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Understanding Heparan Sulfate Biosynthesis and Functional Implications of Heparanase

TIANYI SONG

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Abstract

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Heparan sulfate (HS) proteoglycans are present on the plasma membrane of all animal cells studied so far and are a major component of extracellular matrices. Heparanase is an endo-ß-glucuronidase that cleaves HS chains at internally located ß-glucuronidic linkages[1]. Except its conventional enzymatic function, non-enzyme function and its function in the nucleus have been demonstrated recently.

Regulation of HS biosynthesis has long been a myth. By generating HEK293 cells that overexpress different doses of C5-epimerase and a mutant without catalytic activity we created tools to analyze HS chain structure. We show that HS chain length increases in a dose dependent manner, dependent on the enzymatic activity of C5-epimerase, which can be reversed by co-overexpresssing with 2-*O*-sulfotransferase.

Lipoprotein lipase (LpL) is a HS-bound enzyme that processes the triglycerides of lipoproteins from both exogenous and endogenous sources. Underdeveloped adipose tissue was revealed in heparanase transgenic mice, due to compromised LpL activity. Mechanistically, a likely possibility was demonstrated due to HS shedding related to overexpressed heparanase.

The finding that breast cancer T47D cells which overexpress heparanase adopt a higher rate of aerobic glycolysis fueling cell proliferation and cell survival added up to the diverse functions of heparanase.

Transcriptomic analysis after knockdown of heparanase in melanoma cells confirmed involvement in inflammatory responses as studied previously, and regulation of cell adhesion, ECM components, apoptosis and nucleosome assembly emerged as novel functional and mechanistic indications of heparanase. Supported by experimental evidence, heparanase's effect in cell adhesion and apoptosis validated its role in cancer progression. Moreover, regulation of nucleosome assembly prompts further investigation into its action mode in the nucleus.

Taken together, the present study adds to the elucidation of HS biosynthesis and depicts novel roles of heparanase, highlighting its multifaceted roles in cancer and providing exciting notions for future studies.

Keywords: Heparan sulfate, biosynthesis, heparanase, lipid metabolism, cancer, cell adhesion, apoptosis.

Tianyi Song, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden.

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To

my past 30 years……

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Fang J, Song T, Lindahl U, Li JP. Enzyme overexpression–an exercise toward understanding regulation of heparan sulfate biosynthesis. *Scientific reports. 2016;6.*

II Song T,et al.. Heparanase disrupts Lipoprotein lipase function in heparanase transgenic mice. *Preliminary manuscript*.

III Song T, Spillmann D. Heparanase modifies glucose metabolism in breast cancer T47D cells. *Manuscript*.

IV Song T, Spillmann D. Transcriptomic analysis reveals multifaceted roles associated with heparanase expression in melanoma cells. *Submitted*.

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Contents

Abbreviations

2-OST	2-*O*-sulfotransferase
AKT	Protein kinase B
Bax	Bcl2 Associated X
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma extra large
Bim	Bcl-2-like protein 11
BMPs	Bone morphogenetic proteins
CASK	Calcium/Calmodulin-dependent Serine protein kinase
CS	Chondroitin sulfate
DS	Dermatan sulfate
ECM	Extracellular matrix
EGF	Epidermal growth factor
Ets1	Protein C-ets-1
EXTs	Exostoses
FAK	Focal adhesion kinase
FDG	Fludeoxyglucose
FGFs	Fibroblast growth factors
GAGs	Glycosaminoglycans
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
GlcA	Glucuronic acid
Glce	C5-epimerase
GlcNAc	*N*-acetylglucosamine
GlcNS	*N*-sulfoglucosamine
GPI	Glycosylphosphoinositide-linked
HAT	Histone acetyltransferase
HGF	Hepatocyte growth factor
HSPGs	Heparan sulfate proteoglycans
IdoA	Iduronic acid
IGF1	Insulin like growth factor 1
IGFBP	IGF-binding protein
IL-6	Interleukin 6
IR	Insulin receptor
LpL	Lipoprotein lipase

NDST	*N*-deacetylase/*N*-sulfotransferase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K	Phosphoinositide 3-kinase
PtdIns(4,5)P_2	Phosphatidylinositol-4, 5-bisphosphate
Puma	P53 upregulated modulator of apoptosis
Sp1	Specificity protein 1
Src	Steroid receptor co-activator
TCA	Tricarboxylic acid
TFIID	Transcription factor II D
TGFs	Transforming growth factors
TNF-α	Tumor necrosis factor α
Xyl	Xylose

Introduction

1 Heparan sulfate and its functions

Heparan sulfate proteoglycans (HSPGs) are composed of a core protein and one or more linear heparan sulfate (HS) glycosaminoglycan (GAG) chains composed of alternating *N*-acetylated (*N*-acetylglucosamine, GlcNAc) or *N*-sulfated glucosamine units (*N*-sulfoglucosamine, GlcNS) and uronic acids (glucuronic acid, GlcA, or iduronic acid, IdoA).

1.1 Biosynthesis

The biosynthesis of HSPGs is initiated by the translation of a proteoglycan core protein and the assembly of the linkage region tetrasaccharide on specific serine residues[2]. Two classes of GAG chains, chondroitin sulfate (CS)/Dermatan sulfate (DS) and HS/heparin, are linked to serine residues in core proteins by xylose. After the synthesis of the linker tetrasaccharide, two types of reactions can occur: addition of a β1–4 linked GalNAc (initiation of CS) or addition of GlcNAc in α1–4 (initiation of HS)[2,3]. The addition of β4 *N*-acetylgalactosamine (GalNAc) or α4 GlcNAc appears to be regulated at the level of enzyme recognition of the polypeptide substrate. In HS formation, Exostose 3 (EXT3) recognizes the linear amino acid sequence immediately adjacent to the attachment site in the core protein. Attachment sites for HS formation usually contain a cluster of acidic residues within seven to nine residues from the serine. Several HSPGs contain multiple contiguous Ser-Gly attachment sites[4]. As HS chains polymerize, they undergo a series of modification reactions catalyzed by at least four families of sulfotransferases and one epimerase. GlcNAc *N*-deacetylase/*N*-sulfotransferases (NDST) act on GlcNAc residues to generate *N*-sulfated glucosamine units, some of which occur in clusters along the chain. A small number of glucosamine residues with free amino groups are present, which may arise from incomplete *N*-sulfation. C5-epimerase, then acts immediately on glucuronic acid residues on the reducing side of *N*-sulfated glucosamine units, followed by 2-*O*-sulfation of some of the resultant iduronic acid. Some glucuronic units also undergo 2-*O*-sulfation. The addition of 2-*O*-sulfate

groups to glucuronic acid or iduronic acid blocks further epimerization reactions. Next, a 6-*O*-sulfotransferase (6-OST) adds sulfate groups selectively to glucosamine residues. Occasionally, a 3-*O*-sulfotransferase (3-OST) adds sulfate groups to glucosamine residues[2]. The highly regulated biosynthesis results in HS chains composed of different domains that vary in the extent of modification by sulfation and epimerization. *N*-sulfated domains (NS); domains of variable sulfation (NA/NS); *N*-acetylated domains (NA)[2]. Modified domains make up binding sites for various ligands, for example, fibroblast growth factors (FGFs)[5] and antithrombin[6].

