Shed proteoglycans in the tumor stroma

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Abstract

Cancer cell behavior is not only governed by tumor cell-autonomous properties, but also by the surrounding tumor stroma. Cancer-associated fibroblasts, blood vessels, immune cells and the extracellular matrix of the tumor microenvironment have a profound influence on tumor progression. Proteoglycans control various normal and pathological processes, modulating cell proliferation and motility, cell-matrix interactions, immune cell recruitment and angiogenesis. They are major mediators of cancer cell behavior though a dynamic interplay with extracellular matrix components. During cancer progression, their altered expression can promote the activation of several signaling cascades regulating crucial functional properties of cancer cells. Notably, the function of cell surface proteoglycans can be altered by ectodomain shedding, which converts membrane-bound coreceptors into soluble paracrine effector molecules. In this review, we highlight the importance of proteoglycans and their soluble counterparts in cancer progression and the consequences if their interactions with the adjacent stroma. The dynamic interplay among shed proteoglycans and proteolytic enzymes has a significant impact both on tumor cells and their surrounding stroma, with important implications for the diagnosis of this disease and novel therapeutic approaches.

Keywords: proteoglycans, syndecans, shedding, tumor microenvironment, stroma

The tumor stroma – a permissive environment for cancer progression

Tumorigenesis and tumor progression are complex events which occur when physiological control mechanisms within a given cell are inactivated as a result of a series of gene mutations (Vogelstein et al. 1993). In fact, in their highly influential conceptual review 'Hallmarks of Cancer', Hanahan and Weinberg included the properties of an evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals and unlimited proliferative potential as self-autonomous properties which enable and promote malignant growth of tumor cells (Hanahan et al. 2000). However, already in this initial concept, it was clear that cancer progression does not only depend on the tumor cell itself, but also on its microenvironment. For example, tumor cells interact with their environment to stimulate the process of angiogenesis, the outgrowth of blood vessels from existing vasculature, in order to be supplied with nutrients and oxygen, and they need to interact with and overcome their extracellular matrix (ECM) environment in order to move to distant sites in the process of metastasis (Hanahan et al. 2000). In an update of their concept, the authors stressed the importance of tumor cell interactions with stroma cells and with immune cells of the tumor microenvironment, as the tumor has to evade destruction by the immune system, and may even influence immune cells in a way that promotes tumor growth (Hanahan et al. 2011). While these publications nicely delineate key cell biological events governing tumorigenesis and cancer progression, the idea of permissive environment that promotes tumor growth has indeed historic roots. Already in the 1850, the German pathologist Virchow described a possible influence of inflammatory cells and of aberrant ECM biosynthesis on tumor growth, whereas the English surgeon Paget coined the, "Seed and Soil" hypothesis, according to which tumor cells "can only live and grow if they fall on congenial soil", aptly describing an important role of the tumor stroma in the pathogenetic process (Paget 1889). Nearly a century later, several of these elements were put into the context of a pathophysiological process when Harold Dvorak described tumors and their surrounding stroma as 'wounds that do not heal' (Dvorak 1986). In fact, the granulation tissue that is formed during the physiological process of wound healing includes the key elements of the reactive stroma, or 'desmoplastic stroma' surrounding solid tumors. These include: cancer-associated fibroblasts (CAFs), inflammatory cells, cells constituting blood vessels (endothelial cells, pericytes), and the ECM, which is synthesized and remodeled by both the tumor cells and their cellular microenvironment. Moreover, the ECM can also be derived from plasma proteins

escaping leaky tumor blood vessels (Dvorak 2015). The mutual interactions and crosstalk between tumor cells and the stroma promote tumor progression in various ways. Under the influence of growth factors secreted by tumor cells, including transforming growth factor (TGF) beta, epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2) or vascular endothelial growth factor (VEGF), cancer cells change the properties of their microenvironment (Mueller et al. 2004). Fibroblasts are converted to large, spindle-shaped CAFs characterized by expression of alpha-smooth-muscle actin, an increased proliferative index, and a high rate of collagen biosynthesis, which results in an altered ECM deposition and composition (Lazard et al. 1993). This altered ECM has a profound influence on the properties of the tumor cells, as exemplified by increased synthesis of Tenascin-C by CAFs, which promotes tumor cell motility and metastasis by providing a low-adhesive substrate (De Wever et al. 2010, Lange et al. 2007). Recent results indicate that an altered ECM biosynthesis within the tumor microenvironment may also modulate the properties of tumor-initiating cells, or cancer stem cells, a particularly therapy-resistant subpopulation of cancer cells which shares similarities with both tumor cells and stem cells (Greve et al. 2012). An aberrant expression of tenascin C, of proteoglycans (decorin, biglycan, versican syndecan-1), and of the glycosaminoglycan hyaluronan – the ligand for CD44 – have been discussed as having important signaling functions in the cancer stem cell (Bourguignon et al. 2016, Fanhchaksai et al. 2016, Farace et al. 2015, Ibrahim et al. 2013, Jachetti et al. 2015). Moreover, CAFs secrete proteases which degrade the ECM and free cytokines from their ECM storage, thus facilitating tumor angiogenesis and metastasis (Olumi et al. 1999, Sieuwerts et al. 1998). Furthermore, CAFs secrete cytokines such as insulin-like growth factor (IGF) and hepatocyte growth factor (HGF, ‘scatter factor’) which increase tumor cell survival and promote cell motility in a vicious feed-forward cycle (Mueller et al. 2004). With respect to the interaction with inflammatory cells, tumor cells secrete cytokines and chemokines which attract various groups of leukocytes, including tumor-associated macrophages (TAMs) (Coussens et al. 2002). This process may at first appear counter-intuitive, as leukocytes may attack and destroy tumor cells. However, recruitment of immune cells also facilitates tumor growth and metastasis via secretion of matrix-degrading enzymes such as matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA), and heparanase (Coussens et al. 2002, Gotte et al. 2006). Moreover, TAMs, monocytes, mast cells and neutrophils secrete a vast array of cytokines which exert a proangiogenic effect on endothelial cells and pericytes by inducing signaling events via cell surface receptor tyrosine kinases and serine/threonine kinases expressed by these cell types.

