling pathways and triggers cellular transformation in the recipient cells [20].

The transfer of the oncogenic phenotype via EVs derived from cancer cells also affects heterotypic cells in the microenvironment during cancer progression, including endothelial cells, fibroblasts, and immune cells. For example, EVs derived from cancer cells overexpressing wild-type EGFR can induce angiogenesis by transferring the receptor to nearby endothelial cells, triggering the release of vascular endothelial growth factor (VEGF) and subsequent autocrine signaling activation via its receptor VEGFR2 [22]. Similarly, expression of tissue factor (TF) (a primary cellular initiator of blood coagulation and modulator of angiogenesis and metastasis) in colorectal cancer cells directly links the genetic status of the cells, such as an activated *Kras* gene or a loss-of-function mutation in the *p53* gene, to their *in vivo* angiogenesis and growth capacity. These genetic alterations influence the level and activity of TF not only at the cell membrane, but also on EVs. Such TF-containing EVs were suggested to upregulate angiogenesis and stimulate tumor growth *in vivo*, although *in vitro* they do not directly increase cancer cell proliferation and a direct interaction with endothelial cells remains to be demonstrated [23].

Escaping from Homeostasis and Growth Suppression

The elimination of damaged cells is important to maintain normal tissue architecture and function. To sustain tissue homeostasis, somatic epithelial cells use a ‘cell competition’ mechanism between surrounding cells, originally described in *Drosophila melanogaster*, that selects the fittest cell clones during development [24]. During cell competition, cell populations that are lost through damage (losers) are rapidly replaced by proliferating healthy cell populations ('winners'). Through local interactions, cells sense their relative metabolic status, triggering apoptosis in the losers and their phagocytosis by winners [25]. Signals emanating from apoptotic cells may stimulate the growth of winner cells within a given region [24]. The molecular mechanisms detecting metabolic aberrations and translating these signals into apoptosis remain largely elusive [26]. However, certain growth-regulating genes, including *Myc*, *Warts/Hippo*, and *p53*, are involved in cell competition, suggesting that these genes contribute to this homeostatic process. For example, normal prostate cells secrete EVs containing miRNAs, including miR-143, which can attenuate but not completely suppress the growth of abnormal prostate cancer cells [27]. In another case, EVs derived from bone marrow mesenchymal stem cells (BM-MSCs) also contain tumor-suppressive miRNAs, including miR-23b, and can suppress proliferation and invasiveness of bone marrow-metastatic CD44⁺/CD24⁻ breast cancer stem cells (CSCs) while decreasing their susceptibility to the chemotherapy drug docetaxel [28]. In contrast to cell competition in the epithelium that leads to apoptosis, metastatic breast cancer cells in the bone marrow undergo quiescence-like changes that are thought to contribute to cancer dormancy in response to EVs from BM-MSCs [29]. How tumor cells evade growth suppression via EVs derived from neighboring normal cells remains largely unknown [28].

Resisting Programmed Cell Death

For a cancer to survive and grow, tumor cells must evolve strategies that limit or circumvent apoptosis [30]. EVs have been shown to transfer an antiapoptotic phenotype to neighboring cells [31]. For example, EVs derived from highly aggressive MDA-MB231 breast cancer cells and U87 glioma cells can confer malignancy characteristics, such as anchorage-independent growth and survival capability in nutrient-limiting conditions, onto normal fibroblasts and epithelial cells [31]. Proteomic analysis of MDA-MB231 and U87 EVs revealed that the effect requires EV-mediated transfer of the protein cross-linking enzyme tissue transglutaminase (tTG) and dimerization of its substrate fibronectin on the EV surface. Furthermore, tumor cell-derived EVs stimulate kinases, such as focal adhesion kinase (FAK) and ERK, that are downstream of integrin activation, suggesting this as a possible mechanism of EV signaling [31]. Transfer of anchorage-independent growth capacity has also been shown in glioma cells expressing EGFRVIII [20].

