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Tumorigenic and adhesive properties of heparanase

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Abstract

Heparanase is an endo- β -glucuronidase that cleaves heparan sulfate side chains presumably at sites of low sulfation, activity that is strongly implicated with cell invasion associated with cancer metastasis, a consequence of structural modification that loosens the extracellular matrix barrier. In addition, heparanase exerts pro-adhesive properties, mediated by clustering of membrane heparan sulfate proteoglycans (i.e., syndecans) and activation of signaling molecules such as Akt, Src, EGFR, and Rac in a heparan sulfate-dependent and -independent manner. Activation of signaling cascades by enzymatically inactive heparanase and by a peptide corresponding to its substrate binding domain not only increases cell adhesion but also facilitates cancer cell growth. This notion is supported by preclinical and clinical settings, encouraging the development of anti-heparanase therapeutics. Here we summarize recent progress in heparanase research emphasizing the molecular mechanisms that govern its pro-tumorigenic and pro-adhesive properties. Pro-adhesive properties of the heparanase homolog, heparanase 2 (Hpa2), are also discussed. Enzymatic activity-independent function of proteases (i.e., matrix metalloproteinases) is discussed in the context of cell adhesion and tumor progression. Collectively, these examples suggest that enzyme function exceeds beyond the enzymatic aspect, thus significantly expanding the scope of the functional proteome. Cross-talk with matrix metalloproteinases and the role of heparanase in pathological settings other than cancer is also described.

Keywords

Heparanase; Hpa2; Akt; Src; MMP

I. Introduction

Cancer cells tend to adhere poorly to each other, detach from neighboring cells, invade blood and lymphatic vessels and spread to distant organs. Loose cell-cell contacts, often because loss of E-cadherin, a direct mediator of cell-cell adhesive interactions [1], are necessary but not sufficient to activate tumor metastasis. In addition, cell dissemination is associated with increased migratory capacity as part of the invasive process. Reduced cell-cell contact is thus accompanied by enhanced interaction between cells and the extracellular matrix (ECM). During this process, expression of several classes of proteases which degrade the ECM is induced. These includes serine proteases [2], cathepsins [3], and

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metalloproteases [4]. The tissue-invasive program includes, in addition, activity responsible for cleavage of heparan sulfate (HS). HS is a linear polysaccharide assembled from repeating units of glucuronic/iduronic and D-glucosamine disaccharide units that are modified at various positions by sulfation, epimerization and N-acetylation, yielding clusters of sulfated disaccharides separated by low or non-sulfated regions [5,6]. The HS chains are covalently O-linked to a proteoglycan core protein (HSPG). Two main types of cell-surface bound HSPG core proteins have been identified: the transmembrane syndecan with four isoforms, carrying HS near their extracellular tips and occasionally also chondroitin sulfate chains near the cell surface [5], and the glycosylphosphatidyl inositol-linked (GPI) glypican with six isoforms, carrying several HS side chains near the plasma membrane and often an additional chain near the tip of its ectodomain [7]. Two major types of ECM-bound HSPG are found: agrin, abundant in most basement membranes, primarily in the synaptic region [8]; and perlecan, with a widespread tissue distribution and a very complex modular structure as an important ECM component [9].

From mice to worms, embryos that lack HS die during gastrulation [10], suggesting a critical developmental role for HSPGs. HSPGs function is not limited to developmental processes but play key roles in numerous biological settings, including cytoskeleton organization, cell-cell and cell-ECM interactions [11–13]. HSPGs exert their multiple functional repertoires via several distinct mechanisms that combine structural, biochemical and regulatory aspects. By interacting with other macromolecules such as laminin, fibronectin, and collagens I and IV, HSPGs contribute to the structural integrity, self-assembly and insolubility of the ECM and basement membrane, thus intimately modulating cell-ECM interactions [5,14,15]. Biochemically, HSPGs often facilitate the biological activity of bound ligands by actively participating in receptor-ligand complex formation [16]. In other cases, HSPGs mediate cellular uptake and catabolism of selected ligands [16], and/or sequester polypeptides to the ECM and cell surface, generally as an inactive reservoir [17–21]. Cleavage of HSPGs would ultimately release these proteins and convert them into bioactive mediators, ensuring rapid tissue response to local or systemic cues.