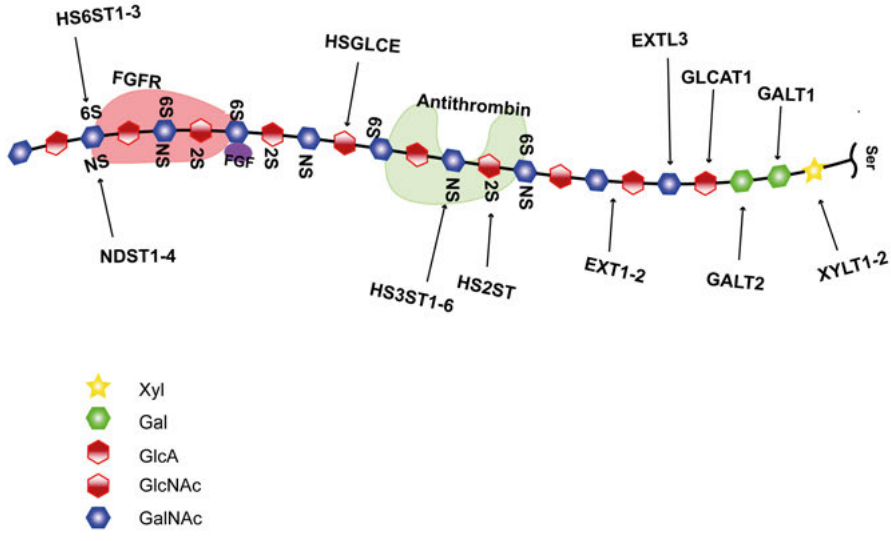

Figure 1. Biosynthesis of heparan sulfate and binding sites for FGF, FGFR and antithrombin, according to Bishop et al., 2007[7].

D-Glucuronyl C5-epimerase (Glce) is a key enzyme in HS/heparin synthesis, it converts GlcA to IdoA by C5 epimerization at the polymer level[8]. The epimerization increases the HS chain flexibility and is essential for HS-ligand binding and cell signaling[9]. The reaction is reversible *in vitro* but irreversible *in vivo*[10].

Disrupting the Glce gene (Hsepi) in mice results in HS chains lacking L-iduronic acid, with a phenotype of neonatal death and defects in kidney, lung, and skeletal development[11]. Analysis of MEFs derived from Hsepi$^{-/-}$ mouse reveals disturbed binding of FGF-2 and HS lacking L-iduronic acid[9].

The interaction between Glce and 2-*O*-sulfotransferase (2-OST) in mammalian cultured cells has been reported, and lack of the 2-OST caused a decrease of epimerase activity and protein stability[2]. Furthermore, it has been reported that Glce, 2-OST, and 6-OST could physically interact[12,13].

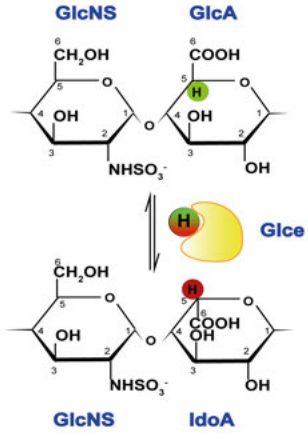

Figure 2. Glce converts GlcA to IdoA during HS biosynthesis.

1.2 HS processing and turnover

Cells secrete matrix PGs directly into the extracellular environment (*e.g.* the basement membrane PGs and members of the aggrecan family)[14–16]. Others may be shed from the cell surface through proteolytic cleavage of the core protein (*e.g.* the syndecans). Cells also internalize a large fraction of cell surface and ECM PGs by endocytosis[17]. Internalized PGs first encounter proteases that cleave the core protein and heparanase that cleaves the HS chains at a limited number of specific sites. The resultant smaller fragments eventually undergo complete stepwise degradation by a series of exoglycosidases and sulfatases in the lysosome [18].

Apart from intracellular heparanase, cells secrete heparanase as well. Extracellular heparanase can cleave HS chains at restrictively specific sites, resulting in shorter HS chains with differently modified domain distribution and sulfation pattern[19].

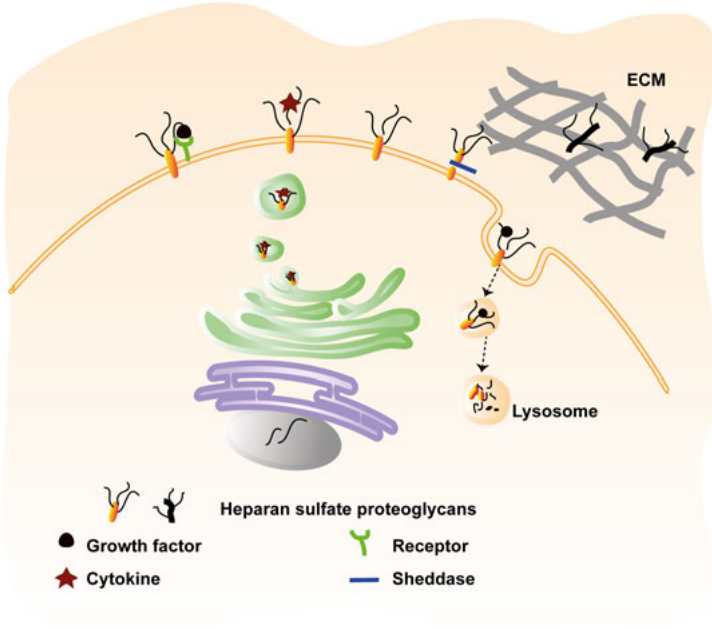

Figure 3. Cellular distribution and metabolism of heparan sulfate.

1.3 HS in cell signaling

Cell surface HSPGs are divided into mainly two types: the transmembrane syndecans and the glycosylphosphoinositide-linked (GPI) glypicans. These bind to growth factors and enhance formation of ligand-receptor complexes. These HSPGs also immobilize and regulate the turnover of growth factors that act at the cell surface. Moreover, the extracellular domains of these HSPGs can be shed from the cell surface, thus affecting the availability of growth factors and ligand-receptor interaction. The FGFs are small globular proteins with distinct binding domains for HS and FGF receptors. Heparin or HS is not required for receptor binding but enables the formation of a high-affinity complex that potentiates receptor signaling at low concentrations of growth factor[20,21]. Hepatocyte growth factor (HGF) is a growth factor with pleiotropic effects. It binds heparin more tightly than FGF and in a different manner. HS is not required for HGF binding to its receptor know as c-Met, but HS can stabilize HGF oligomers, thus facilitating c-Met receptor dimerization and activation[22].

