Role of extracellular vesicles in cancer and potential implications towards cancer care

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Abstract

Sustained growth, invasion and metastasis of cancer depend upon bidirectional cell communication within complex tissue environments. Such communication has been associated with the secretion of soluble factors (e.g., cytokines, chemokines, and growth factors) by cancer/stromal cells within the tumour microenvironment. Recently it has become clear that tumour and stromal cells also export membrane encapsulated particles containing regulatory elements that augment cell-cell communication. These particles known as extracellular vesicles (EVs) include species of exosomes as well as shed microvesicles. EVs carry molecules such as oncoproteins and peptides, RNA species (e.g., microRNA, mRNA, lncRNAs), lipids, and DNA fragments that shuttle from donor to recipient cells to initiate profound phenotypic changes in the microenvironment. The emerging picture suggests that EVs play a critical role in cancer development and metastasis. It is now recognized that cancer cells secrete more EVs than their non-tumour cell counterparts and that these elements can be isolated from body fluids. Thus, EVs have strong potential as blood or urine markers for diagnosis, prognosis and surveillance of cancer. In this Review, we discuss the biophysical properties of EVs and their physiological functions, particularly with regard to pro-metastatic effects. The utility of EVs in development of cancer diagnostics and potential for exploitation in cancer therapeutics is discussed.

Key Points

- Exosomes and shed microvesicles represent two classes of small lipid-encapsulated extracellular vesicles (EVs) that convey information between cells through the transfer of functional protein and genetic information to alter phenotype and function of recipient cells
- Within each class there are subtypes (sub-populations) which can be distinguished by their distinct protein and RNA signatures
- Exosome participation in signalling between tumour cells and the microenvironment aids establishment of the pre-metastatic niche (PMN) and tumour progression
- Circulating exosomes containing tumour-specific molecular signatures (oncoproteins, mRNA transcripts for fusion genes/ alternative splice variants/ spliceosome subunits, lncRNA and double-stranded DNA fragments with cancer-driver mutations) underlie their clinical utility as next generation biomarkers for liquid biopsy in cancer diagnostics and management
- Standardized isolation protocols for EV populations are required to provide for inter-laboratory data comparison and advancement of clinical utility
- Exosomes have potential as a new source of therapeutic targeting which could possibly guide changes in clinical practice

Bidirectional communication between cells and their microenvironment is critical for both normal and pathological physiology. While such cross-talk is traditionally known to occur via direct cell-cell contact or the secretion of soluble factors[1-3], a new paradigm involving extracellular vesicle (EV) trafficking has recently emerged (as previously reviewed[4-10]). EVs are secreted by many eukaryotic cell types *in vitro* and have been found in body fluids including blood, urine, bile, ascites, breast milk, synovial lacrimal and seminal fluids, as well as bronchoalveolar lavage and faeces[11]. EVs can be released in response to cell activation, pH changes, hypoxia, irradiation, injury, exposure to complement proteins, and cellular stress[12-14]. Interestingly, EVs are also secreted by plant cells[15,16], and pathogens[17,18], including bacteria, mycobacteria, archaea, and fungi[19,20], suggesting an important evolutionary conserved mechanism of intercellular signaling.

Role of EVs in normal and pathological processes

Normal physiological processes. EVs participate in a variety of normal physiological processes including blood coagulation[21], innate/acquired immunity and immunomodulation[7,22], stem cell differentiation[23], tissue regeneration and angiogenesis[24], autophagy[25], implantation[26-28], placental physiology[29], semen regulatory function[30], and pregnancy[31,32]. Further, EVs have been proposed to be novel mediators during normal development and physiology of the nervous system and regeneration of normal neurons[33,34].

Pathological processes. In addition to their roles in normal physiology, EVs also participate in pathological processes such as the progression of neurodegenerative

diseases[35] and cancer. In the context of cancer, EVs are involved in a wide range of processes that underlie cancer progression – the so-called 'hallmarks of cancer'[36] - including inflammatory responses[37], angiogenesis[38], lymphogenesis[39], cell migration[40], cell proliferation[41], immune suppression[42], invasion[43], epithelial-mesenchymal transition[44] and metastasis[45]. Further, cancer-derived EVs have been shown to modulate somato-to-germ-line transmission of cargo{Cossetti, 2014 #1699}. Unsurprisingly, because exosome biogenesis shares many similarities to virion assembly, the host exosome pathway is manipulated for pathogenesis[46] by viruses such as human immunodeficiency virus[47], influenza A virus[48], hantavirus[49], respiratory syncytial virus[50], human papilloma virus[51], and herpesviruses[52].

There are two major EVs classes: exosomes and shed microvesicles

At least two different classes of EVs have been identified based on their mechanism of biogenesis: exosomes and shed microvesicles (sMVs) – sMVs are also referred to as microvesicles, ectosomes, and microparticles – but, for clarity, we will use the term sMVs throughout this Review. While it has been widely reported that these two classed of EVs can be discriminated based on their size[5,53] – exosomes averaging between 30 and 150 nm, and sMVs typically ranging in size from 50 to ~2000 nm – it has been thought that size discrimination alone is a useful definition of vesicle type, however, there is an increasing awareness of size overlap between the two EV classes, especially in the smaller particle range[53,54]. It should be noted that the size of some EVs classes is also affected by storage conditions; for example, in contrast to exosomes which are stable to repeated freeze-

thawing, the size of sMVs is significantly reduced upon storage at -80ºC and repeated free-thawing (AR & RJS unpublished observations). A list of biophysical attributes (e.g., range of particle diameter, and buoyant density) is shown in **FIG. 1A**. Accumulating evidence from *in vitro* studies using cell grown in culture and *ex vivo* body fluids indicates the existence of more than one exosome subtype (**TABLE 1**). While comprehensive protein and RNA profiling studies reveal distinct differences in cargo between exosome subtypes, the question of detailed functional differences between the subtypes is unclear and must await further experimentation using highly-purified and well characterized EVs. Other EV types such as platelet-derived microparticles (~130-500 nm diameter, also referred to as 'platelet dust'), which are released upon platelet activation[21,55], are by far the most abundant vesicle type in blood. Platelet-derived vesicles and another other vesicle types such as apoptotic bodies (50-2000 nm), generated from cells undergoing programmed cell death[56], will not be covered in this Review.

Biogenesis of exosomes. Exosomes and sMVs differ not only in content but also in mechanism of formation (**FIG. 1 B**). Exosomes originate by inward budding of the plasma membrane to form a membrane-bound vacuole (early endosome) which undergoes several changes as it matures to form a late endosome. The limiting membrane of late endosomes then buds inward and pinches off to form membrane-enclosed vesicles (intraluminal vesicles, ILVs) within the late endosome - now referred to as a multivesicular body (MVB)[57]. At this juncture, the primary role of ILV-loaded MVBs is to act as intermediates in the degradative lysosomal pathway whereby MVBs undergo fusion with lysosomes resulting in the discharge and degradation of their ILV content by nucleases, proteases,

lipases and other hydrolytic molecules within the lumen of the lysosome (reviewed[58]). MVBs targeted for the lysosome degradative pathway display surface proteins such as the tumour suppressor phosphatase HD-PTP, the HOP complex (co-chaperone of Hsp70/Hsp90-complex), the GTPase Rab7, and members of a membrane-fusion ***s***oluble ***N***SF attachment protein ***re***ceptor (SNARE) complex including VAMP7, Vti1b, syntaxins-7 and -8[59,60]. Alternatively, MVBs destined for the formation of exosomes traffic to and fuse with the plasma membrane whereupon the MVB limiting membrane integrates with the endosomal recycling system and their ILV contents are released into the extracellular space (now referred to as exosomes)[61,62]. For some time it has been known that the four endosomal sorting complexes required for transport (ESCRTs-0, -I, -II, and -III)[63-66] are key drivers of MVB/ILV exosome formation, a process that utilizes reversible protein ubiquitination in ILV protein cargo selection[67-69]. In addition to ESCRT-dependent MVB/exosome formation, ESCRT-independent pathways involving neutral sphingomyelinase (N-SMase)/ ceramide formation[70], as well as ARF6/PLD2[71] have been reported. It is likely that ESCRT-dependent and ESCRT-independent MVB/exosome biogenesis machineries vary from tissue to tissue (or even cell type) depending on specific metabolic needs, but this critical aspect of EVs has not been well-explored and warrants further study.

Shed microvesicle biogenesis. Compared to biogenesis of exosomes, much less is known about sMV formation. In contrast to exosomes, sMV release is MVB-independent, a process not requiring exocytosis. They egress from the cell by direct budding from the plasma membrane through ARF6[72] and RhoA-dependent rearrangement of the actin

cytoskeleton[73]. Like the budding viruses, sMVs plasma membrane budding exploits the TSG101/ ESCRT machinery (**FIG. 1C**). For example, the ESCRT-I subunit TSG101 has been shown to traffic to the plasma membrane and interact with accessory proteins Alix and ARRDC1 (arrestin-domain containing protein-1) during the later stages of sMV release, a process also implicated in sMV cargo sorting[74]. ESCRT-III and Alix are also implicated in Gag-mediated HIV budding from cells[75] as well as in cytokinetic abscission[76,77]; based on our recent data (AR & RJS unpublished data) we speculate that this generic mechanism also prevails in budding of sMVs into the extracellular space. Interestingly, activation of acid sphingomyelinase (A-SMase), a downstream event in ionotropic ATP receptor P2X7 activation, triggers release of sMVs from glial cells and astrocytes thought to be a critical effector of neuro-inflammatory disease[78]. This observation, along with the demonstration that N-SMase modulates release of exosomes from oligodendrocytes[70], indicate that different members of the SMase family are key molecular effectors of EV formation and that inhibitors of these enzymes may provide new strategies treatment of neuro-inflammatory disease. Despite differences in their mechanism of biogenesis and membrane of origin - limiting MVB membrane in the case of exosomes, and plasma membrane for sMVs - the two classes of EVs appear to function similarly when released into the extracellular space[79]. However, this aspect of vesicle biology is far from being resolved and must await further experimentation.

