Heparan sulfate dependent cell signaling and associated pathophysiology

Implications in tumorigenesis and embryogenesis

TAHIRA BATOOL
Dissertation presented at Uppsala University to be publicly examined in Thursday, 6 December 2018 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor, MD Liliana Schaefer (Department of General Pharmacology and Toxicology, Goethe University, Frankfurt, Germany).

Abstract

Heparan sulfate proteoglycans (HSPGs) consist of a protein core to which several linear, negatively charged heparan sulfate (HS) chains are covalently attached. HSPGs are expressed on the cell surface and in the extra-cellular matrix (ECM) where they have diverse biological functions, for example co-receptor functions. The diverse functions of HS are linked to structural variability of the polysaccharide. In this thesis, I investigated HS structure-function relationship by using different cell and animal models of one HS-biosynthetic enzyme, glucuronyl C5-epimerase (Hsepi) and one enzyme responsible for post synthetic modification, heparanase.

Deletion of Hsepi in mice resulted in neonatal lethality, with multiple organ defects, indicating the importance of HS in embryogenesis. Up-regulated expression of heparanase is found in most human tumor tissues, correlating with increased metastatic potential and decreased survival of cancer patients.

In the first project, I focused on the effects of HS on cancer associated cell signaling and found that heparanase overexpression attenuated TGF-β1 stimulated Smad phosphorylation in tumor cells because of increased sulfation degree and turnover rate of HS.

Heparanase role in cancer progression has led to clinical trials where inhibition of heparanase activity is currently being evaluated as a potential cancer treatment. Heparin, a HS-related polysaccharide, is being used to inhibit heparanase activity. In my second project, we studied the effect of low molecular weight heparin (LMWH) on cisplatin resistance of ovarian cancer cells (A2780cis). LMWH treatment of A2780cis cells reduced Wnt-activity in these cells and consequently reduce the drug resistance.

In paper III, we continued exploring the HS/heparanase role in cancer by using heparanase overexpressing mice (Hpa-tg). We found Lewis Lung Carcinoma (LLC2) cells showed faster growth, bigger tumors and more metastasis in the Hpa-tg mice as compared to wild-type (WT) mice, because of suppressed antitumor immunity in the Hpa-tg mice.

In paper IV and V, we studied the structure-function relationship of HS by using Hsepi-/- mice model. Hsepi-/- results in HS-chains lacking IdoA, which makes the chain rigid and consequently affects its co-receptor function. Skeletal malformation in Hsepi-/- mice, led us in paper IV to investigate bone morphogenic protein (BMP), an important signal molecule during embryogenesis and known to interact with HS. We found upregulation of a number of BMPs and expression of P-smad1/5/8, but reduced expression of inhibitory Smads and Gremlin1 in the Hsepi-/- MEF cells. The study indicated that the developmental defects in Hsepi mice could be contributed by a higher BMP signaling. In paper V we investigated the lung of the Hsepi-/- mice. The distal lung of 17.5 days old embryos remained populated by epithelial tubules, because of impaired differentiation of type I cells of the lungs. Potential mechanisms behind the failure of type I cell formation was identified to be reduced vascularization and a sustained signaling of Smad pathways.

Keywords: Heparanase, Glucuronyl-C5 epimerase, Heparan Sulfate, Heparin, Lung Cancer, Cancer cell Singling, TGF-Beta, BMP, VEGF, Notch Signaling, Tumor growth, Metastasis, embryonic development

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

© Tahira Batool 2018

ISSN 1651-6206
ISBN 978-91-513-0478-6
urn:nbn:se:uu:diva-363258 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-363258)
“There are only two ways to live your life. One is as though nothing is a miracle. The other is as though everything is a miracle”. Albert Einstein

“The intimacy that arises in listening and speaking truth is only possible if we can open to the vulnerability of our own hearts. Breathing in, contacting the life that is right here, is our first step. Once we have held ourselves with kindness, we can touch others in a vital and healing way”. Tara Brach

Dedicated to my loving father and angelic mother
List of Papers

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


IV **Batool, T.**, Fang, JP., Jansson, V., Zhao, H., Gallant, C., Moustakas, A., Li JP. Upregulated BMP-Smad signalling activity in glucuronyl C5-epimerase knock out MEF cells. *Cellular Signaling. Submitted manuscript*.

V Cui, H., **Batool, T.**, Zhang, X., Li, JP. Glucuronyl C5-epimerase is crucial for epithelial cell maturation during embryonic lung development. *Manuscript*.

Reprints were made with permission from the respective publishers.
Contents

Introduction .................................................................................................................. 11
1. Proteoglycans ........................................................................................................... 11
   1.1 Heparan sulfate proteoglycans (HSPGs) ......................................................... 11
   1.2 Heparan sulfate (HS) ....................................................................................... 13
   1.3 HS biosynthesis ............................................................................................... 15
   1.4 Mouse models defective in HS biosynthetic enzymes .................................... 16
2. Heparanase ............................................................................................................. 17
   2.1 Functions of heparanase ................................................................................... 18
3. HS functions in cell signaling .................................................................................. 20
   3.1 Transforming growth factor (TGF-β) Signaling ............................................. 22
   3.2 Bone Morphogenetic Proteins (BMP) signaling ........................................... 23
   3.3 Wnt signaling ................................................................................................. 24
   3.4 Notch signaling ............................................................................................... 25
   3.5 VEGF signaling .............................................................................................. 25
4. Implications of heparanase in tumorigenesis ......................................................... 27
   Present Investigation ............................................................................................. 31
   Aim ......................................................................................................................... 31
   Paper I .................................................................................................................... 31
   Paper II ................................................................................................................... 32
   Paper III ............................................................................................................... 33
   Paper IV ............................................................................................................... 34
   Paper V ............................................................................................................... 34
Concluding remarks and Future perspectives ......................................................... 36
Acknowledgements .................................................................................................... 39
References .................................................................................................................. 42
Abbreviations

CS    Chondroitin sulfate
CSL   (CBF1/Suppressor of Hairless/LAG-1)
CTLA4 cytotoxic T-lymphocyte-asscoaited protein4
DS    Dermatan sulfate
Erk   Extracellular Signal-regulated Kinase
FGF   Fibroblast growth factor
GlcN  Glucosamine
GlcA  Glucuronic acid
Hsepi Glucuronyl C5 epimerase
GPC3  Glypican 3
HBD   Heparin Binding Domain
HGF   hepatocyte growth factor
Hh    Hedgehog
HPSE  Heparanase (Hpa)
HS    Heparan sulfate
HSPGs Heparan sulfate proteoglycans
IdoA  Iduronic acid
KS    Keratan Sulfate
LLC2  Lewis Lung carcinoma
MAPK  mitogen activated protein kinases
MEF   Mouse embryonic fibroblast
Ndst  N-deacetylase/N-sulfotransferases
PD1   Programmed cell death protein1
PGs   Proteoglycans
PI3K  Phosphoinositide 3-kinase
Src   sacro/cma/ protooncogene tyrosine-protein kinase
TGF-β Transforming growth factor-beta
TLR   Toll like receptor
VEGF  Vascular endothelial growth factor
Introduction

1. Proteoglycans

Proteoglycans, the sweet mystery of life, are far more complex than the genome or proteome because of dynamic structural changes that occur during development, homeostasis and pathological conditions. Thus, a given cell type in a given species has a unique “glyco-fingerprint”.

