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Abstract: Cancer cells emit a heterogeneous mixture of vesicular, organelle-like structures (microvesicles) into their surroundings including blood and body fluids . Microvesicles (MVs) are generated via diverse biological mechanisms triggered by pathways involved in oncogenic transformation, microenvironmental stimulation, cellular activation, stress, or death. Vesiculation events occur either at the plasma membrane (ectosomes, shed vesicles) or within endosomal structures (exosomes). MVs are increasingly recognized as mediators of intercellular communication due to their capacity to merge with and transfer a repertoire of bioactive molecular content (cargo) to recipient cells. Such processes may occur both locally and systemically contributing to the formation of microenvironmental fields and niches. The bioactive cargo of MVs may include growth factors and their receptors, proteases, adhesion molecules, signalling molecules, as well as DNA, mRNA and microRNA (miRs) sequences. Tumour cells emit large quantities of MVs containing procoagulant, growth regulatory, and oncogenic cargo (oncosomes), which can be transferred throughout the cancer cell population and to non-transformed stromal cells endothelial cells and possibly to the inflammatory infiltrates (oncogenic field effect). These events likely impact tumour invasion, angiogenesis, metastasis, drug resistance, and cancer stem cell hierarchy. Ongoing studies explore the molecular mechanisms and mediators of MV-based intercellular communication (cancer vesiculome) with the hope of using this information as a possible source of therapeutic targets and disease biomarkers in cancer.

MICROVESICLES AS MEDIATORS OF INTERCELLULAR COMMUNICATION IN CANCER - THE EMERGING SCIENCE OF CELLULAR 'DEBRIS'

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Abbreviations

MVs – microvesicles; TF – tissue factor; EGFR- epidermal growth factor receptor; EGFRvIII – mutant EGFR (variant III); GBM – glioblastoma multiforme; IL-6 – interleukin 6; IL-8 – interleukin 8; mRNA- messenger ribonucleic acid; PS – phosphatidylserine; RTK – receptor tyrosine kinase; VEGF – vascular endothelial growth factor, VEGFR-2 – VEGF receptor 2;

Abstract

Cancer cells emit a heterogeneous mixture of vesicular, organelle-like structures (microvesicles) into their surroundings including blood and body fluids . Microvesicles (MVs) are generated via diverse biological mechanisms triggered by pathways involved in oncogenic transformation, microenvironmental stimulation, cellular activation, stress, or death. Vesiculation events occur either at the plasma membrane (ectosomes, shed vesicles) or within endosomal structures (exosomes). MVs are increasingly recognized as mediators of intercellular communication due to their capacity to merge with and transfer a repertoire of bioactive molecular content (cargo) to recipient cells. Such processes may occur both locally and systemically contributing to the formation of microenvironmental fields and niches. The bioactive cargo of MVs may include growth factors and their receptors, proteases, adhesion molecules, signalling molecules, as well as DNA, mRNA and microRNA (miRs) sequences. Tumour cells emit large quantities of MVs containing procoagulant, growth regulatory, and oncogenic cargo (oncosomes), which can be transferred throughout the cancer cell population and to non-transformed stromal cells endothelial cells and possibly to the inflammatory infiltrates (oncogenic field effect). These events likely impact tumour invasion, angiogenesis, metastasis, drug resistance, and cancer stem cell hierarchy. Ongoing studies explore the molecular mechanisms and mediators of MV-based intercellular communication (cancer vesiculome) with the hope of using this information as a possible source of therapeutic targets and disease biomarkers in cancer.

Modes of intercellular communication – the emerging role of microvesicles.

In a multicellular organism, biological functions are executed by complex assemblies of cells, the actions of which must be coordinated by intercellular communication. In this regard, the exchange of signals is usually ascribed to specific molecules (soluble or immobilized) and their corresponding cognate receptors. This exchange may entail a direct cell-to-cell contact (adhesion, juxtacrine interactions), or gradients formed by soluble (paracrine) mediators, which may also circulate in blood and body fluids and act in a regional or systemic (endocrine) manner. Such information translates into activation of intracellular signalling networks and changes in the behaviour of individual cells and their populations[1;2]. Indeed, molecular pathways of cell-cell communication play an important role in development, health, and disease including cancer [3-5].

It is increasingly clear, however, that cells may also communicate *via* supramolecular complex mechanisms involving the exchange of cellular fragments, membranes, or specialized organelles. The latter could be of vesicular (microvesicles)[3], tubular (nanotubes/TNTs)[8;9] or filopodial (cytoneme)[4] nature, depending on their biogenesis and whether they are separated or contiguous with the emitting cell. The underlying process of the intercellular transmission of proteins, lipids, or nucleic acids encapsulated in plasma membranes is often referred to as trogocytosis, or cellular synapse[5;6]. Notably, this transfer entails 'pre-programmed' combinations of soluble or insoluble molecules, which are uniquely protected from degradation and dispersion in the extracellular space[7].

Of particular interest are mechanisms involving microvesicles (MVs), spherical or cup-shaped membrane structures that originate from 'donor' cells and may travel considerable distances in the interstitial space until they undergo uptake, fusion, or interaction with a range of 'acceptor' cells [3;8-14]. MVs can also reach cells located at a distance by being released into the circulating blood, lymph, cerebrospinal fluid (CSF), urine, glandular secretions, and other fluids. The effects MVs exert on various 'acceptor' (target) cells are rather diverse and may include sharing of interactive, signalling and enzymatic activities that would otherwise be compartmentalized to individual cells by their gene expression patterns [14]. This mechanism may explain a level of coordination and molecular integration within multicellular populations, as is often observed in heath and disease. In this article, we will consider the various possible roles of MVs in intercellular communication in general and especially as it relates to pathogenesis, progression, and therapeutic responses in cancer, recognizing that profound qualitative and quantitative differences may exist between various specific disease contexts [7].