EV classes and their respective subpopulations display unique cargo profiles. During biogenesis, EVs selectively enrich an array of cellular bioactive cargo molecules (**FIG. 1D**). Interestingly, in the human colorectal carcinoma cell lines SW480/ SW620 we

observed many miRNAs (by high-throughput NGS) that are non-detectable in parent cell lysates (i.e., <5 transcripts per million reads, TPM) but are uniquely enriched (>1,000 TPM) in exosomes and sMVs secreted by these cell lines (MC & RJS manuscript submitted). This observation is important given current global efforts to identify RNA signatures in cancer biopsy tissues by NGS deep sequencing for the purpose of developing diagnostic markers of disease. In general, while much is known about trafficking of cellular cargo to EVs[80], our understanding of the underlying mechanism of cargo selection is still very much in its infancy. In addition, during EV biogenesis diverse surface proteins characteristic of the parent cell are selectively displayed on secreted EVs (**FIG. 1E**). These include signaling receptors, integrins, RNA-binding proteins and ribonucleoproteins which play a critical role in recipient target cell recognition and uptake[81,82] by various endocytic processes including direct fusion[12,83], lipid raft-, clathrin- and calveolae-dependent endocytosis, micropinocytosis, phagocytosis[84-89], and antigen presentation[7,22,90] (**FIG. 1F**) (see review[91]).

Why the need to purify EVs?

Over the past decade there has been a greater awareness in the EV community of the need to rigorously isolate specific populations of EVs for research purposes. Foremost, highly-purified EVs are crucial if we are to better understand fundamental biochemistry (e.g., define bioactive cargo) - as a first step towards better establishing mechanisms of biogenesis and functionality. Additionally, there is a pressing need to define EV surface-exposed proteins for the purpose of generating mAbs that would allow –i) discrimination

of EV class/ subtype (i.e., stereotypical markers) and –ii) large-scale purification for clinical applications such as transfusion/ EV vaccines[92] and for the presentation of tumour-associated antigens to the immune system[93,94]. (For a summary of commonly-used methods for purifying EVs for the purpose of stringent biochemical analyses – see **BOX 1**, and for diagnostic application (e.g., isolation of EVs from body fluids), and large-scale production for therapeutic studies - see **BOX 2**, and reviews[5,10].

Evidence for functional effects of exosomal cargo on constituent recipient cells in the tumour microenvironment

Although it has been known for some time that the molecular composition of EVs secreted by diverse cell types varies markedly, it is only recent that – especially, in the case of exosomes – phenotypic changes in recipient cells induced by exosomal uptake have been directly attributed to the action of specific exosomal proteins and RNA molecules. (At this juncture there is a paucity of functional data for sMVs.) Evidence supporting a role of specific exosomal cargo in crosstalk between constituent cells of the tumour microenvironment (e.g., cancer cell-stromal cell/ cancer cell- cancer cell / stromal cell-cancer cell communication) is given in **TABLE 2**.

The presence of occult tumours *in situ* is a widespread phenomenon (e.g., breast[95], thyroid[96], lung[97] and pancreatic cancer[98]) and has been extensively studied over the past 50 years – particularly, with respect to why they don't necessarily become malignant. It has been demonstrated that despite possessing oncogenic mutations, when injected into normal microenvironment (e.g., developing embryos) cancer cells lose metastatic capability[99] (reviewed[100]). This is thought to be due to the ability of normal

tissue to restrain aberrant growth and progression to malignancy[100,101]. This phenomenon led to a paradigm shift where cancer is now thought to be more than a disease defined - by molecular (genetic and epigenetic) events within the cell – but, also an "ecological disease" modulated by components of the tumour microenvironment (TME)[102,103].

Over the past decade, it has been shown that tumour cell-derived exosomes influence non-cancer cells to generate a permissive TME (**TABLE 2**). For instance, exosomes influence endothelial cells to support neo-angiogenesis that fuels tumour growth[104] and induce vascular permeability to support metastasis[105]. Exosomes also trigger fibroblasts differentiation towards pro-angiogenic and pro-tumorigenic CAFs[41,106]. Moreover, tumour exosomes can initially suppress immune cells to evade detection[107,108] and then, as cancer progresses, modify immune cells towards pro-tumorigenic[109] and pro-metastatic[105] phenotypes. Exosomes accomplish this by transferring functional oncoproteins to recipient cells where they activate downstream signaling pathways such as MAPK and AKT (**TABLE 2**). Exosomes also transfer miRNAs to recipient cells resulting in attenuation of gene expression[110-113]. More recently, tumour exosomal lncRNAs have also been shown to be functionally important[114]. Taken together, the above-mentioned studies – albeit, conducted using mouse models – demonstrate that "exosome-transformed" non-cancer cells enhance primary tumour growth[41] and can even push non-malignant cells towards malignancy[115].

Exosomes also transfer oncogenic entities such as mutated proteins[116,117], fusion gene mRNA (EML4-ALK)[118] and oncogenic lncRNA[114] to neighboring cells in the TME. For instance, glioblastoma-derived exosomes transfer mutated EGFRvIII receptor - and its associated oncogenic signaling - to other indolent cancer cells to drive malignancy[116].

Apart from cancer 'hallmark'-enabling capabilities, exosomes can also transfer miRNAs to confer drug resistance[119]. In fact, the cargo content of cancer exosomes can change in response to external cues, such as hypoxia[104] to induce angiogenic responses in endothelial cells, and therapeutic agents (e.g., sunitinib)[114], to provide drug resistance to neighboring cells

It is also becoming increasingly evident that intra-tumour heterogeneity (both spatial and temporal)[120] is not only restricted to cancer cells. For example, stromal cells are reported to co-evolve with the tumour cells, so that both continuously participate to drive cancer progression[121]. During this process, stromal cell-derived exosomes continually traffic to cancer cells to transfer functional proteins and RNAs to support tumour growth, invasion and metastasis (**TABLE 2**). Moreover, stromal cell-derived exosomes can dictate progression to malignancy[40,122] and successful metastatic dissemination[123], and also confer drug resistance[124,125] thereby interfering with therapeutic outcomes in clinical settings.

It should be noted that most studies directed towards the role of exosomes in generating a permissive TME used mouse models and/or human cancer cell line-derived exosomes isolated from cancer cells grown *in vitro*. However, emerging studies support that exosomes isolated from cancer patients appear to be functional. For example, exosomes isolated from tumour interstitial fluid[126], malignant ascites[127] and sera of cancer patients[44,107,128] have been shown to be functional in that they modified normal non-cancer/stromal cells to support cancer progression.. Taken together, these studies indicate that signaling reciprocity between cancer and non-cancer cells is an important aspect of cancer biology[102], with exosome-mediated signaling now attracting increasing attention[129] (**TABLE 2**).

Role of exosomes in pre-metastatic niche generation

How does a primary tumour prepare the pre-metastatic niche? A major breakthrough in our understanding of pre-metastatic niche generation arose from the pivotal observation that primary tumours can secrete factors (i.e., 'seeds') that migrate to preferred metastatic sites, *prior* to dissemination of cancer cells to actively remodel these sites (i.e., generate favorable 'soil') to enhance metastasis[130]. (See **BOX 3** for Paget's 'seed and soil' hypothesis.) Unlike healthy tissues, which have an innate ability to resist outgrowth of tumour cells[100], predetermined metastatic microenvironments (referred to as 'pre-metastatic niches', PMNs[131,132]) are shaped by the primary tumour secretome and acquire traits that enable the distant site to recapitulate the primary tumour microenvironment - these traits include vascular leakiness[133-135], inflammation[136], immune suppression[137], coagulation[138], stromal cell activation amongst others that support tumour outgrowth.

Numerous tumour-secreted factors[130,139] have been implicated in PMN generation. For example, primary tumour-derived soluble factors (vascular endothelial growth factor (VEGF)-A, placental growth factor (PlGF)[130], chemoattractants S100A8/A9[139], and tumour-secreted granulocyte colony-stimulating factor (G-CSF)[140] are implicated in lung PMN generation (reviewed[3,132,141]). In this Review we restrict our remarks to the contribution of primary tumour-derived exosomes to PMN generation[45,105,142]; for involvement of soluble protein/peptide factors, see[3,131,132].

