Newly uncovered novel properties of heparanase in the heart and pancreas

by

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Abstract

Heparanase, a protein with enzymatic and non-enzymatic properties, contributes towards disease progression and prevention. We have reported that high glucose (HG) stimulates active heparanase secretion from endothelial cells (EC) to cleave cardiomyocyte heparan sulfate and release bound lipoprotein lipase (LPL) for transfer to the vascular lumen. We examined whether heparanase also has a function to release cardiomyocyte vascular endothelial growth factor (VEGF), and whether this growth factor influences cardiomyocyte fatty acid (FA) delivery. HG promoted both latent and active heparanase secretion into EC conditioned medium, an effective stimulus for releasing cardiomyocyte VEGF. Intriguingly, latent heparanase was more efficient than active heparanase in releasing VEGF from a cell surface pool. VEGF augmented cardiomyocyte intracellular calcium, AMPK phosphorylation and heparin-releasable LPL. Our data suggest that the heparanase-LPL-VEGF axis amplifies FA delivery, an adaptive mechanism that is geared to overcome the loss of glucose consumption by the diabetic heart. If prolonged, the resultant lipotoxicity could lead to cardiovascular disease in humans. Therefore, we globally overexpressed heparanase and evaluated whether excessive heparanase would exacerbate the development of diabetic cardiomyopathy. The transgenic mice (hep-tg) showed normal life span and fertility, with improved glucose homeostasis. Heparanase overexpression was associated with enhanced GSIS and hyperglucagonemia, in addition to changes in islet composition and structure. Strikingly, the pancreatic islet transcriptome was greatly altered in hep-tg mice with over 2000 genes differentially expressed. The upregulated genes were enriched for diverse functions including cell death regulation, extracellular matrix component synthesis, and pancreatic hormone
production. The downregulated genes were tightly linked to regulation of the cell cycle. In response to multiple low-dose STZ, hep-tg animals developed less severe hyperglycemia compared to WT, an effect likely related to their remaining beta cells that were more functionally efficient. In animals given a single high dose of STZ, causing severe hyperglycemia related to the catastrophic loss of insulin, hep-tg mice continued to have significantly lower blood glucose. In these mice, protective pathways were uncovered for managing hyperglycemia and include augmentation of FGF21 and GLP-1. Overall, this thesis uncovers opportunities to utilize both enzymatic and non-enzymatic properties of heparanase in managing diabetes and its complications.
Preface

This thesis is written by D. Zhang and reviewed by Dr. Rodrigues. All the experiments in this thesis were designed and conceived by D. Zhang under the supervision of Dr. Rodrigues.

Studies in chapter 3 has been previously published (D. Zhang, A. Wan, AP. Chiu, Y. Wang, F. Wang, K. Neumaier, N Lal, MJ. Bround, JD. Johnson, I. Vlodavsky, B. Rodrigues. 2013. Hyperglycemia-induced secretion of endothelial heparanase stimulates a vascular endothelial growth factor autocrine network in cardiomyocytes that promotes recruitment of lipoprotein lipase. Arterioscler Thromb Vasc Biol. 33(12): 2830-8). D. Zhang designed all the experiments and wrote the manuscript with Dr. Rodrigues. A. Wan, AP. Chiu, Y. Wang, F. Wang, K. Neumaier, N Lal and Dr. Johnson participated in data analysis and manuscript editing. MJ. Bround performed the calcium detection in cardiomyocytes and Dr. Vlodavsky provided the recombinant heparanase.

Data in chapter 4 is currently in press (D. Zhang, F. Wang, N. Lal, AP. Chiu, A. Wan, J. Jia, D. Bierende, S. Flibotte, S. Sinha, A. Asadi, X. Hu, F. Taghizadeh, T. Pulinilkunnil, C. Nislow, I. Vlodavsky, JD. Johnson, TJ. Kieffer, B. Hussein and B. Rodrigues. 2016. Heparanase overexpression induces glucagon resistance and protects animals from chemically-induced diabetes. Diabetes). F. Wang, N. Lal, AP. Chiu, A. Wan, J. Jia, D. Bierende, S. and Dr. Nislow contributed in editing the manuscript. S. Flibotte and S. Sinha performed the RNAseq of pancreatic islets and analyzed the data. X. Hu and F. Taghizadeh isolated the mouse islets. Dr. Vlodavsky provided the heparanase transgenic mice. Dr. Johnson and Dr. Kieffer assisted in conceiving some of the experiments. B.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bCAECs</td>
<td>Bovine coronary artery endothelial cells</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Ctsl</td>
<td>Cathepsin L</td>
</tr>
<tr>
<td>CPT</td>
<td>Carnitine palmitoyltransferase</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECCM</td>
<td>Endothelial cell Conditioned medium</td>
</tr>
<tr>
<td>F-1,6-Pase</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GCG</td>
<td>Glucagon</td>
</tr>
<tr>
<td>GCGR</td>
<td>Glucagon receptor</td>
</tr>
<tr>
<td>GK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>Hep</td>
<td>Heparanase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycans</td>
</tr>
<tr>
<td>Hs3st</td>
<td>Heparan sulfate 3-O-sulfotransferases</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>M6PR</td>
<td>Mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Proprotein convertase</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK</td>
<td>Pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor α</td>
</tr>
<tr>
<td>SLC2A1</td>
<td>Solute carrier family 2, facilitated glucose transporter member 1</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Xyl</td>
<td>Xylose</td>
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</table>
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Chapter 1: Introduction

1.1 Heparan Sulfate Proteoglycan

1.1.1 Location and Distribution

Heparan sulfate proteoglycans (HSPG) are glycoproteins that are mainly found on the plasma membrane and in extracellular matrix (ECM) (1), with certain subtypes being discovered in intracellular space and nucleus. Syndecan-1, which was the first identified HSPG, is a type-I trans membrane protein that connects ECM components to epithelial cells during development (2; 3). The name “syndecan” comes from the Greek word “syndein,” which means to bind together, because of its function of linking the ECM to the cytoskeleton. HSPGs share a common tree-shape structure of a core protein with one or more covalently attached heparan sulfate (HS) side chains (4). HS is a highly negatively charged sulfated glycosaminoglycan (GAG) polysaccharide, with the capability of capturing a great number of positively charged proteins (with more than 100 molecules identified so far) (5). Normally, HSPG contains a few HS side-chains; in some cases though, proteoglycan can function even without a GAG chain. Depending on its location, there are three types of HSPGs: i) Cell surface HSPGs including trans membrane HSPGs, such as syndecans and glycosylphosphatidylinositol (GPI)-anchored proteoglycans (glypicans), which are anchored in plasma membrane (PM) or lipid rafts, interacting with membrane surface proteins and cytoskeleton proteins (6). ii) Secreted extracellular matrix HSPGs (agrin, perlecan, type XVIII collagen), present in tissues and circulating in blood, which can function as ligands to initiate signaling cascades or act as ligand modulators (7-9). iii) Secretory vesicle proteoglycan, mainly serglycin, which is critical for vesicle formation and secretion (10). In addition, the nuclear presence of multiple HSPGs has been identified recently, which extended the scope of HSPG’s
functions (11; 12). Collectively, HSPG’s ubiquitous presence defines their profound roles at the cellular, tissue and organismal levels. Additionally, besides its function to provide cellular and tissue structural support, most of HSPG’s effects are delivered through HS.

### 1.1.2 Heparan Sulfate

HS is a polysaccharide that is made up of repeating N-acetyl glucosamine (GlcNAc)-d-glucuronic acid (GlcA) disaccharide units, which are amongst the most negatively charged biopolymers in nature. HS has no template for its synthesis; therefore the length and number of chains varies greatly, resulting in enormous functional diversity in numerous physiological and pathological processes.

The synthesis of HS starts in the endoplasmic reticulum (ER), where the chains are initiated by attaching xylose (Xyl) to serine residues in HSPG core proteins. In the Golgi apparatus, two galactose (Gal) residues and one GlcA is attached to Xyl to complete the formation of a core protein linkage tetrasaccharide (GlcA-Gal-Gal-Xyl). Elongation of the chain is assisted by a number of exostoses enzymes in a template independent manner, resulting in huge heterogeneity in their sequences (13). Furthermore, to obtain a fully functional product, HS has to undergo several modifications including N-sulfation/N-acetylation on glucosamine residues, C-5 epimerization on GlcA and O-sulfation at different sites. The outcome of these reactions is an extensive structural heterogeneity, providing specific sites for protein binding and HS cleavage. In mammals, there could be 26 enzymes involved in HS formation and modification. Of particular interest, 3-O sulfation of HS is achieved by the largest enzymatic family consisting of 7 heparan sulfate 3-O-sulfotransferases (Hs3st). The 7 members of each subgroup may create unique 3-O-sulfated sequences at C3 position, generating distinct biological properties of HS. This modification of HS is mainly responsible for antithrombin binding.
and Herpes simplex virus glycoprotein D infection (14), in addition to providing selectivity for a number of fibroblast growth factors (FGF) (15) and their receptors (16). The patterns of modification and organization of the residues are generally determined by the cell type in which HS is expressed, rather than by the nature of the core protein it is attached to. Thus, the overall composition of HS on different core proteins expressed by the same cell is almost consistent, but great variation occurs between cell types (17).

HSPGs bind to macromolecules (growth factors, enzymes, protease inhibitors, mitogens, extracellular proteins etc.) through HS. The heparin-binding sites of the macromolecules are usually positively charged amino acids such as arginine and lysine, which are structurally located on the external surface or in shallow grooves of the proteins (18-20). The spacing between the residues and the proximity of the spatial distance of the residues determine the binding capability of the proteins (20). Cations that are released from GAGs generate free energy for electrostatic interactions, which is the dominant force for the attachment. In addition, hydrogen bonding, van derWaal interactions, and hydrophobic effects also contribute to the interactions (20; 21). HS and protein interaction is clearly crucial for cellular activity and physiology; therefore, this relationship is utilized extensively as a therapeutic target for diseases.

1.1.3 Physiological and Pathological Functions

Given HSPGs’ universal presence, they are broadly involved in intra- and inter- cellular activities. The fundamental function of HSPGs occurs in the extracellular space. In this regard, its members (e.g. perlecan, agrin, and collagen XVIII) collaborate with other matrix components such as laminin to support basement membrane structure and to provide a matrix for cell-cell interaction and migration (22; 23). Secondly, located on the cell surface, HSPGs can bind cytokines, chemokines, growth factors, and morphogens
through HS (24). These interactions provide a reservoir for the regulatory factors that can be liberated by selective degradation of the HS chains (25). This allows for a rapid responsive source of these factors, rather than a delayed release from an intracellular pool (26). The extracellular pool of molecules also facilitates the formation of morphogen gradients that is essential for regulating cellular activities during development (27; 28). On the endothelial cell (EC) surface, HSPG can act as receptors for proteases like antithrombins and its inhibitors, regulating their spatial distribution and activity (29). In addition, endocytosis for macromolecules like apolipoprotein E (ApoE) is mediated by cell surface HSPGs (30). Finally, HSPGs on plasma membrane can directly initiate signaling cascades by cooperating with integrins and other cell surface receptors (31). One established model is the transactivation of VEGFR2 by HSPGs on the smooth-muscle cells that leads to enhanced signal transduction in ECs by facilitating the formation of receptor–ligand complexes and trapping the active VEGFR2 signaling complex on ECs (32).

Equally important but less noticed functions of HSPG are their activities in the intracellular spaces, including in cytosol and nucleus. HSPGs that are found in secretory vesicles, most notably serglycin and syndecan-1 (33), assist in packaging granular contents, maintaining proteases activities, and regulating various biological activities after secretion. In the nucleus, the first discovery of HS dates back to 1974 (34), when the mucopolysaccharide was found to localize in the template section of chromatin and augment RNA synthesis (35). Then it was reported that mucopolysaccharides stimulate transcription by making new RNA polymerase binding sites available on the chromatin (36). In addition, it was also found that the GAGs might interact with histone proteins or its regulatory enzymes (37). The mechanism by which HSPG enters into the nucleus was
deciphered later. HSPG’s nuclear entry normally requires a nuclear localization signal (NLS) that is positioned upstream of HS attachment sites (38). The NLS of glypican is blocked by HS chain and removal of this inhibitory HS exposes the NLS that mediates nuclear entry of HSPGs in a tubulin dependent manner (39). In addition, matrix protein such as collagen and fibronectin can enhance nuclear entry of cell surface HSPG involving protein kinase C (PKC) mediated signaling (11). It has also been reported that FGF2 can mediate nuclear translocation of HSPGs that is dependent upon dephosphorylation of the protein core (40).

The nuclear presence of HS and HSPGs revealed novel regulatory functions of these “non-traditional nuclear proteins” in cell proliferation (41), transcription (42) and nuclear cargo transportation (40). Similar to their roles in endocytosis, cytosol HSPGs also function as receptors/vehicles for intracellular delivery of genes and macromolecules into nucleus (43). FGF2 is one of the macromolecules that escape lysosomal degradation by being hidden in the nucleus (44). The nuclear FGF2 and HS not only co-localize but also seem to regulate cell-cycle progression of various cells in a coordinated and dose-dependent manner (45). Regarding the gene expression regulation, HS is capable of affecting the interaction of transcription factors such as AP-1, SP-1, ETS-1 and nuclear factor κB (NF-κB), with their consensus oligonucleotide elements (42; 46). The DNA binding domains of these transcription factors contain sequences similar to proteins that exhibit high affinity to heparin. This inhibition of topoisomerase-I activity by HS is dose-dependent and its efficacy depends upon the source of GAG (47). The nuclear functions of HSPGs broaden the additional regulatory mechanism of gene expression and provide valuable information for developing treatments for diseases like cancer. The overall HSPG cellular function in the heart is described in Figure 1.1. Heparanase, as a
specific enzyme that cleaves HS, is such a target in cancer research due to its close relationship with intra and inter cellular HSPGs functions (48).

![Diagram](image)

**Figure 1.1** HSPG’s cellular functions in the heart include 1) Cell surface uptake of heparanase, 2) cell surface co-receptors, 3) bind functional enzymes such as LPL at the cell surface, 4) function as a barrier at the cell surface and mediate cell adhesion to ECM, 5) cell surface receptors that can trigger downstream signaling such as Src, 6) endocytosis of other proteins such as ApoE present on circulating lipoproteins, 7) sequester growth factors and morphogens in ECM, and 8) regulation of nuclear activities.

1.2 Heparanase

1.2.1 Synthesis and Processing

Heparanase is an endo-β-D-glucuronidase that belongs to the glycoside hydrolase 79 family (49). It is ubiquitously expressed in most mammalian cells, with platelets, activated immune cells and endothelial cells having the highest expression (50). So far only one gene with heparanase-like catalytic activity has been identified in mammals (51). Regulation of its expression has been studied intensively in cancer, which is shortlisted as a treatment target (48). So far multiple mechanisms in regulating its
expression have been revealed, which include demethylation of its gene promoter (52), transcriptional activation of two E26 transformation-specific binding sites by GABP, and transcription factor Sp1 and Sp3 binding to their respective receptors (53), which altogether help in increasing its promoter activity. Hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) have also been reported to induce heparanase expression through the PI3K-Akt pathway (54). As induction of heparanase occurs in pathological conditions like tumor cells, activated immune cells (55) and diabetic pancreatic islet cells (56), it has been closely linked to disease initiation and progression. However, additional functions of heparanase have been discovered recently, leading to a broader understanding of this protein, particularly its non-enzymatic property.

Regarding heparanase synthesis and activation, this protein is initially translated as a preproenzyme containing a signal sequence spanning Met1–Ala35. Cleavage of this signal sequence by signal peptidase leaves an inactive 65-kDa proenzyme, which must undergo further processing for activity. Interestingly, the proenzyme has to be secreted out of the cell as the first step of activation, followed by a re-uptake via cell surface HSPG (57) or other molecules such as mannose-6-phosphate receptor (M6PR) or lipoprotein receptor-related protein (LRP) (58; 59). Packaged in endosome, latent heparanase is finally activated in the lysosome by Cathepsin L (60). Proteolytic removal of a linker peptide in the proenzyme generates a N-terminal 8-kDa subunit and a C-terminal 50-kDa subunit, which remain associated as a non-covalent heterodimer in mature active heparanase (HepA) (61). Upon activation, HepA could reach many destinations depending on the stimulating signals. Soluble HepA exhibits maximal endoglycosidase activity between pH 5.0 and 6.0, and is inactivated at pH greater than 8.0 (62). Therefore extracellular heparanase remains moderately active at pH 6.7 (63), but
the non-vascularized core of tumor masses might provide the acidic environment that is
required for heparanase degradation of ECM.

