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Review

The pleiotropic role of proteoglycans in extracellular vesicle mediated communication in the tumor microenvironment

M. Cerezo-Magaña[a], A. Bång-Rudenstam[a], M. Belting[a,b,c,*]

[a] Department of Clinical Sciences, Lund, Section of Oncology and Pathology, Lund University, Lund, Sweden
[b] Department of Hematology, Oncology and Radiophysics, Skåne University Hospital, Lund, Sweden
[c] Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden

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ABSTRACT

Compartmental exchange between cells through extracellular vesicles (EVs), including exosomes and microvesicles, has emerged as a central mechanism that coordinates the complex communication between malignant and stromal cells during tumor initiation and evolution. Some of the most critical processes of EV-mediated communication, including EV biogenesis and EV uptake, can be mediated by heparan sulfate proteoglycans (HSPGs) that reside on the surface of producer and recipient cells as well as on EVs. With interestingly similar, HSPG-dependent, pathways as the ones exploited by some viruses, EVs may, in an evolutionary perspective, be viewed as endogenous counterparts of viral particles. Cancer cell-derived EVs exert their protumorigenic effects by direct interactions of biologically active surface molecules, by transfer of proteins and nucleic acids into recipient cells or by transfer of metabolites that can be utilized as an energy source by the recipient cell. Here, we discuss the pleiotropic role of the HSPG family in these different contexts of EV communication with a specific focus on tumor development. We propose EV-associated PGs as dynamic reservoirs and chaperones of signaling molecules with potential implications in ligand exchange between EVs and tumor target cells. The protumorigenic consequences of EV mediated communication through HSPG should motivate the development of therapeutic approaches targeting EV-HSPG interactions as a novel strategy in cancer treatment.

1. Extracellular vesicle mediated communication in cancer

Eukaryotic cells regulate many of their basic processes by coordinated, reciprocal communication with neighboring as well as distant cells. Together with soluble factor-mediated cell-to-cell signaling, extracellular vesicle (EV)-mediated communication has been established in the last decades as a key process in different pathological conditions with a special relevance in cancer biology [1]. EV secretion is a common mechanism for all cell types, and is particularly active and relevant in the context of cancer development and diagnostics [2]. Different EV populations are actively secreted by cancer cells [3] and they differ in protein markers, size and density [4]. These EVs include mainly microvesicles, 100–1000 nm in diameter, which arise after plasma membrane budding, and exosomes, 30–50 nm in diameter, which are derived from the endosomal compartment and hence are enriched in endosomal markers [5]. Additionally, dying cells release a variety of EVs, together defined as apoptotic cell-derived EVs, that include large membrane-bound vesicles as well as smaller vesicles that participate in apoptotic cell clearance and intercellular communication (Table 1). Here we will use the common term EVs for both types of particles, unless otherwise stated. The evolutionary benefit that eukaryotic cells attain from EV-mediated communication, which resulted in a complex system of compartmentalization, dispersion and exchange of cellular material, constitutes an interesting food for thought. The characterization of EV-dependent cellular crosstalk has resulted in the realization that defining a eukaryotic cell as a compartment delimited by its plasma membrane that prohibits intercellular compartmental exchange is no longer strictly valid. Instead, it should be understood that a cell's range of action is hard to delimit due to diffused borders of membrane-enclosed cell-derived compartments.

Abbreviations: EV, extracellular vesicle; MVB, multivesicular body; PGs, Proteoglycans; GAG, glycosaminoglycan; HSPGs, heparan sulfate PGs; CSPGs, chondroitin sulfate PGs; ECM, extracellular matrix; GF, growth factor; SDC, syndecan; PIP2, phosphatidylinositol 45-bisphosphate; ESCRT, endosomal-sorting complex required for transport; GPC, glypican; MMP, metalloprotease; ctDNA, circulating tumor DNA TLR, toll like receptor; HCV, hepatitis C virus

* Corresponding author at: Department of Clinical Sciences, Lund, Section of Oncology and Pathology, Lund University, Barngatan 4 SE-221 85, Lund, Sweden.
E-mail address: mattias.belting@med.lu.se (M. Belting).

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Table 1
Characteristics of major EV subclasses. To date, no protein markers have been identified that can reliably distinguish between different EV categories. Therefore, the general term "EVs" is preferred in cases where EVs cannot be defined according to their size, density and/or mechanism of biogenesis.

