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The influence of tumour-derived extracellular vesicles on local and distal metastatic dissemination

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ABSTRACT

Extracellular vesicles (EVs) are key mediators of intercellular communication that have been ignored for decades. Tumour cells benefit from the secretion of vesicles as they can influence the behaviour of neighbouring tumour cells within the tumour microenvironment. Several studies have shown that extracellular vesicles play an active role in pre-metastatic niche formation and importantly, they are involved in the metastatic organotropism of different tumour types. Tumour-derived EVs carry and transfer molecules to recipient cells, modifying their behaviour through a process defined as "EV-driven education". EVs favour metastasis to sentinel lymph nodes and distal organs by reinforcing angiogenesis, inflammation and lymphangiogenesis. Hence, in this review we will summarize the main mechanisms by which tumour-derived EVs regulate lymph node and distal organ metastasis. Moreover, since some cancers metastasize through the lymphatic system, we will discuss recent discoveries about the presence and function of tumour EVs in the lymph. Finally, we will address the potential value of tumour EVs as prognostic biomarkers in liquid biopsies, specially blood and lymphatic fluid, and the use of these tools as early detectors of metastases.

1. Introduction: extracellular vesicles as key vehicles of cell-cell communication

Multicellular organisms rely on cell-cell communication to guarantee tissue homeostasis. There are different ways that neighbouring cells can communicate, through cell-cell contacts, gap junctions, extracellular vesicles and tunnelling nanotubes (McMillen and Holley, 2015; Nawaz and Fatima, 2017). Of these, extracellular vesicles (EVs) have emerged as key messenguers for the intercellular communication that regulates both physiological and pathological processes (Becker et al., 2016; Maas et al., 2017). EVs are released by most cell types and they carry different molecules that influence the activity of the surrounding cells, including proteins, RNA, DNA, lipids and metabolites (Abels and Breakefield, 2016; Tkach and Thery, 2016; Valadi et al., 2007). EVs are a heterogeneous group of membrane vesicles mainly comprised of exosomes (40–100 nm diameter multivesicular vesicles of endocytic origin) and microvesicles (MVs, 100–1000 nm diameter that bud directly from the plasma membrane (Raposo and Stoorvogel, 2013). Nevertheless, recent studies suggest that smaller vesicles (<100 nm) derived from plasma membrane protrusions can be isolated together with exosomes (Colombo et al., 2014). Larger vesicles are also actively secreted by some cell types, like cytoplasts (Headley et al., 2016) and large oncosomes (Di Vizio et al., 2012), further demonstrating that EVs are a heterogeneous population of vesicles that influence different biological processes.

Different techniques can be used to isolate EVs, such as ultracentrifugation, filtration, size exclusion chromatography, immunoaffinity isolation and microfluidic approaches (Li et al., 2017). As such, the International Society for Extracellular Vesicles (ISEV) has published guidelines in order to standardize EV isolation methods across the research community (Witwer et al., 2013, 2017). Ultracentrifugation is considered the gold-standard purification method to isolate exosomes and MVs, and it is one of the most commonly

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used techniques (Li et al., 2017). Polymeric precipitation has raised concerns in the EV research community due to the contamination with protein or soluble factors (Helwa et al., 2017; Lobb et al., 2015). The further development of protocols to isolate and characterize EVs, adapting microfluidic isolation (Gholizadeh et al., 2017), nanoplasmonic sensors (Im et al., 2014; Liang et al., 2017; Maiolo et al., 2015) or asymmetric flow fractionation (Petersen et al., 2014; Sitar et al., 2015), should shed light on their true complexity and heterogeneity (Kowal et al., 2016).

Exosome shedding is normally dependent on canonical pathways regulated by the Rab family of proteins, including Rab27, Rab11 and Rab35 (Hsu et al., 2010; Ostrowski et al., 2010; Savina et al., 2002, 2005). There are common molecules involved in the biogenesis of both MVs and exosomes, such as the VPS4 ATPase (Jackson et al., 2017). MVs are normally formed by direct budding of the plasma membrane (Muralidharan-Chari et al., 2010) and changes in Ca^{2+} seems to be critical for these membrane lipid rearrangements (Minciacchi et al., 2015). Although there has not been extensive research into the mechanisms that control MV release, the calcium dependent enzyme Calpain regulates MV biogenesis in platelets (Crespin et al., 2009) and malignant breast cancer cells, its inhibition blocking their secretion by the latter (Taylor et al., 2017). ADP-ribosylation factor 6 (ARF6) also control MV formation and membrane release (D'Souza-Schorey and Chavrier, 2006), and it has been implicated in regulating ERK-MLCK (myosin light-chain kinase) activation-dependent MV shedding from breast cancer cells (Muralidharan-Chari et al., 2009). In other human cancer cells, small GTPases like RhoA, Rac and Cdc42 are key players in MV biogenesis, not least because they regulate actin dynamics (Antonyak et al., 2012; Li et al., 2012). Through independent and non-redundant mechanisms, Ubiquitin ligase adaptors like the arrestin domain-containing proteins Arrdc1 and Arrdc4, influence the release and sorting of the EV cargo in HEK293 cells and gut explants (Mackenzie et al., 2016). It has also been hypothesized that the MV cargo is recruited to specific foci at the plasma membrane, increasing the local pressure at the membrane to force its curvature and posterior budding. Hence, the enrichment of the protein cargo at sites of future MV formation could be sufficient stimulus to generate extracellular MVs (Stachowiak et al., 2012). Interestingly, novel pathways of vesicle release have also been described, such as hyaluronan-coated EVs (Rilla et al., 2014). Due to this heterogeneity, it is sometimes not clear what kind of vesicles are referred to in the literature and therefore, in this review we will use the general term EV when the nomenclature is ambiguous or not defined.

