Slit2-Robo Signaling Pathway as a Potential Target for
Attenuating Pathological Diabetic Glomerular Angiogenesis

by

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A thesis submitted in conformity with the requirements
for the degree of Medical Science

Institute of Medical Sciences
University of Toronto

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Abstract

A hallmark of diabetes, the leading cause of kidney failure, is glomerular endothelial cell injury, where pathological angiogenesis contributes to hyperfiltration, a common feature linked to progression of renal injury. No clinically available therapies safely target this process. Slit2 has emerged as a regulator of endothelial function, eliciting either pro- or anti-angiogenic effects through its Robo1 and Robo4 receptors respectively.

Glomerular endothelial cells grown in high glucose medium expressed lower levels of Robo4, but not Robo1, and had greater response to vascular endothelial growth factor, while Robo1 knockdown inhibited VEGF-induced tube formation and migration. While healthy glomerular endothelial cell expressed high levels of Robo4 and low levels of Robo1 in vivo, Robo4 levels fell while Robo1 did not significantly change. Four weeks
post-streptozotocin-induced diabetes, Robo4 knockout mice kidneys demonstrated increased glomerular endothelial cell density.

Our observations suggest that diabetic glomerular angiogenesis is promoted by a reduction in anti-angiogenic Slit2-Robo4 activity.
Acknowledgments

I have been so very blessed to be surrounding by amazing personalities that have shaped me for the best throughout these past two years. First and foremost, this thesis and project would not have been possible without the wonderful support I received from my supervisor and mentor, Dr. Darren Yuen. His style of mentorship let me to not only design and lead my own experimental projects, but also allowed me to build on my interpersonal and leadership skills. For your patience, support, and your guidance, I wish to extend to you my sincerest gratitude.

As a researcher, I have met several collaborators, and none of them have been as dedicated to the development of my project as has the members on my advisory committee, Dr. Andrew Advani and Dr. Philip Marsden. I have been very fortunate to have such experts helping me perfect my work. For your open-door-policy, and for your invaluable advice, I humbly thank you.

Late nights, long days, and frustrating experimental results are common occurrences in the laboratory. These were made far more bearable, and even enjoyable, with the help of my good friends Stephen Szeto and Hoyee Wan. You guys have made the slow days fly by, and have been pivotal in me achieving joy every day in the lab. Thank you for your insightful and wonderful discussions on both research and non-research related topics. Thank you for pushing and helping me achieve so much in these past couple of years. Thank you for celebrating my accomplishments, and for encouraging me to learn from my shortcomings. And very importantly, thank you for making us the dominant trio we are in basketball courts near and far!
I at last speak about my most valuable asset, and the very foundation upon which I have developed my love for research, community service, and medicine and science. I have been immensely blessed to have been provided with such an incredibly, supportive, encouraging, and inspirational family.

My late father, Dr. Mohammad Omar Sidiqi, may Allah (SWT) shower him with his mercy, was my role model, my inspiration, and the physician and scientist I emulate to be. He was first to show me how medicine and science are two faces of the same coin, and how wonderfully rewarding it is to connect with patients and to take part in their healing. My values, my beliefs, my achievements, and who I am today, is because of my dear and beloved father. It is to Padar Jan, and to his memory, that I dedicate this thesis.

My beloved mother, Shafika Sidiqi, who has been a warm and caring mother, who now also plays the role of my father, has been my biggest fan and supporter. You have supported me in ways no one else can, without which I would have collapsed before I even began this project. Your bravery and warmth is truly inspirational, and I strive to have the kind of heart you have. Thank you for everything Mawdar Jan.

My amazing sisters, Aysha, Fatima, Maryam, Amena, and my fantastic brothers, Jamal Jan and Abduwlahab, you all have helped me grow and develop into the person I am today. I especially want to thank my nephew Zacharia, who has filled my life with happiness, and whose escapades always keep me on alert! Nothing is dearer to me than your wellbeing and your happiness. Thank you for being there for me when I have
needed you most. Thank you for filling my life with love and joy. And thank you for always believing in me and for inspiring me to achieve my potential.

To my family all over the world, your outpouring of support and encouragement has been nothing but amazing. My respected Grandfather’s academic achievements taught me the importance of contributing to scholarly knowledge. My dear Engineer Kaka and Mama Jans, Dr. Kaka Jans, Lala Kaka Jan, Dada Kaka Jan, Shireen Kaka Jan, Khala Jan and Ama Jans, thank you all for your support over the years, and especially over the last two years. You have been exemplary Uncles and Aunts. I cannot thank you enough for your wisdom, advice, encouragement, and for your support. I especially want to sincerely thank my honourable grandmother, who has been like a second mother to me, and whose unwavering encouragements and support has allowed for her children and grandchildren to achieve success in this life, and in the hereafter. Thank you for providing us the foundation upon which we build ourselves on.
Contributions

In this thesis, I was responsible for all the *in vitro* and *in vivo* experimental designs and execution, ordering of reagents, data analysis, and figure and text preparation.

The migration and network formation was modified from Dr. Darren Yuen’s protocol, with input by Kerri Tai and Yi-wei Huang.

The Western immunoblotting experiments were performed with the training and support from our lab manager, Mingliang Lu.

The flow cytometry experiment and image was generated with the help of Dr. Christopher Spring.

The confocal microscopy images were generated with the assistance and the guidance of Caterina Di Ciano-Oliveira.

Krystale De Freitas and Melissa Mitchell were responsible for breeding the animals, maintaining the newborn pups, and notching the mice for identification purposes. I was responsible for genotyping the animals, conducting metabolic caging and collecting the relevant urine and blood samples, collecting weekly blood-glucose measurements, inducing diabetes to the mice, providing daily administration of mashed foods, cage replacement, and saline treatments to the diabetic mice.

Dr. Golam M. Kabir conducted the invasive blood pressure measurements, perfusion of the animals, and collection of organs at end study. I processed and embedded the kidney tissue into paraffin wax.
Dr. Rohan John and his team were responsible for cutting and staining the kidneys with PECAM-1 antibody.
# Table of Content

Acknowledgments ........................................................................................................ iv

Contributions .................................................................................................................. vii

Table of Content ............................................................................................................. ix

Abbreviations .................................................................................................................. xii

List of Tables .................................................................................................................. xv

List of Figures .................................................................................................................. xvi

Chapter 1 ......................................................................................................................... 1

## Introduction

1.1 Diabetes .................................................................................................................... 2

   1.1.1 Background: Definitions .................................................................................. 2

   1.1.2 The cost of diabetes ....................................................................................... 3

1.2 Diabetic vascular complications ............................................................................. 4

   1.2.1 Diabetic Nephropathy .................................................................................... 5

1.3 DIABETIC GLOMERULOPATHY .......................................................................... 14

   1.3.1 Major cell types in glomeruli ......................................................................... 14

   1.3.2 Glomerular ECs in diabetic nephropathy ....................................................... 17

1.4 Slit-Robo signaling: an important pathway in development and disease ............ 22

   1.4.1 Slit – isoforms, structure and localization ...................................................... 22

   1.4.2 Robo receptors – isoforms, structure and localization .................................... 24

   1.4.3 Slit-Robo signaling – role in embryogenesis, and in adult organisms .......... 25

1.5 The use of HRGECs as an in vitro model .............................................................. 32

1.6 Diabetic nephropathy animal models .................................................................. 33

   1.6.1 STZ model of diabetes .................................................................................... 34

   1.6.2 The db/db mouse model of diabetes ............................................................... 35

   1.6.3 Diabetic-renal complications of animal models ............................................. 35

1.7 Preliminary work .................................................................................................... 36

   1.7.1 Microvascular human glomerular renal ECs express more Robo4 than Robo1.. 37

   1.7.2 Robo4 and Robo1 expression in diabetic animal models ......................... 40
1.8 HYPOTHESIS.................................................................................................................................43

i) HRGEC Responsiveness to VEGF is Regulated by Robo1 and Robo4 ..................44

Chapter 2: ........................................................................................................................................45

HRGEC Responsiveness to VEGF is Regulated by Robo1 and Robo4 ..................45

2 **In vitro HRGEC Experiments** ........................................................................................................46

2.1 Introduction ...................................................................................................................................46

2.2 Specific Aims and Hypothesis ......................................................................................................46

2.3 Materials and Methods .................................................................................................................48

2.3.1 Human renal glomerular endothelial cell (HRGEC) culture .............................................48

2.3.2 Glucose stimulation ....................................................................................................................48

2.3.3 Immunoblotting ........................................................................................................................49

2.3.4 Flow Cytometry ........................................................................................................................49

2.3.5 Robo1 silencing ..........................................................................................................................50

2.3.6 Matrigel™ Network Formation Assay ......................................................................................50

2.3.7 Boyden-Chamber HRGEC Migration Assay ..........................................................................51

2.4 Statistical analysis ..........................................................................................................................52

2.5 Results ..........................................................................................................................................53

2.5.1 Characterization of primary HRGECs .....................................................................................53

2.5.2 High glucose exposure enhances VEGF-induced HRGEC migration and network

formation ...............................................................................................................................................55

2.5.3 Altering the levels of Robo4 and Robo1 regulates angiogenesis in HRGECs .....................59

2.6 Discussion ......................................................................................................................................66

2.7 Limitations ......................................................................................................................................68

2.8 Conclusions ....................................................................................................................................69

3 **In vivo Robo4 KO mice Experiments** ..........................................................................................71

3.1 Introduction ...................................................................................................................................71

3.2 Specific Aims and Hypothesis ......................................................................................................71

3.3 Materials and Methods .................................................................................................................73

3.3.1 Breeding of Robo4 WT and KO mice .......................................................................................73

3.3.2 Diabetes induction .......................................................................................................................74

3.3.3 Metabolic monitoring .................................................................................................................75
3.3.4 Invasive blood pressure measurements ................................................................. 75
3.3.5 End study sample collection .................................................................................. 76
3.3.6 Tissue preparation and histology .......................................................................... 77
3.3.7 Renal fluorescence microangiography .................................................................... 77
3.3.8 Confocal microscopy of FMA sections ................................................................... 79
3.3.9 Statistical analysis ................................................................................................. 80
3.4 Results ....................................................................................................................... 80

3.4.1 The role of Robo4 in the regulation of glomerular angiogenesis in experimental
diabetic nephropathy ................................................................................................. 80
3.4.2 Generation of male Robo4 knockout and wild type mice ....................................... 81
3.4.3 Metabolic parameters of Robo4 KO and wild type mice ....................................... 82
3.4.4 Robo4 deficiency enhances diabetic glomerular angiogenesis ............................. 86
3.5 Discussion .................................................................................................................. 92
3.6 Limitations ............................................................................................................... 92
3.7 Conclusions .............................................................................................................. 94

4 Overall Discussion ..................................................................................................... 95

4.1 Summary of Results ............................................................................................... 95
4.2 Limitations ............................................................................................................... 95

5 Conclusions and Future Directions ......................................................................... 98

5.1 Future Directions .................................................................................................... 98
5.2 Overall Conclusion ................................................................................................. 103

References ................................................................................................................... 104
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin II Converting Enzyme</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycosylation Products</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II Receptor Blocker</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double Distilled Water</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>CC</td>
<td>Conserved Cytoplasmic</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-Regulated Kinase</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>Eph</td>
<td>Ephrin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FMA</td>
<td>Fluorescence Microangiography</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAP</td>
<td>Guanosine Trinucleotide Phosphatase Activating Protein</td>
</tr>
<tr>
<td>GDM</td>
<td>Gestational Diabetes Mellitus</td>
</tr>
<tr>
<td>GEC</td>
<td>Glomerular Endothelial Cell</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Trinucleotide Phosphate</td>
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</tbody>
</table>
GTPase  Guanosine Trinucleotide Phosphatase
HG      High Glucose
HMVEC  Human Microvascular Endothelial Cells
HRE    Hypoxia Response Element
HRGEC  Human Renal Glomerular Endothelial Cells
HS     Heparin Sulfate
HUVEC  Human Vein Umbilical Endothelial Cells
IgG    Immunoglobulin
I.P    Intra-Peritoneal
KDR    Kinase Insert Domain Receptor
KO     Knockout
LRR    Leucine Rich Repeats
MAP    Mitogen-Activated Protein
Man    Mannitol
NG     Normal Glucose
NO     Nitric Oxide
NOS    Nitric Oxide Synthase
PBS    Phosphate Buffered Saline
PDGF   Platelet Derived Growth Factor
PECAM  Platelet Endothelial Cell Adhesion Molecule
PI3K   Phosphotidylinositol-3-Kinase
PVDF   Polyvinylidene Fluoride
qRT-PCR Quantitative Real Time Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Robo</td>
<td>Roundabout</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per Minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Mean Error</td>
</tr>
<tr>
<td>srGAPS</td>
<td>Slit-Robo Guanosine Trinucleotide Phosphatase Activating Proteins</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 Diabetes Mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TGH</td>
<td>Toronto General Hospital</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
List of Tables

Table 1: Parameters of Mice at end-study (12 Weeks of Age)................................. 83
List of Figures

Figure 1: Robo4 mRNA levels drop, while Robo1 levels remain unchanged after 48 hours of high glucose (HG) treatment, relative to the housekeeper transcript Rpl13a..................................................................................................................................................38

Figure 2: Robo4 protein levels are diminished after 72 hours of HG treatment in HRGECs..................................................................................................................................................39

Figure 3: Diabetes is associated with a reduction in renal Robo4 to Robo1 mRNA ratio in two rodent models...............................................................................................................................................41

Figure 4: Diabetes is associated with reduced glomerular Robo4 protein levels, while Robo1 levels remain unchanged in STZ-Wistar rats..................................................................................................................42

Figure 5: Overall schematic for in vitro..................................................................................................................................................47

Figure 6: Primary HRGECs express cell surface markers characteristic of microvascular endothelial cells..................................................................................................................................................54

Figure 7: Exposure to high glucose enhances VEGF-induced HRGEC migration. ..................................................................................................................................................................................56

Figure 8: Exposure to high glucose enhances VEGF-induced HRGEC network formation..................................................................................................................................................................................58

Figure 9: Confirmation of specific Robo1, but not Robo4, knockdown..................................................................................................................60
Figure 10: Robo1 deficiency abrogates the high glucose-induced augmentation of VEGF-induced HRGEC migration.................................................................62

Figure 11: Robo1 deficiency abrogates the high glucose-induced augmentation of VEGF-induced HRGEC network formation.................................................................64

Figure 12: Overall schematic for in vivo.................................................................72

Figure 13: Sample genotyping results showing Robo4 WT, KO and +/- mice.................................................................81

Figure 14: Diabetes is associated with renal hypertrophy in both Robo4 knockout (KO) and wild type (WT) mice.................................................................85

Figure 15: Robo4 deficiency is associated with enhanced diabetic glomerular angiogenesis.................................................................87

Figure 16: Robo4 deficiency enhances diabetic glomerular angiogenesis.................................90

Figure 17: Robo4-GFP plasmid map.................................................................99

Figure 18: Rat kidney fibroblasts transfected with Robo4-GFP plasmid.................................100
Chapter 1

Introduction
1.1 Diabetes

1.1.1 Background: Definitions

Diabetes mellitus (DM) is a collection of diseases characterized by elevated blood glucose levels (hyperglycemia). This hyperglycemia classically manifests as polydipsia (excessive thirst), polyphagia (excessive hunger), polyuria (excessive urination), glucosuria (excessive glucose in the urine), and/or weight loss, amongst other symptoms. Accordingly, the pathogenesis of diabetes typically involves defects in insulin production and/or responsiveness (Daneman, 2006; DeWitt & Hirsch, 2003). Diabetes can be categorized into three major categories: (1) Type 1, (2) Type 2, and (3) gestational.

Type 1 diabetes mellitus (T1DM) is characterized by idiopathic autoimmune-related destruction of β-cells, leading to impaired insulin secretion. T1DM accounts for roughly 5% of all cases of diabetes worldwide (Meltzer et al., 1998). Patients with T1DM frequently experience weight loss, and are typically diagnosed during childhood. Given that insulin production is their primary defect, these patients require daily administration of insulin for survival (Daneman, 2006).