Furthermore, the cancer cells themselves promote tumor angiogenesis by secreting these cytokines, as a prerequisite for enhanced supply of the tumor with nutrients, and for providing access to the vasculature, thus facilitating metastatic spread (Carmeliet et al. 2000, Rapraeger 2013). In summary, these data clearly indicate that interactions between tumor cells and the surrounding stroma make a considerable contribution to tumor cell survival and tumor growth, as well as metastatic spread. Remarkably, all of the functions of the tumor stroma mentioned above are modulated by glycoproteins of the proteoglycan family, as will be outlined in detail in the following sections.

Proteoglycans – multifunctional modulators of cell physiology

Proteoglycans (PGs) are important functional components of the stroma- and cancer- derived ECMs, as well as the cell surfaces (Sofeu Feugaing et al. 2013, Theocharis et al. 2010). They are composed of a core protein substituted with one or more covalently linked carbohydrate chains of the glycosaminoglycan (GAG) type. GAGs form long chains of linear repetitive disaccharide units of an amino sugar and one uronic acid (Yip et al. 2006) (Fig. 1). GAGs of particular relevance for this review are the heparin-related heparan sulfate (HS; N-acetylglucosamine-alpha-L-iduronic acid/beta-D-glucuronic acid), chondroitin sulfate (CS; N-acetyl-D-galactosamine-D-glucuronic acid, and dermatan sulfate (DS), which is derived from CS by C5-epimerization of the beta-D-glucuronic acid residue. As the names indicate, the basic disaccharide pattern of these GAG chains is subject to post-translational modifications with sulfate residues, which occurs as a non-template-driven enzymatic process during biosynthesis in the Golgi apparatus, thus providing the GAG chain not only with a high negative charge, but also with a high degree of structural diversity and functional heterogeneity (Theocharis et al. 2010). This is exemplified by the HS 3-O-sulfotransferase HS3ST2, which is epigenetically silenced in numerous tumor entities, and which has been shown to modulate signaling via the MAPK and Wnt-pathways in human breast cancer cells (Vijaya Kumar et al. 2014). The various modifications that occur in PGs structure are cell- and tissue- specific but they also depend on the cell stimuli and differentiation stage (Theocharis et al. 2015b). According to their localization, PGs are classified into four major families, including intracellular, cell surface, pericellular and extracellular members. Each family can be further divided into subgroups according to the following criteria: gene and protein homology, modular composition, protein core properties and molecular size (Iozzo 1998, Iozzo et al. 2015, Schaefer et al. 2010). Apart from the fact that they

contribute to the necessary mechanical structure for the cellular components embedded in the stroma, PGs can functionally interact with various ECM components and matrix-associated proteins, including growth factors, growth factor receptors and cytokines (Iozzo et al. 2011). Due to the fact that PGs bind numerous secreted proteins and cell surface co-receptors, they can serve several morphogens and signaling molecules to interact with other matrix components thus modulating their activities and affecting cell-matrix dynamics (Theocharis et al. 2014, Theocharis et al. 2015a, Theocharis et al. 2010). Due to their structural complexity, PGs contribute in several processes that are crucial for homeostasis, differentiation and tissue morphogenesis, participating in various normal and pathological processes, such as wound repair, inflammation and tumor development (Elenius et al. 2004, Frantz et al. 2010, Lu et al. 2011). In cancer, altered expression of PGs in the stroma affects cancer cell signaling resulting in the disruption of critical processes during tumor progression (proliferation, adhesion, migration, invasion, angiogenesis and metastasis) (Bouris et al. 2015, Gialeli et al. 2013, Piperigkou et al. 2016, Tsonis et al. 2013). There is only one intracellular PG, serglycin, which is involved in the formation of secretory granules as well as in the synthesis of various ECM components. Serglycin is expressed in hematopoietic and non-hematopoietic tumors and it is reported to promote migration and invasion of low aggressive breast cancer cells (Korpetinou et al. 2015, Korpetinou et al. 2014, Korpetinou et al. 2013). PGs located in the ECM of cancer cells, such as versican, can increase their proliferation and motility through modulating the adhesion of cancer cells to ECM (Iozzo et al. 2011, Skandalis et al. 2011, Wight et al. 2014). Moreover, as a member of small leucine-rich matrix PGs, decorin is a key player in tumor growth and progression; it inhibits angiogenesis as well as it induces tumor cell arrest (Sofeu Feugaing et al. 2013). Of note, soluble decorin is considered to be anti-oncogenic and anti-metastatic (Csordas et al. 2000, Neill et al. 2012, Neill et al. 2016). Regarding cell surface PGs, it is well established that syndecans are the major representatives of the transmembrane HSPGs. They have important roles in several biological processes, such as wound healing, development, stem cell differentiation, inflammation and tumorigenesis (Gotte 2003, Ibrahim et al. 2014b). In cancer, syndecans are considered to be cell surface mediators as they modulate cell-cell and cell-matrix interactions (Lim et al. 2015, Manon-Jensen et al. 2010). Although they are expressed in several cancer types, each syndecan member has diverse role based on the type and the stage of cancer, acting as promoters or inhibitors of tumor progression (Barbouri et al. 2014, Nikolova et al. 2009). During the last years, the research interest focuses in the field of epigenetics as it is reported that the expression of syndecans in cancer is regulated by specific