1.2.2 Heparanase Functions

1.2.2.1 Enzymatic Activity of Heparanase

The only activity of heparanase is to cleave HS at D-glucuronic acid residues, an action
associated with extracellular matrix disruption and release of cell surface-bound
molecules (64). Due to the success of human heparanase crystallization, the biochemical
details of binding and cleaving HS by heparanase are clearly elucidated (49). Along the
whole process of initial touching, binding, structural distortion and final cleavage, various
sulfation on HS chain plays an irreplaceable role. Not only does sulfation serve as a
molecular signal that directs the enzyme to strictly cleave glycan sites, but N-sulfate and
6-O-sulfate also act as mechanistic handles for heparanase to enter into anomeric center
of the –1 residue. Furthermore, the 6-O-sulfate and N-sulfate may also stabilize the
heparanase-bound trisaccharide through electrostatic interactions with basic residues in
the active site cleft. Once bound, heparanase induced a distortion of HS chain, which
allows heparanase access to the cleavage site (49).

1.2.2.2 Non-enzymatic Activity of Heparanase

Recently, new properties in addition to its enzymatic activity have drawn attention to
heparanase. The latent protein is secreted physiologically, and also in response to
hyperglycemia. The fact that this enzyme has to undergo secretion and then re-uptake
would be a waste for the usage of cell resources, unless the latent enzyme also has a
functional role. Heparanase contains a heparin-binding site that makes it have a high
affinity to ECM (65). The consequence of this function is to enhance cell mobilization
during embryonic development (66), neovascularization (67) and cancer metastasis (68).
Additional cell surface function of heparanase is that in tumor cells, cell surface syndecan 1 and 4 could be clustered by latent heparanase, resulting in recruitment and activation of PKC and Rac1 (69). Moreover, by binding to cell surface molecules, this latent enzyme can directly trigger numerous signaling events including Erk1/2 via insulin receptor (70), PI3K-AKT and RhoA via integrin (71). In the cell cytosol, one key cellular activity that heparanase is involved in is stimulating the exosomal secretion of syntenin-1, syndecan and CD63, in a concentration-dependent manner (33). In endocrine cells, this property of heparanase could enhance the secreting process such as in insulin secreting beta cells.

1.2.3 Heparanase in the Nucleus

Though heparanase is highly active on the cell surface, it is also detected in the nucleus (72), probably by a chaperon-facilitated mechanism. It is generally thought that heat shock protein 90 (Hsp90) is the molecule that facilitates its cytosol to nucleus shuttling (73). The existence of heparanase in the nucleus provides an insight on this protein’s regulatory effect on gene expression, directly or indirectly. The decrease of nuclear syndecan-1 in heparanase overexpressing myeloma cells is concomitant with a simultaneous increase of histone acetyl-transferase (HAT) activity (74), in which the inhibitory effect on HAT is exerted through HS (37). Removal of the inhibition on HAT by elevated heparanase will in turn enhance the transcription of a number of proteins such as MMP-9, VEGF, HGF and RANKL, most of which are contributing factors in tumor invasion(75; 76). A more direct effect of heparanase on gene expression manipulation is through histone H3, which is phosphorylated by heparanase treatment (77). In addition, nuclear heparanase appears to regulate histone 3 lysine 4 methylation by influencing the recruitment of demethylases to transcriptionally active genes (78). In active immune cells, heparanase is bound to chromatin and co-localizes with RNA polymerase II,
leading to a significant influence on a number of immune-related target genes (78). Collectively, heparanase regulates gene expression in a cell specific and environment specific manner.

1.3 Glucose Metabolism

Glucose is a key substrate for energy production to maintain the function of an organism, as well as for lipid and protein synthesis. Except a few organs that require special resources for ATP production, most of the organs in mammals utilize glucose, whereas the brain and red blood cells only use glucose for energy (79). Glucose is mainly provided by food and the surplus is stored as glycogen in the liver (80). When glucose level in the blood is low, the endogenous sources of glucose are from liver through glycogenolysis and gluconeogenesis (80). The signals that regulate glucose metabolism in the liver come from other organs, mainly pancreas, which keeps the circulating glucose concentration at a stable level, named normoglycemia. Other organs that coordinate glucose homeostasis besides the liver and pancreas include skeletal muscle and adipose tissue. Of these, pancreas is the main digestive organ, providing numerous types of hormones from its endocrine sections to regulate whole body metabolism.

1.3.1 Islet of Langerhans

1.3.1.1 Islet Structure

Islet of Langerhans is a micro-endocrine organ that scatters throughout the pancreas. It clusters mainly five types of cells, alpha, beta, delta, gamma and epsilon cells, with each containing their own special hormones (81). Islet is highly vascularized due to the high demand for oxygen, nutrient and hormone delivery. Typically islets making up only ~1% of the total volume of the pancreas, whereas more than 3% of pancreatic blood vessels are condensed in these micro-organs, carrying 5-10% of total pancreatic blood flow.
Physiologically, each islet can vary significantly in size and cell population. In rodents, 90% of islets cells are insulin secreting beta cells (82), whereas this cell type only makes up for 60% in humans (83-85). In addition, most islets in rodents form a mantle shell structure, whereas human islet has no particular pattern (82). This difference between species may result in completely distinct secreting profiles and islets’ response to stress, such as diabetogens like streptozotocin (STZ) (86).

Many models of islets structure have been proposed based on different methodology, such as the mantle-core structure (82), ribbon-like structure (87), cloverleaf structure (88) etc. Most observed islet structure in rodents are the mantle-core arrangement, in which the islets present a well-defined shape with a central core of beta cells and a layer of other endocrine cells surrounding the core peripherally (82). The alpha cells appear to be the dominant type in the mantle around the beta cell core, which may count for up to 30% of islet cells. Though this is the most acceptable cytoarchitecture in rodent islets, the structure is subject to dynamic changes when needed, such as during development or pregnancy. In addition, the composition of an islet is not fixed, as non-beta cells are able to proliferate and transform into insulin producing cells when there is a dramatic beta cell loss. Even the beta cell population themselves demonstrate heterogeneity. Mapping of islet functional architecture revealed the presence of hub cells with pacemaker properties, which control the electric pace of other types of beta cells. The complexity of islet biology is set to satisfy its fundamental duty, hormone secretion to maintain normoglycemia.

Blood perfusion is essential to islet function. Vasculature formed during development is stabilized in adults. Inhibiting VEGF expression in pancreas in embryo impairs vascularization and islet function, but doesn’t affect islets function in adult mice,
suggesting that islets produce more than required vasculature upon maturation. In the situation of partial damage, islets can still guarantee the transportation of oxygen, nutrients and hormones. In islet transplant, revascularization is a key determinant for the success of the operation because isolated islets loose much HS during isolation and are very fragile to hypoxia. Delayed and insufficient revascularization can deprive islets from oxygen and nutrients, resulting in islet cell death and early graft failure.

1.3.1.2 Islet Function

In beta cells, active insulin is stored in dense core secretory granules, each containing 300,000 or more molecules of insulin. The glucose-induced release of insulin accounts for only a small fraction of the insulin granules released through exocytosis, which is sufficient to maintain normoglycemia and leaves the islets extra capacity for urgent need. After a meal, plasma glucose increased, and is taken up through facilitated diffusion by the high $K_m$ GLUT2 transporter in rodent and the low $K_m$ GLUT1 in human beta cells. In the beta cells, glucose undergoes glycolytic and oxidative metabolism to generate ATP, resulting in elevated ATP. The increased ATP/ADP ratios then shut down the ATP-sensitive potassium ($K_{ATP}$) channels (89). Followed by influx of positively charged ions(90), which leads to PM depolarization that in turn activates voltage-gated $Ca^{2+}$ channels(91), and prompts the activation of secretory granule-associated small N-ethylmaleimide-sensitive factor receptor (SNARE) proteins and vesicle fusion with the PM (92). The whole process described above is essential for insulin secretion in response to glucose. The glucose-stimulated insulin release proceeds in two distinct temporal phases in both humans and rodents, including a fast, transient first phase and a slow, sustained second phase (93). Together both phases maintain the basal insulin level and a pulsatile release in response to glucose and other signals. The impairment of first phase
insulin release is an early detectable symptom for impaired islet function, insulin resistance and Type 2 diabetes (T2D).

The islet function is strictly regulated by numerous autocrine and paracrine signals, however, extracellular matrix that serve as scaffold for islets is further identified as providing additional regulatory roles in islet survival and function. Macromolecules in extracellular matrix such as collagen, HSPGs and integrin not only have a tight connection with islet cells, but also provide growth factors and signaling molecules to islet cells, which are necessary for normal islet functions. Among all the molecules in ECM, HS is an irreplaceable component that plays a key role in islet activities. For instance, HS facilitates alpha cell derived FGF1 and FGF2 action of inducing signaling in beta cells, which regulates beta cell proliferation and maturation during development. Attenuation of signaling via FGFR1 causes decreased number of beta cells and impaired pro-insulin processing. In adult beta cells, heparin treatment of islet can improve its survival and proliferation in vitro. Additionally loss of HS is observed in non-obese diabetic mouse islet, which contributes to ROS induced damage and insulitis. The understanding of ECM and HS in islet function is of great importance in searching for diabetic therapy, particularly in islet transplantation.

1.3.2 Pancreatic Hormones

1.3.2.1 Insulin and Its Functions

Insulin is a 51-residue peptide that contains two chains (A and B), which are connected by two disulfide bonds (94). In beta cells, insulin mRNA transcript encodes a preproinsulin, which is translated on the endoplasmic reticulum (ER) (95) and subsequently translocated into the reverse ER with the assistance of a signal peptide (94). The signal peptide is then cleaved by a signal peptidase to generate proinsulin (94).
Unlike the preproinsulin, in some cases, the proinsulin, which contains a C-peptide that links the A and B chains, can be secreted (96). Predominately however, proinsulin is folded to generate the native tertiary structure, the direct precursor of insulin. Proinsulin is transported to the Golgi apparatus where it is packaged into secretory granules (94; 97). The conversion into mature insulin is achieved by the action of proprotein convertases (PC1/3, PC2) during trafficking through the secretory pathway. C peptide is a byproduct of beta cells, and has multiple clinical values, one as an assessment of endogenous insulin production in diabetic patients. The mature insulin can be self-assembled to a dimer and further form a hexamer with the assistance of zinc. Nevertheless, the predominant form of insulin in circulation is a monomer, which is the sole form that binds the receptor.

The insulin receptor gene locates on chromosome 19 and is composed of 22 exons. Alternative splicing of the 36 base pair exon 11 results in two isoforms that differ in sequence at the C-terminal end of the insulin-binding alpha-subunit. Insulin receptor is a trans membrane tetramer that contains two alpha and two beta subunits. The beta subunits have the tyrosine kinase activity, which is inhibited by the alpha subunits in basal conditions (98; 99). The inhibitory effect of alpha subunits is removed once they bind to insulin, and the beta subunits are mutually activated. Insulin receptor is not limited for binding to insulin as insulin-like growth factor (IGF) I and II can also recognize this receptor (100; 101). The major downstream substrates of insulin receptor are insulin receptor subunit (IRS) that contains four members (102), whereas 5 other substrates can also pass the insulin receptor signaling down depending on the cell types and situations (103). Phosphatidylinositol-3-OH kinase (PI(3)K), Akt and fork head transcription factors are the key downstream signaling events that accomplish metabolic and mitogenic actions of insulin and IGF-I (104; 105). The predominant Akt downstream signaling is
regulating intracellular glucose transporter vesicle movement through the actions of two intermediate protein kinases, PDK1 and Rictor/mTOR (106-108).

Glucose enters into cells by facilitated transport through glucose transporters, which consists of 13 members. Based on the structure similarity, these 13 members were classified into 3 groups (109). In most insulin sensitive organs, the principal glucose transporter protein that mediates this uptake is GLUT4 (110). GLUT4 is an insulin dependent glucose transporter, and a major participant of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis (108; 110). GLUT4 displays a unique characteristic of a predominantly intracellular disposition in the resting state that is acutely redistributed to the PM in response to insulin and other stimuli (108). Insulin stimulates GLUT4 exocytotic pathways while also inhibiting its endocytosis from the PM, which together cause the overall redistribution of GLUT4 to the cell surface (108).

Insulin sensitivity refers the whole body’s response to insulin, which is reflected by the rate of clearing circulating glucose (111). Weakened insulin sensitivity, also recognized as insulin resistance, is the key stage of the metabolic syndrome and T2D development (112). Insulin sensitivity is determined by all the components in the system including expression of insulin receptor (113), its downstream signaling elements, and insulin dependent glucose transporters (114), etc. Any malfunction of these components such as spontaneous or inherited gene mutation, or chemical induced abnormality (115), would cause metabolic disorders and diabetes (116). FA and inflammatory cytokines are the culprits for insulin resistance and T2D (117), where adipocyte derived cytokines dramatically reduced insulin sensitivity in peripheral organs. Clinically, insulin sensitivity can be improved by chemicals (118), controlled diet and exercises. In rodents,
gene manipulation such as glucagon receptor (GCGR) knockout (119) or pore-forming K⁺-channel alpha-subunit knockout also results in improved insulin sensitivity (120), which provide valuable information for finding treatment of diabetes. In addition to glucose metabolism, insulin also profoundly affects lipid metabolism, by increasing lipid synthesis in liver and fat cells, and attenuating FA release from TG in fat and muscle (121).

1.3.2.2 Glucagon and Its Functions

Glucagon is a 29 amino acid peptide produced and secreted from alpha cells. It is one of the key hormones that maintain glucose homeostasis, exerting the opposite metabolic effect to insulin (122). Glucagon is secreted at low levels in the basal non-fasting state, whereas during long-term fasting or hypoglycaemia, secretion of glucagon is increased. Glucagon acts through the GCGR, which is a class B G protein-coupled receptor expressed in the liver as well as multiple other tissues, such as the brain, heart, kidney, gastrointestinal tract and adipose tissues (123). Therefore, glucagon has a wide range of regulatory functions on glucose and FA metabolism (124).

In the alpha cells, Gcg gene encodes proglucagon (125), a 160 amino acid precursor polypeptide that has to be processed by prohormone convertase 2 (PC2) to yield glucagon, which contains unprocessed GLP-1 and GLP-2 (126; 127). However, depending on the situations or cell types, such as in L-cells, this precursor is cleaved by PC1 to generate glicentin, oxyntomodulin, glucagon-like peptide (GLP)-1 and GLP-2 (128). Alpha cells can also express PC1 to produce GLP-1 when insulin production is not sufficient, such as after STZ treatment (129). At the cellular level, the mechanism of glucagon secretion is similar to insulin secretion, which is mediated by glucose derived ATP, potassium channel closure, cAMP activation and Ca²⁺ influx (130). The properties
in alpha cell and beta cell that determine the hormone secretion at different glucose levels are determined by: 1) the differential expression of glucose transporter (alpha cell, Glut1; beta cell Glut2) which makes the glucose transport 10 fold lower in alpha cells than in beta cells (131); 2) glucose utilization is aerobic in beta and anaerobic in alpha cells (132); 3) low $K_m$ hexokinase that helps alpha cells detect low glucose (133). Overall, glucose induces much smaller changes in cytoplasmic ATP in alpha cells than in beta cells, which make glucagon secretion possible under low glucose levels.

Glucagon is a hyperglycemic hormone; and dysregulation of this hormone is considered to be more devastating to diabetes progression than deficiency of insulin (134). Physiologically, the secretion of glucagon is triggered by hypoglycemia, amino acids and FA, as well as by adrenergic stimulation and by some regulatory peptides (135-137). On the other hand, its counter partner insulin from beta cells, together with other secretory products, zinc and $\gamma$-aminobutyric acid (GABA) can inhibit glucagon secretion (138; 139). Besides insulin, delta cell derived somatostatin is a potent inhibitor of both insulin and glucagon secretion and was proposed to be a paracrine regulator of glucagon secretion (140). Distal regulation of glucagon secretion is from the central nervous system (CNS), whose function is heavily dependent on glucose provision (141). CNS is sensitive to ambient glucose levels and sends neural signals to control glucagon secretion during hypoglycaemia. The regulatory system in glucagon secretion is complicated, with autocrine (142), paracrine (140) and distal influences being involved (141). This strict regulation is important not only to prevent hyperglycemia, but also to suppress the potentially lethal hypoglycemia that is attributed to over secretion of insulin.