Exosomal construction of the pre-metastatic niche occurs in a stepwise manner. While our understanding of the biology of PMN is very much in its infancy, transgenic and syngeneic mouse models suggest that PMN construction takes place in a step-wise manner – a process that is similar across different cancer types[131]. Construction begins with primary tumour exosomes entering the circulation, whereby they encounter vascular beds of distant secondary organs; potentially future metastatic site(s). While hematologic vessels draining tumours are likely to influence the choice of secondary organs, exosomes have been shown to display organ tropism - for instance, melanoma and breast cancer exosomes home primarily to lung, liver, bone and brain, whereas colorectal cancer exosomes primarily home to liver **(FIG. 2)**. While very little is known about exosome surface proteins that dictate this homing behavior, emerging evidence implicates integral membrane proteins such as the integrins[45]. Intriguingly, systemic biodistribution studies in mice show specific human cancer cell-derived exosomes disseminate to organs that mirror parental cancer-type metastases in the clinic **(FIG. 2A)**. Moreover, all cancer cell-derived exosomes distribute to bone marrow, suggesting exosomal/ BMDC engagement may be a common denominator of metastasis[45,105,131,142,143].

A critical initial step in generation of PMN in target organ tissues involves vascular leakiness induced by a combination of disseminated cancer cell-derived exosomes[45,105] and soluble protein factors[133-135] acting on local stromal cells. The nature of recipient cell-type varies depending on cancer type and is secondary organ-specific. For example, pancreatic cancer-derived exosomes are taken up by Kupffer cells in the liver[45,142], breast cancer-derived exosomes by fibroblasts[45,143]/epithelial cells[45] in the lung, and astrocytes[143]/endothelial cells[45] in the brain. Uptake of exosomes by stromal cells results

in their reprogramming[105,136,142,143], and activation of signaling pathways[136] which, in turn can alter the local chemokine repertoire[105], and remodel extracellular matrix composition[136,142,144], increase nutrient availability[143], neo-angiogenesis[145] and lymphogenesis[144].

During the initial phase of PMN development, a pool of circulating tumour exosomes home to bone marrow where they are taken up by BMDCs, enhancing their mobilization and entry into the peripheral circulation[105,136,142]. The altered microenvironment in secondary organs attracts circulating BMDCs[105,142] whereupon infiltration they secrete soluble factors that generate local inflammatory millieu[105,142] and/or exert pro-tumorigenic immunosuppression. Cumulatively, these events result in the generation of a receptive PMN that attracts circulating cancer cells to enhance metastasis.

What do we know about exosomal cargo in PMN development? The molecular nature of the PMN is directly influenced by exosomal cargo such as signaling proteins (e.g., receptor tyrosine kinase Met[105], macrophage inhibitory factor MIF[142]), and by RNA [136,143] and, potentially, DNA[146] . For example, in an experimental model of melanoma metastasis[105], mouse melanoma cells (BF10) - capable of metastasizing to the lung – released Met-containing exosomes (**FIG. 2B)**. These exosomes when taken up by BMDCs transferred Met receptor, resulting in enforced expression of c-Kit/Tie2 and subsequent mobilization of Met-containing BMDCs into the circulation[105]. Circulating tumour exosomes also generate vascular leakiness in the lung, dysregulate ECM remodeling and inflammatory genes (e.g., S100A8 and S100A9) which, resulting in recruitment of c-Kit/Tie2 BMDCs cells creating a PMN to support lung metastasis. Genetic ablation of Met levels in exosomes or interfering with Met function using the pharmacological Met

inhibitor crizotinib reduced the level of circulating c-Kit/Tie2 BMDCs cells in mice and attenuated exosome-dependent metastatic burden. In another experimental mouse model of pancreatic cancer metastasis to the liver[142], exosome-containing MIF was shown to establish PMN by causing release of TGF-β expression in recipient Kupffer cells; TGF-β then activated hepatic stellate cells to deposit fibronectin which, in turn, recruited bone marrow macrophages (F4/80+) increasing liver metastatic burden **(FIG. 2C)**.

Exosomal miRNAs are also important in PMN generation. For example, in an experimental mouse model of breast cancer metastasis to brain and lungs[136], circulating breast cancer exosomes contained high levels of miR-122 have been shown to attenuate levels of pyruvate kinase in recipient astrocytes in brain and fibroblasts in lungs. This caused reduction in expression levels of the glucose transporter GLUT1 and suppression of glucose uptake in adjacent stromal cells. In turn, this increased glucose availability to cancer cells resulting in increased metastatic outgrowth **(FIG. 2D)**.

Sentinel lymph node pre-metastatic niche development

Most mouse models of metastasis focus on PMN generation via hematologic circulation[131]. However, metastasis also occurs via local lymphatic drainage with histologically- positive sentinel lymph nodes an indicator of poor prognosis. In melanoma tumour-bearing mice, exosomes released by primary tumour (as opposed to intravenously injected exosomes released by melanoma cells grown in culture[105]) were most abundantly detected in tumour draining lymph nodes[147] when compared to blood and other organs such as bone, lung and

liver (also observed in a hematologic metastasis model of melanoma[105]). Alteration in pre-metastatic sentinel lymph node microenvironment exists in many cancer types[148-150] and may be necessary before lymph node metastases can successfully develop[151]. Indeed, melanoma cell-derived exosomes injected into mice via the foot pad generated lymph node PMN[144]. In the primed lymph node, exosomes upregulated genes implicated in cell recruitment, ECM modelling and angiogenesis, as well as influencing the distribution pattern of melanoma DTCs.

In a pancreatic cancer mouse model, injection of exosomes - from highly-metastatic pancreatic cancer cells - into the footpad induced metastasis in the popliteal lymph node of poorly metastatic tumour cells[152]. This is thought to occur via transfer of functional miRNAs to stromal cells resulting in alteration of their adhesion profile, chemokine ligands, proteases, and cell cycle/angiogenesis-promoting genes[153].

Non-cancer cell derived exosomes can regulate metastasis and cancer cell dormancy

A pre-metastatic site primed for metastasis may not always require generation by the primary tumours. For example, physiological changes in tissue environment due to aging[154], pregnancy[155], or infections[156] can potentially foster niches permissive to metastasis. Some organs are intrinsically capable of supporting metastasis of DTC (disseminated metastatic tumour cells), without primary tumour influence. For example, direct injection of cancer cells into non-tumour bearing mice have been shown to successfully establish metastatic foci – "active metastatic niches" - which don't require prior conditioning by a tumour.

Physiological exosomes contribute towards 'active metastatic niche' formation. One key line of evidence supporting non-tumour cell exosome involvement in 'active metastatic niche' formation is outlined in the study of Zhang et al.,[123]. Using a mouse metastasis model it was shown that breast cancer cells lose expression of an important tumour suppressor PTEN[157] - after dissemination to the brain, but not to other organs[123]. This loss of PTEN in glioma cells was shown to be mediated by transfer of an exosomal PTEN-targeting miR19a released by resident astrocytes. This adaptive PTEN loss in brain metastatic cells leads to an increased secretion of the chemokine CCL2, which recruits IBA1-expressing myeloid cells that reciprocally enhance the outgrowth of brain metastatic cells via enhanced proliferation and reduced apoptosis. Such niches exist independent of influence of primary tumours and represent "active niches".

Exosome involvement in cancer cell dormancy. Because tumorigenic potential of oncogenes is context dependant[100], and secondary organs are generally inhospitable to DTCs, the PMN microenvironment attempts to recapitulate hallmark-enabling traits of the primary tumour microenvironment that initially supported expansion of the tumour cells. However, successfully disseminated cancer cells do not always land in PMN or 'active niches'. As a result, tumour cells can enter prolonged dormancy[158].

Secreted factors that regulate dormancy appear to be organ specific[159,160]. Additionally, the extracellular matrix composition that DTCs encounter at distinct sites also influences dormancy[161]. (Such sites are often referred to as "sleepy niches"[131].) There is a growing body of evidence that highlights the role of physiological exosomes in this process[158,162]. For example, exosomes released from bone marrow mesenchymal stem cells

have been shown to induce dormancy in breast cancer cells disseminated to the bone by transferring miR-23b that targets the MARCKS gene, which encodes cell division and motility-related protein[162].

Clinical utility of exosomes

Finding reliable biomarkers for early detection of cancer is the holy grail in diagnostic cancer research. Ideally, a useful biomarker must be specific for a given tumour type and universally detectable in pre-metastatic stages using non-invasive techniques. However, despite a steadily increasing number of biomarker reports, few FDA approved biomarkers have thus far reached the clinic[163,164]. With the aim of improving biomarker identification, stringent guidelines for sample number, target specificity and sensitivity in biomarker discovery research have recently been formulated[165] (also reviewed elsewhere[164]).