Type	Density (g/ml)	Size(nm)	Biogenesis	Cargo/markers
Exosomes	1.13-1.19	~20-100	Inward budding of endolysosomes leads to multivesicular bodies formation, which fuse with the plasma membrane to release exosomes	Tetraspanins (CD9, CD81, CD63), RNA, DNA, lipids, raft proteins (caveolin-1, flotillin), alix, TSG101
Microvesicles	1.032-1.068	~50-1000	Direct budding of the plasma membrane	CD40 ligand, ARF6, VAMP3, integrins, phosphatidylserine, annexin V, tissue factor, RNA.
Apoptotic bodies	1.16-1.28	~50-5000	Direct budding of the apoptotic cell membrane	Phosphatidylserine, annexin V, nuclear content, cell organelles

1.1. EV mediated messages come in many flavors: Waste containers, signalosomes or food trucks?

Initial discoveries of EV/exosome biology described multivesicular body (MVB)-derived vesicles as a way for reticulocytes to eliminate unnecessary transmembrane proteins [6]. EVs have also been found to facilitate the removal of misfolded proteins or metabolic waste products that are harmful to the cell [7]. More interestingly, bioactive molecules such as miRNA [8], proteins and macromolecular metabolites have been shown to be sorted into EVs, thus defining EVs as complex organelles with extraordinary signaling transduction potential [9]. Early studies on EV-mediated signaling transduction and molecule transfer furthered the interest on EV communication especially in cancer by demonstrating the transfer of mutated epithelial growth factor receptor (EGFR) through EVs and consequent oncogenic signaling in recipient glioma cells [10]. We and others have shown that the EV composition depends on the status of the secreting cell, where hypoxic glioma cells reflect their status on the content of their EVs, and EVs secreted by hypoxic cells harboring *e.g.* tissue factor on their surface could initiate angiogenesis and accelerated tumor growth [11–13].The transfer of active miRNAs through EVs, which can regulate gene expression by direct binding to target mRNAs in the recipient cell has been extensively studied [8,14]. However, quantitative analysis of the amount of miRNAs contained per vesicle suggested very few miRNAs copies per EV, which has raised doubts on the efficacy of EVs as vehicles of miRNA transfer [15].

Apart from proteins and nucleic acids, other types of metabolites are sorted to EVs, including lipids like cholesteryl esters and fatty acids. It has been shown that lipid sorting to EVs is somewhat specific, as some lipid classes are selectively enriched in EVs as compared to cellular lipid composition [16–18]. The rich metabolite composition of EVs opens the possibility of EVs constituting a local nutrient source in the tumor microenvironment. Deregulated lipid metabolism has been shown in different tumor models like prostate [19], breast [20] or glioblastoma [21], where metastasis-initiating cells were shown to be craving for lipids. Under circumstances of aberrant lipid metabolism, it is expected to find a different lipid composition in EVs, and cells containing high levels of intracellular lipids are potentially more likely of secreting lipid-rich vesicles. Notably, different lipid signatures have been found on EVs derived from adipocytes [22]. The possibility that some of these intracellular lipid storages are leaked into EVs sets up a new perspective in the cancer-cell lipid metabolism field. Following EV internalization, EV-containing endosomes will fuse with late endosomes and in lysosomes, the acidic pH can enhance membrane fusion of the otherwise rigid EV membrane. This membrane fusion process would allow luminal content of EVs to be directly released into the cytoplasm of recipient cells. The released EV cargo could then serve as substrates for metabolic enzymes or be sorted to intracellular storage compartments, such as lipid droplets. Apart from nutrient sources, lipid classes contained in EVs can additionally serve as metabolites for lipid signaling involved in inflammation and immune regulation [23]. Further, the differential composition of EV-lipids has been investigated as a potential source of circulating biomarkers [24]. Importantly, the specificity and underlying regulatory mechanisms of lipid sorting to EVs are key questions that remain to be further investigated. It is generally conceived that certain membrane proteins, such as tetraspanins, are sorted to EVs due to their preference for cholesterol-rich raft domains enriched in the EV membrane [25,26]. However, the lipid composition of secreted EVs is clearly highly dynamic and, consequently, the identities of accompanying membrane proteins can vary. The multiple roles of tumor EVs discussed in this section are represented in Fig. 1.