Type 2 diabetes (T2DM) is characterized by peripheral insulin resistance and at later stages, a relative insulin deficit. Accounting for nearly 95% of all cases of diabetes worldwide, the prevalence of T2DM has risen exponentially over the last 2 decades, in part due to the growing incidence of obesity worldwide (Goeree et al., 2009).
The third and final form of diabetes is Gestational Diabetes Mellitus (GDM), which affects 2-10% of pregnant women (Boney, Verma, Tucker, & Vohr, 2005). Most women, diagnosed with GDM have β cell dysfunction and chronic insulin resistance (Boney et al., 2005). About 5-10% of women who develop GDM also have circulating antibodies against β cells, similar to T1DM patients (American Diabetes Association, 2004). Insulin treatment, monitoring of diet, and use of oral hyperglycemic drugs, before and after pregnancy, are used to maintain the health of the mother and the child (Glueck, Goldenberg, Streicher, & Wang, 2003; Hellmuth, Damm, & Mølsted-Pedersen, 2000; Jacobson et al., 2005). While this form of diabetes is typically transient, it is important to treat it early because the developing fetus is also at risk of poor perfusion from diabetes-related vascular impairment, and over-nutrition (Bellamy, Casas, Hingorani, & Williams, 2009).

1.1.2 The cost of diabetes

The prevalence of diabetes in Canada has increased exponentially over the past two decades (Dawson, Gomes, Gerstein, Blanchard, & Kahler, 2002). It is estimated that by 2020, 1 in 10 Canadians will be diagnosed with this disease (Dawson et al., 2002). Alarmingly, in every age group, the risk of death is twice as high for diabetic patients, and nearly 30% of all Canadians who died in 2011 had diabetes (Public Health Agency of Canada, 2011). In particular, people of Aboriginal descent are three to five times more likely to develop T2DM (Goeree et al., 2009). However, the burden of diabetes is shared amongst all Canadians, as the cost of treating diabetic patients is
four times greater than in non-diabetic groups (Public Health Agency of Canada, 2011). Indeed, diabetes now accounts for 3.5% of public health care spending in Canada, and these costs are expected to increase in the coming years (Dawson et al., 2002). With such a significant socio-economic impact, the development of effective preventative measures, as well as efficient and novel treatments for diabetes, will be critical to curb its effects.

1.2 Diabetic vascular complications

One of the earliest manifestations of uncontrolled hyperglycemia is endothelial injury. Classically, diabetic vascular injury has been divided into microvascular (neuropathy, retinopathy, and nephropathy) and macrovascular complications (coronary artery disease, cerebral vascular disease, and peripheral vascular disease). Each type is associated with increased morbidity and mortality (Klein, 1995).

In diabetic neuropathy, poor perfusion leads to nerve injury and associated numbness, especially in the peripheral appendages. This, combined with retarded wound healing from cuts and scrapes, can lead to serious infections, which may lead to gangrene and eventual amputation (Duby, Campbell, Setter, White, & Rasmussen, 2004).

Diabetes is also one of the leading causes of blindness in the developed world. Diabetic retinopathy is characterized initially by pathologic hypoxia in the retina that leads to up-regulation of angiogenic growth factors as a response to promote new blood
vessels formation. These new vessels, however, tend to be leaky, and the resulting retinal edema leads to progressive loss of vision (Cooper et al., 1999; Goldin, Beckman, Schmidt, & Creager, 2006).

1.2.1 Diabetic Nephropathy

1.2.1.1 Background

Diabetes accounts for nearly 50% of all cases of end-stage renal disease (ESRD) in the United States and Canada (Lippert, Ritz, Schwarzbeck, & Schneider, 1995). Approximately 20-30% of all diabetic patients will have some form of nephropathy, with nearly 50% of T1DM patients progressing to ESRD (Jorge L. Gross et al., 2005). Long recognized as one of the earliest features of diabetic kidney disease, hyperfiltration usually precedes the development of albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis (Jerums, Premaratne, Panagiotopoulos, & Maclsaac, 2010; Vora & Ibrahim, 2003). Multiple epidemiologic studies have linked the presence and/or degree of hyperfiltration to the development of later manifestations, including microalbuminuria (Magee et al., 2009; Ruggenenti et al., 2012; Yip, Jones, Wiseman, Hill, & Viberti, 1996), decline in glomerular filtration rate (GFR) (Ruggenenti et al., 2012), and even death (Groop et al., 2009). Pathophysiologically, hyperfiltration of individual nephrons has been linked to subsequent kidney injury, including the development of renal fibrosis and impaired renal function (Anderson, Rennke, & Brenner, 1986). Over time, the kidney develops progressive glomerulosclerosis and
tubulointerstitial fibrosis, along with a steady decline in glomerular filtration rate (GFR), podocyte loss and endothelial injury (Ernst, 1993; J. L. Gross et al., 2004; Lippert et al., 1995).

The mechanisms that promote hyperfiltration in the diabetic kidney are manifold. Historically, attention has focused on tubuloglomerular feedback as a major hemodynamic mechanism that enhances glomerular filtration in diabetes (Hryciw, Lee, Pollock, & Poronnik, 2004; Thomson et al., 2001; Vallon, RICHTER, BLANTZ, THOMSON, & OSSWALD, 1999). Specifically, in the diabetic kidney, excess sodium/glucose reabsorption in the proximal tubule, driven by the increased glucose filtered into the urine, reduces the sodium/chloride concentration detected by the macula densa of the juxtaglomerular apparatus, leading to afferent arteriolar dilation and increased glomerular perfusion (Vallon et al., 1999). Superimposed upon this phenomenon is activation of the renin-angiotensin system, which leads to preferential efferent arteriolar constriction, and enhanced glomerular hypertension and filtration. This enhanced filtration, in turn, promotes enhanced proximal tubular sodium reabsorption, which exacerbates the tubuloglomerular feedback mechanism described above (Chiolero, Maillard, Nussberger, Brunner, & Burnier, 2000)

Conventional treatment of diabetic nephropathy has focused primarily on controlling hypertension (using angiotensin converting enzyme (ACE) inhibition and angiotensin II receptor blocker (ARB), and blood glucose levels (through diet, exercise, oral agents, and insulin injections) (Ruggenenti et al., 2012). Unfortunately, despite these therapies, many patients continue to experience GFR decline, with a significant number progressing to ESRD, at which point renal replacement therapy is necessary for
survival. The cost goes beyond the physical and mental trauma to the patient, as the healthcare system pays nearly $100,000/patient/year to care for patients with ESRD (Menzin et al., 2011).

1.2.1.1.1 Angiogenesis

Angiogenesis is the process of forming new blood vessels from pre-existing ones (J Folkman, 1995). As the ability of oxygen to diffuse into tissue is limited to a distance of 100-200µm (Judah Folkman & Kalluri, 2003), angiogenesis is important for normal development, as a highly vascularized system is necessary for overcoming the oxygen demands of complex organisms. It is also crucial for both physiologic and pathophysiologic processes in adult organisms (Judah Folkman & Kalluri, 2003). Not surprisingly, angiogenesis is a highly regulated process, controlled by a balance of positive and negative growth stimuli.

The first step in angiogenesis is characterized by degradation of the vascular basement membrane in response to angiogenic stimuli. Neighbouring endothelial cells (ECs) migrate into this opening to begin formation of the branching neovessel (Risau, 1997). Several ancillary cell types, such as pericytes, smooth muscle cells, fibroblasts, and epithelial cells are also recruited in order to provide support for these new vessels (Ribatti & Crivellato, 2012; Risau, 1997).

Within a newly forming vessel, the migrating ECs differentiate into tip and stalk cells that are critical in the sprouting process (Lobov et al., 2007). Tip cells are found at
the tip of the sprouting neovessel. They are migratory and polarized, extend filopodia
towards the angiogenic stimuli, proliferate minimally, and are highly branched. In
contrast, stalk cells are found behind the tip cells along the recently formed neovessel.
They generally do not migrate, are highly proliferative, and generate the vessel lumen
through a process called tube formation (Thurston & Kitajewski, 2008). Stalk cells can
differentiate into tip cells and vice versa, through a process that is controlled by Dll4-
Notch signaling (Lobov et al., 2007; Thurston & Kitajewski, 2008). A reduction in Notch-
1 leads to tip-like ECs, whereas the reverse is true for stalk cells. Cells closer to the
angiogenic source have upregulated Dll4 ligand production, and have a reduction in
Notch-1. The Dll4 produced by the tip cells increases Notch signaling in the neighboring
cells, promoting them towards a stalk cell phenotype (Lobov et al., 2007; Thurston &
Kitajewski, 2008).

1.2.1.1.2 VEGF

VEGF is a secreted growth factor that plays a prominent role in angiogenesis. As
part of the platelet derived growth factor superfamily (PDGF), there are several splice-
variants of this secreted glycoprotein. There are eight isoforms of VEGF, the most
prominent of which is VEGF$_{165}$ (named after the number of amino acids in the final
structure) or VEGF-A (Drake, LaRue, Ferrara, & Little, 2000). VEGF-A elicits its effects
by binding to VEGF receptors 1, 2, and 3 (VEGFR1, VEGFR2, VEGFR3) (Achen et al.,
1998). Upon binding of VEGF, these single-pass transmembrane receptors dimerize
and become active through transphosphorylation of their cytosolic tyrosine-kinase
domains, allowing downstream signaling effects of VEGF, one of which is the angiogenic effects on ECs as described above (Ferrara, Gerber, & LeCouter, 2003). In 2002, an antagonist to VEGF-A was discovered: VEGF\textsubscript{165}b. This isoform is generated through distal alternative splicing of the pre-mRNA at exon 8 (Bates et al., 2002; Woolard, Bevan, Harper, & Bates, 2009).

1.2.1.1.3 VEGF Receptors and their interaction with VEGFA and VEGF\textsubscript{165}b

VEGF receptors are single-pass transmembrane proteins, which consist of 7-immunoglobulin (IgG)-like extracellular domains (there are only 6 in VEGFR3), and a cytosolic tyrosine-kinase domain (Ferrara et al., 2003).

VEGFR1 (also known as fms-like tyrosine kinase receptor/Flt-1) is a receptor for VEGF-A, VEGF-B, and placental growth factor (PGF) (Ferrara et al., 2003). VEGFR1 is expressed in ECs, osteoblasts, monocytes/macrophages, pericytes, placental trophoblasts, and renal mesangial cells (Zachary & Gliki, 2001). It is enriched in normal ECs, vascular smooth muscle cells and lipopolysaccharide (LPS)-induced monocytes (Ferrara et al., 2003). VEGFR1 is necessary for the developing embryo, as VEGFR1 knockout mice are embryonically lethal (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006). Additionally, VEGFR1 regulates angiogenesis, monocyte migration, and macrophage function (Olsson et al., 2006). VEGFR1, along with its soluble isoform (s-Flt-1) are thought to be decoy receptors for VEGF, sequestering its signaling effects.
(Eremina et al., 2003; Shalaby et al., 1995; Zhang et al., 2014). Interestingly, in combination with VEGFR2, VEGFR1 it is thought to enhance VEGF signaling (Ferrara et al., 2003). This is due to its very strong affinity to VEGF-A, while having weak cytosolic tyrosine-kinase activity (Kendall & Thomas, 1993). The expression of sFlt1 is important in maintaining foetal vasculature during pregnancies, as significant levels of sFlt1 mRNA is expressed by the extravillious trophoblasts of the placenta. Dysregulation of sFlt1 levels is associated with preeclampsia, a serious pregnancy complication that is more prevalent in diabetic patients (Fan et al., 2014; M Shibuya et al., 1990).

VEGFR2 (also known as kinase-insert-domain-containing receptor, KDR) is a receptor for VEGF-A, VEGF-B, and VEGF-C. VEGFR2 is widely expressed, is enriched in ECs and is also expressed in podocytes (Ferrara et al., 2003). VEGFR2 plays an important role in regulating angiogenesis, and in maintaining vascular density and endothelial cell permeability. VEGFR2 mediates its effects by activating pro-survival signaling cascades such as mitogen activated protein kinase (MAPK) / extracellular-signal-regulated kinase (ERK), Akt, protein kinase-C (PKC) and Phosphotidylinositol-3-Kinase (PI3K) (Ferrara et al., 2003). Despite having lower affinity for VEGF than VEGFR1, VEGFR2 exhibits much more robust tyrosine-kinase phosphorylation, making it a more efficient effector of VEGF signaling (Masaubmi Shibuya, 2006). Mouse VEGFR2 mutation leads to failure of vascular development, leading to pre-natal death (Shalaby et al., 1995).

VEGFR3 is expressed in all endothelial cells during development, but is restricted to lymphatic ECs in the adult organism (Kaipainen et al., 1995). When bound
to its VEGF-C and VEGF-D ligands, VEGFR3 elicits similar effects, as when VEGFR2 is bound to VEGF-A (Kukk et al., 1996). As such, VEGFR3 deficient mice die pre-natally due to defective vessel formation (Dumont et al., 1998).

In addition to the three VEGF receptors, neuropilin-1 and 2 are also important receptors for VEGF-A signaling (Robert, Zhao, & Abrahamson, 2000). ECs, as well as podocytes, express these co-receptors, which increase the affinity of VEGF binding to VEGFR2 (Robert et al., 2000).

Interestingly, VEGF-A promotes angiogenesis through activating VEGFR2, and VEGF₁₆₅b appears to inhibit angiogenesis by interacting with the same receptor. Both ligands have similar affinity with VEGFR2, but differ in the way they elicit downstream signal transduction (Hua et al., 2010; Kawamura, Li, Harper, Bates, & Claesson-Welsh, 2008). While VEGF-A strongly promotes cytosolic autophosphorylation and thus activation of the tyrosine kinase domain of VEGFR2, VEGF₁₆₅b is inefficient and transient in its effect (Bates et al., 2002; Hua et al., 2010; Kawamura et al., 2008). Additionally, VEGF₁₆₅b does not have the amino acid sequence necessary for binding neuropilin-1 and 2 (which increase the affinity of VEGF binding to VEGFR2), which reduces the activation of VEGFR2 (Cébe Suarez et al., 2006; Kawamura et al., 2008). VEGF₁₆₅b also directly inhibits VEGF-A effects in a dose dependent manner (Bates et al., 2002; Hua et al., 2010). The balance of the pro and anti-angiogenic effect on endothelial cells is based on growth factors regulating the expression of VEGF-A and VEGF₁₆₅b (Nowak et al., 2008).
1.2.1.1.4 VEGF-A Signaling Transduction

When VEGF-A binds to its receptors and co-receptors, the cytosolic tyrosine-kinase domain of VEGFR2 dimerizes and auto-phosphorylates (Ferrara et al., 2003; Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999). This autophosphorylation recruits VEGFR2-associated proteins to the activated tyrosine-kinase domain. A number of intracellular signaling cascades are then activated, including PKC, PI3K/Akt, MAPK/ERK, and small GTPases (Gélinas, Bernatchez, Rollin, Bazan, & Sirois, 2002; Podar et al., 2001). This leads to elevated production of nitric oxide (NO) through nitric oxide synthase (NOS) (which increases vascular permeability, vasodilation and endothelial cell migration), promotion of VEGFR2 production and stabilization, increase in Notch-Dll4 signaling behind the leading cells, promotion of endothelial cell survival and cytoskeletal rearrangement through VEGFR2 activation of p38 MAPK and focal adhesion kinase, together with its substrate paxilin, an important regulator of neuropilin 2 (a protein that enhances VEGFR2 signal transduction) (Breier, Albrecht, Sterrer, & Risau, 1992; Risau, 1997; Takahashi, Ueno, & Shibuya, 1999).

1.2.1.1.5 VEGF expression in disease

The most well studied stimulus for increased VEGF signaling is hypoxia. This is a pathological phenomenon associated with many diseases, and is well studied in tumour development, as well as in diabetic retinopathy (Pe’er et al., 1995). Rapidly growing tumour cells necessitate a high metabolic and oxygen demand, leading to increased
vascularization requirements, which in turn are met by upregulating VEGF signaling. In diabetic nephropathy, ischemia-induced hypoxia is combated through VEGF-induced neovascularization (Aiello et al., 1995; Y. Liu, Cox, Morita, & Kourembanas, 1995; Pe'er et al., 1995; Stellmach, Crawford, Zhou, & Bouck, 2001).