microRNAs (Asuthkar et al. 2014, Ibrahim et al. 2014a, Ibrahim et al. 2014b, Ibrahim et al. 2012, Li et al. 2014a). Another transmembrane PG is the melanoma-associated chondroitin sulfate PG (NG2), which promotes vascularization, cell survival, migration and adhesion, and modulates the signal transduction of fibroblast growth factor receptor (Cattaruzza et al. 2013, Garusi et al. 2012, You et al. 2014). Betaglycan also belongs to the superfamily of transmembrane PGs and it is a modulator of epithelial-to-mesenchymal-transition, acting as co-receptor for TGFβ members (Diestel et al. 2013). Moreover, this PG is necessary for reproduction and fetal growth, while it is generally considered as a tumor suppressor (Bernabeu et al. 2009, Bilandzic et al. 2011). Taking under consideration the above data, it is plausible to suggest that due to their well-established involvement in cancer progression, PGs are of relevance as potential targets for cancer therapy. In many cases, these PGs are expressed by the tumor cells themselves, however, as will be discussed below, an aberrant and functionally relevant expression of selected PG members has also been observed in the tumor stroma (Ahmed Haji Omar et al. 2013, Farnedi et al. 2015, Li et al. 2014b, Szarvas et al. 2014, Takahashi et al. 2012). Intriguingly, in the case of several cell surface-bound PGs, the proteolytic (or in some cases – lipolytic) mechanism of ectodomain shedding is capable of converting the membrane-bound form of the proteoglycan into a soluble, paracrine effector molecule (Nam et al. 2012). As one can imagine, shedding can have a profound effect on the tumor microenvironment, as formerly membrane-bound PGs at the tumor or stroma cell surface may no longer be able to act as co-receptors for a wide range of ligands in this cell type, while they may either enhance or competitively inhibit processes such as proteolysis, angiogenesis and cytokine signaling within the paracrine diffusion range of the ectodomain. Before we discussing selected examples of shed proteoglycans in the tumor stroma, and their functional implications, the basic mechanisms of PG ectodomain shedding are briefly presented and discussed below.

A primer on proteoglycan ectodomain shedding

Cell membrane proteoglycans have crucial roles in many steps of tumor progression, as interactions with the surrounding stroma have been correlated with differential PG effects in cancer cells. Several membrane PGs are known to undergo controlled enzymatic cleavage of their ectodomain by certain proteases, the so called sheddases. Shedding is a mechanism by which the intact extracellular domain of a membrane protein is converted into a soluble molecule. Notably, shedding does not result in complete degradation of the ectodomain, but

rather leaves it intact, allowing for functional ligand interactions of this domain as a paracrine effector molecule (Manon-Jensen et al. 2010, Nam et al. 2012).The shedding of membrane-bound proteoglycans constitutes an important and clearly controlled post-translational procedure that regulates several normal and pathological conditions. It can be a critical mechanism for the onset of infectious and non-infectious diseases, therefore the development of reliable tools to measure the presence of shedding PGs in such conditions. With the exception of the glycosylphosphatidyl-inositol (GPI)- anchored glypicans, which can be shed by a phospholipase (Traister et al. 2008), all PGs of the tumor stroma are shed by proteases. Shedding usually occurs at a juxtamembrane site and is mediated by members of the matrix metalloproteinase family (matrilysin, collagenases, gelatinases), by membrane-type metalloproteinases (MT1-MMP, MT3-MMP), and members of the ADAM (a disintegrin and metalloproteinase) and ADAM-TS (ADAM with thrombospondin motif) families (ADAM-10, ADAM-17 (TACE), ADAMTS-1, ADAMTS-4). In addition, serine proteases such as plasmin and thrombin have been shown to induce shedding of proteoglycans (Manon-Jensen et al. 2010, Nam et al. 2012). An overview of relevant sheddases can be found in Table I. Shedding of PGs can be regulated at several levels. Regulatory mechanisms include the induction or repression of sheddases and protease inhibitors such as tissue inhibitors of metalloproteinases (TIMPs), and the modulation of enzyme activity via binding of sheddases and protease inhibitors to glycosaminoglycan chains, which can either stabilize these proteins in an active or inactive conformation, or protect them from degradation (Bernfield et al. 1999, Elenius et al. 2004). While shedding can occur constitutively, it can be substantially enhanced by exogenous stimuli, including growth factors, chemokines, bacterial virulence factors, cell stress, phorbol esters, trypsin, insulin and the HS degrading enzyme, heparanase (Bernfield et al. 1999, Manon-Jensen et al. 2010, Nam et al. 2012). We will discuss selected examples in the following section, which addresses different members of shed proteoglycans in the tumor stroma.

Shed proteoglycans in the tumor stroma

Recent studies have categorized cell surface proteoglycans into different families. Membrane-bound PGs include syndecans (-1 to -4), the GPI-anchored glypicans (-1 to -6), the TGF beta Type III receptor betaglycan, melanoma-associated chondroitin sulfate proteoglycan (MCSP/NG2) and phosphacan / protein tyrosine phosphatase gamma (PTPgamma). Syndecans and glypicans are HSPGs, NG2 and phosphacan are CSPGs, whereas the GAG

chains of betaglycan is composed of CS and HS chains (Iozzo et al. 2015). Notably, members of all of these PG families are known to be substrates for sheddases. In this section, we will present the process of ectodomain shedding using selected examples, and discuss their pathophysiological relevance within the tumor stroma.