2. Tumor cell-associated PGs in EV mediated intercellular communication

Proteoglycans (PGs) are complex macromolecules which are

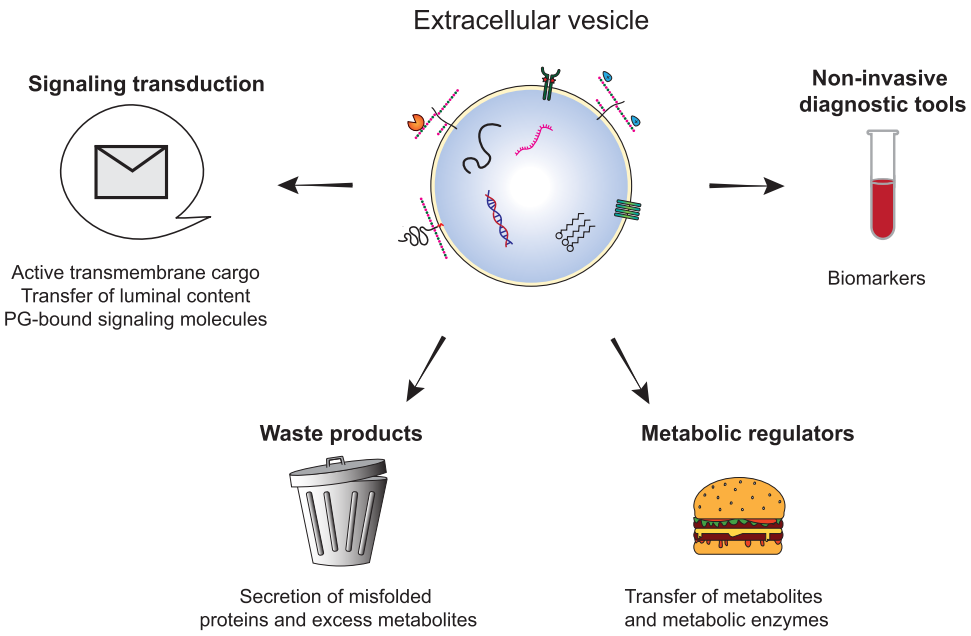

Fig. 1. The multifaceted role of EVs in tumor cell communication. Tumor-derived EVs carry active signaling molecules in their luminal compartment, as transmembrane proteins and as extraluminal cargo bound to transmembrane proteins (*e.g.* proteoglycans). Signaling activation can occur by direct EV-tumor cell interactions or by transfer of EV-cargo after internalization. Initial studies pictured EVs as cell waste products because they can expel misfolded proteins and drain out excess metabolites from the secreting cell. If regarded from a different perspective, their rich macromolecular composition can serve as a nutrient source for recipient cells of especial relevance in the generally nutrient-deprived tumor environment. Importantly, because EVs are present in bodily fluids and carry all classes of macromolecules, they are strong biomarker candidates for non-invasive diagnostics.

constituted by a core protein covalently decorated with linear glycosaminoglycan (GAG) chains including alternating glucuronic acid and *N*-acetylglucosamine or *N*-acetylgalactosamine residues, giving rise to heparan sulfate PGs (HSPGs) and chondroitin sulfate PGs (CSPGs), respectively. The polysaccharide chains are further modified by sequential epimerization of glucuronic acid into iduronic acid and by sulfation. The specific sulfation pattern together with the carboxyl groups lead to a highly polyanionic structure that mainly determines their interactions with functional ligands [27,28]. PGs are thus prone to interactions with polyamines and domains of basic amino acids present in a wide variety of protein ligands [29,30]. PGs are key components of the extracellular matrix (ECM) [27] in virtually all mammalian tissue compartments, and have important functions in a wide variety of pathophysiological contexts. The well-established role of heparan sulfate (HS) PGs in cancer, both in the ECM and as cell-surface receptors, involves the sequestration and binding of diverse pro-tumorigenic factors including growth factors (GFs), cytokines, and chemokines, as extensively reviewed elsewhere [31–33]. More recently, HSPGs have been placed as key players in exosome biogenesis as well as in EV uptake [34,35]. The importance of PG remodeling regarding alterations in their GAG content and structure, and core protein expression, has therefore become an important aspect when we try to understand the basic principles of EV-mediated cell-to-cell signaling. In the following sections we will present and discuss available lines of evidence that establish membrane PGs as important constituents in EV formation and function.

2.1. PGs in exosome biogenesis

Exosomes originate in the endosomal compartment after inward budding of the plasma membrane with subsequent endosome self-invagination, which gives rise to MVB. MVB fusion with the plasma membrane leads to exosome exocytosis into the extracellular compartment. Particularly, a role for membrane-associated PGs in the biogenesis of exosomes has been described. The cell-surface PG syndecan-1 (SDC1), which can be decorated both by HS and CS chains, is involved in exosome biogenesis as part of the SDC1-syntenin-alix axis. Syntenin is essential for the membrane availability of SDCs by controlling their endocytosis and recycling to the cell membrane. The recycling of SDC1 through syntenin occurs *via* its direct interaction with phosphatidylinositol 4,5-bisphosphate (PIP2) and depends on the activation of the small GTPase ARF6 [36,37]. The cytoplasmic protein syntenin binds to the cytoplasmic domain of SDC1 with one of its PDZ domains and can bind to alix with its other PDZ (Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein) domain [38]. Alix associates with the endosomal-sorting complex required for transport (ESCRT) through interactions with TSG101 and CHMP4, which have been shown to be important for exosome cargo selection and loading [39,40].

Apart from the involvement of the cytoplasmic domain of SDC1 in exosome biogenesis, the trimming of its extracellular HS chains by heparanase has been linked to exosome biogenesis [41]. Heparanase mediated exosome production may occur through enhanced SDC1 clustering and formation of the SDC1–syntenin–alix complex. In this context, it is of interest that EV-associated SDC1 (EV-SDC1) was recently identified as a plasma biomarker that could non-invasively differentiate malignant, high grade gliomas (glioblastoma) from low grade gliomas [42]. It is conceivable that the increased secretion of EV-SDC1 by malignant tumors relates to the direct role of SDC1 in EV biogenesis. Future studies should explore whether SDC1-mediated induction of the EV biogenesis pathway confers a more aggressive tumor phenotype. If so, SDC1 would appear as an interesting target for perturbation of the EV machinery, and EV-SDC1 as an attractive biomarker of such strategies.