Heparanase is an endo- β -glucuronidase that cleaves HS side chains presumably at sites of low sulfation [22], releasing saccharide products with appreciable size (4–7 kDa) that can still associate with protein ligands and facilitate their biological potency. Heparanase activity has been traditionally correlated with cell invasion associated with cancer metastasis, a consequence of structural modification that loosens the ECM barrier [23–25]. Interestingly, Lider and coworkers have noted that depending on the local pH, heparanase can function either as an enzyme or as adhesion molecule [26]. The mechanism underlying pro-adhesive properties of heparanase has been elucidated following cloning of the gene [27–30] and subsequent generation of specific molecular tools. Studies utilizing recombinant heparanase, anti-heparanase antibodies, peptides, and site-directed mutagenesis and deletion approaches have reinforced heparanase as a pro-adhesive molecule for normal (T-cells, endothelial cells) and tumor-derived cells. Indeed, enzymatic activity was not required for this function of heparanase [31–33], as predicted by the early studies [26]. Instead, pro-adhesive function of heparanase has been shown to result from clustering of membrane HSPGs (i.e., syndecans) and activation of signaling molecules such as Akt, Src, EGFR, and Rac in a HS-dependent and -independent manner. Here we summarize recent progress in heparanase research emphasizing the molecular mechanisms that govern its pro-tumorigenic and pro-adhesive properties. Pro-adhesive properties of the heparanase homolog, heparanase 2 (Hpa2), are also discussed. Aspects such as heparanase gene regulation, proteolytic processing, cellular localization, and the development of heparanase inhibitors have been the subject of several recent review articles [34–38] and will not be discussed in detail here.

II. Heparanase in tumor progression and metastasis

Enzymatic activity capable of cleaving glucuronidic linkages and releasing polysaccharide chains resistant to further degradation by the enzyme was first identified by Ogren and Lindahl [39]. The physiological function of this activity was initially implicated in degradation of macromolecular heparin to physiologically active fragments [39,40]. The activity of the newly discovered endo- β -glucuronidase, referred to as heparanase, was shown soon after to be associated with the metastatic potential of tumor-derived cells such as B16 melanoma [24] and T-lymphoma [25]. These early observations gained substantial support when specific molecular probes became available shortly after cloning of the heparanase gene. Both over-expression and silencing of the heparanase gene clearly indicate that heparanase not only enhances cell dissemination, but also promotes the establishment of a vascular network that accelerates primary tumor growth and provides a gateway for invading metastatic cells [36,38]. These studies enabled researchers to critically approve the notion that HS cleavage by heparanase is required for structural remodeling of the ECM underlying tumor and endothelial cells, thereby facilitating cell invasion, and providing a proof-of-concept for the pro-metastatic and pro-angiogenic capacity of heparanase. The clinical significance of the enzyme in tumor progression emerged from a systematic evaluation of heparanase expression in primary human tumors. Immunohistochemistry, *in situ* hybridization, RT-PCR and real time-PCR analyses revealed that heparanase is up-regulated in essentially all human carcinomas examined [36,38]. Notably, increased heparanase levels were most often associated with reduced patients' survival post operation, increased tumor metastasis and higher microvessel density [36–38], thus critically supporting the intimate involvement of heparanase in tumor progression and encouraging the development of heparanase inhibitors as anti-cancer therapeutics [22,34,35,41]. Somewhat surprising, heparanase up-regulation in head & neck, tongue, hepatocellular, breast and gastric carcinomas was associated with tumor larger in size [42–46]. Likewise, heparanase over-expression enhanced [33,47–50], while local delivery of anti-heparanase siRNA inhibited [51] the progression of tumor xenografts. These results imply that heparanase function is not limited to tumor metastasis but is also engaged in accelerated growth of the primary lesion.