Reprogramming Energy Metabolism

Under hypoxic conditions, glucose transporters and multiple enzymes of the glycolytic pathway are upregulated. Consequently, increased levels of metabolic acids are produced and secreted resulting in the acidification of the tumor microenvironment [32]. To survive abnormally low pH conditions, cancer cells upregulate proton pumps that correct their intracellular pH and render them resistant to changes in extracellular pH [33]. Tumor acidosis can increase the secretion and uptake of EVs, thus enhancing cell-to-cell communication within tumors [34]. Hypoxia increases shedding of EVs by breast cancer cells via the HIF-dependent expression of RAB22A, a small GTPase that colocalizes with budding MVs at the cell surface. These EVs can promote focal adhesion formation and invasion in the same cells *in vitro*, enhance extravasation *in vivo* after intravenous injection, and facilitate spontaneous metastasis to the lungs after mammary fat pad implantation [35]. Thus, metabolic changes in cancer cells within a hypoxic and acidic microenvironment promote active cell-to-cell communication via EVs that results in further cancer invasiveness and progression.

Promoting Genomic Instability

Normal cells strictly regulate the replication and repair of their genome during the cell cycle to maintain genetic integrity. The induction of genomic instability may be EV mediated because certain retrotransposon RNA transcripts, including HERV, LINE-1, and Alu elements, are enriched in tumor-derived EVs and can be transferred to normal endothelial cells [36]. However, it remains unclear whether these EV-encased retrotransposons are functionally delivered and inserted into the genome of the recipient cells.

In some tumor types, amplified chromatin DNA is present in the form of extrachromosomal circular fragments called ‘double minutes’ [37]. Lacking centromeres, these fragments may become separated from chromosomes during mitosis and left in the cytoplasm at G1 phase, from where they can be eliminated by extrusion out of cells within micronuclei [38]. Some studies have shown that tumor cell-derived EVs can functionally transfer fragments of genomic DNA [21]. EVs derived from rat epithelial cells transformed by forced expression of H-ras enclose chromatin-associated double-stranded genomic DNA fragments containing full-length H-ras [21]. Furthermore, these EVs can transiently transform normal epithelial cells. However, this and other studies [39] have demonstrated that the EV-loaded genomic DNA fragments cover the entire host genome. Thus, the mechanism of EV-mediated functional DNA transfer must be more complex than mere inclusion of double minutes. Similar genomic oncogene transfer was shown using plasma from patients with colon cancer that contained mutated K-ras [40], although whether this transfer was mediated by EVs was not examined.

It has been shown that EVs derived from transiently transfected tumor cells can functionally transfer plasmid DNA (pDNA) encoding reporter molecules to recipient cells while the

corresponding mRNA molecules are not expressed in recipient cells [41]. Therefore, the transfer of pDNA may have been facilitated by the same mechanism seen for genomic DNA fragments, suggesting that the underlying mechanism of EV-mediated transfer of pDNA and genomic DNA fragments will take us closer to understanding cell-to-cell communication via DNA contained in EVs.

Manipulating the Tumor Microenvironment, Heterotypic Interactions, and Invasion

Over the course of multistep tumorigenesis, cancers develop a complex supporting stroma [18]. In many solid tumors, the predominant stromal cells are fibroblasts that are ‘reprogrammed’ and show an altered phenotype, hence are distinguished as ‘cancer-associated fibroblasts’ (CAFs). CAFs include at least two distinct fibroblast cell types: one has similarities to regular fibroblasts that serve as the structural foundation for normal epithelial tissues; the other are myofibroblasts, normally involved in wound healing and inflammation and often defined by their expression of α -smooth muscle actin (α -SMA). In some carcinomas, the interstitial stroma is abnormally rich in myofibroblasts, which support cancer cell proliferation and enhance angiogenesis [42,43]. EVs containing TGF- β can drive differentiation of fibroblasts into myofibroblasts, as detected by the expression of α -SMA [44,45]. However, myofibroblasts generated with recombinant soluble TGF- β are not pro-angiogenic or tumor promoting, suggesting that EVs must transfer other biomolecules that shape these and other cells in the tumor microenvironment.