Thomson and colleagues [43] were among the first to suggest the importance of heparanase in EV/exosome regulation by stimulating EV release as well as influencing EV cargo and function. A study by Roucourt et al. [44] further showed that heparanase, by trimming the HS-chains of SDCs, acts as a fundamental regulator of exosome biogenesis *via* the SDC1-syntenin-alix pathway. The importance of heparanase as a key enzyme in ECM remodeling, affecting tumor progression both through enzymatic as well as non-enzymatic function, has been extensively studied [45]. For example, heparanase expression and activity tailors growth factor (GF) availability in the tumor microenvironment by trimming the GF-binding GAG-chains of PGs, with consequences in tumor progression. Further, as discussed in [46], tumor microenvironmental factors like hypoxia and acidosis can alter heparanase expression and activity, enhancing its pro-metastatic features. To what extent hypoxia and acidosis regulate other aspects of SDC1-dependent exosome biogenesis remains to be elucidated. Notably, SDC1 can carry both HS and CS chains, and it is conceivable that the relative abundance of these types of GAGs is sensitive to stress factors with potential consequences for exosome formation. How GAGs and the PG machinery

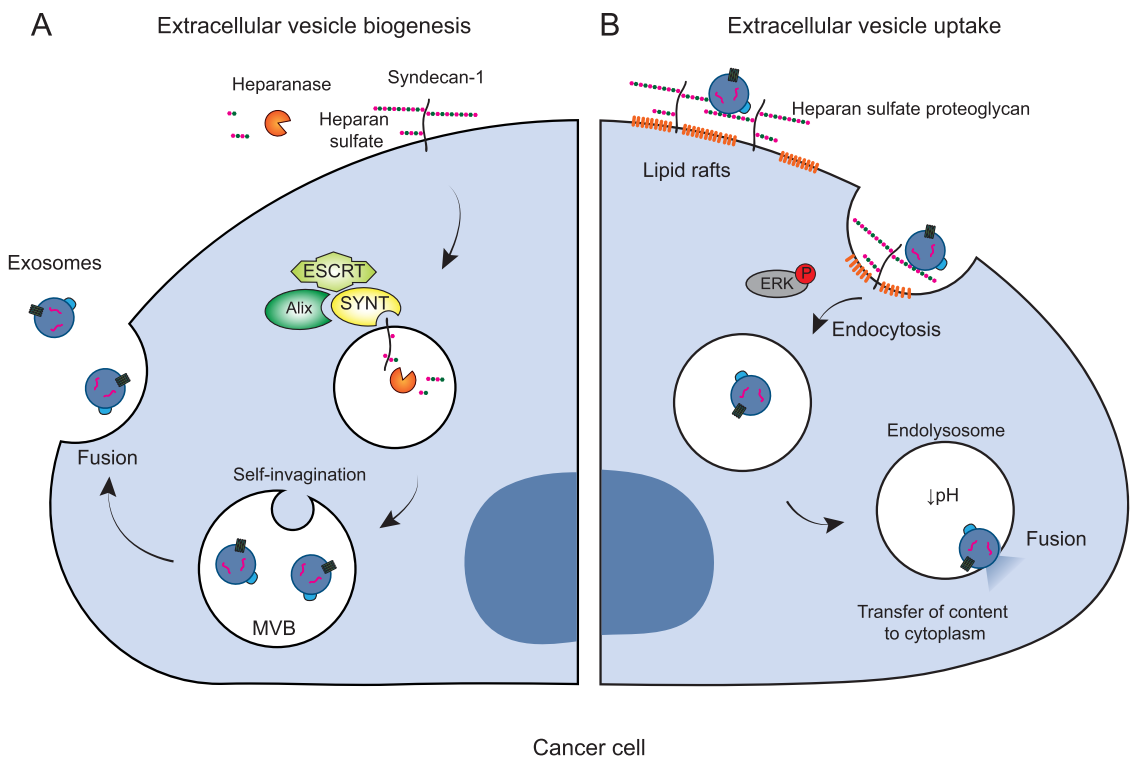

Fig. 2. Cancer cell surface proteoglycans are implicated in EV biogenesis and uptake. (A) Cell surface proteoglycans (PGs), particularly the heparan sulfate (HS) proteoglycan (HSPG) syndecan-1 (SDC1) has been implicated in exosome biogenesis through the SDC1-syntenin-alix pathway. After SDC1 internalization, the cytosolic domain of SDC1 binds to syntenin (SYNT), which can bind simultaneously to alix protein. ESCRT members bind to alix and induce self-invagination of endosomal compartment and intraluminal body (ILV) formation, giving rise to multivesicular bodies (MVB). After fusion of MVB with the plasma membrane, exosomes are released to the extracellular compartment. (B) HSPGs are involved in extracellular vesicle (EV) uptake by serving as receptors for the binding of the vesicles to the cell surface. After EV binding to the HS chains of PGs, simultaneous binding to several receptors can trigger HSPG clustering and induce internalization. This route of EV uptake has been shown to occur in cholesterol-rich domains of the plasma membrane called lipid rafts and to be dependent on phospho-ERK activation. After internalization, the low pH in endosomal compartments enhances heparanase activity, which may serve to enhance EV release from the HS chains. Similarly, the low pH in these compartments enhances EV-endosome membrane fusion, allowing cytoplasmic release of EV content.

may fine-tune EV-dependent signaling in the tumor microenvironment should be an interesting area of future investigations (Fig. 2A).