In normoxia, the hypoxia response element (HRE), an enhancer region upstream of the VEGF promoter, is not bound by the heterodimer co-transcription factor hypoxia inducible factor – 1 (HIF-1) (Y. Liu et al., 1995). This is because the von Hippel-Lindau (VHL) tumour suppressor promotes ubiquitination of HIF-1α, and subsequent proteasomal degradation in normoxia. In hypoxia, on the other hand, VHL is downregulated, and HIF-1α binds to HRE, which enhances VEGF mRNA expression and stabilization, leading to neovascularization through angiogenesis (Banai et al., 1994; Mahon, Hirota, & Semenza, 2001; Semenza, 2002, 2007; Tuder, Flook, & Voelkel, 1995).

VEGF expression is also upregulated in inflammatory diseases such as rheumatoid arthritis, where cytokines such as tumour necrosis factor-alpha (TNF-α), a potent upregulator of VEGF mRNA expression, is increased (Ferrara et al., 2003).

1.2.1.2 Diabetes induced glomerular angiogenesis

Nyengaard and Rasch have described extensive glomerular neovascularization occurring in the early stages of diabetes in animal models (J R Nyengaard & Rasch, 1993). Both streptozotocin-induced diabetic rats, which best model insulin-deficient
diabetes, and the db/db mouse which models insulin-resistant diabetes, demonstrated increased glomerular capillary length, size, and area (J R Nyengaard & Rasch, 1993; Y. Yamamoto et al., 2004). Additionally, between 10-50 days after diabetes induction, elevated levels of glomerular ECs were detected histologically (J. R. Nyengaard & Rasch, 1993).

1.3 DIABETIC GLOMERULOPATHY

1.3.1 Major cell types in glomeruli

Glomeruli are the filtration units of the kidney, and are commonly the site of diabetic vascular injury. Three main cell types are found in the glomerulus, including podocytes, mesangial cells, and the glomerular ECs (GEC).

Podocytes are epithelial cells that make up the outer part of the filtration barrier, and are critical for maintaining the permeability of the glomerular filter (Pavenstädt, 2000). Neighboring podocytes have integrating foot processes, which generate filtration slits that selectively allow the filtrate into the proximal tubule of the nephron (Pavenstädt, 2000). As they are the final barriers for glomerular filtration, podocyte injury typically leads to proteinuria (Mundel & Shankland, 2002). In addition to this, podocytes are the cell group that predominately expresses VEGF within the glomeruli, which is necessary for normal glomerular endothelial development (F. Guan, Villegas,
Teichman, Mundel, & Tufro, 2006). Moreover, podocyte-specific inhibition of VEGF expression in adult mice resulted in marked glomerular endotheliosis and renal failure, further highlighting the importance of this cell-type within glomeruli and in particular for maintaining glomerular endothelial homeostasis (Eremina et al., 2003).

Interestingly, podocyte loss has been detected in the glomeruli of both T1DM and T2DM patients, regardless of age (Pagtalunan et al., 1997; Steffes, Schmidt, Mccrery, & Basgen, 2001). Podocyte loss is associated with progression of renal injury as indicated by increased albuminuria (White, Bilous, & Group, 2004), and is characterized histologically also by broadening of the foot processes of the remaining podocytes (Pagtalunan et al., 1997). Podocytes can be lost via two major mechanisms, including cellular detachment from the glomerular basement membrane due to reduced integrin levels mediated by hyperglycemia-induced increase in angiotenisin II (eliciting its effect through angiotensin receptor I), as well as increased podocyte apoptosis (Durvasula & Shankland, 2008; Regoli & Bendayan, 1997) (Regoli & Bendayan, 1997). Enhanced podocyte apoptosis in the diabetic glomerulus is thought to be mediated by TGF-β, which promotes programmed cell death through the activation of the P38 MAPK and Caspase-3 pathways (Schiffer et al., 2001). Importantly, podocyte apoptosis has been shown to preceed early signs of renal injury (albuminuria, and mesangial matrix expansion) in both T1DM and T2DM murine models, highlighting the importance of these cells in maintaining renal function (Susztak, Raff, Schiffer, & Böttinger, 2006).

Mesangial cells cover about 30% of the glomerular cell surface area. Intraglomerular and extraglomerular mesangial cells are found inside and outside (at the vascular pole) of glomeruli respectively (Mene, Simonson, & Dunn, 1989).
Intraglomerular mesangial cells play a role in filtration, structural support, and phagocytosis. Extraglomerular mesangial cells are important in regulating blood pressure, and monitoring blood glucose levels (Hugo, Shankland, Bowen-Pope, Couser, & Johnson, 1997). Mesangial cells respond to stretching stress by reducing the filtration rate through contracting and reducing the glomerular surface area (Goligorsky et al., 1997).

Mesangial cells also play a role in the development of diabetic nephropathy. In both animal models of diabetes and in patients with T1DM and T2DM, there is evidence of mesangial cell hypertrophy, proliferation, and expansion of mesangial cell matrix (Steffes, Østerby, Chavers, & Mauer, 1989). These pathologic mesangial changes are driven by hyperglycemia-induced activation of PKC, and increased levels of advanced glycation end products (AGEs), processes which promote mesangial matrix synthesis (KOYA et al., 2000; Tamsma et al., 1994; Vestra, 2001). Additionally, hyperglycemia increases expression of transforming growth factor-β (TGF-β), which promotes mesangial cell expression of matrix proteins such as collagen IV, laminin and fibronectin, each of which are protein families with multiple genes and splice variants (Ayo et al., 1991; T. Yamamoto, Nakamura, Noble, Ruoslahti, & Border, 1993). Moreover, periods of enhanced synthesis of renal angiotensin-II also promote laminin and collagen I production (Wolf, Haberstroh, & Neilson, 1992). This new matrix protein synthesis, along with altered mesangial cell metabolism of pre-existing matrix, leads to a significant accumulation of fibrotic proteins. The resulting progressive glomerulosclerosis can lead to downstream tubulointerstitial ischemia, which in turn can initiate tubular injury and interstitial fibrosis.
Renal vascular GECs are the major cell type (accounting for >50% of the cells in
glomeruli) found on the inner lining of the glomerular capillaries (Mene et al., 1989). These cells have fenestrations, or pores, that selectively allow the passage of fluids, plasma solutes, and protein, but not cells. The GECs also proliferate and expand under the influence of VEGF, as they are enriched in VEGFR1 and VEGFR2 (Section 1.2.1.2.4).

These three major cell types within glomeruli have also been shown to communicate, influence, and function together in a coordinated unit. This is especially evident in the setting of disease. Diabetic injury of ECs, for example has been associated with podocyte injury and subsequent albuminuria (Darren A Yuen, Stead, et al., 2012). Similarly, VEGF inhibition in podocytes has been linked to glomerular endotheliosis (Eremina et al., 2008). Additionally, mesangial cell overexpression of platelet-derived growth factors leads to endothelial cell injury, and precedes glomerulosclerosis (Floege et al., 1992). Together, this demonstrates how the glomerular cell types regulate one another, and how injury to one can lead to dysfunction of the other cells, highlighting the complexity and the delicate balance within glomeruli.

1.3.2 Glomerular ECs in diabetic nephropathy

Diabetes causes a number of changes to the GEC phenotype, which differ between early and late stages of diabetes. In late-stages of diabetes, there is loss of GECs and a reduction in VEGF-A mRNA and protein levels, which was linked with
enhanced proteinuria (Lindenmeyer et al., 2007). This may result in more leakiness to the glomerular barrier due to the reduction of total GEC fenestrations. These fenestrations are necessary for high-water permeability, and are covered in negatively charged proteoglycans, which, along with other glycoproteins, make up the glycocalyx, a significant contributor to the glomerular barrier (Satchell, 2012). In diabetes, there is significant glycocalyx loss, enhancing glomerular barrier disruption and promoting albuminuria. The reduction in glomerular cell cross-talk may exacerbate this effect (Six, Kureishi, Luo, & Walsh, 2002; D. A. Yuen et al., 2012).

On the other hand, in early diabetes described in 1.3.1, neoangiogenesis is one of the first observable phenotypes of diabetic nephropathy, with evidence of this phenomenon having been shown in both diabetic patients and in animal models of diabetes. These rodent models demonstrate enhanced glomerular capillary growth within weeks (10-50 days) of disease onset, characterized by an increase in total capillary length, and a larger glomerular capillary filtration surface area (J. R. Nyengaard & Rasch, 1993). While similar data in humans is not as strong, Osterby and Nyberg interestingly showed impaired vasculature within 5% of the capillary area in the glomeruli (Østerby & Nyberg, 1987). Follow up work by Min and Yamaka, using computational modeling of 94 diabetic patients showed extravessels at the glomerular vascular hilum (Min & Yamanaka, 1993). Importantly, these vessels were shown to exist during the first two years of diabetes, demonstrating impairment in early stages of diabetes, as was shown in diabetic animal models. As evidence exists in human models, and certainly in animal models of diabetes, ameliorating pathological
angiogenesis may be a potential therapeutic target for regulating renal injury in diabetic nephropathy patients.

1.3.2.1 VEGF in diabetic nephropathy

Podocytes are responsible for the majority of VEGF expression in the kidney (F. Guan et al., 2006). While it remains unclear how a large protein like VEGF can cross the glomerular basement membrane to interact with GECs in a paracrine fashion (Eremina et al., 2003), elegant podocyte-specific knockout studies have demonstrated that some form of VEGF-based podocyte-GEC crosstalk is clearly necessary to promote glomerular endothelial formation in embryogenesis, promoting biogenesis and maintenance of GEC fenestrations, and also inducing endothelial proliferation and angiogenesis following certain types of glomerular endothelial injury (Cooper et al., 1999; Satchell, Anderson, & Mathieson, 2004; Sivaskandarajah et al., 2012).

In diabetic animal models, VEGF mRNA and protein overexpression has been detected and linked with glomerular basement membrane thickening, hyperfiltration, and neoangiogenesis (de Vriese et al., 2001; Flyvbjerg et al., 2002; Ku et al., 2008; Sung et al., 2006). The pathogenic role of VEGF-A in this experimental setting has been supported by VEGF inhibition studies. Indeed, VEGF-A blockade using neutralizing VEGF antibodies or small molecular VEGF-A inhibitors blocked glomerular basement thickening, glomerular capillary growth, and hyperfiltration in various diabetic rodent models (de Vriese et al., 2001; Flyvbjerg et al., 2002; Ku et al., 2008; Sung et al., 2006).
Whether VEGF plays a similar role in human diabetic nephropathy, however, is not as clear. Interestingly, renal biopsies of patients with diabetic nephropathy have shown both a reduction and increased expression in VEGF-A (Shulman, Rosen, Tognazzi, Manseau, & Brown, 1996). The reduction in VEGF-A expression in this study may have been due to selection bias of renal biopsies collected from patients in advanced stages of diabetic nephropathy, with sclerotic regions having reduced podocytes expressing VEGF-A (Baelde et al., 2007). At the same time, another study found VEGF-A protein as well as mRNA levels were significantly elevated only in patients with nephrotic range proteinuria, supporting a role for upregulation of VEGF-A as an important mediator of diabetes-induced renal complication in humans (Hohenstein et al., 2006; Kanesaki et al., 2005; Zdarska, Zavadova, & Kvapil, 2007).

More recent work has added a further layer of complexity to the role of VEGF in glomerular physiology. VEGF$_{165}$b, a VEGF-A splice variant, was recently shown to act as an anti-angiogenic protein (Bates et al., 2002; Konopatskaya, Churchill, Harper, Bates, & Gardiner, 2006; Varey et al., 2008). Interestingly, however, complete lack of VEGF$_{165}$b leads to significant mesangial sclerosis and ultimately to renal failure, in Denys-Drash syndrome (Schumacher et al., 2007). Similarly, recently published work points to the reno-protective role of VEGF$_{165}$b in diabetic nephropathy, possibly through maintaining the synthesis of endothelial glycocalyx important in maintaining the renal filtration barrier (Oltean et al., 2014). Together, these findings point to the many varied roles that VEGF isoforms play in regulating glomerular physiology in both health and disease.
1.3.2.2 VEGF inhibition is associated with glomerular injury

While it has been demonstrated that VEGF expression levels are generally upregulated in the setting of diabetes, total VEGF inhibition may not be the best strategy for attenuating diabetic neovascularization because VEGF is necessary for several aspects of GEC development, sustainability, and function. Advani et al. elegantly demonstrated this in 2007, in a study of pharmacologic VEGF receptor inhibition in non-diabetic rats. Not only did these rats develop significant renal injury, as demonstrated by proteinuria and severe glomerulosclerosis, but also many of the rats developed a thrombotic microangiopathy, dying before end-study (Advani et al., 2007).

Consistent with this potential for harm, pharmacological VEGF inhibition as an anti-cancer agent has been associated with significant renal complications. Indeed, in cancer patients treated with the VEGF inhibitor bevacizumab, 21-64% of patients developed severe proteinuria, while 3-36% of the patients developed hypertension. Similar to findings in experimental rodents, biopsies from these patients revealed extensive glomerular endotheliosis (swollen capillaries and deposition of fibrotic material beneath the cells of the capillaries), and classic features of thrombotic microangiopathy (clotting of the capillaries) in focal areas of podocyte injury (Eremina et al., 2008).

Additionally, the earlier diabetic rodent studies that had suggested that a VEGF inhibition strategy for diabetic nephropathy might be beneficial had examined only short-term effects of pharmacologic VEGF blockade. More recent studies employing a podocyte-specific VEGF conditional knockout approach have demonstrated that complete VEGF inhibition in diabetic mice actually exacerbated proteinuria and
accelerated glomerular injury, leading eventually to glomerular endothelial cell loss in later stages of the disease (Sivaskandarajah et al., 2012). Taken together, these studies indicated that while upregulation of glomerular VEGF likely contributes to diabetic glomerular neovascularization, complete inhibition of VEGF, both in the diabetic and non-diabetic kidney, leads to glomerular endothelial pathology. Thus, as with most biological systems, VEGF signaling must be finely regulated to maintain endothelial homeostasis in the kidney. In addition, given the multifunctional role of VEGF in controlling not only the angiogenic responses of GECs but also many other aspects of their phenotype, these studies also suggest that VEGF blocking strategies may not be viable for the treatment of diabetic renal microvascular disease.

1.4 Slit-Robo signaling: an important pathway in development and disease

1.4.1 Slit – isoforms, structure and localization

The secreted Slit family of glycoproteins was initially described in Drosophila as a group of axonal repellents, which elicit their effects through binding to their cognate Roundabout (Robo) receptors, and activation of intracellular signaling cascades. Slit has three known isoforms (Slit1-3) (Brose et al., 1999; Kidd, Bland, & Goodman, 1999). Each of the isoforms of Slit has a N-terminal signal peptide, four leucine-rich repeats
(LRRs), nine epidermal growth factor (EGF) repeats and a C-terminal cysteine knot (Jonathan Marc Rothberg & Artavanis-Tsakonas, 1992). The LRR domains of Slit2, the most well studied Slit isoform, are critical for binding of Slit2 to its target receptors. In vivo, Slit2 is cleaved at a point just C-terminal to the EGF repeats, to create a bio-active N-Slit2 and a bio-inactive C-Slit2 peptide (Brose et al., 1999; J M Rothberg, Jacobs, Goodman, & Artavanis-Tsakonas, 1990). Additionally, extracellular heparan sulfate (HS) polysaccharides promote binding of Slit to its target receptors, and deficiency of specific HS polysaccharides has been linked to impairment in Slit-Robo signaling and downstream effects on cell proliferation, apoptosis, adhesion, and migration (Hu, 2001; Inatani, Irie, Plump, Tessier-Lavigne, & Yamaguchi, 2003).

The distribution of the Slit proteins varies between the isoforms. Slit1 is localized in the brain (Brose et al., 1999), Slit2 is predominantly expressed in the spinal cord, but has also been found in lungs, kidneys, and the brain (Wong et al., 2001), and Slit3 is predominantly expressed in cells of the thyroid, and in the ECs of mouse diaphragm and lungs (Wong et al., 2001). In the kidney, in situ hybridization studies have revealed that Slit2 is expressed in both tubular epithelial cells and endothelial cells lining renal capillaries and venules. These same studies also showed that Slit2 is expressed in the glomerulus, being found not only in glomerular mesangial cells, but also in parietal epithelial cells lining Bowman’s capsule (Kanellis et al., 2004; Wu et al., 2001).