In contrast to traditional 'solid biopsies', which are impractical for screening or prognostic assay, liquid biopsies that focus on circulating tumour cells, cell-free tumour DNA, cell-free tumour RNA and, more recently, exosomes - are rapidly gaining recognition in precision or personalized medicine due to ease and non-invasive nature of sample collection (reviewed in[166-168]). The great strength of liquid biopsy is the ability to provide clinical information prior to and during treatment for therapeutic planning and monitoring. Over the last decade, circulating exosomes have been shown to be a good source of cancer-associated molecules (typically, miRNAs) with potential as biomarkers for many cancer types, including hepatocellular carcinoma (HCC)[169], lung cancer[170-173], gastrointestinal cancer[174], colorectal cancer[175], pancreatic cancer[176], melanoma{Alegre, 2014 #1674;Ragusa, 2015 #1675;Fleming, 2015 #1676}{Logozzi, 2009 #1700}, breast

cancer[128], ovarian cancer[180] and prostate cancer[181] (for a detailed review, see Ochiya and colleagues[182]). In addition to miRNAs, other exosomal cargo molecules such oncogenic mRNAs (including fusion gene, and splice-variant transcripts), double-stranded DNA fragments (including cancer driver mutation genes), lipids and lncRNAs are gaining much attention as potential biomarker candidates (**BOX 4** and **TABLE 3**). Key positional papers discussing the use of EV-based clinical trials and diagnostic and therapeutic (i.e., theranostic) clinical utility (reviewed{Lener, 2015 #1188}{Fais, 2016 #497}). Over the past 20 years there has been much interest in the application of exosome-based cell-free vaccines as alternative approaches to dendritic cell adoptive therapy for suppressing tumour growth[183]; **BOX 5** provides an update on the current status of EV vaccine clinical trialing.

Two seminal reports of EV-derived biomarkers potentially enabling detection of pancreatic cancer (PC) have generated much interest and discussion[184,185]. The potential implications of these studies are enormous given that PC is currently the third leading cause of cancer death in the United States[186] but early detection has been hampered by the lack of sufficiently specific and sensitive biomarkers. The first report from Melo and colleagues[184] involved antibody-based detection of glypican1 (GPC1) expressed on the surface of cancer-associated EVs circulating in the bloodstream. GPC1 is a membrane anchored heparin sulphate proteoglycan that is overexpressed in diverse tumours including glioma, breast, colorectal, and pancreatic tumours[184,187-190]. Indeed, it has been recently reported that exosomal GPC1 and its regulatory miRNAs are specific markers for the detection and target therapy of colorectal cancer[190].

Using blood samples from 251 patients divided into discovery and validation cohorts, EV isolation and GPC1 identification allowed partition of patients with late-stage PC from those with benign pancreatic disease with reported 100% accuracy. However, in a more recent study, using 12 samples, Lai et al.,[185] report that exosomal GPC1 is not diagnostic for PC whereas an exosomal miRNA signature comprising high expression of miRs-10b, 21, 30c, and 181a and low expression of miR-let7a readily differentiates PC and normal samples. Further, in contrast to exosomal GPC1, the signature miRNA levels reverted to normal values within 24h following PC resection. Interestingly, while both groups assayed exosomal GPC1 using proteomics platforms, Melo et al.,[184] employed an antibody-based assay system whereas Lai and colleagues[185] used quantitative mass spectrometry-based (MS) methodologies. The MS method quantitatively analyzed exosomal GPC1 by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) to assay peptide QIYGAK that is unique to GPC1 and not shared with GPC-2, -3, -4, -5, or -6 or with other human proteins. It is conceivable that the discrepancies in these important biomarker studies could be due to differences in patient cohorts, sample numbers or reagent specificity, however given the urgent need for biomarkers for PC surveillance such studies, warrant further research employing uniform methodologies. The later study also highlights the utility of MRM, also referred to as selective reaction monitoring (SRM), as a novel LC-MS/MS based method that is rapid, sensitive and robust enabling efficient high-throughput analysis of clinical samples (LC-MS/MS and MRM is reviewed elsewhere[191,192]).

Conclusions

With the emergence of new biological concepts relating to EV involvement in many biological processes, this is an exciting time for the EV field. Of particular interest for the present discussion is the bi-directional transfer of molecules between tumour cells and the microenvironment, including the role of EVs in establishment of the PMN. It is clear that specific bioactive molecules contained in circulating EVs have great promise as creditable surrogates of tumours thus presenting a new paradigm as diagnostic, prognostic and therapeutic indicators. However, with increased focus on cancer-derived EVs come new challenges including standardization of methods for isolation, quantification and analysis of EVs from complex tissues such as blood. While great advances have been made in ability to accurately determine EV particle numbers[193], notwithstanding EV size diminution upon storage at -80ºC or freeze-thawing, especially for sMVs, there remains a pressing need to standardize blood-based EV-enumeration procedures. This is critical for inter-laboratory comparisons of clinical data and determination of EV dosages for clinical trial purposes. Another largely unexplored question in the field is how EVs and their contents should best be quantified: vesicle number, protein content, a ratio of the two[193] or classical mAb microarray-based surface profiling. Hence the need for stereospecific EV-surface directed mAbs[194,195] - especially those that can enable tumour staging.

A further challenge in clinical application of EV technologies is the fact that even for advanced cancers, the percentage of total blood vesicle content[196] that might represent tumour-derived EVs (1% , 0,1% , 0.01% --?[197]) is completely unknown. Thus it is not clear if any given sample will be within the range of our current technological capabilities. Furthermore little is currently known about constituent and functional differences between EV classes (exosomes and sMVs) or their half-life in biological samples. The answer to

these questions will dictate the *degree* of enrichment required to realistically employ EV-containing biomarker candidates as cancer diagnostics. However, unlike protein biomarkers, RNA markers can be readily amplified and, coupled with high-throughput multiplexed RNA profiling, offer a promising way forward as cancer diagnostics[198]. **BOX 4** in this Review lists a number of 'rare' RNA species[116] and DNA fragments containing cancer driver mutations[199] that are uniquely found in EVs - and afford an exquisite level of biomarker specificity. However, given the likelihood of platelet 'dust' contaminating blood-derived EV samples, any cancer-based EV candidate biomarker or therapeutic target must be checked for specific presence in tumour derived EVs versus platelet-derived material.

Beyond these practical considerations, current research into the role of EVs in the tumour microenvironment and pre-metastatic niche discussed earlier will undoubtedly alter our view of cancer biology and present new targets for therapeutic intervention. Looking forward, perhaps a future paradigm in EV biology may lie in the notion that blocking cancer cell-derived EV release and/or uptake by recipient cells in conjunction with extant cancer adjuvant therapies may add another weapon to the armory for cancer treatment.

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Competing interests statement

The authors declare no competing interests

Author contributions

All authors contributed to researching the data for the article, and to discussion of the contents of content. All authors reviewed/ edited the manuscript before submission.

Figure Legends

Figure 1 | **Physical properties and characteristics of extracellular vesicles. (A)** EVs are heterogeneous in diameter and buoyant density; the major classes include exosomes (Exos) and shed microvesicles (sMVs). Other entities co-isolated include lipoprotein particles (light, LDL; heavy, HDL), viral particles (VIR), apoptotic blebs (Abs), bacteria (BAC), and cells (CELL). Because lipids have a density of ~1 g/cm, and proteins/ RNA >1.3 g/cm, density gradients can be used to separate subpopulations of EVs with differing ratios of lipids/ RNA/ and proteins. Density gradients can be used to purify EV classes away from soluble proteins, free RNA, and protein–RNA complexes. In addition to particle diameter and buoyant density other physical parameters of EVs, such as light scatter, which is correlated to size - but also to geometry and composition - can be measured by flow cytometry **(B)** Exosome biogenesis and release is coordinated through select intracellular pathways. Endocytosis is an active process by which cells internalize material in the extracellular fluid by invagination and pinching of the plasma membrane to from internal vesicles (endosomes). Exosomes form by inward budding of endosomal membranes (early endosome, EE; late endosome, LE). The formation of exosomes within a LE occurs via a multivesicular body (MVB) that fuses with the plasma membrane to release exosomes into the extracellular space. The exosome generation pathway can be regulated by either ESCRT (endosomal sorting complex required for transport) complexes or ESCRT- and protein-independent pathways, which are thought to operate in parallel with ESCRT-dependent mechanisms. Following their assembly, release of exosomes from the cell occurs via endosomal fusion with the plasma membrane, under the regulation of several

Rab-GTPases (including RAB11, RAB35, RAB27A, and RAB27B). Fusion of MVBs with lysosomes results in degradation of the intraluminal vesicles **(C)** The biogenesis and release of sMVs follow a distinct pathway; key events include plasma membrane organization and redistribution of phospholipids, repositioning of phosphatidyl serine to the outer leaflet of the plasma membrane, local disassembly of the cytoskeleton network, and contraction of the actin– myosin machinery by the activation of myosin light chain kinase/ESCRT-1/ ADP-ribosylation factor 6 (ARF6) components **(D)** EVs can deliver DNA, nucleic acids, proteins, and lipids including various oncoproteins, fusion/splice variant genes, transcriptional factors, and RNA binding proteins that can be functional in recipient cells. For a publicly-accessible, and fully annotated, database of exosomal proteins, RNA and lipids refer to Exocarta2012[200] and Vesiclepedia (a compendium of EVs with continuous community annotation[201]). **(E)** The specificity of EV-recipient cell targeting most likely occurs by the EV-cell surface ligand interactions (e.g., lipid rafts, tetraspanins, adhesion molecules (integrins for example), signaling receptors, and components important in antigen presentation and membrane trafficking). **(F)** EVs can be taken up via different mechanisms, including dynamin-, PI3-kinase-, and actin polymerization–dependent phagocytosis, micropinocytosis, clathrin-/ caveolae-dependent endocytosis, direct membrane fusion, or phagocytosis. Ligand–receptor interactions on the cell surface can also result in biological effects and help to target vesicles to specific cell types.