II.1. Heparanase signaling

The cellular and molecular mechanisms underlying enhanced tumor growth by heparanase are only starting to be revealed. At the cellular level, both tumor cells and cells that comprise the tumor microenvironment (i.e., endothelial, fibroblasts, tumor-infiltrating immune cells) are likely to be affected by heparanase. Pro-angiogenic potency of heparanase was established clinically [36–38] and in several *in vitro* and *in vivo* model systems, including Matrigel plug assay [52], tube-like structure formation [53], wound healing [52,54] and tumor xenografts [33,48]. In addition, heparanase augmented signaling cascades leading to enhanced phosphorylation of selected protein kinases and promoted gene transcription associated with aggressive tumor progression [36,55]. Heparanase signaling can practically be divided into HS-dependent and – independent pathways.

II.1.1. HS-dependent signaling

II.1.1.1. Clustering and activation of syndecans in cell adhesion: Syndecans are a family of four transmembrane proteins having covalently attached HS chains. The HS chains mediate syndecan interactions with a large number of proteins, including plasma proteins (i.e., antithrombin), ECM constituents (i.e., fibronectin), and a multitude of biological mediators. By doing so, syndecans function as co-receptors, facilitating growth factors, morphogens, and integrin activity [56–58]. In addition to its co-receptor properties, clustering of syndecan-4 has been shown to directly initiate signaling cascades that result in

PKC α and Rac1 activation which appears instrumental for cell adhesion and directional migration [59–62]. Syndecan clustering was mainly investigated, however, in the context of its interaction with matrix proteins such as fibronectin, while clustering by soluble proteins has not been demonstrated.

II.1.1.2. Heparanase-mediated syndecan clustering augments Rac activation and cell adhesion: Heparanase interacts with syndecans by virtue of their HS content and the typical high affinity that exists between an enzyme and its substrate. This high affinity interaction directs rapid and efficient uptake of heparanase [63,64] considered a pre-requisite for the delivery of latent heparanase to late endosomes/lysosomes and its subsequent proteolytic processing and activation by cathepsin L [63,65–70]. Notably, syndecan-1 and 4 are internalized by cells following addition of exogenous heparanase, and colocalize with heparanase in endocytic vesicles [63] (Fig. 1A, second panel, Merge). As a result, syndecans apparently disappear from the cell surface [63] (Fig. 1A, upper panel, Hepa), altering the ability of cells to communicate with each other and with their immediate extracellular micro-environment.

In-depth appraisal of heparanase-syndecan interaction and its significance was accomplished when the heparin-binding domains of heparanase were identified. Based on consensus sequences considered to mediate the interaction between polypeptides and heparin, three potential heparin-binding domains have been recognized [68]. Particular attention was given to the Lys¹⁵⁸-Asp¹⁷¹ domain since a peptide corresponding to this sequence [KKFKNSTYSRSSVD(C); termed KKDC] physically interacts with heparin and HS with high affinity and inhibits heparanase enzymatic activity [68]. Notably, the KKDC peptide associates with the plasma membrane and induces clustering of syndecans [68] (Fig. 1A, Upper panel, KKDC). The clustering of syndecan-1 induced by heparanase or the KKDC peptide was associated with improved spreading of primary and tumor-derived cells (Fig. 1A, lower panel). Remarkably, syndecan clusters formed by the KKDC peptide were exceptionally large and failed to be internalized at the time points examined [68] (Fig. 1A, upper panel, KKDC), while the heparanase-syndecan complexes are rapidly and efficiently internalized and accumulate in endocytic vesicles perinuclearly [63,68] (Fig. 1A, second panels, Merge). Mechanistically, syndecan clustering by wild type heparanase, enzymatically inactive double mutated (DM; Glu²²⁵, Glu³⁴³) [71] heparanase, or the KKDC peptide, enhanced cell spreading (Fig. 1A, lower panel) and was associated with PKC, Src, and Rac1 activation [68] (Fig. 1C), molecular determinants shown to be induced by syndecans [59,61,62,72]. Thus, while heparanase has been reported to facilitate the adhesion, spreading and migration of several cell types in a manner that appeared independent of its enzymatic activity [31,33,53,73] and to induce Rac1 activation [33], studies applying the KKDC peptide combined these observations in a linear mode in which syndecan clustering by the heparin binding domains of heparanase initiates signaling cascades, resulting in enhanced cell spreading [32]. This mode of action likely represents a non-enzymatic function of heparanase in its simplest term (Fig. 2A).