Regarding glucagon’s physiological effect, liver is the primary target of this hyperglycemic hormone, where glucagon concentration is two to three times higher than
its presence in other organs. During fasting or exercises, glucagon increases hepatic glucose output and modulates glycogen synthesis, in addition to modulating hepatic FA metabolism (124). Details of these effects include increase of gluconeogenesis and glycogenolysis and inhibition of glucose uptake (143), while elevation of FA oxidation and inhibition of TG secretion (124). In addition to regulating metabolism, glucagon signaling promotes hepatocytes survival through cAMP (144). Therefore, disruption of GCGR signaling in mice results in impaired hepatic lipid oxidation, which predisposes these animals to hepatic steatosis and liver injury (145). Classic pharmacological actions of glucagon in other organs include the induction of satiety in the brain, control of heart rate, inhibition of insulin secretion from the pancreas and enhancement of gastric emptying, which supports the investigation of glucagon agonist for the treatment of obesity.

By far the most astounding observation in the GCGR knockout (GCGR KO) mice was the fact that glycaemia and glucose tolerance remained normal, despite destruction of virtually 90% of beta cells by STZ (146). Similar protective effects were observed in GCGR KO mice with high fat diet treatment further confirming the necessity of glucagon in overt diabetes (147). Detailed mechanism of glucagon deficiency in impeding diabetes development is not very clear, however, as a compensation mechanism to disrupted Gcgr gene, beta cell content remain the same while there is a profound glucagon cell hyperplasia and hyperglucogranemia (146; 148). Even the Gcgr liver specific KO mice share similar characteristics to the GCGR KO model (149), suggesting that besides glucagon’s direct effect in islet formation, an additional circulating factor produced by the in liver can also determine the islet physiology and overall metabolism. Hepatic FGF21 and GLP-1 were suggested to contribute to the diabetes resistant feature of these
animals in an insulin independent manner (150). Overall, disruption of the glucagon receptor gene or signaling appear to be a potential strategy to manage hyperglycemia, under the condition of insulin deficiency; however, glucagon signaling removal has little effect in normalizing glucose level or extending animals’ lifespan when insulin is completely absent. Studies from Kieffer’s lab suggests that glucagon is not the contributing factor that causes insulin deficient death, raising questions as to the therapeutic strategy targeting glucagon signaling (151).

1.3.3 Glucose Metabolism in Peripheral Organs

1.3.3.1 Glucose Metabolism in the Liver

In the hepatocyte, GLUT2 transporter is responsible for glucose uptake and release (152). Glucokinase (GK) in hepatocytes converts glucose to glucose 6-phosphate (G6P), leading to a reduction in intracellular glucose concentrations, which further increases glucose uptake (153). G6P is a precursor for glycogen synthesis and also a metabolite for pyruvate production (153). Pyruvate is oxidized in mitochondria through tricarboxylic acid (TCA) cycle or can be used to synthesize FA through lipogenesis (154). Insulin initiates the metabolism and storage of glucose, while repressing glucose synthesis and release. The overall glucose lowering effect of insulin is accomplished through a coordinated regulation of enzyme synthesis and activity, which is mediated by a series of transcription factors and co-factors, including sterol regulatory element-binding protein-1, hepatic nuclear factor-4, the fork-head protein family and PPAR co-activator 1 (155-157). In the fasted state, glucagon mediated hepatic gluconeogenesis contributes to normalizing euglycemia, which is regulated by CREB, FOXO1, and C/EBPα/β (155).
1.3.3.2 Glucose Metabolism in the Skeletal Muscle and Adipose Tissue

Though muscle cells use FA as their primary energy source, glucose is preferred for rapid energy generation, a mechanism shared by skeletal and cardiac muscle. The muscle cells (myocyte) cannot release glucose into the circulation; however, its ability to rapidly increase its glucose uptake is critical for handling sudden increases in plasma glucose. The uptake of glucose in skeletal myocytes is facilitated by transporters GLUT1 and GLUT4(158), which function differently. Basal glucose uptake is maintained by GLUT1 that has a predominant PM localization, whereas GLUT4 in the cytosol relies on insulin stimulation to relocate to PM. Once stimulated by insulin or by contraction-associated activation of AMP-activated protein kinase (AMPK), GLUT4 is relocated to the PM for glucose uptake (159). In the cells, glucose undergoes glycolysis (catalysed by phosphofructokinase-1) to form pyruvate, which then enters into the mitochondria for oxidation, a process controlled by the activity of pyruvate dehydrogenase (PDH) (160).

Besides direct glucose clearance, skeletal muscle has an additional role in maintaining plasma glucose levels by providing free amino acids as substrates for hepatic gluconeogenesis (161). Similar to liver, myocytes normally contain some amount of stored glycogen, FA and a large pool of protein that can be broken down in emergency situations (162).

Differently, the adipose tissue serves as the major organ for nutrient storage, in addition to its function of thermogenesis and hormone secretion. In adipose tissues, glucose is not only used for generation of ATP by glucose oxidation, but also provides the backbone for FA synthesis and the three carbon chain required for the esterification of FAs (163). Regulation of glucose entry into the adipocytes serves as a primary regulatory site for the control of both nutrient storage and mobilization, which is also mediated by the insulin
dependent GLUT4 (164). Though white adipose tissue is deemed to be detrimental to glucose clearance, brown adipose tissue (BAT) is exceptional due to its function in improving whole body glucose homeostasis (165). BAT is a metabolically active organ that generates heat using glucose and fat (165), which is found consistently in mice but mostly in human infants. Mice receiving transplanted BAT showed a significant decrease in body weight and an improvement in insulin sensitivity and glucose metabolism (166); even the harmful effects of a high-fat diet are ameliorated in these mice (167). The metabolic effects of the transplanted BAT originated from a paracrine or endocrine action, by increasing circulating norepinephrine, IL-6, and FGF21 (168). Among these, FGF21 is an insulin independent hormone, which relies on UCP1 dependent thermogenesis as a method to increase glucose disposal (169).

1.3.3.3 Glucose Metabolism in the Heart

Heart is a unique organ that undergoes uninterrupted contraction; therefore it is not surprising to see that it has a high demand for energy. The heart demonstrates substrate promiscuity, enabling it to utilize multiple sources of energy. Regular heart metabolism produced ATP that is 30% from glucose and lactate, whereas the rest 70% of ATP generation is through FA oxidation (FAO) (170). Regarding glucose metabolism, similar to the skeletal muscle, glucose utilized in the heart requires GLUT1 and GLUT4 facilitated uptake first. The delivered glucose undergoes glycolysis to generate pyruvate, which has to be converted to acetyl-CoA that then enters the mitochondria for oxidation. This process is regulated by the activity of pyruvate dehydrogenase complex including PDH and its inhibitor pyruvate dehydrogenase kinase (PDK) (171). The increase of pyruvate and reduction of acetyl-CoA suppress PDK and leaves PDH activated, resulting in an increase of glucose and pyruvate oxidation. The activity of PDH and glucose
oxidation in the heart is decreased by elevated rates of FAO, a mechanism that is named as Randle Cycle (172). A different feature from skeletal muscle is that cardiomyocytes have less content of intracellular glycogen storage, probably due to the heart’s highly mechanical activities (173). It should be noted that functions of glucose extend beyond its primary use for energy generation and glycogen storage in the cardiomyocytes. In the heart, glucose can also be used for ribose formation or enter into the hexosamine biosynthetic pathway (174).

1.4 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder that is featured with high blood glucose. Diabetes is caused either by insufficient insulin production or by impairment in insulin action. Although the direct cause for diabetes is not completely deciphered, it is generally considered as genetic and life style related issues. According to the World Health Organization report, the worldwide diabetic population has been rising since World War II and has already reached 422 million by 2016, with even more people living with pre-diabetes (175). The overall death related to diabetes is approximately 1.5 million people each year. Moreover, people who are living with diabetes could suffer from multiple organ complications include heart attack, stroke, kidney failure, leg amputation, vision loss and nerve damage (176). Diabetes and its complications produce substantial financial burden to the patients, their families, health systems and national economies through direct medical costs and loss of work and wages. Based on the etiology of diabetes, there are many types of diabetes, among which type 1, type 2 and gestational diabetes are the most prevalent subtypes (177).
1.4.1 Type 1 Diabetes (T1D)

This type of diabetes that arises predominantly in juvenile or childhood is characterized by deficient insulin production in the body. People with T1D require administration of insulin to regulate the levels of glucose in their blood and whole body metabolism. Though the exact cause for T1D still requires more research, both genetic and environmental risk factors are generally considered to be involved (178). The most pivotal genes contributing to T1D susceptibility are located in the HLA-DQ locus on the chromosome 6, which accounts for 40% of the incidence (179; 180). In addition, there are over 50 non-HLA regions that significantly affect the risk for T1D (181). Although T1D is mostly considered as a consequence of inheritance, only a small portion of the genetic polymorphism carriers indeed proceed to overt disease (178). In addition to genetic reasons, environmental triggers for beta cell destruction including viruses, bacteria, and dietary factors are increasingly recognized as triggers for this disease (178). Regardless of its initiators, T1D is essentially an autoimmune disease, in which the immune system destroys its own insulin producing beta cells, whereas cells secreting glucagon, somatostatin, and pancreatic polypeptide are generally preserved. Naive CD4\(^+\) T cells that circulate in the blood and lymphoid organs may recognize major histocompatibility complex and beta cell peptides presented by dendritic cells and macrophages in the islets (182). Beta cell antigen-specific CD8\(^+\) T cells are activated by IL-2 that is produced by the activated CD4\(^+\) T cells, differentiate into cytotoxic T cells and are recruited into the pancreatic islets (183). These activated TH1 CD4\(^+\) T cells and CD8\(^+\) cytotoxic T cells are involved in the destruction of beta cells through releasing antibodies (182). Recently the last key piece of islet cell antibodies was identified, making a total of five candidates confirmed, which are insulin, glutamate decarboxylase,
IA-2, zinc transporter-8 and Tetraspanin-7 (184). In a regulated immune system, T-cells are inactivated by increased apoptosis of the target cells. However, it is thought that in T1D this inhibition disappears and T-cells stay chronically activated leading to an expansion and enrichment of auto-reactive T-cells against β-cells.

Primary treatment of T1D is using insulin and its analogs, administered either by pen or insulin pump. However, poor compliance of using insulin yields unstable blood glucose levels, even lethal hypoglycemia. Pancreatic islet transplantation is a better option for managing T1D, however, the major limitations of this therapy is lack of donors and immune suppression in patients. Stem cell therapy appears as a promising method for T1D in future, but currently it is not practical and intensive research is still undergoing (185; 186).

### 1.4.2 Type 2 Diabetes

Type 2 diabetes is generally due to insulin resistance, and gradual development of severe loss of pancreatic beta cells. T2D is much more prevalent than T1D and harder to be diagnosed because the symptoms are not obvious until complications have already arisen. The cause of T2D is more complex than T1D, determined by interplay of genetic and metabolic factors. Ethnicity, family history of diabetes, previous gestational diabetes combined with older age, overweight and obesity, unhealthy diet, physical inactivity and smoking are all contributing factors for T2D (187). Genetically, hepatocyte nuclear factor (HNF)-1 alpha is a typical mutant found in T2D patient (188). Overweight and obesity, which are mainly due to inactive life style (physical inactivity, sweetened diet and smoking), are the main environmental culprits for insulin resistance and T2D (187). Currently, children overweight, infant overweight and early childhood over-nutrition are contributing to the overall increased incidence of diabetes in youths (189). Chronically
excessive fat induces hypertrophy and inflammation in adipose tissue, which in turn disturbs its metabolic function and secretory capability. Elevated circulating tryglyceride and FA, together with inflammatory cytokines such as TNF alpha are the main culprits for insulin resistance (190). Insulin resistance alone is not sufficient to make this disease overt, as pancreas has a compensatory mechanism to overcome the deficient insulin action by releasing more insulin (191). If hyperinsulinemia persists, the pancreatic islet gradually loses its function due to multiple toxic issues and developing hyperglycemia. Currently, T2D can be managed by combining a healthy lifestyle including physical exercise and dietary control with oral medications. The latter currently in use include: 1) alpha glucosidase inhibitors (prevent calories absorption), 2) thiazolidinediones (enhance insulin sensitivity), 3) sulfonylureas (promote insulin secretion), 4) biguanides (reduce hepatic glucose output), (192) 5) DDP4 inhibitors (increase incretins by inhibiting its enzyme dipeptidyl peptidase-4), 6) GLP-1 agonists (increase glucose dependent insulin secretion) and (193) 7) SGLT2 inhibitors (decrease the re-absorption of glucose from the kidney) (194). In addition, bariatric surgery is proven to be clinically beneficial for glycemic control, cardiovascular diseases, even cancer and all-cause mortality in severe obese patients (195).

1.4.3 Other Types of Diabetes

In rare cases, diabetes is a consequence of single gene polymorphism that is inherited or by a spontaneous mutation. These types of mutations normally induce impairment either on beta cell function or on insulin's action, and the emergence of the symptom occurs at an early age. Many types of rare diabetes arise from defects in insulin secretion that is caused by the impairment of single or multiple steps in the insulin granule trafficking
cycle, including granule biogenesis from the Golgi apparatus, subsequent maturation, recruitment to the PM, exocytosis, and endosome-to-TGN movement (196).

The most common types of monogenetic diabetes are neonatal diabetes mellitus (NDM) and maturity-onset diabetes of the young (MODY) (197), both of which cause beta cell defects. More than 50% of diabetes that occurs before 6 months of age is NDM. The mutation on the $K_{ATP}$ potassium channel of the $\beta$-cells gene occurs spontaneously; therefore insulin secretion from these carriers is diminished (198). MODY is the most common monogenetic diabetes that is seen in the second or third decade of life (197).

There are multiple forms of MODY, whose polymorphisms are dedicated on 6 genetic loci on different chromosomes. The genes that translate the functional proteins include hepatocyte nuclear factor-4$\alpha$ (MODY1), -1$\alpha$ (MODY3) and -1$\beta$ (MODY5), glucokinase (MODY2), insulin promoter factor 1$\alpha$ (MODY4) and NEUROD1 (MODY6) (199). Five of them are transcription factors present in the $\beta$-cell nucleus; the only exception is glucokinase, which is the enzyme that metabolizes glucose in beta cells. All of these defects lead to less insulin secretion capability and uncontrolled glycaemia at early age.

The insulin receptor is the first and most critical element in insulin action. Mutations affecting the alpha subunit of this receptor are associated with a severe insulin resistant phenotype, Leprechaunism syndrome and the mutations affecting the beta subunit refer to Rabson-Mendenhall syndrome (200). In Leprechaunism, most of the single amino acid mutations are localized in the alpha subunit of the receptor and therefore likely negatively affect the binding and subsequent signaling of insulin, causing severe insulin resistance in the patient. In contrast, the Rabson-Mendenhall syndrome appears less severe and could be controlled with IGF-1, metformin or leptin (201). Individuals with Leprechaunism usually die within the first two years of life.
Another form of diabetes is gestational diabetes, which affects approximately 7% of pregnancies (202). The condition is present when blood glucose values vary between normal and diagnostic of diabetes in pregnant individuals, which is caused by hormone profile changes and thus insulin action is affected by these hormones (203). Women with gestational diabetes are at increased risk of some complications during pregnancy and delivery, the same as their infants. To compensate for the reduced insulin sensitivity, there is an increased beta cell proliferation and elevated insulin synthesis in pregnancy (204). Gestational diabetes mellitus is often reversible and rarely affects the newborn severely given an appropriate blood glucose level management during pregnancy.

