Role of VEGF and VEGF Receptors in the Glomerulus

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

Doctor of Philosophy Graduate

Department of Institute of Medical Science

University of Toronto

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ABSTRACT

VEGF is a potent angiogenic and endothelial cell growth factor that is key for the development of the glomerulus, the main filtration unit of the kidney. It is continued to be expressed in the mature glomerulus, with podocytes being the major site of production. VEGF binds to two receptors, VEGFR-1 and VEGFR-2, which are expressed by the adjacent endothelial cells (ECs). VEGFR-2 is the primary mediator of VEGF signaling while VEGFR-1 is thought to function as a ‘decoy’ receptor, sequestering VEGF away from VEGFR-2. Gene targeting studies in mice show that VEGF loss from the podocyte results in profound defects of the ECs, consistent with a paracrine signaling loop. However, the identification of VEGF receptors on podocytes in vitro suggests an additional autocrine signaling pathway for VEGF may exist.

To further study the role of VEGF in the glomerulus and to address whether a VEGF autocrine loop is functional in vivo, we generated a transgenic mouse model with inducible VEGF upregulation in the podocyte and genetically deleted VEGFR-2 and VEGFR-1 from the podocyte using the Cre-loxP system. Increased VEGF production from the podocyte leads to increased glomerular permeability and ultrastructural changes in the glomerular filtration barrier depending on the time and length of induction.
Podocyte-selective deletion of VEGFR-2 did not cause glomerular disease. In contrast, VEGFR-1 loss from the podocyte led to proteinuria and glomerular defects at 6 weeks of age with extensive podocyte foot process effacement. In keeping with the model that VEGFR-1 functions as a VEGF trap, similarities were observed between the glomerular lesions of VEGFR-1 mutant mice and transgenic mice that overexpress VEGF within podocytes. Strikingly, in vitro studies also revealed an increase in podocyte cell adhesion to sVEGFR-1, suggesting additional roles for sVEGFR-1.

Together, these data suggest that a tight regulation of VEGF must be maintained in the adult glomerulus. Furthermore, these findings provide the first genetic evidence that VEGF autocrine signaling loop through VEGFR-2 is dispensable in normal glomeruli. In addition, podocytes express sVEGFR-1 and is required in podocytes in vivo to maintain glomerular integrity by regulating VEGF availability and podocyte cell adhesive properties.
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ATTRIBUTIONS</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>ABBREVIATION</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES AND FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>HYPOTHESES</td>
<td>xiv</td>
</tr>
<tr>
<td><strong>CHAPTER ONE: INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 RATIONALE</td>
<td>2</td>
</tr>
<tr>
<td>1.2 VASCULAR ENDOThelial GROWTH FACTOR</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Gene structure and alternative splicing</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2 Expression</td>
<td>8</td>
</tr>
<tr>
<td>1.2.3 Regulation</td>
<td>8</td>
</tr>
<tr>
<td>1.2.4 Function</td>
<td>10</td>
</tr>
<tr>
<td>1.3 VEGF-RELATED PROTEINS</td>
<td>13</td>
</tr>
<tr>
<td>1.4 VEGF RECEPTORS</td>
<td>15</td>
</tr>
<tr>
<td>1.4.1 VEGF-1</td>
<td>17</td>
</tr>
<tr>
<td>1.4.1.a Gene and protein structure</td>
<td>17</td>
</tr>
<tr>
<td>1.4.1.b Expression</td>
<td>19</td>
</tr>
<tr>
<td>1.4.1.c Signal transduction</td>
<td>20</td>
</tr>
<tr>
<td>1.4.1.d Function</td>
<td>22</td>
</tr>
<tr>
<td>1.4.2 VEGF-2</td>
<td>23</td>
</tr>
<tr>
<td>1.4.2.a Gene and protein structure</td>
<td>23</td>
</tr>
<tr>
<td>1.4.2.b Expression</td>
<td>24</td>
</tr>
<tr>
<td>1.4.2.c Signal transduction</td>
<td>24</td>
</tr>
<tr>
<td>1.4.2.d Function</td>
<td>26</td>
</tr>
<tr>
<td>1.4.3 VEGF-3</td>
<td>26</td>
</tr>
<tr>
<td>1.4.4 NEUROPILIN-1</td>
<td>28</td>
</tr>
<tr>
<td>1.4.5 NEUROPILIN-2</td>
<td>30</td>
</tr>
<tr>
<td>1.5 Glomerular biology</td>
<td>31</td>
</tr>
<tr>
<td>1.5.1 Glomerular development</td>
<td>31</td>
</tr>
<tr>
<td>1.5.2 Components of the glomerular filtration barrier</td>
<td>35</td>
</tr>
<tr>
<td>1.5.2.a Podocytes</td>
<td>37</td>
</tr>
<tr>
<td>1.5.2.b Glomerular basement membrane</td>
<td>41</td>
</tr>
<tr>
<td>1.5.2.c Endothelium</td>
<td>43</td>
</tr>
<tr>
<td>1.5.3 Role of VEGF in glomerular function</td>
<td>44</td>
</tr>
<tr>
<td>1.6 VEGF and disease</td>
<td>47</td>
</tr>
<tr>
<td>1.6.1 Diabetic nephropathy</td>
<td>47</td>
</tr>
<tr>
<td>1.6.2 Eye disease</td>
<td>50</td>
</tr>
<tr>
<td>1.6.3 Cancer</td>
<td>51</td>
</tr>
<tr>
<td>1.6.4 Autoimmune disease</td>
<td>52</td>
</tr>
</tbody>
</table>
1.7 VEGFR-1 and disease................................................................. 53
1.7.1 Pre-eclampsia................................................................. 53
1.7.2 Placental malaria............................................................. 54
1.7.3 Sepsis.............................................................................. 55

CHAPTER TWO: AGE-DEPENDENT RESPONSE TO VEGF UPREGULATION OF PODOCYTES

2.1 Abstract............................................................................. 57
2.2 Introduction....................................................................... 58
2.3 Materials and Methods...................................................... 60
2.4 Results............................................................................... 67
2.5 Discussion......................................................................... 83

CHAPTER THREE: LOSS OF VEGFR-1 DISRUPTS GLOMERULAR FUNCTION IN MICE

3.1 Abstract............................................................................. 88
3.2 Introduction....................................................................... 89
3.3 Material and Methods......................................................... 93
3.4 Results............................................................................... 98
3.5 Discussion......................................................................... 121

CHAPTER FOUR: SUMMARY AND GENERAL DISCUSSION............ 125

CHAPTER FIVE: FUTURE DIRECTIONS........................................... 133

REFERENCES.......................................................................... 141
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>AP-1/2</td>
<td>activating protein-1/2</td>
</tr>
<tr>
<td>ARE</td>
<td>adenylate/uridylate-rich elements</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>camp</td>
<td>cyclic adenosine monophosphate</td>
</tr>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
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<td>CLG</td>
<td>capillary loop glomeruli</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
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<td>doxycycline</td>
</tr>
<tr>
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<td>diabetic retinopathy</td>
</tr>
<tr>
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<td>diabetic nephropathy</td>
</tr>
<tr>
<td>E7.0</td>
<td>embryonic day 7.0</td>
</tr>
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<td>ECM</td>
<td>extracellular matrix</td>
</tr>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>Flk-1</td>
<td>fetal liver kinase-1</td>
</tr>
<tr>
<td>Flt-1</td>
<td>fms-like tyrosine kinase-1</td>
</tr>
<tr>
<td>FP</td>
<td>foot process(es)</td>
</tr>
<tr>
<td>FSGS</td>
<td>focal segmental glomerulosclerosis</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HIVAN</td>
<td>human immunodeficiency virus associated nephropathy</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyltransferase</td>
</tr>
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<td>HSPG</td>
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</tr>
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<td>GBM</td>
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<tr>
<td>GFB</td>
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<tr>
<td>GFP</td>
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</tr>
<tr>
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<tr>
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</tr>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MG</td>
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</tr>
<tr>
<td>MM</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
</tbody>
</table>
MS  multiple sclerosis
NFκB  nuclear-factor κB
Np-1  neuropilin-1
Np-2  neuropilin-2
N-WASP  Neuronal Wiskott-Aldrich syndrome protein
PAE  porcine aortic endothelial
PAN  puromycin aminonucleoside nephrosis
PAS  periodic-acid Schiff
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDGF  platelet-derived growth factor
PECAM  platelet and endothelial cell adhesion molecule
PHN  passive Heymann nephritis
PIGF  placental growth factor
PLC  phospholipase C
RA  rheumatoid arthritis
RNA  ribonucleic acid
RT-PCR  real-time polymerase chain reaction
RTK  receptor tyrosine kinase
rtTA  reverse tetracycline transactivator
SD  slit diaphragm
SDS PAGE  sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM  scanning electron microscopy
SH2  src homology 3
SG  S-shape glomeruli
STZ  streptozotocin
TEM  transmission electron microscopy
TGF  transforming growth factor
TMA  thrombotic microangiopathy
TSH  thyroid stimulating hormone
UAE  urinary albumin excretion
UB  ureteric bud
U-P/C  urine protein:creatinine
VEGF  vascular endothelial growth factor
VEGFR-1  vascular endothelial growth factor receptor-1 (Flt-1)
VEGFR-2  vascular endothelial growth factor receptor-2 (Flk-1)
VPF  vascular permeability factor
VSMA  vascular smooth muscle actin
VVO  vesiculo-vacuolar organelles
VHL  von-hippel lindau
WT-1  wilms tumor suppressor gene
ZO-1  zona occludens-1
µg  microgram
LIST OF TABLES AND FIGURES

CHAPTER 1

Table 1.1 Phenotypes of VEGF and VEGF receptor mutant mice

Figure 1.1 Schematic representation of human VEGF isoforms.

Figure 1.2 Schematic representation of VEGF ligands, their receptors and co-receptors.

Figure 1.3 Schematic representation of VEGFR-1 gene, mRNA and protein structure.

Figure 1.4 VEGFR-1 protein structure, phosphorylation sites and signal transduction.

Figure 1.5 VEGFR-2 protein structure, phosphorylation sites and signal transduction.

Figure 1.6 A schematic of glomerular development.

Figure 1.7 The glomerulus and the glomerular filtration barrier.

Figure 1.8 Schematic representation of the podocyte foot process-associated proteins.

Figure 1.9 Paracrine vs. autocrine VEGF signaling in the glomerulus.

CHAPTER 2

Table 2.1 Primers used for real-time PCR analysis.

Table 2.2 Numbers of affected vs. unaffected mice.

Figure 2.1 FACS sorting of primary podocytes.

Figure 2.2 Expression of VEGF receptors in the glomerulus.

Figure 2.3 Breeding scheme to generate mouse models.

Figure 2.4 Glomerular VEGF mRNA and western blotting of VEGFR-2 phosphorylation in podocyte-specific VEGF overexpressors.

Figure 2.5 Urine protein quantification.
Figure 2.6 Podocyte-specific overexpression of VEGF at birth leads to proteinuria and ultrastructural changes.

Figure 2.7 Podocyte-specific overexpression of VEGF in adult glomeruli leads to ultrastructural changes depending on stage of induction.

Figure 2.8. Loss of VEGFR-2 from podocytes does not affect glomerular function.

Figure 2.9 Loss of VEGFR-2 from the podocyte does not rescue glomerular phenotype in podocyte-specific VEGF overexpressors.

CHAPTER 3

Table 3.1 Numbers of affected vs. unaffected mice.

Figure 3.1 Generation of a podocyte-specific VEGFR-1 knockout mice.

Figure 3.2 Urine protein quantification by protein-creatinine ratio (U-P/C Ratio) over time in VEGFR-1 fl/fl, Neph Cre and control mice.

Figure 3.3 Loss of VEGFR-1 from podocytes leads to glomerular disease.

Figure 3.4 Course of disease in podocyte-specific VEGFR-1 knockout mice

Figure 3.5 Molecular analysis of podocyte and endothelial cells of podocyte-specific VEGFR-1 knockouts.

Figure 3.6 Glomeruli from 12 week-old podocyte-specific VEGFR-1 knockouts.

Figure 3.7 mRNA expression levels VEGF (A) and sVEGFR-1 (B) in FACS-sorted primary podocytes by RT-PCR.

Figure 3.8 Double mutant mice (podocyte-specific VEGF overexpressors and VEGFR-1 knockouts) develop glomerular disease.

Figure 3.9 Quantification of urine protein:creatinine (U-P/C) ratio.
Figure 3.10 Light micrographs of mutant and control glomeruli.

Figure 3.11 Podocyte cell adhesion.

Figure 3.12. Light and electron micrographs of glomeruli from 7 week-old podocyte-specific VEGFR-1 knockouts (Pod-VEGFR-1) and podocyte-specific VEGFR-1 and VEGFR-2 double mutants (Pod-VEGFR-1, VEGFR-2 DM).

CHAPTER 5

Figure 5.3.1. Breeding scheme to generate double mutant mice that lack VEGFR-1 from the podocytes and VEGFR-2 from the glomerular endothelial cells (ECs).