CAFs have key roles in cancer cell invasion and metastasis [46]. During cancer–stroma interactions, CAF-secreted EVs may promote the protrusive activity and motility of breast cancer cells via Wnt-planar cell polarity (PCP) signaling [47], which is transduced by a core module of conserved proteins, including Fzd6, Dvl1, Pk1, and Vangl1 [48]. Due to acylation and glycosylation, Wnt proteins associate tightly with the plasma membrane and the extracellular matrix, but how they function as long-range signaling molecules is still an outstanding question. One possible way to mobilize autocrine Wnt-PCP signaling and drive invasive behavior implicates Cd81-positive EVs secreted by CAFs and taken up by breast cancer cells. Autocrine Wnt proteins are tethered to the CAF-derived EVs that then mediate enhanced cancer cell motility and metastasis [47]. Thus, autocrine Wnt proteins can hitchhike on stroma-secreted EVs within the endocytic pathway to activate PCP signaling and dramatically change the phenotype and aggressiveness of breast cancer cells.

The crosstalk between stromal and breast cancer cells via EVs is also important for resistance to radiation and chemotherapy [49]. It was observed that EVs containing retrotransposon RNAs with 5'-triphosphate are uniquely produced from stromal fibroblasts if these are co-cultured with breast cancer cells that are resistant to radiation and chemotherapy, but not if the latter cells are therapy-sensitive. These retrotransposon RNAs activate the pattern recognition receptor RIG-I, which induces upregulation of interferon-related DNA damage resistance signature genes (*STAT1*, *ISG15*, *IFIT1*, *MX1*, and *OAS1*) in therapy-sensitive cells [49]. Thus, stromal cells can enable information flow between breast cancer cells by providing EVs to mediate stress response signaling and expand the pool of therapy-resistant tumor cells.

Metastasis: From Preparing Premetastatic Niches to Establishing Colonies

A novel EV-mediated mechanism appears to upregulate cancer cell motility. In a fibrosarcoma model, autocrine secretion of EVs coated with fibronectin (FN)-integrin $\alpha_5\beta_1$ complexes promoted directionally persistent cell migration at the leading edge in culture and *in vivo* [50]. The proposed model suggests that these FN-integrin-coated EVs bind to both collagen fibrils and cellular integrins to facilitate integrin clustering and strong adhesion formation [50]. Furthermore, EVs derived from different tumor types have distinct integrin expression patterns and those integrin types may determine organ-specific metastasis. EVs containing integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$

are associated with lung metastasis, whereas expression of integrin $\alpha_v\beta_5$ on EVs is linked to liver metastasis [51].

Bone marrow-derived cells (BMDCs), including macrophages, neutrophils, mast cells, myeloid cell-derived suppressor cells (MDSCs), and MSCs, contribute to malignant progression and are critical components of the premetastatic niche [52]. Kaplan *et al.* showed that bone marrow-derived hematopoietic progenitor cells expressing VEGFR1 home to sites with potential for metastasis and demonstrated that BMDCs form cellular clusters, thus initiating premetastatic niches before the arrival of tumor cells [53]. EVs also have a key role in the creation of the premetastatic niche. For example, EVs derived from highly metastatic melanoma cells increase the metastatic behavior of primary tumors by reprogramming bone marrow progenitor cells toward a pro-angiogenic phenotype dependent on the receptor tyrosine kinase MET [54]. Pancreatic cancer-derived EVs containing the macrophage migration inhibitory factor (MIF) induce TGF- β secretion in liver Kupffer cells, leading to the activation of hepatic stellate cells and extracellular matrix remodeling. This microenvironmental remodeling promotes an influx of bone marrow-derived macrophages, providing a favorable niche for pancreatic metastasis in the liver [55]. In addition to BMDC-associated premetastatic niche formation, cancer cell-derived EVs may directly contribute to the early phase of metastasis. Metastatic breast cancer cells, for instance, secrete EVs containing miR-105 that targets the mRNA encoding the tight junction protein ZO-1 in endothelial cells [56]. Thus, these EVs trigger the opening of the junctions between endothelial walls, increasing vessel leakiness, and facilitating cancer cell extravasation, which promotes metastasis to the liver and brain. In brain metastasis, astrocytes may epigenetically downregulate the expression of tumor suppressor PTEN in metastatic cancer cells by transferring a set of miRNAs via EVs, leading to increased secretion of the chemokine CCL2, which recruits IBA1-expressing myeloid cells that reciprocally enhance the outgrowth of brain metastatic cells [57].