It has been shown that TGFβ receptor III (betaglycan) is a PG containing both HS and CS[23,24]. It is traditionally thought that TGFβ signals through the sequential activation of two serine/threonine kinase receptors, the type I and

type II receptors; betaglycan serves as a co-receptor for TGFβ in most cell types, enhancing TGFβ mediated signaling[25]. But there is increasing evidence pointing to other functions of betaglycan. It was demonstrated that in LLC-PK1 cells, expression of betaglycan prevents association between the type I and type II TGFβ receptors, inhibiting TGFβ signaling[26]. A report suggests that betaglycan enhancement of TGF-β2 signaling in L6 cells is a function of interactions between the cytoplasmic domain of betaglycan and the autophosphorylated TβRII kinase domain[27].
Insulin like growth factor 1 (IGF1) and IGF2 are potent stimulators of diverse cellular activities such as proliferation and differentiation. IGF2 acts on the insulin receptor (IR) and the IGF receptor 1 (IGF1R) and is cleared from circulation by the IGF2R[28,29]. There are six IGF-binding proteins (IGFBP1–IGFBP6) that regulate IGF half-life and receptor availability. IGF2 binding to IGFBP2 greatly increases the affinity for GAGs rich in 2- or 3-*O*-sulfation such as heparin and HS[30], likely undergoing a unique conformational change that unmasks GAG binding sites. It has been shown that IGFBP2 complexed with IGF2 increases binding to human osteoblast ECM, and in a matrix-rich environment, the IGF2/IGFBP2 complex is as effective as IGF2 alone in stimulating osteoblast proliferation, but the mechanism is still unclear[31].

1.4 Function in physiology and cell biology

HSPGs reside on the plasma membrane of all animal cells studied so far and are a major component of extracellular matrices. Studies of model organisms and human diseases have demonstrated their importance in development and normal physiology. There are extensive studies on phenotypes of mice or humans carrying mutations in core proteins or biosynthetic enzymes responsible for assembly of HS chains.

These studies focus on interactions between HSPGs and protein ligands by electrostatic interaction. HS chains bind to a broad group of proteins, including the members of the FGF family[32,33] and their receptor tyrosine kinases, transforming growth factors (TGFs)[23,34], bone morphogenetic proteins (BMPs)[33], wnt proteins[35], chemokines and interleukins[36], as well as enzymes and enzyme inhibitors, lipases and apolipoproteins[37,38], and extracellular matrix (ECM) and plasma proteins. HSPGs function as co-receptors for growth factors and their receptor tyrosine kinases, which are present either on the same cell or on adjacent cells[39]. The most studied is the interaction between HS with FGFs and their receptors[40]. In this case, the GAG chain enhances the association of FGF to its receptor leading to a ternary complex, thereby lowering the ligand concentration required to initiate signaling and extending the duration of the response[41,42]. Other growth fac-

tor/receptor interactions may follow similar binding and activation mechanisms. HSPGs also facilitate cell adhesion to the extracellular matrix and form bridges to the cytoskeleton[43]. The cytoplasmic domains of syndecans interact with cytoskeletal proteins, as well as kinases (steroid receptor co-activator (Src), and calcium/Calmodulin-dependent serine protein kinase) and phosphatidylinositol-4, 5-bisphosphate (PtdIns (4,5) P_2)[44]. Cell secreted HSPGs are involved in the formation of organized extracellular matrix that form physiological barriers and sequester growth factors and morphogens for later release[42].

In fact, almost every ECM molecule contains binding sites for HS[45]. For example, the ECM protein fibronectin contains domains that simultaneously bind to the HS chains of syndecans and one or more integrins to induce cell spreading and focal adhesion formation[46,47]. For hematopoietic cells, serglycin carrying highly sulfated heparin chains is tightly packed into secretory granules[46,48]. HS are also involved in transporting chemokines across cells and present them at the cell surface[49]. Finally, some experiments suggest that HS chains exist in the nucleus[17,50].

1.5 Nuclear location of HS

The presence of HSPGs in the cell nucleus has been reported for a long time. Ishihar *et al.* have shown the kinetics of HS turnover in cell nucleus in a model of immortalized rat hepatocytes. Approximately 10 % of internalized exogenous HS is transported into the nucleus where it appears as free chains in a lysosome independent manner. The lysosome compartment seems to then be involved in exit of HS from the nucleus[51]. The functional significance of such a process is not yet understood. A multitude of growth factors, morphogens, peptides, nucleic acid complexes[52,53] lipoproteins and exosomes[54] enter the cells through HSPG mediated endocytosis[55].

The functions of the nuclear HS and HSPGs are, however, still far from completely understood. Many of the known nuclear functions of HS are mainly attributed to its interaction with growth factors, various nuclear structures[56] and nuclear proteins and transcriptional factors.

The mechanism of FGF-2 shuttling involves both FGF receptor[146] and HSPG mediated nuclear translocation[57–60]. Syndecan-1 is clearly implicated in the nuclear delivery of FGF-2, the two compounds sharing the same tubulin-mediated transport route to the nucleus, where they co-localize with heparanase.

It has been demonstrated that HS bind to and inhibit topoisomerase-I activity *in vitro*, thereby potentially inhibiting gene transcription, given that topoisomerase-I activity is important for the unwinding of supercoiled DNA during transcription[56]. HS in nucleus may regulate nuclear proteins and tran-

scription factors. Casein kinase II, midkines and histones have all been reported as target molecules[61]. HS interacts with activator protein 1, Ets1, Sp1, TFIID and NFkB and inhibits the binding of consensus oligo-DNA, however, binding specificity depends to a large extent on the HS fine structure that differs from tissue to tissue[62]. Mounting evidence suggests a correlation between nuclear HS or HSPGs and cell cycle distribution, proliferation and migration[63–66].

2 Heparanase and its functions

2.1 Synthesis and processing

Heparanase 1 (heparanase) of animal origin cleaves glucosiduronic bonds between GlcA/IdoA and GlcN residues. It has first found in human placenta and platelets, and later in murine metastatic melanoma cells, human hepatoma. Heparanase is restrictively expressed in placenta, keratinocytes, platelets and activated cells of the immune system, with little or no expression in most normal epithelial and connective tissue cells[67,68].

Only one gene has been found to encode the heparanase protein, although several transcript variants encoding different isoforms have been found for the human gene heparanase and one single heparanase cDNA was cloned independently in several groups around the same time[69–72]. The availability of recombinant heparanase provides further insight into the enzyme specificity as well as an additional tool for structural studies. The human heparanase cDNA contains an open reading frame that encodes a polypeptide of 543 amino acids with a predicted molecular size of about 65 kDa. An intervening 6 kDa peptide (Ser110–Gln157) is excised by proteolysis and the active heparanase forms a heterodimer consisting of a 50 kDa subunit (Lys158–Ile543) non-covalently associated with an 8 kDa peptide (Gln36–Glu109)[73–75].