Biogenesis and heterogeneity of microvesicles.

MVs have long been regarded as 'cellular debris', but this view is rapidly changing[7;13;16-20]. The release of MVs was first described by Wolf in 1967 who noted procoagulant particulate matter around activated blood platelets [15]. Subsequently, similar organelles, referred to as exosomes, were implicated by Trams as carriers of 5' exonucelotidase associated with glioma cells [16]; furthermore, the groups of Johnstone [17] and Stahl [18] established exosomes as a mechanism involved in the removal of spent transferrin receptors from differentiating reticulocytes [25;26].

Different biological circumstances under which formation of MVs (vesiculation) has been observed reflect the diversity of their biogenesis, structure, and function (Figure 1). Thus, cellular activation, transformation, stress, or programmed cell death are associated with a different output and nature of vesicular structures [7]. Indeed, it is clear that microvesicles are heterogeneous, and this has led to the usage of multiple names for their designation under different experimental settings [7;19]. Some of the most frequently encountered descriptors are: microvesicles, microparticles, ectosomes, exosomes, exosome-like vesicles, shed vesicles, and most recently oncosomes [13;25;26]. Other names have also been used in various specific settings, including: argosomes, promininosomes, P4 particles, prostasomes, and several others [15;25;26]. To some extent this diversity reflects the culture of different fields in which MVs have been studied, but also substantial biological diversity of the underlying biological process. Indeed, MVs originate through at least three distinct mechanisms: (i) breakdown of dying cells into *apoptotic bodies*, (ii) blebbing of the cellular plasma membrane (*ectosomes*), and (iii) the endosomal processing and emission of plasma membrane material in the form of *exosomes* [13;25-27].

Apoptotic bodies are relatively large (up to 4000 nm in diameter) and contain genomic DNA and intact organelles. Since they result from cellular breakdown, their generation has self-limiting dynamics, but is not devoid of biological influences[20]. Indeed, the cargo of apoptotic MVs remains protected from degradation and is often ingested by tissue phagocytes or neighbouring cells.

Ectosomes are MVs that emerge from the outward blebbing of the cellular plasma membrane [7;13]. These MVs (also known as shed vesicles, or microparticles) may range in sizes between 100 nm and 1000nm in diameter, and are characterized by the prominent exposure of phosphatidylserine (PS) residues on their outer surfaces, among other markers (Table 1). Ectosome-like MVs have been commonly associated with membrane regions containing high levels of cholesterol and signalling complexes, often referred to as lipid rafts [13;29]. Indeed, certain lipid raft-associated molecules can be found in the cargo of these MVs including tissue factor (TF) and flotillin-1[21]. Depending on the cell type, membrane MVs may be rich in cellular lineage markers, β1 integrin, matrix metalloproteases (MMPs) and their activators (EMMPRIN), P-selectin glycoprotein ligand 1 (PGSL1), cytokines and chemokines (e.g. interleukin 1 β, IL1β; interleukin 8, IL-8), vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF2)[19;31;32;32-34]. Their relatively large size, surface PS, and the presence of specific molecules are often used to distinguish these MVs from exosomes (Table 1).

Biogenesis of ectosome-like MVs has been analysed in various settings. Paradigmatic, in this regard, are studies demonstrating the release of procoagulant microparticles (MPs) from activated platelets engaged in processes of haemostasis and thrombosis[22]. The significance of this process is illustrated by a rare congenital bleeding disorder, known as Scott syndrome, in which platelet microvesiculation is permanently altered[23]. This results from a defect in the enzymatic activity responsible for the maintenance of phospholipid asymmetry in the plasma membrane [22] such that the active translocation of PS residues from the inner to the outer leaflet of the surface bilayer is impaired causing a deficiency

in vesiculation. It is now understood that enzymes directly involved in ectosomal vesiculation of platelets include aminophopholipid translocase, scramblase, floppase, and calpain [22].

Another cellular paradigm that may be informative with respect to ectosome-like vesiculation has been recently described in the context of inflammatory responses. For instance, in the case of CNS phagocytes (microglia), acidic sphingomyelinase (aSMase) is both necessary and sufficient for ectosome release. In this case, activation of the purinergic P2X7 receptor upon exposure to ATP acts as a triggering stimulus[24]. It is proposed that dying cells release ATP, which stimulates microglia to release MVs containing proinflammatory cytokines (IL1β) and to orchestrate the clearance of cellular debris. These processes are blocked by inhibitors of p38 MAPK and Src kinase [24]. This is intriguing as the respective signalling modules (e.g. src) are also involved in oncogenic signalling events. Whether vesiculation of cancer cells involves a similar src/aSMase-dependent mechanism is currently being explored[25].

Exosomes are markedly different from membrane MVs and ectosomes with respect to their mechanism of generation, structural properties, and molecular cargo [7;13;15;62]. The phospholipid composition of exosomes is distinct from that of ectosomes such that a lower abundance of PS residues is exposed on the outer leaflet. Exosomes are also smaller than membrane MVs with a diameter ranging from 30 nm to 100 nm [26]. Arguably, smaller vesicles may also be generated by mechanisms separate from endocytic pathway formation of exosomes, for example, the biosynthesis of CD133-

postive promininosomes [27]. However, these distinctions require further analysis. Exosomes transport different cargo compared to other MVs emanating from the same cell; indeed, reports confirm the selective enrichment of specific tetraspanins (CD63; Tspan8) and heat shock proteins (HSP70) in exosomes [53;73]. These differences in size, membrane composition, and cargo are often used for preparation and characterization of exosomes and other MVs (Tables 1 and 2).