Figure 5.4.1. Breeding scheme to generate double mutant mice that lack VEGFR-1 and VEGF from podocytes.
HYPOTHESES

1) VEGF production by the podocyte is required to maintain the integrity of the GFB in the mature glomerulus.

2) VEGF paracrine signaling predominates in the glomerulus.
Chapter One

Introduction
1.1 RATIONALE

Kidney disease affects approximately 2 million Canadians and is rapidly increasing worldwide. In the majority of cases, the glomerular filtration barrier (GFB) is the main target of injury that in turn leads to the development of proteinuria (protein in the urine). The GFB is a special filter that allows the free passage of salts and small molecules while preventing the leakage of macromolecules. It consists of two cell types, the visceral epithelial cells (podocytes) and the endothelial cells. One of the key signaling pathways involved in the formation and physiologic function of the glomerulus is vascular endothelial growth factor (VEGF) acting through the vascular endothelial growth factor receptors (VEGFRs). VEGF signaling between podocyte precursors and endothelial cells is critical for glomerular development as loss of VEGF production by the podocyte in mice results in embryonic death due to renal failure [1]. These mice lack mature endothelial cells and hence do not form functioning glomeruli. Interestingly, podocytes continue to express VEGF at high levels in the mature glomerulus. The paradoxical presence of a potent-angiogenic factor in an anti-angiogenic, quiescent vascular bed has initiated several scientific inquiries on the exact role of VEGF in the adult glomerulus. Recent work from Eremina et al. showed that VEGF production by the podocyte is not only required for the development but is also necessary to maintain the integrity of the GFB once fully formed [2]. Pharmacologic and genetic inhibition of VEGF in the mature glomerulus results in defects in the glomerular endothelium, demonstrating a major role for VEGF paracrine signaling. However, the question remains whether an additional autocrine signaling for VEGF is also present. A number of groups have identified the expression of VEGF receptors in podocytes in vitro including, Neuropilin-1 (Np-1),
VEGFR-1, VEGFR-2 and VEGFR-3 [3-5]. Functional studies in cell culture models suggest that VEGF is a survival factor for podocytes but there is disagreement about which VEGF receptor, VEGFR-1 or VEGFR-2, is responsible. Of the two receptors, VEGFR-2 is believed to be the main signaling receptor for VEGF. The precise role of VEGFR-1 still remains elusive. Since VEGFR-1 exhibits weak phosphorylation in response to VEGF in basal conditions, it is thought to be a negative regulator of VEGF activity similar to the decoy function attributed to its soluble form, sVEGFR-1.

The studies undertaken in this thesis work towards two specific aims: 1) To assess the function of endogenous VEGF in the adult glomerulus and 2) To investigate whether an autocrine signaling pathway for VEGF plays a role in glomerular biology. To address these goals, a genetic approach is used to generate mouse models with a podocyte-specific gain of function mutation in VEGF and loss of function mutations for the various VEGF receptors, including VEGFR-1 and VEGFR-2. Double mutant mice are also generated to further study the ligand-receptor interactions and the possible interactions between receptors. These include: mice with both podocyte-specific VEGF upregulation and VEGFR-2 knockout, podocyte-specific VEGF upregulation and VEGFR-1 knockout as well as podocyte-specific knockouts for both VEGFR-1 and VEGFR-2. The renal phenotypes of all mice are characterized by a combination of physiologic, histologic and molecular methods that include urine assays, light and electron microscopy, real-time (RT) PCR analysis, in situ analysis and immunohistochemistry.

The work presented in this thesis extends the findings of other groups, validating the essential role of VEGF signaling in the glomerulus. In addition, novel perspectives regarding the function of VEGF receptors on podocyte biology were provided. Together,
the results provide additional insight into the mechanisms that regulate glomerular function which may be beneficial for the development of therapeutic strategies for kidney disease.

1.2 VASCULAR ENDOTHELIAL GROWTH FACTOR

VEGF belongs to a family of growth factors that include, placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and VEGF-F [6]. The members of this family are characterized by the presence of eight conserved cysteine residues which are important for the formation of the typical cysteine knot structure, a dimer of two cysteines linked by a disulfide bond. All of these growth factors are endogenously expressed in mammals except for VEGF-E and VEGF-F. The former is encoded by the double stranded DNA virus, orf, while VEGF-F is found in snake venom. In 1983, groups led by Donald Senger and Harold Dvorak identified and partially purified a protein that induced vascular leakage, naming it vascular permeability factor (VPF) [7]. In 1989, another group purified a protein from bovine pituitary follicular cells and found it to display mitogenic activity on endothelial cells in culture, hence naming it vascular endothelial growth factor [8]. Further tests reveal that VPF and VEGF are the same secreted protein and since then has been shown to play a predominant role in the formation, growth and maintenance of blood vessels [9].
1.2.1 Gene structure and alternative splicing

The human VEGF gene is located on chromosome 6 and comprises 8 exons. Alternative splicing of the VEGF transcript gives rise to at least 9 different isoforms that include VEGF 121, VEGF 145, VEGF 165, VEGF 189 and VEGF 206 (figure 1.1) [10-12]. Additional splice variants, VEGFxxx, have also been identified and function as endogenous inhibitors of VEGF [13, 14]. In mice, VEGF isoforms lack a glycine residue at position 8 of the mature protein and are therefore one amino acid shorter than their corresponding human isoforms. All transcripts contain exons 1-5 and 8, with diversity arising from alternative splicing of exons 6 and 7 [6]. Secretion of VEGF is mediated through a hydrophobic signal sequence that is encoded within exon 1 and a small region of exon 2. Exon 6 and 7 encodes a heparin-binding domain.

The various isoforms display markedly different properties with regards to diffusibility and heparin-binding affinities. More specifically, VEGF 121, which lacks the heparin-binding domain, is acidic and highly diffusible whereas VEGF189, a highly basic molecule, is mostly found associated and sequestered in the extracellular matrix (ECM) [15]. VEGF 165 on the other hand has intermediate properties, being both soluble and matrix-bound. Exposure to heparin or heparinases or cleavage by plasmin or urokinase can release ECM-bound isoforms [16, 17].

These characteristics reflect the distinct function of the various VEGF isoforms in vivo. VEGF165 is the predominant isoform expressed in most VEGF-producing cells and is secreted as a 45 kDA homodimer [8]. It is considered to be the master isoform, playing the central role in vascular development. In fact, approximately 50% of mice that exclusively express VEGF 120 die a few hours after birth due to ischemic
cardiomyopathy and impaired myocardial angiogenesis [18]. Mice that express only VEGF188 develop a number of abnormalities, including dwarfism, knee joint dysplasia and disrupted bone and cartilage development due to alterations in epiphyseal vascularization [19]. Mice that only express the VEGF164 isoform; however, develop normally [20].
Figure 1.1. Schematic representation of human VEGF isoforms. Alternative splicing of the VEGF gene containing 8 exons results in multiple isoforms including VEGF121, 145, 165, 189 and 206. All isoforms contain exons 1-5 and 8 but differ in exons 6 and 7. Alternative stop codons within exon 8 result in the production of the anti-angiogenic VEGFxxxb family of isoforms.
1.2.2 VEGF Expression

VEGF is robustly expressed embryologically. During mouse development, VEGF mRNA is detected as early as embryonic day (E) 7.0 in extra-embryonic and embryonic endoderm which is adjacent to the mesodermal layer of cells that express VEGFR-2 [21]. At later developmental stages, VEGF mRNA is observed in the choroid plexus epithelium, the vertebral column, the ventricular layers of the brain, the myocardium of the heart and in the glomerular epithelium (podocytes) of the kidney. In the adult mouse, VEGF mRNA expression is detectable in virtually all tissues and is abundant in the brain, lung, liver, spleen and kidney [22].

1.2.3 VEGF regulation

The regulation of VEGF is controlled at multiple levels including transcription, mRNA stability and translation. Among the primary regulators of VEGF gene expression is oxygen tension, with VEGF expression being highly stimulated by hypoxia. The VEGF gene contains hypoxia-responsive enhancer elements (HRE) in its 5′ and 3′ UTR regions [23-25]. In hypoxic conditions, the transcription factor, hypoxia-inducible factor (HIF)-1 binds to the HRE. HIF-1 is a heterodimer consisting of one HIF-1α and one HIF-1β subunit. At normal oxygen levels, HIF-1α is hydroxylated at proline residues and is targeted for proteosomal degradation. Since prolyl hydroxylases require molecular oxygen, HIF-1α accumulates under hypoxic conditions. Another protein required for proteosomal proteolysis is the Von Hippel-Lindau (VHL) tumor-suppressor protein. A genetic deficiency of this protein causes VHL disease in humans, a condition characterized by retinal and cerebral capillary hemangioblastomas. Deletion of VHL in
the podocyte cell of the glomerulus leads to severe kidney disease in mice, characterized by crescentic formation [26].

VEGF mRNA stability is also increased in hypoxic conditions due to the binding of HuRs, mRNA stability proteins, and other unidentified factors to the adenylate/uridylate-rich elements (AREs) in its 3’ UTR. VEGF mRNA is extremely labile in normoxic conditions, with a half-life of less than 1 hour as compared with the average half-life of 10–12 hour for eukaryotic mRNA. In hypoxic conditions, the half-life of VEGF mRNA increases by 2–3-fold due to a stabilizing effect of HuR. [27]. This protein binds with high affinity to AREs in the 3’ UTR of VEGF mRNA and protects it from degradation by endonucleases [27].

Further analysis of the promoter region of the VEGF gene revealed other transcriptional regulatory elements. Both the human and mouse VEGF gene contain multiple binding sites for SP-1, AP-1 and AP-2 [28, 29]. AP-1 activity is stimulated by phorbol esters and growth factors while AP-2 is activated by cAMP-dependent kinase and protein kinase C (PKC) pathways. Phorbol esters, peptide growth factors, and intracellular elevation of cAMP induced levels of VEGF mRNA, suggesting that the AP-1 and AP-2 consensus sites present in the VEGF promoter may mediate VEGF transcriptional activation in response to these effectors [30]. Despite the significant similarity in sequence and organization of the mouse VEGF and human homologue, there are certain differences. NFKB, a transcription factor implicated in the regulation of inflammation and stress response genes, binds to the mouse VEGF promoter but there are no consensus sites found in the human VEGF gene.

A number of growth factors and cytokines also affect VEGF gene expression. For
instance, insulin growth factor (IGF), PDGF-ββ, transforming growth factor (TGF)-β, basic fibroblast growth factor (FGF-2), interleukin-1β and interleukin-6 can upregulate VEGF expression, with some acting synergistically with hypoxia [29, 31-35]. Hormones such as thyroid stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) also induce VEGF expression in several thyroid carcinoma cell lines and cultured human fetal adrenal cortical cells respectively [36-38].

1.2.4 VEGF function

VEGF is a well-established factor in vasculogenesis and angiogenesis.

Vasculogenesis comprises the initial steps that lead to the formation of the embryonic vasculature and begins with the differentiation of endothelial cell precursors, the angioblasts, from the mesodermal hemangioblasts [39]. As the endothelial cells invade and fuse together, a primitive capillary plexus is assembled. Subsequently, angiogenesis, the growth of new blood vessels from the pre-existing vasculature follows and is characterized by the expansion of the endothelium by proliferation, migration and modeling.

As the name suggests, VEGF is a mediator of angiogenesis, both in physiological and pathological states. Among its major biological activities is the capacity to stimulate endothelial cell proliferation in vitro as well as in vivo models. For example, treatment of microvascular lymphatic endothelial cells with VEGF induces the cells to invade a collagen gel and form capillary-like structures [40]. The addition of VEGF to human umbilical vein endothelial cells (HUVEC) also resulted in increased angiogenesis which was inhibited by VEGF neutralizing antibodies [41]. VEGF also elicits a potent
angiogenic response in vivo including the rabbit cornea and chorioallantoic membrane (CAM) of the chick embryo [42, 43]. Furthermore, the generation of VEGF-deficient mice demonstrates the essential role of VEGF in embryonic vasculogenesis and angiogenesis. Loss of a single VEGF allele results in embryonic lethality at E11-12 (table 1.1) [44]. The heterozygous VEGF embryos displayed a defective vasculature in a number of organs. Conversely, a two-three fold increase in VEGF gene expression results in abnormal heart development and embryonic lethality at E12.5-14 [45]. In contrast, genetic inactivation of other members of the VEGF family such as PIGF or VEGF-B did not result in major developmental abnormalities, further indicating the critical role of VEGF in vascular formation [46, 47].

More recently, Lee et al. have shown that endothelial cells also produce VEGF and acts in an autocrine manner to maintain vascular homeostasis [48]. Genetic deletion of VEGF specifically in endothelial cells in mice results in cardiovascular abnormalities due to hemorrhages, micro-infarcts, endothelial cell rupture and vascular constriction. These data offer a novel view for VEGF which was originally thought to be produced by other vascular support cell types and not endothelial cells.