Since Valadi *et al.* proposed that ‘exosomal shuttle RNA’ could mediate genetic exchange between cells [58], several studies have shown that the transfer of EV content can result in changes in the levels of miRNAs in recipient cells [59]. In addition, sorting miRNAs to EVs is modulated by cell activation-dependent changes in miRNA target levels in the producer cells [60]. This suggests that such a mechanism of miRNA compartmentalization also has implications for EV-mediated miRNA transfer and cell-to-cell communication from and to cancer cells. However, the significance of miRNA-mediated suppression of gene expression via EVs is still debated. Chevillet *et al.* [61] quantified the number of miRNA molecules per EV in EVs derived from diverse sources, including cancer patient plasma, and concluded that, on average, over 100 EVs would need to be examined to observe a single copy of a given abundant miRNA. However, accurate analysis of EVs is one of the biggest challenges in the field because different instruments show different EV size distributions and counts [62]. Although the abundance of miRNAs in EVs appears to be low, we need to consider that cells *in vivo* experience a constant flux of EVs and that the sheer numbers of EVs taken up over time by recipient cells may more than compensate for the inefficiency of packaging of miRNAs and other nucleic acids into EVs, such that EVs will, sooner or later, effect biological change.

Inducing Angiogenesis

As solid tumors grow, the cells most distant from the nearest blood vessel become nutrient deficient, hypoxic, or outright necrotic [63]. The hypoxic stress triggers neo-angiogenesis. Classically, angiogenesis is attributed to secreted pro-angiogenic factors, such as VEGF, that the hypoxic cells and tumors release to stimulate adjacent endothelial cells and to recruit mast cells and macrophages from the bone marrow. However, this mechanism may be incomplete. As noted above, glioma-derived EVs carrying EGFRvIII can induce autocrine VEGF-VEGFR activation in endothelial cells and subsequent neo-angiogenesis [22]. EV proteins and/or mRNAs

derived from the plasma from patients with highly malignant brain tumor glioblastoma multiforme (GBM) have a molecular signature that reflects the hypoxic status and aggressiveness of these tumors [64]. EVs derived from hypoxic GBM cells activate surrounding endothelial cells to secrete soluble factors that trigger PI3K/AKT signaling in pericytes and accelerate angiogenesis and tumor growth. Furthermore, in patients with GBM, EVs were found to be released by the tumors into the serum, enriched in angiogenic proteins, and able to stimulate angiogenesis *in vitro*, as measured by tubule formation in endothelial cells [65].