Recent studies have shown that Slit3 is important for non-neuronal development processes such as the development of the diaphragm and the kidney (J. Liu et al., 2003). Slit3 expression was also detected in the vasculature, with small amounts being found in endothelial cells, and expression also detected in vascular smooth muscle cells.
(Zhang et al., 2009). Interestingly, Slit3 interacts with Robo4, and mediates endothelial angiogenesis through Rho GTPase activation, in a non-VEGF stimulating fashion (Zhang et al., 2009).

1.4.2 Robo receptors – isoforms, structure and localization

In mammals, the Robo proteins are a set of 4 single-pass transmembrane receptors (Robo1-4) belonging to the IgG receptor superfamily that bind Slit and N-Slit. Human Robo1-3 contain five extracellular IgG repeats and three fibronectin type III (FN) domains, which are required for interaction with the LRR repeats of Slit and N-Slit. Robo1-3 contain four conserved cytoplasmic domains (CC0, CC1, CC2 and CC3), which are thought to mediate at least some of the responses to Slit ligands (Bashaw, Kidd, Murray, Pawson, & Goodman, 2000; Marlow et al., 2010; Wong et al., 2001).

As described in 1.4.2, Robo1 is expressed in neuronal cells, dendritic cells, glial cells, leukocytes, platelets, megakaryocytes, and vascular and lymphatic ECs (Wong et al., 2001; Yu et al., 2014). Robo2 and Robo3 are generally expressed during embryogenesis and are important regulators of axonal navigation at ventral midline and neural tube (Vargesson, Luria, Messina, Erskine, & Laufer, 2001; Zlatic, Landgraf, & Bate, 2003). Recent studies have shown that Robo2 is also expressed in endothelial cells upon loss of Robo1 expression (Rama et al., 2015).
1.4.2.1 Robo4 – structure, and localization

Robo4 is dubbed the “magic roundabout” protein because it is markedly different in both structure and distribution compared to the other Robo receptors. Robo4 consists of only two Ig and FN domains, whereas the other Robo receptors (Robo1-3) have 5 Ig and 3 FN domains. (Huminiecki, Gorn, Suchting, Poulsom, & Bicknell, 2002). Despite this difference, Robo4 has been shown to bind to Slit2 and Slit3, including the bioactive region of N-Slit2 (Huminiecki et al., 2002; Legg, Herbert, Clissold, & Bicknell, 2008).

Through differential DNA methylation, Robo4 is expressed only in ECs, and has been detected in both macrovascular human vein umbilical ECs (HUVEC) and in human microvascular ECs (HMVEC) (Okada et al., 2014). Robo4 expression is detected in the ECs of the kidney, liver lungs, heart, liver, lungs, muscle, small intestine, and in the placenta (C. A. Jones et al., 2008; Sheldon et al., 2009).

1.4.3 Slit-Robo signaling – role in embryogenesis, and in adult organisms

During development, the Slit proteins play a key role in guiding the outgrowth of central nervous system neurons (Kidd et al., 1999). Slits are expressed along the midline to prevent midline crossover of neurons, except at certain highly regulated locations (J M Rothberg et al., 1990). This process helps ensure that the left hemisphere of our brain to control the right side of our body, and vice versa (Inatani et al., 2003; Kidd et al., 1999; J M Rothberg et al., 1990).
The persistent expression of Slits and Robos in the mature organism, however, suggests biologic functions that extend beyond development (Dickinson & Duncan, 2010). Indeed, growing evidence suggests that Slit-Robo signaling is an important regulator of the structure and function of many cell types (Dickinson et al., 2010; H. Guan et al., 2003; Prasad, Qamri, Wu, & Ganju, 2007). In particular, Slit-Robo signaling has been shown by multiple groups to regulate hematopoietic cell function, including the recruitment of both leukocytes and platelets (Patel et al., 2012; Darren A Yuen & Robinson, 2013), inhibiting leukocyte migration, chemotaxis, and adhesion (Prasad et al., 2007) and platelet spreading (Patel et al., 2012). In neuronal cells, Slit2-Robo1 signaling also inhibited dendritic chemotaxis (H. Guan et al., 2003).

In most cell types, Slit-Robo signaling has been shown to mediate most of its effects through regulation of small GTPase activity. Slit2, for example, binds to Robo1, and elicits the activation of small guanosine trinucleotide phosphatase (GTPase) – activating proteins (GAPs). These Slit-Robo GAPs (srGAPs) are responsible for regulating actin cytoskeletal rearrangement through regulating Akt, Enabled, Abelson, and small GTPases such as RhoA, Rac, and Cdc42 of the Rho-family of GTPases, and Arf6 (C. a Jones et al., 2009; Patel et al., 2012; Wong et al., 2001).

1.4.3.1. Slit-Robo signaling: control of endothelial function

Aside from its role in regulating cells of hematopoietic lineage, mounting evidence suggests that Slits also regulate endothelial function. In particular, Slit2, the most well studied Slit isoform, has been shown to have effects on ECs, including
regulation of migration, proliferation, network formation, and inflammatory activation (Fujiwara, Ghazizadeh, & Kawanami, 2006; Legg et al., 2008; K. W. Park et al., 2003)

1.4.3.1.2. Slit2 promotes VEGF-induced pro-angiogenic signaling through Robo1

Section 1.4.3 demonstrates the effects of Slit2-Robo signaling outside of development, and highlights its role in cell motility. Because of this, and the fact that Robo1 was detected in endothelial cells, early studies focused on the role that Slit2-Robo1 signaling had on endothelial function (K. W. Park et al., 2003; Seth et al., 2005). In these studies, it was found that, unlike its effects on leukocytes and neuronal cells, Slit2 promoted migration and network formation of human umbilical vein ECs (HUVEC) in a Robo1-dependent manner (K. W. Park et al., 2003; Wang et al., 2003). Importantly, it was later shown that Slit2-Robo1 signaling promoted VEGF-induced angiogenesis in an ERK-dependent manner (Fish et al., 2011). Further, Slit2-Robo1 signaling enhanced VEGF signaling through promoting VEGFR2 autophosphorylation (Fish et al., 2011). Therefore, in at least macrovascular HUVECs, Slit2-Robo1 signaling appears to enhance the pro-angiogenic effects of VEGF.

1.4.3.1.2. Slit2 also regulates endothelial VEGF signaling through Robo4

Elegant studies by Dr. Dean Li’s group have found that Slit2, through its Robo4 receptor, significantly blocks VEGF- stimulated microvascular endothelial cell migration and network formation (C. A. Jones et al., 2008). The in vivo relevance of these findings
was confirmed following the generation of Robo4-knockout (Robo4 KO) mice, which, after undergoing oxygen-induced retinopathy, a model of post-natal VEGF-induced neovascularization, had increased retinal neovascularization which could not be rescued with Slit2 administration, unlike their Robo4 wild-type littermates. Importantly, Robo4 KO mice demonstrated normal levels of expression of Slit2 and Robo1-3, suggesting that the observed effects were mediated solely by the loss of Robo4. Furthermore, Robo4 restricted VEGF signaling in murine breast tissue, as Robo4 KO breast tissue demonstrated enhanced vascularization when implanted into pregnant mice (Marlow et al., 2010). Later studies demonstrated that this effect is mediated by the Slit2-induced formation of a Robo4–paxillin complex at the cell surface, which subsequently blocks activation of the small GTPase Arf6 and, consequently, Rac, a small GTPase whose activity is necessary for endothelial cell migration (1.4.3.3) (C. a Jones et al., 2009). Interestingly, independent of Slit2, Robo4 was shown to elicit anti-angiogenic effects by acting as a ligand for another endothelial guidance receptor, UNC5B, leading to inhibition of VEGF signaling (Koch et al., 2011).

Recent work has also demonstrated that Slit2-Robo4 signaling dysfunction may play a role in regulating the endothelial inflammatory response to endotoxemia. Following endotoxin exposure, endothelial cells release a number of pro-inflammatory cytokines. Interestingly exogenous Slit2 and N-Slit2 were found to cause a reduction in endothelial cytokine expression via signaling through the Robo4 receptor, and not through Robo1. Slit2 elicited this effect by inhibiting endotoxin-induced pyk2 activation, which in turn inhibited nuclear translocation of NF-κB, a potent transcription co-factor that promotes cytokine expression (Zhao, Anand, & Ganju, 2014). Following endotoxin
exposure, endothelial Slit2 and Robo4 expression was greatly reduced, suggesting that endotoxin-induced downregulation of Slit2 and Robo4 may be at least partly responsible for the resultant pro-inflammatory endothelial responses typically seen in this setting, such as cytokine production and increased endothelial permeability (Zhao et al., 2014). Whether the effects of endotoxin exposure were mediated also through associated changes in VEGF signaling were not investigated in this report. Taken together, these studies demonstrate that in the adult microvascular endothelium, Robo4 serves primarily as an anti-angiogenic and anti-permeability receptor, mediating the effects of ligands such as Slit2 and UNC5B, at least in part through the inhibition of VEGF signaling (C. A. Jones et al., 2008).

Interestingly, in contrast to these findings, others have shown that Robo4 may be necessary for angiogenesis in some settings, as Robo4 knockdown in HUVEC impairs the basal ability of these cells to migrate and form networks (Sheldon et al., 2009). Similarly, knockdown of Robo4 in zebrafish embryos leads to multiple vascular abnormalities (Bedell et al., 2005). However, unlike the previous studies mentioned above, a limitation of these reports was a failure to document whether Robo4 knockdown altered the expression of Slit2 or the other Robo receptors. Thus, it is difficult to interpret these results as the effect of Robo4 knockdown may have been masked by changes in other relevant components of Slit2-Robo signaling (eg. Robo1).

1.4.3.1.1 The dual role of Slit2-Robo signaling in the endothelium

How can Slit2-Robo signaling be pro-angiogenic in some settings, and anti-
angiogenic in others? One possible explanation for these apparently conflicting results may be differences in the endothelial cells used in the various studies described above. Interestingly, in studies where Robo4 was found to act as a pro-angiogenic receptor, the cell type used was usually the human umbilical vein endothelial cell (HUVEC) (Sheldon et al., 2009; Suchting, Heal, Tahtis, Stewart, & Bicknell, 2005) a macrovascular venous endothelial cell. In contrast, groups using microvascular endothelial cells, such as human microvascular lung endothelial cells (HMVEC-L) have generally reported an anti-angiogenic role for Robo4 (C. A. Jones et al., 2008; C. a Jones et al., 2009; K. W. Park et al., 2003). Importantly, macrovascular ECs are enriched in both Robo1 and Robo4, while microvascular ECs are predominantly enriched in Robo4, rather than Robo1 (London & Li, 2011). As a result, Robo4 expression is significantly greater in microvascular ECs as compared to their macrovascular counterparts. As Robo1 and Robo4 mediate the pro- and anti-angiogenic effects of Slit2 respectively, this difference in Robo4 to Robo1 expression ratio may therefore explain how Slit2 can play a dual role as both a promoter and inhibitor of angiogenesis (Dickinson & Duncan, 2010; C. A. Jones et al., 2008; London et al., 2010; Marlow et al., 2010; Sheldon et al., 2009).

Another possible explanation for the varying effects of Slit2 on endothelial cells may be due to differences in other signaling pathways. Recently, Ephrin-A1, a transmembrane endothelial protein, together with its cognate Eph receptors, was shown to modulate endothelial cell response to Slit2 (Dunaway et al., 2011). In this report, Slit2 stimulated microvascular endothelial cell angiogenic activity in the absence of Ephrin-A1, but when Ephrin-A1 was added, Slit2 inhibited angiogenesis through activation of Robo1 (Dunaway et al., 2011). Interestingly, Ephrin-A1 levels are not
equally expressed between micro and macrovascular endothelial cells, an observation which further highlights the potential impact of phenotypic differences between endothelial cells from different vascular beds on responses to Slit2.

1.4.3.2 Angiogenesis assays

The endothelial cell is the primary driver of new blood vessel formation. These cells form the innermost layer of all capillaries, including the vessels that make up the glomeruli. Understanding how these cells function is pivotal in generating new therapies for pathological microvascular renal injury. To test endothelial function, several in vitro assays have been established and validated by multiple groups. Since each assay determines one aspect of endothelial angiogenesis, it is typical to use a combination of these assays to determine the pro or anti-angiogenic effects of a given treatment.

In vivo, ECs must migrate in response to chemoattractant gradients during the sprouting phase of angiogenesis. Such chemotaxis can be measured using a modified Boyden-Chamber migration assay. Similarly, during angiogenesis, ECs must form three-dimensional networks that ultimately serve as the framework for new capillary formation. The ability of such ECs to form extensions and networks can be measured using a Matrigel™ network assay.

A recent modification of the standard Matrigel network assay is the spheroid-based sprouting assay (Vinci et al., 2012). In this assay, cells are aggregated into a spheroid, which is then embedded in a Matrigel™ matrix. The cells are then incubated
with a pro-angiogenic stimulus, and later analyzed for extensions protruding from the spheroid body (Vinci et al., 2012). The sprouting and invasion of cells into the surrounding matrix from the seeded spheroid may better emulate the \textit{in vivo} growth patterns of ECs as they form new vessels. However, the reproducibility of the assay is dependent on the passage of the cells that are used, and importantly this difference in response between cell populations is exacerbated with VEGF stimulation (Heiss et al., 2015). Moreover, cell responsiveness is significantly reduced in immortalized cell lines (Heiss et al., 2015). Additionally, due to its novelty, several aspects of the assay remain to be optimized for a range of ECs.

1.5 The use of HRGECs as an \textit{in vitro} model

Admittedly, there are many downsides to using a primary cell model instead of an established transformed cell line for \textit{in vitro} studies. Immortalized cell lines are financially easier to maintain, experimentally easier to handle, and genetically easier to modify. Primary cells also lose their phenotype after a few passages, and come from different donor sources, adding variability between cells.

Despite these disadvantages, we opted to use primary human renal glomerular endothelial cells for a number of reasons. First, because of the differences in Robo4 and Robo1 expression between micro- and macrovascular endothelial cells, we wanted to use a microvascular endothelial cell type for our studies. Second, HRGECs were also used over more widely used and characterized endothelial cells, like HUVECs. While no
primary cell makes for an ideal in vitro model, because of the reasons in the above paragraph, we wanted to use cells that best mimicked the glomerular environment. HRGECs are capillary microvascular endothelial cells, while HUVECs are not only macrovascular endothelial cells (see Section 1.4.3.4 for the heterogeneity between endothelial cells), but are also derived from a venous source, which experience a different blood flow than arterial endothelial cells (Bouïïs, Hospers, Meijer, Molema, & Mulder, 2001).

To minimize variability introduced by the use of primary endothelial cells, for our experiments we used HRGECs from a single lot. Similarly, to avoid loss of phenotype, we only used cells between passages 2-6, after determining that HRGECs lost their endothelial phenotype after passage 7.

### 1.6 Diabetic nephropathy animal models

Translating in vitro findings towards the clinic requires the use of animal models that emulate not only diabetes, but also renal complications of this disease. Unfortunately, no animal model perfectly recapitulates the functional and structural injury found in patients. Given that the primary goal of this thesis was to study the roles that Robo1 and Robo4 play in the regulation of diabetic glomerular neovascularization, one of the objectives of this study was to examine the effects of diabetes on Robo1 and Robo4 expression in animal models of diabetic kidney disease. While nearly all rodent models of diabetic nephropathy fail to recapitulate the late stages of human disease
such as reduced GFR and progressive interstitial fibrosis, most of these models do develop the hyperfiltration and neovascularization characteristic of early human diabetic kidney injury (Breyer et al., 2005). The following sections will briefly describe the two models chosen for these studies, including the STZ-induced diabetic rodent (a model of insulin-deficient diabetes), and the db/db mouse (a model of insulin-resistant diabetes).