VEGF was initially identified based on its ability to stimulate vascular permeability and it can do so by opening inter-endothelial junctions, induction of fenestrations and through the formation of vesicu-lo-vacuolar organelles (VVOs) [49]. Topical application of VEGF on rodents rapidly resulted in the appearance of fenestrations in vascular beds which normally do not contain fenestrae, namely the muscle and skin [50]. It also induces the formation of endothelial fenestration in cultured adrenal endothelial cells and glomerular endothelial cells in vitro [51, 52]. In the blood-retinal barrier endothelium,
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>VEGF +/-</td>
<td>Die between E11-12 with impaired vascular development.</td>
<td>44</td>
</tr>
<tr>
<td>VEGF -/-</td>
<td>Embryonic lethal at E9.5-10.5 with more severe defects in vascular formation than VEGF heterozygotes.</td>
<td>44</td>
</tr>
<tr>
<td>VEGF-B -/-</td>
<td>Viable and fertile but abnormal heart development.</td>
<td>46</td>
</tr>
<tr>
<td>VEGF-C -/-</td>
<td>Pre-natal death with a failure to form lymphatic vessels.</td>
<td>71</td>
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<tr>
<td>VEGF-D -/-</td>
<td>Normal development but displays subtle defects in lymphatic vessels.</td>
<td>72</td>
</tr>
<tr>
<td>PIGF -/-</td>
<td>Viable and fertile with no overt vascular defects.</td>
<td>47</td>
</tr>
<tr>
<td>VEGFR-1 -/-</td>
<td>Embryonic lethal at E8.5-9 due to disorganized vasculature as a result of endothelial cell overgrowth.</td>
<td>100</td>
</tr>
<tr>
<td>VEGFR-1 (TK) -/-</td>
<td>Normal vascular development but displays defects in VEGF-induced migration of macrophages.</td>
<td>104</td>
</tr>
<tr>
<td>VEGFR-2 -/-</td>
<td>Embryonic lethality at E8.5-9.5 with defective vascular development, lacked mature endothelial cells.</td>
<td>101</td>
</tr>
<tr>
<td>VEGFR-3 -/-</td>
<td>Embryonic lethal at E9.5 due to cardiovascular failure, fluid accumulation in the pericardium.</td>
<td>122</td>
</tr>
<tr>
<td>Np-1 -/-</td>
<td>Die between E12-13.5 with extensive cardiovascular and neuronal defects.</td>
<td>130</td>
</tr>
<tr>
<td>Np2 -/-</td>
<td>Embryos survive into adulthood, but display abnormal neural patterning and fewer small lymphatic vessels and capillaries.</td>
<td>135</td>
</tr>
<tr>
<td>Np1 -/-, Np2 -/-</td>
<td>Embryonic lethal at E8.5 with defective vascular development, resembling VEGF-/- and VEGFR-2-/-..</td>
<td>136</td>
</tr>
</tbody>
</table>

**Table 1.1.** Phenotypes of VEGF and VEGF receptor mutant mice.
VEGF was reported to increase vascular permeability for plasma proteins due to increased trans-endothelial transport through pinocytotic vesicles but not through endothelial fenestrations or VVOs [53].

While VEGF is primarily an endothelial-specific factor, it has been shown to play a role in non-vascular cell types. For example, VEGF displays neurotrophic and neuroprotective activities in vitro and in vivo [54]. VEGFR-2 is detected on cultured superior cervical ganglia (SCG) and dorsal root ganglia (DRG) from adult mice. VEGF was found to stimulate axonal outgrowth from DRG and this effect was blocked by the VEGFR-2 inhibitor SU5416 [54]. Moreover, VEGF has also been shown to stimulate macrophage migration, which express VEGFR-1 [55] and also plays multiple roles in bone development [56, 57]. Other non-endothelial cell targets of VEGF may include haematopoietic stem cells and myoblasts [58, 59].

1.3 VEGF-RELATED PROTEINS

Other members of the VEGF family share significant sequence homology to VEGF and may share the same receptors as VEGF, consequently influencing VEGF activity. These include placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and VEGF-F.

PIGF shares 53% amino acid sequence identity with human VEGF [60]. It is highly expressed in the placenta and through alternative splicing, exists in four different isoforms, PIGF-1, PIGF-2, PIGF 3 and PIGF-4. Angiogenesis during embryonic development is unaffected in PIGF-deficient mice; however, angiogenesis is impaired in pathophysiological conditions such as ischemia, wound healing and cancer, suggesting
that PIGF contributes to vessel repair rather than vascular development [47]. PIGF is able to modulate VEGF bioactivity in a number of ways. First, PIGF binds to and signals via VEGFR-1 and binding to VEGFR-1 prevents the association of VEGF to the same receptor, increasing the amount of VEGF available for VEGFR-2-mediated signaling [61]. Secondly, PIGF has been shown to form heterodimers with VEGF in vivo. These heterodimers; however, display a weaker mitogenic activity compared with VEGF homodimers [62].

VEGF-B expression is prominent in the heart and skeletal muscle [63]. It exists as two isoforms, both of which bind VEGFR-1 and Np-1 [64]. VEGF-B knockout mice show no obvious abnormalities, implying that it does not play a major role in vascular development [46]. One VEGF-B isoform, however, forms heterodimers with VEGF and may indirectly control VEGF release and bioavailability [63]. More recently, VEGF-B has been suggested to play a role in neurogenesis and neural protection [65].

VEGF-C shares 30% amino acid identity with VEGF165 but is more closely related to VEGF-D [66]. Human VEGF-C and -D bind and activate VEGFR-2 and VEGFR-3 [67-69]. Mouse VEGF-D is specific to VEGFR-3 in the mouse [70]. VEGF-C-deficient mice do not form lymphatic vessels and die before birth with accumulation of fluid in tissues [71]. VEGF-C thus plays a role in vascular and lymphatic development and its action becomes more centralized in the adult, participating in maintenance of the lymphatic vessels [67]. Loss of VEGF-D in mice only leads to a subtle lymphatic phenotype, suggesting that VEGF-D is dispensable for lymphatic vessel development [72].
VEGF-E consists of a group of proteins that are encoded by particular strains of the orf virus, a pathogen found in sheep, goats and occasionally in humans. VEGF-E only binds VEGFR-2 and stimulates endothelial cell proliferation and vascular permeability in primary endothelial cells [73, 74].

VEGF-Fs were identified in 1995 and are found in venom of 2 snake species [75, 76]. It specifically recognizes VEGFR-2 and has been shown to preferentially induce the formation of fenestrae over VVOs in guinea pigs, resulting in increased vascular permeability [77].

### 1.4 VEGF RECEPTORS

VEGF mediates its function through two type III receptor tyrosine kinases, VEGFR-1 (Flt-1: fms-like tyrosyl kinase-1, where fms refers to feline McDonough sarcoma virus) and VEGFR-2 (Human KDR: kinase domain receptor; murine Flk-1: fetal liver kinase-1) (figure 1.2). Binding of the VEGF ligand to the receptors leads to receptor dimerization, protein kinase activation, trans-autophosphorylation and initiation of intracellular signaling. VEGF also binds to two non-protein kinase co-receptors, Neuropilin-1 (Np-1) and Neuropilin-2 (Np-2).
**Figure 1.2.** Schematic representation of VEGF ligands, their receptors and co-receptors. VEGF binds to two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. VEGF-C and VEGF-D are specific for VEGFR-3. Specific isoforms of VEGF and other members of the VEGF family also bind to Np-1 and Np-2.
1.4.1 VEGFR-1

1.4.1.a Gene and Protein Structure

VEGFR-1 was the first VEGF receptor (VEGFR) to be identified [78, 79]. It exists as a transmembrane receptor as well as a soluble form (sVEGFR-1) and binds to VEGF, VEGF-B and PIGF [61, 79-81]. The human VEGFR-1 gene maps to chromosome 13 and contains 30 exons. The mRNAs for the full length VEGFR-1 and sVEGFR-1 arise from the VEGFR-1 gene and share a common transcription start site but have distinct 3’ ends. The full length VEGFR-1 transcript contains all 30 spliced exons and is translated into a nascent 150 kDA protein (figure 1.3) [80]. This protein is rapidly glycosylated to an intermediate form of ~170 kDA and is further glycosylated to the mature form of ~180-185 kDA. This mature form is expressed on the cell surface and contains three regions - the extracellular N-terminal ligand-binding region containing 7 immunoglobulin-like (IgG) domains, a transmembrane domain and a C-terminal intracellular segment that contains two tyrosine kinase domains. sVEGFR-1 shares the first 13 exons with VEGFR-1 and is produced by reading through intron 13 and utilizing an alternate poly(A) signal sequence to create a shorter transcript that lacks exons 14-30 (figure 1.3) [82]. This transcript is translated into a ~100 kDA truncated protein that contains the ligand binding domain consisting of 6 IgG repeats followed by a unique 31 amino acid polypeptide; it lacks the membrane-spanning and C-terminal tyrosine segments present in the full-length form. Additional soluble forms of VEGFR-1 have also been detected but their biological significance remains to be determined [83, 84].

Compared to VEGFR-2, VEGFR-1 binds VEGF with at least 10 fold higher affinity (VEGFR-2: Kd=750 pM vs VEGFR-1: Kd=10-20 pM) [81]. VEGF and PIGF bind
Full-length VEGFR-1 mRNA contains all 30 exons and is translated into a protein containing 7 IgG repeats, a transmembrane (TM) domain, 2 tyrosine kinase (TK) domains and a C-terminal tail. sVEGFR-1 mRNA shares the first 13 exons of full-length VEGFR-1 mRNA and a region of intron 13 and is translated into a soluble protein consisting of 6 IgG repeats and a unique C-terminus.
directly to the second IgG domain of VEGFR-1 [85]. The affinity of PIGF for VEGFR-1 is somewhat lower than VEGF (Kd=170). Further analysis reveal that several peptide sequences surrounding the second domain are important in the formation of the tertiary structure at the ligand binding site of the receptor [86, 87]. The fourth IgG-like domain is required for receptor dimerization and heparin binding.

1.4.1.b Expression

The embryonic vasculature is established at E7.5-12.5 of mouse development. At this stage, VEGFR-1 mRNA expression moderately increases in vascular endothelial cells until E14.5-16.5 when expression decreases [88]. At this timepoint, endothelial differentiation is complete but blood vessels continue to grow. VEGFR-1 expression once again increases in newborn mice and continues to be expressed in the quiescent endothelium of adult organs including the brain, lung, heart and kidney as well as in vessels surrounding healing wounds.

VEGFR-1 is also found in non-endothelial cells. Full length and soluble forms of VEGFR-1 are found in monocytes [55]. VEGFR-1 is also found in haematopoietic progenitor cells, choriocarcinoma cells, epidermal keratinocytes and vascular smooth muscle cells [89-92]. Several forms of sVEGFR-1 have been found in human placenta [84, 93]. In mice, sVEGFR-1 is also highly expressed in the placenta especially in later stages of gestation [94]. Expression is also observed in the adult lung, kidney, liver and uterus.
1.4.1.c Signal Transduction

Despite the weak tyrosine kinase activity of VEGFR-1 (approximately one tenth of VEGFR-2), six residues of VEGFR-1 have been identified as sites of phosphorylation (figure 1.4) [80]. By expressing the intracellular domain of hVEGFR-1 in baculovirus, Tyr1213 and Tyr 1242 have been identified as major sites of phosphorylation as well as Tyr 1327 and Tyr 1333 as minor sites [95]. PLCγ binds to both Tyr1213 and Tyr1333, whereas Grb2 and SH2-containing tyrosine protein phosphatase (SHP-2) bind to Tyr1213, and Nck and Crk bind to Tyr1333.

Another group also overexpressed VEGFR-1 in insect cells and found that Tyr 1169 and Tyr 1213 on VEGFR-1 were phosphorylated in response to VEGF treatment and replacement of Tyr 1169 with phenylalanine strongly suppressed the association with PLCγ [96]. Fyn and Yes, members of the Src family of protein kinases, have also been found to preferentially associate with VEGFR-1 in VEGF-treated HUVECs [97]. In primary human monocytes, VEGF or PIGF treatment resulted in the activation of PI3K/AKT and MAPK pathways which mediate monocyte migration [98]. Although the binding site for PI3K was not identified, Tyr 1213 may be a potential candidate. Tyr 1309 has also been shown to be phosphorylated in response to PIGF and the activation of AKT/protein kinase B (PKB) [99].
Figure 1.4. VEGFR-1 protein structure, phosphorylation sites and signal transduction. PLCγ, phospholipase C-γ; PI3, phosphatidylinositol 3’ kinase; AKT, protein kinase B; MAPK, mitogen-activated protein kinase. Adapted from A.K. Olsson et al., Nat Rev Mol Cell Biol 2006.
1.4.1.d Function

VEGFR-1 is essential during vasculogenesis and angiogenesis as shown by gene knockout studies in mice. Although heterozygous VEGFR-1 knockout mice are healthy and display normal blood vessel formation, homozygous VEGFR-1 null mutants are embryonic lethal at E8.5 due to a severely disorganized vasculature with overgrowth of endothelial cells [100]. This is in contrast to the VEGFR-2 knockout mice which lack mature endothelial cells [101]. Based on this observation, VEGFR-1 was originally believed to be a negative regulator in vessel formation. Selective activation of VEGFR-1 through the use of VEGFR-1 chimeras or VEGF mutants that only bind to VEGFR-1 did not result in proliferation or migration in endothelial cells [102, 103]. The lack of significant endothelial responses to VEGFR-1 and the phenotype seen in the VEGFR-1 null mice have led to the idea that it primarily functions as a decoy receptor that binds to and sequesters the VEGF ligand from signaling through VEGFR-2. Indeed, the deletion of the VEGFR-1 tyrosine kinase domain does not affect vascular development as demonstrated by the VEGFR1 (TK) -/- mice [104]. The soluble form of VEGFR-1, sVEGFR-1, also performs this function. Recently, Ambati et al. has shown that sVEGFR-1 plays an essential role in preserving avascularity in the cornea by binding endogenous VEGF [105].

