Message in the bottle: Regulation of the tumour microenvironment via exosome-driven proteolysis

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Abstract

Exosomes comprise a subtype of extracellular vesicles involved in cell-to-cell communication, specifically by transporting biological molecules, such as proteins and nucleic acids, to either local or more distant recipient cells, thus triggering distinct biological behaviours. Included in the exosome cargo is frequently a wide range of proteolytic enzymes, such as the matrix metalloproteinases (MMPs), the disintegrin and metalloproteinases (ADAMs), and the ADAM with thrombospondin-like motifs (ADAMTSs), whose functions contribute to the development and progression of cancer. In recent years, extensive research on the potential use of exosomes in diagnostic and therapeutic applications for personalized medicine has emerged, but the targeting of the proteolytic cargo of exosomes has not been fully exploited in this direction. In this review, we aim to explore both the mechanistic and the translational importance of proteolytic enzymes carried by the tumour-cell derived exosomes, as well as their role in the acquisition and support of certain hallmarks of cancer.

Keywords: exosomes, proteolysis, MMPs, metastasis, immunosuppression, biomarkers

Introduction

Cell-to-cell communication is required for proper coordination among different cell types within tissues. Cells may interact with each other by diverse types of communication, which primarily include endocrine, autocrine, and paracrine modes of action [1]. The endocrine mode in particular, involves the secretion of molecules, such as hormones, directly into the bloodstream, which travel long distances and eventually reach their target cells [1]. Exosomes, which are small extracellular vesicles (EVs), comprise a recently described mode of endocrine communication, and involve a more organized and sophisticated "packaging" of biomolecules into vesicles, as opposed to the more "naked" nature of hormones [1]. Since exosomes are difficult to discriminate from other microvesicles, they are both usually referred to as simply EVs [2]. Apart from their action as a newly described endocrine system, exosomes may also act locally to provide autocrine or paracrine signals when taken up by the target cells shortly after release [3].

Exosomes are released by almost all cell types, including cancer cells. The latter typically release a higher number and variety of exosomes compared to normal cells, which are referred to as "tumour cell-derived exosomes" [4-6]. Ranging from 30 to 150 nm in size, exosomes are formed by the inward budding of the late endosomal membrane which encloses cellular biomolecules within multivesicular bodies (MVBs), later released as exosomes into the extracellular environment following the fusion of MVBs with the plasma membrane [7]. Although the common term 'exosome' was not devised until 1987, these small extracellular vesicles were first discovered in 1981 by *Trams et al.* Because their sole purpose was believed to remove undesirable material from cells via exfoliation of the plasma membrane [8]; they were often referred to as the 'garbage of the cells' [9]. Following this, extensive research in exosomes provided novel insights into their function and potential use in therapeutics, justifying their role as biological messengers and carrying a "message in a bottle" [10].

Exosomes may contain multiple biomolecules, such as microRNAs (miRNAs), mRNAs, proteins, lipids, and other molecules which are transferred either locally or to distant sites. The composition of exosomes varies significantly, depending on the cell type of origin [1]. Upon their release, exosomes and their cargo travel either short or longer distances to directly affect recipient cells, frequently altering their normal or even pathological behaviour and functions, such as gene expression, cell signalling, adhesion, and proliferation [11]. The process of transferring proteins and nucleic acids to recipient cells has provided evidence for the important role of exosomes in regulating the "hallmarks of cancer", as they can contribute towards tumorigenesis, metastasis, tumour-induced immunosuppression, as well as drug resistance of cancer cells [12,4]. Several signalling cascades have been found to use EVs as message carriers in tumour–stroma interactions [13]. Cancer cells send exosomes to distant sites to 'educate' their future microenvironment and stromal cells with tumour-supportive signals. Therefore, exosomes play a crucial role in the preparation of the pre-metastatic niche and the ensuing metastatic process [12,14-16]. In this review, we describe the association between the cancer cell exosome cargo, especially when including proteolytic enzymes, and the regulation/acquisition of the hallmarks of cancer.

Proteolytic enzymes, produced either by tumour cells or their surrounding stroma cells, play critical roles in cancer invasion and metastasis [17] and angiogenesis [18]. Their primary functions comprise of extracellular matrix (ECM) remodelling, activation of adhesion molecules and growth factors, suppression of cell apoptosis, and finally, contextual regulation of the antitumor immune response [17]. It has been previously estimated that enzymes account for almost a third of all proteins contained within exosomes, with exosome proteases being a major constituent of their functional properties [19]. For instance, certain members of the Metzincin family, the

secreted and membrane-anchored matrix metalloproteinases (MMPs), have been identified at the surface of tumour-derived exosomes [20]. Likewise, the proteolytic enzymes disintegrin and metalloproteinases (ADAMs) and ADAMs with thrombospondin motifs (ADAMTS) [21], have also been found in tumour-derived exosomes [22], whereby they actively contribute to cancer progression, via regulating cell migration, adhesion, ECM modification, and most importantly, targeted shedding of membrane proteins, such as receptors and other signalling molecules [11]. Regardless of these observations, there is a limited understanding on the distinction between proteases secreted freely in the microenvironment and those included in the exosome cargo.

Certain investigations have reported promising clinical significance of exosomes and their respective cargos, including therapeutic, diagnostic, or prognostic potential [23,24]. In addition, the natural characteristics of exosomes, such as their small size, stability in peripheral circulation, constant formation in the human body, and cargo transfer mediating cell-cell communication, have further supported their potential as a drug delivery system [25,26]. Exosomes have also been identified in easily accessible body fluids, such as blood serum and plasma, cerebrospinal fluid (CSF), urine, saliva, and breast milk [27-29], thus constituting a potential, non-invasive source of diagnostic, prognostic, and predictive cancer biomarkers using 'liquid biopsies' [27,30].

The aim of this review is to foremost explore the mechanisms via which proteolytic enzymes are carried by tumour cell-derived exosomes within the tumour microenvironment (TME), including their roles in cancer progression, invasion, metastasis, and immunomodulation. In addition, the importance of exosome-derived proteolytic enzymes in cancer diagnostics and therapeutics is explored, with a particular focus on targeted cancer treatment and their potential as biomarkers in personalized cancer medicine.