1.4.4 Heparanase and Diabetes

Progressive beta cell failure and a decrease in the number of functional insulin-producing cells are fundamental to both Type 1 and Type 2 diabetes. The mechanisms leading to beta cell failure are complex and include multiple factors (cytokines, oxidative stress and islet amyloid formation) at different stages of the diseases. Of interest, tumor marker heparanase levels are elevated in the urine and plasma of T2D patients (205), together with its increased presence in multiple organs including pancreas and kidney (56; 206). Moreover, heparanase levels are associated with glucose levels because heparanase secretion is reported to be regulated by intracellular ATP and P2Y receptors (207). All the above observations suggest heparanase’s role in diabetes and its complications. It should be noted that within the BM of mouse pancreatic islets, HSPG is expressed at surprisingly high levels, which constitutes a primary defense mechanism against peri-islet invasion by mononuclear cells (56). Thus, the structural integrity of HSPG is essential for beta cell survival (208). In addition, in vitro experiments indicate that beta cells rapidly lost their HS and died (56), an effect that could be prevented by HS replacement. Loss of
intra-islet HSPG and HS due to hepananase cleavage is identified in non-obese diabetic (NOD) mice, resulting in migration of inflammatory cells to individual islets and beta cell death (56). In addition to the direct damage elicited by immune cells, inflammatory cytokines such as TNF-alpha, IFN-gamma and interleukins that are released from immune cells also contribute to beta cell death (209). Although these cytokines are secreted mainly from immune cells and ECs, HSPG on the cell surface could serve as an auxiliary pool for these cytokines (210). Therefore, hepananase cleavage of HSPG could promote beta cell death by creating a burst of cytokines release. As the hepananase inhibitor PI-88 preserved intra-islet HS and protected NOD mice from T1D, hepananase inhibition is claimed to be useful against T1D progression (211; 212). However, contrasting results were observed in NOD mice injected with exogenous recombinant hepananase, which reduced the occurrence of diabetes (213). In addition, amyloid formation in the islets as a hallmark of T2D, could be ameliorated by hepananase-induced matrix metalloproteinase-9 expression (214; 215). In conclusion, though inhibiting this protein in a wild type background could reduce the occurrence of diabetes, overexpression of this protein may also have a beneficial effect, which requires further investigation.

1.5 Diabetic Complications

1.5.1 Diabetic Heart Disease

Cardiovascular disease is the primary cause (~70%) of diabetes-related death, with atherosclerosis being the primary reason for an increased incidence of cardiovascular dysfunction. However, a predisposition to heart failure may also reflect abnormalities in left ventricular diastolic function as an outcome of specific impairment to the heart muscle (termed diabetic cardiomyopathy) (216). The etiology of diabetic cardiomyopathy
is complicated including the dysfunction of calcium transit and extracellular matrix remodeling etc. (217). Among all the factors, the change in metabolism is considered to be a key culprit (216).

1.5.1.1 Fatty Acids Metabolism in the Healthy Heart

Though 70% of ATP generated in the heart is from FAO, the cardiomyocyte has a limited capacity to synthesize FA, and thus relies on an exogenous supply. FA delivery to the heart involves (a) release from adipose tissue and transport to the heart after conjugating with albumin, (b) provision through breakdown of endogenous cardiac TG, and (c) lipolysis of circulating TG-rich lipoproteins (VLDL, chylomicrons) to FA by endothelial cell presented lipoprotein lipase (LPL) (218). Two essential enzymes involved in TG hydrolysis include adipose TG lipase (ATGL) and hormone-sensitive lipase (HSL) (219). Delivered FA can enter the biosynthetic or oxidative pathways. In the latter, FAs are oxidized by converting to fatty acyl-CoA, which is transported into the mitochondria through carnitine palmitoyltransferase (CPT1/CPT2) (220). Inside the mitochondria, fatty acyl-CoA undergoes β-oxidation to generate acetyl-CoA, which goes through the TCA cycle to yield ATP. When activated, AMPK, peroxisome proliferator-activated receptor-α (PPARα), and malonyl-CoA decarboxylase, all have substantial roles in modulating FA delivery and oxidation (221; 222). A general comparison of glucose and FA metabolism in the heart is listed (Table 1.1).
## Table 1.1 Comparison of glucose and FA metabolism

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major consuming organs</td>
<td>Brain, endothelium</td>
<td>Skeletal Muscle, heart</td>
</tr>
<tr>
<td>Oxygen needed (mol/mol)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>ATP yield (mol/mol)</td>
<td>38</td>
<td>129</td>
</tr>
<tr>
<td>Trans membrane transporter</td>
<td>GLUTs</td>
<td>CD36, FATPs, FABPs</td>
</tr>
<tr>
<td>Insulin dependence</td>
<td>Yes*</td>
<td>No</td>
</tr>
<tr>
<td>Intracellular storage</td>
<td>Glycogen</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>Metabolic pathway</td>
<td>Glycolysis/oxidation</td>
<td>Oxidation</td>
</tr>
</tbody>
</table>

* Insulin independent metabolism presents in certain tissues

### 1.5.1.2 Alterations in Cardiomyocyte Metabolism following Diabetes

During diabetes, the balance in glucose and FA metabolism in the heart is lost, which is considered as the geneses of diabetic cardiomyopathy. Multiple adaptive mechanisms, either the whole body or intrinsic to the heart, collaborate to make the metabolic switching achievable. These include augmented adipose tissue lipolysis, which elevated circulating FA that is delivered to the heart (223). For the FFA delivered to the liver, it triggers the synthesis of VLDL by raising lipoprotein concentrations. VLDL-TG is an additional and major resource to increase FA supply to the heart for oxidation. Innate to the cardiac muscle, the rate-limiting enzyme for plasma VLDL-TG clearance, LPL, is secreted and moved to endothelial lumen to release FFA (216). In addition, the uptake of albumin-bound FA is driven by plasma membrane FA transporters (e.g. CD36), which are increased following diabetes (224). The mechanisms that regulate above changes in cardiac FA are AMPK and PPARs respectively (225). AMPK is an immediate responsive mechanism to ensure adequate cardiac energy production following acute diabetes.
AMPK increases FA delivery through its activation of LPL as well as repositioning CD36 to the sarcolemma (226). AMPK also participates in the intracellular oxidation of FA by phosphorylating and inhibiting acetyl-CoA carboxylase, relieving its inhibition on CPT1, hence promoting FAO (226). However, in chronic diabetes, with the persistence of augmented plasma lipids, AMPK activation is lost, in turn, PPARα takes control of FAO. Cardiac PPARα is activated by elevation of intracellular FA, which can significantly reduce cardiac glucose oxidation, and to a lesser extent glycolysis and glucose uptake (227). Consequently, intracellular concentrations of glucose and its metabolites accumulate (glucotoxicity) to potentiate O-linked protein glycosylation and interfere with protein functionality (228).

Evidence suggests that, at given oxygen supply, heart contractility is greater when it is using glucose, compared to using FA (229). A similar observation is that in healthy human, elevated plasma FFA is associated with decreased cardiac mechanical efficiency (230). The mechanism of impaired heart function with high FAO is probably due to its higher oxygen consumption than glucose, for generating the same amount of ATP (231). The diabetic cardiomyopathy is also characterized by decreased capillary density and reduced myocardial perfusion, in addition to a significant decrease in PPARα expression and its associated genes (232). These changes create a setting where oxygen delivery and augmented FA uptake exceed its utilization, leading to increased intramyocardial storage of TG (233). Chronically, the accumulation of intracellular TG, FA and potentially toxic metabolites from FAO, including ceramides, diacylglycerols, and acylcarnitines can provoke cardiomyocyte death (lipotoxicity) (234).
1.5.2 Heparanase and Diabetic Cardiomyopathy

During diabetes, the major FA provision to the heart is through hydrolysis of plasma VLDL-TG and chylomicrons by EC surface bound LPL. However, LPL is not produced by EC (235). A tightly regulated system is in control of LPL trafficking from an intracellular pool to myocyte surface HSPGs, followed by further translocation to the vascular lumen (235). Our lab has described the whole process from LPL production, maturation, translocation and degradation (236), among which, LPL intracellular and intercellular translocations are mediated by heparanase (237). Previously our lab has observed the prompt secretion of heparanase, both latent and active forms, from ECs in response to hyperglycemia (237). The secreted active form of heparanase can detach the LPL from surface bound HSPGs, whereas the latent heparanase promotes the LPL replenishment from cytosol to the cell surface (237). This close circle provides a mechanism that assures the acute energy provision to the heart. However, if prolonged, excessive usage of FA is likely to be catastrophic to the heart. It should be noted that heparanase expression in long-term diabetic heart dropped, suggesting that heparanase’s role is limited within acute conditions, which is more beneficial than harmful to the heart.

1.6 Hypothesis and Research Objectives

Heparanase is a key mediator in cardiac metabolism, not only through its enzymatic activity, but also through its non-enzymatic properties such as signaling transduction and transcriptional regulation. In the heart, these properties of heparanase assist in the acute metabolic adaptation during energy shortage, a mechanism that could be harmful if prolonged. However, preliminary data indicated that overexpression of heparanase indeed improved overall glucose metabolism in mice and protected the animals from chemically induced diabetes. I hypothesize that heparanase, through its enzymatic and non-
enzymatic properties, exhibits protective roles in correcting diabetes development and ensuring energy provision to the heart during diabetes.

The objectives of my research proposal were to:

1. Investigate the mechanism by which heparanase acutely facilitates cardiac metabolic switching during diabetes, using VEGF as its surrogate.

2. Characterize the metabolic features and changes of islet genome and function in heparanase overexpression mice.

3. Determine the mechanism by which overexpression of heparanase protects animals from chemically induced diabetes.
Chapter 2: Materials and Methods

2.1 Experimental Animals

Adult male Wistar rats (260-300 g) were obtained from Charles River Laboratories. Wild type (WT) C57BL/6J mice aged 10 to 12 weeks were purchased from Charles River Laboratories. Heparanase transgenic mice (hep-tg), in which a constitutive β-actin promoter drives the expression of human heparanase gene in a C57BL/6J genetic background, were a kind gift from Dr. Israel Vlodavsky. All animals were housed in 12-h light/dark cycle, and pathogen-free conditions. Hep-tg mice were previously crossed for 10 generations with C57BL/6J mice to produce a stable homozygous background. Male homozygous hep-tg mice aged 12 to 15 weeks were used for all experiments. For confirmation of genotype, genomic DNA was prepared from 21 day-old animal ear punched tissue, and analyzed by PCR (Fig. 4.1 A) as previously detailed. All experiments were approved by the University of British Columbia Animal Care Committee, and were performed in accordance with the Canadian Council on Animal Care Guidelines.

2.2 Diabetes Induction

Streptozotocin (STZ) is a beta-cell selective toxin. Using STZ, we employed two different strategies to induce diabetes. With the first protocol, we injected multiple low doses of STZ (MLD-STZ, 50 mg/kg i.p.) for 5 consecutive days. This procedure induces gradual beta cell death, followed by an immune system response. Body weight and plasma glucose levels were monitored daily after the first STZ injection. Metabolic assessment and organ isolation were performed 1 week after the last STZ injection, following confirmation of hyperglycemia. We also used a single high dose of STZ (SHD-
STZ, 200 mg/kg i.p.), which is directly cytotoxic to beta cells, resulting in robust hyperglycemia within 24-48 h (238).

2.3 Metabolic Assessments and Treatment of Animals

For measurement of basal blood glucose and plasma hormones, animals were fasted for 6 h and blood sampled from the tail vein. Blood glucose was measured using an ACCU-CHEK glucose monitor (Roche, Basel, Switzerland). Blood was also collected using a heparinized micro-tube (Fisher Scientific, Waltham, MA, USA), and centrifuged immediately for separation of plasma. Circulating hormones in the plasma were measured using the following kits: mouse insulin (ALPCO, Salem, NH, USA), glucagon and GLP-1 (Meso Scale Discovery, Rockville, MD, USA), FGF21 (R&D Systems, Minneapolis, MN, USA). For the oral glucose tolerance test (OGTT) or intraperitoneal (i.p.) glucose tolerance test (IPGTT), animals were fasted overnight (16 h), 2 g/kg glucose administered orally or i.p., and blood glucose measured at the indicated times. A different cohort of animals was treated in the same manner for the assessment of insulin following the OGTT. To perform the glucagon challenge, glucagon (1 µg/kg) was administered i.p. into 6 h fasted mice, and blood glucose measured at the indicated times. The L-arginine (2 g/kg, i.p.) test was performed in 6 h fasted mice. Following injection of L-arginine, blood glucose and plasma insulin and glucagon were measured at the indicated times. For evaluation of skeletal muscle sensitivity to insulin, mice were fasted for 6 h prior to an i.p. injection of 2 µg/kg insulin. 10 min post insulin injection, skeletal muscle (soleus and gastrocnemius) was isolated for Western Blot determination of phospho- and total Akt (Cell Signaling, Danvers, MA, USA), with beta actin (Santa Cruz, Dallas, TX, USA) used as an internal control.
2.4 Cardiomyocytes Isolation and Treatments

Ventricular, calcium-tolerant myocytes were prepared by a previously described procedure. In brief, hearts were removed from anaesthetized rats and retrogradely perfused using collagenase. Subsequent to digestion, myocytes were exposed to increasing calcium concentrations to induce calcium-tolerance. Our method of isolation yields a population of calcium tolerant, viable (85%), rod-shaped ventricular myocytes. Calcium intolerant cells lose their rod structure and start to round up. Viable cells can be differentiated from non-viable cells by their rod shape and their ability to exclude 0.2% trypan blue. Myocytes were plated on 100 mm culture plates to a density of 1,000,000 cells/plate. Plated cells were nourished using Medium 199, and incubated at a temperature of 37°C under conditions of 5% CO2. Different agents were added to the incubation media, and cells maintained for the time indicated.

Cardiomyocytes were incubated with medium containing 5 U/mL of heparin, ECCM or 1 µg purified A-Hep and L-Hep for the times indicated. VEGF in the incubation medium was determined using an ELISA (R&D system). In a separate experiment, isolated myocytes from control and DZ animals were also treated with heparin, and VEGF release was determined. FGF released by recombinant heparanase was measured using an ELISA kit (R&D system).

2.5 Endothelial Cell Culture and Treatments

Bovine coronary artery endothelial cells (bCAECs; Clonetics) were cultured in endothelial basal medium (EBM) supplemented with EGM-MV BulletKit (Lonza, Basel, Switzerland) at 37°C in a 5% CO2 humidified incubator. Cells from the 4th to the 12th passage were used for the experiments. Following incubation with high glucose (25 mM) DMEM for 30 min, medium (ECCM) was collected and subjected to TCA precipitation.
Briefly, one fourth volume of TCA was mixed with ECCM and incubated for 10 min, followed by centrifugation, acetone wash and air drying. The dry pellet was dissolved in 2x SDS loading dye and Western blot performed to determine A-Hep and L-Hep1. The high glucose medium, termed endothelial cell conditioned medium (ECCM), was freshly prepared prior to treating cardiomyocytes. A-Hep and L-Hep were also determined in cell lysates (using Western blot). A monoclonal antibody Bevacizumab (200 µg/mL) was added to ECCM to neutralize VEGF.

2.6 Matrigel

BD Matrigel™ matrix (BD Biosciences, San Jose, CA, USA) is a reconstituted basement membrane preparation containing HSPG, laminin, collagen IV, and entactin. A growth factor reduced version of Matrigel (50 µl/cm² growth area) was used, and incubated for 2 h at 37 °C to solidify. Recombinant VEGF was added to the gel at a concentration of 100 ng/ml, and various treatments including heparin and A-Hep and L-Hep were used to release bound VEGF.

2.7 LPL Measurement

Medium 199 used to culture cardiomyocytes was collected from control and experimental groups. After loading the sample medium into a VIVASPIN filter tube and centrifugation at 4000 xg for 20 min, the final volume of the sample was made up to 150 µl and loaded for SDS-PAGE. LPL activity was also determined by measuring in vitro hydrolysis of [3H] triolein substrate.

2.8 Western Blot

Western blot was carried out as previously described6. Briefly, cells were lysed with 20 mM pH 7.5 Tris, 0.36% β-Mercaptoethanol, 250 mM EGTA, 0.2 M EDTA, 1% NP-40, 0.1% SDS, 0.5% DCA, 1 mM PMSF, and 1 mM NaF, followed by centrifugation at 12k
for 15 min. Protein concentrations were measured using a Bradford assay. Samples were adjusted and boiled with loading dye, and 20 to 50 µg of the total protein was used for SDS-PAGE. After blotting, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were incubated with primary antibodies and subsequently with secondary antibodies.