The heparanase molecule appears, nevertheless, versatile and can function in a manner that operates beyond merely interaction with HS. For example, heparanase appeared more efficient in stimulating the adhesion of floating ARH-77 leukemia and U266 myeloma cells compared with the KKDC peptide [32]. This result indicates that syndecan activation by the KKDC peptide may not be sufficient for efficient cell adhesion, and that cooperation with additional adhesion molecules such as integrins is required [58]. In fact, β 1-integrin activation was noted upon heparanase over expression or exogenous addition [33,73] in a manner which does not involve enzymatic aspects since mutated, inactive heparanase was as potent as wild type heparanase in facilitating cell adhesion [32]. Although the molecular mechanism underlying integrin activation by heparanase is yet to be defined, enhanced

adhesion of cancer cells is thought to confer cell adhesion mediated drug resistance (CAM-DR). This aspect, mediated in part by β 1-integrins, appears particularly relevant to multiple myeloma [74], where an emerging role of heparanase has been revealed [55,75].

II.1.1.3. Pro-adhesive properties of heparanase 2 (Hpa2): Cloning of a single human heparanase cDNA sequence independently reported by several groups [27–30] implied that one active heparanase enzyme exists in mammals. Further analysis of human genomic DNA led researchers to conclude that the heparanase gene is unique, and that the existence of related proteins is unlikely [76]. Based on amino acid sequence, McKenzie and colleagues nonetheless reported the cloning of heparanase homolog termed heparanase 2 (Hpa2) [76]. Wild type Hpa2 (Hpa2c) [76] is secreted and markedly accumulates in the cell conditioned medium following the addition of heparin or HS but not hyaluronic acid (HA; Fig. 1D), indicating that Hpa2 retains the capacity to interact with HS despite the lack of HS-degrading activity [38]. In fact, Hpa2c exhibits even higher affinity towards heparin and HS than heparanase (Fig. 1D). Immunofluorescent staining illustrates Hpa2c localization on the cell surface following its exogenous addition, co-localizing with and clustering of syndecan-1 and -4 (Fig. 1B,2c). Unlike heparanase, Hpa2c does not appear to get internalized into endocytic vesicles but rather remains on the cell surface for a relatively long period of time. This result clearly indicates that the rapid and efficient internalization of heparanase together with syndecans (Fig. 1A) [32,63,68] is unique and not purely a consequence of HS-ligand binding. Since syndecans mediate the uptake of a large number of molecules including atherogenic lipoproteins [77,78] and microorganisms such as bacteria and viruses [79], mechanisms that mediate internalization of syndecan ligands are of interest and clinical significance. Notably, immobilized Hpa2c facilitates cell adhesion and spreading. U87 glioma cells loosely adhere to BSA-coated dishes and assume round morphology. In contrast, U87 cells plated on heparanase-, or Hpa2c-coated dishes appeared nicely spread. Interestingly, U266 multiple myeloma cells which grow in suspension efficiently adhered to Hpa2c-coated dishes while adhesion to heparanase was minimal, indicating that Hpa2c exerts even higher pro-adhesive properties than heparanase (our unpublished results). Both U87 and U266 cell adhesion was inhibited by heparin, reflecting the involvement of HS in these adhesion processes. Thus, secreted heparanase or Hpa2c can cluster membrane HSPGs, or can get incorporated into the ECM and facilitate the adhesion and tissue retention of metastatic and immune cells.