Cancer cells adapt to hypoxia primarily by HIF signaling, which regulates critical aspects of cancer progression, including angiogenesis, metabolic reprogramming, invasion, and metastasis [66]. Recently, a novel mechanism regarding HIF-1 α expression via EVs was found in nasopharyngeal carcinoma (NPC) cells infected with Epstein–Barr virus (EBV). The principal oncoprotein of EBV, LMP1, has been shown to associate and traffic with the tetraspanin CD63 into EVs, and restrain downstream NF- κ B activation by promoting trafficking in the endosomal-exosomal pathway inside infected lymphoblastoid cell lines (LCLs) [67]. LMP1 can be functionally transferred to stromal cells via EVs derived from NPC cells [68]. Surprisingly, these LMP1-positive EVs also contain HIF-1 α and actively transfer it to LMP1-negative recipient cells, resulting in the activation of the epithelial-mesenchymal transition (EMT) program with N-cadherin expression [69]. These studies show not only that human tumor viruses can utilize EVs for intercellular communication, but also that active transcription factors can be transferred to neighboring cells via EVs to modify the tumor microenvironment by controlling expression of target genes in stromal cells.

Fueling Chronic Inflammation while Blunting the Innate Immune Response

Tumor progression is closely related to chronic inflammatory processes and involves dysregulated activity of various types of immune cell [70,71]. Clinical and preclinical studies indicate that tumor-associated macrophages (TAMs) provide important protumoral growth and survival factors, pro-angiogenic factors, and extracellular matrix-modifying enzymes [72]. Tumor-derived EVs promote the induction and persistence of inflammation that often contributes to cancer progression [73] but whether and, if so, how TAMs are affected by these is unclear. The activation of TAMs in the tumor stroma is also implicated in cancer cell intravasation and metastasis. EVs carrying damage-associated molecular pattern (DAMP) molecules that act as danger signals [74] are released from stressed or injured tissues and participate in the induction and persistence of inflammation [75]. However, the relative biological importance of signaling via EV-associated DAMPs remains to be established.

Similar to EV-associated DAMPs, miRNAs can also bind to a type of pattern recognition receptor, the single-stranded RNA-binding Toll-like receptor (TLR) family [76]. Since TLR signaling can activate NF- κ B signaling and induce the secretion of proinflammatory cytokines, miRNAs, and other cargo elements in EVs, it may significantly contribute to inflammation and tumor progression. For instance, miR-21 and -29a contained in EVs derived from lung cancer cells may serve as ligands to murine TLR7 and human TLR8 in immune cells, including macrophages, triggering inflammatory responses that may ultimately enhance metastatic tumor growth in the lung [73]. Alternatively, NF- κ B activation in macrophages may be triggered through TLR2 via palmitoylated proteins, as has been shown for breast cancer cell-derived EVs [77].

Evading Immune Surveillance

The immune system can initially restrict tumor progression. However, these defense mechanisms are progressively disabled, such as by T cell inhibitory signals on cancer cells, including PD-L1 [71], which limit T cell effector function during direct cell–cell contact [78]. However, an emerging alternative mechanism of immune escape involves the active release of immunosuppressive EVs by cancer cells. For example, tumor-derived EVs were identified as immune

regulators that may help suppress the immune response against tumors [79,80]. EVs derived from human melanoma or colorectal carcinoma cells inhibit the differentiation of monocytes toward dendritic cells (DCs) and induce a TGF- β -secreting MDSC subset [81]. Moreover, various tumor-derived EVs have been shown to contain Fas Ligand (FasL), which may induce apoptosis in activated antitumor cytotoxic T lymphocytes (CTLs) and decrease the cytotoxicity of natural killer cells [79,80].

Concluding Remarks

Hanahan and Weinberg provided a highly useful conceptual framework for understanding the complex biology of cancer: the hallmarks of cancer [18,19]. While our knowledge of the many roles of EVs in biology in general and in cancer biology in particular is far from complete, the evidence we have gathered here already suggests that EVs are critical for some, if not all, cancer hallmarks. EVs contain various markers that are potentially involved in these hallmarks, such as oncogenes, mutated tumor suppressor genes, hypoxia-related molecules, angiogenic factors, immune regulatory proteins and/or noncoding RNAs, and various metabolites. The field of EV research has by now attracted the curiosity of many investigators and the number of studies investigating the role of EVs in cancer biology is expanding fast. However, a consensus regarding the nomenclature, standard isolation, or composition of EV subtypes has yet to be established. Even the current 'state-of-the-art' isolation methods are less than ideal [5,82]. By solving these and related questions (see Outstanding Questions), we anticipate that EV research will help clarify the complex biology of cancer and contribute to the design of improved diagnostics and therapies.