Biogenesis of exosomes is controlled by a distinct cellular pathway [7;16;26] the initial steps of which are controlled by the endosomal sorting complex required for transport (ESCRT)[28]. This, signalling events involved in the recycling of membrane receptors lead to formation of inward invaginations of plasma membrane microdomains coated with clathrin protein (clathrin-coated pits) [29]. These evolve into intracellular vacuoles (early endosomes) that under control of ESCRT mature into the late endosome/multivesicular bodies (MVBs). At this stage, the endosomal cargo has 4 potential fates, it can be (1) recycled back to the plasma membrane; (2) sequestered in intraluminal vesicles (ILVs) within MVBs [7;26;27]; (3) degraded upon fusion of MVBs with lysosomes; and (4) released as exosomes following the redirection and fusion of MVBs with the plasma membrane.

Out of four distinct ESCRT complexes (ESCRT-0, ESCRT-1, ESCRT-2, and ESCRT-3), involved in endosomal pathway, ESCRTs-0, 1, and 2 have ubiquitin-interacting modules that are necessary for the sequential sorting of cargo destined for degradation[28], while exocytic MVBs may form in unbiquitination-independent manner[26]. An alternative pathway of exosome formation may involve bioactive membrane lipids such as the

sphingomyelin metabolite, ceramide, the synthesis of which is catalyzed by neutral sphingomyelinase (nSMase2) [30].

Mechanisms involved in the assembly of the microvesicle cargo

The molecular content of MVs is defined by processes of their formation as well as the state and nature of their parent cell. MV cargo includes a variety of molecular entities, which are not a random sample of the molecular repertoire of the originating cell. Instead, these include a distinct combination of lipids, proteins, and nucleic acids (mRNA; microRNA, miR; and DNA) [17;53;88;94]. It is generally believed that lipid rafts give rise to the formation of ectosomes while endocytic clathrin pits are representative of at least one mechanism that initiates the formation of exosomes. Studies demonstrating reduced ectosome release following depletion of plasma membrane cholesterol support ectosome biogenesis from lipid rafts [7;30;97]. Alternative pathways have also been proposed to function in the sorting of cargo into ectosomes such as endosomal recycling [98-101].

On the other hand, the content of exosomes follows the aforementioned endocytic pathway, which can be subdivided into ubiquitin-dependent and independent mechanisms[16;102;103]. It is well-described that ESCRT complexes sort ubiquitinated cargo into ILVs targeted for lysosomal degradation[31]. While the role of this pathway in exosome formation and sorting of mRNA and miRNA is unknown, the ESCRT 2 complex can bind directly to RNA independent of endosomal sorting and ESCRT 1/3 [32]. In spite of the recent progress in cataloguing the content of MVs and dissecting the processes involved in their formation, the specific cellular mechanisms that mediate the

sorting of molecular species into distinct classes of MVs is unknown. Nonetheless, many components of MV cargo have been implicated in cancer (Table 3), especially since cancer cells have a particularly high rate of vesiculation [6;13;19;110-112].

Microvesicle function.

The wealth of molecular cargo contained in MVs raises the question as to their biological role. In this regard, several mutually non-exclusive hypotheses have been put forward to explain the functional importance of vesiculation in various cellular contexts. It should be mentioned that while these concepts are reinforced by compelling molecular and cellular data, direct evidence for the requirement of MV formation *in vivo* is presently rather scarce. In this regard, it is thought provoking that null mutations affecting molecules strongly implicated as key biological effectors within MV cargo (e.g. TF, FGF, MMPs or VEGF) usually lead to different and often more severe consequences than deficiencies affecting the vesiculation process itself. The latter is exemplified by the genetic disruption of sphingomyelinases (asmase, nsmase)[33], scramblase, and other enzymes implicated in MV formation [34]. Likewise, clinical conditions (Scott, Castaman, or Griscelli syndromes) involving various aspects of impaired vesicle formation do not necessarily recapitulate deficits in what is often viewed as key cargo molecules found in various MVs. Furthermore, *in vivo* administration of drugs that either block MV generation (Imipramine)[24] or their uptake (Diannexin)[35] often leads to effects that may be reminiscent of, but not identical to, those observed during *in vitro* studies on MV function[6;15;115]. The same cells may exhibit different patterns of vesiculation *in vivo* and

in vitro[36] wich further argues that our understanding of what MVs actually do under realistic conditions remains to be studied more carefully.

However, it is becoming increasingly clear that the networks of intercellular communication via MVs have a potential to influence processes, as diverse as cell polarity, differentiation, migration, chemotherapy resistance, immunoregulation, inflammation, coagulation, angiogenesis and cancer metastasis [6;15;16;26;117;118]. Several scenarios have been proposed to explain the biological roles of various MVs. First, MVs could be viewed as a highly efficient mechanism of molecular 'dumping'[37]. Indeed, removal of superfluous or harmful molecules by exosome formation is well described; for instance, in the case of transferrin receptors that must be rapidly removed from reticulocytes to allow their differentiation into mature red blood cells [38]. Similarly, MVs allow rapid, 'defensive' shedding of complement attack complexes from the plasma membrane of cells that have undergone opsonization, thereby protecting them from destruction[13]. Removal of β-catenin from cells by production of exosomes under control of terraspanins has also recently been described as an alternative and unconventional pathway that regulates Wnt signalling [39].

MVs have been implicated as a unique vehicle for the release of soluble molecules, which are otherwise unable to interact with the classical secretory pathway due to the absence of a signal peptide in their sequences (IL-1β or basic FGF)[24;40;40]. In addition, microvesicular transport extends the extracellular half-life of secretable molecules (e.g.

VEGF), alters their gradient formation (Wnt, MMPs), and concentrates their activities at specific sites[59;117;120].