The study of proteoglycans dates back to the beginning of the 20th century. The field has expanded tremendously from basic understanding to trying to find cure for complex diseases. For example; Roneparstat (a modified heparin) has ended a phase I clinical trial in myeloma patients.

All mammalian cells produce and secrete proteoglycans (PGs), that refers to a group of glycoconjugates composed of a core protein to which several glycosaminoglycan (GAG) chains are attached. GAGs are linear polysaccharides in which repeating disaccharide building blocks consist of amino sugars (N-acetylglucosamine/GlcNAc or N-acetylgalactosamine/GlaNAc) and an uronic acid (glucuronic acid/GlcA or iduronic acid/IdoA) or galactose. The main GAG chains attached to PGs are of five types based on disaccharide composition, glycosidic linkage and degree of sulfation: chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS) and heparin. PGs are differentiated with regard to core protein and substitution with one or two types of glycosaminoglycan chains. Because of their abundance on the cell surface and in the extracellular matrix (ECM), PGs are of prime importance for studying in relation to tumor growth, metastasis and tumor/host interactions via signaling.

This thesis has elaborated some of the complex roles that PGs play in cancer pathology (tumor growth) and during the embryonic development of organisms with particular focus on HS signaling activities.

1.1 Heparan sulfate proteoglycans (HSPGs)

Heparan sulfate proteoglycans (HSPGs) are one of the most important PGs with common characteristic of having one or more covalently attached heparan sulfate (HS) chains. They are ubiquitously expressed on the cell surfaces and in the extracellular matrix of most animal tissues and are classified into 3 groups based on their location: 1: Plasma-membrane-bound HSPGs such as
syndecans and the glycosylphosphatidylinositol-anchored glypicans. **2:** HSPGs secreted into the ECM e.g., agrin, perlecan, and type XVIII collagen. **3:** Intracellular, secretory vessel proteoglycans, serglycin (Figure 1). In all these locations, they function through interactions of either HS chains or core protein with protein ligands. For example, membrane HSPGs act as co-receptors for various tyrosine kinase-type growth factors and their receptors, controlling signaling transduction. In the ECM, they act as storage depot for cytokines, growth factors and morphogens. Cleavage of HSPGs (or HS chains) leads to release of the cytokines, modulating the biology of cells.

Syndecans are of particular importance for tumor progression studies because many questions about their role in tumor development are still unanswered. Redundancy is an important issue. Syndecans (-1 and -4) null mice are viable and fertile whereas deletion of some HS biosynthetic enzymes is lethal. Similarly, cleavage of HS chains by heparanase on syndecans altered the glyco-fingerprint of cells in tumor microenvironment. High levels of shed HS-rich ectodomain of syndecan-1 are present in sera of patients with myeloma and predict poor prognosis.

In addition to syndecans, small leucine-rich proteoglycans (SLRPs; Decorin and Biglycan) are emerging subset of matrix-derived, soluble regulators, that are important for their role in cancer and inflammation. Under certain conditions, matrix components in their soluble form may act as endogenous “danger” signals. These molecules are recognized by innate immunity receptors and are capable of triggering inflammatory response reactions.
Figure 1: Illustration of HSPGs at various locations in cell and in ECM. HSPGs consist of core proteins linked through a linker tetrasaccharide to one or more HS/heparin chains. Some subtype of syndecans can also carry other types of GAGs including Chondroitin sulfate (CS). Reprinted with permission from Digre A., 2017.

1.2 Heparan sulfate (HS)

Heparan sulfate (HS) is composed of repeating disaccharide units of β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) that are 1, 4 glycosidically linked to D-glucosamine (GlcN).
The uronic acid residues can be IdoA2S and rarely GlcA2S, but also occur nonsulfated. The GlcN residue can contain an N-acetyl or N-sulfate group (GlcNAc or GlcNS), as well as modification by 6-O-sulfation\(^\text{12}\). 3-O sulfation is a very rare phenomenon mainly seen in heparin (a highly sulfated GAG)\(^\text{13}\). HS generally displays a domain structure, involving NS-domains and NAc domains, which is determined by the presence or absence of GlcNS residues, respectively\(^\text{11}\). HS biological properties are largely dictated by the structure and sulfation pattern of NS-domains, which comprise the binding sites for most protein ligands\(^\text{12}\) (Figure 2).

**Heparin**

Heparin is produced by limited number of cells, notably connective tissue-type mast cells, in contrast to that HS is made by virtually all types of cells\(^\text{3}\). Heparin is known for its anticoagulant activity\(^\text{13}\). Heparin and HS can be compared based on following structural differences: first, heparin has mostly IdoA, whereas HS has mainly GlcA residues\(^\text{14}\); Second, heparin features 80-90% N-sulfated GlcN units and a total (N- and O-) average sulfate content typically greater than two residues per disaccharide unit, whereas HS is 30-60% N-sulfated with a total sulfate content of approximately one residue per disaccharide unit. HS has domain structure as described above in figure 2,
whereas Heparin is more uniformly sulfated and resembles a continuous NS domain \(^{11, 15}\) (Figure 3).

Low molecular weight heparins are derived from commercial unfractionated heparin. Some of them lack anticoagulant activity but still retain other properties e.g., inhibition of inflammation, proliferation and antimetastatic activity \(^3\).

1.3 HS biosynthesis

The biosynthetic enzymes for HS chain synthesis are present in Golgi apparatus and assumed to form a GAGosome (complexes of enzymes interacting with each other, their substrates and potentially additional auxiliary proteins) \(^{16}\). The sequential order of reaction shows that the biosynthetic enzymes are substrate specific (Figure 3) \(^{17}\). Synthesis begins with the stepwise addition of four mono-saccharides to form a tetra-saccharide (Xyl-Gal-Gal-GlcA) linked to a serine residue in the core protein. Then special glycosyltransferases (EXT) polymerize and elongate the chain by adding alternating GlcA and GlcNAc residues, forming repeating disaccharide unit \(^{16}\). After that, chain modification enzymes come into action to give HS chains distinct and diverse structural features, described below. With 32 possible modifications for each di-saccharide unit the structure of HS is very complex and is information rich \(^{18}\).

First chain modification enzyme, N-deacetylase/N-sulfotransferases (Ndst) removes N-acetyl groups from subsets of GlcNAc residues followed by sulfation of free amino groups, a critical step for the formation of domain structure and also a key step in overall polymer modification because further modifications of HS chain by glucuronyl C5-epimerase and by sulfotransferases requires the presence of GlcNs residues (NS-domains). Glucuronyl C5-epimerase (Hsepi) converts glucuronic acid residues (GlcA) into iduronic acid (IdoA) followed by consecutive action of 2-O and 6-O sulfotransferases (2-OST, 6-OST), rarely also 3-O sulfotransferase (3-OST) \(^{19}\). Iduronic acid provides flexibility to the HS chain and facilitates its interaction between the GAGs and proteins \(^{20}\). 2-O sulfation locks iduronic acid residues in interaction-prone configuration. 2-O sulfated and nonsulfated iduronic acid and 6-O sulfated glucosamine are abundant modification products, whereas 2-O sulfated glucouronic acid and 3-O sulfated or N-substituted glucosamine units are scarce \(^{15}\). Different tissues have different ratios of component di-saccharides in HS, which is regarded as “glyco-fingerprint” and can show distinctive changes in certain diseases, for example in cancers \(^{18, 21, 19}\).