Considering the plethora of bioactive molecules associated with HS on the cell surface and in the ECM and their potential to profoundly affect cell function and development, heparanase activity and bioavailability should be tightly regulated. Many studies have pointed out that regulation at the transcriptional level is one of the important control mechanisms for heparanase. Regulation at the post-translational level, namely heparanase processing, cellular localization and secretion are now recognized as additional key regulatory mechanisms. Extracellular cathepsin L has been shown to cleave the linker domain (Ser110–Gln157) of latent heparanase secreted first by cells, followed by endocytosis of the resultant active heparanase; the linker domain has been shown to inhibit heparanase activity[76]. Later findings indicated heparanase processing to occur intracellularly because of the detection of both latent and active heparanase in endocytic vesicles in cells[77]. The en-

dosome/lysosome was found to be capable of heparanase processing and the processing by a lysosomal/endosomal preparation was most efficient at acidic pH conditions (pH 4-5), typical of the lysosomal compartment[78]. Given the studies above were all conducted in transfected cell systems, the scenario of heparanase processing in physiological conditions needs to be future elucidated.

Heparanase 2 shows ~35 % homology with heparanase 1 and has 3 splicing variants. It was cloned just after heparanase 1, although no enzymatic activity could be demonstrated. Distinct from heparanase, heparanase 2 mRNA expression shows a wide distribution in normal tissues[75].

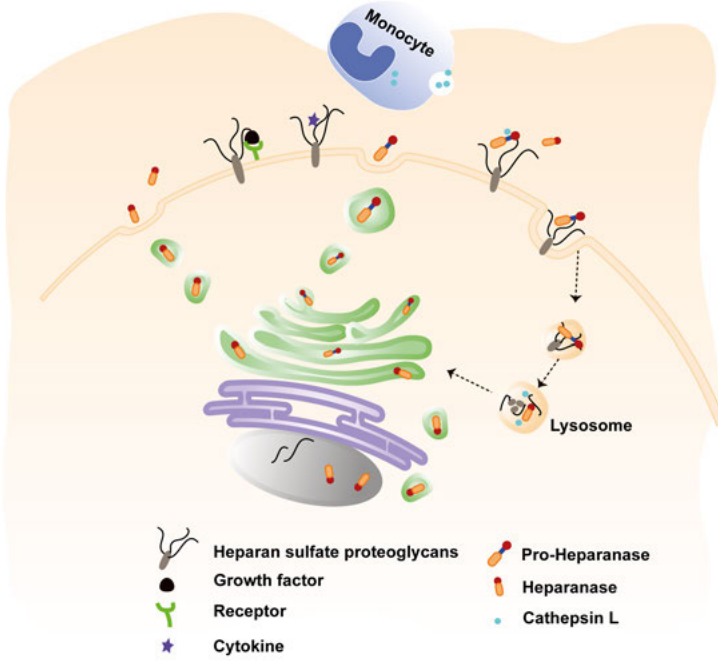

Figure 4. Schematic illustration of heparanase synthesis, activation and enzyme activity.

2.2 Heparanase in physiology

Given the ubiquitous distribution and multiple functions of HSPGs, cleavage of HS side chains by heparanase is expected not only to alter the integrity of the ECM, but also to release sequestered cytokines, growth factors and chemokines in the ECM[79]. Moreover, heparanase may act together with ma-

trix metalloproteases to remodel the ECM. In particular, invading cells secrete heparanase in order to degrade the ECM. It acts as procoagulant by increasing the generation of activation factor X in the presence of tissue factor and activation factor VII[80].

In addition to extensive studies on the enzymatic activity of heparanase, an increasing number of investigations have appeared on non-enzyme functions of heparanase. Cell surface heparanase augments cell adhesion regardless of whether the cells are transfected with active or point mutated, inactive enzyme, indicating that heparanase functions as an adhesion molecule independent of its endoglycosidase activity[81.]

2.3 Heparanase in cell signaling

Heparanase overexpression has been found to induce cell invasion in the human glioma cell line, U87. Heparanase expression levels correlated with increased U87 cell spreading and decreased proliferation rates accompanied by β1-integrin activation, focal adhesion kinase (FAK) and Protein kinase B (PKB/Akt) phosphorylation[82]. In addition to its enzymatic activity, studies in cultured cells have shown that the 65 kDa latent heparanase enhances Akt phosphorylation in a phosphoinositide-3-kinase (PI3K) dependent manner, promoting endothelial cell migration and invasion. This is independent of cell membrane HSPG and can be augmented by heparin[83]. Observed effects of heparanase on Akt may provide a potential mechanism how overexpressed heparanase can elicit an anti-apoptotic phenotype in tumor cells[84]. Akt phosphorylation after addition of exogenous 65 kDa latent heparanase has been further confirmed in MCF 7, SYF and MEF cell lines, with time after addition of the latent heparanase, Akt signaling decreased, accompanied by an increase in Erk phosphorylation. An explanation for the observation could be the effect of internalization of heparanase, resulting in its processing by the cells to an active heparanase which may have more complex effects in the cells[83,85].

It has been shown that heparanase acting on insulin receptor can induce growth of myeloma cells[86]. Insulin is as potent as IGF-1 in triggering the phosphorylation of insulin receptor, Akt, and Erk in myeloma cells[87]. Recent studies support the role of insulin as an important growth factor acting through the tyrosine kinase growth factor cascade to enhance tumor cell proliferation[88].

2.3 Nuclear location of heparanase

Heparanase has been detected in nuclei in cancer tissue by immunohistochemical staining. In some cell lines, it mainly localizes in the nucleoplasma (The Human Protein Atlas). It colocalizes with nuclear HS and is enzymatically active[17]. Moreover, following uptake of latent 65 kDa heparanase by cells that do not express the enzyme, an active 50 kDa heparanase can be detected in the cell nucleus[17]. A number of growth factors and molecules can induce heparanase nuclear localization. Epidermal growth factor (EGF) induces heparanase nuclear localization where heparanase augments DNA Topoisomerase I activity in brain metastatic breast cancer[89]. It is likely that this occurs via cleavage of HS by heparanase considering that nuclear HS has been reported to inhibit DNA topoisomerase I[56,90]. Moreover, several studies show that nuclear heparanase promotes the activity of histone acetyltransferase (HAT) and enhances acetylation of histones, subsequently upregulating the transcription a cohort of genes[70–72]. However, there is evidence showing nuclear heparanase down regulates a number of relevant genes by binding to DNA in metastatic melanoma cell lines[94].

3 Heparanase in lipid metabolism

3.1 Fat metabolism

Adipose tissue is the main tissue for storage of energy in the body. In addition to its roles in energy storage and as an endocrine organ, adipose tissue is increasingly recognized as an important immune organ, it produces hormones such as leptin, estrogen, resistin, adiponectin and the cytokines IL-6, TNF-α[95]. Obesity is accounted for a number of metabolic disorders, including type 2 diabetes and insulin resistance, and mechanistically due to hypertrophy of adipocytes and increase in pro-inflammatory adipokines like TNF-α and IL-6. As obesity is now accepted as a low grade, chronic inflammatory disease [96], it has been demonstrated that the infiltration and accumulation of macrophages in adipose tissue was associated with the increased TNF-α and IL-6 secretion, contributing to insulin resistance[97]. Moreover, TNF-α impairs adipocyte differentiation of new adipocytes from precursor cells[98].