1. Exosomes and exosome-derived proteases in the tumour microenvironment (TME)

Direct exosome-driven proteolysis within the TME

Based on accumulating data that proteolytic systems are found functioning either as free-form (secreted) proteins, or packaged within exosomes, a key question discussed here is whether either form offers any advantage over the other. Indeed, exosome-derived proteolytic enzymes have distinctive features compared to the respective free proteins that are employed as cancer biomarkers [31]. These enzymes demonstrate greater specificity in terms of mode of action, compared to their equivalent free forms. Of note, protein encapsulation inside the exosomes increases their stability as they are sheltered from degradation by other enzymes until reaching their target [31,32]. Alternatively, exosomes can burst and deliver their content within the TME under abnormal acidic conditions. Therefore, molecules carried by exosomes, such as growth factors, angiogenic factors, and proteases, can have a strictly localized impact within the ECM and the TME to support tumour growth [33]. Through proteases, exosomes can also provide paracrine or endocrine signals to more distant cells within the TME, but such mechanisms of action are poorly understood. For instance, by packaging together proteases that function in a proteolytic "cascade" (e.g. urokinase-type plasminogen activator uPA, MMP2, and MMP9), it is ensured that once released, these proteases will find each other in the TME, facilitating degradation of and invasion to the ECM, leading to cancer progression and metastasis. Therefore, carriage and secretion of those factors by exosomes might have been selected to be part of their synchronous secretion in addition to their local enrichment that might altogether, be more effective. Traditionally, the TME plays a crucial role in cancer progression by providing inhibitory or stimulatory signals [34]. Since exosomes represent a relevant cell-to-cell communication,

they contribute to the cross talk between cancer and stromal cells (e.g. fibroblasts, endothelial cells, immune cells), or various ECM components (collagen, fibronectin, laminin, etc), to establish the critical hallmarks of cancer within the TME [35]. It is also believed that exosomes serve as a platform for ectodomain shedding (i.e. conversion of transmembrane molecules into their soluble form) and as a vehicle for cellular export of soluble molecules [36]. Cleavage of ectodomains is mediated by MMPs and proceeds in a constitutive or inducible fashion. A recent study in ovarian cancer cells indicated that ADAM-10 is necessary for CD41 and L1 cleavage inside endosomes and subsequent exosome secretion in the extracellular space, while ADAM-17 proceeds to cleave the same molecules on the cell membrane [36]. Likewise, *Gutwein et al.,* further supports that constitutive cleavage of L1 in ovarian cancer cells by ADAM-10 is responsible for vesicle secretion [37].

Exosomes themselves and/or the exosome-contained active proteases, (such as MMPs and ADAM/TSs), via direct or indirect exosome-driven proteolysis, can also promote tumour growth and metastasis [20,38,11]. For instance, EVs derived from primary or metastatic brain tumour cells can increase ECM degradation directly through activation of serine protease tissue plasminogen activator (tPA), or indirectly via increase in MMP expression [39]. Moreover, a comprehensive analysis of EVs from pancreatic adenocarcinoma with different ECM components indicated that these EVs contain active proteases, such as MMPs and ADAMs, promoting tumour growth and invasion. [40]. MMPs and ADAM/TSs play an important role in cancer progression, mainly via the direct degradation of the ECM, leading to exposition of hidden ECM binding sites for interaction between cancer cells and their matrix via integrins, and to the release of active pro-oncogenic growth factors that are sequestered to the ECM [41,42]. Although proteases directly secreted from cancer cells are important, recent evidence suggests that focal action of proteases in the ECM must be frequently mediated by extracellular vesicle secretion (**Figure 1**) [21,22]. Membrane type 1 matrix metalloproteinase (MMP14) detected in fibrosarcoma and melanoma cell-derived exosomes is responsible for activation of other proteases and growth factors, via direct proteolytical cleavage within the ECM, thus promoting the conversion of pro-MMP2 into an MMP2 active form, capable of degrading collagen fibers (35). It has also been shown that ARF6-positive EVs contain MMP14 along with beta 1 integrin, which allows the binding to the ECM and its direct degradation in the immediate surroundings of those EVs [43]. ARF6 is a small GTPase involved in EV secretion through kinesin-dependent transport of MMP14 from the cytoplasm to the plasma membrane. The phosphorylation of ARF6 by ERK leads to actin-myosin-mediated constriction of the membrane and the release of the exosome cargo in the extracellular space [44,41,43] (**Figure 1A**).

MMPs and ADAM/TSs are either tethered to the exosome membrane or contained within the exosome cargo in several types of cancer cells. It was first discovered that fibrosarcoma cells in culture secrete vesicles containing membrane-anchored gelatinases MMP2 and MMP9, as well as the urokinase-type plasminogen activator (uPA) [45]. The uPA/uPA receptor (uPAR) complex can activate gelatinases both directly via proteolytic cleavage, and indirectly via conversion of plasminogen to the serine protease plasmin [46]. Thus, the presence of these three proteases on the vesicle's membrane could be responsible for ECM degradation in the surrounding environment of neoplastic cells.

Several studies have provided insights on the mechanisms by which vesicles containing gelatinases can be transported and released. In melanoma cells, two types of intracellular vesicles containing MMP2 and MMP9 respectively, have been identified. These vesicles were shown to use the microtubule network as a transport substrate. Disruption of the microtubular system by the chemotherapeutic drug Paclitaxel led to reduced secretion

of these vesicles along with decreased invasion ability [47] (**Figure 1B**). In another study, vesicles carrying MMP2 and MMP9 together, were shown to be actively transported to invadopodia in a Rab40b-dependent fashion in breast cancer (BCa) cells. When reaching the tip of invadopodia, the interaction between Rab40b and Tks5 triggered the release of the vesicle content into the ECM, causing its local degradation. Importantly, the knockdown of Rab40b was sufficient to reduce tumour growth *in vivo* [48] (**Figure 1C**). Exosomes released by tumour cells have been found to contain MMP14, which can degrade type 1 collagen in the ECM and also cleave pro-MMP2 in parallel, leading to the remodelling of the ECM and subsequent invasion [49] (**Figure 1D**). MMP2 was also found present in BCa-derived exosomes, where upon activation by the chaperone protein HSP90a, resulted in ECM degradation and growth factor activation in the surrounding tumour microenvironment, which eventually contributed to tumour invasion [50]. Other proteases have also been linked with exosome-mediated ECM degradation. It was shown that microvesicles secreted from oligodendroglioma had proteolytical activity that was associated with the presence of ADAMTS and responsible for aggrecan degradation and increased invasiveness of cancer cells [51]. In another study, exosomes containing MMP13, along with tetraspanins CD9 and CD63, were found overexpressed in nasopharyngeal cancer (NPC) cells and in serum from NPC patients, and demonstrated the ability to enhance invasion, migration and epithelial-mesenchymal transition of NPC cells *in vitro*. [52] (**Figure 1E**).