The epimerase and sulfotransferase reactions along the GAG chains are not uniform, resulting in a domain-type arrangement with some areas being highly modified and some with low density of modifications. To understand the mechanism that control this, is a challenge for the field \(^{22}\).
1.4 Mouse models defective in HS biosynthetic enzymes

Numerous mutational studies of genes involved in biosynthesis of HS, e.g. Hsepi (Glce) have demonstrated the essential role of specific structural requirement of HS in developmental processes 23. Mice lacking Hsepi gene (Glce) express altered HS structure lacking IdoA and die neonatally with several defects in lung, kidney and skeletal formations 24.

Figure 3. The HS biosynthesis. HS and Heparin follow the same biosynthetic pathway but heparin is more sulfated than HS. (Modified with permission from Digre A., 2017).
In contrast to the gestation failure of embryos entirely lacking HS, heparan sulfate 2-O sulfotransferase deficient mice (2-OST -/-) mice survive until birth but die prenatally due to complete absence of kidneys. The observation of 2-OST -/- mutant kidneys suggests that signalling between two tissues, ureteric bud and metanephric mesenchyme, is disrupted. Two classes of growth factor was assumed to be possible candidates for further investigation in this interaction, Glial derived neurotrophic factor (GDNF) and members of the FGF family 25.

Ndst1 -/- mice also show severe defects of multiple organs including skeleton, brain and lungs and die shortly after birth due to respiratory failure 26. Lung development starts in mice at embryonic day 9.5 and by E16.5 airways have grown and branched to form bronchial tree. Between E16.5 and E20.5 distal lung undergoes differentiation to form elongated type I and cuboidal surfactant filled type II cells. Differentiation of epithelial cells accompanied by vascular remodeling and thinning of the mesenchyme results in enlargement of the diameter and surface area of the alveolar sacs known as saccula- 

2. Heparanase

Heparanase (HPSE) cleaves HS side chains by hydrolysis of internal D-glucuronic acid (GlcUA) (β1→4) and glucosamine (GlcNS) bond and yields fragments of 10-20 sugar units or 5-10 kDa degradation fragments (Figure 4) 37, 38.
Heparanase catalytic reaction: Heparanase cut the HS/Heparin between GlcA and GlcN. Main determinants for recognition and binding stability include N-sulfation and 6-O-sulfation, two and one monosaccharide away from cleavage site.

Heparanase is initially synthesized as a preproenzyme, containing a signal sequence that is cleaved by peptidase to yield an inactive 65-kDa proheparanase in the Golgi apparatus. Further processing occurs in lysosomes/endosomes by cathepsin L that liberates a linker peptide, an N-terminal 8 kDa and a C-terminal 50 kDa subunit, which remain associated as a noncovalent heterodimer in mature active heparanase. This active heparanase can be trafficked to the cell surface or ECM to breakdown extracellular HS. The enzyme can also remain in lysosomes to process internalized HSPGs or help in exosome formation.

Crystal structure of heparanase shows that it is composed of a TIM barrel fold which contains two catalytic glutamic acids and two HS/heparin binding domains along with a C-terminal domain that is important for secretion and non-enzymatic signaling function of the protein. The crystal structure confirmed the requirement of sulfation for heparanase interaction with HS.

2.1 Functions of heparanase
Heparanase is expressed at low levels in normal tissues. It plays roles in wound repair, tissue regeneration, and in hair follicle growth. It also regulates gene expression and activates cells of innate immune system. In this way, the normal functions are presumably associated with HS turnover. Heparanase presence in lysosomes indicates that this enzyme has important role in regulating the function of lysosomes as it has been found that lysosomal heparanase regulates the basal level of autophagy and resides within autophagosome.

When heparanase is overexpressed, it leads to rapid turnover of HSPG with upregulation of N- and O-sulfation degree of HS. Also, when heparanase is added exogenously to cells it leads to colocalization of syndecans with heparanase in endocytic vesicles, as a result syndecans disappear from the cell sur-
face that affects the cell communication with each other and with the surrounding microenvironment \(^{48}\). Moreover, elevated levels of heparanase was not only found in cancer but also in the diseases of inflammation, autoimmunity, fibrosis, kidney failure and diabetes \(^{38}\).

2.2 HSPG turn over

HS chains of both membrane-intercalated and secreted PGs can be modified by heparanase and endosulfatases or surface bound PGs can be shed by proteases. Eventually, endocytosis of PGs leads to degradation of HS by exoenzymes in lysosomes or alternatively to recycling \(^{49}\).

Infact, this process of HSPGs turnover is tightly related to heparanase synthesis and trafficking \(^{38}\). Intracellular degradation is a relatively fast process and the half-life of HSPGs found at plasma membrane or in recycling compartments was estimated to be 2-3 h \(^{50}\). This, normal process can be upregulated in some pathologies such as cancer, because of heparanase overexpression, that can lead to rapid turnover of HSPGs and in turn can affect signaling in cancer.
3. HS functions in cell signaling

The most important role of HS is in cell signaling through interaction with growth factors, chemokines, immune modulators, morphogens and plasma proteins. In ECM, HS act as reservoir for these molecules and at cell surface, HS act as co-receptor to mediate the interaction between cytokines and their receptors through binding to both the ligands and the receptors.\(^5\)

The precise mechanism of HS modulation of the functions of these growth factors is not clear yet. Apart from functioning as a co-receptor for growth factors (GF), HS also exert other functions in modulating cellular signaling. One possible mechanism is that HS stabilize gradient of signaling molecules, which provide spatial information to cells.\(^51\) HS chains have been found to promote ligand oligomerization, which is prerequisite for certain cytokine interaction with their receptors.\(^52\) (Figure 5). Other mechanism is to internalize the signaling molecule or store it so it can be released by heparanase cleavage.\(^53,54\) The direct participation of GAG chains in signaling complexes with some cell surface receptors has been demonstrated for example, highly sulfated polyanions such as heparin and synthetic polysulfonates, but not HS that is less sulfated can activate FGFR4 in the absence of FGF ligand.\(^55,56,15,57\) Low molecular weight are more active in triggering danger responses via binding to Toll-like receptors (TLR) responses.\(^58\)

Interactions between proteins and HS primarily depend on charge distribution on the HS chains and may require the presence of specific saccharide components and or sulfation patterns.\(^23\) The major example of HS-protein interaction that requires highly specific epitopes for interaction is, antithrombin interacting with heparin. The rare 3-O sulfation is important determinant in this interaction.\(^59,13\) On the other extreme, a typical example of low specificity interaction is shown by hepatocyte growth factor (HGF), that binds various GAGs.\(^60,61\)
Figure 5: GAGs as co-receptors in ligand/receptor complex formation. GAG chains can act as an activator and promote the formation of a ligand/receptor complex. GAG chains can block the ligand-binding site on a receptor and act as a repressor. It can act as a concatenator that strings receptors or ligands together at the cell surface. By limiting the space available to these proteins from three-dimensional space of extracellular fluid and the ECM to the one-dimensional space along the chains, the chance of encounter among ligand and its receptor may be enhanced. Thus, the critical role of HSPGs may be in controlling the kinetics of protein-protein interactions rather than the thermodynamics of such encounters.62