Syndecans

Syndecans are prototypical transmembrane HSPGs which have several functions relevant to tumor progression (Fig. 1). For example, they are involved in the regulation of cell proliferation by acting as co-receptor for several growth factor receptors; they act as a cell adhesion molecules, and modulate proteolysis, chemokine action, angiogenesis, and cancer stem cell function (Ibrahim et al. 2013, Manon-Jensen et al. 2010, Yip et al. 2006). Notably, all syndecans are shed *in vitro* and *in vivo* (Bernfield et al. 1999). Proteolytic cleavage of syndecans is mediated by plasmin, thrombin, MMPs, ADAMTs (Kim et al. 1994) (Table I) and can be modulated by interfering with several signaling cascades including protein kinase C (PKC), protein tyrosine kinase (PTK) and the MAP kinase pathway (Bernfield et al. 1999). It has been previously demonstrated that a substitution of the glycine residue at position 245 of syndecan-1 with a leucine reduces the extent of MT1-MMP mediated shedding by ~50% in human fibrosarcoma cells (Endo et al. 2003). On the other hand, the ectodomain shedding of murine syndecan-1 and -4 is inhibited by TIMP-1 and TIMP-2, revealing that the responsible sheddase belongs to the ADAM family of metalloproteinases (Fitzgerald et al. 2000). Syndecan shedding has bimodal effects under several pathological conditions *in vivo* (Fig. 2). Soluble syndecan-1 was shown to be required for the formation of chemotactic gradients in a lung inflammation model in mice, and may be implicated in the regulation of leukocyte-endothelial interactions and angiogenesis (Gotte 2003, Gotte et al. 2003). Moreover, shedding of syndecan-3 ectodomain appears to modulate feeding behavior and body weight in mice (Reizes et al. 2003). On the other hand, overexpression of soluble syndecan-1 delays wound repair and induces abnormal blood vessel morphology, due to the increased amount of soluble syndecan-1 observed in skin wound fluids (Elenius et al. 2004). These high amounts have been shown to increase elastolytic activity, which may also be of relevance in the tumor stroma. Once syndecan-1 is shed from the cell surface, the remaining transmembrane fragment (cCTF) undergoes intramembrane proteolysis by γ-secretase and it is cleaved by the proteasome (Pasqualon et al. 2015). This fragment is able to inhibit cell migration and invasion in A549 lung cancer cells through the phosphorylation/activation of Src kinase, focal adhesion kinase and Rho-GTPase, whereas it

blocks syndecan-1-dependent lung tumor cell migration and invasion. Moreover, lung tumor formation of A549 cells in mice was reduced by overexpression of syndecan-1 cytoplasmic fragment, enhancing the crucial role of syndecan-1 in tumor cell migration. The cleavage sites for syndecan shedding seem to be conserved, and have been identified in the case of human and murine Syndecan-1, and in Syndecan-4 (Manon-Jensen et al. 2010, Wang et al. 2005). It has been suggested that the potential cleavage sites may located within the 15-amino acid juxtamembrane region near the plasma membrane and it is confirmed that the shedding is a membrane surface event. In the case of murine Syndecan-1, for example, the cleavage site of syndecan-1 is located between Ala243 Ser244, nine AA from the membrane, as determined by domain swapping experiments and mass spectrometric sequencing (Wang et al. 2005). Clearly, several functions of syndecans in an oncological context are mediated by the membrane-bound form. Moreover, a large part of these functions have been shown to be tumor cell-autonomous. However, there are also indications for important roles of syndecans in the tumor stroma, and for soluble syndecan ectodomains in the stroma, which can be either generated by cleavage from the tumor cell surface or from other cell types such as CAFs, endothelial cells or leukocytes. Indeed, syndecan-1 is expressed and dysregulated in the stromal of several types of cancers, including breast carcinoma, colon cancer, gastric cancer, bladder cancer and squamous cell carcinoma (Ahmed Haji Omar et al. 2013, Hashimoto et al. 2008, Mukunyadzi et al. 2003, Szarvas et al. 2014, Yang et al. 2002). An aberrant stromal expression has also been noted for syndecan-2 in different tumor entities (Ahmed Haji Omar et al. 2013, Farnedi et al. 2015, Hrabar et al. 2010). Tumor promotion is counted among its main functions. This may be correlated with the existence of its soluble form in some cancers (Miles et al. 2014, Nikolova et al. 2009). To note, the expression levels of syndecan-1 distributed from epithelium to stroma, were by far higher in breast cancer tissue compared to the normal one (Lofgren et al. 2007). While it is difficult to assess the contribution of soluble syndecan ectodomains in these histopathological studies, functional studies do indeed point at a mechanistic role for soluble syndecans in tumor progression. For example, soluble syndecan-1 promotes growth of myeloma tumors *in vivo*, and it has been shown that tumors producing soluble syndecan-1 ectodomain grow significantly faster than tumors expressing syndecan-1 on the cell surface (Yang et al. 2002). Moreover, breast cancer cells overexpressing soluble syndecan-1 show higher cell proliferation rates and higher matrigel invasiveness compared to cells overexpressing a non-cleavable, constitutively membrane-bound form of this PG (Nikolova et al. 2009). This change in cell behavior could be attributed to a downregulation of the antimetastatic