Perhaps most important and intriguing is the possibility that MVs may serve as a unique mechanism (or set of mechanisms) for the release of proteins that are *bone fide* insoluble. This includes membrane antigens involved in immunomodulation[121-123], transmembrane receptors (CCR5, TF, EGFR, HER-2, AXL) [32;39;124;125], transmembrane ligands (Dll4[41]), , and other cell surface molecules [111;128]. As these molecules are involved in a number of crucial biological processes, their release in MVs may place them in the context of other cells, with which they can change/expand the scope of their intrinsic biological activities (Table 3 [13;15;27;117]). Furthermore, MV-mediated release provides a platform for controlled enrichment, assembly of multimolecular complexes, and molecular combinations[42] with a pre-programmed composition of proteins, lipids and nucleic acids. In this regard, the biological activities encapsulated in MVs may result in effects that are quantitatively and qualitatively different from the sum of effects predicted for their individual molecular constituents. Such combinatorial interactions between elements of MV cargo and target cells may potentially lead to outcomes otherwise impossible to achieve.

Microvesicles as mediators of intercellular communication

With the possible exception of procoagulant MVs (microparticles) harbouring tissue factor (TF) or mucins [23;43], which mainly interact with 'soluble' components of the coagulation system, other biological effects of MVs are related to their interaction with

cells.[117;130]. Such interactions may occur locally, regionally or systemically and are often of an 'external' nature namly they entail a simple surface-to-surface contact and stimulation (between the target cell and the MV surface). Instead of physical contact the influence of MVs on the target cell may also involve pericellular discharge/activation of the bioactive cargo[18;19;59]. For instance, this may involve proteolytic remodelling of the extracellular microenvironment, modulation of ligand-receptor interactions, and a variety of other effects that could change the behaviour of target cells and properties of their surroundings (niches)[42]. In some instances such interactions could be rather complex and multifactorial. The recently described Rab27B-regulated exosomal release of MMPs and HSP90a from metastatic cancer cells is believed to control invasive cellular behaviour by inducing changes in the extracellular matrix (ECM), as well as through modification of growth factor responses[42]. Likewise, procoagulant MVs may facilitate tumour initiation, invasion, and dissemination by activating the clotting cascade extracellularly, and coagulation-dependent signalling of intracellularly[131]. MV-mediated emission of various factors including tetraspanins, chemoattractants, adhesion molecules, and proteases from cancer cells, platelets, and other cellular sources contributes to metastatic regulation in several experimental systems [111;132]. As mentioned earlier, MVs may also act as important reservoirs of cytokines and mediators of inflammatory and immune responses [33;133;134].

Bioactive ligands exposed on the MV surface are thought to be responsible for several important regulatory processes; for instance, direct stimulation of endothelial cells with MV-associated CD40 ligand (CD40L) may provoke angiogenic responses at sites of atherosclerosis [135]. Recent evidence suggests that delta-like 4 (Dll4), a transmembrane

Notch ligand, is also exposed on the surface of exosomes and thus may evoke angiogenic changes by interacting with Notch receptors expressed by endothelial cells[41]. Contact with the cell death ligand (FasL) exposed on certain tumour cell-derived MVs is lethal for Fas-expressing lymphoid cytotoxic effector cells, a process implicated in the induction of immunotolerance in colorectal cancer and possibly other malignancies[44]. In all of these instances, vesiculating cells generate a field of biological influence by extending the reach of molecular mediators, which would otherwise be confined to their cellular sources.

These influences may affect recipient cells via a random distribution of MVs in tissue and body fluids, or more directional MV homing/uptake mechanisms. For instance, an acidic pH commonly present in hypo-perfused areas of solid tumours may lead to localized disruption of MVs and consequent discharge of their proangiogenic and pro-inflammatory cargo, such as VEGF and other factors [45]. MVs may also be directed to specific sites due to the molecular addresses they carry on their surfaces (below) [46;47].

MV-mediated intercellular communication extends far beyond external contact. Indeed, one of the most tantalizing consequences of cellular vesiculation is the physical transfer of bioactive molecules between cells *via* MV-based mechanisms [13;20;115;117]. Such MV uptake may entail a physical integration of the MV and target cell plasma membranes, or penetration of intact MVs into the cell interior [18;115]. These processes may allow the exchange and 'sharing' of molecules (proteins, nucleic acids and lipids) that would

otherwise be sequestered by MV-manufacturing 'donor' cells , and by propagation within cellular populations may affect their collective phenotypes and properties.

The nature, directionality, and efficiency of this molecular exchange depends on several factors. For instance, the physical properties of vesicular plasma membranes affect the fusion rate between MVs and target cells, which may increase their MV uptake under acidic pH [140]. In some instances, MV transfer could also be directed by specific molecular addresses; for example, a high concentration of PS on the surface of certain MVs (e.g. ectosomes or procoagulant microparticles) may enable their recognition by PS receptors (PSRs) on the surface of specific types of target cells. Many of such PSRs have been described, mainly within the context of phagocytosis of apoptotic cells by mononuclear cells, Examples of such PSRs include: Tim1, Tim4, stabilin 2, and BAI1[138;141] at least some of of which could be expressed more widely, and may be involved in the uptake of MVs[46]. Indeed, blocking PS often obliterates MV incorporation by endothelial cells, platelets, and cancer cells[14;30;32;78]. A corollary to this point would be that phagocytes could be particularly susceptible to molecular influences of PS-positive MVs, beyond their simple destruction. It has also been proposed that Tim1/4 receptors on two adjacent cells could allow formation of MV/exosome bridges thereby promoting additional (indirect) intercellular interactions[46]. Similarly, the presence of PSGL-1 (P-selectin ligand) on the surface of procoagulant MVs directs them to P-selectin expressing platelets and endothelial cells[142].