2.2. Heparan sulfate PGs as major receptors for exosome internalization

Because of the polyanionic charge of their GAG chains, PGs serve as “landing tracks” for a wide variety of polybasic ligands, including polyamines, nucleic acid-peptide complexes, cationic lipids, viral capsid proteins, apolipoproteins and GFs. HSPGs have been described as necessary for efficient GF signaling by presentation to high affinity tyrosine kinase receptors [47–49], to serve as co-receptors for the internalization of highly relevant molecules like lipoproteins [50] as well as to serve as *bona fide* internalizing receptors [51,52]. The importance of HSPG not only as initial attachment sites but also as true internalizing receptors of macromolecular ligands, including EVs, has been extensively explored in our lab (for reviews [31,34],). By screening a library of phage-display derived, anti-HS antibodies we provided first solid evidence that HSPGs act as independent, internalizing receptors of macromolecular cargo. We could further show that HSPGs, of both SDC and glypican (GPC) type, were able to mediate nanoparticle uptake [52]. In line with this, we explored and showed the importance of HSPGs as a major internalizing receptor involved in cancer cell uptake of EVs with exosome-like characteristics [53]. Notably, we could also observe the importance of HSPGs regarding the functional effects of exosomes. Both exosome-induced ERK1/2 signaling as well as exosome-dependent cancer cell migration were attenuated in PG-deficient mutant cells as well as when parental cells were treated with PG inhibitory xylosides. Further, there was no evidence for an involvement of chondroitin sulfate (CS) PGs in exosome uptake, pointing at the specificity of HSPGs as an important player in EV-cell interaction (Fig. 2B). Interestingly, clustering of SDCs in lipid raft domains has been shown to be an important step for their internalization [54,55]. The relatively large size of EVs can potentially facilitate the binding of multiple cell-surface SDCs to several EV proteins, bringing them into proximity and facilitating their clustering and scaffolding to intracellular downstream mediators for internalization.

HSPGs are mainly internalized through a clathrin and caveolin-independent endocytic pathway, which is exploited by some pathogens like viruses to enter eukaryotic cells (see further below). [56]. In most cases, the ligand responsible for lipoprotein and virus uptake through HSPGs has been delineated. By contrast, the surface ligands of EVs involved in HSPG interaction and uptake have yet not been characterized. Partly due to the polyvalent binding capacities of the relatively large and heterogeneous HS chains, it is challenging to specify interacting ligands on the surface of the likewise heterogeneous EVs. In a myeloma model, fibronectin was found to mediate EV-cell interactions serving as a bridge between EV-PGs and cell membrane-PGs [57]. However, the involvement of these interactions in EV uptake was not explored. In other model systems of glioma, we concluded that EV-associated HSPGs were not essential for EV uptake into recipient cells, although both SDC and GPC PGs were shown to be associated with EVs. The relative abundance of EV-PGs, EV-PG ligands, together with differences in cell surface HSPG structure and cell-specific EV-uptake mechanisms can orchestrate EV-cell interactions and can explain differences between different cell types. Therefore, further investigations aimed at finding the key EV-associated ligands involved in EV-cell interaction and cellular uptake through HSPGs, remains an area of high interest. We are currently characterizing the global surface proteome of glioblastoma

cell-derived EVs according to high or low affinity for heparin/HS, with the aim to better understand the mechanism behind EV uptake and in the search of potential protein targets to block EV communication in the tumor microenvironment.