homotypic cell adhesion molecule E-cadherin, and a downregulation of the MMP inhibitor TIMP-1 in cells overexpressing soluble syndecan-1. Moreover, it has been recently shown that MT1-MMP produced by stromal fibroblasts releases syndecan-1 ectodomain as a paracrine mediator by the direct cleavage of syndecan-1 at the surface of these cells (Table I). CAFs stimulate breast cancer cell growth through MT1-MMP enzymatic activity. Together, these data pinpoint the novel role of fibroblast-derived MMPs and their proteoglycan targets in stromal-epithelial signaling in carcinomas. A mechanistically highly relevant study employing a tumor cell- stromal fibroblast co-culture system has demonstrated that stromal syndecan-1 mediates breast cancer cell proliferation *in vitro* and *in vivo* and that this regulation requires the HS chains and the presence of the stromal cell-derived factor 1 (SDF1) and fibroblast growth factor 2 (FGF2) (Su et al. 2008). Strikingly, syndecan-1 expressed by stromal fibroblasts has apparently a profound effect on matrix organization within the tumor stroma, and can thus be expected to modulate tumor cell motility and signaling via integrins (Yang et al. 2002). However, the potential roles of syndecans are not restricted to cancer-associated fibroblasts: Data from a syndecan-1 transgenic mouse skin wound model suggest an influence of the HS chains of syndecan-1 on the proteolytic milieu, as soluble syndecan-1 increased elastolytic activity in the wound fluid, leading to delayed repair and abnormal angiogenesis (Elenius et al. 2004). Indeed, several studies have demonstrated that syndecans are capable of binding proteases, including different members of the MMP family (Manon-Jensen et al. 2010). While these data are in accordance with Dvorak's paradigm of tumors as wounds that do not heal (Dvorak 1986), there are additional indications for a role of syndecan-1 in tumor angiogenesis; Syndecan-1 is a modulator of integrins relevant for this process (Rapraeger 2013) and heparanase-accellerated shedding of syndecan-1 ectodomains has been shown to modulate angiogenesis in myeloma via binding of the PG to VEGF (Purushothaman et al. 2010). Apart from these processes, data in animal models point at a role for syndecan-1 in leukocyte recruitment, thus including potential roles in endothelial and inflammatory cells. For example, lack of syndecan-1 on leukocytes enhances integrin-ICAM-1 interactions and leads to increased leukocyte recruitment during inflammation, whereas lack of heparan sulfate on endothelial cells interferes with leukocyte recruitment (Kumar et al. 2015). Similar findings can be expected regarding leukocyte recruitment in the tumor stroma; however, no functional studies have been performed so far. Mechanistically, a striking finding has been the observation that shed syndecan-1 can be transported to the nucleus, where it can influence transcription via inhibition of histone acetylation, thus broadening the functional impact of the molecule (Stewart et al. 2015) (Fig. 2).

In summary, these data indicate that the soluble ectodomains of syndecans have a profound influence on the tumor microenvironment, thus emerging as potential therapeutic targets.

Glypicans

The glypican family of cell surface proteoglycans consists of six members in mammals (Bernfield et al. 1999). The core protein of glypicans is attached to the cell surface through a glycosyl-phosphatidyl-inositol (GPI) anchor. Several members of the glypican family influence tumor development, and their expression is dysregulated in a context-dependent manner in different tumor entities (Ibrahim et al. 2014b, Theocharis et al. 2015b). For example, glypican-3 has been proosed as a prognostic marker for melanoma and human hepatocellular carcinoma (HCC) (Haruyama et al. 2015). Notably, stromal expression of glypican-1 is increased in prostate cancer (Suhovskih et al. 2013) and in ovarian cancer, where it is a poor prognostic indicator for survival (Davies et al. 2004), indicating a possible important role in the tumor microenvironment. At the functional level, glypicans modulate numerous signaling pathways (FGF, Wnt, Hedgehog, BMP), thus regulating cell proliferation, cell survival, cell migration, cell differentiation and angiogenesis (Filmus et al. 2013). Similar to syndecans, glypicans can be shed, however, by necessity the molecular mechanism differs, as the HSPG is GPI-anchored (Fig. 2). Glypican shedding includes cleavage of their GPI anchor by phospholipases (Traister et al. 2008). Moreover, they can be dually processed by partial cleavage via proteases and lipases (Filmus et al. 2013). The high levels of shed glypican may arise either from high amounts of this PG or from increased enzymatic activity of proteases. Therefore, glypican-3 may be an important candidate antigen for immunotherapy of HCC, where it is strongly expressed. Various clinical approaches include the glypican-3 peptide vaccination and the anti-glypican-3 antibody in patients with melanoma and HCC (Ishiguro et al. 2008, Nakatsura et al. 2004). Moreover, the suppression of glypican-1 in pancreatic cancer leads to decreased cytotoxic effects of natural killer cells (NK) (Bloushtain et al. 2004, Hershkovitz et al. 2007).

Melanoma associated chondroitin sulfate proteoglycan-nerve/glial antigen 2 (MCSP/NG2)

NG2 is a large multidomain cell surface CSPG, which is of particular relevance for the tumor stroma, as it is expressed by mural cells (and thus relevant for angiogenesis) and is widely used as a molecular marker for CAFs (Balzarini et al. 2012, Kim et al. 2015, Park et al. 2015).