2.9 Ca^{2+} Imaging

Ca^{2+} was measured using the Fura 2-AM method as previously described (7). Briefly, cardiomyocytes were incubated with 1 µM Fura-2-acetoxymethyl in Media 199 for 30 min, and subsequently rinsed. Cells (3-10 per image field) were excited at 340 and 380 nm, and monitored through a 20× objective. Ca^{2+} signal quantification was performed using waveform statistics by calculating 340/380 ratios. Ca^{2+} levels 2 min prior to treatment were averaged to give a pretreatment mean for each cell. The maximum Ca^{2+} determination during the treatment period was subtracted from the pretreatment average to give the maximal amplitude of the Ca^{2+} signal.

2.10 Staining and Quantification

The pancreas from normal or MLD-STZ mice was perfused with PBS, harvested, fixed in 4% paraformaldehyde, and stored in 70% ethanol before paraffin embedding. All paraffin sections (5-µm thickness) were processed by Wax-it Histology laboratories (Vancouver, BC, Canada). Immunofluorescence staining and quantification of insulin (Cell Signalling Technology, Beverly, MA, USA), glucagon (Sigma-Aldrich, St. Louis, MO, USA), GLUT2 (Chemicon International Inc, Temecula, CA) and synaptophysin (Novus Biologicals, Littleton, CO, USA) were performed as previously described (239). Alcian blue (0.65 M MgCl₂, pH 5.8; Sigma-Aldrich, St. Louis, MO, USA) staining and imaging was used to visualize HS.
2.11 Isolation of Mouse Islets

Pancreatic islets were isolated using collagenase as previously reported (56; 240). Briefly, the pancreas was perfused with collagenase through the pancreatic duct. Additional collagenase digestion was performed in a water bath. Following digestion, the tube containing the tissue sample was shaken vigorously. Individual islets were handpicked under a bright-field microscope. Islets were cultured for 3 h (37 °C, 5% CO$_2$) in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) with 5 mM glucose (Sigma, St. Louis, MO, USA), 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 10% FBS (Invitrogen). For RNA sequencing, 100 islets were selected, rinsed with PBS and snap frozen for later isolation of RNA. For glucose-induced insulin secretion, 10 islets were rinsed three times with PBS to remove glucose and insulin, and then transferred to RPMI1640 medium with either 3 mM or 16.7 mM glucose for 15 mins, followed by medium collection and insulin determination.

2.12 RNA Sequencing and Analysis

RNA from 5 hep-tg and 5 WT mice was isolated using an RNeasy purification kit (Qiagen, Hilden, Germany). Sequencing libraries were prepared from 400 ng total RNA using the TruSeq Stranded mRNA Sample Preparation kit according to the manufacturer’s instructions (Illumina, San Diego, USA). Samples were checked for quality using a Bioanalyzer (Agilent, CA, USA) and quantified using Qubit fluorometer (Thermo Fisher, MA, USA). Libraries were then multiplexed and sequenced over one rapid run lane on the HiSeq2500 (Illumina), collecting 89 million 100 bp paired-end reads. Kallisto version 0.42.4 was first used to build an index file for the mouse reference transcriptome GRCm38 as downloaded from the Ensembl web site (www.ensembl.org). The sequence reads for each sample were then quantified with the quant function of
Kallisto. In house Perl scripts were used to sum the read counts at the transcript level for each gene and to create a matrix comprising the read counts for all the genes for all the samples. Differential expression analysis was then performed on the data from that matrix using the R package DESeq2 (241). Each sample was assessed using the quality control software RSeQC version 2.6.3 (242) and the PtR script from the trinity suite (243). One potential outlier was detected when clustering the samples and was therefore removed for the differential expression analysis. RNA sequencing transcriptomic data were analyzed using Panther and DAVID. Network analysis was conducted using STRING.

2.13 Statistical Analysis

Statistics were performed using Sigma Plot (Systat Software Inc., Chicago, IL, USA). For all analysis, Student’s t-test or one-way ANOVA was used to determine differences between group mean values. Values are presented as means ± SEM with individual data points. \( p<0.05 \) was considered statistically significant.
3.1 Premise

Atherosclerotic cardiovascular disease is a leading cause of diabetes-related death (216; 244; 245). However, patients with diabetes also demonstrate a specific impairment of the heart muscle (diabetic cardiomyopathy) (216), with changes in cardiac metabolism having a significant impact on its development. In this regard, cardiac glucose uptake, glycolysis, and pyruvate oxidation are impaired. Thus, the heart rapidly adapts to predominantly use FA for ATP (245). In the short term, this adaptation might be beneficial. Chronically, increased FA conversion to potentially toxic FA metabolites (ceramides, diacylglycerols and acylcarnitines), paired with an increased formation of reactive oxygen species secondary to elevated FA oxidation, can promote cardiac cell death (lipotoxicity) (246).

Multiple adaptive mechanisms operate to make FA available to the diabetic heart, with cardiac lipoprotein lipase (LPL) being a major contributor, through its ability to hydrolyze lipoprotein-TG into free FA (245). Dr. Rodrigues lab was the first to report a robust expansion in the coronary pool of LPL after diabetes. This increase in LPL activity was immediate and unrelated to LPL gene expression (247). Its prelude included accelerated LPL processing to a dimeric, catalytically active enzyme, an obligatory step for ensuing secretion (236). Active enzyme transfer to the cardiomyocyte plasma membrane then involved LPL vesicle formation and actin cytoskeleton polymerization,
thus providing LPL cargo with an appropriate transport infrastructure for secretion onto myocyte plasma membrane heparan sulfate proteoglycans (HSPG) (236).

HSPG are ubiquitous macromolecules consisting of a core protein to which several linear heparan sulphate (HS) side chains are covalently linked. They function not only as structural proteins but also as anchors (248). In cardiomyocytes, the latter property is used to bind a number of different proteins (including enzymes like LPL, and growth factors like VEGF) (248). Attachment of these bioactive proteins is an efficient arrangement, providing the cell with a rapidly accessible reservoir, precluding the need for de novo synthesis when the requirement for a protein is increased. Heparanase is an endoglycosidase, exceptional in its ability to degrade HS and release its associated proteins (249).

In endothelial cells (EC), heparanase is initially synthesized as an inactive (latent, L-Hep) 65 kDa enzyme that is secreted followed by reuptake (57). After undergoing proteolytic cleavage in the lysosome, a 50 kDa polypeptide is produced that is approximately 100-fold more active than its latent form (250; 251). In the presence of high glucose (25 mM), we have previously reported that EC released heparanase caused LPL detachment from the myocyte cell surface (252). Given their similar localization on the cardiomyocyte cell surface, it is possible that heparanase releases not only LPL, but also VEGF, as observed in cancer cells (253). Such a function would be beneficial to the diabetic heart, given the capability of VEGF to induce angiogenesis, thus providing the heart with sufficient O₂ to accommodate increased FA oxidation (paracrine function) (254). As VEGF’s promotion of angiogenesis has been linked to AMPK activation in EC (255), an AMPK activating property of VEGF in cardiomyocytes (autocrine function) could also directly affect FA delivery (AMPK promotes LPL recruitment to cardiomyocyte cell surface) (256).
Interestingly, cardiac-specific overexpression of LPL is associated with severe myopathy, characterized by both muscle fiber degeneration and extensive proliferation of mitochondria and peroxisomes (257; 258). In genetically engineered mice that specifically overexpressed an anchored form of cardiomyocyte surface-bound LPL, lipid oversupply and deposition was observed, together with excessive dilatation and impaired left ventricular systolic function. Interestingly, loss of cardiac LPL also causes cardiomyopathy (259). Hence, although specific knockout of cardiac LPL in adult mice increased glucose metabolism, neither this effect, nor albumin-bound FA could replace the action of LPL, and cardiac ejection fraction decreased. These experiments in genetically modified mice demonstrate that cardiac LPL is of crucial importance, and disturbing its innate function is sufficient to cause cardiac failure. The present study describes a novel pathway by which high glucose induced secretion of endothelial heparanase stimulates a VEGF-LPL autocrine network in cardiomyocytes.

3.2 Results

3.2.1 High Glucose Promotes Secretion of Latent and Active Heparanase from EC

ECs are the predominant cell type in the heart to express heparanase. Using bCAECs incubated with high glucose, we measured heparanase release into the incubation medium. Within 30 mins, high glucose released approximately 7-fold more L-Hep and A-Hep as compared to control, with L-Hep being the major form secreted (Fig. 3.1A). Measurement of residual heparanase revealed that ECs have a larger reserve of L-Hep as compared to A-Hep (Fig. 3.1B, inset). On incubation with high glucose, although the intracellular content of both heparanase forms were reduced, this decrease was only statistically significant with A-Hep (Fig. 3.1B). The mannitol osmolality control had no effect on medium or intracellular heparanase (data not shown).
A. To study heparanase release by high glucose in vitro, bCAECs were incubated with DMEM containing 25 mM glucose. After an incubation period of 30 min, medium (endothelial cell conditioned medium, ECCM) and cells were separated. Medium was concentrated and Western blot used for detection of latent (left panel) and active (right panel) heparanase.

B. bCAECs were washed with PBS and intracellular latent (left panel) and active (right panel) heparanase determined using Western blot (right panel). 5 mM glucose DMEM was used as control. n=3-5 animals in each group. L-Hep, latent heparanase; A-Hep, active heparanase. *Significantly different from Con, P<0.05.
To model the in vitro observation of heparanase release following high glucose, we used DZ to induce acute hyperglycemia. Blood glucose levels increased within 30 min, and remained elevated for at least 2 h after DZ injection (Supplemental Fig. 3.1A). Assuming that high glucose stimulates heparanase secretion from the basolateral side of EC to affect HSPG cleavage on the underlying myocytes, we used the modified Langendorff perfused heart to separate the coronary perfusate from the interstitial fluid, and measured interstitial heparanase. There was a robust secretion of interstitial A-Hep within 30 min of DZ, an effect which closely mirrored the changes in blood glucose. Extending the duration of DZ for 2 h lowered this active heparanase to values below control. Interestingly, this pattern of release was also observed with L-Hep (Supplemental Fig. 3.1B).

### 3.2.2 ECCM is an Effective Stimulus for Releasing Cardiomyocyte VEGF

As high glucose was effective in releasing both L-Hep and A-Hep into the incubation medium, we collected this ECCM after a 30 min exposure to 25 mM glucose. Exposure of cardiomyocytes to this ECCM caused a 5-fold release of VEGF into the medium, an effect that was only observed after 30 min of incubation (Fig. 3.2A). Previously, we used heparin, a highly negatively charged molecule, to release proteins like LPL from cell surface HSPG9. Heparin treated cardiomyocytes were also capable of releasing VEGF, but unlike ECCM, this effect of heparin was rapid, observed as early as 3 min after incubation, and suggested an extracellular localization of VEGF. Extending the duration of heparin incubation further increased the amount of VEGF released (Fig. 3.2B).
Figure 0.2 ECCM and heparin are capable of releasing cardiomyocyte VEGF.

A. Following the isolation of cardiomyocytes, cells were incubated with ECCM for 3 and 30 min respectively. At the indicated time points, medium was separated and used to determine VEGF using an ELISA assay. *Significantly different from all other groups. B. In a different experiment, cardiomyocytes were also incubated with 5 U/mL heparin for 3 and 30 min, medium separated and used for determination of VEGF. *Significantly different from Con. C. To determine in vivo release of VEGF in hyperglycemic animals, cardiomyocytes were isolated from normal and DZ-treated rats. The amount of VEGF remaining on the cardiomyocyte cell surface was evaluated using heparin (5 U/mL) for 5 min. *Significantly different from Con; #Significantly different from +Heparin, P<0.05.
To relate these findings to an in vivo model of hyperglycemia, cardiomyocytes were isolated from control and DZ-treated animals. As basal and heparin releasable VEGF released into the medium was lower in DZ myocytes as compared to control, our data suggests that a relationship exists between acute hyperglycemia and VEGF secretion from the cardiomyocytes, possibly secondary to secreted EC heparanase (Fig. 3.2C). It should be noted that increasing the duration of hyperglycemia (7 days) using STZ, increased the expression of cardiac VEGF (Supplemental Fig. 3.3).
Figure 0.3 Latent heparanase has a more pronounced effect in releasing VEGF from cardiomyocytes than active heparanase.
A. Recombinant latent (L-Hep, 65 kDa) and active (A-Hep, 50 kDa) heparanase were produced, and purity confirmed using Western blot (A, right-top inset). FGF release from cardiomyocytes was used as a surrogate marker of enzymatic activity for these proteins (right-bottom inset). 1 µg of A-Hep and L-Hep were used to treat cardiomyocytes for 15 min, and medium collected for determination of VEGF using ELISA. *Significantly different from all other groups. The central inset (A) illustrates cardiomyocyte VEGF released by A-Hep using a different scale. B. As latent heparanase had a more predominant effect in releasing VEGF, we tested the concentration dependence of this observation. *Significantly different from all other groups. C. A time dependent release of VEGF from cardiomyocytes using 1 µg latent heparanase was also carried out. *Significantly different from Con, P<0.05.

We hypothesized that the delay of up to 30 min required for ECCM to release cardiomyocyte VEGF represented the time needed for A-Hep to hydrolyze heparan sulfate side chains on the cardiomyocyte cell surface to liberate VEGF. Interestingly, even though A-Hep was enzymatically active in releasing FGF (Fig. 3.3A, right inset), its effect on releasing VEGF was statistically insignificant (Fig. 3.3A, central inset). Remarkably, it was L-Hep that produced the most dramatic release of VEGF (Fig. 3.3A), especially at concentrations between 0.1 and 1 µg/mL (Fig. 3.3B). In addition, the release of VEGF with 1 µg L-Hep was unexpectedly fast, with significant amounts of VEGF being detected as early as 1 min, increasing with the duration of incubation (Fig. 3.3C).

3.2.4 Latent Heparanase Can Release VEGF from a Unique Cell Surface Pool

We treated myocytes with heparin, followed by L-Hep, anticipating a reduced release by the latter if the two treatments released VEGF from the same pool. L-Hep was still capable of releasing VEGF under these conditions (Fig. 3.4A). This release was rapid, and unexpectedly, the amount of VEGF released by L-Hep was almost 2-fold higher than heparin (Fig. 3.4A). To address incomplete displacement of VEGF by heparin, we used pharmacological concentrations of heparin and multiple treatments. Interestingly, neither the higher concentration (data not shown) nor the addition of a second incubation with
heparin was able to release VEGF to the same extent as L-Hep (Fig. 3.4B). Using an alternative approach, we observed that adding increasing concentrations of recombinant VEGF yielded proportional cell surface binding following release by heparin or L-Hep (Supplemental Fig. 3.2). However, it should be noted that compared to heparin, L-Hep released higher amounts of recombinant VEGF at all concentrations used (Supplemental Fig. 3.2). Interestingly, prior incubation with heparin lowered the heparin releasable pool of VEGF, but had a limited effect on the L-Hep releasable pool, suggesting that multiple VEGF cell surface binding domains exist (Fig. 3.4C). Whether these pools originated extracellularly was tested using Matrigel to which recombinant VEGF was attached. Under these conditions, L-Hep still induced the highest amount of VEGF release compared to heparin and A-Hep (Fig. 3.4D), suggesting a predominantly extracellular effect, and a unique L-Hep releasable pool of VEGF on the cardiomyocyte cell surface. As L-Hep was more effective than heparin in releasing collagen-bound VEGF (Supplementary Fig. 3.2), our data suggest that VEGF could also be bound to non-heparan sulfate binding sites on the cardiomyocyte cell surface.
Figure 3.4 Latent heparanase can release VEGF from a unique cell surface pool.

A. Cardiomyocytes were isolated, treated with 5 U/mL of heparin for 30 min, and the VEGF released into the medium assayed using ELISA. Following a PBS wash, 1 µg/mL L-Hep was added to these same cells, and medium collected at the indicated times for determination of VEGF. *Significantly different from Con, #Significantly different from all other groups.