Most of the proteins that bind to HS and heparin seems to have the heparin binding sites which is characterized by arrangements of basic amino acids in linear peptides, (-X-B-B-X-B-X-), where B is a basic and X is a hydrophobic residue.61,63 However, there is no single protein motif to define binding sites. There are relatively short, widely spaced sequences in heparin-binding proteins that are arranged to generate heparin-binding sites on protein surfaces.60,64,61

The GAG-protein interaction specificity depends on GAG structure and in broader perspective depends on regulation of GAG biosynthesis. Overall, a HS pool generated through strictly regulated biosynthesis may be designed to accommodate a single protein ligand with defined affinity or more commonly, more than one protein species, often with variable affinities.61 There are no general relations between binding affinity, binding specificity and functional importance in HS-protein interactions.

Care should also be taken in extrapolating data obtained with heparin versus binding to HS and HSPGs; binding to heparin can occur owing to the high charge content of the polysaccharide, whereas the same factor might bind to HS with lower affinity or not at all. On the other hand, specific protein-binding
motifs expressed in subspecies of HS may occur also in heparin, although concealed by additional, redundant, sulfate residues.

3.1 Transforming growth factor (TGF-β) Signaling

TGF-β family consist of 33 pleiotropic factors divided into two groups. The first group includes TGF-β and other group comprise of BMPs. TGF-β signal via heterotetrameric complexes of type I (ALK5) and type II serine/threonine kinase receptors. Members of smad family (Smad2, Smad3 and co-Smad 4) are important mediators TGF-β signaling.

TGF-β was the first member in the family identified as a HS binding protein. Several studies have elaborated the involvement of HS in TGF-β signaling. Regarding TGF-β interaction with HS, it is known that this interaction is isoform specific. Both TGF-β1 and 2 possess affinity for heparin and for highly sulfated HS but not TGF-β3. The relative lack of effect of CS and DS on TGF-β1 along with the marked reduction in activity of heparin after removal of its N-sulfate groups, indicates a strong requirement for N-sulfated GAGs and the requirement is also sequence and domain specific.

The main function of TGF-β is to maintain tissue homeostasis via tight regulation of cell proliferation, survival and adhesion or through microenvironment communication, but in cancer this tumor suppressor function is disturbed either through inactivation of core signaling components or alternation of main pathway regulatory components. Smad dependent pathways induce growth arrest or apoptosis by activation of cell cycle inhibitory genes or pro-apoptotic genes and these pathways are disturbed during cancer growth. Smad 6 and 7 (inhibitory smads) are upregulated upon TGF-β stimulation as negative feedback mechanism. TGF-β R-smads are phosphorylated in the linker region by MAP kinases preventing their accumulation in nucleus.

Diversity of TGF-β signaling responses is because of cross-talk of core pathway components with other signaling pathways and it also depend on the ability of TGF-β receptors to activate other signaling modules in addition to Smads, to reinforce, attenuate, or otherwise modulate downstream cellular responses. For example, in non-smad pathways, TGF-β induces ERK activation by inducing phosphorylation of tyrosine residues on both type I and type II receptors. The phosphorylated tyrosines then recruit Grb2 and activate Ras, Raf and MAPK cascade. ERK can also inhibit R-smad by phosphorylating them. Similarly, AKT signaling can either synergize or antagonize TGF-β pathway in context and cell dependent manner. The challenge is to accurately predict biological outcomes from these combinatorial signaling activities.
3.2 Bone Morphogenetic Proteins (BMP) signaling

BMPs are mainly known for their critical roles in skeletal development, especially in mammals. Total 15 BMPs have been identified in vertebrates and are classified into many subgroups: the BMP2/4 subgroup, the BMP-5/6/7/8 subgroup, the GDF (growth and differentiation factor)-5 (also termed as BMP14), GDF-6 (BMP-13)/GDF-7 (BMP-12) subgroup and the BMP-3 subgroup. All BMPs are members of TGF-β superfamily, except BMP-1.

BMP signal through two types of transmembrane serine/threonine receptors. BMP receptors IA (ALK-3), IB (ALK-6), ALK-1 and ALK-2 function as type I BMP receptors, whereas BMP receptor II, activin receptors II and IIB work as type II receptors which are constitutively active kinases. First type II receptors phosphorylate type I receptors and then type I receptor phosphorylate Smad1/5/8 transcription factors in cytoplasm (Figure 6). P-smad1/5/8 accumulate in nucleus within 1 hr after BMP stimulation to regulate expression of primary target genes for example, Runx2.

BMP signaling is regulated by intracellular or extracellular molecules. Most importantly, Noggin, chordin, follistatin and the DAN family proteins bind to BMPs in the extracellular space and inhibit binding of ligands to cell surface receptors. In contrast, heparin and other types of sulfated polysaccharides have been shown to enhance BMP-2 signaling and it depends on the size and number of their sulfated residues. Cells lacking Ndst1 produced HS with reduced N-sulfation, which impaired BMP internalization in lung development.
3.3 Wnt signaling

Wnt (Wingless/integrated) signaling pathways are important in cancer stem cells (CSCs) and carcinogenesis of ovarian cancer. Ovarian CSCs are involved in chemoresistance, metastasis and tumor recurrence. In epithelial ovarian cancer cells, Wnt/beta catenin target genes regulate cell proliferation and apoptosis, thereby cause initiation and progression of cancer.

In the canonical Wnt/beta catenin pathway, in the absence of ligand the pathway is “off” and leads to degradation pathways. When a ligand binds to cell surface receptor; Frizzled(Fzd) family of G-protein coupled receptors and low-density lipoprotein receptor-related proteins (LDLR=LRP6/LRP5) then LRP6/LRP5 is phosphorylated by the kinases casein kinase1 (CK1) and glycogen synthase kinase 3β (GSK3- β). Dishevelled (Dsh) molecules interact with Fzd and then interaction of Axin with phosphorylated receptors and Dsh leads to inactivation of degradation pathway. β- Catenin accumulates in the cytoplasm, enters the nucleus and activates Wnt target genes by binding to the transcriptional factors of the TCF/LEF family by displacing Groucho.