1.6.1 STZ model of diabetes

Originally developed in the 1950s as an antibiotic extracted from *Saccharomyces achronomggenes*, STZ was found to be toxic to the beta cells of the islets of Langerhans in the 1960s (Stauffacher et al., 1970). As described in Section 1.1.1, these cells produce insulin, and are therefore critical in maintaining normoglycemia. When administered as a high dose intra-peritoneal (i.p) injection (160-240 mg/kg), rats develop hyperglycemia, weight loss, and other physiological changes typical of T1DM (Section 1.1.1) (Junod, Lambert, Stauffacher, & Renold, 1969). When administered as low-dose (~50mg/kg) daily i.p injections over a five-day period, rodents similarly develop hypoinsulinemia and hyperglycemia (Ventura-Sobrevilla et al., 2011)(Section 1.1.1). Corresponding injections of the solvent for STZ, Tris-citrate, generates non-diabetic controls.

Similarly, in mice, administration of a single high-dose intra-peritoneal STZ (130-150 mg/kg) injection generates insulin-deficient diabetes in mice 3 days post injection (Ventura-Sobrevilla et al., 2011). Concerns regarding direct STZ nephrotoxicity and mortality however, have been raised (Ventura-Sobrevilla et al., 2011). For this reason, a
5 day regimen of daily low-dose (50 mg/kg) intra-peritoneal STZ injections has also been used, producing mice with insulin-deficient diabetes with reduced mortality rates 2 – 7 days after the last STZ injection (Ventura-Sobrevilla et al., 2011).

1.6.2 The db/db mouse model of diabetes

In 1966, Jackson Labs identified a mouse with hyperphagia and obesity. Analysis of this mouse revealed a spontaneous G to T point mutation in the mouse leptin receptor (OB-R), leading to a premature truncation of this pivotal protein (Chen et al., 1996). Without the cytosolic domain of the receptor, leptin signaling is defective. As leptin is enriched in the hypothalamus, the region of the brain responsible for regulating hunger, mutations in leptin signaling lead to hyperphagia and obesity in db/db homozygous mice. db/db mice at 8 weeks of age develop hyperglycemia (peaking at 5-6 months of age at 40 mmol/L) and other physiological conditions typical of T2DM (see 1.1.1). As the leptin receptor gene is haplo-sufficient, the heterozygous db/m mice littermates are used as non-diabetic controls (Breyer et al., 2005; Chen et al., 1996).

1.6.3 Diabetic-renal complications of animal models

Both the STZ and db/db animal models of diabetes mimic the early manifestations of human diabetic nephropathy. For example, at 8 weeks of age, db/db mice develop hyperglycemia, and by 12 weeks of age, they demonstrate glomerular hypertrophy, increased levels of urinary albumin excretion, and glomerular basement
membrane thickening. They also demonstrate glomerular angiogenesis, as detected by an increase in length and surface area of glomerular capillaries (Breyer et al., 2005). Renal functional changes accompany these structural alterations, as diabetic animals demonstrate a significant two-fold increase in glomerular filtration rate, and albuminuria (Breyer et al., 2005).

Unfortunately, neither of these animal models mimic the late stages of human diabetic-renal disease. Indeed, common features of late-stage human diabetic nephropathy, such as loss of the microvasculature and the development of tubulointerstitial and glomerular fibrosis do not develop in these rodents (Breyer et al., 2005). Although these limitations render these models less clinically relevant for the study of late stage diabetic complications, they are suitable for the study of glomerular angiogenesis and other events that occur early in the course of diabetic renal disease.

1.7 Preliminary work

Preliminary work has examined the effects of high glucose and diabetes on Robo1 and Robo4 expression in glomerular endothelium. This work serves as the basis for my thesis and is summarized in the following sections.
1.7.1 Microvascular human glomerular renal ECs express more Robo4 than Robo1

As Robo1 and Robo4 seemingly play antagonistic roles in the regulation of Slit2 activity in endothelial cells (Section 1.4.3.1.2), it was important to determine the relative expression levels of these two receptors in microvascular HRGECs. Consistent with the notion that microvascular ECs express higher levels of Robo4 relative to Robo1 compared to macrovascular HUVECs (Aird, 2007; Fish et al., 2011), in preliminary work it has found that human renal glomerular ECs (HRGECs) express 6000 fold more Robo4 than Robo1, using quantitative real time polymerase chain reaction (qRT-PCR) coupled with standard curve analysis (data not shown).

1.7.1.1 High glucose alters Robo4 expression in HGRECs

To test the effect of high glucose on HGREC expression of Robo1 and Robo4, cells were cultured in growth media containing normal glucose concentrations (5 mmol/L), high glucose concentrations (25 mmol/L), or normal glucose supplemented with mannitol (20 mmol/L) as an osmotic control. Exposure to high glucose, but not mannitol, resulted in a dramatic reduction in both Robo4 mRNA and protein levels, while Robo1 levels remain unchanged (Figures 1 and 2). No significant changes in the housekeeper Rpl13a transcript were noted.
Figure 1: Robo4 mRNA levels drop, while Robo1 levels remain unchanged after 48 hours of high glucose (HG) treatment, relative to the housekeeper transcript Rpl13a.

Passage 2-6 HRGECs were stimulated with medium containing normal glucose (NG), high glucose (HG), or mannitol (HM). RNA was isolated using TriZol reagent at 0 and 48 hours post-stimulation, reverse transcribed, and amplified using sequence-specific primers for (A) Robo4, (B) Robo1, or the house keeper transcript Rpl13a. * p < 0.05 vs t=0. † < 0.05 vs. HM osmotic control at 48 hours, n=3.
Figure 2: Robo4 protein levels are diminished after 72 hours of HG treatment in HRGECs.

Passage 2-6 HRGECs were stimulated with endothelial growth medium containing normal glucose (NG) levels, or supplemented with extra glucose (high glucose, HG) or mannitol (Man) as an osmotic control. Cell lysates were prepared at 24 and 72 hrs post-stimulation, separated by SDS-PAGE, and immunoblotted for Robo4 and Robo1, along with the α-tubulin and GAPDH loading controls. Representative immunoblots and quantification at 24 hrs and 72 hours are presented. * p < 0.05 vs NG control medium, n=3.

1.7.2 Robo4 and Robo1 expression in diabetic animal models

Our preliminary in vitro data demonstrated the effects of diabetic-like conditions on HRGEC expression of Robo4 and Robo1. To determine if similar effects are observed in vivo, renal Robo4 and Robo1 mRNA and protein expression levels were examined in two established rodent models of diabetic kidney disease.

In the first model, db/db mice, which develop spontaneous hyperglycemia at 8 weeks of age, and glomerular hypertrophy and hyperfiltration shortly thereafter, were compared with their non-diabetic db/m littermates at 12 weeks of age. Using qRT-PCR, it was determined that the diabetic kidneys had reduced Robo4 mRNA transcript levels, while Robo1 mRNA transcript levels remained unchanged, compared to their non-
diabetic db/m littermates. Taken together, the Robo4:Robo1 ratio was significantly lower in db/db mice, compared to their db/m littermates (Figure 3).

In the second model, male Wistar rats were injected with a single dose of STZ or citrate buffer as a control. Similar to our findings in db/db mice, STZ-induced diabetic rats also showed a reduction in glomerular Robo4 staining with no change in Robo1, as measured histologically via antibodies directed against Robo4 and Robo1 (Figure 4).
Figure 3: Diabetes is associated with a reduction in renal Robo4 to Robo1 mRNA ratio in two rodent models.

RNA was isolated from snap-frozen kidney tissue from both diabetic (DM) db/db (n = 8) and non-DM db/m mouse littermates (n = 6) (A), as well as STZ-induced DM (n = 6) and non-DM citrate buffer-injected Wistar rats (n = 8) (B). The RNA was reverse transcribed, and amplified using sequence-specific primers for Robo4, Robo1, or the housekeeper transcript Rpl13a. * p < 0.05 vs non-diabetic control
Figure 4: Diabetes is associated with reduced glomerular Robo4 protein levels, while Robo1 levels remain unchanged in STZ-Wistar rats.

Formalin-fixed kidneys were collected and paraffin wax embedded for histological purposes from STZ-Wistar rats 3 weeks after STZ induction. 30 glomeruli cut through the macula densa were randomly selected and digitally analyzed for positive immunosignal for Robo4 and Robo1. A and B show Robo4 and Robo1 expression respectively of health rat glomeruli at 400X magnification. Arrows and arrowheads mark Robo1 expression in endothelial and non-endothelial cells respectively. Shown in the graphs (C and D) are the quantitative analysis for staining density of Robo4 and Robo1, respectively. * p < 0.05 vs. non-DM controls.

1.8 HYPOTHESIS

Preliminary data has demonstrated that the high glucose levels of diabetes are associated with a reduction in levels of the anti-angiogenic Robo4 receptor, but a non-significant yet increasing trend in Robo1 mRNA. This thesis will explore the importance of the Robo1 and Robo4 receptors in HRGEC responses to high glucose in vitro and in vivo.

Overall Hypothesis:

Slit2-Robo signaling is an endothelial pathway that regulates the glomerular
angiogenesis that occurs in early diabetic kidney disease, and therefore may be a potential target for generating novel therapies.

I will be exploring this overall hypothesis in the following chapters:

i) HRGEC Responsiveness to VEGF is Regulated by Robo1 and Robo4.

In this chapter, I explore the effects of high glucose and Robo1 knockdown on HRGECs in migration and network formation assays, in the presence of VEGF.

My hypothesis is that a reduction of the pro and anti-angiogenic Robo1 or Robo4 receptors will inhibit or promote VEGF-mediated endothelial angiogenic functions, respectively.

ii) Robo4 KO Mice have Increased Diabetes Induced Glomerular Angiogenesis

In this chapter, I explore the effects of high glucose and Robo4 knockout on glomerular angiogenesis in mice.

My hypothesis is that diabetic mice that lack Robo4 will have more glomerular angiogenesis as measured by PECAM-1 staining and fluorescence microangiography.
Chapter 2:

HRGEC Responsiveness to VEGF is Regulated by Robo1 and Robo4
2 In vitro HRGEC Experiments

2.1 Introduction

Acting through its pro-angiogenic receptor Robo1, Slit2 promotes HUVEC migration and network formation in the presence of VEGF. In contrast, Slit2 acts through its receptor Robo4 to antagonize VEGF signaling in microvascular ECs, through recruitment of intracellular proteins that ultimately inactivate small GTPases necessary for VEGF-induced angiogenesis (Fish et al., 2011; C. A. Jones et al., 2008; C. a Jones et al., 2009). As HUVEC and microvascular ECs express varying levels of Robo4 and Robo1, a potential explanation for these contrasting effects is that the relative levels of these two receptors may determine the ultimate response of the cell to Slit2.

2.2 Specific Aims and Hypothesis

Preliminary work has demonstrated reduced GEC expression of Robo4 following culture in high glucose (Figures 1 and 2), and diminished renal Robo4 expression in two independent rodent models of diabetic kidney disease (Figures 3 and 4), with relatively unchanged levels of Robo1 at time points when glomerular neovascularization is apparent. Taken together, these results suggest that diminished Robo4 expression in diabetic glomeruli may contribute to reduced anti-angiogenic signaling, permitting the uncontrolled VEGF-driven glomerular angiogenesis seen in diabetes. To date, however,
no published reports have investigated whether changes in Robo isoform expression in GECs are responsible for the enhanced angiogenesis observed in diabetes. In this study, we will investigate whether the levels of Robo1 and/or Robo4 are important in regulating the VEGF-driven GEC angiogenic functions in both normal and high glucose settings. We hypothesize that Robo1 promotes, while Robo4 antagonizes, the VEGF-induced glomerular angiogenesis that occurs in the diabetic kidney.

**Hypothesis:** Reduction of the pro and anti-angiogenic Robo1 or Robo4 receptors will inhibit or promote VEGF-mediated endothelial angiogenic functions, respectively.

![Overall schematic for in vivo.](image)

HRGEC will be cultured in HG, NG and mannitol conditions, and silenced for Robo4 and Robo1 before undergoing network formation and migration angiogenesis assays.
2.3 Materials and Methods

2.3.1 Human renal glomerular endothelial cell (HRGEC) culture

Primary human renal glomerular endothelial cells (HRGECs) were purchased from Sciencell (Carlsbad, California, catalogue # 4000), and cultured in standard Endothelial Culture Medium (Sciencell) on human fibronectin (1 mg/mL)-coated plates (Sigma, Oakville, Ontario, catalogue # F2006). Cells between passages 2 and 7 were used for experiments. When cells reached 80-90% confluency, they were lifted from surfaces with Trypsin – EDTA (Fisher Scientific, Waltham, Massachusetts, catalogue # 25200-056), or in the case of flow cytometry, StemPro Accutase Cell Dissociation Reagent (Innovative Cell Technologies, San Diego, California, catalogue # A11105), before being quantified with a hemocytometer.

2.3.2 Glucose stimulation

To simulate the high glucose environment in the diabetic kidney, HRGECs were grown in endothelial culture medium (ECM) supplemented with 20 mmol/L D-glucose (high glucose, HG). Control HRGECs were grown in normal ECM (containing 5 mmol/L D-glucose), or ECM supplemented with 20 mmol/L D-mannitol as an osmotic control.
2.3.3 Immunoblotting

HRGECs were lysed using a Triton X-100 lysis buffer (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1% triton X-100, and protease inhibitors). Total cellular protein levels were measured using the Bradford Assay (Sigma, catalogue # B6916). Lysates were then separated by SDS-PAGE, and following transfer, membranes were blotted with primary antibodies directed against: human Robo1 (Rockland, catalogue # 600-401-692), human Robo4 (Abcam, Cambridge, Massachusetts, catalogue # ab103674), α-Tubulin (Abcam, catalogue # ab4074), and GAPDH (Abcam, catalogue # ab8245). Primary antibodies were detected using horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse secondary antibodies (Santa Cruz).

2.3.4 Flow Cytometry

After 24 hours of cell culture, HRGECs were collected using Accutase (Innovative Cell Technologies, catalogue # A11105), as described in Section 2.1.1. Five individual cell pellets consisting of 1 x 10^6 cells were re-suspended in 400 µL of fresh ECM + 1% fetal bovine serum albumin (FBS) and 1% EDTA. The cells were then incubated with 0.01 µg/µL of FITC-conjugated isolectin-B4 (VWR, Radnor, Pennsylvania, catalogue # 89153-050), PE-conjugated anti-human VE-Cadherin antibody (Abcam, catalogue # ab33321), or PE-conjugated anti-human CD31 antibody (Abcam, catalogue # ab46733) for 20 minutes on ice in the dark. FITC- and PE-conjugated secondary antibody alone controls were also used (Abcam). Stained cells
were analyzed using a MACS Quant flow cytometer (Miltenyi Biotec, Bergisch, Germany). Cells incubated with irrelevant IgGs conjugated to the appropriate fluorophore were used as negative isotype controls and gating was further validated using the appropriate Fluorescence Minus One controls. MACSQuantify software (Miltenyi Biotec) was used to determine the percentage of cells that were positively fluorescent for either FITC or PE.

2.3.5 Robo1 silencing

HRGECs at 60-80% confluency were transfected with siRNA with Lipofectamine RNAiMAX according to manufacturers recommended suggestions, targeting human Robo1 (RNAiMAX and Silencer Select from Invitrogen, Carlsbad, California, catalogue # 13778030 and s12093). Following transfection, HRGECs were cultured in standard ECM for 72 hours. In some cases, ECM was supplemented with D-glucose or D-mannitol, as described in Section 2.1.2. to simulate high glucose or mannitol osmotic control conditions, respectively. Following this 72 hr culture period, cells were collected and tested for functionality or protein quantification. As lipofectamine is toxic to HRGECs, the cells were exposed to the transfection reagent for no longer than 4 hours.

2.3.6 Matrigel\textsuperscript{TM} Network Formation Assay

HRGECs were subjected to a network formation assay, as previously described (Darren A Yuen, Zhang, et al., 2012). Briefly, growth-factor reduced basement
membrane matrix Matrigel (Corning, New York, New York, catalogue # 354320) was thawed on ice overnight to a liquid-like consistency. A 96-well plate, along with p200 pipette tips, were also chilled overnight at 4°C. The wells of the 96-well plate were then coated with 50 µl of ice-cold Matrigel. The plate was spun down at 300g to remove any bubbles from the bottom of the well before being placed into a 37°C incubator for 30 minutes, to solidify the Matrigel.