Biological consequences of microvesicle-mediated molecular transfer

There is mounting evidence for the biological impact of microvesicular transfer of several classes of molecules, the examples of of which deserve some commentary.

Proteins are amongst the most studied functional elements of MV cargo. In this regard, chemokine receptors, especially CCR5 [48] or CXCR4 [143;144], which are known as portals for viral (HIV) infection, have recently been shown to undergo vesicular transfer to heterotypic cells. Such transfer engenders susceptibility to viral infection on cells that are normally resistant to HIV penetration, such as monocytes and endothelial cells[26;124;143;144]. Moreover, transfer of growth factor as well as cytokine and chemokine receptors may alter cellular responsiveness to their respective ligands, albeit often in a complex fashion, for example, by promoting receptor turnover rather than protracted signaling[14]. Conversely, MVs may also contain and transfer regulatory polypeptides such as IL-1β [33;46;145-147] and CCL5/RANTES[49] to cells that do not express these proteins resulting in changes in cellular responses (e.g. during inflammation). Microvesicular sharing of lineage markers (GpIIb/IIIa [149;150]) between platelets and neutrophils as well as the transfer of MHC molecules between dendritic cells [151-153] are examples of intercellular sharing of molecules involved in inflammation and antigen recognition, respectively.

Proteins contained in MVs are proposed to exert a multiplicity of effects during complex processes such as angiogenesis [77;132;154]. For example, a regulated dissolution of the vascular basement membrane and surrounding extracellular matrix is thought to be facilitated by MV-mediated delivery of proteases (e.g. MMP9, MMP2, MT1-MMP)[50] and their activators (EMMPRIN[51]). MVs also carry soluble, pro-angiogenic regulators

including VEGF [137;154;157], bFGF [154;157], PDGF [52], and other polypeptides [53]. Pro-angiogenic interleukin 8 (IL-8) and hepatocyte growth factors (HGFs) can be induced in various cells upon their uptake of platelet-derived MVs[132]. The aforementioned trafficking of exosomes containing Tspan8[54] or Dll4 [41], may affect vasculoar sprouting and endothelial tip cell formation, respectively. Moreover, MVs released from endothelial progenitor cells may instruct resident vascular cells to initiate angiogenesis [55], while in other instances MVs were found to contain oncogenic proteins (EGFR)[56] capable of modulating and reprogramming endothelial cell responses *in vitro*.

The examples of molecular transfer via MVs have also been documented during developmental and differentiation processes[20;144;159;160]. Thus, vesicular distribution of wingless (Wnt) in the developing Drosophila wing has been implicated in formation of morphogenic gradients[57], while intercellular transfer of MV-associated hedgehog (Hh) protein was proposed to induce leukemic stem cells to differentiate[58].

Spreading drug resistance within the cancer cell population could be one of the most tantalizing examples of multicellular phenotypic adaptation influenced by the MV transfer. Thus, MVs are thought to act as carriers of proteins involved in multidrug resistance, such as ABC transporters (e.g. P-glycoprotein, Pgp)[59] and drug metabolizing enzymes[60]. Passage of these proteins from cell-to-cell could serve to rapidly change the responses of tumours to anticancer chemotherapeutics. Also under physiological conditions MVs mediate transfer of phenotype modifying enzymes, including the passage of carbonic anhydrase from epithelium to Payer's patches in the intestine[61]. MVs are also

involved in the cellular exchange of transcription factors, such as peroxisome proliferator-activated receptor gamma (PPAR) and retinoid receptor (RXR) [62], which may profoundly alter the gene expression profile of the recipient cells.

The aforementioned MV-mediated exchange of growth factors [63], their receptors [6;32] and survival molecules [32;167] may promote cooperative events and affect 'collective' viability within the heterogenous cellular populations. On the other hand, contact with MVs harbouring FasL [122] or caspase 1 may have the opposite (competitive and pro-death) effect [64].

MVs present in the circulating blood are an important mechanism of locating coagulation effector molecules in their relevant cellular contexts. Indeed, the MV-mediated transfer of tissue factor (TF) between monocytes, cancer cells, platelets, and the endothelium represents one of the best characterized processes in this regard [14;30]. Normally, circulating MVs originate mainly from platelets; however, they may also emanate from inflammatory cells, cancer cells, and other sources [65]. Exposed PS TF, epithelial mucins, and other MV cargo influence the clotting cascade in multiple ways [50;169]. This is exemplified by the aforementioned bleeding disorder, Scott syndrome, which is associated poor PS exposure and platelet vesiculation [31;50;129;169-171]. MV-related effects can also be a part or response to certain anticoagulants[66]. It is noteworthy that MVs may contain proteins with anticoagulant activity, such as tissue factor pathway inhibitor (TFPI) and activated protein C (APC) [173-175].

Nucleic acids. As mentioned earlier MVs participate in the intercellular exchange of several nucleic acid species including DNA [20], mRNA [158;160], and miRs [162;176-178]. The 'packing' mechanism responsible for the inclusion of these molecules into the MVs cargo is poorly understood, but there is considerable evidence for the biological relevance of horizontal transmission of this cargo between cells during processes of inflammation [67], cellular differentiation[68], maintenance of the stem cell hierarchy[11], and cancer [69]. For instance endothelial cells have been shown to respond to MV-mediated transfer of various mRNA and microRNA species[69], which may promote formation of vascular networks in cancer. Another captivating experiment recently reported by Ratajczak et al employed pluripotent embryonic stem (ES) cells as a source of nucleic acid containing MVs, which were incubated with more lineage-restricted hematopoietic stem cells (HSCs)[68]. In this case, the apparent transfer of mRNA triggered profound reprogramming of HSCs to a more pluripotent state, characterized by enhanced clonogenic growth and the expression of genes associated with stemness, such as Oct4, Nanog, Rex, and others [68]. Vesiculation of ES cells has also been explored as a mechanism mediating the transfer microRNA to other cells[70].