2.3. EV-PGs as chaperones, dynamic signaling reservoirs and cancer biomarkers

The potential of EVs as versatile signaling entities is not restricted to the transfer of luminal content and cis-trans activation of target cells by membrane-intercalated EV proteins. Clearly, EV cargo that decorates the outer part of the EV lipid bilayer through *e.g.* electrostatic interactions with HSPGs can have an important signaling role in the tumor microenvironment [58]. As described above, both GPC and SDC members of membrane-bound HSPGs have been found on the surface of EVs [53,59] and have great potential as biomarkers for liquid biopsy cancer diagnostics. Circulating exosomal GPC1 could detect early stages of pancreatic cancer development [60], and SDC-1 in patient-derived plasma EVs can be used as a biomarker to discriminate high grade, malignant glioblastoma tumors from low grade gliomas [42]. Apart from their potential as biomarkers, EV surface PGs, in analogy with cell-surface and ECM-resident PGs, constitute promiscuous binding platforms for polybasic ligands, including well-stablished binding partners like GFs (*e.g.* VEGF, FGF and PDGF) and cytokines (*e.g.* IL-8 and TGF-β) among others. HS-binding proteins could thus be opsonized to the EV surface either during circulation or in the vicinity of the tumor, resulting in an increased circulating half-life by protecting them from proteolytic degradation. In this context, it is notable that hyaluronan (HA), *i.e.* a nonsulfated GAG commonly overexpressed in malignant tissue, has been shown to cover the surface of EVs to act as a potential carrier of pro-tumorigenic ligands [61]. The low affinity, high binding capacity of HA and polysulfated GAG chains of PGs display high association rates with polybasic ligands, which together with equally high dissociation rates allows efficient exchange of interacting ligands, providing a highly dynamic, mobile reservoir of signaling molecules [62][48]. A classic example of macromolecular exchange between a secreted PG (serglycin) and cell-surface PGs is represented by the serglycin-granzyme B-perforin complex where the cargo molecules favor binding to the more highly sulfated PGs of target cells, further resulting ingranzyme B-perforin internalization and induction of apoptosis [63]. Differences in sulfation of the GAG chains of EV-associated PGs compared to neighboring target cells and the ECM could determine cargo exchange between different compartments allowing spatial flow of signaling ligands. In addition, EV-mediated signaling can similarly occur *via* direct ligand exchange between high affinity receptors. For example, free proinflammatory interferon gamma (IFN-γ) bound to the high affinity interferon gamma receptor 1 (Ifngr1) on the EV surface was shown to be recycled and transferred to its plasma membrane cellular counterpart on neural stem cells to trigger STAT1 signaling activation and consequent nuclear transfer [64]. Therefore, the relative abundance of high affinity, low capacity ligand receptors (*e.g.* tyrosine kinase receptors) on EVs would also be a determining factor of how tumor promoting molecules are distributed in the tumor microenvironment.

Interestingly, the binding of fibroblast growth factor (FGF) to HSPGs can trigger conformational changes that enhance the affinity for the signaling receptor [48]. Moreover, IL8 and other proinflammatory, HSPG-binding chemokines like TNF-α have been found enriched in EVs [65], implicating a role for EVs in immune modulation through chemokine presentation and oligomerization. Similarly, TGF-β binds to the CS chains of betaglycan (or TGFbR3) and the HS chains of SDCs [66]. An EV-associated form of TFG-β has indeed been found to trigger immunomodulatory responses in tumors by suppressing natural killer cell function or delaying T cell activation [67–69], and it is conceivable that EV-TGF-β is sequestered and transported by PGs on the EV surface, due to its lack of a transmembrane domain.

These facts define EV-PGs as extracellular chaperones that can increase the dynamics of GF presentation and provide conformational advantages with potential consequences in cell signaling. Apart from electrostatic exchange of PG-bound ligands, the ectodomain of SDC1 can be shed upon heparanase [70] and matrix metalloproteases (MMP) activity [71], which are known to be increased in tumors. The shedding of the extracellular domain can further facilitate distribution and signaling of PG-bound signaling ligands. It may be envisaged that systemic EVs loaded with HSPG-binding GFs and cytokines are recruited to and unleashed in the tumor microenvironment by the action of *e.g.* heparanase and proteases. Similar mechanisms, may play a role in the establishment of pre-metastatic niches (for review see [72]). The inactive, proheparanase form is translocated from the Golgi to endolysosomes where it is processed into active heparanase prior to further sorting to the cell-surface. In a recent study by the Sanderson group, it is suggested that both the pro-form and active form of heparanase is bound to surface HSPGs of melanoma cell-derived exosomes. The functional activity of EV-bound heparanase was supported by the release of HS fragments from the ECM. Further, functional EV-mediated heparanase transfer was shown to activate cell signaling, migration and cytokine expression in tumor cells and macrophages exposed to melanoma derived EVs [73].

In addition to the role of EV-PGs in GF delivery and dynamics in the extracellular space, EV release may similarly be linked to FGF secretion. According to this model, HSPGs are essential for unconventional secretion of FGF to the extracellular compartment by forming a molecular trap that exports FGF by membrane translocation [74]. Other studies found an involvement of exosomes in FGF secretion, although the role of HSPG was not specifically explored [75]. However, later studies found no role of plasma membrane derived EVs in the shedding of FGF [76], and it remains to be further elucidated how EVs, and in particular EV-associated HSPG may be involved in the secretory route of leaderless proteins.

As we have discussed, the polyanionic GAG chains of EV surface PGs are efficiently opsonized by polybasic protein domains. DNA and RNA species represent other polyanions widely implicated in EV cell-cell communication, and that in analogy with GAGs have high binding preference for positively charged proteins embedded in the EV lipid bilayer or bound to surface EV-PGs. Circulating tumor DNA (ctDNA), and miRNAs secreted by tumor cells are currently under intense investigation because of their potential as non-invasive biomarkers for tumor mutation profiling [77]. However, it is highly unlikely that these macromolecules travel alone in the circulation, owing to their sticky properties provided by the high negative charge, and the degradative activity of serum nucleases. Binding to circulating EV partners with polybasic motifs *via* electrostatic interactions would offer a more stable and rational way of transport. Similarly, the plethora of studies claiming a role of EV-encapsulated miRNAs in regulating gene expression in target cells would be easier to conceptualize by a model in which extraluminal transport of electrostatically bound miRNAs on the outer part of the EVs is transferred to the endosomal compartment following their internalization. Consistent with this idea, a mechanism has been proposed where EV-associated miRNAs bind to and activate members of the toll like receptor (TLR) family in endosomes of the recipient cell, which can trigger pro-tumorigenic effects [78]. Extraluminal miRNAs stuck to polybasic domains of EV surface proteins could similarly activate TLRs presented by recipient cells. See Fig. 3 for an overview of the EV-PG functions in tumor communication.