Indirect exosome-driven proteolysis within the TME through regulatory factors

As already mentioned, there is on the one hand, a direct exosome cargo delivery of proteases that is released in the ECM and facilitates tumour progression. On the other hand, exosome-mediated transportation of regulatory factors (such as miRNAs or mRNAs) regulating the transcription and translation of proteolytic enzymes in distant recipient cells has also been documented. miRNAs conveyed within exosomes can mediate communication between cancer cells and their milieu and can increase the level of translation of their target mRNAs in recipient cells [53], while mRNAs transferred to cells by exosomes can result in the production of proteins with novel biological significance. For instance, exosomes from BCa patients and cell lines were demonstrated to contain miRNA processing machinery proteins, such as Dicer, and induced epithelial transformation and tumour formation in non-tumorigenic mammary cells through transcriptional modulation [54]. In addition, glioma-derived exosomes have been shown to transfer functional mRNAs, such as EGFR mRNA, that could further promote tumour growth [55].

Exosomes secreted from multiple myeloma were shown to increase MMP9 and the cysteine protease cathepsin K levels in human primary osteoclasts via direct transfer of MMP9 mRNA (**Figure 2A**). This pathway resulted in enhanced migratory ability of osteoclasts, responsible for bone resorption. These exosomes were also found in the blood serum of patients and were able to increase MMP9 expression in targeted macrophages and induce their differentiation into osteoclasts [56]. In another study, miRNAs (miR-100-5p, miR-21-5-p, and miR-139-5p) were found increased in prostate cancer (PCa)-derived exosomes. These exosomes were enriched in miRNAs involved in osteoblast differentiation, which in turn increased RANLK production, triggering osteoclastogenesis and contributing to the preparation of the pre-metastatic niche (**Figure 2B**). Purification and transfection of these exosomes in fibroblasts led to their activation and increased migratory ability, achieved via upregulation of

MMP2, MMP9, and RANKL [57]. Therefore, cancer cell-derived exosomes can modulate transcription via transferring miRNAs in stromal cells, setting up a pro-tumorigenic environment and pre-metastatic niche [57].

Most recently, *Wang et al.,* studied the function of miR-4443 derived from exosomes in breast cancer (BCa) metastasis *in vitro* and *in vivo*. The authors showed that miR-4443 promotes metastasis of BCa cells through downregulation of the tissue metalloproteinase inhibitor (TIMP2) and upregulation of MMPs [58] (**Figure 2C**). More specifically, highly invasive BCa cells can activate MMPs by delivering exosomal miR-4443 to stromal cells of the primary tumour and by impairing TIMP2 [58]. Exosomes secreted from cancer cells have been shown to trigger MMP2 expression in cancer-associated fibroblasts in several cancers [59-61]. In all cases, the exosomes were positive for the MMP inducer EMMPRIN (CD147). In some instances, exosomes were shown to enhance MMP expression in the target cells, but the regulatory factors were unknown. Finally, it was shown that EVs carrying MMP1 mRNA facilitate peritoneal dissemination in ovarian cancer (OC) [62] (**Figure 2D**). MMP1 mRNA enriched in the EVs as well as in the ascites of OC patients was responsible for increased cell death in the targeted mesothelial cells, leading to the disruption of the peritoneal-mesothelium barrier, and eventually to peritoneal dissemination [62].

In conclusion, in addition to the direct effect of MMPs released within the ECM, cancer cell-derived exosomes can modulate transcription via transferring miRNAs/mRNA or other regulatory factors in stromal cells, again, setting up a pro-tumorigenic environment to prepare the metastatic niche [56].

2. Exosomes and exosome-driven proteases in the hallmarks of cancer

Exosome-mediated proteolysis in cancer progression, invasion, and metastasis

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Tumour cells send signals to distant sites through EV secretion, to prepare the environment towards which they metastasize. EVs and their content have the ability to set up the TME before tumour cells by transforming macrophages and stromal cells into tumour-supporting cells, to enhance tumour cell dissemination [63]. Annexin A2 protein is found in exosomes and is increased in tumour-associated exosomes. Exosomal annexin A2 promoted angiogenesis and metastasis in an *in vivo* BCa model. This process was dependent on tPA and thus probably plasmin generation. Plasmin is involved in angiogenesis and MMP activation. Therefore, exosomal AnnexinA2 could act as co-receptor for tPA and plasminogen and, as such, could be indirectly involved in MMP activation [64].

Vesicle trafficking of proteases through exosome secretion, is a crucial factor in the regulation of ECM degradation, cancer progression, and metastasis [65,47,14,66] and MMPs are among factors secreted in the context of pre-metastatic niche formation [67]. For example, the BCa cell line MCF-7 releases MMP9-containing vesicles in a Rab26a/b-dependent manner, contributing to tumour progression [65]. In addition, exosomal proteases are directly involved in the ability of the cells to migrate and invade through the basement membrane and the stroma tissue. Release of EVs containing MMP14 is necessary to maintain amoeboid cell migration, a type of migration in which the cells move via actin contractility supposedly with virtually no adhesion force and no ECM degradation [68]. On the other hand, invadopodia are protrusions regulated by actin polymerization expressing active ECM-degrading MMP14 at the cell surface. MMP14 is transported via endosomes along the microtubule track, and is then released at the surface of the cells in an ARF6-dependent mechanism [44]. Invadopodia act as docking and secretion sites for exosomes from intracellular multicellular endosomes.