Important functions of NG2 include a role in pericyte recruitment during (tumor) angiogenesis (Gibby et al. 2012) and as a mediator of growth-stimulatory and pro-survival effects, which are due to activation of the Akt and beta-catenin signaling pathways following the interaction of NG2 with its matrix substrate collagen VI (Iyengar et al. 2005, Cattarruzza et al. 2013a). Moreover, NG2 is capable of modulating FGF2 signaling in perivascular cells (Cattarruzza et al. 2013b). Its role as a pericyte marker has recently been exploited in a therapeutic study where a fusion peptide comprised of an NG2 targeting peptide and tissue factor was utilized to iduce tumor cell infarction in a xenograft model (Brand et al. 2016). Importantly, a clinicopathological study on head and neck cancer revealed that NG2 expression in neoplastic cells and in the intralesional stroma was strongly associated with loco-regional relapse, emphasizing the relevance of aberrant NG2 expression in an oncological context (Farnedi et al. 2015). Moreover, a predictive value regarding metastasis formation has been assigned to NG2 in soft tisue carcinoma patients (Benassi et al. 2009). The juxtamembrane domain of NG2 contains several protease cleavage sites and the diffusible ectodomain can be detected in the blood stream of melanoma patients (Price et al. 2011). Moreover, NG2 has been detected in unique glial cells receiving synaptic input from neurons. Interestingly, its enzymatic cleavage by ADAM-10 and γ-secretase yields an active ectodomain associated with the extracellular matrix thus modulating neuronal networks (Sakry et al. 2014). Soluble NG2 has been detected in vitro and in vivo. Pharmacological inhibition of NG2 shedding resulted in impaired behavior and sensory data in mice that is correlated with various human diseases such as schizophrenia (Geyer et al. 2001), paving the way for similar therapeutic approaches in an oncological context.

Betaglycan

Betaglycan - the proteoglycan form of the TGFbeta receptor type III – has been identified as a suppressor of cancer progression in several epithelial tumors (Dong et al. 2007, Hempel et al. 2007, Sun et al. 1997), although its function is context-dependent in some tumor entities (Criswell et al. 2008). Mechanistically, betaglycan plays an important role as a receptor in TGFbeta and TGFbeta family member - mediated signaling, leading to a suppression of the transcription factor NFkB and downstream targets such as MMP2, and to a modulation of Rho-GTPase-mediated signaling cascades involved in cytoskeletal remodeling (Bilandzic et al. 2014, Oh et al. 2013, You et al. 2009). In accordance with a potential tumor suppressor role, betaglycan expression is downregulated in CAFs in oral squamous cell carcinoma (Meng et al.

2011). Notably, betaglycan inhibits heterotropic adhesion between myeloma cells and bone marrow stromal cells, indicating an important function in the tumor microenvironment (Lambert et al. 2011). While betaglycan may be transported from tumor cells to fibroblasts via exosomes, inducing their differentiation to a myofibroblast-like-state (Webber et al. 2010), some functions of this proteoglycan depend on shedding of its ectodomain, which is mediated by MT1-MMP (Velasco-Loyden et al. 2004). Using betaglycan mutants which displayed either reduced or increased shedding rates, Elderbloom et al. demonstrated that inhibition of betaglycan shedding increased TGF-β responsiveness and abrogated the ability of the PG to inhibit breast cancer cell migration and invasion, whereas expression of a mutant recaprot showing increased ectodomain shedding resulted in the opposite effect (Elderbroom et al. 2014). Importantly, soluble betaglycan reduced breast cancer metastasis in an in vivo model. Using a similar experimental approach and pharmacological shedding inhibitors, the same research group demonstrated that the balance of cell surface and soluble type betaglycan regulates BMP signaling in normal and cancerous murine mammary epithelial cells. The impact BMP/Smad signaling resulted in a comparable effect on breast cancer cell proliferation and migration and was attributed to ligand sequestration by the soluble ectodomain (Gatza et al. 2014).

Outlook: prognostic and therapeutic considerations

The generation of soluble PG ectodomains within the tumor stroma has a considerable impact on several relevant non-cancer cell-autonomous processes, including regulation of the proteolytic milieu, of cytokine signaling, of matrix remodeling as a prerequisite for metastasis, and of angiogenesis (Figure 2). Based on these important functions, the question arises if this knowledge could be utilized in a translational or clinical setting. One possibility is the detection of PG ectodomain levels in the serum as a diagnostic tool. Elevated levels of soluble proteoglycans in biological fluids, such as blood serum, have been detected in patients with sepsis, ischemia or reperfusion injury, graft-versus-host disease as well as in various cancer types (Joensuu et al. 2002, Kliment et al. 2009, Rehm et al. 2007, Seidel et al. 2003, Steppan et al. 2011). For example, the presence of the soluble syndecan-1 in the blood stream is indicative for poor prognosis in several malignancies (Joensuu et al. 2002, Lovell et al. 2005, Szarvas et al. 2014, Vassilakopoulos et al. 2005). A diagnostic or prognostic value for additional shed proteoglycan ectodomains in hepatocellular carcinoma has also been attributed to glypican-3 (Haruyama et al. 2015) and NG2 (Lu et al. 2015). Finally, *in vivo* studies have correlated the