3. Final remarks - is there an evolutionary conserved system of EV-virus codependence in cancer development?

The fact that various pathogens, including viruses, bacteria, parasites and fungi exploit a variety of GAG structures to facilitate and host cell infection has long been known [79], [80], [81]. In particular, the overlap in physical properties of EVs and some viruses, as well as their

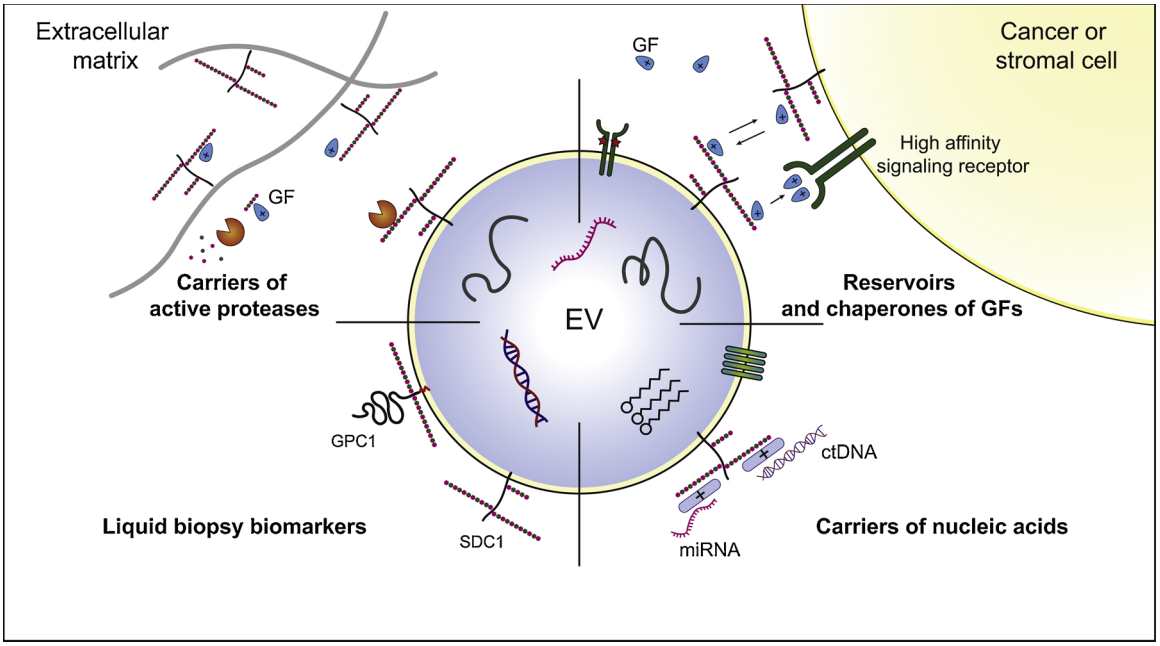

Fig. 3. EV-PGs as circulating polyvalent molecular carriers and cancer biomarkers. The highly anionic glycosaminoglycan (GAG) chains of PGs present on the EV surface represent low affinity, high capacity binding surfaces for a wide variety of proteins containing polybasic domains. Different matrix degrading enzymes like heparanase and matrix metalloproteases (MMPs) have been found present on EVs, either as transmembrane proteins or electrostatically bound to PGs. EV-associated matrix proteases have the potential to remodel the tumor extracellular matrix, and regulate growth factor signaling, which could have consequences in tumor cell invasiveness and proliferation (top left). HSPGs on the surface of EVs can act as dynamic molecular reservoirs of *e,g,* growth factors (GFs) and other signaling molecules (top right). Similarly, polybasic proteins bound to EV-PGs can serve as carriers for nucleic acids such as miRNA and circulating tumor DNA (ctDNA) that can trigger intracellular signaling responses and have biomarker potential (bottom right). Finally, EV-PGs have themselves great potential as cancer biomarkers for liquid biopsy diagnostics (bottom left). GPC1, glypican-1; SDC1, syndecan-1.

common dependence on HSPGs for efficient binding and uptake into cells, is worth some further consideration.