Further work has revealed that VEGFR-1’s role in vessel formation may not be limited by its VEGF ligand trapping mechanism. VEGFR-1 has been shown to dimerize with VEGFR-2, resulting in autophosphorylation and transactivation of VEGFR-2 and downstream angiogenic pathways [106]. In this setting, VEGFR-1 positively regulates angiogenesis when paired with VEGFR-2. Orecchia and colleagues have also reported
that sVEGFR-1 is deposited in the ECM and is involved in cell adhesion and migration through its interaction with $\alpha5\beta1$ integrins [107]. In this manner, sVEGFR-1 also positively modulates the angiogenic process.

Despite the notion that direct signaling via VEGFR-1 is minimal in endothelial cells, it has been shown to transduce signals in the monocyte/macrophage cell lineage which differentiates into many cell types including bone osteoclasts, dendritic cells, Kupfer cells in the liver and mature macrophages [80]. These cells express high levels of VEGFR-1 mRNA but little VEGFR-2 mRNA. In monocytes, VEGFR-1 has been shown to mediate VEGF-induced migration [98, 108]. Interestingly, despite the lack of an overt vascular phenotype in the VEGFR-1 TK (-/-) mice, the VEGF-dependent migration of macrophages are also reduced [104].

1.4.2 VEGFR-2

1.4.2.a Gene and Protein Structure

VEGFR-2 binds VEGF as well as VEGF-C, VEGF-D and VEGF-E. The human VEGFR-2 gene is located on chromosome 4, contains 28 exons, and encodes a full-length receptor of 1356 amino acids as well as a soluble form [109-111]. It contains an extracellular region consisting of 7 IgG domains, a transmembrane domain and an intracellular region with two tyrosine kinase domains. VEGFR-2 protein is initially synthesized as a 150 kDA protein within the cell but is rapidly glycosylated to a 200 kDA intermediate form that is further glycosylated at a slower rate, resulting in a mature 230 kDA form that is expressed on the cell surface [112]. Flk-1 is the murine homologue of human KDR, sharing 85% sequence homology and being 2 amino acids shorter [113].
1.4.2.b Expression

Similar to VEGFR-1, VEGFR-2 mRNA is localized in blood vessels and capillaries of developing organs throughout embryogenesis and its expression is complementary to that of VEGF [114, 115]. VEGFR-2 mRNA is detected at E7.0 in mouse when vascularization begins. It is first found in the allantois and blood islands of the yolk sac and by E8.5, it is found throughout the entire vasculature. By E12.5-15.5, its expression becomes restricted to blood vessels. VEGFR-2 expression, although downregulated, is still present in the quiescent adult vasculature including the liver, lung, adipose and kidney [116].

1.4.2.c Signal Transduction

Despite the weaker binding affinity of VEGFR-2 for VEGF, VEGFR-2 is responsible for transducing that majority of VEGF-induced activity. Human VEGFR-2 is phosphorylated at a number of tyrosine residues that include Tyr 951, 996, 1054, 1059, 1175 and 1214, some of which serve as docking sites for SH2 domain-containing proteins (figure 1.5) [117]. Knock-in mutant mice carrying a Tyr-to-Phe substitution at residue 1173 (1175 in humans) died between E8.5-9.5 as a result of defects in vascular development, strongly suggesting that signaling through Tyr 1173 of VEGFR-2 is essential for endothelial development during embryogenesis [118]. In primary endothelial cells, VEGF stimulation leads to the activation of various signal transduction molecules including PI3K; PLCγ; the Src family of tyrosine kinases; Ras GTPase-activating protein (Ras GAP); Nck; focal adhesion kinase; Akt/protein kinase B (PKB);
Figure 1.5. VEGFR-2 protein structure, phosphorylation sites and signal transduction. PLCγ, phospholipase C-γ; PKC, protein kinase C; PI3, phosphatidylinositol 3’ kinase; AKT, protein kinase B; MEK, MAPK, mitogen-activated protein kinase; eNOS, endothelial nitric oxide synthase; HSP27, heat-shock protein-27. Adapted from A.K. Olsson et al., Nat Rev Mol Cell Biol 2006.
protein kinase C (PKC); Raf-1; MEK; extracellular signal-regulated kinase (ERK); and p38 mitogen-activated protein kinase (MAPK) (Reviewed in [99, 117, 119]). These pathways lead to cell proliferation, cell survival, cell migration and increased permeability.

1.4.2.d Function

The key role of this receptor in development is demonstrated by VEGFR-2 knockout mice which fail to form blood vessels, resulting in embryonic death between E8.5-9.5 [101]. Unlike the VEGFR-1 null mutants which develop endothelial cells, VEGFR-2 knockout mice lack mature endothelial cells. VEGFR-2 activation mediates the mitogenic and permeability-enhancing actions of VEGF which are discussed in 1.5.2.c.

A soluble truncated form of VEGFR-2, sVEGFR-2, has been found in mouse and human plasma [111]. Its precise function still remains to be determined but it may regulate VEGF-mediated actions similar to sVEGFR-1.

1.4.3 VEGFR-3

VEGFR-3 or Flt-4 was cloned from placenta cDNA libraries and was found to be related to the VEGFR-1 and VEGFR-2 [120]. The VEGFR-3 gene maps on chromosome 5 in humans and contains 31 exons which are alternatively spliced to yield two isoforms that differ in their C-terminal tails [121]. VEGFR-3 is similar in structure to VEGFR-1 and VEGFR-2, containing an extracellular, ligand-binding region with 7 IgG-like domains, a tyrosine kinase domain and an intracellular C-terminal tail. Unlike the other
VEGF receptors, the extracellular domain of VEGFR-3 is cleaved to yield two polypeptides that are held together by a disulfide bridge.

VEGFR-3 mRNA signals are first detected in the angioblasts of the head mesenchyme and the cardinal vein at E8.5 of mouse development [67]. By E12.5, VEGFR-3 is more prominent in the developing veins and presumptive lymphatic endothelia and during later stages of development, its expression becomes restricted to the lymphatic endothelium.

In VEGFR-3-deficient mice, vasculogenesis and angiogenesis are still initiated; however, large vessels become abnormal, leading to fluid accumulation in the pericardial cavity and cardiovascular failure by E9.5 [122]. This phenotype and the relatively restricted expression of VEGFR-3 in lymphatic endothelium strongly suggest its role in the regulation of the lymphatic vasculature.

Similar to VEGFR-1 and VEGFR-2, ligand binding to VEGFR-3 results in receptor dimerization and activation of intrinsic kinase activity and autophosphorylation. Five of the six tyrosine residues in the C-tail of VEGFR-3 have been identified as potential phosphorylation sites and these include Tyr 1230, 1231, 1265, 1337, and 1363 [123]. Phosphorylation at Tyr1337 creates docking sites for the adaptor proteins Shc and Grb2. VEGFR-3 activation also results in PKC-mediated cell proliferation and PKB-dependent cell survival. Treatment of primary lymphatic endothelial cells with VEGF-C, but not VEGF, also resulted in the formation of VEGFR-3/VEGFR-2 heterodimers.
1.4.4 NEUROPIILIN-1

The human Np-1 gene is located on chromosome 10 and contains 17 exons [124]. Np-1 exists as a full-length, membrane-bound form and as soluble forms. The full-length protein is translated from all 17 exons while the soluble isoforms are generated by reading through into introns, creating shorter transcripts that either contains the first 11 exons and a small region of intron 11 or the first 12 exons and a small region of exon 12. Additional isoforms have been identified more recently from normal and cancerous tissues [125].

Np-1 was originally identified on developing neurons and plays a role in neuronal cell migration and axon guidance [126]. It mediates the repulsive cues of the semaphorin family of proteins but due to a short cytoplasmic domain, does not signal directly and instead, couples with other signaling plexins on the cell surface. Np-1 is also expressed in endothelial cells and binds to the 165 isoform of VEGF. In vitro models have shown that Np-1 can couple with VEGFR-2, potentiating the VEGF-induced VEGFR-2 signaling. More specifically, co-expression of Np-1 and VEGFR-2 in PAE cells enhances binding of VEGF165 to VEGFR-2 by 4-6 fold compared to cells expressing VEGFR-2 alone [127]. The chemotaxis of these cells is also enhanced when treated with VEGF165 but not with VEGF121. In contrast, a GST fusion protein (contains VEGF exon 7 and 8 which encodes for the domain responsible for Np-1 binding) that directly inhibits VEGF165 from binding to Np-1, substantially inhibited the association of VEGF165 to VEGFR-2 as well as its mitogenic activity on endothelial cells. Additional VEGF family members such as the heparin binding form of placenta growth factor (PIGF-2), VEGF-B, VEGF-C, VEGF-D and VEGF-E also bind to Np-1 [128, 129].
Targeted inactivation of Np-1 results in embryonic lethality at E12.5-13.5 due to a number of vascular defects in addition to a disorganized neural network [130]. Np-1-deficient embryos display an impaired vascularization in the central and peripheral nervous system and insufficient development of the vascular network in the yolk sac. On the other hand, overexpression of Np-1 in transgenic mice leads to excess blood and capillary formation [131].

In 2000, the first soluble form of Np-1 was identified. It is a 90 kDA secreted protein that lacks the transmembrane and cytoplasmic domain of full length Np-1 [132]. Consistent with the isoform specificity of Np-1, it also binds to VEGF165 but not VEGF121. Through in situ hybridization, Np-1 and sNp-1 localization was found to be differentially expressed and does not overlap [132]. For example, sNp-1 mRNA is detected in human liver hepatocytes and kidney proximal tubules but Np-1 is not. Conversely, Np-1 is detected in liver veins, kidney glomeruli and blood vessels of the heart, brain and placenta and but not sNp-1. The expression of Np-1 in blood vessels supports its role as an endothelial-associated co-receptor for VEGF165. The precise role of cell-specific sNp-1 remains to be established. Cancer cells expressing recombinant sNp-1; however, show that VEGF165 access to VEGF receptors is inhibited, suppressing the VEGF165-elicited cell migration and proliferation, implicating sNp-1 as a VEGF165 antagonist.
1.4.5 NEUROPILIN-2

The human Np-2 gene is on chromosome 2 and contains 17 exons [124]. VEGF165 and VEGF145 bind to Np-2 while VEGF121 does not [133]. VEGF-C and VEGF-D also bind to Np-2 [128, 129]. Np-2 is closely related to Np-1, sharing a common structure and amino acid sequence with Np-1 [134]. It also plays a role in axon guidance during neuronal development with expression overlapping with Np-1. In contrast to Np-1 knockout mice which die during mid-gestation, mice deficient in Np-2 are viable and survive into adulthood [135]. Defects were, however, observed in the hippocampus neural connectivity and organization of cranial and spinal nerves. Interestingly, transgenic mice with a targeted deletion of both Np-1 and Np-2 died at E8.5 with avascular yolk sacs [136]. Mice lacking Np-1 but heterozygous for Np-2 or mice lacking Np-2 but heterozygous for Np-1 survived up to E10-10.5. These mutant embryos were growth-retarded, displayed diminished yolk sac vasculature and disorganized blood vessels, demonstrating that both Np-1 and Np-2 are required to mediate normal vasculogenesis and angiogenesis in the developing yolk sac and embryo. The abnormal vascular phenotype of the Np-1/Np-2 double knockouts resemble that of the VEGFR-2 knockout mice which died in utero at E8.5-9.5 and VEGF-deficient mice which died at E9.5-10.5, both of which displayed abnormalities in angiogenesis.
1.5 GLOMERULAR BIOLOGY

1.5.1 Glomerular development

The mammalian kidney derives from the intermediate mesoderm and gives rise to three types of kidney structures: the pronephros, the mesonephros and the metanephros [137, 138]. The pronephros is a primitive structure that is fully functional in lower vertebrates but is a transient embryonic structure in humans. It regresses as the mesonephros, the functional embryonic kidney, forms. The metanephros is the final stage which differentiates into the permanent adult kidney and it arises from reciprocal inductive signaling between the metanephric mesenchyme and the adjacent ureteric bud. At 10.5 post-coitum in mice, the ureteric bud extends into and invades the surrounding metanephric mesenchyme (figure 1.6). As the ureteric bud undergoes a series of branching, it also induces the mesenchymal cells that are located at the tips of the newly formed branches to begin the mesenchymal-to-epithelial conversion and form a polarized renal vesicle. A single cleft forms in the vesicle, generating the comma-shaped body, and is followed by the formation of a second cleft, leading to the s-shaped body. At the s-shaped stage, visceral epithelial cells or podocyte precursors begin to express VEGF and podocyte-specific proteins including podocin, WT-1 and Tcf21/Pod-1. Nephrin is expressed by the presumptive podocytes in the capillary loop stage [137]. VEGF secretion from the podocyte precursors attracts the VEGFR-2-positive endothelial cells which migrate into the cleft and proliferate to form the capillary loops of the glomerulus. Once recruited, the endothelial cells begin to produce platelet-derived growth factor
Figure 1.6. A schematic of glomerular development. Reciprocal interactions between ureteric bud (UB) and the metanephric mesenchyme (MM) leads to the condensation and epithelialization of the MM which undergoes a series of developmental stages including the comma-shape, s-shape and capillary loop stage. At the S-shape stage, podocyte precursors appear and express VEGF attracts the VEGFR-2 positive endothelial cells. Adapted from A. Harkin and S. Quaggin. Fetal and Maternal Medicine Review 2003.
(PDGF)-B and the PDGF Receptor-β becomes expressed by mesangial precursor cells. Communication between these two cell types initiates the proliferation and assembly of the mesangium, stabilizing the glomerular vasculature. Interactions between endothelial cells and the differentiating podocytes also lead to the formation of the glomerular basement membrane (GBM) which is first generated as two separate layers by the two cell types but fuses together during maturation. Podocytes produce GBM components and the replacement of laminin-1 (α1β1γ1) with laminin-11 as well as the replacement of α1 and α2 chains of type IV collagen by collagen-α 3, 4, 5 (IV) chains mark the maturation of the GBM [139-141]. It has been reported that developing endothelial cells also contribute to the production of laminins and secrete factors that mediate the switch in laminin and collagen production by podocytes [142-144].