Lipids in their various bioactive species represent both structural and functional components of all MVs. One of the best known examples of their horizontal exchange is the transfer of arachidonic acid (AA) between activated and resting platelets that results in the modulation of their procoagulant responses[179-181]. Platelet activation is also at the heart of procoagulant effects associated with MV-mediated release of another lipid, known as platelet activating factor (PAF), e.g. from endotoxin stimulated neutrophils [71].

Interestingly, the transfer of PS from vesiculating cells to erythrocytes was implicated in tagging these cells for destruction by phagocytes[72].

Thus, vesicular transfer of several molecules emerges as a relatively wide spread process that may complement intercellular communication by other mechanisms. One of the most intriguing questions in this regard is how, and to what extent, is this process involved in various forms of cellular pathology. Of particular interest is the role of MVs in cancer [19].

Oncogene-driven vesiculation – oncosomes

Cellular vesiculation is linked with cancer progression in at least three major ways, through (i) distinct mechanisms of MV generation; (ii) cancer-specific MV properties and content, and (iii) involvement of MVs in multiple cancer-related processes such as angiogenesis, migration, metastasis, niche effects, and other events already alluded to in the prior sections.

During malignant transformation, the action of mutant oncogenes, such as K-ras, epidermal growth factor receptor (EGFR), or its constitutively active mutant EGFRvIII, as well as several others appear to stimulate the formation and release of MVs [32;184]. Similarly, the activation or loss of specific tumour suppressor proteins appears to impact cellular vesiculation either positively or negatively [184;185]. While the exact nature of the signalling pathways involved in oncogene-driven MV biogenesis remains largely unknown, a handful of recent studies have begun to shed more light on the underlying processes. For instance, in cultures of prostate cancer cells, elevated MV (ectosome)

production was detected in association with increased oncogenic activity of protein kinase B (PKB/Akt), or upon stimulation with growth factors (EGF), and depending on the status of the actin regulating protein known as Diaphanous Related Formin 3 (DRF3) [89]. In this case, inhibition of DRF3 expression through RNA interference enhanced the rate of MV formation, and membrane blebbing activity, suggesting that DRF3 may be an inhibitor of ectosome release [89]. Interestingly, DRF3 expression is lost during the progression of prostate cancer to metastatic disease, which suggests an intriguing link between oncogenesis, vesiculation, and metastasis [89].

MV release by colorectal cancer cells is a function of K-ras and p53 status [184]. A recent study links p53 activation after the DNA damage, to the formation of secretory exosomes containing several p53-regulated proteins[73]. This effect is mediated by the p53 target protein, tumour suppressor-activated pathway 6 (TSAP6)[73]; mice lacking this protein develop microcytic anemia and signs of abnormal reticulocyte maturation [74], which is consistent with earlier studies implicating exosome formation with erythropoiesis[37]. Another report has demonstrated that vesiculation of LOX melanoma cells is controlled by a cascade involving ARF6 GTP-ase, phospholipase D, Erk, and MLCK. This pathway triggers phosphorylation of myosin light chain (MLC), which leads to MV production, proteolysis, and increased cellular invasiveness[75].

It is noteworthy that oncoproteins not only stimulate MV formation, but also become incorporated into their cargo [32;78]. As a result, oncogene-containing MVs (*oncosomes*) may serve as vehicles that carry oncogenic cargo and mediate its transfer between cells

[19]. At least four different modes of such oncogenic transfer have been described: (i) intercellular passage of active oncoproteins [56], (ii) transfer of oncogenic mRNA transcripts [69]; (iii) exchange of oncogenic microRNA[76], and/or (iv) passage of genomic sequences containing oncogenic DNA[20]. In many instances this horizontal transfer may have marked biological (transforming) consequences. Thus, oncosomes containing EGFRvIII may emanate from malignant tumours cells and be taken up by their indolent counterparts indicing their growth, survival, clonogenic, and angiogenic capacity[56]. Oncosomes may also act on endothelial cells and reprogram their responses such that they exhibit an increase in angiogenic activity [69], or switch to an autocrine mode of secretory pathway, e.g. by turning on VEGF production [35]. Indeed, blocking oncosome uptake using the Annexin V analogue (Diannexin) is associated with a measurable anti-angiogenic affect *in vivo*[35]. In chronic lymphoblastic leukemia (CLL), oncosome-like vesicles containing AXL kinase conditioned the bone marrow stroma to support disease progression[40]. These and similar effects identify oncosomes as possible effectors of oncogenic and proangiogenic *field effects*, long postulated to exist in cancer [13;187;188] and viewed as a mechanism of cell recruitment to the malignant process.

Translational implications of microvesicle generation, shedding and transfer

The emerging intense interest in MV biology stems from the realization that these particles are not just a "functionless debris", but rather represent a distinct biological phenomenon of notable functional and translational importance in cancer. In this regard, there are at least two important considerations. First, since different types of MVs may contribute to cancer progression as mediators of intercellular communication and

'communal effects', agents that block MV shedding as well as MV interaction with target cells and molecular transfer may possess hitherto unsuspected anticancer properties[35].

Moreover, unique, cancer-specific of functionally important cargo (molecular biomarkers) can be recovered from MVs shed into blood stream and body fluids of cancer patients. This includes certain effector proteins (e.g. TF), oncoproteins (e.g. EGFRvIII) , cancer-related transcripts and miRs [32;38;51;88;194;197]. Of particular interest is the fact that MVs may preserve the functional state of cancer-related proteins (e.g. their phosphorylation), which may serve as a means to follow the effects of targeted anticancer agents [77]. Thus, MVs represent an integral part of both physiological regulation and disease pathogenesis, and their exploration may inspire new therapeutic and diagnostic approaches.