Lipoprotein lipase (LpL) is synthesized by parenchymal cells of several extra hepatic tissues such as heart, muscle and adipose tissue and then transferred to the local vascular endothelial cells[99]. This enzyme is thought to be bound and retained on vascular endothelium via interaction with HSPG. The action of LpL hydrolyses triglycerides in chylomicrons and very-low-density lipoproteins[100] from both exogenous and endogenous sources, with the product fatty acid to be taken up by the local adipose tissue or muscle tissue ei-

ther for energy storage or usage for ATP production. LpL enzyme activity is reduced drastically by fasting and immediately activated postprandial.

Adipocytes have an important physiological role in maintaining triglyceride and free fatty acid levels in the plasma. In the circulation, free fatty acids are liberated from lipoproteins by LpL and enter the adipocyte, where they are reassembled into triglycerides by esterifying to glycerol. Disturbance in LpL function may cause abnormalities in the plasma lipoprotein levels. Heparin has a higher affinity of binding to LpL than HS due to its highly sulfated structure[101], therefore heparin provides an approach to strip LpL off from cell surfaces and to stabilize the enzyme activity to allow for LpL activity measurement and quantification.

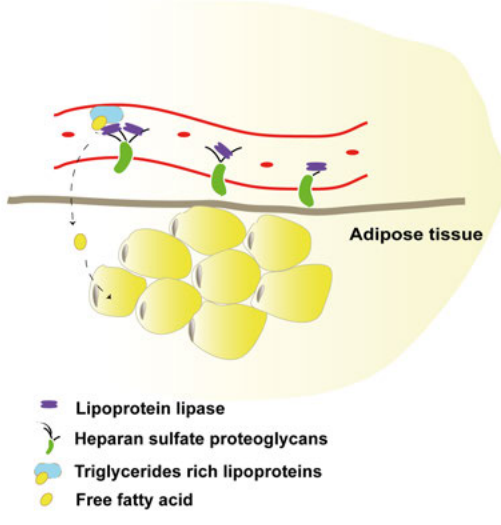

Figure 5. The location and activity of LpL in the adipose tissue.

3.2 Mouse model

Homozygous transgenic mice (Hpa-tg) overexpressing human heparanase (endo-β-glucuronidase) in all tissues have been generated previously, the mice appear normal, fertile, and have a normal life span[102]. No significant difference of body weight is detected between C57BL/6 and Hpa-tg mice of 12 weeks. However, Hpa-tg mice display a reduced fat mass, even though they eat moderately more chow diet[103].

4 Heparanase and cancer

4.1 Hallmarks of cancer

The hallmarks of cancer comprise eight biological capabilities acquired during the multiple steps of development of human tumors. They include sustaining proliferative signaling, resisting growth suppressors, invading cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability and mutation, and deregulated energetics[104]. The hallmarks provide constitutive principles that rationalize the complexities of neoplastic diseases.

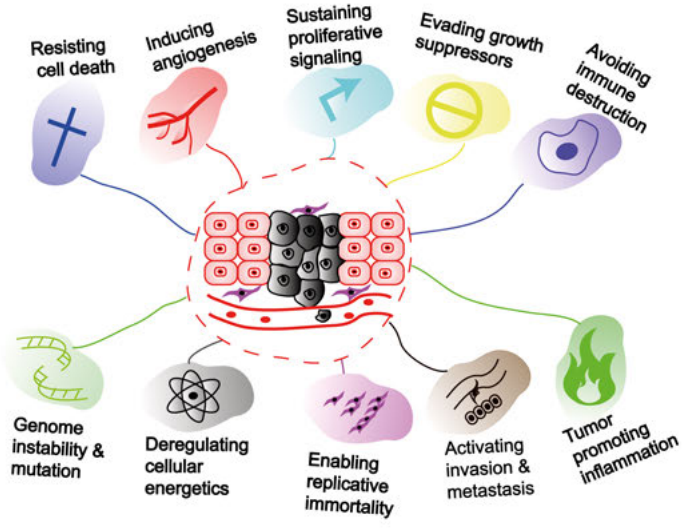

Figure 6. Hallmarks of cancer, according to Hanahan, et al., 2011[104].

4.1 Glycolysis

In the presence of oxygen, most differentiated cells primarily metabolize glucose to carbon dioxide by oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle. In this way, the cells produce reduced nicotinamide adenine dinucleotide (NADH) that fuels oxidative phosphorylation to maximize ATP production. Differentiated cells produce large amounts of lactate only under anaerobic conditions[105]. In stark contrast, as an emerging hallmark, most cancer cells produce large amounts of lactate despite of available oxygen. So their metabolism is referred as "aerobic glycolysis" or "Warburg effect"[106].

Given the fact that the metabolism of one molecule of glucose to lactate generates only 2 ATPs whereas complete oxidation of one glucose molecule via oxidative phosphorylation generates up to 36 ATPs[107], one question is raised: Why would a less efficient metabolism, in terms of ATP production, be selected for in proliferating cells? One possible explanation is that inefficient ATP production is a problem only when nutrients are scarce. But for proliferating mammalian cells that are exposed to a constant supply of glucose and other nutrients in blood circulation, this seems never the case. Moreover, no matter how much they are stimulated to divide, cells undergoing aerobic glycolysis still exhibit high ratios of ATP/ADP and $NADH/NAD^+$. So, instead of converting all glucose to CO_2 in mitochondria to maximize ATP production, proliferating cells need large quantities of glucose to divert to synthesis of macromolecular precursors such as acetyl-CoA for fatty acids, glycolytic intermediates for nonessential amino acids, and ribose for nucleotides[108].

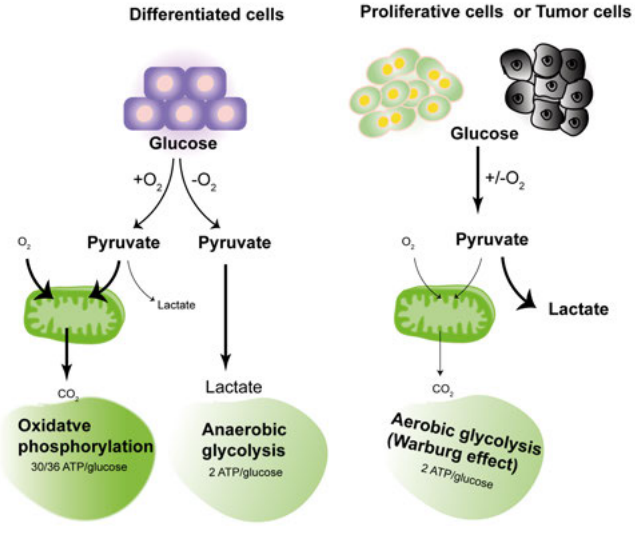

Figure 7. Glucose metabolism of differentiated, proliferative and tumor cell, according to Heiden, *et al*., 2009[105].