Glomerular development continues for 2-3 weeks after birth in mice. Once formed, the growth of capillaries must cease and instead stabilize to properly perform its function in selective filtration. Interestingly, podocytes continue to produce large amounts of VEGF in the mature glomerulus despite the absence of active angiogenesis. Mechanisms that regulate VEGF action and/or availability must therefore exist. Since VEGF contains a heparin-binding domain, it is able to bind HSPGs and other matrix-associated proteins. It has been suggested that most of the VEGF pool remains biologically inactive when sequestered to GBM. The matrix is thus an extracellular store for the growth factor that must be broken down by enzymes to increase VEGF bioavailability. Matrix metalloproteinases (MMPs) have been shown to regulate VEGF bioavailability from the matrix reservoir by the degradation of the matrix proteins or by directly processing the VEGF protein [145]. Although VEGF 165 does not require
activation by proteolysis, the larger isoforms, VEGF 189 and 206, may require cleavage to induce the activation of VEGF receptors [11, 145, 146].

Another potential mechanism is the presence of endogenous anti-angiogenic factors that counteract VEGF action. For example, angiopoietins are endothelial growth factors which act in concert with VEGF in vascular formation and maintenance. Ang-1 binds to the Tie-2 receptor to promote endothelial survival and restrict permeability while Ang-2 opposes Ang-1 activity. Similar to VEGF, Ang-1 is also expressed in developing and mature podocytes while its receptor, Tie-2 is localized to the glomerular endothelial cells [147, 148]. Ang-1 is thus a likely candidate in the regulation of VEGF action.

Furthermore, inhibitory isoforms of VEGF such as VEGF165b have been identified in normal kidney and isolated human glomeruli [149]. VEGF165b prevents VEGF165-mediated endothelial cell migration and proliferation in culture. Using cultured human podocytes, Cui et al. found that differentiated podocyte cells mostly express the inhibitory VEGF165b isoform; however, if dedifferentiated, a splicing event occurs leading to a switch to the exon 8a-containing VEGF165 stimulatory form [150]. They propose the same event to occur in glomeruli in vivo. Under normal conditions, VEGF165b is expressed, regulating VEGF165-induced endothelial proliferation and permeability. With glomerular disease, injured podocytes dedifferentiate and respond by switching from the inhibitory exon 8b-containing VEGF isoform expression to the stimulatory exon 8a-containing VEGF isoform expression. Since VEGF has also been shown to act as an autocrine survival factor in vitro, this event is beneficial for both endothelial cells and podocytes. The inhibitory isoform is clearly another good candidate as an endogenous inhibitor of VEGF.
The presence of sVEGFR-1 may also explain why the high concentration of VEGF in adult glomeruli does not cause angiogenesis. Whittle et al. examined the expression levels of the VEGF isoforms and VEGF receptors in biopsies of healthy human glomeruli [151]. Using RT-PCR, they found sVEGFR-1 to be the most prominent VEGF receptor expressed, followed by VEGFR-1 then VEGFR-2. Whether sVEGFR-1 is produced by podocytes or endothelial cells was not addressed.

1.5.2 The Glomerular Filtration Barrier

The adult human kidney contains approximately 1 million glomeruli, where filtration of the blood plasma occurs. Specifically, filtration takes place across the glomerular capillary wall into the Bowman’s space and the wall comprises three layers: podocytes on the urinary side, an inner layer of fenestrated endothelial cells and an intervening GBM (figure 1.7). Each layer has unique structural features and together forms a selective filtration barrier that is charge, shape and size-dependent, allowing the free passage of water and small molecules while preventing that of albumin and other larger molecules.
Figure 1.7. The glomerulus and the glomerular filtration barrier. A) A light micrograph of a mature glomerulus, a specialized vascular bed responsible for the filtration function of the kidney (H&E). B) The GFB consists of 3 layers: podocyte foot processes (FP) that interdigitate and is spanned by a slit diaphragm (SD), the glomerular basement membrane (GBM) and the fenestrated endothelium (EC fen) (TEM).
1.5.2.a Podocytes

The podocyte is a highly differentiated cell type that has a cell body with long primary processes that extend toward the glomerular capillaries [142]. The primary processes further divide into secondary foot processes which interdigitate with those of neighbouring podocytes. Studies on podocyte biology and glomerular permeability have suggested that the ultimate barrier in restricting the leakage of proteins from the vasculature is the slit diaphragm, a porous membrane that bridges the narrow spaces (30-40 nm) between inter-digitating foot processes. The identification of podocyte-specific genes as underlying causes of inherited kidney diseases has also emphasized the importance of podocyte function of the GFB. In 1998, Trygvason and colleagues made a break-through discovery and reported that mutations in the NPHS1 gene causes congenital nephrotic syndrome of the Finnish type [152]. The disease is characterized by the absence of podocyte foot processes and slit diaphragms and is manifested at the fetal stage, with heavy proteinuria in utero, leading to death during the first two years of life. The gene product is nephrin, a transmembrane protein that contains a large extracellular region that self-associates in a zipper-like arrangement, forming a key structural component of the slit diaphragm (figure 1.8). In addition, nephrin is also involved in the signaling processes that are critical in podocyte survival and differentiation. Work from two separate groups have shown that nephrin tyrosine phosphorylation affects podocyte morphology through the recruitment of Nck adaptor proteins [153, 154]. Specifically,
**Figure 1.8.** Schematic representation of the podocyte foot process-associated proteins.

ZO-1, Zona occluden 1; Syn, synaptopodin P-cad, P-cadherin; u, utrophin; T, talin; V, vinculin; P, paxillin; α,β,γ; α,β,γ-catenin, α3,β1, α3β1-integrin; αD, βD, αβ-dystroglycan. Adapted from S. Somlo and P. Mundel. Nature Genetics 2000.
nephrin phosphorylation through Fyn creates a binding site for the SH2 domain of Nck. This in turn recruits N-WASP, activating the Arp2/3 complex which subsequently regulates podocyte actin dynamics. Selective deletion of both members of the Nck family, Nck1 and Nck2, from podocytes in mice prevents the formation of a functional GFB [153]. These mice fail to form foot processes, resulting in nephrotic-range proteinuria.

Podocin is another protein that is localized in the slit diaphragm. Mutations in the nphs2 gene that encodes for podocin leads to an autosomal-recessive steroid-resistance nephrotic syndrome that is also characterized by childhood onset of proteinuria with disruptions in the slit diaphragms and foot process fusion [155]. Podocin is first detected in the S-shaped stage of glomerular development and its expression is maintained in the mature glomerulus. Podocin may serve two functions: the organization and stabilization of the slit diaphragm complex by interacting with nephrin and CD2AP [156, 157] and by augmenting nephrin signaling [158]. CD2AP is expressed simultaneously with nephrin and synaptopodin at the capillary loop stage [159]. It is a cytoplasmic adaptor protein that binds to podocin and nephrin, anchoring them to the actin filaments of the actin cytoskeleton. CD2AP-deficient mice develop proteinuria and kidney failure and exhibit extensive foot process effacement [160].

Other proteins localized in the slit diaphragm includes FAT, P-cadherin, ZO-1 and NEPH-1. FAT is a member of the cadherin superfamily that mediates cell-cell adhesion and is also involved in actin dynamics [161]. FAT plays an essential role in the GFB in mice as FAT null mice develop proteinuria and die shortly after birth [162]. P-cadherin may play a role in mediating cell adhesion between neighbouring foot
processes; however, mice deficient in P-cadherin are viable [163]. The intracellular domain of P-cadherin is associated with β- and γ-catenin and this complex is linked to the actin cytoskeleton through α-catenin. ZO-1 is also located on foot processes and interacts with the actin based cytoskeleton [164]. Phosphorylation of ZO-1 has been shown in newly formed tight junctions of foot processes, suggesting its participation in signaling events during glomerular development [165]. Furthermore, ZO-1 binds to the Neph family members which are homologues of nephrin. NEPH-1 is a transmembrane protein that colocalizes at the slit diaphragm, interacting with nephrin and podocin [166-168]. Disruption of the NEPH-1 gene in mice results in foot process effacement, massive proteinuria and early postnatal death, suggesting that NEPH1 plays a role in maintaining the structure of the GFB [169].

Podocytes possess a highly dynamic cytoskeleton which accounts for its unique shape and architecture and provides stability to support the mechanical processes acting on the cell. The cell body and primary processes contain microtubules and intermediate filaments whereas microfilaments and actin filaments are found in the secondary foot processes [170]. Regulation of this cytoskeleton plays an important role in normal renal function and disease. Healthy podocyte foot processes are characterized by contractile actin filament bundles that are organized in a parallel manner and dysregulation of this flexible and dynamic network is closely associated with renal dysfunction. In effaced foot processes, the parallel contractile bundles become dense, losing their capacity to respond to the mechanical stresses encountered. Mutations in components of the podocyte actin cytoskeleton such as the actin filament cross-linking protein, α-actinin 4, have been linked with an adult-onset autosomal dominant focal segmental glomerulosclerosis.
(FSGS) [171]. The role of this protein in glomerular function has also been shown in mice as mice that lack α-actinin 4 also develop proteinuria and glomerular disease that is accompanied by podocyte foot process effacement [172]. α-actinin 4 also colocalizes with synaptopodin, another actin-associated protein [173]. Although synaptopodin-deficient mice are viable and fertile and show no defects in podocyte formation and structure, these mice exhibit poor recovery from protamine sulfate (PS)-induced acute foot process effacement [173, 174].

1.5.2.b Glomerular Basement Membrane

The GBM located between the fenestrated endothelial cells and the podocytes has also been proposed to be an important component of the GFB due to its characteristic negative charge that is imparted by heparan sulfated glycosaminoglycan (GAG) side-chains of proteoglycans (HSPGs). It is approximately 300 nm thick and contains four major components: type IV collagens, laminins, nidogen/entactin and sulfated proteoglycans [139]. The importance of the basement membrane components in the formation and maintenance of the GFB has been shown by gene targeting studies in mice. Collagen IV is a trimeric protein consisting of 3 α chains. At present, 6 genetically distinct α chains have been described. Mice that lack α3 collagen IV chains result in progressive renal disease with proteinuria and Alport syndrome-like abnormalities in the GBM [175]. Laminins are also heterotrimeric proteins that consists of α, β and γ chains and lack of a single laminin monomer is sufficient to disrupt the GFB. Mice with a targeted mutation in the laminin β2 gene develop massive proteinuria and die within 3-5 weeks of age [176]. In this mutant, the GBM remains intact but the podocyte foot
processes are fused.

The attachment of podocyte foot processes to the GBM is critical for a functional filtration barrier. One of the major structural defects that occur during proteinuria is the retraction and flattening of the foot processes. Among the molecules important for anchoring the podocytes to the underlying matrix in the basement membrane are integrins and dystroglycans which are expressed on the basolateral surface of podocytes [177]. Integrins are heterodimeric transmembrane receptors that bind to a number of ligands found in the GBM including fibronectin, type IV collagen and laminin. Integrins regulate actin dynamics in two ways – through ‘outside in’ signaling and through ‘inside out’. In the former, extracellular events activate integrins, triggering a number of downstream signaling pathways that affect actin polymerization and cell morphology including FAK, ILK and/or Arp2/3 [170]. In the latter, integrins change their adhesive properties in response to intracellular stimuli. The predominant integrin expressed by podocytes is $\alpha_3\beta_1$ integrin which is linked to the podocyte actin-cytoskeleton by paxillin, talin and vinculin [178]. $\alpha_3\beta_1$-deficient mice fail to form foot processes, with the podocytes appearing flat against the fragmented GBM [179]. Increased adhesion or a failure of cytoskeletal organization may underlie this phenotype.