B. In a separate experiment, cardiomyocytes were treated with heparin (5 U/mL, 30 min) and medium was collected for VEGF determination. Following a PBS wash, cells were treated either with a second round of heparin (5 U/mL, 30 min) or L-Hep (1 µg, 30 min) respectively, and VEGF released into the medium was assayed. *Significantly different from Con, #Significantly different from all other groups.

C. To test exogenous binding, myocytes were incubated with recombinant VEGF (10 ng/mL), followed by incubation with heparin (5 U/mL, 30 min) or L-hep (1 µg, 30 min). The above experiment was also repeated following pre-incubation with heparin (5 U/mL, 30 min).
3.2.5 Cardiomyocyte Intracellular Calcium and AMPK Phosphorylation are Augmented by VEGF and ECCM

Elevation in cytosolic calcium is known to increase CaMKKβ phosphorylation and hence, AMPK activation. Cardiomyocytes treated with VEGF displayed a robust increase in intracellular free calcium, an effect that was immediate and returned to baseline within 5 min (Fig. 3.5A, left panel). Although the amplitude of the peak calcium response induced by ECCM was comparable to that demonstrated by VEGF, the response was delayed (Fig. 3.5A, right panel), and likely related to a requirement of ECCM to promote prior release of myocyte VEGF. As predicted, AMPK phosphorylation followed the rise in intracellular calcium observed with both VEGF and ECCM. High glucose itself had no influence on AMPK phosphorylation (Fig. 3.5B). Bevacizumab reduced activation of AMPK observed with ECCM (Fig. 3.5B, inset).

3.2.6 Cardiomyocyte Heparin-releasable LPL is Increased By VEGF

Following AMPK activation, p38 MAPK is a downstream signaling molecule that regulates LPL transport in myocytes by facilitating rearrangement of the actin cytoskeleton18. In cardiomyocytes treated with VEGF, there was a robust phosphorylation of p38 MAPK (Fig. 3.6A, left panel), associated with an increase in the
Figure 0.5 VEGF and ECCM activate cardiomyocyte AMPK through calcium signaling.

A. Cardiomyocytes were plated on glass bottom dishes coated with laminin, and maintained using Medium 199 at 37°C for 3 h. In cardiomyocytes exposed to VEGF (1 µg/mL) or ECCM, changes in cytosolic Ca²⁺ over time was measured using Fura-2. Representative traces of individual cardiomyocytes are shown (lower panel). The horizontal bars indicate an average response of 15-25 cardiac cells of 3 separate preparations. **Significantly different
from Con, $P<0.01$ (upper panel). B. To evaluate effects on AMPK, myocytes were treated with high glucose, VEGF or ECCM for 1 h, and protein extracted to determine phosphorylated and total AMPK using Western blot. *Significantly different from untreated control, $P<0.05$. Results are mean ± SE of three myocyte preparations in each group. The monoclonal antibody Bevacizumab (Avastin, 200 µg/mL) was used to neutralize VEGF in ECCM, and AMPK activation in myocytes subsequently determined (inset).

**Figure 0.6 VEGF augments LPL trafficking in cardiomyocytes.**

A. In cardiomyocytes exposed to VEGF (1 µg/mL), samples were probed for total and phosphorylated p38 MAPK (A, left panel). In the same experiment, F-actin and G-actin ratio were measured using a G-Actin/F-actin *In Vivo* Assay Biochem Kit (right panel). B. To measure LPL (heparin-releasable), cardiomyocytes were treated with VEGF at the indicated times, and medium was collected and replaced with medium containing 5 U/mL heparin for 30 min. Heparin-released medium was concentrated using a VIVASPIN filter system, and
Western blot (left panel) and radiometric assay (right panel) performed to detect LPL protein and activity respectively. *Significantly different from Con, \( P<0.05 \). **Significantly different from Con, \( P<0.01 \).

F/G actin ratio (Fig. 3.6A, right panel). As this effect would be expected to accelerate LPL transport to the cell surface, we measured heparin-releasable enzyme. Interestingly, VEGF after 2 or 4 h was capable of increasing heparin-releasable LPL secretion (Fig. 3.6B, left panel). The increase in LPL protein with VEGF correlated to an increase in LPL activity, both enzymatic (Fig. 3.6B, right panel) and functional (Supplemental Fig. 3.4).

3.3 Discussion

Given their strategic location, ECs are the first to detect and inform underlying myocytes about hyperglycemia after diabetes. In this regard, we have reported that high glucose is a potent stimulator of A-Hep secretion from the EC (252), which stimulated the myocyte to transfer LPL to the vascular lumen. In the current study, we demonstrated that in addition to A-Hep, L-Hep can also be secreted in response to high glucose to release cardiomyocyte HSPG-bound VEGF as a consequence. In doing so, VEGF facilitated cardiomyocyte LPL movement, an adaptive mechanism that is geared to overcome the loss of glucose consumption by the diabetic heart.

In hearts from diabetic animals or ECs exposed to high glucose, we reported elevated A-Hep secretion into the interstitial space and incubation medium, respectively (254). We also determined that ATP release, purinergic receptor activation, cortical actin disassembly, and stress actin formation were essential for high glucose induced A-Hep secretion from lysosomes (260). As lysosomes contain both A-Hep and L-Hep, an anticipated consequence of high glucose ought to be their dual secretion. Indeed, the
current study also identified higher amounts of L-Hep in the interstitial fluid of hearts from hyperglycemic animals, likely a result of polarized secretion towards the basolateral rather than the apical side of ECs. As high glucose promoted an increase of L-Hep in ECCM, our data suggests that ECs can rapidly secrete both A-Hep and L-Hep after diabetes.

Present on the cardiomyocyte surface are HSPGs, ubiquitous macromolecules that can strongly bind a number of proteins like LPL, which has a heparin-binding domain (261). Such a location would allow for a rapid release when there is a requirement for LPL. We have reported that myocyte LPL can be released by A-Hep, facilitating its forward movement across the interstitial space to the apical side of vascular EC, where LPL provides the heart with FA by breaking down circulating TG (Fig. 3.7). Like LPL, VEGF also has a HBD, and thus an affinity to HSPG (262). Using the property of heparin to enable ionic displacement of HSPG bound proteins; we observed a rapid release of VEGF from the cardiomyocytes, suggesting that it resides extracellularly. Although ECCM had a comparable effect in releasing VEGF, the time to release was prolonged and likely related to a requirement for proteolytic displacement of VEGF by active heparanase. To confirm that this process occurs in an in vivo setting of hyperglycemia, myocytes from DZ animals were treated with heparin, and VEGF release was determined. As acute hyperglycemia caused a significant reduction in the heparin-releasable pool of VEGF, our results suggest that after diabetes, heparanase is a likely candidate that increases the availability of free VEGF, an effect that is supported by the ability of chronic hyperglycemia to increase the cardiac expression of VEGF.

To validate whether heparanase is responsible for releasing cardiomyocyte VEGF, we successively purified A-Hep and L-Hep, with expected molecular weights and activity.
Unexpectedly, L-Hep produced a greater release of VEGF, with A-Hep having a minimal effect. Retrospectively this was explainable, given that L-Hep requires HSPG binding to undergo internalization and subsequent activation (57). As the release of VEGF by L-Hep was rapid and sustained, our results imply that L-Hep, through its time-dependent occupation of myocyte HSPG, is able to displace cell surface VEGF.

Considering an extracellular location for VEGF, we assumed that prior removal of this growth factor using heparin will lower the amount released by L-Hep. Intriguingly, L-Hep was still able to release VEGF after heparin, an effect that was rapid, and even more robust than the initial heparin treatment. Assuming incomplete displacement of VEGF with heparin, we employed higher concentrations of heparin, together with multiple heparin treatments, but were still unable to release comparable amounts of VEGF as seen with L-Hep. In addition, as prior incubation with heparin inhibited the amount of recombinant VEGF that could be released by heparin and not L-Hep, our data suggests that either heparin or L-Hep releases VEGF through distinct mechanisms or from different pools. To address the latter question, we used Matrigel to which VEGF was bound. In this setting, L-Hep still had the most robust effect in releasing VEGF compared to heparin or A-Hep, which was similar to our observations in the cardiomyocytes. Given that Matrigel consists of collagen IV, laminin, entactin and HSPG (simulating the extracellular construct of cardiomyocytes), it is possible that any one of these components may bind VEGF and be accessible only to L-Hep. Indeed, compared to heparin, L-Hep was effective in releasing collagen-bound VEGF. Altogether, our results demonstrate a function of L-Hep that is unique and not simply limited to its reuptake into EC to generate active enzyme. It also addresses a fundamental
question as to why a cell would secrete an inactive protein for reuptake and activation, which appears counterintuitive and wasteful.

Following its release from the myocyte surface, VEGF has a paracrine function in EC where it stimulates new blood vessel formation (to provide O$_2$ needed for mitochondrial oxidative phosphorylation of FA), or promotes FA uptake and transport across the endothelial layer (263). However, this growth factor is also competent to provide autocrine signaling. For example, cardiomyocytes express VEGF receptors, and in these cells, VEGF induces a gene expression program of hypertrophy (264), in addition to evoking cardiomyocyte plasticity to match changes in capillary density (265). To test the existence of an autocrine pathway for VEGF control of metabolism, we focused on AMPK, a pivotal cellular energy sensor and regulator (266). At least in EC, which are quiescent, AMPK can be stimulated by VEGF, an effect that would be more relevant in the highly metabolically active cardiomyocytes. Our data indicates that recombinant VEGF also has a capacity to induce AMPK activation in cardiomyocytes. Multiple pathways produce AMPK activation including an elevation in AMP levels or a Ca$^{2+}$ dependent process involving phosphorylation by an upstream kinase, CaMKKβ. Given that VEGF increases calcium mobilization in a number of cells (267), we determined intracellular Ca$^{2+}$ concentrations in cardiomyocytes treated with VEGF. Interestingly, this growth factor caused significant Ca$^{2+}$ influx in a rapid, but transient manner. As similar Ca$^{2+}$ responses were also observed with ECCM, albeit delayed (likely due to a prior requirement for L-Hep to release VEGF), together with AMPK activation, our data for the first time suggest that cardiomyocyte derived VEGF has a novel autocrine role in stimulating AMPK.
AMPK activation governs LPL recruitment to the myocyte surface for forward movement to vascular lumen (256). The mechanisms underlying LPL recruitment embraces p38 MAPK activation with subsequent phosphorylation of the heat shock protein (Hsp). Actin monomers are released from phosphorylated Hsp25 and self-associate to form fibrillar actin. Vesicles containing LPL then move along the actin filament network, to bind to HSPG on the cardiomyocyte plasma membrane. Strikingly, using this described mechanism, we observed that VEGF was able to increase LPL translocation to the myocyte cell surface. Our data on the ability of VEGF to promote LPL movement, implicate this growth factor in the cascade of expanding actions that are geared to help the diabetic heart switch its substrate selection to predominately FA. Although Heinonen et al. recently reported a negative correlation between LPL activity and VEGF, their study differs from ours in that LPL was measured in the plasma, mouse models of atherosclerosis fed a Western-type diet were used, and VEGF-A was overexpressed by adenovirus gene transfer (261).

In summary, heparanase, LPL and VEGF together, can amplify FA delivery to the diabetic heart in the short term (T1D patients who have poor glucose control, which leads to bouts of hyperglycemia) (Fig. 3.7). If these events are prolonged, the resultant lipotoxicity could lead to cardiovascular disease in humans. Gaining more insight into the heparanase-LPL-VEGF axis may assist in devising novel therapeutic strategies to restore metabolic equilibrium, curb lipotoxicity, and help prevent or delay heart dysfunction seen during diabetes.
Figure 0.7 The summary diagram describes the mechanism of endothelial heparanase regulation of cardiac metabolism.

After diabetes and the development of hyperglycemia, there is an increased translocation of LPL from the cardiomyocyte cell surface to the apical side of endothelial cells. This process is dependent on the ability of high glucose to rapidly release EC active heparanase into the interstitial space to splice cardiomyocyte HSPG, and release bound LPL. Cardiomyocyte cell surface VEGF is also liberated by high glucose, but principally by latent heparanase. This cytokine could be a significant contributor towards enabling FA delivery (through LPL-induced breakdown of circulating triglycerides) and utilization in cardiomyocytes using AMPK as its instrument of control.
Supplementary Figure 3.1 Acute hyperglycemia augments the amount of active and latent heparanase in cardiac interstitial fluid.

A. Rats were treated with diazoxide (DZ, 100 mg/kg, i.p.), and blood samples were collected at various times from the tail vein to determine blood glucose. B. At the indicated time points, hearts were also isolated and perfused using the modified Langendorff technique,
which separates interstitial fluid from the coronary effluent. The amount of heparanase (active, A-Hep; latent, L-Hep) in the interstitial fluid was determined using Western blot. Normal rats were used as control (Con).

Supplementary Figure 3.2 Latent heparanase releases more recombinant myocyte bound VEGF than heparin.

Cardiomyocytes were isolated and incubated with increasing concentrations of recombinant VEGF (15 min). Following this time, cells were treated with either 5 U/mL of heparin or 1 µg/mL of L-Hep (30 min), and VEGF released into the medium assayed using ELISA. *Significantly different from Con, #Significantly different from the heparin-treated group, P<0.05.

Supplementary Figure 3.3 Increased VEGF expression is observed in STZ-induced chronically diabetic hearts.

Male Wistar rats (250-320 g) were injected i.v. with 55 mg/kg STZ, and animals kept for 7 days before hearts were removed. Whole heart homogenates of control (Con) and STZ animals were used to determine VEGF expression by Western blot.
**Supplementary Figure 3.4** VEGF-induced increase in cardiomyocyte LPL is catalytically active and competent to breakdown VLDL-TG.

Primary cardiomyocytes were treated with 100 ng/mL VEGF for 4 h, and heparin-releasable medium subsequently collected. This medium (rich in LPL) was then concentrated using VIVASPIN tubes, and incubated with increasing concentrations of VLDL-TG (0-0.8 mM). The concentration of released free fatty acids was determined after 30 min.
Chapter 4: Heparanase Overexpression Induces Glucagon Resistance and Protects Animals from Chemically-induced Diabetes

4.1 Premise

Heparan sulfate proteoglycans (HSPG), located mainly on the cell surface and in the extracellular matrix, are composed of a core protein to which one or more heparan sulfate (HS) side chains are attached (268). HSPGs function, not only as structural proteins, but also as anchors for bioactive molecules, as HS is negatively charged. Highly expressed in pancreatic islets, HS binds and guides the signaling and distribution of fibroblast growth factor (FGF) family members, which regulate pancreatic endocrine cell differentiation, clustering and development (269). It has been suggested that the presence of HSPG in the nucleus has a suppressive effect on histone acetyltransferase activity and may therefore modulate gene expression (270).

Heparanase is an endo-β-D-glucuronidase that is ubiquitously expressed in many organs, with blood and endothelial cells having the highest expression. Heparanase is encoded as a 65-kDa latent precursor (HepL) that requires proteolytic cleavage to form an active enzyme (HepA) (49). Functionally, HepA cleaves HS at D-glucuronic acid residues, an action associated with extracellular matrix disruption (271) and release of cell surface-bound molecules such as FGF (269). Aside from the function of HepA in cleaving HS, HepL can also activate numerous signalling elements including Erk1/2, PI3K-AKT, RhoA and Src (70; 272; 273). These signalling events, in addition to the entry of HepA into the nucleus to regulate histone acetylation by cleaving HS, have been suggested as mechanisms for modulating gene expression (270).
Heparanase has physiological functions in wound healing and hair growth (274). However, intensive research has also hinted towards an additional role of heparanase in both disease progression and prevention. Thus, in cancer, degradation of HS chains by the increased expression of heparanase is associated with ECM and BM disruption; loss of this physical barrier facilitates tumour cell invasion (253). In the pancreas, islet-enriched HS blocks immune cell infiltration (275), reduces T-cell mediated inflammation, and supports beta cell survival and function (56). Thus, removal of HS by heparanase is critical for the initiation and progression of autoimmune (Type 1) diabetes (275). Additionally, heparanase expression is induced in many organs during diabetes. In the heart, such inappropriate expression may amplify fatty acid delivery and utilization. These events, if sustained, can lead to lipotoxicity and cardiovascular disease (276). In contrast to these observations, heparanase has also been shown to have beneficial effects in some diseases. For instance, overexpression of this protein is protective against adriamycin-induced kidney injury (277). In the brain, fragmentation of HS as a result of heparanase cleavage reduced amyloid deposition in Alzheimer’s disease (278). Thus, precise temporal and spatial control of heparanase may be essential for normal cell physiology.