Three-hour serum starved HRGECs were collected using Trypsin-EDTA, and spun down at 1400 RPM for 5 minutes. Between 8 - 10 x 10³ cells were resuspended in 100 µl of basal ECM (with no added factors, except 5% FBS), supplemented additionally with or without 100 ng/mL of VEGF-165, and seeded into their respective Matrigel-coated wells. Cells were then incubated for the indicated times, and imaged using a phase-contrast light microscope (Zeiss Axio Observer Live Cell, Oberkochen, Germany). Five non-overlapping images were taken in a standardized fashion per well. The total summed length of the networks was analyzed using Fiji image analysis software (ImageJ software, NIH)

2.3.7 Boyden-Chamber HRGEC Migration Assay

HRGECs were subjected to a Boyden-Chamber migration assay as previously described (Li & Zhu, 1999). Briefly, HRGECs were first serum-starved for 6 hours, and then collected using Trypsin-EDTA. 2.5 x 10⁵ cells were then resuspended in 100 µl of 5% FBS-supplemented basal ECM, and seeded into human fibronectin-coated 8 µm pore transwell filters (Corning, catalogue # 3422). The transwell filters were then placed
into wells of a 24 well plate, with each well containing 600 µL of basal ECM supplemented with 100 ng/mL of VEGF-165 where indicated. After a 3 hour incubation at 37°C, the transwell filters were gently washed with PBS, and the upper chamber was cleared of any excess cells by using a cotton swab. The transwell filter was then placed into a 4% paraformaldehyde (PFA) solution for 15 minutes, before being washed with PBS. The filter was then stained with a 5 µg/mL crystal violet stain (solubilized in 70% ethanol) for 5 minutes. Excess crystal violet was washed with PBS, before the filter membrane was removed from the transwell via a razor blade, mounted on a slide, and air-dried for 24 hours.

Slides were imaged using bright field microscopy at 200X magnification using a brightfield microscope (Nikon Upright E800, Tokyo, Japan). Five 2.5 x 10^5 µm^2 non-overlapping regions of each filter were imaged in a standardized fashion. The total number of cells in these five images was manually counted per filter, and normalized to the number of loaded cells.

2.4 Statistical analysis

All data are shown as mean ± SEM unless otherwise specified. A minimum number of 3 independent replicates were conducted per experiment. Between group differences were analyzed using one-way ANOVA with a post-hoc Fisher’s least significant difference test. All statistical analyses were performed using GraphPad Prism 6.00 for MacOS X (GraphPad Software). A statistically significant difference was
considered to be $p < 0.05$.

2.5 Results

2.5.1 Characterization of primary HRGECs

Since primary HRGECs have been characterized in less detail than other standard endothelial cell types such as human umbilical vein endothelial cells, I first examined whether these cells express cell surface markers characteristic of microvascular endothelial cells. Flow cytometric analysis of passage 2 – 6 HRGECs demonstrated that these cells expressed high levels of CD31 (99.6%), VE-Cadherin (97.1%), and plasma membrane polysaccharides with terminal α-D-galactosyl residues that bind to *Bandeiraea simplicifolia* isoelectin B4 (Figure 6).
Figure 6: Primary HRGECs express cell surface markers characteristic of microvascular endothelial cells.

Passage 2-6 HRGEC were collected and stained with PE-conjugated anti-VE-cadherin or anti-CD31 antibodies, or with FITC-conjugated isolectin B4. When compared to cells stained with FITC- and PE alone (A, C), > 95% of the HRGECs were positive for VE-Cadherin, Isolectin B4, and CD31 (B, D, E). The percentage of positively stained cells is listed at the top left corner of each panel.
2.5.2 High glucose exposure enhances VEGF-induced HRGEC migration and network formation

VEGF is a major driver of glomerular angiogenesis in the diabetic rodent kidney. In preliminary work, diminished Robo4 transcript and protein levels were found in HRGECs following high glucose stimulation (Figures 2 and 3). Similarly, in rodent models of insulin-deficient (STZ-Wistar rat) and insulin-resistant diabetes (db/db mouse), glomerular Robo4 RNA and protein levels were decreased compared to non-diabetic controls (Figures 4 and 5). Our finding that levels of the anti-angiogenic Slit2 receptor Robo4 fall in high glucose/diabetic conditions suggested that glucose-induced Robo4 deficiency may enhance VEGF-induced HRGEC angiogenic activity. To test this hypothesis, HRGEC responses to VEGF were measured using two established in vitro assays of angiogenic activity: the Boyden-chamber migration assay and the Matrigel network formation assay.

To test the effect of glucose concentration on VEGF-induced migration, HRGECs were first incubated for 72 hours in complete Endothelial Cell Medium containing either normal glucose (NG, D-glucose 5 mmol/L), high glucose (HG, D-glucose 25 mmol/L), or normal glucose supplemented with mannitol as an osmotic control (Man, D-glucose 5 mmol/L + D-mannitol 20 mmol/L). Cells were then loaded into transwell filters, and their VEGF-induced chemotaxis measured in a modified Boyden chamber migration assay. In this experiment, high glucose-cultured HRGECs migrated to a greater degree than HRGECs maintained in normal glucose-containing medium. In contrast, mannitol exposure as an osmotic control did not enhance VEGF-induced chemotaxis (Figure 7).
Figure 7: Exposure to high glucose enhances VEGF-induced HRGEC migration.

HRGECs were grown in normal glucose- (NG, D-glucose 5 mmol/L), high glucose- (HG, D-glucose 25 mmol/L), or mannitol- (Man, D-glucose 5 mmol/L + D-mannitol 20 mmol/L) containing complete Endothelial Culture Medium (ECM) for 72 hours. After 6 hours of serum starvation in basal ECM supplemented only with glucose or mannitol as described above, 1 x 10^5 cells were loaded into transwell filters (8 µm pore size) placed in wells containing basal endothelial culture medium (+ 5% FBS) supplemented with VEGF 10 ng/mL and glucose or mannitol. After 3 hours, the number of cells which had migrated through the filter were counted. VEGF-induced migration was enhanced with high glucose exposure (C), when compared with cells grown in normal glucose-containing medium (B) or mannitol-supplemented medium (D). (G) Quantification of cell migration, presented as number of cells migrated per 10^5 cells loaded. Arrows identify migrated cells. Scale bar: 100 µm. Original magnification 200X. * p < 0.05, n=3.

To characterize the effects of glucose exposure on VEGF-induced HRGEC network formation, cells were again cultured in normal glucose, high glucose, or mannitol-containing Endothelial Culture Medium for 72 hours. Following this culture period, 8 x 10^3 cells were seeded in Matrigel-coated wells and stimulated with VEGF 100 ng/mL. Following a 6 hour incubation period, network formation was assayed by measuring cumulative branch length. Similar to the results obtained with the VEGF-induced migration assay, high glucose-cultured HRGECs formed more extensive networks compared with both normal glucose and mannitol-cultured cells (Figure 8).
Figure 8: Exposure to high glucose enhances VEGF-induced HRGEC network formation.

HRGECs were grown in normal glucose- (NG, D-glucose 5 mmol/L), high glucose- (HG, D-glucose 25 mmol/L), or mannitol- (Man, D-glucose 5 mmol/L + D-mannitol 20 mmol/L) containing medium for 72 hours. 8 x 10^3 cells were loaded onto Matrigel™ after being resuspended in culture medium (+5%FBS) supplemented with or without VEGF 100 ng/mL. After 6 hours, cumulative branch length was measured using ImageJ. VEGF-induced network formation was enhanced with high glucose exposure (C), when compared with cells grown in normal glucose-containing medium (B). Cells grown in mannitol-supplemented medium did not exhibit enhanced VEGF-induced network formation (D). (E) Quantification of network formation is presented as total length of networks formed normalized to NG without VEGF. Scale bar: 100 µm. Original magnification 200X. * p < 0.05, n=3.

2.5.3 Altering the levels of Robo4 and Robo1 regulates angiogenesis in HRGECs

The results presented above suggest that high glucose exposure enhances the VEGF responsiveness of cultured HRGECs. As described in Section 2.1.5., preliminary work has also demonstrated that exposure of glomerular endothelial cells to high glucose is associated with a reduction in the levels of the anti-angiogenic Robo4 receptor relative to its pro-angiogenic Robo1 counterpart. To determine if glucose-
mediated changes in VEGF responsiveness are regulated by the ratio of these two receptors, siRNA-mediated knockdown of the pro-angiogenic Robo1 receptor was performed, followed by culture in normal glucose, high glucose, or mannitol-containing Endothelial Culture Medium for 72 hours. At the end of the 72 hour culture period, specific Robo1 knockdown was confirmed via immunoblotting. In contrast, no significant difference in Robo4 levels was noted (Figure 9).

![Immunoblot images for Robo1, Robo4, and α-Tubulin](image)

**Figure 9: Confirmation of specific Robo1, but not Robo4, knockdown.**

HRGECs were grown to 60-80% confluency, and transfected with 25 pmol/L of Robo1 siRNA, 25 pmol/L of a non-targeting control siRNA, or lipofectamine only (Ctrl). Seventy-two hours after transfection, Robo1 siRNA-transfected cells demonstrated a
near complete knockdown of Robo1 protein expression, without altering Robo4 or α-Tubulin protein levels, highlighting high transfection efficiency.

Since HG treatment caused a reduction in Robo4 and pro-angiogenic-like response from HRGECs, we were interested to see if reducing levels of Robo1 would also have a dramatic, yet opposite, effect on HRGEC response to VEGF. We decided to reduce Robo1 levels using siRNA targeting human Robo1 in our HRGECs. Importantly, we also made sure that our siRNA only knocked down Robo1 levels, without altering its “antagonist” Robo4 protein levels. Accounting for this allowed us to better postulate the importance of the Robo4 and Robo1 receptors in VEGF-induced angiogenesis.

Following confirmation of specific Robo1 knockdown, Robo1-deficient cells cultured in normal glucose, high glucose, or mannitol-containing Endothelial Culture Medium for 72 hours were subjected to VEGF-induced migration. Consistent with its role as a pro-angiogenic mediator, loss of Robo1 led to a dramatic impairment of both basal and VEGF-induced chemotaxis (Figure 10). In contrast, cells transfected with a non-targeting control siRNA demonstrated robust VEGF-induced chemotaxis that was further augmented in the setting of high glucose exposure (Figure 10). Trypan blue exclusion assay confirmed HRGEC viability prior to being plated onto the migration assay (data not shown).
Figure 10: Robo1 deficiency abrogates the high glucose-induced augmentation of VEGF-induced HRGEC migration.

HRGECs were transfected with a Robo1 siRNA (siRobo1), or a non-targeting siRNA (NT-siRNA), and then cultured in ECM containing normal glucose (NG), high glucose (HG), or mannitol (Man) for 72 hours. When compared to NT-siRNA-transfected cells grown in medium containing normal glucose or mannitol, NT-siRNA transfected cells grown in high glucose conditions migrated in greater numbers in response to a VEGF chemotactic gradient. Following Robo1 knockdown, however, HRGECs did not migrate in response to VEGF, irrespective of glucose concentration. Scale bar: 100 µm. Original magnification: 200X. Arrows identify migrated cells. * p < 0.05 vs non-VEGF treated cells. † p < 0.05 vs. NG+VEGF and Man+VEGF, n=3.

Following confirmation of specific Robo1 knockdown, Robo1-deficient cells cultured in normal glucose, high glucose, or mannitol-containing Endothelial Culture Medium for 72 hours were subjected to VEGF-induced network formation assay. Consistent with its role as a pro-angiogenic mediator, loss of Robo1 led to a dramatic impairment of both basal and VEGF-induced chemotaxis (Figure 11). In contrast, cells transfected with a non-targeting control siRNA demonstrated robust VEGF-induced chemotaxis that was further augmented in the setting of high glucose exposure (Figure 11). Trypan blue exclusion assay confirmed HRGEC viability prior to being plated onto the matrigel (data not shown).
Figure 11: Robo1 deficiency abrogates the high glucose-induced augmentation of VEGF-induced HRGEC network formation.

HRGECs were transfected with a Robo1 siRNA (siRobo1), or a non-targeting siRNA (NT-siRNA), and then cultured in ECM containing normal glucose (NG), high glucose (HG), or mannitol (Man) for 72 hours. When compared to NT-siRNA-transfected cells grown in medium containing normal glucose or mannitol, NT-siRNA transfected cells grown in high glucose conditions formed more total networks in response to VEGF. Following Robo1 knockdown, however, HRGECs formed minimal networks, irrespective of glucose concentration. Scale bar: 100 µm. Original magnification: 200X. * p < 0.05 vs non-VEGF treated cells. † p < 0.05 vs. NG+VEGF and Man+VEGF, n=3.
2.6 Discussion

Diabetic nephropathy, one of the most serious complications of diabetes, is the leading cause of chronic kidney disease in Canada (Public Health Agency of Canada, 2011). As in other targets of diabetic end-organ damage, endothelial injury plays a key role in mediating kidney disease (Nakagawa, Kosugi, Haneda, Rivard, & Long, 2009). One of the earliest manifestations of renal endothelial injury in diabetes is new capillary growth within the glomerulus. Diabetic glomerular neovascularization has classically been thought of as driven by the increased glomerular production of pro-angiogenic stimuli such as vascular endothelial growth factor (VEGF) (Cooper et al., 1999). Preliminary data, however, have suggested that high glucose exposure leads to alterations in Slit-Robo signaling pathways that have been shown by other groups to be important in regulating angiogenesis (C. A. Jones et al., 2008; C. a Jones et al., 2009; Sheldon et al., 2009). Specifically, culture in high glucose conditions lead to reduced expression of the anti-angiogenic receptor Robo4, but a non-significant increase in the levels of the pro-angiogenic receptor Robo1, resulting in a diminished Robo4 expression (Figures 1 and 2). Similarly, induction of diabetes in both STZ-Wistar rats and db/db mice was associated with a similar reduction in Robo4 expression (Figures 3 and 4). Taken together, these results suggested that diabetes may induce changes in endothelial Slit-Robo signaling that could modulate VEGF activity and glomerular angiogenesis.

Building upon this preliminary data, in this thesis, I explored the role of the Slit-Robo signaling pathway in the regulation of VEGF-driven diabetic glomerular
angiogenesis. Using in vitro and in vivo model systems, I first demonstrated that high glucose augments the VEGF responsiveness of glomerular endothelial cells, leading to enhanced migration and network formation in vitro, and increased PECAM-1 glomerular density and total capillary length in vivo. These results suggested that high glucose promotes glomerular angiogenesis not only by increasing renal VEGF production (Cooper et al., 1999), but also by enhancing the VEGF responsiveness of glomerular endothelial cells.

Having documented the augmenting effects of high glucose, I next examined whether changes in Robo4 and Robo1 expression induced by high glucose exposure may regulate glomerular endothelial VEGF responsiveness. I showed that HRGECs raised in HG migrated more and formed more networks in the presence of VEGF (Figures 7 and 8). However, when Robo1 was knocked down using siRobo1 (which did not alter Robo4 levels) (Figure 9), I showed that the VEGF and HG induced angiogenesis was diminished (Figures 10, 11).
2.7 Limitations

The importance of Robo1 and Robo4 in regulating glomerular endothelial responses to VEGF

The experiments described in this thesis suggest that Robo1 and Robo4 protein levels in glomerular endothelial cells are important in regulating their responses to VEGF. In the context of diabetes in particular, I demonstrate that high glucose-induced reduction in Robo4 expression is associated with enhanced glomerular endothelial cell responsiveness to VEGF.

In my experiments, I demonstrated that Robo1 knockdown in human glomerular endothelial cells completely abrogates VEGF-induced migration and network formation, both in normal and high glucose conditions. However, the role of glucose on Robo1 and its effects on VEGF signaling remains untested.

Although these results suggest that Robo1 is critical for VEGF-induced glomerular endothelial responses, it is interesting to note that while Robo1 knockout mice die prenatally (Andrews et al., 2006), mice with severely reduced Robo1 expression (Robo1 hypomorphs) were reported to be viable and grossly normal in appearance (Long et al., 2004). While in this report the kidneys were not specifically commented on, the normal development of these mice would suggest that very low levels of Robo1 are sufficient to sustain the VEGF-induced glomerular endothelial responses necessary for normal renal development (Cooper et al., 1999). Clearly, formal study of these mice, both in the absence and presence of diabetes, would
provide important insights into the role that Robo1 plays in regulating VEGF-mediated glomerular endothelial responses.