In addition to integrins, dystroglycans mediate podocyte-GBM interactions [177]. They are composed of $\alpha$ and $\beta$ subunits, with the $\alpha$ unit found in the GBM and the $\beta$ subunit linked to the podocyte actin cytoskeleton through utrophin.
1.5.2.c Endothelium

The third component of the GFB is the fenestrated endothelium and it has only been until recently when more research is conducted to further characterize its properties. More specifically, there is a growing amount of interest in studying the endothelial cell surface coat – the glycocalyx. The glycocalyx is a dynamic, hydrated layer that consists of glycoproteins and proteoglycans with absorbed plasma proteins [180, 181]. Similar to the GBM, it has a negative charge imparted by the abundant HSPGs. Dehydrating effects of standard electron microscopy (EM) fixation and processing has precluded the visualization of the glycocalyx but newer fixation protocols have enabled the demonstration of a glomerular endothelial glycocalyx of 200–400 nm in thickness [182]. Scanning EM studies have revealed that the glycocalyx is present on the luminal side of glomerular endothelial cell and covers the fenestrae [183]. Jeansson et al. reported that enzymatic digestion of GAG decreased the thickness of the glycocalyx and increased albumin excretion in mice, providing evidence that endothelial cell glycocalyx is an important component in the glomerular barrier [184].

In 2006, Satchell and colleagues generated a conditionally immortalized human glomerular endothelial cell line that expressed endothelial specific markers. These cells responded to VEGF treatment by forming fenestrations [185]. EM studies revealed that the same cell line contained a 200 nm-thick glycocalyx which contributed to the prevention of albumin flux across the cell layer [186]. This cell line may be of potential use for further studying endothelial cells in vitro.

Ample clinical evidence has supported that damage to the podocyte or GBM components leads to proteinuria but there is also evidence that glomerular endothelium
damage or dysfunction alone results in increased permeability. Patients with preeclampsia develop proteinuria and is associated with endotheliosis, characterized by endothelial swelling and loss of endothelial fenestrae [187]. This glomerular lesion is mediated by an increase in sVEGFR-1 levels that subsequently deprives glomerular endothelial cells of VEGF, leading to the disruption of selective filtration [188]. Endothelial damage is also seen in patients with hemolytic uremic syndrome [189]. In both disorders, the podocytes and GBM initially remain intact, indicating that the glomerular endothelial cells is an important feature of the GFB.

1.5.3 Role of VEGF in glomerular function

Podocyte-derived VEGF plays a key role in the development and maintenance of the glomerular endothelial cells. Genetic deletion of VEGF from the podocyte results in dramatic and distinct glomerular phenotypes in mice. Loss of both VEGF alleles results in a marked reduction in endothelial cell migration in the developing glomerulus [1]. Consequently, the GFB fail to form and the mice die at birth due to renal failure. Loss of only one VEGF allele results in glomerular endotheliosis at 2.5 weeks of age, the classic lesion found in pre-eclamptic patients. Mice with an intermediate dose of VEGF (between podocyte-specific heterozygotes and homozygous VEGF nulls) result in the loss of endothelial cells followed by rapid lysis of mesangial cells [190]. On the other hand, a 15-20 fold increase in VEGF production during glomerular development leads to a collapsing glomerulopathy that resembles HIV-associated nephropathy [1]. These findings demonstrate that the glomerular endothelial cells are highly dependent on VEGF production by the podocyte during glomerulogenesis. The persistent expression of VEGF
in the adult glomerulus suggest that VEGF may be involved in the maintenance of the fenestrated, differentiated glomerular endothelium. Using an inducible tet-on system whereby the gene of interest is deleted only in the presence of doxycycline, a tetracycline derivative, Eremina et al. found that selective deletion of VEGF from the podocyte in adult mice results in proteinuria, hypertension and thrombotic microangiopathy [2].

Whether VEGF acts on podocytes still remain to be clearly established; however, several lines of evidence support the existence of a functional VEGF autocrine loop in cultured podocytes (figure 1.9). First, a number of groups have reported the presence of VEGFR-1 and VEGFR-3, but not VEGFR-2 in immortalized human podocytes and found that VEGF exerts an anti-apoptotic effect upon serum starvation [4]. Since treatment with tyrosine kinase inhibitors abolished this effect, they suggest that VEGFR-1 and/or VEGFR-3 mediates podocyte cell survival. Ziyadeh et al. found similar expression patterns and detected VEGFR-1 mRNA and protein by real-time PCR, immunoblotting and immunohistochemistry in cultured mouse podocytes [3]. In addition, they demonstrated that VEGF induces phosphorylation of VEGFR-1 in a dose and time-dependent manner. Using the same mouse podocyte cell line, Guan et al. reported the presence of Np-1, Np-2, VEGFR-1 and VEGFR-2 mRNA in undifferentiated and differentiated states; however, upon differentiation, VEGFR-2 levels increase [5]. They also extend Foster et al.’s previous findings that VEGF is a survival factor for podocytes. However, they suggest that VEGF’s anti-apoptotic activity is mediated by VEGFR-2, not VEGFR-1.
Figure 1.9. Paracrine vs. autocrine VEGF signaling in the glomerulus. Podocyte-derived VEGF acts in a paracrine manner towards the glomerular endothelium which are known to express VEGF receptors in vivo. Whether VEGF acts in an autocrine fashion towards the podocytes has been suggested by in vitro studies but in vivo data are lacking.
Of note, VEGF has been suggested to inhibit apoptosis in cultured human podocytes by stimulating nephrin phosphorylation; however, the interaction between VEGF and nephrin is unclear [191]. The cytoplasmic tail of nephrin can be phosphorylated by the Src family members, Fyn and Yes, which preferentially bind to VEGFR-1 [97]. Since VEGFR-1 has been detected in the same podocyte cell line, it can be inferred that VEGF phosphorylation of VEGFR-1 leads to the activation of Fyn or Yes which subsequently phosphorylates nephrin.

In addition, VEGF may also be involved in the homeostasis of podocyte slit diaphragms. Exogenous treatment of cultured mouse podocytes with VEGF leads to a four-fold increase of podocin and its association with CD2AP, suggesting that VEGF promotes the structural integrity of the slit diaphragm [5]. Furthermore, exogenous VEGF164 treatment of cultured podocytes leads to an increased α3 (IV) production which was blocked by SU5146, a tyrosine kinase inhibitor [3]. Here, podocyte-derived VEGF is suggested to operate in an autocrine loop through VEGFR-1 while VEGF is believed to regulate podocin/CD2AP interactions through VEGFR-2.

1.6 VEGF AND DISEASE

1.6.1 Diabetic Nephropathy

VEGF’s permeabilizing effects has been implicated in the proteinuria seen in diabetic nephropathy (DN). DN is one of the microvascular complications of diabetes and is currently the leading cause of end-stage renal disease in North America. The long-term hyperglycemia that results from the diabetic setting leads to various pathologic changes and in early stages, there is glomerular and renal hypertrophy, microalbuminuria
glomerular hyperfiltration, increased GBM thickness, mesangial matrix expansion and podocyte damage [192, 193]. As the disease progresses, renal insufficiency, proteinuria, interstitial fibrosis and glomerulosclerosis develop. High glucose has been shown to increase VEGF mRNA and protein expression in cultured mesangial cells and cultured differentiated mouse podocytes [194-198]. An increased expression of VEGF mRNA and protein in the glomerular, tubulointerstitial, and vascular compartments was shown in streptozotocin (STZ)-induced type 1 diabetic rats [199]. A similar increase in VEGF has also been described in experimental models of type 2 diabetes such as the OLETF rats and the db/db mouse which carries a mutation in the leptin receptor gene [200, 201]. These reports have led to the idea that VEGF contributes to the pathogenesis of DN and blocking VEGF action may prevent or normalize DN. A number of studies support this notion and have shown that VEGF inhibition abolishes diabetes-associated structural and functional alterations in the mouse kidney. Interestingly, administration of a neutralizing VEGF antibody attenuated the increase in renal/glomerular volume, BMT, and UAE and abolished the increase in creatinine clearance in db/db mice [202]. Administration of the same VEGF antibody in STZ-induced diabetic rats, also abolished the diabetes-associated hyperfiltration and partially blocked the increase in UAE [203]. Inhibition of VEGF signaling using the pan-VEGF receptor tyrosine kinase inhibitor, SU5416, also abolished albuminuria and basement membrane thickening in the db/db mouse but did not prevent mesangial matrix expansion [201]. Others however did not observe a beneficial effect of VEGF inhibition on disease progression [204]. Ku et al. took a genetic approach to determine the role of VEGF in DN in mice [205]. They overexpressed sVEGFR-1 specifically in the podocyte using the tet-On inducible system and induced diabetes using
Interestingly, they found the amelioration of diabetes-associated mesangial expansion, GBM thickening and podocyte foot process fusion in mice with elevated sVEGFR-1 levels, suggesting that VEGF plays a pathogenic role in the disease.

In the human kidney, dysregulation of VEGF expression has been also associated with DN although data are conflicting as both increased and decreased levels of VEGF have been reported. Immunohistochemical staining of glomeruli from biopsies from type 2 diabetic patients revealed an increase in VEGF expression compared to biopsies from individuals without kidney disease [206]. On the other hand, Bortoloso et al. examined the relationship between VEGF and the severity of DN in microdissected glomeruli type 2 diabetic patients and found that glomerular VEGF mRNA levels were inversely related to albuminuria and mesangial and mesangial matrix expansion [207]. These results were consistent with studies using gene expression profiling and RT-PCR that found a decrease in VEGF expression in renal biopsies and isolated glomeruli of patients with DN [208, 209].

The apparent contradiction in experimental models of diabetes and human DN may be explained in part by the stage of disease. Animal models do not fully develop progressive DN and only demonstrate early signs of DN [210]. In contrast, the renal biopsies that are often examined are from patients with late DN. Results from the human studies suggest that a loss of VEGF contribute to the progression of DN. Baelde at al. posit that the diabetic setting injures the podocyte, resulting in podocyte loss and subsequent reduction in expression of angiogenic factors such as VEGF [208]. Since VEGF is critical in the maintenance of the endothelial cells, endothelial cell dysfunction and capillary loss occurs. Preservation of the renal microvasculature should therefore be
considered, which is in contrast to the therapeutic VEGF inhibition that is suggested by studies that use experimental animal models of diabetes.

1.6.2 Eye Disease

VEGF has also been implicated in highly prevalent retinal diseases including age-related macular degeneration (AMD) and diabetic retinopathy (DR). AMD is a leading cause of vision loss in people over 60 [211]. It is characterized by degenerative changes in the outer portion of the retina and the retinal pigment epithelium (RPE). In late stage AMD, neovascularisation ensues due to a diminished function of the RPE and reduced oxygen concentrations. These new blood vessels are highly permeable, allowing blood and fluid to leak into the retina, leading to poor vision and scarring [212]. DR is a slow, progressive disease which, if left untreated leads also to visual impairment and blindness [213]. DR is characterized by increased retinal vascular permeability, ischemia and retinal neovascularization. Macular edema, bleeding and fibrosis may follow. VEGF is believed to initiate the abnormal vessel growth and hyperpermeability of the retinal vessels in AMD and DR. VEGF is normally produced by a number of retinal cell types including RPEs, astrocytes, Müller cells, vascular endothelium, ganglion cells [214]. In DR, VEGF levels are elevated and is associated with increased vascular permeability and increases with disease severity [214]. In a primate model, injection of VEGF into the eye was sufficient to produce many of the vascular abnormalities seen in DR such as hemorrhage, edema, capillary closure, microaneurysm formation, and intraretinal vascular proliferation [215]. In addition, transgenic mice that overexpressed VEGF in the photoreceptor layer developed intraretinal and subretinal neovascularization [216].
Current strategies to prevent visual loss associated with AMD and DR focus on blocking VEGF’s activity. In June 2006, the drug Lucentis (Ranibizumab) was approved by the US FDA for treatment of AMD. Lucentis is a humanized antibody fragment that binds to and inhibits VEGF-induced blood vessel growth and leakage. Monthly injections into the eye prevented vision loss and was the first treatment for AMD that was associated with vision improvement in a remarkable proportion of patients [212].

1.6.3 Cancer

Angiogenesis is also a vital process in the development and progression of cancer. By forming new vasculature from existing blood vessels, small localized neoplasms obtain oxygen and nutrients and acquire the capacity to grow and the potential to metastasize. A number of studies demonstrate VEGF mRNA expression in lung, breast, pancreatic, gastric, ovarian and renal carcinomas, implicating VEGF in the promotion of tumor growth and metastasis [217-224]. Clinical observations have not only demonstrated that VEGF levels are significantly correlated with neovascularization in solid tumors but also serve as a predictive measure of the resistance to various therapies. At present, a number of clinical trials in cancer patients are underway to test several VEGF inhibitors including bevacizumab; Avastin, a humanized anti-VEGF monoclonal antibody generated by Genentech, an anti-VEGFR-2 antibody, small molecules inhibiting VEGFR-2 signal transduction, and a VEGFR chimeric protein [225]. The use of bevacizumab in combination with chemotherapy has been shown to statistically increase survival in patients with metastatic colorectal cancer [226]. In addition, the combinatorial use of bevacizumab with interferon as first-line treatment in patients with metastatic renal
cell carcinoma also results in significant improvement of progression-free survival [227]. Although a number of bevacizumab-related side effects have been identified (thrombosis, bleeding, proteinuria, and hypertension) [2, 228], the overall successful clinical outcomes provides evidence for the anti-vascular effects after clinical blockade. In February 2004, bevacizumab was approved by the US FDA as a first-line treatment for metastatic colorectal cancer [228]. The role of bevacizumab in other tumor types is currently under investigation and Phase III clinical trials in non-small cell lung cancer, renal cell cancer and metastatic breast cancer are ongoing.