Exosomes, in turn, can function to promote invadopodia formation and stabilization [69]. MMP14 cytosolic tail was demonstrated to be anchored to the F-actin filament beneath the invadopodium plasma membrane, which makes it quite stable and restrains its endocytosis uptake, allowing active ECM degradation [44]. Therefore, both types of vesicles - the ones released by amoeboid cells and the ones released from invadopodia - contain MMP14 and might contribute to matrix degradation and stabilization of nascent invadopodia.

Regulation of cell invasion by MMP14 is not exclusively related to its catalytic activity against ECM components or activation of pro-MMP2. Recent evidence indicates that MMP14 can also cleave the multifunctional adhesion molecule CD44,promoting cancer cell migration [70]. In another study, astrocytes were found to release MMPs in response to the MMP inducer EMMPRIN (CD147) contained in glioblastoma-derived EVs and, radiation treatment enhanced MMP release [71]. It was suggested that GBM cells secrete the transmembrane glycoprotein CD147 in EVs, in a manner capable of enhancing release of MMPs by astrocytes. Specifically, astrocytes receive GBM EVs and subsequently increased secretion of active MMP9, a phenomenon that was also exacerbated by irradiation of GBM cells. Evaluating the MAPK pathway activation, which also regulates MMP expression, showed that JNK signalling was increased in astrocytes incubated with EVs from irradiated compared to non-irradiated GBM cells [37]. Knockout of CD147 in GBM cells suppressed JNK signalling and active MMP9 secretion [71]. Thus, by means of their specific cell-surface localization and substrate profiling, membrane-anchored MMPs modulate the necessary molecular mechanisms that control the migratory and invasive phenotype of tumour cells.

Cancer-associated fibroblasts (CAFs) play an important role in tumour progression by secreting exosomes, which can in turn modulate tumorigenicity via regulating the levels of MMPs [72] Secretion of exosomes carrying MMP11 by CAFs was responsible for the acquisition of metastatic properties in gastric cancer cells [73]. An example of EMT driven mechanism by exosome-derived proteases was found in oral squamous cell carcinoma (OSCC), whereby cancer cells were delivered exosomes devoid of miR-34a-5p from CAFs, while normal cells received exosomes containing miR-34a-5p from normal fibroblasts [74]. When transfected in cancer cells, miR-34a-5p reduced tumorigenicity through direct antagonization of AXL tyrosine kinase receptor. In cancer cells, AXL was not antagonized by miR-34a-5p and induced upregulation of the transcriptional factor SNAIL, which in turn activated both MMP2 and MMP9. In parallel, AXL promoted EMT via augmentation of vimentin and reduction of E-cadherin [74]. This is consistent with previous studies showing that SNAIL induces cell invasion via the activation of MMP2 and MMP9 [75]. Concomitant EMT with increased secretion of proteases in tumour-derived exosomes have been described in several other studies. For example, exosomes derived from H-Ras-transformed cells display EMT markers (e.g. decrease in E-cadherin and increase in vimentin), while being enriched in MMP1, MMP14, MMP19, ADAM-10, and ADAMTS1 proteins [76].

EV-mediated MMP expression has been shown to contribute to the acquisition of long-distance migrating abilities by cancer cells and thus, of a pro-metastatic phenotype. Platelet-derived microvesicles (PMV) as well as carrying MMPs, induced MMP9 and MMP14 mRNA expression, and MMP2 activation, in recipient lung cancer cell lines, resulting in increased invasion capacity. *In vivo*, when cells covered by PMV were injected in mice, increased metastasis was observed in the bone marrow [77]. Exosomes derived from PCa cells grown under hypoxic conditions increased stemness and invasive ability of normal cells and converted stromal cells in CAFs. These exosomes contained an increased activity of MMP2 and MMP9 as well as increased levels of diverse growth factors and cytokines involved in pre-metastatic niche set-up [78]. More recently, it was shown that exosomes

secreted from PCa cell lines increased MMP2 and MMP9 activity in different distant organs that represent potential pre-metastatic sites, such as kidneys and spleen [79].

Finally, proteases released in the context of senescence can exert their pro-tumorigenic effect in the TME. Recently, senescence and the senescence-associated secretory phenotype (SASP) have been added to the very new list of hallmarks of cancer [80]. SASP is the secretion by senescent cells of a multitude of proteases, chemokines, and growth factors, that have been traditionally regarded as having a role in tumour suppression [80]. Senescence is now believed to have a more complex panel of consequences on the surrounding non-senescent cells, including the promotion of EMT, invasion, and angiogenesis, through the paracrine transport of exosomal cargos containing, amongst others, MMPs [80-82]. Further research will be warranted to determine whether the long list of factors, amongst which proteases, secreted in the context of SASP, have implication in tumorigenicity over tumour protection.

Exosome-mediated immunomodulatory mechanisms

Cancer can trigger alterations in the immune system through a variety of immunomodulatory mechanisms, essential for tumour development and progression. Three major lines of immunomodulation have been identified in the tumour microenvironment: i) evasion of cancer cell recognition by the immune system, ii) suppression of immunological reactions, and iii) survival advantage of cancer cells in inflamed microenvironments [83]. Of those, exosomes may be involved in the latter two mechanisms. For example, exosomes secreted from cancer cells could prevent the maturation of antigen-presenting cells and suppress the activity of T and NK cells [84].

Of note, there is clear evidence of immunomodulating effects by MMPs within the TME [85]. MMP9 is required by lymphocytes for basement membrane infiltration and by NK cells for migration. Importantly, neutrophils contain granules filled with MMP9 that are ready to be released in the TME [85]. MMP9 secreted by tumour-associated neutrophils (TANs) had actually a higher contribution to angiogenesis *in vivo* than the ones secreted by TAMs, since the latter need time to polarize and inhibit their TIMP1 expression [86]. Besides the outdated concept that MMPs exert their functions exclusively via ECM degradation, it has now been shown that chemokines and cytokines are directly cleaved by MMPs, suggesting that MMPs can directly regulate cancer-associated immunological reactions. In addition, MMPs and ADAMs can play a more indirect role in the modulation of immune response through regulation of transcription, processing of receptors, and shedding of membrane-anchored factors [87]. Therefore, they are true conductors of the communication between cells, stroma, and the ECM in the context of the immunologic response. Tumor-associated macrophages (TAMs) are primarily found at the leading edge of the tumour and play an active role in degrading the ECM through direct secretion of diverse factors [88]. Tumour cells need TAMs along the way, as they maintain their pro-tumorigenic phenotype and help suppress immunologic responses, which can be facilitated by exosome secretion [89,90]. For example, MMP9 secreted by macrophages is coordinated with production of elastin fragments, which activates and guides monocyte migration. TAMs also secrete other MMPs, uPA, cathepsins, and serine proteases, which contribute to ECM degradation, as well as IL-10 and TGF-β in parallel, which contribute to immunosuppression [91].