Acknowledgments

This work was funded in part through a generous gift from the Chambers Family Foundation for Excellence in Pediatric Research, and the Child Health Research Institute at Stanford University. We are grateful to the reviewers for their kind efforts and helpful comments.

References

1. Raposo, G. and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383
2. EL Andaloussi, S. et al. (2013) Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* 12, 347–357
3. Barteneva, N.S. et al. (2013) Circulating microparticles: square the circle. *BMC Cell Biol.* 14, 23
4. Bergsmedh, A. et al. (2001) Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6407–6411
5. Jia, S. et al. (2014) Emerging technologies in extracellular vesicle-based molecular diagnostics. *Expert Rev. Mol. Diagn.* 14, 307–321
6. Melo, S.a. et al. (2015) Glycan-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 523, 177–182
7. Esselman, W.J. and Miller, H.C. (1977) Modulation of B cell responses by glycolipid released from antigen-stimulated T cells. *J. Immunol.* 119, 1994–2000
8. Freimuth, W.W. et al. (1979) Soluble factors containing Thy-1 antigen shed from lymphoblastoid cells modulate in vitro plaque-forming cell response. *J. Immunol.* 123, 201–208
9. Harding, C. et al. (1983) Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97, 329–339
10. Pan, B.T. and Johnstone, R.M. (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 33, 967–978
11. Colombo, M. et al. (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* 30
12. Johnstone, R.M. et al. (1987) Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262, 9412–9420
13. Lo Cicero, A. et al. (2015) Extracellular vesicles shuffling intercellular messages: for good or for bad. *Curr. Opin. Cell Biol.* 35, 69–77
14. Boulanger, C.M. et al. (2006) Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension* 48, 180–186
15. Morello, M. et al. (2013) Large oncosomes mediate intercellular transfer of functional microRNA. *Cell Cycle* 12, 3526–3536
16. Minciucchi, V.R. et al. (2015) Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. *Semin. Cell Dev. Biol.* 40, 41–51
17. Gupta, G.P. and Massagué, J. (2006) Cancer metastasis: building a framework. *Cell* 127, 679–695
18. Hanahan, D. and Weinberg, R. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
19. Hanahan, D. and Weinberg, R. (2000) The hallmarks of cancer. *Cell* 100, 57–70
20. Al-Nedawi, K. et al. (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* 10, 619–624
21. Lee, T.H. et al. (2014) Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells. *Biochem. Biophys. Res. Commun.* 451, 295–301
22. Al-Nedawi, K. et al. (2009) Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3794–3799
23. Yu, J.L. et al. (2005) Oncogenic events regulate tissue factor expression in colorectal cancer cells: Implications for tumor progression and angiogenesis. *Blood* 105, 1734–1741
24. Johnston, L. (2009) Competitive interactions between cells: death, growth, and geography. *Science* 324, 1679–1682

Outstanding Questions

Are there unique EV profiles for each cell type, and for each phase of cellular differentiation?

Once released, are EVs from specific cells directed to specific tissue locations with tropisms for specific recipient cell types, or is the delivery of EV cargo random and nontargeted? If there is tropism, is it determined by membrane components, by the cargo, or by both?

Does one type of EV evoke the same or a different response in different recipient cell types? Do different fractions of EVs have different functions?

How are EVs released, how is this release regulated, and what is the EV concentration within tumors, their microenvironment, and at premetastatic sites?

What is the life span of EVs in the tumor microenvironment and in circulation?

What are the signals of MVB fusion with plasma membranes?

Which cargos loaded into tumor-derived EVs have biologic properties that contribute to the cancer hallmarks, and which are adventitious accessories?