1.6.4 Autoimmune Disease

VEGF also plays a pathological role in autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS). RA is characterized by a dysfunctional cellular and humoral immunity, vascular proliferation and blood vessel invasion of the joint that ultimately results in joint destruction. VEGF has been linked to the initiation and progression of RA [229]. In MS, the break down of the blood-brain barrier, the upregulation of adhesion molecules on capillary endothelial cells, and the occurrence of perivascular inflammation, suggests that altered vessel permeability and activated endothelial cells are involved in the pathogenesis of the disease. VEGF has been shown to be upregulated in MS plaques, exacerbating the inflammatory response in autoimmune diseases of the CNS by inducing focal BBB breakdown and migration of inflammatory cells into the lesions [230].
VEGFR-1 and Disease

1.7.1 Pre-eclampsia

Pre-eclampsia affects ~5% of pregnant women and is characterized by the new onset of hypertension and proteinuria after 20 weeks of gestation [231]. Placental ischemia has been postulated to be the initiating event in preeclampsia, leading to the production and secretion of soluble factors into the maternal vasculature that in turn induces endothelial dysfunction, which results in the clinical features of pre-eclampsia. A number of groups have reported that placental sVEGFR-1 is upregulated in preeclamptic patients, leading to increased circulating levels of sVEGFR-1 and an associated decrease in circulating levels of VEGF and PIGF. After delivery, sVEGFR-1 levels decline and corresponds to the resolution of the syndrome. These initial observations have led to the hypothesis that sVEGFR-1 plays a causal role in the pathogenesis of preeclampsia. Interestingly, administration of sVEGFR-1 to pregnant rats induced hypertension, proteinuria and glomerular endotheliosis, a classic lesion of preeclampsia [232]. In addition, podocyte-specific deletion of a single VEGF allele also resulted in proteinuria and glomerular endotheliosis, providing genetic evidence that VEGF deficiency leads to preeclamptic symptoms [1]. Together, these data suggest that excess placental production of sVEGFR-1 contributes to the pathogenesis of preeclampsia. More recently, novel isoforms of sVEGFR-1, sVEGFR-1-14, have been identified in pre-eclamptic patients [233].
1.7.2 Placental Malaria

Elevated sVEGFR-1 has also been associated with placental malaria (caused by *Plasmodium falciparum*) which is highly prevalent in Sub-Saharan Africa [234]. Upon infection, malarial parasites become sequestered in the intervillous space of the placenta and induce an inflammatory reaction which may lead to adverse pregnancy outcomes including fetal growth restriction, premature birth or still birth [235]. In 2008, Muenlebachs et al. suggested that the infant FLT1 (VEGFR-1) genotype is related to the pregnancy outcome during placental malaria [236]. Specifically, they detected a dinucleotide repeat polymorphism in the 3’ UTR of the VEGFR-1 gene that affects the expression levels of VEGFR-1 in blood monocytes, with the S alleles associated with greater expression compared to the L alleles [236]. SS infants, compared with LL or SL, have lower birth rates and the highest rate of prenatal loss in the presence of placental malaria but have heavier birth weights in its absence. In addition, the SS fetal genotype was associated with increased expression of inflammatory markers in the placenta and increased sVEGFR-1 in the maternal plasma. Whether VEGFR-1 genotype or sVEGFR-1 levels have a causal role in pathogenesis remains unknown. VEGF’s pro-inflammatory effects are believed to be mediated through VEGFR-1 in monocytes while sVEGFR-1 has an opposite, anti-inflammatory effect by anatohizing VEGF. sVEGFR-1’s increased expression may then be a response to the greater inflammation of SS placentas rather than a cause of the inflammation. It remains to be determined how the polymorphism regulates VEGFR-1 vs. sVEGFR-1 expression. It may affect the secondary structure of the RNA, mRNA stability or processing that in turn affects VEGFR-1 production.
1.7.3 Sepsis

Increased sVEGFR-1 levels have also been reported in a nonpregnant setting in humans. VEGF has been shown to play a role in the pathophysiology and prognosis of sepsis, also known as a systemic inflammatory response syndrome. A recent study by Shapiro et al. evaluated the levels of VEGF and sVEGFR-1 in the blood of 83 patients with early infection and determined whether an association existed between the levels and the severity of illness and inflammation [237]. They found both VEGF and sVEGFR-1 levels to be highest in septic shock patients and both VEGF and sVEGFR-1 significantly associated with the activation of the inflammatory cascade. An important distinction was, however, stated between the roles of VEGF or sVEGFR-1. Although both circulating VEGF and sVEGFR-1 correlate with disease severity, VEGF has been suggested to contribute to sepsis pathogenesis while sVEGFR-1 may have a protective effect in sepsis by inhibiting free VEGF or may be a compensatory anti-inflammatory response. sVEGFR-1 may thus represent a potential diagnostic marker in patients with sepsis.
Chapter Two

Age-dependent response to VEGF upregulation in podocytes

A version of this chapter is in preparation for publication.

Sison, K, Eremina, V, Rossant, J, Quaggin S. Age-dependent response to VEGF upregulation in podocytes. To be submitted.
2.1 ABSTRACT

VEGF is a potent growth factor that promotes endothelial cell migration, proliferation and differentiation in developmental and pathological angiogenesis. In the kidney, podocytes produce large amounts of VEGF during glomerular development and continue to express VEGF in fully formed adult glomeruli. Increased levels of VEGF expression have been reported in glomerular disease that include diabetic nephropathy. Although most studies have focused on the role of VEGF signaling in the vascular compartment (glomerular endothelial cell (EC)), in vitro data has suggested that an additional autocrine signaling loop through VEGFR-2 exists in the podocyte. In this study, we used genetic models to upregulate VEGF expression selectively in podocytes during development and in adult mice. Proteinuria was associated with different ultrastructural findings depending upon the age of induction. Specifically, developmental increase was associated with foot process effacement, while induction in the adult mice resulted in proteinuria with no gross structural defects. To determine if this phenotype results from disruption of a constitutively activated VEGFR-2-mediated autocrine signaling loop within the podocytes, we genetically deleted VEGFR-2 from podocytes. No defects were observed in the 12 weeks following birth, demonstrating no essential role for VEGF-mediated VEGFR-2 signaling during glomerulogenesis. In addition, loss of VEGFR-2 from the podocyte did not rescue the glomerular defects in the VEGF overexpressor models. Taken together, our data show that balance of VEGF is required in the glomerulus. How increased levels of VEGF lead to proteinuria and altered podocyte effects is not clear but no evidence for VEGFR-2-mediated autocrine loop was observed.
2.2 INTRODUCTION

VEGF was initially discovered as a tumor permeability factor in 1989 [8, 9]. Subsequently, it has been shown that VEGF has major angiogenic stimulatory properties in development and pathologic angiogenesis of tumours, wound healing and arthritis. VEGF is a secreted dimeric glycoprotein that exists in a number of splice variants, each displaying different properties with regards to diffusibility and heparin-binding affinities [11, 12, 17, 149]. The most abundant isoform expressed by the kidney is VEGF165 (164 in mouse) [238, 239]. Two receptor tyrosine kinases have been identified for VEGF, VEGFR-1 (Flt1) and VEGFR-2 (Flk1) [6, 99]. The majority of VEGF signaling described to date is mediated through VEGFR-2 while the signaling capacities of VEGFR-1 are still debated. VEGF also binds to two non-protein kinase co-receptors, Neuropilin-1 (Np-1) and Neuropilin-2 (Np-2), both of which may function to enhance VEGFR-2 signaling [22, 240].

In the glomerulus, podocytes function as vasculature support cells and produce VEGF in vast amounts. Podocyte-specific deletion or overexpression of VEGF during development leads to dramatic and distinct glomerular phenotypes [1, 190]. Here, the major defects occur within the glomerular endothelium, indicating that VEGF paracrine signaling is critical for the development of a functional glomerular filtration barrier (GFB). Within the past ten years, VEGF receptors have also been identified in the podocyte. In particular, VEGFR-2 expression has been detected in a cultured mouse podocyte cell line and its expression increases upon differentiation [5]. Immunogold staining and electron microscopic studies have also revealed the presence of VEGFR-2 in the podocyte foot processes [205]. In contrast, other groups have identified VEGFR-1,
but not VEGFR-2, in cultured podocytes and have shown that VEGFR-1 mediates podocyte cell survival and production of alpha3 (IV) collagen [3, 4].

Increased levels of VEGF expression have been reported in a number of glomerular diseases. In patients with diabetic nephropathy, VEGF levels correlate with glomerular hypertrophy and degree of proteinuria, suggesting that VEGF inhibition may be a potential therapeutic strategy to restore normal renal function [206, 241]. Passive Heymann nephritis (PHN) and puromycin aminonucleoside nephrosis (PAN) rodent models of proteinuria also exhibit increased VEGF mRNA and protein, implying a role for VEGF in pathogenesis [242]. Clearly, upregulated VEGF expression contributes to the loss of permeaselectivity and increased permeability of the GFB. The strict control of VEGF action is therefore crucial for the maintenance of glomerular function.

Although the existence of a paracrine cell-cell interaction between podocytes and endothelial cells is well established, it is unclear whether VEGF signals in an autocrine fashion in vivo. To begin to address this question, we generated transgenic mice with an inducible and podocyte-specific VEGF overexpression and found that these mice develop proteinuria and ultrastructural changes in the GFB depending on the age of induction. Since VEGFR-2 is believed to be responsible for transducing VEGF action and has been identified in immortalized podocyte cell lines and in vivo, we investigated whether VEGF operates in an autocrine signaling loop through VEGFR-2 by generating another mouse model that specifically lacks VEGFR-2 in the podocyte. We also sought to determine whether the genetic deletion of VEGFR-2 could rescue the glomerulopathy seen in the VEGF overexpressors. Interestingly, loss of VEGFR-2 from the podocyte alone does not affect glomerular development and function and does not prevent the
glomerular abnormalities seen in the VEGF overexpressors. Together, these results provide further evidence that a critical balance in VEGF must be maintained in both developing and mature glomeruli. In addition, VEGF autocrine signaling through VEGFR-2 was not observed.

2.3 METHODS

2.3.1 Transgenic lines

Transgenic lines used include: tetO-VEGF164, Nephrin-Cre, ROSA26 rtTA, Nephrin-CFP, VEGFR-2-GFP and floxed VEGFR-2 mice. tetO-VEGF164 mice carry the cDNA for the murine VEGF 164 isoform that is driven by the tetO-CMV minimal promoter (J. Whitsett, Cincinnati, Ohio). Generation and characterization were previously described [243, 244]. In ROSA26 rtTA transgenic mice, the coding sequence for reverse tetracycline transactivator (rtTA) was targeted into the ubiquitously expressed ROSA26 locus. Since a floxed STOP codon precedes the coding sequence for rtTA, expression is dependent on a Cre excision (A. Nagy Samuel Lunenfeld Research Institute, Toronto, Canada). Presence of doxycycline (DOX) results in the formation of an active transcriptional activator and the activation of the tet-responder transgene, allowing for temporal regulation [245]. In Nephrin-Cre and Nephrin-CFP transgenic mice, Cre and cyan fluorescent protein (CFP) expression is under the control of the podocyte-specific Nephrin promoter, allowing for spatial regulation [246]. Floxed VEGFR-2 mice contain loxP sites around exon 1 (J. Rossant, Hospital for SickKids, Toronto, Ontario, Canada). VEGF receptor 2–green fluorescence protein (VEGFR-2–GFP) mice were generated by replacing exon 1 of the endogenous VEGFR-2 gene with EGFP cDNA and has been
validated to be equivalent to a null allele (J. Rossant, Hospital for SickKids, Toronto, Ontario, Canada) [247].

2.3.2 Generation of inducible VEGF mouse model and podocyte-specific VEGFR-2 knockouts

To generate a transgenic mouse model with inducible VEGF overexpression within the podocyte, we bred podocyte-specific Nephrin Cre mice to another line that carries a tetO-regulated VEGF 164 gene. This bistransgenic line was bred to mice that bear the tetracycline-controlled rtTA that is driven by the ROSA26 promoter. DOX was administered in the drinking water (2mg/ml) at P0 and 3 weeks of age. Podocyte-specific VEGFR-2 knockout mice were generated by breeding mice carrying one copy of the floxed VEGFR-2 allele and one copy of the Nephrin-Cre transgene with homozygous floxed VEGFR-2 mice.