Wnt interaction with HSPGs was first established in Drosophila models. Syndecan 4 has been shown to regulate the Wnt-dependent early shaping of xenopus embryos. In addition, syndecan 4 has been shown to promote bone fracture reconstruction in mouse models by Wnt activity.
3.4 Notch signaling

The evolutionary conserved Notch signaling pathway functions as a mediator of short-range cell-cell communications. Notch signalling has emerged as a specific target in cancer and as a potential therapeutic target in efforts to curb tumor angiogenesis. The core Notch pathway contains a limited set of components: Notch ligands, Notch receptor and transcription factor CSL (CBF1/Suppressor of Hairless/LAG-1). Some components like furin, ADAM secretase, and MAML are not part of conveying the signal but are nevertheless crucial for allowing the signal to be transmitted from one step to next in the pathway. Briefly, Furin cleave the Notch receptor (which is synthesized as a single transmembrane receptor) into a heterodimeric notch receptor which is expressed on the cell surface of a “receptor-expressing” cells. Notch ligand on the cell surface of a “ligand-expressing” cells can activate the notch receptor after binding to it. This results in removal of the extracellular domain of Notch, which is then targeted for lysosomal degradation. The remaining portion of the receptor undergoes endocytosis and sequential cleavage by other components and is called Notch Intracellular domain (NICD). NICD then regulates the transcription of target genes after binding to DNA-binding protein CSL. There is no direct evidence of involvement of HS in this important developmental signaling pathway.

3.5 VEGF signaling

Vascular endothelial growth factor (VEGF) signaling is crucial for vascular development in physiological and pathological processes in both embryo and adult life. Earlier study reported that the signaling activity of VEGF is through binding to both the receptors and the cell surface HSPGs and depends on sulfation degree in HS. One important role of HS is to spatially restrict VEGF to create a concentration gradient that is essential for blood vessel branching.

The VEGF family consist of five homodimeric glycoproteins named as: VEGF-A, -B, -C, -D and placenta growth factor PLGF. Their complexity is enhanced further by splicing variants and thereby, the ability to bind VEGF receptors and HS. VEGFs act through three structurally related VEGF receptor tyrosine kinases, denoted VEGFR1 (Flt1), VEGFR2 (Flk1), and VEGFR3 (Flt4). The receptors show an overlapping but distinct expression patterns. However, VEGFR2 is more common in vascular endothelial cells and VEGFR1 in monocytes and macrophages, similarly VEGFR3 in lymphatic endothelial cells. Most biological functions of VEGF are mediated through VEGFR2. VEGFR2 activation after ligand binding can activate several downstream signaling pathways for example, activation of the mitogen-
activated protein kinase, protein kinase C and Akt pathways are all strongly involved in carrying out diverse cellular functions of VEGF, including proliferation, survival, the production of nitric oxide and angiogenesis 87.

Regarding specific roles in embryonic development, it has been found that VEGFR2−/− mice die at E8.5 from impaired development of hematopoietic and endothelial cells, a phenotype similar to that of VEGF-A−/− mouse 91-93.
4. Implications of heparanase in tumorigenesis

Hallmarks of cancer include several aspects; first cancer cells themselves de-
velop the properties of self-sufficiency in growth signals, insensitivity to anti-
growth signals and apoptosis. Secondly, tumor cells interact with their micro-
environment to stimulate the process of angiogenesis, and overcome their ex-
tracellular matrix (ECM) to move to distant sites in process of metastasis.
Conceptual progress in last decades has added two emerging hallmarks in the
list of all the above-mentioned hallmarks and heparanase is important player
in these contexts $^{94}$ (Figure 7).

![Figure 7: Emerging Hallmark of cancer: One involves the capability to modify, or
reprogram, cellular metabolism in order to most effectively support neoplastic pro-
liferation. The second allows cancer cells to evade immunological destruction, in
particular by T and B lymphocytes, macrophages, and natural killer cells. Modified
from $^{94}$.](image)
In contrast to limited knowledge about the role of heparanase in normal conditions, it has been consistently demonstrated that heparanase expression is enhanced in almost all types of cancers including ovarian, pancreatic, gastric, renal, head and neck, colon, brain, breast and liver carcinomas. This up-regulation is correlated with increased tumor size, angiogenesis, enhanced metastasis and poor prognosis. Heparanase expression is not only upregulated in tumor cells but it has been also found that non-tumor cells like immune cells (T, B cells, neutrophils, macrophages), endothelial cells and fibroblasts over-express heparanase upon activation by danger signal (Figure 8). Thus, heparanase expressed by tumor cells and by cells of microenvironment is the master regulator of the aggressive phenotype of cancer, through degradation of HSPGs and release of the growth factors from storage.

When heparanase is overexpressed, it leads to increased colonization of cancer cells in lung, liver and bone. By extensive degradation of HS heparanase destroys ECM integrity and facilitates tumor cell migration. According to the crystal structure of heparanase, the C-terminus of the protein forms a discrete domain, which has non-catalytic properties and is involved in heparanase-mediated signaling function that is distinct from its enzymatic activity.

4.1 Enzymatic actions

Heparanase cleavage of HS alters the signal transduction in various ways. First, heparanase modifies the HS structure, affecting the interaction between signaling molecules and their receptors; secondly, heparanase enhances syndecan shedding, especially syndecan-1; and thirdly heparanase regulates the expression of HSPG.

HS chain modification by heparanase results in increase of solubility of a variety of signaling molecules including VEGF and FGF2, Wnt, HGF and in this way, increase the interaction of these molecules with their receptors. For example, HS enhances FGF2-FGFR ternary complex formation. This effect is not only based on the total mass of HS but is also contributed by increased N- and O-sulfation of HS due to the action of heparanase. By shortening the HS chains on syndecans, heparanase increases access of sheddases to the syndecan 1 core protein and enhances the formation of complex between VEGFR2 and integrins. Because of these properties, heparanase is attributed to pro-tumorigenic processes (like angiogenesis, proliferation, apoptosis and metastasis). Another mechanism by which heparanase enhances tumor metastasis is by stimulation of exosome secretion. Exosomes communicate intercellularly in tumor microenvironment by releasing their RNA and protein cargo to recipient cells and affecting cell signaling, and by this way transforming the whole cell behavior.
4.2 Non-enzymatic actions

Heparanase also shows non-enzymatic action by enhancing signal transduction of Akt, Src, Erk, HGF, VEGF and other mitogenic signaling pathways, which in turn increases metastasis and angiogenesis\textsuperscript{38,100}.

Nuclear located heparanase has been shown to control the transcription of immune response genes by controlling histone H3 methylation\textsuperscript{46}.

![Figure 8: Heparanase affects tumor microenvironment by releasing growth factors, chemokines, cytokines from HS and by activating immune response.](image)

4.3 Heparanase and immunology

The role of heparanase in inflammatory setting is multidimensional as it is involved in both pro- and anti-inflammatory mechanisms, because of broad functional range of its substrate HS. Heparanase itself is proinflammatory because it stimulates the production of proinflammatory cytokines (IL6, TNF-alpha) mediated by MyD88 (a key TLR-4 adaptor molecule) and NF-KB pathway\textsuperscript{101}. 


One example of involvement of heparanase in inflammation is pancreatic ductal carcinoma. Overexpression of heparanase directs the protumorigenic behavior of tumor associated macrophages (TAM). First it results in increase infiltration of TAM and then TAM augmented the expression of several key cytokines like CCL-2, IL-10, VEGF, IL-6. TLR4 receptor on macrophages are known to involve in this mechanism 102,103, 38,58.