Over the years, EVs have been given a variety of names including exosomes, microvesicles, viral-like particles, virosomes and oncosomes, to mention a few. However, the exact distinction between different types of EVs is still incomplete, partly due to inconsistent methods of purification, and limited understanding of their biogenesis [5,82]. Notably, several studies have reported that the EVs biogenesis pathways converge with the biogenesis and release of a variety of viruses [83,84]. The resemblance of EV and enveloped virus characteristics is striking. Indeed, both particles share membrane lipid composition, constituting a phospholipid bilayer membrane with generally higher cholesterol and glycosphingolipid content compared to the plasma membrane. They also share several proteins at their surface (*e.g.* integrins, MHC-proteins, tetraspanins and heat shock proteins) as well as in the cytosol (*e.g.* nucleic acids and their interacting proteins) (reviewed in [85,86],). Further, although depending on the EV source as well as virus subtype, EVs and viruses share similar size as well as density [87]. These morphological similarities can to a high degree be explained by the fact that EVs and viruses, mainly retroviruses, both use cellular vesicular pathways for their biogenesis, including the ESCRT machinery [88]. The fact that EVs have been shown to transfer genetic material between cells [8] further underlines that these vesicular particles can be intriguingly alike. Several theories considering an evolutionary conserved system of virus–EV codependence have been suggested. These include both the possibility of a viral origin of the microvesicle system [84] as well as the “Trojan horse exosome hypothesis”, according to which viruses, mainly retroviruses, utilize the vesicular machinery of the host for their biosynthesis, release and propagation, suggesting that retroviruses could be regarded as “viral exosomes” [83,89].

For the purpose of this review, the similarities in cell-entrance mechanisms of EVs and viruses during the initiation and propagation of malignant tumors are of particular interest. Some enveloped viruses enter host-cells *via* fusion with the target cell plasma membrane. Other enveloped as well as non-enveloped viruses enter target cells through endocytic pathways, including clathrin and caveolar/lipid raft-mediated endocytosis, macropinocytosis and phagocytosis [90,91]. Mechanism redundancy, where some viruses use more than one cell-entry route, also seems to be a common trait for viral-to-cell entry [90]. As previously discussed, EV-to-cell entry mechanisms are still under investigation. We have provided evidence that EVs enter cells mainly *via* HSPG-dependent, lipid raft-mediated endocytosis through activation of a MAPK-dependent route, and that this pathway is negatively regulated by caveolin-1 [92] in accordance with the global role of caveolin-1 in receptor internalization [93]. However, multiple processes, including direct membrane fusion have been suggested as possible EV-cell entry routes. As discussed in the previous sections, the fact that EVs comprise such a heterogeneous mix of vesicles, limits our current understanding of the precise mechanisms involved. However, it is reasonable to suggest, considering the similarities in physical properties and morphology of EVs and viruses, that these two nanoparticles could choose their target cell and deliver their cargo in a similar fashion.

Others have suggested the cell entry mechanism of hepatitis C virus (HCV) as one possible model to study EV uptake [91]. The complex process of HCV cell entry includes cell attachment to several cell-surface receptors including HSPGs, followed by a complex internalization cascade through clathrin-mediated endocytosis [94]. Another virus family that also could be considered in the study of EV uptake mechanisms is the herpesvirus family. Here, the importance of HSPGs for efficient surface binding as well uptake represents an interesting feature. Apart from the Epstein-Barr virus, it seems as most herpes viruses to some degree involve HSPGs for cell-surface interaction and cell entrance [95]. For example, already 20 years ago it was shown that specific modifications, more precisely the rare 3-O-sulfated HS epitope generated by the D-glucosaminyl 3-O-sulfotransferase (3-OST), could serve as an independent receptor for HCV-1 binding and fusion *via* the

interaction with the viral glycoprotein gD [96]. The importance of specific HS epitopes, including differences in sulfation pattern as well as GAG chain length, as important factors influencing virus binding and infectivity [44], should thus be integrated with future studies on EV entry mechanisms. However, despite the several layers of resemblance between EVs and viruses, it is important to bear in mind that EVs do not replicate and have no infectious capacity *per se*. However, in cases when EVs derive form virus-infected cells, it is with current analytical technologies rather impossible to separate EVs (especially exosomes) from virus particles. Moreover, one should consider the propagation of EVs from one cell to another as a multi-step evolutionary process, resulting in the generation of compound vesicles that carry mixed information from a multitude of cells, some of which may be virus infected. In this scenario, the HSPG entry pathway would be a key player in a multi-dimensional system for information transfer with a blurred border between endogenous cell-cell communication and infection by foreign genetic material.

The overall role of viruses in global cancer pathogenesis is still a matter of vigorous debate that has been intensified by compelling evidence demonstrating that viral components manipulate host microRNA expression to their advantage [97]. How virus/microRNA pathway interaction depends on the intermingling within the EV compartment by common transport mechanisms *via* HSPG-dependent internalization, and how this contributes to creating a malignant cell phenotype will be an interesting avenue for future research. Moreover, an improved understanding of the mechanisms of EV transfer and target cell specificity are essential for the prospect of therapeutic targeting of EVs, and for the exploitation of EVs as therapeutic delivery vehicles. The PG expression pattern and type of glycosylation are known to be altered in various pathological conditions, including in cancer. However, the implications for EV tumor tropism and biodistribution remain to be elucidated. Future studies should clarify whether heparin and other inhibitors of HSPG function, *e.g.* xylosides and sulfotransferase inhibitors, can attenuate the tumor promoting effects of EVs *in vivo*.

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