Figure 2 | **Systemic bio-distribution (homing) of cancer exosomes to distant organs in mouse model parallels common metastatic site(s) for different human cancer-types.** **(A)** Systemic bio-distribution of cancer exosomes in mice during pre-metastatic niche preparation, indicating organotropism (left) and metastatic grounds for different cancers in humans (right). Exosome biodistribution (homing) correlates with the organotropic metastatic spread *in vitro* in cell lines from various types of cancer including breast (●)[45,143], colorectal (●) (unpublished data, Simpson et al.), melanoma (●)[105], pancreatic (●)[142], and prostate (●)[45] cancer. The biodistribution of exosomes is at least in part mediated by integrins[45]; ITGβ4 promotes lung tropism and ITGβ5 promotes exosome adhesion in the liver. **(B)** Exosomes from highly metastatic melanoma increases the metastatic behavior of primary tumours by permanently 'educating' bone marrow progenitors through the receptor tyrosine kinase MET[105]. Melanoma exosomes transfer MET to bone marrow progenitor cells (BMPCs) in experimental mouse model to promote BMDC mobilization to premetastatic sites and mediate premetastatic niche formation (lungs). Metastases were significantly increased in these mice where MET knockdown reversed these effects. Further, knockdown of Rab27a, a protein important for exosome biogenesis, decreased exosome secretion and consequently perturbed metastatic potential of the malignant cells. Total levels of MET and MET phosphorylation were both higher in exosomes derived from patients with stage 3 and 4 melanoma, and MET expression was increased in vasculogenic BMPCs from patients with stage 4 melanoma. **(C)** Primary pancreatic ductal cancer (PDAC)-derived exosomes induce liver pre-metastatic niche formation in naive mice and consequently increase liver metastatic burden[142]. Uptake of circulating PDAC cancer

exosomes by Kupffer cells caused release of transforming growth factor (TGF)-β that activated hepatic stellate cells to deposit fibronectin, which in turn enhanced recruitment of BMDC and metastatic burden. Macrophage migration inhibitory factor (MIF) was highly expressed in PDAC-derived exosomes, and its blockade prevented liver pre-metastatic niche formation and metastasis. Compared with patients whose pancreatic tumours did not progress, MIF was markedly higher in exosomes from stage 1 PDAC patients who later developed liver metastasis. These findings suggest that exosomal MIF primes the liver for metastasis and may be a prognostic marker for the development of PDAC liver metastasis. **(D)** Breast cancer exosomes containing miR-122 generate pre-metastatic niche by suppressing glucose uptake by astrocytes in the brain and fibroblasts in the lungs by downregulating the glycolytic enzyme pyruvate kinase and consequently increase metastatic burden[143]. High miR-122 levels in the circulation have been associated with metastasis in breast cancer patients.

Footnotes*: BMDCs, bone marrow-derived dendritic cells; BMPCs, bone marrow-derived progenitor cells; MET, tyrosine-protein kinase Met; MIF, migration inhibitory factor; miR, microRNA; PDAC, pancreatic cancer; Rab27a, Ras-related protein Rab-27A; TGF-β, transforming growth factor-β*

Box 1 | **Commonly-used methods for purifying extracellular vesicles for biophysical studies and clinical utility**

The fact that there is such a large body of literature describing protocols for purifying EVs attests to the technical challenges associated with this task[5,10] and the lack of a universally-accepted approach (i.e., gold standard method). This problem is further confounded by emerging evidence that there are at least two major classes of EVs (exosomes and shed microvesicles), based upon mechanisms of biogenesis, and that subtypes exist within each class (**TABLE 1**). (The majority of rapid/one-step approaches for isolating EVs do not take cognisance of the fact they are dealing with a possible mixture of vesicle classes/subtypes and co-isolated contaminants such as high-M_r protein oligomer and protein-RNA complexes (e.g., HDL/ LDL/AGO2) complexes.) Varying methodologies for purifying (enriching) EVs include differential centrifugation (DC), density (sucrose, percoll, iodixanol-gradient) centrifugation (DGC), HPLC gel permeation (size-exclusion) chromatography (SEC), affinity chromatography using biospecific reagents (e.g., mAbs) covalently fused to either magnetic or agarose beads (AC), membrane ultrafiltration devices using low-centrifugal force, microfluidic devices, and synthetic polymer based precipitation reagents - for a discussion on application, yield/purity and scalability of these methods, see[5,202]. The choice of these diverse methods for EV isolation very much depends on the research question.

Stringent EV isolation procedures. If the research aim is to purify EVs for the purpose of conducting stringent biochemical analysis (e.g., define their luminal cargo – RNA/ DNA/ lipid/ protein species -and surface-exposed proteins) and specific functionality, then rigorous fractionation strategies are critical. In a recent comparison of commonly-used procedures for isolating exosomes from cell line cultures, using the colorectal cancer cell line LIM1863 culture supernatant as a model source material[203], it was clearly evident that AC using magnetic beads coated with a mAb directed to the exosomal surface was superior to DG and DGC. mAbs that have been successfully employed as bait include those directed against A33[204], EpCAM[203,205], MHC-II antigens[206,207], CD45[208,209], CD63[210,211], CD81[211], CD9/CD1b/CD1a/CD14[212], CD24/SWA11[213], and HER2[214].

In the absence of a suitable mAb, targeted EV capture methods that rely upon bio-specific synthetic peptides with specific affinity for heat shock proteins[215] and vesicle surface heparin sulphate proteoglycans[81,216] have been reported. Other successful methods for enriching EV classes include low-g force sequential centrifugal membrane ultrafiltration[53] and DC when used in combination with DGC (top-loaded) (e.g., OptiPrep™/ iodixanol)[27,39,217]. Interestingly, in the case of B16FO melanoma-derived exosomes, when crude sample was top-loaded onto DGC only one exosome subtype was evident, whereas when sample was loaded on the bottom and allowed to float into the gradient, two distinct populations could be distinguished[218].

Box 2 | **Commonly-used methods for purifying extracellular vesicles for clinical utility**

Clinically-relevant approaches in EV isolation. The demands of clinical applications involving diagnostics and therapeutics such as low cost, reliability, and speed can eventually be met with modifications to existing technologies for improved scalability. Over the past decade there has been much interest in blood-derived EVs because they contain clinically-relevant information – oncoproteins, RNA (miRNA, mRNA, lncRNA, fusion gene mRNA), and lipids (see **BOX 4** and **TABLE 3**). However, the isolation of EVs from blood and urine is a challenge due to the presence of bulk proteins and lipoproteins, which undoubtedly will attenuate intrinsic EV protein/RNA signatures. (This problem is exacerbated by the lack of knowledge of the percentage of disease-derived EVs in the total blood EV pool – at this juncture in time, there is no robust enumeration technique for determining specific EV concentrations in blood or for comparing/ standardizing EV purity that would enable inter-laboratory comparisons – critical for evaluating biomarkers.) Isolation of EVs from blood (plasma or serum) by DC, DGC and precipitation methods (e.g., synthetic polymers such as PEG, acid precipitation etc) have serious shortcomings in that they result in co-isolation of protein aggregates, protein (AGO2-RNA complexes[219], and high-density lipoprotein (HDL)- which attenuate EV cargo signals and confound interpretation of miRNA profile, respectively. (It has been reported that HDL contains bound endogenous miRNAs indicating that HDL may play a role in cell-cell communication by horizontal transfer of miRNAs[220].) Recently, it was shown that EVs can be rapidly isolated from biological fluids, such as plasma, using simple ready-made size-exclusion chromatography columns[221-224]. This one-step isolation procedure for plasma EVs is highly reproducible and an effective means of eliminating >95% of extraneous protein from plasma. Other emerging methods that are scalable include field-flow fractionation[225], sequential low-g force centrifugal ultrafiltration[53] and free-flow electrophoresis (G Weber, personal communication).

Generation of EVs for therapeutic studies. By virtue of their bioactive cargo (see **TABLE 2**) EVs have inherent therapeutic potential[226-228]. For example, exosomes secreted by human mesenchymal stem cells (MSC) have been used in tissue regenerative medicine to reduce infarction size in a mouse model of myocardial ischemia/ re-perfusion injury[229]. For these studies, large-scale production of functional

homogeneous MSC-derived exosomes was accomplished using SEC HPLC fractionation. In another therapeutic application, exosomes from dendritic cells (and tumour cells) have been trialed in cancer vaccine studies[230-233] (see **BOX 5**). Navabi and colleagues described a large-scale production method combining ultrafiltration and sucrose/ deuterium oxide (UC cushion) for generating god manufacturing (GMP)- grade exosomes from ascites fluid of ovarian cancer patients for clinical trialing[234].