Which EVs in the plasma can serve as diagnostic and prognostic biomarkers? Which can report on the state of the hallmarks of a given cancer that is influenced or even controlled by EVs?

25. Dejosez, M. *et al.* (2013) Safeguards for cell cooperation in mouse embryogenesis shown by genome-wide cheater screen. *Science* 341, 1511–1514
26. Vincent, J.-P. *et al.* (2013) Mechanisms and mechanics of cell competition in epithelia. *Nat. Rev. Mol. Cell Biol.* 14, 581–591
27. Kosaka, N. *et al.* (2012) Competitive interactions of cancer cells and normal cells via secretory microRNAs. *J. Biol. Chem.* 287, 1397–1405
28. Ono, M. *et al.* (2014) Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci. Signal.* 7, ra63
29. Aguirre-Ghiso, J.A. (2007) Models, mechanisms and clinical evidence for cancer dormancy. *Nat. Rev. Cancer* 7, 834–846
30. Lowe, S.W. *et al.* (2004) Intrinsic tumour suppression. *Nature* 432, 307–315
31. Antonyak, M.A. *et al.* (2011) Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17569
32. Cardone, R.a. *et al.* (2005) The role of disturbed pH dynamics and the Na⁺/H⁺ exchanger in metastasis. *Nat. Rev. Cancer* 5, 786–795
33. Parks, S.K. *et al.* (2011) pH control mechanisms of tumor survival and growth. *J. Cell. Physiol.* 226, 299–308
34. Parolini, I. *et al.* (2009) Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J. Biol. Chem.* 284, 34211–34222
35. Wang, T. *et al.* (2014) Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3234–E3242
36. Balaj, L. *et al.* (2011) Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat. Commun.* 2, 180
37. Vogt, N. *et al.* (2004) Molecular structure of double-minute chromosomes bearing amplified copies of the epidermal growth factor receptor gene in gliomas. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11368–11373
38. Shimizu, N. (2009) Extrachromosomal double minutes and chromosomal homogeneously staining regions as probes for chromosome research. *Cytogenet. Genome Res.* 124, 312–326
39. Kahlert, C. *et al.* (2014) Identification of double-stranded genomic dna spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J. Biol. Chem.* 289, 3869–3875
40. García-Olmo, D.C. *et al.* (2010) Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. *Cancer Res.* 70, 560–567
41. Kanada, M. *et al.* (2015) Differential fates of biomolecules delivered to target cells via extracellular vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 112, E1433–E1442
42. Orimo, A. *et al.* (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121, 335–348
43. Liang, P. *et al.* (2005) Myofibroblasts correlate with lymphatic microvessel density and lymph node metastasis in early-stage invasive colorectal carcinoma. *Anticancer Res.* 25, 2705–2712
44. Webber, J. *et al.* (2010) Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res.* 70, 9621–9630
45. Webber, J.P. *et al.* (2015) Differentiation of tumour-promoting stromal myofibroblasts by cancer exosomes. *Oncogene* 34, 319–331
46. Bhowmick, N.a. *et al.* (2004) TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 303, 848–851
47. Luga, V. *et al.* (2012) Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151, 1542–1556
48. Gray, R.S. *et al.* (2011) Planar cell polarity: coordinating morphogenetic cell behaviors with embryonic polarity. *Dev. Cell* 21, 120–133
49. Boelens, M.C. *et al.* (2014) Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. *Cell* 159, 499–513
50. Sung, B.H. *et al.* (2015) Directional cell movement through tissues is controlled by exosome secretion. *Nat. Commun.* 6, 7164
51. Hoshino, A. *et al.* (2015) Tumour exosome integrins determine organotropic metastasis. *Nature* 527, 329–335
52. Peinado, H. *et al.* (2011) The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin. Cancer Biol.* 21, 139–146
53. Kaplan, R.N. *et al.* (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 438, 820–827
54. Peinado, H. *et al.* (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* 18, 883–891
55. Costa-Silva, B. *et al.* (2015) Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat. Cell Biol.* 17, 816–826
56. Zhou, W. *et al.* (2014) Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* 25, 501–515
57. Zhang, L. *et al.* (2015) Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature* 527, 100–104
58. Valadi, H. *et al.* (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659
59. Redzic, J.S. *et al.* (2014) Extracellular RNA mediates and marks cancer progression. *Semin. Cancer Biol.* 28, 14–23
60. Squadrito, M.L. *et al.* (2014) Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. *Cell Rep.* 8, 1432–1446
61. Chevillet, J.R. *et al.* (2014) Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 14888–14893
62. Maas, S.L.N. *et al.* (2015) Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. *J. Control. Release* 200, 87–96
63. Vaupel, P. (2004) Tumor microenvironmental physiology and its implications for radiation oncology. *Semin. Radiat. Oncol.* 14, 198–206
64. Kucharzewska, P. *et al.* (2013) Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7312–7317
65. Skog, J. *et al.* (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476
66. Semenza, G.L. (2012) Hypoxia-inducible factors: Mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol. Sci.* 33, 207–214
67. Verweij, F.J. *et al.* (2011) LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-κB activation. *EMBO J.* 30, 2115–2129
68. Meckes, D.G. *et al.* (2010) Human tumor virus utilizes exosomes for intercellular communication. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20370–20375
69. Aga, M. *et al.* (2014) Exosomal HIF1α supports invasive potential of nasopharyngeal carcinoma-associated LMP1-positive exosomes. *Oncogene* 33, 4613–4622
70. Schäfer, M. and Werner, S. (2008) Cancer as an overhealing wound: an old hypothesis revisited. *Nat. Rev. Mol. Cell Biol.* 9, 628–638
71. Chen, D.S. and Mellman, I. (2013) Oncology meets immunology: the cancer-immunity cycle. *Immunity* 39, 1–10
72. Noy, R. and Pollard, J.W. (2014) Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 41, 49–61
73. Fabbri, M. *et al.* (2012) MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.* 109, E2110–E2116

74. Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12, 991–1045
75. Buzas, E.I. et al. (2014) Emerging role of extracellular vesicles in inflammatory diseases. *Nat. Rev. Rheumatol.* 10, 356–364
76. Lehmann, S.M. et al. (2012) An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nat. Neurosci.* 15, 827–835
77. Chow, A. et al. (2014) Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF- κ B. *Sci. Rep.* 4, 5750
78. Pardoll, D.M. (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* 12, 252–264
79. Wieckowski, E.U. et al. (2009) Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T lymphocytes. *J. Immunol.* 183, 3720–3730
80. Zhang, H-G. and Grizzle, W.E. (2011) Exosomes and cancer: a newly described pathway of immune suppression. *Clin. Cancer Res.* 17, 959–964
81. Valenti, R. et al. (2006) Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor- β -mediated suppressive activity on T lymphocytes. *Cancer Res.* 66, 9290–9298
82. Antonyak, M. and Cerione, R. (2015) Emerging picture of the distinct traits and functions of microvesicles and exosomes. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3589–3590
83. Sadovska, L. et al. (2015) Biodistribution, uptake and effects caused by cancer-derived extracellular vesicles. *J. Circ. Biomarkers* Published online March 25, 2015. <http://dx.doi.org/10.5772/60522>
84. Lai, C.P. et al. (2015) Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. *Nat. Commun.* 6, 7029
85. Lai, C.P. et al. (2014) Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter. *ACS Nano* 8, 483–494
86. Takahashi, Y. et al. (2013) Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J. Biotechnol.* 165, 77–84
87. Shen, B. et al. (2011) Protein targeting to exosomes/microvesicles by plasma membrane anchors. *J. Biol. Chem.* 286, 14383–14395
88. Ridder, K. et al. (2014) Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. *PLoS Biol.* 12, e1001874
89. Zomer, A. et al. (2015) In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell* 161, 1046–1057