4.2 Invasion of cancer cells

The process of invasion and metastasis of malignant tumors involves multiple steps, often termed the invasion-metastasis cascade[109,110]. This scheme depicts a succession of biological changes, beginning with cancer cell dissemination, local invasion, then intravasation of cancer cells into nearby

blood and lymphatic vessels, circulation of cancer cells, followed by their extravasation from the lumen of such vessels into the parenchyma of distant tissues, the formation of micrometastasis of cancer cells and finally the growth of micrometastatic lesions into macroscopic tumors, a process termed "colonization."

It is obvious that as carcinomas arise from epithelial tissues and progress to higher pathological grades of malignancy, reflected in local invasion and distant metastasis, the associated cancer cells typically developed alterations in their shape as well as in their attachment to other cells and to the ECM. The expression of genes encoding other cell-to-cell and cell-to-ECM adhesion molecules is demonstrably altered in some highly aggressive carcinomas, with those favoring cytostasis typically being down regulated. Conversely, adhesion molecules normally associated with cell migration that occurs during embryogenesis and inflammation are often up regulated[104].
ECM remodeling is tightly regulated during development and primarily accomplished by controlling the expression or activities of ECM enzymes and growth factors at multiple levels. Tumor progression and invasion are often linked to the up regulated expression of proteolytic enzymes – generated by both cancer cells and the surrounding tumor microenvironment that are capable of degrading the major ECM macromolecules that comprise all connective tissues[111]. Except proteolytic degradation of ECM, cancer invasion may involve other non-proteolytic modes of ECM modification linked to cancer cells and ECM interactions.

4.3 Resistance to cell apoptosis in tumorigenesis

Apoptosis is a process of programmed cell death throughout the life cycle. It participates in development, cellular homeostasis, and maintenance of normal physiology. It serves as a natural barrier to develop cancer[112]. Cancer cells acquire the capability to evade apoptosis**.** The aggressive growing ability of tumor is determined not only by the enhanced rate of cell proliferation but also by the defect in cell death mechanisms. Mounting evidence principally from studies in mouse models and cultured cells, as well as from descriptive analyses of biopsied stages in human carcinogenesis verify that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancers[104].

The apoptotic machinery is divided into two major pathways: the extrinsic apoptotic pathway, involving for example the Fas ligand/Fas receptor, receiving and processing extracellular death-inducing signals, and the intrinsic pathway that senses and integrates a variety of signals of intracellular origin. Both pathways lead to activation of a normally latent protease, caspases 8 and 9, respectively, which proceed to initiate a cascade of proteolysis involving effector caspases 3/7 responsible for the execution phase of apop-

tosis. Currently, the intrinsic apoptotic program is more widely implicated as a barrier for cancer pathogenesis.

Tumor cells evolve a variety of strategies to circumvent apoptosis. Most common is the loss of p53 tumor suppressor function, which compromises its role as critical damage sensor. Alternatively, tumors may achieve similar aims by increasing expression of anti-apoptotic regulators like Bcl-2, Bcl-xl or by down regulating pro-apoptotic factors like Bax, Bim, Puma, or by short-circuiting the extrinsic ligand-induced death pathway[104]. The complexity and diversities of apoptosis invading mechanisms presumably reflects the diversity of apoptosis-inducing signals that cancer cell populations encounter during their evolution to the malignant tumors.

4.4 Heparanase in cancer

Analyses at the RNA or protein level demonstrate that heparanase expression is up regulated in almost all types of malignant tumors examined to date such as ovarian tumors, pancreatic tumors, myeloma, and tumors derived from colon, bladder, brain, prostate, breast, liver [28,29,84,113–117]. For example, expression of the heparanase gene and protein was detected already at the stage of adenoma in human colon carcinoma, whereas the adjacent normal-morphology tissue showed no expression of the enzyme[29]. Gradient increasing amount of the enzyme was noticeable as the cells progressed from severe dysplasia through well differentiated to poorly differentiated colon carcinoma. Deeply invading colon carcinoma cells showed high levels of both heparanase mRNA and protein[29].

Numerous clinical studies have consistently demonstrated that increased heparanase expression correlates with increased tumor size, tumor progression, metastasis and poor prognosis[80,96–100]. Knockdown of heparanase gene expression or treating tumor bearing mice using compounds that inhibit heparanase enzyme activity dramatically impair tumor progression[79,118–123].

In endothelial cells, nuclear translocation of heparanase has been shown to regulate HAT(Histone acetyltransferase) activity and transcription of pyruvate dehydrogenase kinase 2 (PDK2) and LDHA(Lactate dehydrogenase A), thus increasing lactate secretion[91]. Considering the extensive involvement of heparanase in different types of malignant tumors, especially inflammation related tumors, it is reasonable to speculate that heparanase may causatively involve in the initiation and transformation of cancer cells.

It has been reported for long that heparanase promotes tumor cells invasion and *in vivo* metastasis. The straightforward mechanism is to loosen the ECM, but it also promotes the invasion and metastasis of cancer cells, for example, heparanase expressed by the cancer cells contributes to the invasive phenotype of tumor cells, and that antisense-mediated inhibition of heparanase activity can restore its invasive capacity[124]. More convincing, direct

evidence for a role of heparanase in tumor metastasis was provided by the conversion of non-metastatic murine T-lymphoma and melanoma cells into highly metastatic cells, following transfection with human heparanase cDNA[72].

Latent heparanase has been shown to render cell resistant to cell apoptosis induced by ultraviolet or H_2O_2 related to activation of PI3K/Akt signaling pathway[85]. Recent studies reveal the presence of hepranase in the autophagosomes. Autophagy is a cellular defense mechanism that generates metabolic precursors and ATP, clears misfolded proteins and cellular debris, which is important for cell survival during conditions of stress. Heparanase can directly localize to autophagosomes, where it positively stimulates the autophagic process through a non-enzymatic mechanism mediated by down regulation of mTOR1 activity, conferring growth and survival advantages[125]. The evidence that heparanase enhances the growth of invasive breast cancer driven by expression of mutant *H-Ras* gene and 12-dimethylbenz (a) anthracene/12-O-tetradecanoylphorbol-13-acetate induced skin carcinogenesis adds to the argument that heparanase may be also involved in the initiation of tumor formation[126].

Present investigations

Paper I

Aim

The aim of the present study was to examine the effect of Hsepi overexpression on HS biosynthesis in HEK293 cells.

Results and discussion

We stably expressed recombinant human Hsepi in HEK293 cells using a lentivirus system. Metabolic ^{35}S-labeling of HS revealed an unexpected increase in chain length that correlated with Hsepi expression levels analyzed by Superose-6 gel chromatography.