Box 3 | **Paget's 'seed and soil' hypothesis: a basic tenet of metastasis**

Sir Stephen Paget and Dr James Ewing pioneered metastasis research in the late 19th and early 20th centuries by proposing two major theories to explain the organ specificity of metastasis. In 1889 Paget[235] proposed that sites of secondary cancer growth are not a matter of chance – rather, that some organs provide a more 'fertile' environment than others for metastatic growth. This hypothesis – the "seed and soil" hypothesis - was at odds with James Ewing extant theory at the time which stated that metastatic dissemination patterns can be solely accounted for by vascular connections to the primary tumour[236]. Ewing's viewpoint prevailed for several decades and the 'seed and soil' hypothesis languished in the shadows for many years. It wasn't until the 1980s that Isaiah Fidler[237], using radiolabeled cancer cells, showed that while circulating cancer cells equally distribute to all tissues, metastases disseminated only in selective organs (i.e., organotypic metastases), that the 'seed and soil' hypothesis re-gained traction. Since then organotypic metastasis has been a cornerstone in metastasis research, with focus turning to unravelling the molecular mechanisms that interlink 'seed and soil' to promote metastases.

Box 4 | **EVs contain rare mRNA transcripts, DNA fragments and lnc RNAs: a source of novel specific biomarkers of cancer?**

EVs contain DNA fragments with cancer driver mutation genes

- Double-stranded DNA fragments are detected in the exosomes isolated from human cancer cell lines, such as chronic myeloid leukemia, murine melanoma, breast, colon, lung, prostate and pancreatic cancers[238,239]. The amount of exosomal DNA isolated from tumour cells is about 20-fold more than the amount of fibroblast exosomal DNA[239].
- EGFR mutation is detected in the exosomal DNA isolated from four non-small cell lung cancer cell lines[239].
- Higher mutation rates in exosomes isolated from human biofluids probably due to exosomal DNA representing tumour heterogeneity at expression levels not attainable through tissue sequencing[240].
- Oncogene *c-Myc* can be amplified in serum EVs from tumour-bearing mice[146].
- KRAS and p53 are the most frequently mutated genes in pancreatic cancer[241]. Mutated KRAS and TP53 can be detected in the serum exosomes of two pancreatic cancer patients[238]. Digital PCR analyses have identified that 39.6% and 4.2% of the pancreatic ductal adenocarcinoma patients have $KRAS^{G12D}$ and $TP53^{R273H}$ mutations in their serum exosome DNA, respectively[199]. Further, KRAS mutations in exosomal DNA were identified in 7.4%, 66.7%, 80%, and 85% of age-matched controls, localized, locally advanced, and metastatic pancreatic ductal adenocarcinoma patients, respectively[242].

EVs contain oncogenes and tumour suppressor mRNAs

- Exosomal EGFRIII promotes tumour growth transfer and uptake in endothelial cells[116].
- Exosomal H-RAS initiates tumour formation in non-tumorigenic endothelial and fibroblast cells[243].

- Prostate cancer mRNA biomarkers PCA-3 and TMPRSS2/ERG detected in urinary exosomes from prostate cancer patients after mild prostate massage[244].
- Oncogenic KRAS mutations ($KRAS^{G12D}$ and $KRAS^{G12V}$) mRNAs detectable in serum exosomes of pancreatic patients[184].
- sMVs, A33-Exos and EpCAM-Exos released by human colon cancer LIM1863 cells contain mRNAs (e.g., TPT1, ribosomal protein genes, FTL EEF1A1 and EEF1B2) up-regulated in CRC tumour tissues compared to matched normal tissues[245].
- Combination of exosomal RNA and circulating DNA improves sensitivity of EGFR mutation detection in plasma of non-small cell lung cancer patients[246].

EVs provide new resources of neoantigens

- High-resolution profiling of genomic and transcriptomic landscapes identifies plasma exosomes containing copy number variations, point mutations, insertions, deletions, gene fusions and mutational signatures, which are new sources of neoantigens[240].
- Colorectal cancer cell line LIM1863-derived EVs contain 268 novel alternative splicing events and 33 fusion genes, many of which some have been reported in other cancers – e.g., SH3D19/LRBA in primary myelofibrosis, RIPK2/OSGIN2 in primary urethral clear-cell adenocarcinoma, and GOLT1A/KISS1 in bladder cancer[245].

Diagnosis potential of EV lncRNAs

- Exosomal lncRNAs are stable in plasma; exosomal LINC00152 evaluated as biomarker for gastric cancer[247].
- Exosomal lncRNAs, including HOTAIR, MALAT1, and MEG3 differentially expressed in cervical cancer patients and cancer-free volunteers (HPV-positive or HPV-negative)[248].

- Colorectal cancer cell line LIM1863-EVs enriched for lncRNAs SNGH5/6/7/8, ZFAS1, H19 and LINC00116, are up-regulated in CRC tumour tissues[245].
- Combination of two mRNAs (KRATAP5-4 and MAGEA3) and 1 lncRNA (BCAR4) evaluated as potential colorectal cancer biomarker[249].

Complexes in the EVs as potential biomarkers?

mRNAs encoding splicing factors SF3B2, SFRS1, SYF2, SRSF7 and PUF60, potential therapeutic targets due to their regulation of protein expression in cancers[250], are enriched in colorectal cancer cell line LIM1863-derived EVs[245]. Of these, SFRS1 has been reported as a proto-oncogene[251]. U1/U2 protein and snRNA co-exist in LIM1863-derived EVs, which is indicative of exosomal spliceosome pre-complexes; circulating U2 snRNA fragments in bloodstream have been reported as a diagnostic/ prognostic biomarker in lung cancer patients[252].

Box 5 | **EV-based vaccines: an update**

DC-Exos (Dex) as EV vaccine

Pre-clinical study: Dex as cell-free vaccine compared with DCs

- Exosomes secreted by antigenic peptide-loaded dendritic cells (DCs) can induce anti-tumour $CD8^+$ T cell responses in pre-clinical mouse model. They carry functional MHC I/II molecules costimulatory molecules (CD86) that presented tumour-peptides and primed naïve T lymphocytes into cytotoxic T lymphocytes (CTL), which eradicated established mouse tumours *in vivo*[183].
- Interestingly, these exosomes elicited better immune response (60% mice with complete tumour regression at day 60) compared to that elicited by tumour-peptide loaded DC themselves (only 20% mouse with tumour-free)[183].

Phase I clinical trials: Assessing safety and feasibility

- Phase I clinical trials using self-DC-derived exosomes loaded with tumour antigens (first-generation Dex) demonstrated their safety with no serious side effects in patients with metastatic melanoma HLA-$A1^+$/$B35^+$ and HLA-$DPO4^+$ ($MAGE3_{247\text{-}258}DPO4^+$ and $MAGE3_{168\text{-}176}$ $A1^+$/$B35^+$ tumour antigen[93], advanced NSCLC HLA*0201 (MAGE-$A3_{112\text{-}120}$+, -$A4_{230\text{-}239}$+, -$A10_{254\text{-}262}$+, and MAGE-$3DPO4_{247\text{-}258}$ peptides[94], and malignant glioma[253].
- However, those first-generation Dex lack the ability to active $CD8^+$ T cell response while they carry NKG2D ligands to promote NKG2D-dependent NK cell activation[254].

Phase II clinical trial: Second-generation DC exosomes as tumour vaccines

- Second-generation autologous Dex with enhanced immuno-stimulatory properties has been developed for potential peptide-dependent $CD8^+$ T cells[255].
- Phase II clinical trial to test progression-free survival in non-resectable NSCLC patients (n=22) following second-generation Dex vaccination. The median time to progression was 2.2 mo and median overall survival (OS) was 15 mo. Seven of 22 patients (32%) experienced stabilization of >4 mo. The primary endpoint was not reached. This phase II trial confirmed the second-generation Dex did not induce cancer-specific T cell immune response, while did boost the NK cells function to mediate anti-tumour immunity in patients with advanced NSCLC[256].

Tumour cell-derived exosomes as EV vaccine

- Human melanoma exosomes enriched with HSP70 and full-length tumour antigens stimulated dendritic cell activated tumour specific $CD8^+$ T cells *in vitro*. Furthermore, mouse tumour cell-derived exosomes induced $CD8^+$ T cell cross-priming and tumour rejection in preclinical study[257].

Ascites-derived exosomes (Aex) as EV vaccine

Pre-clinical study:

- exosomes harvested from melanoma ascites presented (contained) Mart1/Melna A tumour antigen which enabled monocytes-derived dendritic cells (MoDCs) to induce Mart1/Melan A-specific, HLA-A2 restricted $CD8^+$ T cell responses *ex vivo*. Further, lymphocytes from seven of nine melanoma patients stimulated with MoDCs loaded with Aex expanded into tumour specific cytotoxic T lymphocytes *ex vivo*. Therefore, Aex could be a suitable tumour antigen source[258].

Phase I clinical trials: Assessing safety and feasibility

- In phase I clinical trials, patients in both groups (Aex and Aex plus GM-CSF) received a total of four subcutaneous immunizations at weekly intervals. Both therapies were safe and well tolerated and Aex plus GM-CSF but not Aex alone induced tumour-specific CTL response. Thus, Aex could potentially serve as an alternative choice in the immunotherapy of advanced CRC[259].

Mode of exosomes administration

- DC-based vaccines have been administered subcutaneously over weekly intervals.
- Alternatively, autologous tumour cells encapsulated with a biodiffusion chamber re-implanted in the patient's abdomen could serve as slow-release exosome depot[253].