Additionally, while the work presented in this thesis supports the notion that the relative levels of Robo4 and Robo1 protein are important in regulating VEGF-driven glomerular endothelial responses, it is also possible that post-translational modifications of these receptors, or changes in downstream signaling intermediates, may also play important roles in this regulation.

2.8 Conclusions

In this chapter, we were exploring whether the Slit-Robo signaling pathway plays a role in regulating HRGEC responsiveness to VEGF. We have shown that in HG conditions, Robo4 levels drop and this is associated with promoted response to VEGF in migration and network formation assays. We also showed that Robo1 knockdown has an opposite effect. We have proved that our hypothesis is correct, in that a reduction in the pro or anti-angiogenic Robo1 or Robo4 receptors represses and enhances HRGEC responsiveness to VEGF, respectively. Future directions would look to overexpress Robo4 and Robo1 to see if we can see opposite effects on HRGECs, as well as to explore what happens to downstream signaling proteins in the settings of HG.
Chapter 3

Robo4 KO Mice have Increased Diabetes Induced Glomerular Angiogenesis
3 In vivo Robo4 KO mice Experiments

3.1 Introduction

Mice either globally deficient or with endothelium deficient in Robo4 exhibit enhanced postnatal neovascularization in the setting of pro-angiogenic stimuli. Early diabetic kidney disease is characterized by a significant increase in glomerular production of pro-angiogenic factors such as VEGF, which in turn trigger migration and proliferation of ECs to form new glomerular capillaries (Cooper et al., 1999).

3.2 Specific Aims and Hypothesis

In this study, we will examine the roles that the Slit2 receptors Robo1 and Robo4 play in regulating high glucose-induced glomerular endothelial cell angiogenesis and capillary growth. For this purpose, we have established a colony of homozygous Robo4 KO and WT, as described in 2.2.1. These mice are viable and fertile, and their Robo1 levels are unaffected by the Robo4 ablation.

We will test glomerular angiogenesis in these Robo4 KO mice and compare them to their non-diabetic and diabetic WT littermates using both glomerular PECAM-1 density staining, in formalin fixed tissues, and by measuring total glomerular capillary length using fluorescent beads.
**Hypothesis:**

Robo4 KO mice will have enhanced glomerular angiogenesis compared to their WT littermates, in early diabetes.

**Figure 12: Overall schematic for *in vivo.***

WT and KO littermates will receive STZ or a citrate buffer to generate DM and non-DM controls. These animals will then be sacrificed, and renal structure and function will be accessed.
3.3 Materials and Methods

3.3.1 Breeding of Robo4 WT and KO mice

Robo4 knockout (KO) mice on a mixed CD1/C57BL/6 background were a kind gift from Dr. Susan Quaggin and Dr. Dean Li (who generated the mice). Details of the generation of these mice have been previously published (C. A. Jones et al., 2008). Briefly, exons 1-5 of ROBO4 were excised and replaced with an alkaline phosphatase gene, and a neomycin cassette. Embryonic stem cells (ESC) were then transfected via electroporation with this modified vector, and insertion of the mutated Robo4 gene was accomplished via homologous recombination. Neomycin-transfected ESCs were isolated and placed into a blastocyst before being implanted into a pseudopregnant surrogate mother to generate a chimera mouse. Finally, the chimera mice carrying a germline mutation were bred with normal mice, which generated mice heterozygous for the mutation (Robo4<sup>+/−</sup>).

To generate Robo4 wild type (WT) and knockout (KO) littermates for study purposes, mice heterozygous for Robo4 (Robo4<sup>+/−</sup>) were bred to one another. Only male mice were used for this study, given the known gender differences in renal responses to injury (K. M. Park, Kim, Ahn, Bonventre, & Bonventre, 2004; Wei, Wang, & Dong, 2005). Genomic DNA from the ear-notches of male mouse pups was isolated using a KAPA Mouse Genotyping Kit (KAPA, Wilmington, Massachusetts, catalogue # KK7352).
according to the manufacturer’s protocol. The genomic extracts acted as the template in a polymerase chain reaction (PCR) using the following primers:

**ROBO4 Forward Primer:** 5’ – AGA ACA ACC GGA CAA AAG TGT ATG -3’

**ROBO4 Reverse Primer:** 5’ – GTC TGA GTC CAT AGG TCA AGA TC – 3’

**ROBO4 Alkaline Phosphatase Primer:** 5’ – CAG GGA GAT GAT GAG GTT CTT GG – 3’

The amplicon length for the wild type Robo4 allele was 400 bp, and for the knockout Robo4 allele was 250 bp. The PCR conditions were as follows: 95°C x 2 minutes followed by 30 cycles of (95°C x 30 seconds, 56°C x 20 seconds, 72°C x 30 seconds).

PCR products were loaded onto an ethidium bromide-containing 2% agarose gel, and run at 50V for 1 hour, in a horizontal gel electrophoresis machine. In all cases, one lane was dedicated for a negative control, which used an equivalent volume of ddH$_2$O in place of the genomic template.

### 3.3.2 Diabetes induction

Six-week-old male Robo4 KO mice were randomized to receive a daily intraperitoneal (i.p.) injection of 50 mg/kg streptozotocin (STZ, Sigma-Aldrich, catalogue # S0130) solubilized in citrate buffer (pH 4.5), or citrate buffer alone, for 5 consecutive days as per a published protocol from the Animal Models of Diabetic Complications Consortium (Brosius, 2003), to generate diabetic and non-diabetic Robo4 KO study
mice. Mice were fasted for 4 hours prior to each injection. Male Robo4 WT mouse littermates underwent the same protocol and served as diabetic WT controls. One week after the final i.p injection, tail vein blood sugar levels were measured. Mice that had a blood glucose level > 15 mmol/L were considered diabetic. Diabetic mice were given daily mashed-food diets, routine saline subcutaneous injections as needed, and had their cages changed every day for sanitary purposes, until study end. Similar to other studies of murine diabetic kidney injury, subcutaneous insulin was not administered (Daniel & Wegmann, 1996).

3.3.3 Metabolic monitoring

Body weights were recorded at baseline (prior to STZ or citrate buffer injection), and weekly for four weeks, until study end. Mice also underwent 24 hr metabolic caging for recording of urine output and collection of urine samples at baseline and two days before study end. The lab at TGH, using blood collected at end-study, measured blood-glucose levels.

3.3.4 Invasive blood pressure measurements

At end study, mice were intubated and anesthetized with 2% isofluorane. With the help of a skilled surgeon (Dr. Golam M. Kabir), a 24-gauge angio-catheter was placed inside the ascending thoracic aorta of each mouse, and systolic and diastolic pressures were recorded. To minimize temperature-associated variations in blood
pressure, the room temperature was maintained at 20°C, and the mice were placed on a heated-pad to help maintain constant body temperature.

3.3.5 End study sample collection

At study end, following invasive blood pressure measurement, blood was collected using a 24-gauge angio-catheter inserted into the mouse abdominal aorta, with the tip placed just distal to the origins of the renal arteries. Following collection in ethylenediaminetetraacetic acid (EDTA)-coated tubes, the blood was centrifuged at 3000 RPM for 15 minutes at 4°C. The serum was collected and stored at -80°C for future analysis. The anatomical right kidney of the mouse was collected, weighed, and snap frozen in liquid nitrogen for future molecular analysis. Finally, the animal was perfusion-exsanguinated via cutting open the jugular vein and perfusion of the animal with 20 mL/min PBS through the abdominal aorta to allow for slow and steady perfusion of the abdomen (Abcam protocols). Following exsanguination, whole body fixation was performed with perfusion of 10% neutral buffered formalin at 20 mL/min. The left kidney was then removed from the cadaver, weighed, and placed into formalin for 48 hours before being paraffin embedded. In some animals, fluorescence microangiography of the left kidney was performed.
3.3.6 Tissue preparation and histology

Formalin-perfused kidneys (collected from Section 2.2.6) were transferred to 70% ethanol before being paraffin embedded. Kidneys were cut into 3 µm sections using a microtome (Leica RM series, Wetzlar, Germany), and mounted onto slides. The slides were incubated in a 37°C oven for 48 hours to fix the samples securely to the slides. Kidney sections were stained with a rabbit anti-mouse monoclonal antibody directed against platelet endothelial cell adhesion molecule – 1 (PECAM-1, also known as CD31, Abcam, catalogue # ab28364), by our collaborator, Dr. Rohan John. Stained slides were digitally scanned and glomerular endothelial cell density was estimated by measuring the mean percent positive area for CD31 staining in 50-80 randomly selected glomeruli from each mouse using Aperio imagescope software. Glomeruli with visible macula densa, indicating the middle of the glomerulus, were selected for analysis. This allowed us to extrapolate data to the rest of the glomeruli. Digital analysis was performed in a blinded fashion.

3.3.7 Renal fluorescence microangiography

In some mice, renal fluorescence microangiography was performed as previously described (Advani et al., 2011a; Darren A Yuen et al., 2010). Briefly, a fluorescent solution, containing a 10% solution of 0.02 µm diameter, 505/515 nm fluorescent beads (Life Technologies, Carlsbad, California, catalogue # F-8787) mixed with 1% low-melting agarose dissolved in boiling water was prepared, filtered using a 0.45 µm filter,
and placed in a 70°C bath to maintain in liquid phase. Following exsanguination-perfusion, but prior to removal of the left kidney as described in Section 2.2.6, mice were hand-perfused with 3 mL of this fluorescent bead solution, infused via a 5 mL syringe attached to a 24 gauge angio-catheter placed with its tip just distal to the origin of the renal arteries. Immediately after bead perfusion, ice was placed over the mouse abdominal cavity to rapidly cool and thus solidify the fluorescent bead-agarose mixture with the abdominal vasculature. To ensure adequate solidification, mice were subsequently submerged in ice for 15 minutes, followed by collection of the left kidney as described in Section 2.2.6.

Kidneys were subsequently mounted on to the stage of a vibrotome (Leica VT1200 S) using Krazy glue, submerged in ice-cold PBS, and cut into 175 µm sections using a ceramic blade (Cadence, catalogue # EF-INZ10). The translucency of the kidney sections was then enhanced with 2,2′-Thiodiethanol (TDE) (Sigma, catalogue # 88559) treatment. Briefly, after one wash with PBS, the sections were placed sequentially in PBS-diluted 10% TDE x 10 minutes, 25% TDE x 10 minutes, 50% TDE x 10 minutes, and 97% TDE x 20 minutes to increase tissue translucency. Using a fine paintbrush, TDE-treated sections were then mounted onto microscope slides, covered with a square cover slip, and sealed using clear nail polish. The slides were protected from light by being covered in aluminum foil, and air-dried at room temperature for 24 hours before being imaged.
3.3.8 Confocal microscopy of FMA sections

To examine the glomerular microvasculature, TDE-treated FMA kidney sections were imaged using a confocal microscope (Leica LMS-700). Five glomeruli were randomly selected per kidney. Each glomerulus was imaged sequentially, from the top of the glomerulus to bottom, with images being collected with a 0.7 µm step size, which provides better resolution than what was previously shown (Advani et al., 2011). This step size was chosen based on the limit of the objective, and after optimization of the 3D reconstruction software. Glomeruli ranged in size between 60 - 120 µm in depth. The z-stacks were automatically rearranged to form a 3-dimensional (3D) image using Leica’s native confocal image processing software (Zen Black). The z-stacks were further processed in IMARIS 7.06 (Bitplane, Zurich, Switzerland) to generate 3D reconstruction images that were used for quantitative analysis of total glomerular capillary length.

Briefly, the sequential 2-dimentional images of each glomerulus z-stack were used to determine the thickest and thinnest diameters for the capillaries, in order for the software to distinguish capillaries from beads. Converting the z-stacks into 2D, and manually measuring the diameter of multiple small and large vessels, enabled us to determine the minimum and maximum diameters of the capillaries. These measurements were input into the FilamentTracer program in IMARIS, which then generated a 3D reconstruction of the glomerular capillaries. The program did this by creating small seed points within the capillaries it automatically detected according to the thick and thin regions set earlier. Seed points were automatically rendered, but were
manually removed or added to more accurately represent the capillaries only and to avoid the background.

3.3.9 Statistical analysis

All data are shown as mean ± SEM unless otherwise specified. A minimum number of 3 independent replicates were conducted per experiment. Between group differences were analyzed using one-way ANOVA with a post-hoc Fisher’s least significant difference test. All statistical analyses were performed using GraphPad Prism 6.00 for MacOS X (GraphPad Software). A statistically significant difference was considered to be p < 0.05.

3.4 Results

3.4.1 The role of Robo4 in the regulation of glomerular angiogenesis in experimental diabetic nephropathy

In the above studies, I demonstrated that Robo1 and Robo4 modulate HRGEC responsiveness to VEGF, and that glucose-induced changes in this ratio mediate the enhanced VEGF responsiveness that occurs when cells are grown in high glucose-containing medium. Similarly, in preliminary work, it has been demonstrated that in STZ-
diabetic Wistar rats, a model of insulin-deficient diabetes, marked glomerular angiogenesis occurs in conjunction with a significant reduction in the ratio of renal Robo4 to Robo1 mRNA and protein. To assess the in vivo relevance of my in vitro findings, I utilized Robo4 knockout (KO) mice to determine whether altering Robo1 and Robo4 in vivo would affect glomerular angiogenesis in an experimental model of rodent diabetic nephropathy. Importantly, Robo4 knockout mice have previously been demonstrated to express normal endothelial levels of Robo1 in whole tissue lysates collected from the knockout mouse kidney, heart, spleen, brain and liver (C. A. Jones et al., 2008).

3.4.2 Generation of male Robo4 knockout and wild type mice

Male Robo4 knockout and wild type littermates were generated by breeding Robo4+/− parents. The genotype of the resulting pups was confirmed via PCR (Figure 13).

<table>
<thead>
<tr>
<th>Ladder</th>
<th>WT</th>
<th>WT</th>
<th>+/-</th>
<th>KO</th>
<th>+/-</th>
<th>KO</th>
<th>+/-</th>
<th>+/-</th>
<th>+/-</th>
<th>Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3000</td>
<td>500</td>
<td>400</td>
<td>300</td>
<td>200</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 13: Sample genotyping results showing Robo4 WT, KO and +/- mice.

Genomic DNA, extracted from mouse ear-notches, was used as template in a PCR, to genotype the mouse progeny after breeding. WT and KO amplicons are found at 400 and 250 base pairs, respectively. Robo4 +/- mice have one WT and one KO allele. ddH$_2$O was used as a negative control template (Ctrl).

3.4.3 Metabolic parameters of Robo4 KO and wild type mice.

Following induction of diabetes, blood sugars in both wild type and Robo4 KO mice increased significantly. By study end, all mice, with one exception, had greater than 33.3 mmol/L via glucometer measurements (Table 1). Therefore, serum glucose were measured by TGH, using blood samples collected at end-study. There was no significant difference between WT and KO mice, regardless of their diabetes status (Table 1). Over the course of the 4-week study, Robo4 KO mice did not differ from their wild type littermates with respect to body weight, systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure, regardless of their diabetes status (Table 1). In both Robo4 KO and wild type mice, STZ-induced diabetes was associated with a small, non-significant reduction in body weight (Table 1). A reduced blood pressure was observed likely due to the mice being in surgical plane of anaesthesia, at 2% isoflurane and 2% oxygen.
## Table 1: Parameters of mice at end-study (12 weeks of age)

<table>
<thead>
<tr>
<th>Group</th>
<th>WT</th>
<th>KO</th>
<th>DM-WT</th>
<th>DM-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Males only)</td>
<td>18</td>
<td>6</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>33.1 +/- 1.42</td>
<td>35.3 +/- 1.14</td>
<td>30.6 +/- 1.14</td>
<td>28.3 +/- 0.87</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>77.9 +/- 5.95</td>
<td>79.1 +/- 5.179</td>
<td>77.7 +/- 3.38</td>
<td>79.6 +/- 1.86</td>
</tr>
<tr>
<td>Diastolic Pressure (mmHg)</td>
<td>51.5 +/- 5.96</td>
<td>52.1 +/- 3.11</td>
<td>46.1 +/- 3.11</td>
<td>50.8 +/- 2.21</td>
</tr>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>60.3 +/- 5.93</td>
<td>61.1 +/- 5.63</td>
<td>56.7 +/- 2.98</td>
<td>60.4 +/- 1.89</td>
</tr>
<tr>
<td>Serum Glucose (mmol/L)</td>
<td>11.6 +/- 1.58</td>
<td>7.52 +/- 0.446</td>
<td>51.3 +/- 7.59 *</td>
<td>52.7 +/- 1.54 *</td>
</tr>
</tbody>
</table>
After sacrifice, the right kidneys were harvested and weighed. Absolute kidney weight, and kidney weight indexed to body weight, was significantly increased in both diabetic Robo4 KO and wild type mice compared to their corresponding non-diabetic controls. No significant differences were noted between Robo4 KO mice and their wild type littermates (Figure 14). Taken together, these data suggest that Robo4 deficiency does not alter the metabolic or hypertrophic effects of diabetes in the rodent kidney.
Figure 14: Diabetes is associated with renal hypertrophy in both Robo4 knockout (KO) and wild type (WT) mice.