4.4 Heparanase inhibitors

Because of the uniqueness and overexpression in all types of cancer, heparanase is currently regarded as one important target in cancer therapy. The most studied heparanase inhibitors are HS mimetics that work by blocking the active site of the enzyme 38. The drawback with these inhibitors is that they are not specific in their action as they can bind other protein molecules. Four heparanase inhibitors are under preclinical and clinical evaluation. These are PI-88, Roneparstat, M402 and PG545 95,104,105,96.

Recently, another approach has been adopted for neutralizing heparanase activity, by monoclonal antibodies. For example, 9E8 (IgM) and H1023 (IgG) mAb have been found to not only inhibit enzyme activity but also diminish uptake of heparanase by cells and thus accumulation in lysosomes 106. Another way is to find natural inhibitors of heparanase like heparanase 2. A homolog of heparanase, heparanase 2 (Hpa 2), has been reported having strong binding affinity towards HS/heparin but lacks the catalytic activity, suggesting that the proteins are functionally distinct 107.
Aim

The aim of this study was to examine the effects of altered HS structure in two important aspects of mammalian biology i.e. tumor growth and development defects, with particular focus on HS- dependent cell signaling activities (TGF-β, BMP, Wnt, VEGF signaling).

Paper I:

Overexpression of heparanase attenuated TGF- β stimulated signaling in tumor cells.

Summary: The aim of the study was to see the effects of heparanase overexpression on HS structure and on TGF-β1 signaling. We choose Hpa-tg Fadu (head and neck cancer cell line) and MCF7 (breast cancer cell line) as preclinical studies showed the significance of heparanase in these cancers. Our earlier studies have revealed that heparanase overexpression in mice not only leads to fragmented HS chains but also altered HS structure. Increased sulfation of HS promotes FGF2 binding to its receptors.

We found that overexpression of heparanase through its enzymatic action attenuated the TGF-β1-stimulated Smad, Akt and Erk phosphorylation and resulted in a lower proliferation rate of Hpa-tg tumor cells as compared to Mock (tumor cells transfected with the vector only without Hpa) cells. This effect is associated with an increased sulfation degree of HS in the cells. Moreover, the rapid turnover of HS in the Hpa-tg cells may destabilize the interaction between TGF-β1 and its receptor, resulting in weak signaling activities. Our data suggest that molecular structure of HS on the cell surface plays a role in pluripotent activity of TGF-β1. There is one missing point in our studies that we have not studied the complex interaction of HS, ligand and growth factor complex in Mock and Hpa-tg cells. So, we cannot say for sure that GAG-structure is the main cause of this low signaling response.

Although TGF-β does bind to a number of proteoglycan species in vivo, namely the cell surface HS-containing proteoglycan, betaglycan (the type III TGF-β receptor), as well as various members of the family of small secreted chondroitin/dermatan sulfate (CS/DS) proteoglycans (i.e. decorin, biglycan, and fibromodulin), these associations are mediated principally,
if not solely, by protein-protein rather than protein-GAG interactions. Another study showed that although TGF-β 1 binds strongly to heparin and highly sulphated HS but the potentiation of TGF-β signalling is due to antagonism of the binding and inactivation of TGF-β 1 by alpha2-macroglobulin, rather than by modulation of growth-factor receptor interaction 112.

Another study proposed a model for HS modulation of TGF-β receptor complexes. Absence of HS cause the formation of complex in which TGF-β receptor II is more than TGF-β receptor I and it promotes signalling. While the presence of HS leads to degradation of signalling complex 67. So, we can speculate that more sulfation in HS may bind more strongly to TGF-β and interfere with receptors dynamics in the membrane, consequently affecting the downstream signalling response.

Paper II

**Heparin antagonizes cisplatin resistance of A2780 ovarian cancer cells by affecting the Wnt signaling pathway.**

**Summary:** Heparin is one of the closest mimics of HS and is a natural choice as heparanase inhibitor. We continued our research related to HS structure and signaling, so in this project we studied a modified heparin (LMWH/Tinzaprin) and its effects on Wnt signaling in ovarian cancer (A2780 cell line and A2780 cis cell line).

LMWH affects the Wnt signaling pathway that is of major importance for cisplatin resistance in A2780cis cells. LMWH normalizes this resistance to the level of A2780 cells. We cannot exclude if LMWH targets other processes in sensitizing the A2780 cis cells, but our finding is important indication that LMWH acts via the Wnt pathway for its action. It seems likely that LMWH, based on its GAG structure interferes with the HSPG activities at the cell surface, such as the GPC3 interaction with Wnt ligands and Frizzled. Consequently, A2780cis cells try to maintain the Wnt activity and to antagonize this inhibitory effect by upregulating GPC3 as an auto-regulatory loop. This hypothesis is further supported by the finding of increased HS chain length and total amount in the A2780cis cells, which may have contributed to stabilization of Wnt- receptor complex.
Paper III

Heparanase expression soils the tumor microenvironment for tumor growth by enhancing Notch signaling and by suppressing anti-tumor immune response.

Summary: Much of the impact of heparanase on tumor progression is related to its function in mediating tumor-host crosstalk, priming the tumor microenvironment to better support tumor growth and metastasis. Heparanase accomplishes this by degrading HS which regulates the abundance and location of heparin-binding growth factors thereby influencing multiple signaling pathways that control gene expression, syndecan shedding and cell behavior \(^{38}\).

We aim to investigate this effect of heparanase in Lung cancer (Lewis lung carcinoma, LLC2 model) by using transgenic mice overexpressing human heparanase (Hpa-tg).

We found higher tumor growth in Hpa-tg mice as compared to wild type mice (WT), when LLC2 cells were injected in the right flank of mice. Our data highlight the importance of immune cells (especially T- and B-cells) in cancer cell progression. Heparanase hijack the tumor microenvironment especially immune cells for aggressive tumor growth and metastasis by increasing the proportion of tumor friendly B-cells and T-cells (IL35\(^+\)) and reducing the percentage of proinflammatory or tumor inhibitory Interferon gamma (IFN-Y\(^+\)) T-cells. Moreover, we observed interesting information regarding T-cell number and type in immune organs; for example, the percentage of PD-1\(^+\) and CTLA-4\(^+\) (molecules on surface of T cells that can serve as a brake which can prevent T cells from killing cancer cells) were higher in spleen and lymph node of tumor bearing Hpa-tg mice. We can speculate that because of these immune check point inhibitors, tumor growth is higher in Hpa-tg mice.

Second important finding is the higher Notch signaling in Hpa-tg tumor indicating the interaction of HS in this important signaling pathway. There is no direct evidence of HS and heparanase role in notch signaling. One study shows the role of drosophila HS 3-O sulfotransferase-b (Hs3st-B), which catalyzes 3-O sulfation, in notch signaling, indicating the importance of 3-O sulfation in HS in ligand binding and signaling \(^{113}\). But in another study, it has been shown that notch pathway is not affected in Hs3st-B null mutant \(^{114}\). There are several heparin binding proteins that have been shown to effect notch signaling so it’s possible that heparanase may affect notch signaling through these proteins.
Paper IV

Upregulated BMP-Smad signaling activity in the glucuronyl C5-epimerase knock out MEF cells.