Table 1 | **Selection of exosome subtypes secreted from various cell lines / found in body fluids**

Source (cell line/ body fluid)	Subtype	Features	Comments	Refs
Human colorectal cancer model (LIM1863)	$A33^+$ exosomes	$A33^+$, $CD63^-$, $Alix^+$, $TSG101^+$	$A33^+/A33^-$ exosome subtypes were isolated from cell culture medium of human CRC LIM1863 cells using sequential immunoaffinity capture ($A33^-$ mAB / EpCAM-mAb loaded magnetic beads). Proteome profiling shows distinct protein signatures for $A33^{+/-}$ exosomes; $A33^+$ exosomes are selectively enriched in intracellular apical trafficking proteins and $A33^-$ exosomes, basolateral trafficking proteins. miRNA profiling[260] and mRNA transcriptome profiling[245] reveal distinct signatures.	261
	A33- exosomes	$A33^-$, $CD63^+$, $Alix^+$, $Tsg101^+$		
Human melanoma (B16F10), squamous carcinoma (A431), mouse heart endothelial (H5V), mesenchymal stem cell (immortalized human MSC hTERT), mouse neuroblastoma cells (N2a), human plasma	Low-density (LD) exosomes	Both HD- and LD-exosomes display Alix, TSG101, CD9, CD81 and CD63 on their surface; buoyant density range of both subtypes 1.12-1.19 g/mL	HD- and LD-exosomes from a number of cell lines were isolated using density gradient (sucrose) centrifugation. HD- and LD-exosome subtypes have unique protein and RNA compositions and have different functional effects on recipient cells.	218
	High-density (HD) exosomes			
Mesenchymal stem cells (immortalized E1-MYC ESC-derived)	Cholera toxin B-chain (CTB)+ exosomes	CD81+, CD9+, Alix+, Tsg101+, fibronectin-, actin+	MSC-derived exosomes were isolated based on their respective affinities for the membrane-lipid binding moieties cholera toxin B chain (CTB), annexin V (AV) and Shiga toxin B subunit (ST) respectively. Proteome and RNA cargo of the 3 subtypes are distinctive	262
	Annexin V (AV)+ exosomes	CD81-, CD9-, Alix-, Tsg101-, fibronectin-, actin+		
	Shiga toxin B subunit+ exosomes	CD81-, CD9-, Alix-, Tsg101-, fibronectin+, actin+		
Human monocyte-derived dendritic cells	High density (HD) - exosomes	Buoyant density 1.15g/mL	Density gradient (iodixanol) centrifugation used to separate HD- and LD-exosome subtypes which exhibit differing immune functionalities.	54,263

	Low-density (LD)-exosomes	Buoyant density 1.12g/mL		
Human saliva	Exosome I	Alix+, Tsg101+, CD63+, Hsp70+, CD26-	Human saliva-derived exosome-I and –II were fractionated using gel-filtration on Sephacryl S-500 and shown to have different size and protein composition. Most of CD26 (dipeptidyl peptidase IV (CD26) present in whole saliva is found in exosome II subtype and shown to be metabolically active in cleaving chemokines CXCL11 CXCL12.	264
	Exosome II	Alix+, Tsg101+, CD63+, Hsp70+, CD26+		
Human seminal fluid	Large diameter (105 +/- 25 nm) - exosomes	Buoyant density ~1.15 g/ mL ; CD9+, PSCA+ , annexin A1+	Two distinct exosome (prostasomes) subtypes were isolated from seminal fluid using density (sucrose) gradient ultracentrifugation.	265
	Small diameter (56 +/- 13 nm) - exosomes	Buoyant density ~1.25 g/ mL;CD9+, PSCA+ ,GLIPR2+		
Rat basophilic leukemia-2H3 (RBL-2H3) cells	Exosome I	CD63+	Three distinct exosome subtypes identified by combining protein sorting (CD63, CD81, and MHC II) and different fluorescent lipid (phosphocholine, ceramides) probes that label distinct cell compartments such as plasma membrane outer leaflet and the Golgi apparatus.	266
	Exosome II	MHCII+		
	Exosome III	CD81+		

Table 2 | **Evidence supporting a role of exosomal cargo in cell-cell communication**

Donor cell	Recipient cell	Functional cargo	Interfering functional cargo	Key findings	Refs

Cancer : non-cancer cell transfer	Mouse melanoma (B16F10)	Mouse monocytes	PEDF	i-Ab: anti-PEDF	Pre-metastatic tumours generate innate immune response (e.g., PEDF) which are delivered by exosomes to monocytes in the bone marrow; the loss of these triggers enables immunosuppression and abrogates the immune clearance of cancer cells leading to metastasis. Importantly, exosomes isolated from patients with non-metastatic primary melanomas have a similar ability to suppress lung metastasis.	267
	Mouse melanoma (B16F10)	Mouse BMPCs	Met	Met shRNA, crizotinib (Met-inhibitor)	Met containing Exos activate S6 and ERK phosphorylation in BMDCs that was inhibited by crizotinib. Further shRNA silencing Met in melanoma cells reduced exosome mediated pro-metastatic behavior of BMDCs	105
	Mouse pancreatic ductal adenocarcinoma (PAN02), human pancreatic adenocarcinoma (BxPC-3)	Human/mouse kupffer cells	MIF	MIF shRNA	PDAC exosomes augmented liver metastatic burden in naïve mice. MIF was found to be up-regulated in PDAC exosomes and its inhibition impeded liver metastatic burden	142
	Human prostate cancer (PC3)	Human primary fibroblasts (AG02262)	TGF-β	i-Ab: anti-TGF-β, TGF-beta/Smad inhibitor: SB431542	Cancer exosomes highly expressing TGF-β-triggered SMAD3 signaling and α-SMA expression in fibroblasts, activity of which was blocked by a TGF-β-neutralizing Ab. Conversely, inhibition of TGF-β RI abrogates activation of α-SMA	106
	Human breast cancer (MDA-MB-231), human glioma (U87)	Mouse fibroblasts (NIH3T3)	TG, FN	siRNA: TG, TG inhibitor: T101, RGD (Arg-Gly-Asp) peptide inhibitor: FN	Exosomes induce enhanced anchorage-independent growth, increased survival, and exosome-mediated transfer of TG and FN further led to activation of mitogenic signaling and functional changes in fibroblast transformation	115
	Human gastric cancer (SGC7901)	Mouse kupffer cells, hepatic stellate cells	EGFR	EGFR-miRNA, miRNA26b inhibitor, EGFR overexpression	Tumour-derived EGFR containing Exos regulate liver microenvironment to promote liver metastasis through activation of HGF; upregulated liver paracrine HGF inhibits miR-26a/b expression/binds c-MET receptor on the migrated cancer cells to provide fertile location for metastatic cancer cells.	268
	Human glioblastoma (U87)	Endothelial (HMVEC)	DII4	Dll4 overexpression	Exosomal DII4 transfer to endothelial cells led to the inhibition of Notch signaling (Notch target genes Hes1, Hes2 and loss of Notch receptor) and conferred angiogenic behavior on recipient cells in a 3D microenvironment	269

	Glioblastoma (patient-derived)	Endothelial cells (HMVECs)	Gluc mRNA	Gluc overexpression	Gluc activity in exosome-treated endothelial cells showed a continual increase corroborated by the translation of the Gluc mRNA in recipient cells	270
	Breast cancer cells (MDA-MA-231-D3H2LN, BMD1a, and BMD2a/b)	Endothelial (HUVEC), pericytes, astrocytes	miR-181c	miR-181c transfection/overexpression	Exosomal miRNA-181c promoted ectopic actin filament organization through its target gene, PDPK1. Systemic injection of exosomes obtained from brain metastatic cancer cells augmented the brain metastatic potential of breast cancer cells. Increased level of circulating miR-181c in breast cancer patients with brain metastasis was further observed, suggesting an important role of secretory miR-181c in brain metastasis.	271
	Lung adenocarcinoma (CL1-5)	Endothelial (HUVEC)	miR-23a	miR-23a inhibitor (transfection)	miR-23a, an miRNA upregulated in hypoxia in lung cancer cells, was identified in the cancer-derived exosomes. Hypoxic lung cancer-derived exosomes increase angiogenesis and promote cancer cell intravasation/extravasation. When circulating exosomes were collected from lung cancer patients and healthy control patients' sera, the cancer patient exosomes demonstrated higher levels of miR-23a.	104
	Human prostate cancer (Du145)	Endothelial (HUVEC)	TGFβ1	Inhibitor of TGFβ signaling: SB431542, Rab27a knockdown	Exosomal TGFβ1 facilitated myofibroblast transformation, supporting angiogenesis in vitro and accelerating tumour growth in vivo. Depleting exosomes, targeting Rab27a, abolished differentiation and lead to failure in stroma-assisted tumour growth in vivo.	41
	Human multiple myeloma (RPMI8226, KMS-11, U266)	Endothelial (HUVEC)	miR-135b	miR-135b inhibitor (transfection)	Hypoxia-resistant cancer cells that can mimic in vivo conditions of hypoxic bone marrow, shown to release exosomal miR-135b, subsequently enhanced endothelial tube formation under hypoxia via the HIF-FIH signaling pathway	272