The uptake of EVs by recipient cells has been little studied, although it is thought to involve two main mechanisms: direct membrane fusion or endocytosis (French et al., 2017). The most canonical and best characterized mechanism of EV uptake is endocytosis, an active process of engulfment that includes clathrin-mediated endocytosis, phagocytosis or macropinocytosis (French et al., 2017). However, it remains unclear whether this mechanism is dependent on specific receptors or proteins located on the EV surface that may target them to specific cell types. Interestingly, epithelial cells and astrocytes cannot normally internalize EVs from transformed cells, although they do internalize EVs when transformed through oncogenic Ras or c-Src expression (French et al., 2017). Thus, cellular transformation may reinforce EV uptake. The preferential interactions between EVs and certain cell types have also been observed *in vivo*, and melanoma-derived exosomes accumulate in the lungs and bone increasing the frequency of metastasis at these sites (Peinado et al., 2012). Similarly, integrins at the surface of exosomes and cells also influence exosome targeting to specific cell types, promoting their uptake and reinforcing organ specific metastasis (Hoshino et al., 2015). Exosomes from the lung-tropic 4175-LuT breast cancer cells contain α6β4 and α6β1 integrins, and they accumulate in regions of the lung rich in laminin (a ligand for these integrins), which favours lung metastases. Similarly, exosomes from the liver-tropic BxPC-3 pancreatic cancer cell line contain integrin αvβ5 and they preferentially accumulate in regions of the liver rich in the integrin αvβ5 ligand, fibronectin (Hoshino et al., 2015). Overall, these data suggest that EV localization *in vivo* is determined by adhesion molecules, such as integrins, and specific EV localization to these regions may be responsible for specific EV uptake. Futher studies will determine if these molecules are uniquely drivers of EV uptake or complementary to other receptors.

2. Tumour-derived EVs that remodel the tumour microenvironment at primary sites

Tumour cells release a wide variety of tumour-derived EVs (tEVs) that influence the behaviour of cells in the primary tumour microenvironment (Bobrie and Thery, 2013; Thery et al., 2009). Pioneering studies showed that oncoproteins are shed and transferred from one tumour cell to another via tumour MVs (tMVs) (Al-Nedawi et al., 2008; Rak and Guha, 2012). Thus, epidermal growth factor receptor variant III (EGFRvIII) can be packaged into MVs from EGFRvIII expressing glioma cells and transferred to EGFRvIII-negative cancer cells, activating mitogen-activated protein kinase (MAPK) and AKT signalling pathways in the recipient cells, and thereby enhancing their survival and tumour growth (Al-Nedawi et al., 2008). Similarly, human breast and colorectal cancer cells that harbour KRAS mutations secrete tumour exosomes (tExos) that are enriched in KRAS and EGFR ligands, and that enhance the invasiveness of neighbouring recipient cells (Demory Beckler et al., 2013; Higginbotham et al., 2011).

State-of-the-art technology has recently allowed the *in vivo* transfer of exosomes from highly to less metastatic cells to be visualized. For example, a Cre-LoxP system has been used in tExo-donor cells in association with GFP or Tomato genes to induce a colour switch in the recipient cells upon tExo uptake (Zomer et al., 2015). This approach made it possible to observe multiple non-tumour cells receiving tExos in both the tumour microenvironment and in peripheral tissues (e.g., lymph nodes, the lungs and spleen). These data highlight the ability of tExos to not only transfer information to neighbouring tumour cells but also, to stromal cells within the primary tumour microenvironment and to metastatic organs (Zomer et al., 2015). Endothelial cells have also been described as recipients of tEVs in glioblastoma and pancreatic cancer models, resulting in an activation of the angiogenesis that favours tumour growth and dissemination (Nazarenko et al., 2010; Skog et al., 2008). Fibroblasts can also be transformed into myofibroblasts following the uptake of transforming growth factor beta (TGFβ)-enriched prostate tExos (De Wever et al., 2014), and the tumour progression of these tExo-treated fibroblasts is favoured by vascularization, tumour growth and local invasion (De Wever et al., 2008; De Wever et al., 2010). Moreover, this myofibroblast phenotype is also observed in adipose tissue-derived mesenchymal stem cells when they receive breast cancer-derived tExos (Cho et al., 2013). Similarly, tEVs also help generate the immunosuppressive microenvironments that foster tumour growth, inducing a reprograming of macrophages towards a M2 tumour-supportive phenotype (de Vrij et al., 2015; Shinohara et al., 2017a), cytotoxic $CD8^+$ T cell apoptosis (Wieckowski et al., 2009), a decrease in NK proliferation and a phenotypic shift of $CD4^+$ cells to T regulatory lymphocytes (Whiteside, 2013). Myeloid-derived suppressor cells (MDSCs) can also be reprogrammed through the transfer of glioma and carcinoma EV-mRNAs so that they elicit enhanced immunosuppression (Ridder et al., 2015).

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