Summary: We took another approach to study the effect of HS structure alternation on cell signaling by using Hsepi−/− mice. Hsepi is an enzyme responsible for the transformation of glucuronic acid to iduronic acid in the process of heparan sulfate biosynthesis. Hence, the deletion of the gene would result in the malformation of heparan sulfate (HS), leading to the improper functions of growth factors and morphogens during embryogenesis. Since BMP signaling plays an important role in bone formation so first we studied BMP signaling in MEF cells isolated from Hsepi−/− embryos. We found substantially elevated levels of BMPs and P-smad1/5/8 and reduced levels of Gremlin1 in Hsepi−/− MEF cells as compared to Hsepi+/+ MEF cells.

To confirm the correlation of Hsepi expression and BMP signaling, we have re-introduced the Hsepi gene into the KO cells. Re-expression of the gene in the KO cells reverted the level of p-smad 1/5/8 phosphorylation. One dramatically reduced gene is Gremlin 1, which most likely is responsible, at least partly, for the defect of kidney and bone development. As it was recently proposed that there is a negative cross-talk between slits and BMP2-gremlin signaling115, we examined Slit2 expression. The result shows that the KO cells had a higher level of Slit2, which presumably suppressed Gremlin expression. While the Slit2 receptor Robo2 was slightly lower in the KO, which maybe a negative feedback mechanism.

As Hsepi is a highly specific enzyme involved in HS biosynthesis in the Golgi, and so far, there is no report on its presence in the nucleus. Thus, it is most likely not a direct modulation activity of the enzyme at the genome level. Our hypothesis is that HS-associated signaling activity, including FGF, BMP and Hh, may collectively affect the gene expression, therefore leading to the multiple defects in the KO mice.

Paper V

Glucuronyl C5-epimerase is crucial for epithelial cell maturation during embryonic lung development.

Summary: In parallel with BMP signaling studies, we investigated lung developmental defects in Hsepi−/− mice. There is no difference in lung development in Hsepi+/+ and Hsepi−/− mice until E16.5 but after that Hsepi−/− lungs fail to develop saccular structure of alveoli. We found that VEGF and BMP signaling are responsible for the delayed differentiation of alveolar epithelium in the lungs of Hsepi−/− mice. This highlight the importance of Hsepi and fine
structure of HS in controlling signaling for lung development like VEGF and BMP pathways.

From Ndst⁻/⁻ studies we could speculate that the reason for poor lung development in Hsepi⁻/⁻ mice could be because of dependency of differentiation of type I and type II pneumocytes on sulfation degree of HS. Because of lack of IdoA in Hsepi⁻/⁻ mice, upregulation of the overall sulfation occurs. Also, the disturbance in secretion and level of surfactant are signs of immature lungs.

It is well established that lung morphogenesis is guided by interactions of multiple morphogen signaling pathways in epithelium and mesenchyme, however, their regulation by cell surface HSPGs is still largely unknown. HS has been shown to be critical for maintaining sonic hedgehog (SHH) signaling for branching morphogenesis in Lung. In short, HS in ECM can selectively bind to growth factors (GF) and as a consequence, help determine the binding specificity between ligands and receptors and the signaling direction of epithelial-mesenchymal cross-talk during development. For example, specific HS structure selectively bind FGF 10 derived from the mesenchyme but not FGF8 derived from the ectodermal cells during limb development. So, HS can dictate GF selectivity in a developing organ. The resulting function of a GF during branching morphogenesis is dependent on the context in which it interacts with HS.
Concluding remarks and Future perspectives

This study has brought out some important aspects of HS roles in signaling in cancer growth and during embryonic development. Like normal cells during embryogenesis, tumor cells undergo rapid growth, adhere to other cells and cell matrices and invade tissues. So, it was interesting to see how both processes are affected by structure of HS, as HS act as molecular swatches in all these processes. Heparanase overexpression and Hsepi knock out, both affected the HS fine structure and consequently its biological function. Growth factors and cytokines cannot interact properly with disturbed structure of HS, resulting in abnormal functions of the growth factors and change in cell behavior.

There are certain questions that need to be addressed in the future in order to get full understanding of the mechanisms of HS functions.

The message from my first project is that heparanase over-expressing cells are less responsive to TGF-β, likely due to structural alterations in HS and thereby in its ability to function as a co-receptor. Since this effect involves heparanase enzymatic activity, one may examine the effect of heparanase inhibition (i.e., SST, PG545, or antibody 1453). And that could pave the way towards treatment of hypopharyngeal and breast carcinoma.

We tried to restore the co-receptor function by the addition of heparin but it does not restore the signaling to the same level as in wild type cells, probably because of different sulfation degree in heparin and HS. It could also be because that heparin also has an effect on many other pathways. On the other hand, knock out of heparanase has reverted the signaling to the same level as in wild type cells (unpublished data).

Since TGF-β is a major contributor to EMT (epithelial mesenchymal transition), it would give more information if we look into downstream effects of TGF-β on expression of relevant genes (i.e., smooth muscle actin, vimentin, Snail, Twist) in mock vs. Hpa cells. Next question is, does heparanase affect the expression of TGF-β? Up-regulation of TGF-β in Hpa-tg mice was observed in response to stress (i.e., acute kidney injury) and heparanase is needed for TGF-β overexpression in response to profibrotic factor like FGF2 and lack of heparanase delays TGF-β induced EMT in the tubular cells.

Regarding lung cancer in Hpa-tg mice, the main finding is that tumor growth and metastasis in lung is more advanced in Hpa-tg mice as compared to WT mice. Moreover, the proportions of IL-35+ T cells were increased in Hpa-tg
mice. Notch expression is higher in Hpa-tg mice tumor. We need to further explore the involvement of immune cells in tumor growth for example by taking away these T-cells and see the effect on tumor growth or by using antibodies against the surface markers (PD1 and CTLA4) on T-cells. Furthermore, recent study indicates the role of interleukin-35(IL-35) secreted from tumor associated macrophages(TAM) in tumor cell plasticity. It gives us hints to further explore the role of innate immune cells in primary and metastatic tumors.

Since we hypothesize that tumor microenvironment and heparanase enzymatic action are involved in all these perturbations, next step is to use heparanase inhibitors to reverse the effect. Also, we can use LLC2 cells overexpressing heparanase to inoculate the animals to see the effect of heparanase in tumor cells rather than heparanase from the microenvironment. As it has been reported that heparanase cooperates with Ras to promote mammary tumor progression when Hpa-tg MCF10AT1 cells were injected into mice. So, injecting hpa-tg LLC2 cells will help us to understand the early stages of tumor growth.

Further questions raised by our finding is to see how HS can interact with Notch ligands and receptors as we have found higher Notch signaling in Hpa-tg tumor. We will try to explore this relationship by doing binding assays or using Hpa-KO and or Notch-KO cell model.

Regarding HS/Hsepi role in BMP signaling, the main finding is the endogenous high BMP signaling because of high expression of ligands and low expression of Gremlin in Hsepi−/− MEF cells. Next step is to find the mechanism behind the Hsepi regulation of Gremlin gene. We tried to differentiate MEF cells towards osteoblast in order to know the mechanism behind the intensified calcification in cranium region of Hsepi−/− mice. But MEF cells start dying after day 7 of differentiation. So, next we are going to try to differentiate embryonic stem cells (ESC) from Hsepi−/− mice towards osteoblasts.