Overall, it is not clear whether the proteolytic enzymes are packaged inside the vesicle of the exosome and/or they localize at the cell surface to regulate immunosuppression. There is evidence that macrophages secrete or are affected by proteolytic enzymes, but not necessarily that these are packaged into exosomes. However, we adopt here once again the model of a synchronous and contextual secretion, which gives an advantage for the activation

of proteolytic cascades as well as the enrichment of these factors in very narrow niches. Examples in which MMP expression can be modulated through exosomes in the context of immunomodulation have been reported. For example, exosomes derived from epithelial ovarian cancer have been shown to transfer lncRNAs to TAMs with the consequence of releasing the inhibitory effect of the latter on MMP2 signal in endothelial cells (**Figure 3A**). Therefore, cancer-derived exosomes can increase the migration capacity of endothelial cells through modulation of MMP expression [92].

EVs can be used by cancer cells to dictate the polarization of macrophages that will adopt more tumour-supportive behaviours, including MMP secreting characteristics. Pancreatic ductal adenocarcinoma (PDAC) cell lines secrete exosomes enriched in arachidonic acid that are able to interact and fuse with TAMs. Following this interaction, TAMs adopt an immunosuppressive M2-like phenotype. These M2-like TAMs can in turn secrete pro-tumorigenic factors, such as, VEGF, MMP9, IL-6, and TNFa, which contribute to angiogenesis, invasion, and chemoresistance in pancreatic tumour progression (**Figure 3B**) [93,94]. It was shown that ascites-derived metastatic PDAC cell line (AsPC-1) exosomes incubated with non-polarized macrophages (THP-1 cells) caused higher expression of surface proteins CD14, CD163, and CD206, as well as higher secretion of pro-tumorigenic factors including VEGF, IL-6, MMP9, and TNF-α, which supported a polarization to an M2-like phenotype [93,94]. An *in vitro* study provided another example in which EVs isolated from GBM cell lines have also been able to modulate immunologic responses. Blood-derived macrophages were shown to adopt a M2-like phenotype after incubation with GBM-derived EVs, expressing CD163, secreting more cytokines, presenting with increased phagocytotic activity. In parallel, human primary microglia showed an increased expression of MMP14 after incubation with the GBM-derived EVs, which is thought to happen in the macrophage population as well (**Figure 3C**). Therefore, the authors suggested that inhibiting these tumorigenic EVs could be a therapeutic strategy for GBM patients [95].

Finally, MMP mediated signalling can be modulated by exosomes secreted from TAM. In that case, TAMs serve as exosome-secreting cells that target the cancer cells. In hepatocellular carcinoma (HCC), a population of TAMs released exosomes carrying the integrin alpha-M/beta-2, which activated MMP9 signalling in the recipient HCC cells (**Figure 3D**) [96].

3. Exosome-derived proteolytic enzymes in cancer diagnosis

Overall, exosome-derived MMPs likely account for malignant properties in several types of cancers through either modulation of the tumour microenvironment or directly through affecting tumour cells. Therefore, such proteases represent interesting therapeutic targets as well as potential prognostic and diagnostic biomarkers. Indeed, protease levels increase during tumour progression, and often correlate with poor prognosis. Exosomes have diagnostic/prognostic potential in several cancer types, including colorectal cancer, prostate cancer (PCa), and GBM [97,98,55]. Cancer-derived exosomes carry high concentrations of molecules that are better reflecting the tumour cell molecular signature, unlike free-molecules present in the blood stream. Since exosomes contain proteins, lipids, and nucleic acids, and their levels generally increase in cancer, they also have the potential to serve as a sensitive and specific reservoir of cancer biomarkers. Exosomes can be isolated in liquid biopsies using ultracentrifugation and commercially available extraction kits, from blood serum, or other body fluids, for instance ascites, in the case of ovarian cancer. Detection of tumour-derived exosomes, which are present in easily

accessible body fluids, could therefore constitute a non-invasive tool for early diagnosis and prognosis in cancer patients [99,100].

Accumulated evidence reveals that exosome-derived proteases, principally MMPs as well as ADAMs, could serve as biomarkers and potential therapeutic targets in a variety of cancer types. However, MMPs can have tumour suppressor roles in different tissue contexts, which represents a limitation for their targeting in clinics and use as tumour markers [101]. MMP2, MMP9, and uPA were found to be overexpressed in membrane vesicles from ascites fluid of malignant ovarian cancer. uPA activates plasmin, which in turn activates other MMPs, such as MMP14 [102]. Therefore, the presence of these proteases in membrane vesicles could have diagnostic and prognostic value in ovarian cancer. EVs derived from osteosarcoma cell lines expressed MMP1 and -13, along with TGFb and RANKL. RANKL activates MMPs and stimulates osteoclastogenesis. Therefore, these microvesicles could be responsible for bone destruction, and could serve as prognostic biomarkers in osteosarcoma. This has been confirmed by another study showing that MMP1 and -13 expression was associated with shorter survival in osteosarcoma patients [103]. EVs can also be isolated from ovarian cancer patients using lipid-ligand specificity. Annexin-V, which has a high affinity for phosphatidylserine, can be used to isolate vesicles from ascites fluid. Annexin-V-vesicles from ovarian cancer patients are specifically enriched in MMP9 compared to other types of vesicles secreted by ovarian cancer cells, as well as vesicles secreted in other pathologies [104]. Exosomes isolated from plasma of GBM patients were also found enriched in MMP9 along with markers associated with the hypoxic response. These exosomes have an important role in driving an angiogenesis program, and they could be used as predictors of cancer-related angiogenesis and malignant status in glioma patients [105].