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Figures and Tables

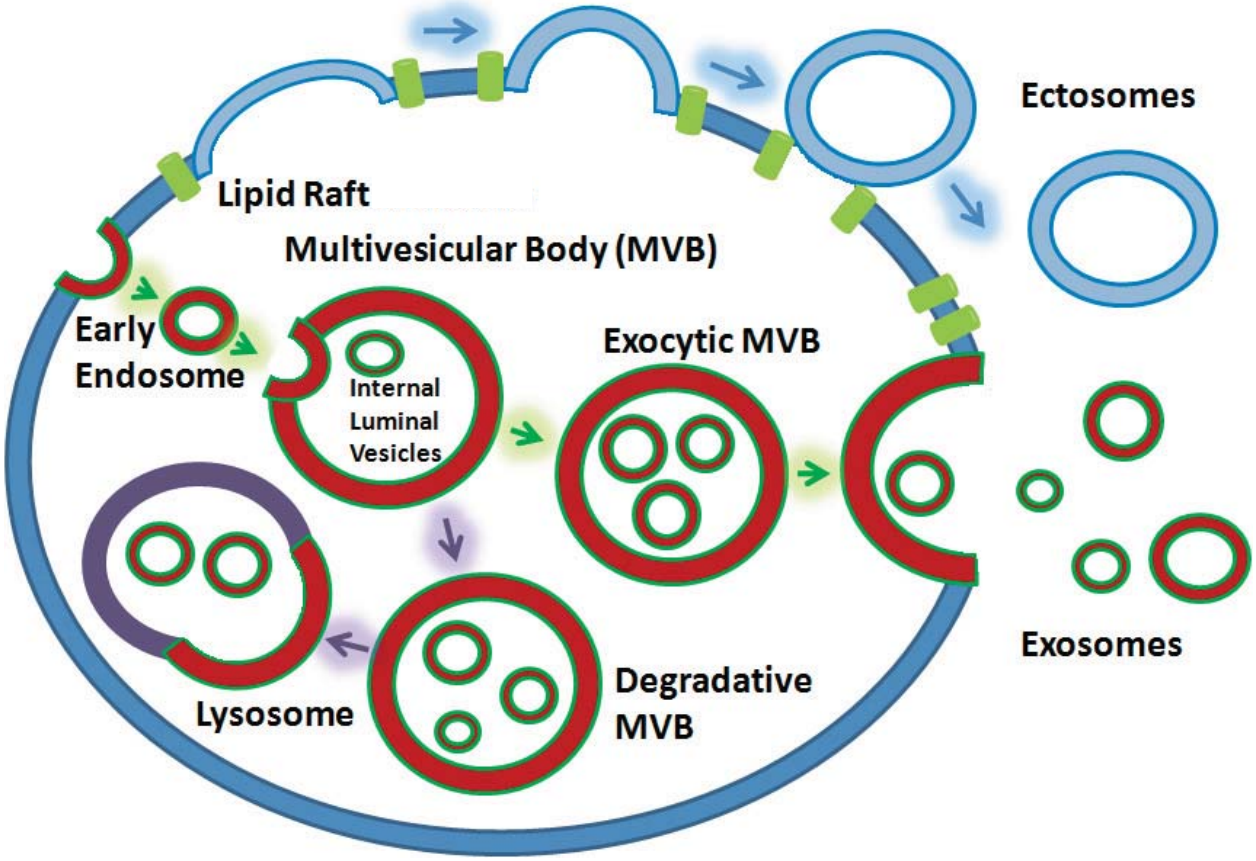

Figure 1. Pathways of cellular vesiculation. Two main types of microvesicles: ectosomes and exosomes emerge from cellular membrane and endosomal system respectively (see text). Ectosomes are thought to be associated with lipid-rafts and are larger in size. Exosomes originate within endosomal multivesicular bodies (MVBs), which are redirected to the cellular surface from the lysosomal degradation pathway.

Table 1. Examples of molecular markers associated with different classes of microvesicles [7;19;25;27;37;38].

Molecular Markers of Microvesicles		
Ectosomes		
Markers	*Functions*	*References*
Tissue Factor (TF)	Coagulation and angiogenesis	[14;30;39;40]
Flotillin-1	Lipid raft molecule	[30;41]
PSGL1	P selectin glycoprotein ligand 1 - cell adhesion	[30;42;43]
β1 Integrin	Cell Adhesion Molecule	[44-46]
Interleukin 1β	Cytokine involved in inflammation	[33;44]
MMP2	Matrix metalloproteinase involved in degradation of the extracellular matrix	[44;44;47]
MMP9	Matrix metalloproteinase involved in degradation of the extracellular matrix	[44;47;48]
EMMPRIN	Extracellular matrix metalloproteinase inducer (CD147/basigin)	[53]
ARF6	GTP-binding ADP ribosylation factor involved in remodelling of membrane lipids and actin	[75]
MUC1	Mucin associated with pathogen protection	[78]
CB1	Cannabinoid G protein coupled receptor	[24]
Lineage markers	CD61 (platelets); glycophorin A (red blood cells);	[50;51]