To exclude nonspecific effects of accumulated protein in the Golgi, a clone was prepared overexpressing a point-mutated (Y168A) Hsepi, devoid of catalytic activity. As a result, HS chains did not differ between the mutant Y168A and mock-transfected cells. Since heparanase, an endo-ß-glucuronidase cleaves HS chains at internally located ß-glucuronidic linkages[1], we assessed whether the exceedingly long HS chains in Hsepi-overexpressing cells could be due to inhibition of the heparanase action. By pulse ^{35}S-labeling for 30 min, HS from were again of high molecular weight, and decreased somewhat during subsequent chase incubation for up to 10 h, which excluded the possibility that Hsepi may have an inhibitory effect on heparanase.

Interestingly, HS chains from Hsepi and Hs2st double-transfected cells did not differ significantly from those of control HS, whereas overexpression of 2OST alone in the HEK293 cells did not appreciably affect HS chain length.

A number of studies have demonstrated interactions between enzymes catalyzing HS biosynthesis. EXT1 and EXT2, form a complex that expresses the combined GlcA- and GlcNAc-transferase activities required for generation of the HS precursor chain[127,128]. EXT2, was further shown to bind NDST1, such that EXT1 and EXT2 have opposing effects on Golgi expression of *N*-deacetylase/*N*-sulfotransferase (NDST)[129]. Importantly, Hsepi and 2OST have been shown to interact with each other during ER-to-Golgi transportation[130].These evidences together predict additional undiscovered

interactions within the GAGosome framework in control of HS chain elongation.

Paper II

Aim

To study the involvement of heparanase in lipid metabolism.

Results and discussions

To study the physiological role of heparanase, the heparanase transgenic mice strain (Hpa-tg mice) had been generated previously[102]. With the question whether those mice have abnormal metabolic capacity, we challenged the mice with high fat food to augment the potential abnormality. Those mice started with comparable body weight, but as feeding with high fat food proceeded, C57BL/6 mice grew in a greater rate with considerable epididymal fat pads, whereas the Hpa-tg mice grew slower, with rather underdeveloped fat layers suggesting that adipose tissue development may be affected by heparanase expression.

Examining adipose tissue revealed that LpL activity was impaired significantly in Hpa-tg mice compared to normal counterpart C57BL/6 mice, which was further supported by the significantly lower level of free fatty acid in Hpa-tg mice plasma.

It has been very well characterized that LpL binds to HS on the cell surface of vascular endothelial cells of organs or tissues involved in lipid metabolism like liver, adipose tissue, and muscle[131]. Using an established protocol to differentiate adipocytes from primary MEF cells, we found those cells to have a similar differentiation capacity (50 % differentiation) detected by oil red staining. Analysis of LpL enzyme that was displaced from cell surfaces by heparin and displaced into the medium revealed a markedly reduced amount of LpL bound to the cell surface of adipocytes differentiated from MEF cells of Hpa-tg mice compared to that of the normal counterpart C57BL/6 mice, suggesting disruption of HS on the cell surface may cause insufficient binding of LpL, thus affecting the lipid metabolism of local adipose tissue.

Further, analyzing the HS chain length and the relative amount of HS chains revealed that the cell surface HS was similar in length to extracellular HS, but with a higher proportion of chains in the medium estimated by the radioactive counts and lower amount of HS in the respective cell surface fraction in the MEF cells of Hpa-tg mice. This may indicate a potentially elevated shedding of HS and HSPGs like syndecans as reported earlier for cells with overexpressed heparanase[132].

Except for affecting LpL activity and thereby lipid storage possibilities in adipose tissue, a recent study revealed another potential mechanism how

heparanase may affect fat metabolism. In the study by He *et al.* it was described that nuclear heparanase is recruited to the promoter and transcribed regions of a distinct cohort of genes, thus regulating transcription of an array of genes in T-cells including fat differentiation related genes[93]. Whether elevation of heparanase in scenarios like inflammatory diseases might affect the differentiation of adipose tissue *in vivo,* remains to be further investigated.

Paper III

Aim:
To study effects of heparanase on aerobic glycolysis in breast cancer T47D cells.

Results and discussions:
Evidence has been documented that heparanase promotes breast cancer MCF 7 cell proliferation *in vitro* and tumor growth *in vivo*[133]. In this study, we found that the lactic acid level in conditioned medium of T47D-HPSE cells was much higher as compared to control T47D-Mock cells. According to the Warburg effect, cancer cells adopt aerobic glycolysis to fulfill the cells' need for ATP, with a great advantage by providing precursors such as acetyl-CoA for fatty acids, glycolytic intermediates for nonessential amino acids, and ribose for nucleotide synthesis for the cells to proliferate. Indeed, the total cell number of T47D-HPSE was moderately higher than that of T47D-Mock, suggesting a higher proliferative capacity of T47D-HPSE cells. When we cultured the cells with serum free low glucose medium, again, we found a significantly higher level of lactic acid in T47D-HPSE cells compared to T47D-Mock cells, together with up regulation of GLUT1 expression and an array of glycolytic genes in T47D-HPSE cells. Comparatively higher levels of GLUT1 expression are seen in cancers of higher grade and proliferative index and in cancers of lower degree of differentiation[134–136]. Accordingly, the expression level of GLUT1 correlates reciprocally with the survival of cancer patients[137–139]. Furthermore, the increased aerobic glycolysis rate and cell viability could be inhibited by 2-deoxy-D-glucose, and independent of PI3K/Akt or MAPK pathways, suggesting alternative mechanisms involved.

Paper IV

Aim

To reveal the gene expression profile associated with heparanase in melanoma cells.

Results and discussions:

While heparanase has been shown to facilitate cancer cell invasion and metastasis, assumedly by degrading ECM structure[140–142], we showed here silencing HPSE also correlates with up-regulation of the synthesis of several cell adhesion proteins and main constitutive adhesion proteins of the ECM such as connective tissue growth factor, cysteine-rich angiogenic inducer 61, thrombospondin 1 and lumican. Morphologically, we observed enhanced cell spreading after silencing of HPSE expression. These observations were also supported by the detection of increased number of focal adhesion complexes and FAK activation after HPSE silencing. Therefore, we reason that elevated heparanase present in malignant tumor cells and surrounding inflammatory cells may orchestrate the down regulation of adhesive protein synthesis apart from degrading ECM structure, contributing to cancer cell dissemination and invasion. In addition, heparanase has been correlated with dedifferentiation of pancreatic ductal adenocarcinoma cells[143]. Interestingly, HPSE silenced cells showed up-regulation of keratin 14 one of the differential markers of melanocytes, suggesting removal of HPSE may wind back cells to a higher differentiated, less aggressive stage. Taken together, these evidences support the role of heparanase in promoting cancer cell invasion and subsequent metastasis.

Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer[144]. Heparanase promotes cell survival in breast cancer cells[145], tumor growth and chemoresistance[125]. Accordingly, silencing of the expression of HPSE induced cells to decease dramatically. Heparanase was reported to down-regulate pro-apoptotic genes such as TNF superfamily 10 and its receptor TNFRSF10B, in rat cardiomyocytes with exogenous addition of latent heparanase, providing protection of the cells against high glucose and H_2O_2 induced cell-death[146]. Consistent with previous studies, silencing of HPSE expression in melanoma cells up-regulated an array of genes involved in positive regulation of cell-death. Detection of DNA fragmentation confirmed apoptosis undergoing in these cells, which was mediated by caspase 3/Parp1 activation. Among the genes, our attention was drawn to cell death receptors TNFRSF10A and TNFRSF12A the expression of which was confirmed by real-time PCR. Additionally, the effects of heparanase on the cell adhesion and invasion of apoptosis appeared correlated with the protein level, further suggesting the potential causative role in tumorigensis and progression.

Heparanase function is not limited to the extracellular surroundings, which has been studied extensively. Heparanase can also interfere with gene transcription directly by binding to nuclear DNA[93,147] or indirectly by controlling histone H3 methylation patterns.[90,148] To our surprise, we found that a number of genes involved in nucleosome and nucleosome assembly were significantly down regulated after removal of heparanase, which might account for the universal regulation of heparanase on gene transcription. Taken together the detection of heparanase in the nucleus, those evidence further add new possibilities to its so far probably underscored role and unclear mode of action in modifying gene transcription, albeit the detailed mechanisms of heparanase action need to be further dissected carefully.

Future perspectives

HSPGs are a group of polysaccharides ubiquitously present on the mammalian cell surface and important components of extracellular matrix. They are composed of one or more HS chains and a core protein. HS has emerged as an attracting research topic due to its immense presence and diverse involvements in normal physiology that any alteration of it can lead to certain pathological abnormality of different extent.

Heparanase, as the only endo-β-glucuronidase that cleaves HS becomes even more interesting with the finding that the protein is elevated in both inflammation and cancer, and later its intracellular presence and functions.

Heparanase is physiologically active in lysosome where the pH is 4-5, prompting the optimal pH of this enzyme. Therefore, heparanase is expected not to be enzymatically active in the extracellular matrix even though it can be secreted to extracellular space. However, in the scenario of inflammation, the acid-base balance slips to acidity. Focal inflammatory situations, such as rheumatoid arthritis[149] and atherosclerotic lesions usually have regions of low extracellular pH[150]. Acidic extracellular pH is also a major feature of tumor tissue, with extracellular acidification being primarily considered to be due to lactate secretion from anaerobic glycolysis[151]. Heparanase is thus reasoned to be active in those scenarios otherwise inactive, resulting in degradation of extracellular matrix, releasing plenty of inflammatory factors and remodeling the matrix in a way that may promote cancer cell invasion and metastasis.

It is believed that heparanase plays an essential role in promoting inflammatory circuits. Heparanase is found in neutrophils and activated T-lymphocytes and contributes to their ability of extravasation and accumulation in the targeted organs[152]. Heparanase is required for macrophage activation resulting in increased production of proinflammatory factors that can in turn induce epithelial heparanase expression via a TNF α dependent way and posttranslational processing of proheparanase via increased secretion of cathepsin L[153].

Recently, the non-enzyme feature of heparanase prompted several studies showing that proheparanase promotes signal transduction, including protein kinase B (Akt), signal transducer and activator of transcription, Src, Erk, HGF, IGF, EGF signaling pathways[154].

Moreover, emerging studies reveal heparanase presence during autophagy and localized to exosome and nucleus, adding tint to potential intracellular

roles beyond its extracellular roles. Taking advantage of heparanase transgenic mice, Shteingauz *et al.* show that significantly more autophagosomes are detected by electron microscope in pancreas tissue sections and in Fadu cells, moreover heparanase is shown to co-localize with autophagosomes in SIHN-013 laryngeal carcinoma cells that overexpress heparanase[125],while PG545, a heparin sulfate mimetic, is shown to impede *in vivo* tumor growth and autophagy formation, it exhibits no effects as such *in vitro* cells. Similarly, heparanase has been shown to stimulate exosome secretion composed of syntenin-1, syndecan, CD63 and heparanase dependent on syntenin-1 and ALIX, reduction of heparanase reduces the secretion of syntenin-1 containing exosomes[155].

Heparanase has been detected in nuclei of malignant tumor cells by immunohistochemical staining. In some cell lines, like epidermoid carcinoma cells A-431, HeLa cells, osteosarcoma U-2 OS cells, it mainly localizes to the nucleoplasma (Human Protein Atlas). Heparanase colocalizes with nuclear HS and is enzymatically active. Moreover, following uptake of latent 65 kDa heparanase by cells that do not express the enzyme, an active 50 kDa heparanase can be detected in the cell nucleus[17]. A number of growth factors can induce heparanase nuclear localization. For example, EGF induces heparanase nuclear localization where heparanase augments DNA Topoisomerase I activity in brain metastatic breast cancer[89]. It is likely that it occurs via cleavage of HS by heparanase considering that nuclear HS has been reported to inhibit DNA topoisomerase I[56,90]. Moreover, several studies show that nulear heparanase promotes the activity of histone HAT and enhances acetylation of histones, subsequently up-regulating transcription of a cohort of genes[70–72]. However, there is also evidence showing nuclear heparanase down-regulates a number of relevant genes by binding to DNA in metastatic melanoma cell lines[94]. The exact action mode of heparanase in the cell nucleus appears specially attracting considering its diverse regulatory roles in many kinds of pathological conditions.

Collective research so far suggests a strong rationale for anti-heparanase therapy. Heparanase inhibiting small molecules have been developed, heparanase neutralizing antibodies are being examined in preclinical studies; heparin mimetics that inhibit heparanase enzymatic activity such as PI-88, SST0001, necuparanid, PG545 are being evaluated in clinical trials for tolerance test. Those heparanase inhibitors and anti-heparanase antibodies have been tested *in vivo* and shown promising effects on impeding tumor growth and metastasis. However, it is not sufficient to attribute the anti-tumor effects to the capability of inhibiting heparanase enzymatic activity given the heparin mimetics have a high affinity for a number of cytokines such as FGF, HGF, VEGF and neither heparin mimetics nor antibodies seen to affect *in vivo* heparanase activity. Accumulated evidence appears to support their extracellular roles instead of entering and acting intracellularly as presumed. Recent pharmacodynamic data reveal PG545 to increase innate immune cell

activation, plasma IFNγ, TNFα, IP-10 and MCP-1 accompanied by disease control in some subjects with solid tumors, supporting the drug mechanism of action in stimulating the immune system, which represents a promising approach for use in combination with existing therapies.

In all, recent emerging studies on intracellular heparanase and its action mode shed light on the potentially underscored face of heparanase. Giving the limitations of the current inhibitors designed based on its conventional roles, it raises novel sights and opens up new gates to better understanding of heparanase for more specific and efficient therapeutic alternatives.

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