In addition, other MMPs derived from exosomes could serve as cancer biomarkers. For example, MMP3 primarily targets ECM components such as proteoglycans, fibronectin, and laminins and its expression correlated with poor prognosis in pancreatic and cervical cancer. Moreover, MMP7 was found among the proteins that were significantly enriched in exosomes derived from pancreatic ductal adenocarcinoma (PDAC) patients in comparison with individuals free of invasive cancer [106].

Membrane vesicles from ascites fluid of ovarian cancer patients contained ADAM10, while the level of pro-heparanase and the MMP inducer EMMPRIN varied between samples. These vesicles were responsible for tumour growth when injected in mice. They tended to be more frequent in the serum of patients, when also found in the ascites fluid of the same patient. Consequently, assessing the vesicles in the serum reflects their presence in ascites fluid and ascites fluid is considered as being very representative of the tumour since it is in close proximity with the TME [107]. ADAM10 expression in non-small cell lung cancer (NSCLC) correlated with poor prognosis. In addition, ADAM10 detected in exosomes derived from blood of patients displayed increased ADAM10 specific sheddase activity compared to non-cancer controls. This entails that the processing of PEPDAB005, a specific and synthetic substrate of ADAM10, could represent an efficient blood test for the diagnosis of NSCLC patients [108].

4. Conclusions and future perspectives

Exosome-driven proteolytic enzymes are of great importance in cancer progression and metastasis. The multifaceted and pleiotropic nature of these proteolytic enzymes, promotes and supports major hallmarks of cancer. The two key families of proteases found in exosomes and discussed here, MMPs and ADAMs, have multiple roles regulating cancer development and progression, including immunomodulation, ECM degradation, shedding and activation of membrane proteins, and angiogenesis. They can also serve as improved prognostic and diagnostic biomarkers for several types of cancer. There is still a lot to learn about exosome-associated proteases, their biological mechanisms of pathogenesis, and their clinical implications. The exosome biology is still a growing field of research, and it is essential to further analyse the roles of exosomes through generation of *in vivo*, and imaging models. *In vivo* models will also serve to increase our understanding on the mechanistic roles of tumour-derived or stromal cell-derived EVs during tumour progression.

In addition to simply facilitating transportation of MMPs/ADAMs to local and distant sites, this review described a working model that posits that exosome packaging of such proteases offers two key advantages for tumour development and progression: (1) Increased concentration of critical proteases at the target sites/tissues/cells, and, (2) Enriched and spatiotemporal secretion of proteases involved in proteolytic cascades. This framework will lay groundwork for future investigations on exosome biology in the tumour microenvironment, using sophisticated and targeted technologies. For example, exploring the current technological applications (such as single cell sequencing, proteomics, intravital imaging etc), would be interesting to see whether these exosome subpopulations with different types of MMPs target specific cell types and facilitate formation of primary and metastatic microenvironments.

Finally, the therapeutic use of exosomes can also justify their use as drug delivery vesicles in cancer treatment. Multiple aspects and properties of exosomes are responsible for their ability to be successfully used in cancer treatment such as their diminished immune-related side effects. Therefore, there are future opportunities for the use of exosomes in personalized medicine. Further investigations into the exosome-derived proteolytic enzymes produced by tumour cells and/or stromal cells within the TME would allow for clarification on whether these could serve as potent biomarkers and potential therapeutic targets in the rationalized treatment of cancer.

Compliance with ethical standards

The authors have no potential conflicts of interest.

Figure legends

Fig. 1 Direct exosome-mediated proteolysis at the protein level within the tumour microenvironment and impact in cancer progression. MMPs are transported from cancer cells to the extracellular space where they target the microenvironment (ECM and stroma cells) leading to tumour-supporting remodelling through different mechanisms: kinesin-dependent transport of MMP14 from the cells to the plasma membrane and release via actin-myosin-mediated constriction **(A)**, MMP2 and MMP9 kinesin-dependant transport through the microtubule network **(B)**, MMP2 and MMP9 Rab40b-dependent transport to invapodia and Tks5-dependant release of the vesicles **(C)**, release of exosomes containing MMP14 leading to collagen degradation and pro-MMP2 cleavage **(D)**, cross talk between cancer cells and stroma cells through MMP13-containing exosomes **(E)**.

Fig. 2 Indirect exosome-driven proteolysis within the tumour microenvironment via regulatory factors in the ECM and impact in cancer progression. Exosomes released from cancer cells mediate protease expression in the targeted stroma cells leading to tumour-supporting remodelling within the microenvironment. Examples shown in multiple myeloma **(A)**, prostate cancer **(B)**, breast cancer **(C)**, and ovarian cancer **(D).** [Figure created with Biorender.com.]

Fig. 3 Exosome-mediated proteases in the context of immunomodulation. Illustrative model with reported examples in which MMP expression and exosomes can impact immunomodulation within the TME. Exosomes released from cancer cells mediate secretion of MMPs by targeted tumour-associated macrophages (TAMs) in the microenvironment **(A-C)**, and MMP expression in cancer cells is triggered via the uptake of exosomes secreted by TAMs **(D)**. Examples shown in ovarian cancer **(A)**, pancreatic cancer **(B)**, glioblastoma **(C)**, and hepatocellular carcinoma **(D)**. [Figure created with Biorender.com.]