	CD66e (granulocytes); CD14 (monocytes); CD62e (endothelium)	
Exosomes		
Markers	*Functions*	*References*
CD9	Tetraspanin – cell surface glycoprotein	[79]
CD37	Tetraspanin – cell surface glycoprotein	[80]
CD63	Tetraspanin – cell surface glycoprotein	[41;53;54]
CD81	Tetraspanin – cell surface glycoprotein	[80]
CD82	Tetraspanin – cell surface glycoprotein	[80]
CD106	Tetraspanin – cell surface glycoprotein	[81]
Tspan8	Tetraspanin – cell surface glycoprotein	[81]
HSP70	Heat shock protein	[56-58]
HSP90	Heat shock protein	[54;57;59]
Caveolin-1	Scaffolding protein of lipid rafts	[60;61]
Rab-5a	GTPase involved in endocytosis	[54;62]
Rab-5b	GTPase involved in endocytosis	[61;63]
Rab27A	Secretory GTPase involved in cell invasion	[42]
PLP	Proteolipid protein of oligodendroglial cells	[54;64]
Alix	Protein involved in late endocytosis	[52;54;65]
TSAP6	P53 transcriptional target involved in exocytosis	[63;66]
Tsg101	Protein involved in ubiquitination-dependent endocytosis	[54;67;68]

MHC Class I/II	Immune recognition/regulation	[69;70]
Flotillin-1 (also present in ectosomes)	Lipid raft molecule	[66;71]

Table 2. Examples of preparative and analytical methods used in studies on microvesicles ([7;17;30;76-78])

Analysis of Microvesicles		
Approach	*Method*	*References*
Separation	Differential Centrifugation Sucrose Gradient Centrifugation Annexin V coated magnetic beads Immunoisolation Precipitation technologies (ExoQuick) Filtration technologies (ExoMir)	33;38;54;79-84
Detection	Scanning Electron Microscope Transmission Electron Microscope (immunogold labelling) Western Blot analysis of MV markers Flow Cytometry (FACS)	14;30;30;32;33;41;43;53;54;68;78;85-89

	Impedance cytometry Cholera Toxin B (CTxB) staining	
Quantification	PKH26/PKH67 Staining FM1-43 Staining DiO and DiD Labelling ELISA Assays (Tissue Factor, Rab-5b/Exotest, GFP) Nanoparticle tracking analysis (NPA) Flow Field-Flow Fractionation (FFFF)	14;32;33;43;61;88;90-93
Uptake	Detection of molecular cargo transfer (FACS, Western, reporter gene expression) Detection of fluorescent tag transfer (PKH26, PKH67) Membrane Fusion Assays (NBD-PE*, Rh-PE*, and DOTAP*)	14;30;32;78;88

Table 3. Examples of microvesicle cargo implicated in cancer [6;15;19;117].

Cargo	*Function*	*References*
(i) Proteins		
Soluble factors		
VEGF, FGF, IL-8	Angiogenic factors	[19;88;137]
IL-6, IL-1	Inflammatory cytokines	[33;88]
MMPs, TIMPs	Regulators of proteolysis	[88;155]
Membrane receptors		
CCR5	Chemokine receptor	[48]
CCR6	Chemokine receptor	[82]
TNFR1 (p55)	Cytokine receptor	[83]
EGFR	Receptor tyrosine kinase	[32;191]
AXL	Kinase involved in leukemia	[40]
FasL (Fas ligand)	Death ligand	[122;192;193]
Oncoproteins and Tumour Suppressors		
EGFR	Oncogenic EGFR	[32;78;88;191]
EGFRvIII	Mutant EGFR	[56]
HER2	Oncogenic RTK	[125;189]
MET	Oncogenic RTK	[189;194]

K-ras	Oncogenic GTP-ase	[77]
Akt	Oncogenic kinase	[89]
PTEN	Tumour suppressor	[77]
(ii) Lipids		
Sphingomyelin	Cell signalling, angiogenesis	[84]
(iii) Nucleic acids		
	mRNA	
Transcripts for VEGF, HGF, IL-8	Angiogenic factors	[53;88;196]
Transcripts for EGFRvIII	Oncogenic receptor	[69]
	microRNA	
Elements of cellular miR-ome:	Several miR sequences are detected in exosomes emanating from cancer cells	[38;53;88]
Oncomirs (miR-520g)	Brain tumour cells release microvesicles containing oncogenic miR520g	[76]
	DNA	
mtDNA	Mitochondrial DNA found in exosomes of tumour cells	[79]
gDNA	Genomic DNA found in apoptotic microparticles	[85]

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CD106	Tetraspanin – cell surface glycoprotein	[81]
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MMPs, TIMPs	Regulators of proteolysis	[88;155]
Membrane receptors		
CCR5	Chemokine receptor	[48]
CCR6	Chemokine receptor	[82]
TNFR1 (p55)	Cytokine receptor	[83]
EGFR	Receptor tyrosine kinase	[32;191]
AXL	Kinase involved in leukemia	[40]
FasL (Fas ligand)	Death ligand	[122;192;193]
Oncoproteins and Tumour Suppressors		
EGFR	Oncogenic EGFR	[32;78;88;191]
EGFRvIII	Mutant EGFR	[56]
HER2	Oncogenic RTK	[125;189]
MET	Oncogenic RTK	[189;194]

K-ras	Oncogenic GTP-ase	[77]
Akt	Oncogenic kinase	[89]
PTEN	Tumour suppressor	[77]
(ii) Lipids		
Sphingomyelin	Cell signalling, angiogenesis	[84]
(iii) Nucleic acids		
	mRNA	
Transcripts for VEGF, HGF, IL-8	Angiogenic factors	[53;88;196]
Transcripts for EGFRvIII	Oncogenic receptor	[69]
	microRNA	
Elements of cellular miR-ome:	Several miR sequences are detected in exosomes emanating from cancer cells	[38;53;88]
Oncomirs (miR-520g)	Brain tumour cells release microvesicles containing oncogenic miR520g	[76]
	DNA	
mtDNA	Mitochondrial DNA found in exosomes of tumour cells	[79]
gDNA	Genomic DNA found in apoptotic microparticles	[85]