The defects that we see in the Hsepi−/− and 2OST−/− mice may be dependent on each other. And the phenotypic effects that we see may not be just because of one enzyme knock out. The lack of Hsepi may disturb other enzymes downstream in the biosynthetic pathway that leads to change in sulfation in the HS domain and consequently affecting the HS-cytokine interaction. The timing and proper availability of the growth factors and morphogen gradients are of utmost importance for normal development of organism. Severely perturbed HS generated by the Hsepi−/− mutant could satisfy at least some essential HS-dependant signaling steps in cerebral patterning and vasculogenesis but question is to what extent this unexpected functional potential relies on the “compensatory” upregulation of N- and 6-O sulfation observed for both Hsepi−/− and 2-OST−/− mutant HS.
In a broader perspective, we aim to analyze the whole transcriptome (or proteome) of Hsepi\textsuperscript{-/-} MEF cells in order to get the overall view of all the genes that are upregulated or downregulated because of knock out of Hsepi and to identify specific signaling and regulatory pathways. This will help us also to understand why some organ development (like kidney, lung and skull) is defective in this mouse but other organs develop normally. Why some organ development is dependent on HS fine structure and while others not. Conditional KO studies of Hsepi would also give valuable insights in HS function from specific organ.

We will also try to see other functional studies like apoptosis in these mouse model because apoptosis plays important role in embryogenesis\textsuperscript{124}. 

Present work has been carried out at the department of Medical Biochemistry and Microbiology at Uppsala University, Sweden. It is supported by scholarship from Erasmus Mundus program “Experts4Asia” and by grants from Swedish Cancer research council.

I would like to express my sincere gratitude to Erasmus Mundus “Experts4Asia” coordination team in University of Göttingen to give me opportunity to fulfill my dream of getting higher education in such a prestigious university of world. Secondly, my warmest thanks goes to my supervisor Jin-Ping Li for her continuous support and guidance, for her patience in training me and being there for me in all the ups and downs of this PhD adventure. Thanks for inspiring me to learn more, do more and become more. You have always given your heart to ensure my continued success. Thank you.

Besides my advisor, I would like to thank my co-supervisor Aristidis Moustakas for his insightful comments, advice and support for experiments. You always made time for me and tried to help and come up with ideas. thank you for being so kind and accessible.

Also, my huge and biggest thanks go to:

JianPing Fang, for giving me helping hand in all laboratory practices. You are a fun and amazing person with all that witty brainy knowledge and expertise. I wish you best of luck for your company.

Andreas Digre, thank you for being such a nice and wonderful sincere friend, helping me with all my problems, sharing discussion, helping me in conducting experiments. You are a very good teacher/adviser. You have helped me a lot in understanding and growing up in Swedish culture. It has been amazing fun to talk with you all the cultural and religious beliefs and differences. Your deep understanding of human behavior is really inspiring. You are very nice, kind and big fan of believing in singularity of human kind. Keep it up good work.

To my collaborators:

Ganlin Zhang: thank you Ganlin for helping me in experiments. It has been fun to work with you in long animal (mice) experiments and sharing our sad feelings together for this small little creature when we treat them with care.

Kailash Singh: thanks for sharing expertise of FACS and helping me understand the complex immunology.

Fabian Baltes: thanks for coming to lab with exciting project.
And to my previous and current Glycobiology lab members:

Xin Liu, Andrew Hamilton, Ramesh Babu Namburi, Tabea Dierker, Honglian Li thank you all for being awesome friends and for your suggestions and help. Especially, to Andrew Hamilton: for being my examiner in my half time and giving me really honest comments. And also for your help in experiments in lab and now your help in reading my thesis. Thanks for being nice friend and always ready to help. To Tabea Dierker and To Paul O Callaghan: for reading my work in your tight busy schedules. Thanks, Tabea also for nice and fun companies on conferences.

To Ulf Lindhal, Lena Kjellen, Dorothe Spillman, Xiao Zhang for being wonderful teachers in IMBIM and for their suggestions and help. Especially to Staffan Johansson: thanks for always giving me time whenever I want to talk to you. You are blessing for international students coming to Sweden. We all love you. And I hope you will keep taking care of them.

To Sher Agha Bakhtiar and Viktor Jansson for coming short terms as witty and sharp läkerprogramm students and help me developing teaching skills.

My sincere thanks also go to my friends outside lab:

Alex Danko: thanks for being my Swedish teacher and really helping me to start learning Swedish language and of course, for wonderful fun traveling trips in Europe. Thanks for being a nice friend and teacher.

Simon Löfgren: thanks Simon for teaching me biking and inviting me to so many fun birthday parties and long hours of discussions of understanding Swedish culture. And to the whole flogsta Gang: Hira, Osama, Kristel, Laura, Madle, Bilal, Gufran, Vira and Dima thanks for fun companionship.

John and Lemarc: Thanks for inspiring me with your positive psychology, mindset and personalities.

Christina Herder: thanks for mentoring me outside lab. It has been wonderful time with you although very short.

Sanea and Zauq: thanks for being with me in happy and sad times and for bringing fun and laughter whenever I am sad and disappointed. You are the besties of besties.

Deepesh Gupta, Siamak, Ramesh, Tianyi Song, Argyris Spyrou and Parisa Missaghian: when I was thinking to write a sentence about you guys, it brings me smile and remembrance of happy moments. Thanks all of you for your kind companies and help. Especially thanks to, Deepesh for bringing so much fun and laughter whenever we talked and for listening all my Ifs and Buts of research. Thanks, Parisa for your free medical consultation and being a kind friend (with big warm heart as you said).

Dan Nörback and Gunilla: thanks for your lovely and kind company, giving me feeling of being part of your family and enthusiasm about my research.
I would like to pay my heartiest gratitude and love for my dear family:

My parents, Barkat Ali Shad and Bushra Khanum for supporting me spiritually throughout my stay here in Sweden and also in my life in general. No matter how far I came, you are always with me in my heart and in my thoughts. Thanks for your continuous prayers for me, believing in me and loving me so much. Mom (Ammi Jan), you are my Angel. My dearest Father (Baba jan) thanks for inspiring me with your love for education and for your persistent nature for learning more and more and achieving higher and higher. I always remember and cherish those moments/days when you gave me hugs, kiss and gifts for coming first in my class in school, college and university and trusting and caring me even more at the time of my failures and insecurities. That was the real source of inspiration for me that I am completing PhD now. Your dream has come true to make one of us doctor.

My Siblings, cousins and brother-in-laws, Amjad, Misbah, Yahya, Faiza, Farzana, Saima, Tahir, Anas Mujahid, Ahmed mujahid, Shahzad Ahmed, for caring/pampering me all the time for being youngest in family and for life-long companionship. My nieces and nephews: Ali, Mariam, Hajra, Zaman, Rafi, Hadia, Ammar and Abdullah. You are sparkling stars and I love all of you. Biggest thanks to my twin brother Tahir Rasool: I have no words to express my sincerest and deepest gratitude to you. Thanks for being with me and caring for me from childhood until now. We will be remembered always together no matter which corner of the world we will live. You are the biggest blessing and gift of God for me in this world.
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-363258