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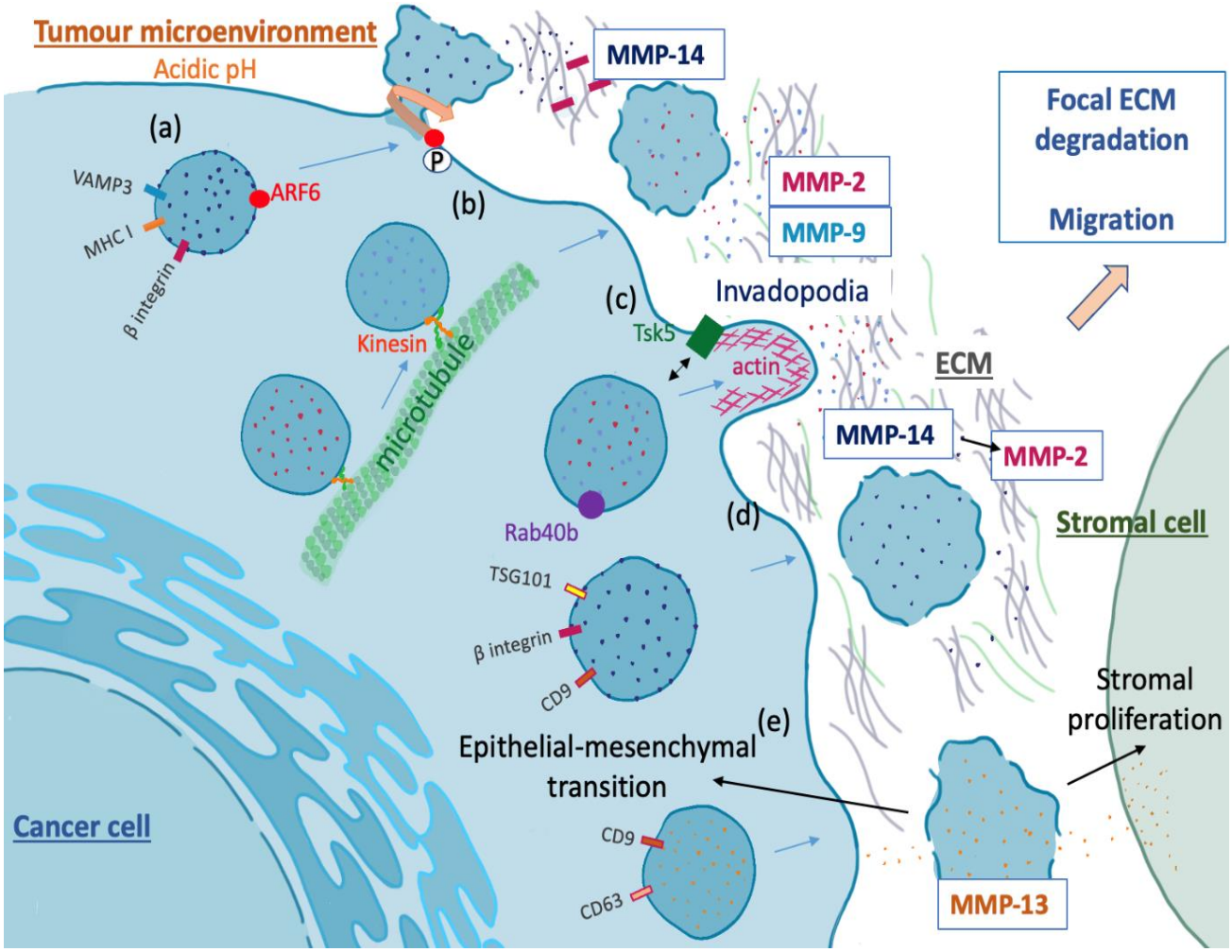
Tumour microenvironment
Acidic pH
MMP-14
Focal ECM degradation
Migration
(a)
VAMP3
ARF6
MHC I
β integrin
(b)
P
MMP-2
MMP-9
Invadopodia
(c)
Tsk5
actin
Kinesin
microtubule
ECM
MMP-14
MMP-2
Stromal cell
Rab40b
(d)
TSG101
β integrin
CD9
Stromal proliferation
(e)
Epithelial-mesenchymal transition
Cancer cell
CD9
CD63
MMP-13

Figure 1.

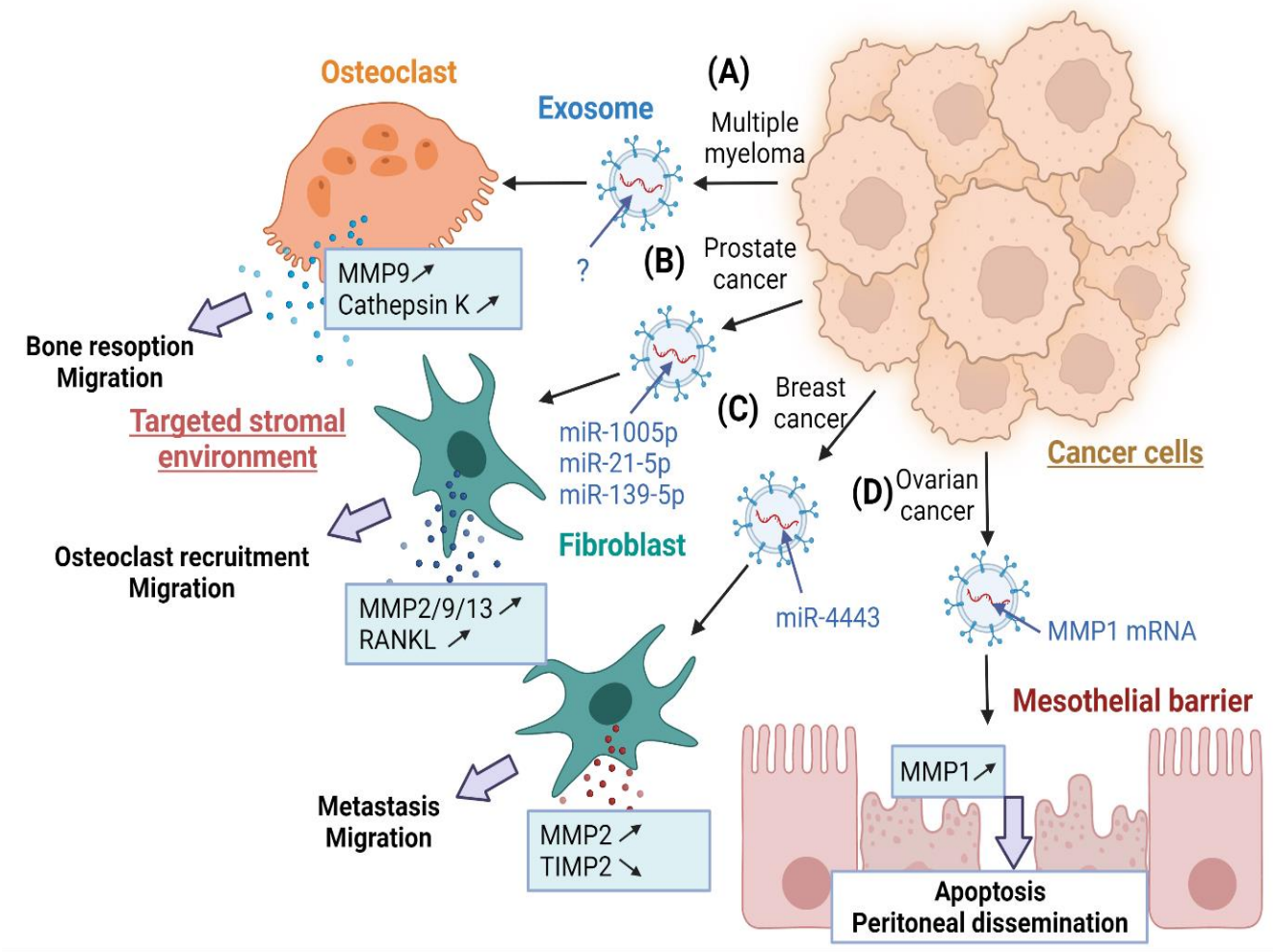
Osteoclast
(A)
Exosome
Multiple myeloma
MMP9↗
Cathepsin K↗
?
(B)
Prostate cancer
Bone resoption
Migration
Targeted stromal environment
miR-1005p
miR-21-5p
miR-139-5p
(C)
Breast cancer
Cancer cells
(D)
Ovarian cancer
Osteoclast recruitment
Migration
Fibroblast
MMP2/9/13↗
RANKL↗
miR-4443
MMP1 mRNA
Mesothelial barrier
MMP1↗
Metastasis
Migration
MMP2↗
TIMP2↘
Apoptosis
Peritoneal dissemination