II.2. HS-independent signaling

The heparanase-syndecan axis described above governs enzymatic activity-independent signaling by heparanase, a result of syndecan clustering and internalization (Fig. 2A). In addition, evidence accumulated in recent years indicate that heparanase also elicits HS-independent signaling (Fig. 2B). Signaling is considered to be HS-independent if it occurs in HS-deficient cells (i.e., CHO 745) or in the presence of heparin, as has been demonstrated for enhanced Akt phosphorylation by heparanase [53]. In fact, laminaran sulfate or heparin, two potent inhibitors of heparanase enzymatic activity, when added together with heparanase augmented Akt phosphorylation [53], thus critically implying that HS and heparanase enzymatic activity are not required for Akt activation. In endothelial cells, latent heparanase added exogenously facilitated cell migration, invasion, and the formation of tube-like structures in a PI 3-kinase-dependent manner [53]. In several cases, where tumor xenograft development was examined, heparanase over-expression resulted in tumors bigger in volume and weight [33,48,49] coupled with Akt induction [33,49]. Importantly, heparanase gene silencing was associated with reduced Akt phosphorylation levels [80], further substantiating a role for endogenous heparanase in Akt modulation.

II.2.1. Signaling via the heparanase-Src axis—Signaling by enzymatically inactive heparanase is not restricted to Akt activation. Activation of ERK was noted following addition of latent heparanase to primary T-cells [73] and p38 phosphorylation was noted to be augmented in cells over-expressing heparanase or following its exogenous addition [81,82]. Heparanase over-expression was also associated with VEGF up-regulation, coinciding with induction of p38 phosphorylation [82]. Notably, however, inhibitors of p38 had no effect on VEGF expression, suggesting modulation by signaling pathways other than p38. Utilizing a screen for small molecule inhibitors of signal transduction, Zetser *et al.* have identified Src as the underlying signaling mechanism responsible for VEGF gene induction by heparanase [82]. Over-expression of heparanase by pharynx (FaDu), myeloma (CAG) and embryonic kidney (293) cells resulted in enhanced proliferation and larger colony formation in soft agar, which was attenuated by Src inhibitor [47]. Likewise, heparanase gene silencing was associated with reduced cell proliferation [47], indicating that endogenous levels of heparanase affect tumor cell proliferation. Given the large repertoire of Src activities and substrates, focus was directed to those most closely associated with tumor progression, such as the EGF receptor (EGFR). EGFR phosphorylation was markedly increased in cells over-expressing heparanase or following its exogenous addition, while heparanase gene silencing was accompanied by reduced EGFR and Src phosphorylation levels [83]. Notably, enhanced EGFR phosphorylation by heparanase was restricted to selected tyrosine residues (i.e., 845, 1173) thought to be direct targets of Src rather than a result of receptor autophosphorylation [84]. Indeed, enhanced EGFR phosphorylation on tyrosine residues 845 and 1173 by heparanase was abrogated in cells treated with Src inhibitors or anti-Src siRNA [83]. The functional significance of EGFR modulation by heparanase emerged by monitoring cell proliferation. Thus, heparanase gene silencing was accompanied by a decrease in cell proliferation, while heparanase over-expression resulted in enhanced cell proliferation and formation of larger colonies in soft agar, in a Src- and EGFR-dependent manner [83]. The clinical relevance of the heparanase-Src-EGFR pathway has been elucidated for head & neck carcinoma. Heparanase up-regulation was found in the majority of head & neck carcinomas. Heparanase induction appeared significant because respective patients that exhibited no or weak heparanase staining were endowed with a favorable prognosis and prolonged survival post operation [42], correlated with phospho-EGFR immunostaining [83]. Even more significant was the correlation between heparanase cellular localization and phospho-EGFR levels. Thus, while cytoplasmic heparanase was associated with elevated EGFR phosphorylation, nuclear localization of heparanase was associated with reduced phospho-EGFR levels [83], in agreement with a favorable outcome of patients exhibiting nuclear heparanase [42]. These studies provide a more realistic view of heparanase function in the course of tumor progression. Hence, while heparanase enzymatic activity has traditionally been implicated in tumor metastasis, the current view points to a multifaceted protein engaged in multiple aspects of tumor progression (Fig. 2).