At the end of the 4-week study, mice were sacrificed and their right kidney was weighed. No significant difference in kidney weight was observed between WT and KO mice, regardless of diabetes status. Diabetic Robo4 WT and KO kidneys were, however, heavier than their non-diabetic counterparts (A), a difference that was enhanced when normalizing for mouse weight (B). * p < 0.05.

3.4.4 Robo4 deficiency enhances diabetic glomerular angiogenesis

To assess the effects of Robo4 deficiency on glomerular angiogenesis in the diabetic kidney, formalin-perfused left kidneys were sectioned and immunostained with an antibody directed against platelet and endothelial cell adhesion molecule-1 (PECAM-1), a marker of endothelial cells. As expected, STZ-induced diabetic animals demonstrated increased glomerular PECAM-1 density when compared to their non-diabetic controls, consistent with glomerular angiogenesis (Figure 15). While Robo4 deficiency was not associated with increased glomerular endothelial density in non-diabetic animals, diabetic Robo4 KO mice exhibited significantly greater glomerular PECAM-1 density than their diabetic WT littermates (Figure 15).
Figure 15: Robo4 deficiency is associated with enhanced diabetic glomerular angiogenesis.

After 4 weeks of diabetes, formalin-perfused kidneys from diabetic and non-diabetic Robo4 KO and WT mice were sectioned and immunostained with an antibody directed against platelet and endothelial cell adhesion molecule-1 (PECAM-1) to identify endothelial cells. Shown are representative images of glomeruli from (A) a non-diabetic WT animal, (B) a non-diabetic Robo4 KO animal, (C) a diabetic WT animal, and (D) a diabetic Robo4 KO animal. (E) Quantitative analysis of glomerular PECAM-1 staining density. Scale bar: 50 µm. Original magnification: 400X. * p < 0.05. See Table 1 for samples per group.

Increases in PECAM-1 staining could be due not only to increased glomerular endothelial cell density, but also to increased glomerular endothelial PECAM-1 expression. Additionally, the rate at which the glomeruli were perfused, at end study, could either inflate or deflate the capillaries, could alter the exposure of the PECAM-1 epitope during immunohistochemical staining.

Therefore, to confirm that Robo4 deficiency enhances diabetic glomerular angiogenesis, I next performed fluorescence microangiography (FMA) of the left kidney of a subset of animals in each group. In this technique, the glomerular microvasculature is perfused with a fluorescent microbead-agarose mixture that, upon hardening, creates a fluorescent cast of the glomerular microvasculature that can be three dimensionally imaged using confocal microscopy. Because the fluorescent beads stain the capillary
lumina rather than the endothelial cells, FMA represents an alternate approach to examine glomerular capillary density that does not depend on recognition of a specific endothelial marker protein. Similar to the PECAM-1 two dimensional immunohistochemical analysis, three dimensional analysis of glomerular FMA z-stack images revealed that diabetes augments total glomerular capillary length, and Robo4 deficiency enhances the angiogenic effects of diabetes (Figure 16).
**Figure 16: Robo4 deficiency enhances diabetic glomerular angiogenesis.**

After 4 weeks of diabetes, diabetic and non-diabetic Robo4 KO and WT mice underwent fluorescence microangiography of their left kidneys (n=2 per group). Shown are representative images of glomeruli from (A and E) a non-diabetic WT animal, (B and F) a non-diabetic Robo4 KO animal, (C and G) a diabetic WT animal, and (D and H) a diabetic Robo4 KO animal. (A – D) are single slices of fluorescent bead-perfused glomeruli. Scale bar: 50 µm. Original magnification: 400X. In (E – H), each glomerular z-stack was converted by IMARIS software (Bitplane, Zurich, Switzerland) into a 3D reconstructed image. Quantitative analysis of total glomerular capillary length from 3D glomerular reconstruction images. A minimum of 5 glomeruli were sampled per group. * is p < 0.05 vs. non-diabetes, † is p < 0.05 vs. diabetes-WT.
3.5 Discussion

Having demonstrated that Robo1 and Robo4 levels are important in regulating HRGECs responsiveness to VEGF in angiogenesis assays in vitro, I next looked to see whether or not these results could be translated in vivo. To test this, I used diabetic and non-diabetic Robo4 KO mice and looked for glomerular angiogenesis using PECAM1 staining and FMA. I showed that Robo4 KO mice are similar to their WT counterparts in metabolic parameters (Table 1). However, diabetic Robo4 KO mice have significantly higher PECAM1 glomerular density (Figure 15) and total capillary length (Figure 16), further demonstrating the role of Robo4 as an antiangiogenic receptor in HRGECs.

3.6 Limitations

Complete loss of Robo4 further augmented glomerular angiogenesis in diabetic mice, whereas deletion of Robo1 in glomerular endothelial cells eliminated the ability of these cells to respond to VEGF. Taken together, these results suggest that Slit-Robo signaling is a novel regulator of diabetic glomerular angiogenesis.

While my results are suggestive of this conclusion, it is possible that other processes may have contributed to my findings. For example, while I demonstrated that Robo4 deficient mice develop augmented diabetic glomerular neovascularization, these mice have Robo4 deleted globally, and as such extraglomerular Robo4 deletion could theoretically have contributed to this phenotype. While Robo4 is endothelial-restricted in
its expression (Huminiecki et al., 2002), due to DNA methylation (Okada et al., 2014), Robo4 deletion in extraglomerular endothelium could conceivably affect glomerular hemodynamics and VEGF expression, leading to changes in glomerular capillary density independent of glomerular endothelial responsiveness to VEGF.

The Table 1 data shows serum glucose levels that were collected at end-study. The very high glucose measurements may be a result of the blood being collected while the mice were not fasting, under anesthesia, and without any preservatives being added to the blood sample.

The FMA data, though novel and in agreement with the PECAM-1 immunohistochemistry data, was generated from n = 2 mice per group. Additional samples to each group would increase the statistical power of our results.

The work described in this thesis also did not examine the potential roles of Slit ligands in diabetic glomerular angiogenesis. In preliminary work, it has been shown that glomerular endothelial Slit2 mRNA levels are decreased following 24 hrs of high glucose exposure. Glomerular Slit2 protein levels were similarly reduced in STZ-Wistar rats, suggesting that high glucose exposure leads not only to alterations in Robo receptor levels, but also in levels of Slit2 ligand. Whether this change in Slit2 plays an important role in the pathogenesis of diabetic glomerular angiogenesis is an important question that needs further examination. Along similar lines, as other Slit ligands have been shown to be important in the regulation of angiogenesis future work examining the effect of high glucose exposure on these Slit isoforms would be useful.
3.7 Conclusions

In this chapter, we were building on our *in vitro* findings that Robo4 plays an anti-angiogenic role in HRGECs, by exploring the glomerular angiogenesis experienced by Robo4 KO mice in diabetic and non-diabetic settings. We conclude that our hypothesis was correct in that complete loss of the anti-angiogenic Robo4 receptor leads to enhanced angiogenesis, as demonstrated by Robo4 KO mice having increased glomerular PECAM1 density, as well as increased total glomerular capillary length. Future directions would explore Robo1 deficient mice, to strengthen our finding that both Robo1 and Robo4 are important in regulating glomerular angiogenesis.
4 Overall Discussion

4.1 Summary of Results

Firstly, using an *in vitro* human glomerular endothelial system, I showed that loss of Robo1 in cultured glomerular endothelial cells prevents the augmenting effects of high glucose on VEGF-induced migration and network formation. Secondly, using mice deficient in Robo4, I demonstrated that loss of Robo4 similarly enhances the effect of diabetes on glomerular angiogenesis *in vivo*. Taken together, these results suggest that Robo4 and Robo1 play antagonizing roles in regulating the augmenting effects of high glucose on glomerular endothelial VEGF responsiveness, with Robo4 acting to inhibit, and Robo1 acting to promote, glomerular angiogenesis in the diabetic kidney.

4.2 Limitations

The mechanisms by which high glucose exposure leads to a reduction in Robo4 expression

The data presented in this thesis suggest that high glucose-induced reductions in Robo4 play an important role in enhancing VEGF-induced glomerular angiogenesis in diabetes. The mechanisms underlying this loss of Robo4, however, remain poorly understood. In preliminary work, high glucose exposure led to reductions in both mRNA
and protein levels of Robo4 (Figures 1 – 4), suggesting that high glucose controls Robo4 protein levels at least in part through regulation of Robo4 mRNA.

The diversity of Slit-Robo signaling

Since the initial discovery of Slit and its Robo receptors in the early 1990s, our understanding of Slit-Robo signaling has grown exponentially. To date, three known Slit and four Robo receptor proteins have been identified in mammalian cells. While the work in this thesis focused on the roles that Robo4 and Robo1 play in the high glucose-mediated regulation of VEGF-induced glomerular endothelial cell responses, other Robo receptors may also be involved. Robo2, for example, was recently found to play an important role in regulating post-natal neovascularization, working in concert with Robo1 to facilitate VEGF-induced endothelial migration and sprouting (Rama et al., 2015). While Robo2 mRNA levels were found to be downregulated in human diabetic nephropathy kidney biopsy samples (Lindenmeyer et al., 2010), little is known about the effects of high glucose on glomerular endothelial Robo2 expression. Future work examining whether Robo2 is differentially regulated following high glucose exposure, and whether alterations in Robo2 levels can modify glomerular endothelial responses to VEGF, would thus be of great interest.

Endothelial heterogeneity and responses to high glucose

As our understanding of endothelial biology continues to expand, endothelial heterogeneity is increasingly being recognized as an important determinant of
phenotypic differences between cells from different vascular beds (Aird, 2007). In this thesis, I focused on the responses of glomerular endothelial cells to VEGF in the context of normal and high glucose conditions, demonstrating that VEGF responsiveness was promoted by high glucose exposure (Figures 7, 8, 10, and 11). This finding, along with my discovery that Robo4 and Robo1 are important modulators of glucose-induced increases in VEGF responsiveness, cannot be generalized to endothelial cells from other vascular beds. Indeed, a recent report demonstrated that high glucose exposure is associated with reduced VEGF responsiveness in cultured human umbilical vein endothelial cells, and in subcutaneously implanted Matrigel plugs (Warren, Ziyad, Briot, Der, & Iruela-Arispe, 2014). This finding suggests that changes in Robo signaling may either be context-specific, or perhaps counteracted by other mechanisms in other endothelial cells.
Conclusions and Future Directions

5.1 Future Directions

The importance of Robo1 and Robo4 in regulating glomerular endothelial responses to VEGF.

Robo4 deletion in extraglomerular endothelium could conceivably affect glomerular hemodynamics and VEGF expression, leading to changes in glomerular capillary density independent of glomerular endothelial responsiveness to VEGF. To address this possibility, I would measure glomerular VEGF levels in Robo4 knockout and wild type mice to examine whether Robo4 deficiency is associated with changes in glomerular VEGF expression. Parallel *in vitro* experiments studying the effects of Robo4 knockdown and overexpression in glomerular endothelial cells cultured under normal and high glucose conditions would also be informative.

In an effort to address these issues, in preliminary work I have generated a plasmid encoding a C-terminally tagged mouse Robo4-GFP fusion protein with the aid of our collaborator (Dr. Lisa Robinson) (Figure 17). To confirm expression of this Robo4 fusion protein, NRK49F rat kidney fibroblasts (which endogenously do not express Robo4) were transfected, and Robo4 was detected, with the Robo4-GFP plasmid (Figure 18). To date, however, transfecting this vector efficiently into HRGECs has proven to be difficult, with no detectable GFP signal in transfected HRGECs. Future work may therefore require using an adenoviral or lentiviral-based delivery system.
Similarly, Robo4 knockdown is also underway using a human Robo4 siRNA sequence (Dharmacon, 5' GCCAAGACUACGAGUCAA 3') (Sheldon et al., 2009).
Figure 17: Robo4-GFP plasmid map.

A pBR322 GFP backbone was used to create a C-terminus GFP-tagged Robo4 expression plasmid using AgeI and KpnI restriction enzymes. Sequencing revealed a plasmid with genes positioned in the correct locations.
**Figure 18: Rat kidney fibroblasts transfected with Robo4-GFP plasmid.**

NRK49F cells were transfected with the Robo4 overexpressing vector using polyethylenimine (PEI) transfection agent. After 3 days, cells were permeabilized and stained with a DAPI nuclear stain, mouse anti-human Robo4 antibody, Alexa 594-conjugated donkey anti-mouse antibody, and WGA conjugated with Alexa 647 (a membrane stain). NRK49f cells that were treated only with PEI showed no Robo4 expression. Similarly, no Robo4 signal could be detected in cells transfected with Robo4-GFP, but stained only with a secondary antibody (2° Ab Ctrl). Cells transfected with Robo4-GFP, and stained with an anti-mouse Robo4 antibody followed by a donkey anti-mouse secondary antibody (Robo4 1° + 2° Ab Ctrl), demonstrated detectable Robo4 signal.

**Regulation of downstream signaling from Robo receptors**

While my work has been focused on the Slit-Robo signaling pathway at the level of the receptors, future experiments could therefore examine potential changes in the levels and activities of the small GTPases Rac, RhoA, and Cdc42 that act downstream of Robo4 and Robo1 receptors.

**The mechanisms by which high glucose exposure leads to a reduction in Robo4 expression**

I have shown that Robo4 levels are regulated by HG conditions. Future experiments will be needed to understand this regulation in greater detail, include RNA polymerase II chromatin immunoprecipitation studies (to assess Robo4 transcription),
qRT-PCR studies following actinomycin D-mediated transcription arrest (to assess Robo4 mRNA stability), $^{[35}S]-$methionine/cysteine pulse-chase experiments and Robo4 immunoprecipitation (to assess Robo4 translation), and Robo4 blotting following lysosomal, proteasomal, and autophagic inhibition (to assess Robo4 protein degradation). Importantly, discovery of the underlying mechanisms by which high glucose reduces Robo4 expression could lead to novel therapeutics that might target the early glomerular angiogenesis that occurs in the diabetic kidney.
5.2 **Overall Conclusion**

The purpose of this thesis was to determine the role that the Slit-Robo signaling pathway plays in regulating glomerular angiogenesis. In our *in vitro* studies we showed that HG conditions reduces the Robo4 expression, which promotes VEGF-induced network formation and migration. This VEGF and HG effect is lost with Robo1 knockdown, highlighting the importance of the balance in Robo4 and Robo1 expression in HRGECs. In our *in vivo* models, we showed that the HG conditions of diabetes reduces Robo4, while not changing Slit2-Robo1 signaling, and this leads to glomerular angiogenesis. Complete systemic ablation of Robo4, which does not alter Robo1 levels, exacerbates these angiogenic effects.

In summary, our data suggest that diabetic glomerular angiogenesis is promoted not only by increased VEGF expression, but also by increased VEGF responsiveness that is mediated by Robo4 and Robo1 in glomerular endothelial cells. Taken together, our results illustrate the potential for Robo signaling as a novel target for attenuating pathological glomerular angiogenesis in early diabetes.


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Roundabout Is a New Member of the Roundabout Receptor Family That Is
Endothelial Specific and Expressed at Sites of Active Angiogenesis. *Genomics,

Mammalian Brain Morphogenesis and Midline Axon Guidance Require Heparan