Figure 2.

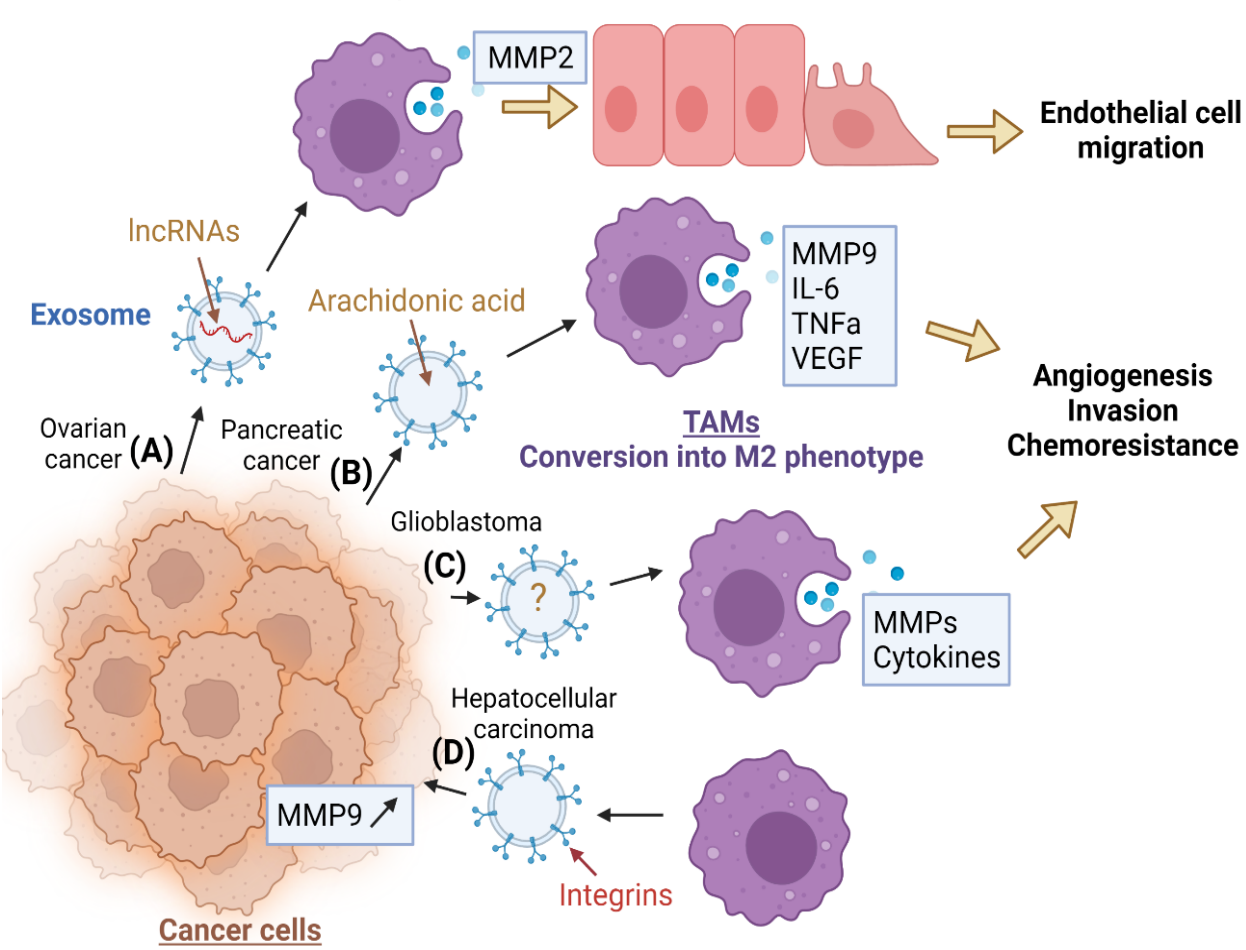
MMP2
Endothelial cell migration
lncRNAs
Exosome
Arachidonic acid
MMP9
IL-6
TNFa
VEGF
Angiogenesis
Invasion
Chemoresistance
Ovarian cancer (A)
Pancreatic cancer (B)
TAMs
Conversion into M2 phenotype
Glioblastoma
(C)
?
MMPs
Cytokines
Hepatocellular carcinoma
(D)
MMP9
Integrins
Cancer cells

Figure 3.