III. Enzymatic activity-independent function: increasing the proteome repertoire

The emerging signaling capacity of heparanase should not come as a surprise. Enzymatic activity-independent function has been described for diverse classes of enzymes including, among others, caspases [85,86], cathepsins [87], plasminogen activator [88], matrix metalloproteinases (MMPs) [89], and even telomerase [90]. MMPs are a family of 23 zinc-dependent mammalian metalloenzymes which, after processing to their active form, are able to cleave all known ECM components. ECM degradation by MMPs has long been implicated in cellular invasion and metastasis, yet MMPs inhibitors failed as anti-cancer therapeutics [91]. The reason behind this disappointing conclusion combines several considerations [91], among which is the increasing awareness of a non-proteolytic function

of MMPs which is not affected by MMP inhibitors [89]. It is now evident that MMPs function is not restricted to cleavage of ECM constituents but rather MMPs are also engaged in multiple signaling pathways that affect the tumor cells and the tumor microenvironment. Non-proteolytic function of MMPs is thought to be executed primarily by their C-terminal, hemopexin-like domain. For example, the hemopexin domain of MMP-9 but not its proteolytic activity is necessary for enhanced epithelial cell migration, mediated by the PI3-kinase pathway [92]. Likewise, the hemopexin domain of MMP-9 attenuated apoptosis of leukemia cells in a Src-dependent manner [93]. Thus, apart from their well characterized enzymatic activity function in cancer metastasis and angiogenesis, the status of heparanase and MMPs research parallels in terms of concept (enzymatic activity-independent function), methodology (i.e., transfection of catalytically-inactive mutants, latent form of the protein either in solution or immobilized, function in the presence of inhibitors of enzymatic activity, etc.), cellular consequences (i.e., increased cell adhesion and migration), and the underlying molecular mechanism (i.e., PI3-kinase and Src activation) executed by the C-terminal domains (hemopexin and C-domain of MMPs and heparanase, respectively) [55]. This and other examples [85,87] suggest that enzyme function exceeds beyond the enzymatic aspect, thus significantly expanding the scope of the functional proteome.

IV. Conclusions and perspective

In addition to the parallels between heparanase and MMPs noncatalytic activities outlined above, recent results implies for co-operation and even compensation between MMPs and heparanase. It was recently demonstrated that enhanced expression of heparanase leads to increased levels of MMP-9, while heparanase gene silencing resulted in reduced MMP-9 activity [94]. In fact, MMP-9 was identified as part of a protease program [which also includes urokinase-type plasminogen activator (uPA) and its receptor (uPAR)] induced by heparanase, leading to enhanced shedding of syndecan-1, thereby promoting aggressive tumor phenotype in myeloma [95]. Cross-talk between heparanase and MMPs also emerged by the recent generation and characterization of heparanase knockout (KO) mice. HS chains isolated from these mice were longer, critically supporting the notion that heparanase is the only functional endoglycosidase capable of degrading HS in mammals [96]. Despite the complete lack of heparanase gene expression and enzymatic activity, heparanase-KO mice develop normally, are fertile, and exhibit no apparent anatomical or functional abnormalities [96]. Notably, heparanase deficiency was accompanied by a marked elevation of matrix metalloproteinase (MMP) family members such as MMP-2, MMP-9, and MMP-14, in an organ-dependent manner, suggesting that MMPs provide tissue-specific compensation for heparanase deficiency. This is likely the reason for over-branching of the mammary gland in heparanase-KO mice [96], a phenotype also noted in transgenic mice over-expressing heparanase [97].