In the current study, when monitoring the metabolism of transgenic mice that globally overexpress heparanase (hep-tg), we made the fortuitous observation that glucose homeostasis was improved in these animals. Compared to wild type (WT) controls, these mice had lower plasma insulin and blood glucose levels; however, they exhibited higher glucagon concentrations, suggesting the presence of glucagon resistance. Given that glucagon receptor knockout (GRKO) mice exhibited increased insulin sensitivity and
resistance to diabetes (119; 147), we followed up these preliminary observations by examining whether heparanase overexpression could contribute towards improving glucose metabolism as well as resistance to chemically-induced diabetes. Our data indicate that heparanase overexpression is associated with dramatic shifts in hormones secreted from the pancreas, reorganization of islet composition and structure, significant changes in islet gene expression, and protection from streptozotocin (STZ)-induced diabetes.
4.2 Results

4.2.1 Heparanase Transgenic Mice have Improved Glucose Homeostasis

At 3 months of age, hep-tg mice had similar body weights compared to WT (hep-tg, 22.03±1.11 g; WT 22.11±1.08 g). However, the determination of circulating plasma insulin in hep-tg animals after a 6 h fast revealed lower levels compared to WT (Fig. 4.1B). Interestingly, the transgenic animals also demonstrated reduced basal blood glucose (Fig. 4.1C), suggesting higher insulin efficiency in these mice. This was tested by administering a bolus dose of insulin and measuring Akt activation in skeletal muscle. Samples of skeletal muscle from hep-tg mice showed an enhanced insulin response (Fig. 4.1D). Similar insulin response was also observed in other organs like heart and pancreas (Appendix B). To pursue this observation, we performed an OGTT after an overnight fast. Unlike in the case of an acute fast, prolonged fasting eliminated the difference in blood glucose between the two genotypes (Fig. 4.1E). In addition, although hep-tg mice tended to clear glucose faster, this improvement was not statistically significant (Fig. 4.1E), suggesting that the improvement in insulin sensitivity observed under basal conditions is not readily apparent during an OGTT. Following an insulin tolerance test, we were also unable to detect any difference in blood glucose lowering between the two groups, up to 30 minutes after insulin injection. Interestingly, after 30 minutes of insulin, we start to see a separation (recovery of glucose levels) between the WT and hep-tg mice (Supplementary Fig. 4.1). Unexpectedly, in response to the glucose challenge, insulin secretion in hep-tg animals was almost 2-fold higher than in WT (Fig. 4.1F).
Figure 4.1 Heparanase overexpression improves glucose homeostasis.

A. PCR amplification of DNA extracted from WT and hep-tg mice ear tissue. L19 is used as an internal control. B-C. Following a 6 h fast, plasma insulin and blood glucose levels were measured in WT and TG mice. n=9-10, *p<0.05 vs. WT mice. D. Intraperitoneal administration of insulin (2 µg/kg body weight) to 6 h fasted mice, followed by skeletal muscle isolation for Western Blot. n=3, *p<0.05 vs. control, "p<0.05 vs. WT insulin. E-F. Following an overnight fast (16 h), WT and TG mice were administered an oral glucose (2 g/kg) gavage. At the indicated times, blood glucose and insulin were measured. n=4-5, *p<0.05 vs. WT. G. Isolated islets from the different groups were exposed to 3 mM and 16.7 mM glucose respectively, and insulin secretion determined. n=3, *p<0.05.
In addition, islets from WT and hep-tg mice were isolated and exposed to high glucose. Hep-tg islets were capable of releasing greater amounts of insulin compared to WT (Fig. 4.1G). Although hep-tg islets appeared to contain slightly more insulin compared to WT, this difference was not statistically significant (Supplementary Fig. 4.2). Similarly, as neither *Ins 1* nor *Ins 2* expression demonstrated any significant difference between the two strains (data not shown), our data suggest that heparanase overexpression influences the secretory process of insulin rather than its synthesis.

### 4.2.2 Heparanase Overexpression Induces Glucagon Resistance and Changes in Pancreatic Islet Structure

Intriguingly, hep-tg mice showed an augmentation in circulating basal glucagon levels (Fig. 4.2A and Supplementary Fig. 4.3). However, these mice demonstrated resistance when given a glucagon challenge after 6 (Supplementary Fig. 4.4) or 16 h (Fig. 4.2B) of fasting. The glucagon resistance in hep-tg mice was evident in the absence of any change in the glucagon receptor in the liver (Supplementary Fig. 4.5). In response to L-arginine, which drives islet hormone secretion independent of glucose sensing, hep-tg mice showed higher glucagon and lower insulin secretion, effects that were reflected in a higher glucose excursion in these animals (Fig. 4.2C). To determine the basis for this observed hyperglucagonemia, we quantified pancreatic alpha cells using fluorescent staining. Expressed as glucagon positive (WT, 0.000465 ± 0.00026; hep-tg, 0.000764 ± 0.000318; p=0.433) and insulin positive (WT, 0.004056 ± 0.000991; hep-tg, 0.004608 ±0.001674; p=0.056) area individually (signal positive area versus total area of the section to represent the alpha and beta cell number in the pancreas), there was no
statistical difference between WT and hep-tg animals in the number of insulin or glucagon positive cells.

Figure 0.9 Glucagon resistance and altered islet cytoarchitecture in heparanase
overexpressing mice.

A. Plasma glucagon levels in the two groups of mice were determined after a 16 h fast. n=4-5, *p<0.05 vs. WT. B. For the glucagon challenge, 16 h fasted mice were administered glucagon (1 µg/kg), and blood glucose measured at the indicated times. n=5, *p<0.05 vs. WT. C. In 6 h fasted mice, 2 g/kg L-arginine was administered i.p. and plasma glucagon, insulin, and blood glucose (fold change in glucose was calculated by normalizing to the corresponding value at t=0) measured at the indicated times. n=5, *p<0.05 vs. WT. D. Higher magnification of insulin and glucagon positive cells in pancreas isolated from the two groups of animals using immunofluorescence staining, together with quantification of the alpha/beta cell ratio, n=3, *p<0.05 vs. WT. E. Whole pancreas sections were stained for insulin (red) and glucagon (green), scanned, and islets counted and quantified by size.

However, when the data was expressed as a ratio of alpha/beta cells, the hep-tg islets demonstrated alpha cells that were more abundant. Additionally, unlike WT islets, wherein alpha cells are mostly found in the periphery and beta cells in the core, hep-tg islets contained alpha cells that were randomly distributed (Fig. 4.2D). Islets in hep-tg mice exhibit similar average size and size distribution (Fig. 4.2E), and no difference in their number compared to WT (Supplementary Fig. 4.6). Overall, heparanase overexpression was associated with dramatic shifts in hormones secreted from the pancreas, in addition to changes in islet composition and structure.

4.2.3 Heparanase Overexpression Greatly Alters the Pancreatic Islet Transcriptome

Entry of heparanase into the nucleus to regulate histone acetylation has been put forth as a mechanism to modulate transcription. Given the impact of heparanase overexpression on insulin and glucagon, we sought to characterize the hep-tg islet transcriptome using RNA-sequencing. As expected, heparanase was the most profoundly altered mRNA in the islet transcriptome, exhibiting a >100 fold change (Fig. 4.3B). This was mirrored by an equally robust increase in heparanase protein expression (Fig. 4.3B, inset). Strikingly,
after correction for multiple testing, over 2000 genes were significantly differentially expressed with $p<0.005$. Of these, 1176 were upregulated and 985 were down-regulated (Fig. 9A, inset). From the mRNAs that increased above a cut-off (>2-fold, $p<0.001$), we identified 350 genes enriched in multiple cellular processes including in development (e.g. Crfl1, Ifrd1), metabolism (e.g. Acacb, Ppargc1a), and cell death regulation (e.g., Npas4, Igfl, P2rx1) (Fig. 4.3A). Of note, glucagon (Gcg) expression increased (Fig. 4.3B), and corresponded to the high plasma glucagon observed in hep-tg mice. Among the 112 genes that decreased (>2-fold, $p<0.001$), the most compelling effect was observed for genes related to the cell cycle (e.g., Cdc20, Ccnb) (Fig. 4.3A). In addition, glucagon receptor (Gcgr) expression was also decreased ($p=0.0004$) (Fig. 4.3B). Combining these data with a protein-protein interaction network model, we identified two functional networks that were upregulated relevant to the current study (Fig. 4.4, circles): one associated with hormone secretion (e.g. Ppy, Pyy), and the other with extracellular structure [e.g. Sdc1 (that encodes for syndecan 1), Hs3st3b1 (that encodes for heparan sulfate-glucosamine 3-sulfotransferase 3B1)] (Fig. 4.3B and Fig. 4.4). The amplification of pancreatic heparan sulfate that was observed in hep-tg mice (Fig. 4.3C) could be attributed, at least in part, to the increase in Sdc1 and Hs3st3b1. Among the downregulated genes, a highly connected network of genes related to the cell cycle function was recognized (Fig. 4.4, box). Altogether, these data suggest that its ability to modulate gene expression is consistent with the role of heparanase as a potent regulator of islet structure and function.
Figure 0.10 Islet transcriptome changes dramatically in hep-tg mice.

A. Islet RNA from WT and hep-tg mice was sequenced and the \((p<0.001)\) upregulated (red) and downregulated (green) genes were categorized based on the functions. Bar represents the enrichment of the genes in particular category over the total genes of input. B. Representative
genes in extracellular matrix, signaling and cell cycle were shown. Heparanase gene was used as positive control of the entire RNA sequencing, together with islet protein detection as inset. C. Pancreas staining for heparan sulfate using alcian blue (pH 5.8, 0.65 M MgCl₂) and its quantification using Western blot. n=3, *p<0.05 vs. WT.

**Figure 0.11 Association network of genes that were significantly different between WT and hep-tg mice.**

Analysis of a protein-protein interaction network assembled from RNAseq data. The blue square depicts the entire down-regulated network that is related to cell cycle. The red circles encompass both the hormone-associated network and the extracellular elements network. Lines represent associations based on differential expression evidence.
4.2.4 Heparanase Transgenic Mice Exhibit Resistance to Multiple Low-dose Streptozotocin

As hep-tg islets are enriched in heparan sulfate, which is important for beta cell survival, we attempted to chemically induce diabetes in these mice. Interestingly, although WT animals showed robust hyperglycemia within one week of STZ injection, hep-tg mice failed to present with plasma glucose levels comparable to WT (Fig. 4.5A). Measurement of plasma insulin provided one explanation for this observation. STZ caused a precipitous drop of plasma insulin in WT animals, an effect that was absent in hep-tg mice (Fig. 4.5B). Prolonging the duration of the study did not change the results-hep-tg mice remained resistant to STZ-induced hyperglycemia for up to 8 weeks following injection (Supplementary Fig. 4.7). This resistance to STZ was likely not due to any difference in expression of pancreatic GLUT2 (Supplementary Fig. 4.8), the transporter required for STZ uptake. To establish whether the failure to develop STZ-induced hyperglycemia was a consequence of beta cell preservation, we quantified beta cell mass. Surprisingly, upon injection of STZ, both the WT and hep-tg mice displayed an identical loss of insulin-positive cells (Fig. 4.5C). This implies that the resistance to experimental diabetes in hep-tg mice is not a consequence of beta cell survival, consistent with observations seen in GRKO mice, which do not develop diabetes when injected with STZ. Given our observation that hep-tg mice secrete more insulin in response to an OGTT (Fig. 4.5F), it is possible that the islets, which survived STZ toxicity in hep-tg diabetic mice, have a greater insulin secretory capacity. In this regard, hep-tg mice injected with STZ were still, albeit in limited capacity, able to secrete insulin in response to an IPGTT (Fig. 4.5D).
Figure 0.12 Hep-tg mice are resistant to multiple low-dose streptozotocin induced diabetes.

A-B. To induce Type 1 diabetes, WT and hep-tg mice were administered 50 mg/kg STZ for 5 consecutive days. 7 days after the last STZ injection and following a 6 h fast, tail vein blood was used for glucose and plasma insulin determination, n=5-10, *p<0.05. C. Pancreas from STZ injected mice was isolated and immune-stained for insulin (red) and glucagon (green), and insulin positive cells were quantified in the different groups. n=3, *p<0.05 vs con. D. Following an IPGTT, plasma insulin in STZ injected WT and hep-tg mice were measured over the indicated times. The data is expressed as a fold change normalized to the corresponding values at t=0 min for each animal within the two groups. n=3-5, *p<0.05 vs WT.

Taken together, our data suggest that the protective effects of heparanase against STZ-induced diabetes is unlikely to be a consequence of HS-mediated beta cell survival, but could be related to the ability of any residual beta cells in these animals to continue their secretion of insulin.

4.2.5 Severity of Diabetes Remains Disparate Between WT and Hep-tg Mice Injected with Single High Dose Streptozotocin

Multiple low-dose STZ induces beta cell death by activating immune mechanisms (279). Hence, we also tested the response of hep-tg mice to a single high dose of STZ, which produces severe diabetes by direct beta cell toxicity via DNA alkylation (238). In contrast to our observation with multiple low-dose STZ, both WT and hep-tg mice injected with 200 mg/kg STZ (single high dose) exhibited a significant reduction in plasma insulin levels (Fig. 4.6B). In agreement with the loss of insulin, WT diabetic mice demonstrated sustained hyperglycemia (Fig. 4.6A). Significantly, the values from day 3 onwards for these mice were beyond the upper limit of detection (33.3 mmol/L) of the glucometer, suggesting that the actual blood glucose concentrations were likely higher. Although high dose STZ was competent to lower insulin and induce diabetes in hep-tg mice, the magnitude of hyperglycemia was lower than that seen in WT animals (Fig. 4.6A),
suggesting the contribution of an insulin mimetic factor. Fibroblast growth factor-21 (FGF21) has significant beneficial effects on glucose homeostasis. Measurement of FGF21 plasma concentration indicated that hep-tg mice had higher concentrations compared to WT (Fig. 4.6C). Moreover, although STZ-induced diabetes caused a significant drop in FGF21 levels in WT mice, its levels in hep-tg mice was strikingly

![Graphs showing blood glucose, plasma insulin, FGF21, and GLP-1 levels in WT and TG mice](image)

**Figure 0.13** Single high dose streptozotocin in hep-tg mice uncovers protective responses to manage hyperglycemia.

**A.** A single high dose (200 mg/kg) STZ (SHD-STZ) was administered i.p., and glucose levels monitored daily over 5 days. n=4-8, *p<0.05.  
**B-D.** One week after injection of SHD-STZ,
insulin, FGF21 and GLP-1 levels from WT and hep-tg mice were determined. n=4-8, *p<0.05. amplified by three-fold (Fig. 4.6C), and was likely related to its increased hepatic production (Supplementary Fig. 4.9). Another hormone that has glucose-lowering effects independent of insulin is glucagon-like peptide 1 (GLP-1). In response to high dose STZ, GLP-1 trended in the opposite direction between these two strains (Fig. 4.6D). Thus, in hep-tg mice, driving circulating insulin concentrations down to very low levels uncovers novel protective pathways for the management of hyperglycemia.

4.3 Discussion

Homozygous transgenic mice overexpressing human heparanase globally are fertile and have a normal life span (274). In addition, physiological functions associated with heparanase in these mice include roles in embryonic implantation, food consumption, tissue remodeling, and vascularization (274). In the current study, an unexpected feature of these animals was our discovery of improved glucose homeostasis, but resistance to glucagon. Strikingly, compared to controls, hep-tg mice manifested significant changes in islet gene expression of over 2000 genes. These mice also demonstrated resistance to chemically induced diabetes. Our data suggest a novel role for heparanase in mechanisms that serve to correct hyperglycemia.