presence of shed syndecan-1 with the inflammatory response (Hayashida et al. 2008, Li et al. 2002), with potential relevance to leukocyte recruitment to the tumor stroma. These data indicate that soluble PG ectodomains that have been released into the blood stream are indeed promising diagnostic markers which can aid therapeutic decisions in a clinical context. Given the functional importance of shed PG ectodomains in the tumor stroma, inhibition of shedding may be a worthwhile therapeutic approach, which may not only have an impact on the tumor cells (which may more easily develop therapeutic resistance due to their genomic instability and cancer stem cell properties), but also on its microenvironment. Therefore, the very promising next step will be the development of specific shedding inhibitors in clinical trials for the focused approach of several pathological situations. Some potential inhibitors that have been recently discussed in the context of syndecan shedding include MT1-MMP inhibitors (GM6001, BB94, TIMP-2), MMP-7 inhibitors (TIMP-1), the FGF2 antagonist PTX3, and all-trans retinoic acid (Choi et al. 2013). While more translational research in this area is warranted, inhibition of shedding may contribute to the exciting therapeutic concept of tumor stroma normalization, which aims at transforming the cancer microenvironment into a non-permissive one with the aim of slowing down or even reverting tumor progression (Mueller et al. 2004).

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Figure legends

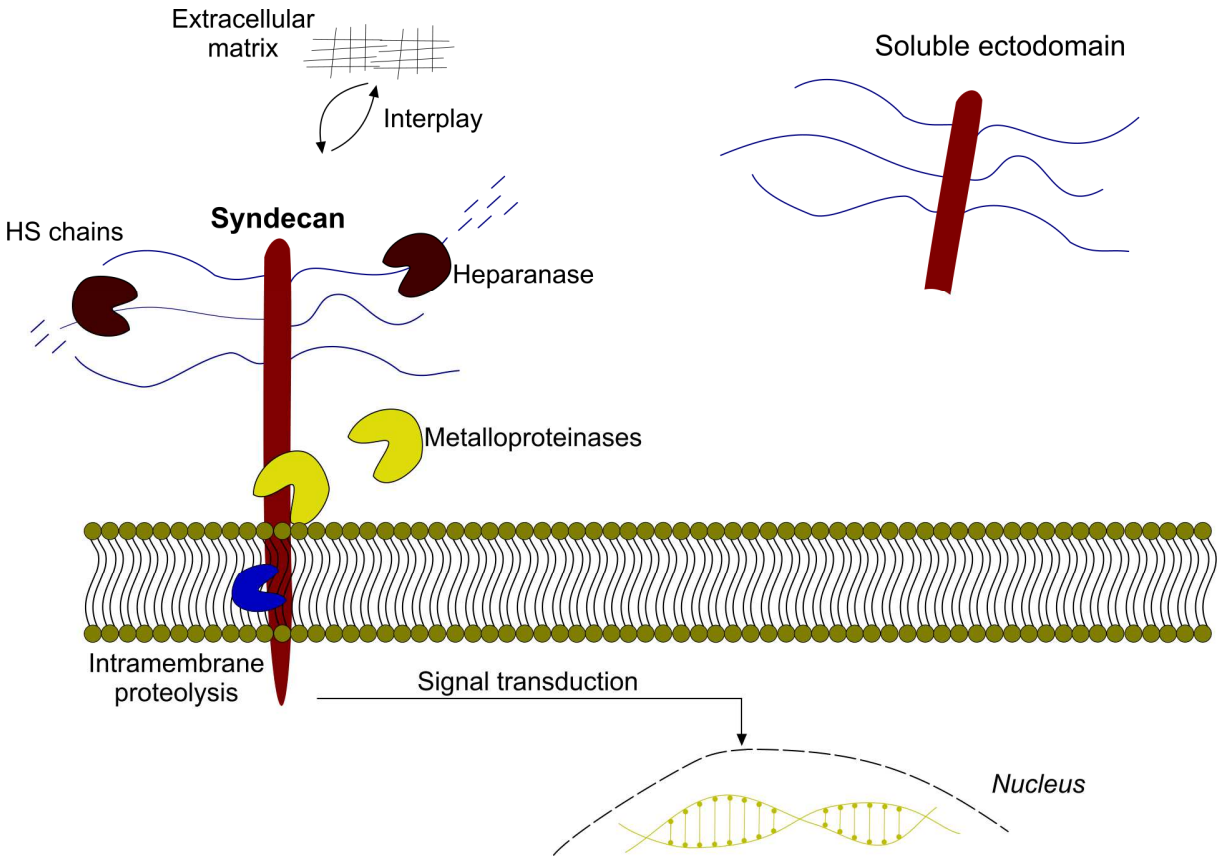

Figure 1: Syndecan shedding. The mechanism of shedding involves the proteolytic cleavage of their ectodomain near the plasma membrane by metzincin enzymes, such as metalloproteinases. HS chains can be additionally cleaved by heparanase. Syndecan core protein can be further processed by intramembrane enzymatic cleavage. Syndecans are in a dynamic interplay with the ECM and several growth factors mediating numerous signaling pathways. See text for details.