While most attention was paid in recent years to heparanase function in tumor biology, emerging evidence indicate that heparanase is also engaged in several other pathological disorders. A most interesting example is the apparent role of heparanase in glomerular diseases [98,99]. HSPGs are important constituent of the glomerular basement membrane (GBM) and its permselective properties [5]. Loss of HSPGs was observed in several experimental and human glomerulopathies, including diabetic nephropathy, minimal change disease, and membranous glomerulopathy [98]. In addition, expression of heparanase was up-regulated in the course of these diseases [100,101], likely destructing the permselective properties of HS. Notably, PI-88 (a heparanase inhibitor) was effective as an antiproteinuric drug in experimental model [102]. This drug, nonetheless, appears nonspecific exerting side effects at high doses. Heparanase is also causally associated with inflammatory conditions such as inflammatory bowel disease [103] and rheumatoid arthritis [104], among other inflammatory conditions (Lerner et al, our unpublished results). Novel heparanase inhibitors

such as glycol-split heparin or more advanced heparin-based compounds [37] are hoped to enter the clinic and relief diabetic, colitis, and cancer patients' condition.

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Figure 1. Heparanase stimulates syndecan clustering, Rac1 activation, and cell spreading

A. Syndecan-1 clustering and cell spreading. U87 glioma cells were serum starved for 24 h and were then incubated with control scrambled peptide (Scr; upper panel, left), KKDC peptide (50 μ M, upper panel, right) or heparanase (Hepa; 1 μ g/ml; upper panel, middle) for 1 h. Cells were then gently washed, fixed with cold methanol and subjected to fluorescent staining with anti-syndecan-4 antibody (green) merged with nuclear staining (red; upper panels). Cells were similarly stained with anti-syndecan-4 (green) and anti-heparanase (red) antibodies, merged with nuclear staining (blue; second panels). Co-localization of syndecan and heparanase in endocytic vesicles appears yellow. Cell spreading. Human fibroblasts were plated on the 110 kDa fibronectin-like protein and incubated with heparanase (Hepa; 1 μ g/ml) or the KKDC/Scr peptides (50 μ M) for 1 h. Cells were then fixed with 4% paraformaldehyde and double stained with phalloidin-TRITC (red) and anti-vinculin (green) antibody (lower panels). Cells treated with heparanase or with the KKDC peptide appeared spread and formed more focal adhesions. **B.** Hpa2c clusters syndecan but fails to get internalized. Cal-27 tongue carcinoma cells were left untreated (Con, upper panels) or incubated with Hpa2c (second panels) protein for 30 min. Cells were then fixed and subjected to immunofluorescent staining applying anti-syndecan-1 (left panels, green) and anti-Hpa2 (Ab58, red). Merge images are shown in the right panels together with nuclear staining (blue). Note, that Hpa2 clusters syndecan-1 but fails to get internalized. **C.** Rac1 activation. U87 cells were kept in serum-free medium for 24 h and were then stimulated

with the KKDC/Scr peptides (50 μ M), wild type (Hepa; 1 μ g/ml) or double mutated (DM; Glu₂₂₅, Glu₃₄₃) heparanase proteins for 30 min. Cell lysates were then subjected to pull-down with GST-PAK-agarose beads for detection of active Rac1 (upper panel) and subjected to immunoblotting with anti-Rac1 antibody (lower panel) (32). **D.** Hpa2c exerts high affinity to heparin/HS. HEK293 cells stably transfected with Myc-tagged heparanase or Hpa2c gene constructs were incubated without (0) or with the indicated concentration of heparin, heparan sulfate (HS), or hyaluronic acid (HA) and the conditioned medium was subjected to immunoblotting applying anti-Myc antibody.

Figure 2. HS-dependent and -independent function of heparanase

A. Clustering of syndecan family members, and possibly glypicans, by the KKDC peptide dimer or the two heparin binding domains of heparanase facilitates cell-adhesion and cell-spreading [32,68]. Enhanced cell adhesion and spreading is mediated by the recruitment and activation of PKC α , Rac1, and Src. **B.** Heparanase is also thought to interact with heparanase-binding cell surface protein/receptor(s), leading to HS-independent Akt, p38, and Src activation. This results in enhanced transcription of genes such as vascular endothelial growth factor (VEGF-A, VEGF-C) [82,105], tissue factor (TF) [81], and Cox2 [106], and further contributes to cell adhesion, spreading and motility. Src activation, in turn, phosphorylates a number of substrates including the EGFR, leading to increased cell proliferation and tumorigenesis [47,83].