In hep-tg mice, both basal insulin and glucose levels were lower than in WT following a 6 h fast, suggesting superior insulin sensitivity in these animals. This idea was reinforced by an enhanced insulin response to activation of skeletal muscle Akt in this study. Supporting these observations is that, in myeloma cells, insulin receptor is the predominant receptor tyrosine kinase activated by heparanase (280). The lower glucose concentration was evident in the presence of high glucagon levels, suggesting a deficit in
glucagon action. Interestingly, an insulin tolerance test used to evaluate insulin efficiency was unable to detect any difference in blood glucose lowering between the two groups. Moreover, the traditional oral glucose challenge test was also unable to confirm a higher rate of glucose disposal in hep-tg mice. We reasoned that the overnight fast prior to performing the OGTT could explain this inconsistency. Following fasting, hypersensitivity of the liver to glucagon accounts for a multi-fold increase in hepatic glucose production (281). Should this happen, even marginally, in hep-tg mice that have higher basal glucagon levels, the advantage of augmented insulin sensitivity in these animals would be dampened, resulting in a similar glucose clearance compared to WT. Remarkably, in hep-tg mice, the OGTT elicited higher insulin release, whereas isolated islets from these animals had a more robust secretion of insulin in response to glucose, even with islet insulin content and gene expression remaining unchanged. Our data suggest that although heparanase appears to enhance insulin signalling, whole body tests to determine insulin sensitivity were unable to confirm this, likely as a consequence of the overriding influence of an insulin counter-regulatory hormone such as glucagon in the hep-tg mice.

In GRKO mice, a prolonged deficiency in glucagon receptor signalling activates alpha cell proliferation, and leads to a compensatory elevation in glucagon production (148). We were surprised to observe that these characteristics in GRKO mice were strikingly similar to features seen in hep-tg animals, which did not respond to a glucagon challenge as well as WT mice. More importantly, hep-tg islets presented an architecture that is conducive to hypersecretion of glucagon, under basal conditions and also subsequent to injection of arginine. In these mice, alpha cells lost their peripheral mantle-like
localization and were distributed randomly throughout the islets. In addition, the alpha/beta cell ratio was higher in hep-tg mice. These changes in alpha cells were evident even though islet size and size distribution remained unaffected. Currently, it is unclear whether these alterations in alpha cell morphology are consequences of glucagon resistance, or of disrupted pancreatic islet development through the action of heparanase on HS.

Measurement of HS in the pancreas produced an unanticipated result; rather than depleting HS, the hep-tg pancreas displayed the opposing phenotype, with increased expression of HS, and the gene \((Hs3st3b1)\) that encodes for its biosynthetic enzyme, 3-O-sulfotransferase, being dominant features. A similar observation has also been made in the hep-tg liver, where the authors described the accumulation of “heparin-like” HS degradation products, both within and between cells, resulting in an accelerated biosynthesis of HS (282). It follows that like in the liver, pancreatic heparanase processes HS into smaller heparin-like oligosaccharides. Regardless of whether a result of its enzymatic activities, or its non-traditional signalling activities, overexpression of heparanase produced a dramatic alteration in the islet transcriptome. Among the clusters of genes that were increased, of particular interest were genes that regulate cell death, synthesize extracellular matrix components, and produce pancreatic hormones. Related to cell death, this was not unusual as cancer cells use the properties of heparanase to induce gene expression and cell survival (283). The impact on the extracellular matrix is especially meaningful, as components like collagen and laminin have roles in islet cell differentiation, proliferation, and hormone secretion (284). Finally, peptide hormones like somatostatin and pancreatic polypeptide hormone are known to influence pancreatic islet
function in a paracrine manner (285; 286). Unlike the upregulated genes that were enriched for diverse functions, the downregulated genes formed a coherent functional group including those tightly linked to the cell cycle. Whether these changes in expression creates an energy-sparing environment that would be conducive to a higher level of hormone production, as recently reported (287), is certainly consistent with our observations and deserving of further investigation. Collectively, our data provide support for the notion that heparanase in the pancreas is particularly beneficial, reinforcing it against the effects of exogenous stress.

Multiple low-dose STZ mimics Type 1 diabetes by stimulating beta cell apoptosis through the recruitment of immune cells. Given the role that HS plays in modulating the innate immune response by effecting immune cell adhesion, cytokine and chemokine binding, and providing a physical barrier against leukocytes infiltration, an increase in HS as seen in hep-tg mice could be potentially beneficial. This, when added to the genomic signature in these mice that is protective against cell death, one would anticipate a resistance to MLD-STZ beta cell cytotoxicity. Indeed, our results, for the first time, demonstrate that hep-tg animals developed less severe hyperglycemia compared to WT, and that this was associated with an almost unchanged circulating insulin concentration. Inexplicably, the preserved plasma insulin in these animals was not a consequence of higher beta cell mass, as quantification of these cells using immunostaining indicated that hep-tg beta cells were destroyed to an equal extent compared to WT. It is possible that, of the beta cells that survived STZ toxicity in both groups, an enhanced insulin secretory capacity in hep-tg animals could explain this anomaly. In support, hep-tg diabetic animals can still increase insulin secretion in response to a glucose challenge, an effect that is lost
in WT diabetic mice. Our data suggest that heparanase produces beta cells that are more functionally efficient, an effect likely related to an elevation of 3-O-sulfotransferases and accumulation of HS in these islets.

STZ in high doses induces diabetes by a mechanism different from MLD-STZ; it directly destroys beta cells by alkylating DNA. Additionally, as there is a comparatively more severe and rapid development of hyperglycemia related to a catastrophic loss of insulin, we tested whether heparanase would continue to correct hyperglycemia in this model. Despite the identical low levels of residual circulating insulin in both diabetic groups, hep-tg mice continued to have significantly lower blood glucose. In GRKO mice, FGF21 and GLP-1 have been suggested to be the hormones responsible for glucose clearance in the absence of insulin (150). This was also true in the hep-tg diabetic mouse. In these animals, regardless of their basal concentrations, SHD-STZ caused an augmentation of both FGF21 and GLP-1, effects that can contribute to their lower glucose levels. Altogether, the results indicate that, with the onset of hyperglycemia, additional glucose lowering mechanisms are triggered in hep-tg mice.

It has been proposed that HS in pancreatic islet BM functions as an obstruction against leukocyte infiltration, in addition to protecting the beta cell against ROS induced cell death (211). Thus, in the NOD mouse, augmented production of active heparanase by immune cells permits destruction of islet BM HS, entry of leukocytes, and degradation of intracellular HS. As the heparanase inhibitor PI-88 preserved intra-islet HS and protected NOD mice from T1D, the authors concluded that heparanase inhibition is useful against T1D progression. Contrasting results were observed in NOD mice injected with exogenous heparanase, which ameliorated the occurrence of diabetes (213). Unlike the
NOD mice or metastatic cancer cells (288) where the overproduction of active heparanase is responsible for disease development, our model is one in which, in a disease free background, latent heparanase is overexpressed, with an associated amplification of islet HS. Thus, although active heparanase has been considered a pathogenic marker, our study has discovered multiple novel properties of latent heparanase related to the control of glycaemia. Overexpression of this protein caused glucose lowering, potentiation of insulin secretion, HS induction, prodigious changes in islet gene expression, and protection against chemically induced diabetes. This study unlocks the possibility of utilizing these properties of heparanase in the management of diabetes.
4.4 Supplementary Materials

Supplementary Figure 4.1 IPITT in WT and hep-tg mice.
I.p. administration of insulin (2 g/kg body weight) to 6 h fasted mice, followed by measurement of glucose at the indicated times. n=7-8

Supplementary Figure 4.2 Insulin content in the islets from WT and hep-tg mice.
10 islets from WT and hep-tg mice were pooled respectively and insulin extracted with Acid-Ethanol. The insulin content was measured with ELISA.
Supplementary Figure 4.3 Glucagon levels and glucagon challenge in WT and hep-tg mice following 6 h fasting.

Plasma glucagon levels from the two groups of mice were measured after 6 h fast. Additionally, 6 h fasted mice were administered glucagon (1 ug/kg), and blood glucose measured at the indicated times. n=5 *p<0.05

Supplementary Figure 4.4 Hepatic glucagon receptor expression remains unchanged in hep-tg mice.

Liver from WT and hep-tg mice was homogenized, and protein extracted from detection of glucagon receptor expression. The antibody is from polyclonal rabbit antiserum raised against an extracellular portion of the glucagon receptor, which is a kind gift from Dr. Kieffer.
Supplementary Figure 4.5 Heparanase overexpression does not change the number of pancreatic islets.

Islet number was determined following synaptophysin (A) and insulin (B) staining of pancreas sections. Absolute islet number per section is presented on the upper row, whereas the ratio normalized to the total pancreas area is on the lower row.

Supplementary Figure 4.6 Hep-tg mice remain resistant to STZ-induced hyperglycemia for up to 8 weeks following MLD-STZ injection.
Blood glucose levels of MLD-STZ treated animals were monitored for 8 weeks.

**Supplementary Figure 4.7 Resistance of hep-tg mice to STZ is not due to difference in expression of pancreatic GLUT2.**

Pancreas sections from WT and hep-tg mice were stained with insulin (red) and GLUT2 (green), and representative images of islets are shown.

**Supplementary Figure 4.8 Increased hepatic production of FGF21 in hep-tg mice following SHD-STZ injection.**

Whole liver lysate was used for detection of FGF21 using an ELISA. N=3-5, * p<0.05 vs. WT; @ p<0.05 vs. TG Con.
Chapter 5: General Discussion

5.1 General Discussion and Conclusion

FA is the predominant substrate that the heart uses for energy during diabetes, and it does so by expanding its coronary LPL pool. This amplification in LPL emerges as an outcome of enzyme movement to the cardiomyocyte cell surface, with subsequent forward transfer to the coronary lumen. The latter event is dependent on EC heparanase secretion, which cleaves heparan sulfate to release bound LPL, but also VEGF. Jointly, heparanase, LPL and VEGF amplify FA delivery and utilization by the diabetic heart in the short term (T1D patients who have poor control of glucose leading to bouts of hyperglycemia), and acts as a compensatory mechanism when the heart cannot utilize glucose. One additional caveat is that to metabolize excess FA, the diabetic heart has a higher demand for oxygen, and angiogenesis is an expected necessity, with the heparanase released VEGF assisting in increasing capillary density. Hence, this growth factor may initially assist with FA oxidation in the diabetic heart by promoting efficient supply of oxygen. One drawback of these effects is excessive production of ROS and progressive oxidative damage (289). In chronic diabetes, down regulation of VEGF and its receptor occurs, leading to impaired myocardial angiogenesis and an insufficient capacity to dispose of FA (290; 291). This, together with a decrease in cardiac heparanase expression, will result in cardiac TG accumulation and the resultant lipotoxicity could lead to cardiovascular disease in humans (264). Therefore, we are proposing that the rapid release of endothelial heparanase following hyperglycemia allows the heart to switch its substrate utilization to FA when glucose use is not possible.
As diabetes decreases the expression of heparanase, we were unable to determine the long-term consequences of heparanase on the cardiac metabolism and the development of cardiomyopathy. We therefore attempted to induce diabetes in mice that have global overexpression of heparanase, expecting a reduction in cardiac TG accumulation and the development of heart failure.

Homozygous transgenic mice overexpressing human heparanase globally not only have normal fertility and life span, but also demonstrated improved glucose homeostasis and resistance to chemically induced diabetes. Under basal condition, although hep-tg mice appeared more insulin sensitive, the traditional IPGTT and IPITT could not detect the difference in vivo, which could be attributed to the presence of hyperglucagonemia. This hyperglucagonemia is based on the higher percentage of alpha cells and disrupted islet structure, a phenomenon that is consistent with a great alteration of transcriptome in the islet. In addition, HS is elevated together with its sulfation enzyme Hs3st3b1. It has been proposed that HS in pancreatic islet BM functions as an obstruction against leukocyte infiltration, in addition to protecting the beta cell against ROS induced cell death. Thus, in the NOD mouse, augmented production of active heparanase by immune cells permits destruction of islet BM HS, entry of leukocytes, and degradation of intracellular HS. It has been claimed that heparanase inhibition is helpful against T1D progression. However, injection of recombinant heparanase to these mice reduced the occurrence of diabetes significantly. Unlike the NOD mice or metastatic cancer cells where the overproduction of active heparanase is responsible for disease development, our model is one in which, in a disease free background, latent heparanase is already overexpressed, with an associated amplification of islet HS. Thus, although active heparanase has been
considered a pathogenic marker, our study has discovered multiple novel properties of latent heparanase that appears beneficial. Overexpression of this protein caused glucose lowering, potentiation of insulin secretion, HS induction, prodigious changes in islet gene expression, and protection against chemically induced diabetes. This study unlocks the possibility of utilizing these properties of heparanase in the management of diabetes.

5.2 Caveats and Limitations

Though a new mechanism of heparanase in mediating heart metabolism is elucidated in Chapter 3, more experiments are needed to closely mimic the in vivo condition. For instance, recombinant heparanase used to release VEGF in this study is much higher than the amount present in vivo, therefore, physiological concentration of heparanase in the plasma following diabetes need to be identified. Although heparanase is mainly from ECs, the in vivo resources of heparanase in the heart could be more complex because blood cells are also a major factory for heparanase production. In addition, there are more than 100 identified molecules bound on myocyte cell surface. Thus there is a significant possibility that factors other than VEGF and LPL are released, which could also participate in heart metabolism. Screening the factors that exert different function in the heart is an experiment designed for the future.

In Chapter 4, the major drawback of the study is the systematic effect of heparanase overexpression that could have influence on organs other than the pancreatic islet. As liver and adipose tissue play critical roles in overall glucose homeostasis, the failure of observing improved insulin sensitivity in vivo could be concluded only after careful examination of heparanase’s effect in these tissues. In addition, the expression of active and latent heparanase in different organs also varies (Appendix A). Hence, the effect of
heparanase is dependent on the tissue specific form of the protein, and requires additional research.

5.3 Future Work

This thesis opened many doors for novel heparanase functions, yet much more investigation is needed for exploring heparanase’s beneficial functions.

Previous studies in our lab have observed that heparanase was detected in cardiomyocyte nucleus; a RNAseq of cardiomyocyte overexpressing heparanase would help to identify this protein’s role in shaping myocyte genome. Data from islet sequencing have demonstrated 350 genes that are influenced by heparanase overexpression enriched in multiple cellular processes including in development (e.g. Crif1, Ifrd1), metabolism (e.g. Acacb, Ppargc1a), and cell death regulation (e.g., Npas4, Igf1, P2rx1). If these genes were also influenced by heparanase overexpression in the heart, the overall heart metabolism and its tolerance to exogenous stress could be greatly affected and is an exciting area of future research. Moreover, as heparanase is not expressed in cardiomyocytes, the mechanism by which this protein is taken up by the cardiomyocytes, activated in lysosome and how it is delivered into nucleus would be an addition to current understanding of heparanase’s cellular processing.

Beyond its function in the heart, how heparanase overexpression influences the whole body metabolism including liver and adipose tissue should be examined in the future. To extend the understanding of heparanase’s effect in the metabolic disorder, high fat diet treatment of hep-tg mice would be a good model.

In my thesis, I used STZ as a diabetes inducer that had limited effect on hep-tg mice. We uncovered FGF21 and GLP-1 as potential insulin independent regulators of whole body
glucose homeostasis in hep-tg diabetic mice. Therefore, knocking down or removal of these two molecules separately following SHD-STZ injection in hep-tg mice would be helpful in deciphering their respective roles in protecting hep-tg mice from chemically induced diabetes. In addition, other models of diabetes should be also applied such as NOD, which is an immune system triggered diabetes model. In these mice, heparanase was found to be low in resting immune cells, but increased dramatically in active immune cells and this elevation is associated with HS loss and beta cell death. It would be interesting to see whether the occurrence of diabetes in NOD mice is reduced in a setting that already contains high amount of heparanase and HS. In the end, overexpression of heparanase has indicated a robust influence on pancreatic islet genome and cytoarchitecture, particularly on alpha cell proliferation, which could contribute to the protection effect against STZ. It is not clear whether this alteration in islet composition is initiated in embryo or happened after birth. Therefore, heparanase and HS function in pancreatic development, particularly in pancreatic islet formation, endocrine cell differentiation and clustering, extracellular matrix protein composition should be examined.
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Appendices

Appendix A  Heparanase expression in various organs, which is used in Chapter 4

![Image of Islets]

![Image of Heart]

![Image of Skeletal Muscle]
Appendix B  Akt activation in the heart and pancreas following insulin injection, which is used in Chapter 4