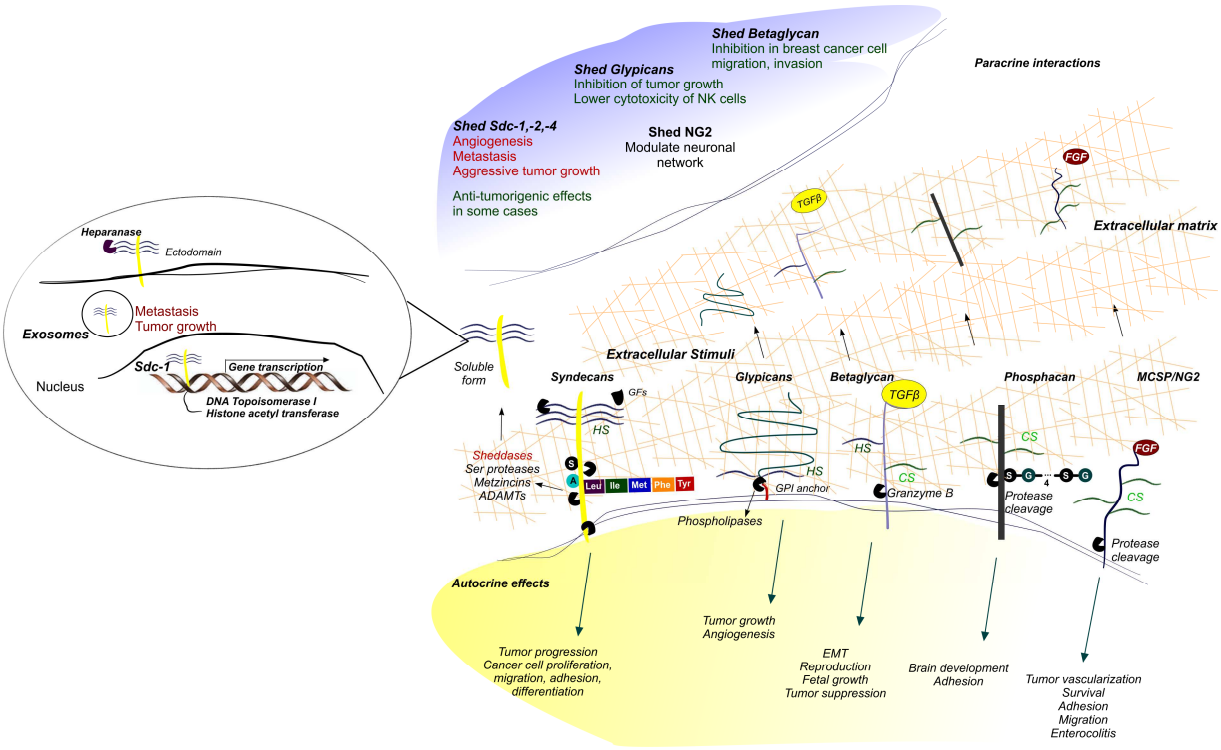

Figure 2: An overview of different cell surface proteoglycans known to be shed, including transmembrane (syndecans, betaglycan, NG2 and phosphacan) and GPI-anchored (glypicans). The GAG chain and the major cleavage sites for selected sheddases are depicted, as exemplified for the syndecan-1 core protein. The GPI-anchored glypicans are shed by phospholipases, whereas the other depicted transmembrane proteoglycans are cleaved by a wide range of proteases (see Table I). Moreover, autocrine and paracrine effects induced by each proteoglycan are shown, affecting important stromal functions in angiogenesis, tumor cell proliferation and survival, cell adhesion, migration and metastatic behaviour. Shed ectodomains are deposited in the ECM where they can influence the function and interactions of large structural matrix glycoproteins, growth factors and cytokines. Ectodomain shedding reduces the available binding sites for such ligands at the cell surface, resulting in altered cytokine responses. Left panel: In the case of syndecan-1, shedding is facilitated by degradation of the HS chains by heparanase. Shed syndecan-1 and betaglycan can be transported in exosomes, which is a means of transferring proteins and microRNAs from tumor cells to stroma cells. Finally, syndecan-1 ectodomains can reach the nucleus and modulate gene transcription (See text and (Theocharis et al. 2015b) for details. While phosphacan is a shed proteoglycan, very

little is known so far about its function in the tumor stroma.

Table I. Selected examples for sheddases and their proteoglycan targets

Shedding enzyme	Classification	Shed proteoglycan (examples)	Reference
MMP-7	Matrilysin	Syndecan-1, Syndecan-2	(Kwon et al. 2014, Li et al. 2002)
MMP-2	Gelatinase	Syndecan-1, Syndecan-2, Syndecan-4	(Brule et al. 2006, Fears et al. 2006)
MMP-9	Gelatinase	Syndecan-1, Syndecan-2, Syndecan-4, NG2	(Brule et al. 2006, Fears et al. 2006, Schultz et al. 2014)
MMP-14	Collagenase	NG2	(Joo et al. 2014)
MT1-MMP (MMP14)	Membrane-type metalloproteinase	Syndecan-1, NG2, Betaglycan	(Brule et al. 2006, Fears et al. 2006, Nishihara et al. 2015, Velasco-Loyden et al. 2004)
MT3-MMP	Membrane-type metalloproteinase	Syndecan-1	(Brule et al. 2006, Fears et al. 2006)
ADAM-10	A disintegrin and metalloproteinase (ADAM)	NG2	(Sakry et al. 2014)
ADAM-17 (TACE)	A disintegrin and metalloproteinase (ADAM)	Syndecan-1, Syndecan-4	(Pruessmeyer et al. 2010)
ADAMTS-1	ADAM with thrombospondin motif (ADAM-TS)	Syndecan-4	(Rodriguez-Manzaneque et al. 2009)
ADAMTS-4	ADAM with thrombospondin motif (ADAM-TS)	Syndecan-4	(Rodriguez-Manzaneque et al. 2009)
Thrombin	Serine protease	Syndecan-4, Syndecan-1	(Schmidt et al. 2005, Subramanian et al. 1997)
Plasmin	Serine protease	Syndecan-4	(Schmidt et al. 2005)
		Glypican-3	

Notum	Phospholipase	Glypican-5 Glypican-6	(Traister et al. 2008)