Cancer : cancer cell transfer	Renal cell carcinoma (786-O)	Renal cell carcinoma (7Su3rd)	LncRNA ARSR	siRNA: LncARSR, mutant lncARSR, to investigate interaction with hnRNPA2B1	Exosome-transmitted lncARSR promotes sunitinib resistance through the competitive binding of miR-34/miR-449 that potentiates AXL and c-Met expression	114
	Human breast (MDA-MB-231)	Human breast (T47D)	Cre recombinase mRNA	Cre transfection, cell independent assays that allow passage of Cre+ EVs	Using a Cre-LoxP system, exosomes secreted by malignant tumour cells were identified to be taken up by less malignant tumour cells located within local and distal sites. These exosomes were also composed of mRNAs involved in migration and metastasis in vivo	273

	Human glioma (U373vIII)	Human glioma (U373)	EGFRvIII (mutant)	Annexin V: blocks recipient cell exosome interaction, CI-1033 (irreversible pan–ErbB inhibitor): inhibits phos-Erk1/2 and Erk1/2	Glioma cells release exosome containing oncogenic EGFRvIII to EGFRvII lacking cancer cell to change cell morphology and increase in anchorage-independent growth capacity	116
	Human colon (DK0–1)	Human colon (DKs-8)	KRAS (mutant)	Transformed (WT), transformed (mutant)	Transfer of mutant KRAS via exosomes induced anchorage-independent growth of recipient non-transformed colon cells, providing a mechanism by which the tumour microenvironment may be influenced by non-cell autonomous signals released by mutant KRAS-expressing tumour cells	117
Non-cancer : cancer cell transfer	Primary: human breast cancer-associated fibroblast; secondary: human breast cancer (MDA-MB-231)	Primary: human breast (MDA-MB-231), secondary: human breast (MDA-MB-231)	CD81	shRNA: CD81	Induction of breast cancer cell protrusion, motility (through PCP-Wnt signaling) and metastasis in an exosomal CD81-dependent manner	40
	Human primary cancer-associated fibroblasts	Human breast (MDA-MB-231)	RN7SL1	POL3 inhibitor, siRNA: POL3	Exosomes containing unshielded RNAs activate pattern recognition receptors in breast cancer and innate immune cells, thereby promoting tumour growth and metastasis. Using chemical and genetic ablation of RNA polymerase III (POL3) function, allowed the specific POL3-driven exoRNA responsible for function (RN7SL1). Examination of exoRNA released by tumour-associated fibroblasts from cancer patients revealed a significant increase in unshielded exoRN7SL1, as compared to healthy control fibroblasts.	124

	Mouse primary dermal fibroblast	Human breast (MDA-MB-231)	ADAM10	ADAM10 inhibitor: GI254023; ADAM17/ADAM10 inhibitor: GW280264; ADAM inhibitor: TAPI-1; metalloproteinase inhibitor: BB94	Exosomes secreted by TIMP knockout fibroblasts were enriched in ADAM10 and increased breast cancer cell motility. Exosomal ADAM 10 enhanced Notch receptor activation and migration in a RHOA-dependent fashion	122
	Human bone marrow mesenchymal stem cells	Human breast (BM2)	miRNA23b	siRNA: MARCKS; overexpression: MARCKS and miRNA23b	miR-23b containing exosomes induced dormant phenotypes through the suppression of a target gene, MARCKS. Further metastatic breast cancer cells in patient bone marrow had increased miR-23b and decreased MARCKS expression	162
	Mouse primary astrocytes	Human breast (MDA-MB-231), mouse breast (4T1)	miRNA19a	Mutant miRNA19a, shRNA: PTEN, CCL2	Astrocyte exosome-derived miRNAs induced loss of PTEN expression in metastatic cells disseminated to the brain and potentiated metastasis. Conversely, silencing of PTEN-targeting miRNAs or inhibition of astrocyte exosome release rescued PTEN depletion and repressed brain metastasis	123

ARSR, lncRNA activated with sunitinib resistance; Dll4, delta-like 4; EGFR, epidermal growth factor receptor; FN, fibronectin; TG, transgutaminase; Met, hepatocyte growth factor receptor; MIF, migration inhibitory factor; PEDF, pigment epithelium-derived factor; T101, 1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride; TGF-β, transforming growth factor β

Table 3 | **Pre-clinical evaluation of selected exosomal cargoes (DNA fragments/ RNA species (lncRNA, mRNA, miRNA) / lipids/ and proteins) as cancer biomarkers: an overview**

Cargo		*Cancer type*	*Patient cohort*	*Exosome source*	*Exosome isolation method*	*Assay used*	*Outcome / Utility*	*Ref*
DNA	KRAS G12V, G12D, G12R, G12C, G12S, G12A, G13D mutant DNA	Pancreatic cancer	Cancer patients= 39, healthy controls = 82	Plasma	UC	ddPCR	Exosomal mutant KRas performed better at predicting PDAC status compared to cell-free DNA mutant Kras.	242
	KRAS G12D and P53R27H mutant DNA	Pancreatic cancer	PDAC = 48, IPMN =7 CP =9, Others =12 Heathy =14	Serum	UC	ddPCR	Circulating exosomal DNA enabled detection of Kras G12D mutant (39.6%) and TP53R273H mutant (4.2%) DNA in In PDAC patients. However, 2.6% healthy individuals were positive for KRas mutation.	199
	activating EGFR mutant and EGFR790M mutant	Non-small cell lung cancer	TIGER-X: n=56; ctDNA low copy number :n=50 intrathoracic disease M0/M1a:n=21	Plasma	Exolutin™ (Exosome Diagnostics Inc)	Exosomal RNA and DNA: EXO100 (NGS) ctDNA: BEAMing (Sysmex Inostics Gmbh)	Compared with ctDNA, Combined exosomal RNA/DNA and ctDNA), increased the sensitivity of activating EGFR mutant and EGFR T790M.	246
RNA	AR-V7 mRNA splice variant	Castration-resistant prostate cancer (CRPC)	CRPC patient receiving hormonal therapy = 36	Plasma	ExoRNeasy™ kit (Qiagen)	ddPCR	Predicts resistance to hormonal therapy. Overall survival significantly shorter in exosomal AR-V7+ participants.	274
	Multiple mRNAs	Prostate cancer	High grade prostate cancer patient = 148, Low grade prostate cancer patient =371	Urine	EXOPRO™ Urine Clinical Sample Concentrator Kit (Exodiagnostics)	RT-PCR	Compared to standard of care (SOC) (AUC 0.63), urine exosome gene expression plus SOC (AUC 0.73) formed better at distinguishing between high grade and low grade/benign disease patients	275

	Neoantigen transcripts / fusion genes	Pancreaticobiliary cancer	Pancreaticobiliary cancer patients =3	Plasma and Pleural effusion	UC	NGS	A wide range of potential cancer-associated biomarkers could be detected, including copy number profiles, point mutations, insertions, deletions, gene fusions and mutational signatures.	240
	lncRNA CRNDE-h	Colorectal caner	Volunteers with normal colonoscopy=80, hyperplastic polyp =80, inflammatory bowel disease=80, adenoma = 80, CRC patients =148	Serum	ExoQuick™ (System Biosciences)	RT-PCR	Distinguishes CRC patients from colorectal benign disease patients and healthy donors (AUC 0.892) with 70.3% sensitivity and 94.4% specificity, which was superior to carcinoembryogenic antigen.	276
	Multiple miRNAs	Lung cancer	Lung adenocarcinoma patients= 50, Lung granuloma patients = 30, healthy smokers = 25	Plasma	ExoQuick™ (System Biosciences)	RT-PCR	Distinguishes lung adenocarcinoma patients from patietns with lung granulomas with AUC 0.76 (sensitivity 96%, specificity 60%)	277
Protein	Glypican 1 (GPC1)	Pancreatic cancer	Pancreatic ductal adenocarcinoma patients = 190, Healthy controls = 100	Serum	UC	FACS	GPC1 positive exosomes distinguishes healthy subjects and patients with a benign pancreas disease from patients with early and late stage pancreas cancer with 100% specificity and 100% sensitivity.	184
	Migration inhibitory factor (MIF)	Pancreatic cancer	PDAC with progression to liver metastasis = 12, PDAC without progression = 10, Healthy control=15, PDAC patients with liver metastasis = 18	Plasma	UC	ELISA	Predicts liver metastasis in stage I PDAC patients ($P < 0.01$).	142
Lipid	Phosphatidylserine (PS) 18:1/18:1, lactosylceramide (d18:1/16:0), PS 18:0-18:2	Prostate cancer	Prostate cancer patients = 15 Healthy control = 13	Urine	UC	Mass spectrometry quantitative lipidomics	Combinations of three lipid species distinguishes prostate cancer patients from healthy control (AUC 0.989) (sensitivity 93%, specificity 100%).	278

Footnote - ddPCR, digital droplet polymerase chain reaction; RT-PCR, real time PCR; UC, ultracentrifugation; FACS, fluorescence-activated cell sorting; ELISA, enzyme-linked immunosorbent assay; AR-V7 androgen receptor splice variant 7; lncRNA, long non-coding RNA; mRNA, messenger RNA; miR, microRNA; AUC, area under curve; PDAC, pancreatic ductal adeno carcinoma. IPMN, intraductal papillary mucinous neoplasm, CP, chronic pancreatitis, NGS, next generation sequencing.

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