2.3.3 Genotyping

Genomic DNA was isolated from mouse tails as previously described [248]. PCR was used to identify triple transgenic mice using the following primers: Nephrin Cre (sense 5’- ATG TCC AAT TTA CTG ACC G- 3’, antisense 5’- CGC CGC ATA ACC AGT GAA - 3’) tetO-VEGF164 (sense 5’-TGG ATC CAT GAA CTT TCT GCT GCT- 3’, antisense 5’-GAA TTC ACC GCC TCG GCT TGT C-3’), ROSA26 rtTA (sense 5’- GAG TTC TCT GCT GCC TCC GC- 3’, antisense 5’- AAG ACC GCG AAG AGT TTG TG -3’). Presence of the floxed VEGFR-2 gene were detected by PCR using the following primers: FlkS15’- TGGAGAGCAAGGCGCTGCTAGC- 3’, FLK1-A - FlkS15’- CTTTCCACTCCTGCCTACCTAG- 3’which generate a 439 bp fragment of the VEGFR-2 allele with 1 loxP site and a 322 bp DNA fragment for the wild-type allele.
2.3.4 Urinalysis

Urine was collected and examined for the presence or absence of protein using a urine dipstick (Chemstrip 5L; Roche Diagnostics Corp., Indianapolis, Indiana, USA) and the standard colorimetric assay was performed according to the manufacturer’s instructions. In addition, total protein levels were assessed by using the Bradford colorimetric-based Bio-Rad assay. Briefly, the protein assay dye concentrate containing the comassie blue dye (Bio-Rad, Cat. No. 500-0006) was added to the urine sample and was incubated at room temperature for 10 min. Absorbance was measured at 595 nm. Protein levels were normalized to the urine creatinine levels as measured by the Jaffé method [249]. Results are expressed as total protein/creatinine ratios (mg/mg).

2.3.5 Histologic and ultrastructural analysis.

Freshly dissected kidneys were fixed in 10% formalin/PBS, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Sections were examined and photographed with a DC200 Leica camera and Leica DMLB microscope (Leica Microsystems Inc., Deerfield, IL). For ultrastructural analysis using electron microscopy, kidney tissue was fixed in 1.5% glutaraldehyde, embedded in Spurr (Canemco Inc., Saint-Laurent, Quebec, Canada), and sectioned.

2.3.6 FACS sorting primary podocytes

Glomeruli were isolated from Nephrin–CFP and VEGFR-2–GFP mice using the sieving method. Nephrin-CFP mice contain a CFP reporter gene that is driven by the nephrin promoter. VEGFR-2-GFP mice carry a GFP cassette that is knocked into the VEGFR-2 locus and is under the control of an endogenous promoter. In these mice, CFP is expressed in Nephrin-expressing podocytes and GFP is expressed in VEGFR-2-positive
endothelial cells respectively [190, 246]. Following enzymatic digestion with collagenase, trypsin and EDTA in PBS for 1 hr, 37°C, cells were washed 3X in PBS. Dissociated glomerular cells were sorted using a BD FACS Aria flow cytometer (BD Biosciences). CFP-expressing (CFP+) primary podocytes and GFP-expressing (GFP+) endothelial cells were separated from glomeruli based on CFP and GFP expression respectively.

**Figure 2.1.** FACS sorting of primary podocytes. Glomeruli were isolated from Nephrin–using the sieving method (Baelde et al. 1994). Nephrin-CFP mice contain a CFP reporter gene that is driven by the podocyte-specific nephrin promoter. CFP is expressed throughout glomerular development including in the S-shaped stage (SG), capillary loop stage (CLG) and in the mature stage (MG). Glomerular cells were dissociated enzymatically and were sorted into two fractions, CFP-expressing (CFP+) primary podocytes and CFP-negative (CFP-) endothelial and mesangial cells. RNA were extracted from each fraction and converted into cDNA which were used for RT-PCR analysis.
2.3.7 Real-time PCR

Total RNA was extracted from FACS sorted primary podocytes and endothelial cells with Trizol reagent (Invitrogen) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Fermentas) and random hexamer primers (Fermentas) according to the manufacturers’ protocol. cDNA samples and standards were amplified in qPCR MasterMix Plus for SYBR Green I (BioRad). Samples were analyzed in triplicate. Primer sequences are listed in Table 2.1. Relative quantification of target gene expression was evaluated using the comparative CT method [250]. The ΔCT value was determined by subtracting the HPRT CT value of each sample from its respective target CT. Fold-changes in gene expression of the target gene were equivalent to 2^-ΔΔCT. The values obtained were then entered into a Student's t test.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrin</td>
<td>5’: ACCCTCCAGTTAACCTTGCTTTGG</td>
</tr>
<tr>
<td></td>
<td>3’: ATGCAGGCAGGCTTTTTGA</td>
</tr>
<tr>
<td>VEGF</td>
<td>5’: TCACCAAGGCCCAGCAGATAG</td>
</tr>
<tr>
<td></td>
<td>3’: AATGCTTTCTCCGCCTCTGAA</td>
</tr>
<tr>
<td>HPRT</td>
<td>5’: GGCTATAAGTTCTTTGCTGACCTG</td>
</tr>
<tr>
<td></td>
<td>3’: AACTTTATGTCCCCCGTTGA</td>
</tr>
<tr>
<td>Np-1</td>
<td>5’: CAGAGTTCCCGACATACGGTTT</td>
</tr>
<tr>
<td></td>
<td>3’: TCCCAGTGGCAGAATGTCTTTG</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>5’: GCTCAGGTGTCTGCTTTTCTC</td>
</tr>
<tr>
<td></td>
<td>3’: CTCAGCCTTTGTCTCCTCTG</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>5’: GGCGGTGTGTCAGTATCTT</td>
</tr>
<tr>
<td></td>
<td>3’: GTCACTGACAGAGCGATGA</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>5’: CGAGCCCACTTGTCCCAA</td>
</tr>
<tr>
<td></td>
<td>3’: TGTAGCATAAGGTCTTTCCATGGT</td>
</tr>
</tbody>
</table>

*Table 2.1.* Primers used for real-time PCR analysis.
2.3.8 Western blot analysis

Glomeruli were isolated from control and mutant mice using the sieving method and were lysed in RIPA lysis buffer. Protein concentration was determined by the Biorad Assay according to the manufacturer’s instructions. Samples were resolved on an 8\% SDS-PAGE gel and were transferred to a PVDF membrane. Membranes were blocked in 5\% non-fat dried milk for 1hr, incubated with the appropriate primary antibodies diluted in blocking solution at 4ºC overnight, and washed 3X in 0.05\% Tween 20 in PBS rinse buffer. Membranes were incubated with HRP-conjugated secondary antibodies for 1hr at room temperature, washed and developed with enhanced chemiluminescence (ECL, Amersham). The following primary antibodies were used for immunoblotting: rabbit anti-VEGFR-2, rabbit anti-PY1054/1059-VEGFR-2 (all used at 1:1000; provided by Dr. Wang Min, Yale University) and mouse anti-\(\beta\)-actin (1:5000, Santa Cruz). Secondary antibodies were goat-anti-rabbit IgG-HRP and goat-anti-mouse-IgG-HRP (1:10000; Santa Cruz).
2.4 RESULTS

2.4.1 Podocytes do not express detectable levels of VEGF-2 protein in vivo

To determine if podocytes express VEGFR-2 in vivo, we took advantage of the VEGFR-2-GFP transgenic reporter strain. Immunostaining for the GFP protein clearly demonstrates expression in endothelial cells at all stages of glomerular development, but never in podocytes (figure 2.2 a). To further analyze the expression levels of VEGF receptors in podocytes, we performed real-time (RT)-PCR on RNA from FACS sorted podocytes from isolated and dissociated glomeruli. Podocytes were identified by expression of CFP fluorescence. As a control, VEGF and nephrin mRNA levels were measured. Since VEGF and nephrin are known to be highly expressed by podocytes, values were expressed as fold change over the CFP+ podocyte fraction while the values for the receptors were expressed as fold change over the CFP- fraction. VEGFR-2 expression was undetectable in podocytes (absolute C_T value for VEGFR-2 in CFP+=30) (figure 2.2 b). Np-1 was expressed at relatively low levels; however, VEGFR-1 displayed the highest level of expression (absolute C_T value for VEGFR-1 in CFP+=26). Expression was also determined in primary FACS sorted glomerular endothelial cells as a comparison and yielded similar results, with VEGFR-2 primarily expressed by GFP-positive endothelial cells (figure 2.2 c).
**Figure 2.2.** Expression of VEGF receptors in the glomerulus. A) GFP is knocked into the VEGFR-2 locus. GFP staining shows that VEGFR-2 is only expressed in endothelial cells in developing and mature glomerulus. B, C) VEGF receptor mRNA expression in FACS-sorted cells was assessed by RT-PCR (CFP+ = podocytes, CFP- = endothelial cells and mesangial cells, GFP+ = endothelial cells, GFP- = podocytes, mesangial cells). Values are expressed as fold increase over CFP-, CFP+ or GFP+ cell fraction and represent mean and standard error of the mean (SEM) (n = 2-3 in all groups). T-test was used for statistical analysis (*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$).
2.4.3 Upregulation of the VEGF ligand in podocytes leads to glomerular abnormalities

The effects of increased VEGF production in developing and adult glomeruli were examined using a triple transgenic animal model with inducible overexpression of the 164 isoform of VEGF (figure 2.3 a). DOX was administered in the drinking water of VEGF overexpressors and control (includes wild type mice and mice that did not carry all transgenes) littermates P0 and 3 weeks of age. Furthermore, as additional controls, uninduced triple transgenics were included and confirmed the non-leaky tet(on) system. To verify increased VEGF levels, glomeruli were isolated from 3 week old mice that were induced at birth. RT-PCR revealed a 5-fold increase in VEGF mRNA in the VEGF overexpressors compared with controls (figure 2.4 a). The upregulated VEGF levels were accompanied by an increase in VEGFR-2 tyrosine phosphorylation as shown by Western blot analysis (figure 2.4 b).

Upregulation of the VEGF ligand in podocytes leads to significant proteinuria at all stages examined. Induction at birth results in proteinuria by 3 weeks of age (figure 2.5) with focal effacement of podocytes (figure 2.6). No defects were observed ultrastructurally in the glomerular endothelial cells. Upregulation of VEGF in the adult also led to proteinuria, although only 50% of mice developed the glomerular phenotype (table 2.2). Incomplete penetrance may be attributable to differing Cre expression and efficiency between the Cre lines utilized; Nephrin Cre were used to generate the developmental VEGF overexpressors while Podocin Cre mice were used for the adult VEGF overexpressors. In the adult overexpressors that developed a glomerular
phenotype, 4 days of induction led to massive proteinuria (5g/L by dipstick) with no
gross ultrastructural defects on transmission electron microscopy (TEM) in podocytes,
endothelial compartment or mesangium (figure 2.7). Following 4 weeks of continuous
induction, structural defects were observed on light microscopy with periglomerular
infiltrates, mesangial expansion and proliferation (figure 2.7).
Figure 2.3. Breeding scheme to generate mouse models. A) Inducible podocyte-specific VEGF164 overexpressors. Podocyte-specific Cre expression leads to the excision of the ‘floxed’ STOP codon, resulting in the expression of rtTA within the podocyte. In the presence of DOX, rtTA is able to bind to the tet(o) sequence located in the 5’ region of the VEGF 164 gene, activating its transcription. In the absence of DOX, rtTA is unable to bind to tet(o), preventing VEGF transcription and expression. Since Cre is under the control of a podocyte-specific promoter, this system is inactive in other tissues. B) Podocyte-specific VEGFR-2 knockout mice. Podocyte-specific Cre leads to the deletion of the VEGFR-2 gene. C) Podocyte-specific VEGF overexpressors and VEGFR-2 knockout double mutants contain 5 transgenes. Cre excision deletes VEGFR-2 and activates rtTA simultaneously in podocytes and with DOX, VEGF 164 expression is upregulated.
Figure 2.4. A) Glomerular VEGF mRNA is increased in VEGF overexpressors compared to controls as assessed by real time PCR analysis. (CON n = 4, VEGF+++ n=10). T-test was used for statistical analysis (**: $P<0.01$). B) Western blotting of lysates of isolated glomeruli show increased phosphorylation of VEGFR-2 in isolated glomeruli from VEGF overexpressors compared to control mice. ($V^+ =$ VEGF overexpressors, $C =$ control).
Table 2.2. Numbers of affected vs. unaffected mice. Affected mice develop proteinuria and glomerular disease.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Number of Affected Mice</th>
<th>Number of Unaffected Mice</th>
<th>Total Number of Mice</th>
<th>% Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podocin-CreTg/+, ROSA26-rtTA Tg/+, tet-O-VEGF-A-164Tg/+ (induced in the adult)</td>
<td>Proteinuria within 1 day of induction, progressive glomerular disease with continuous induction</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Nephrin-CreTg/+, ROSA26-rtTA Tg/+, tet-O-VEGF-A-164Tg/+ (induced at birth)</td>
<td>Proteinuria and glomerular disease at 3 weeks of age</td>
<td>10</td>
<td>1</td>
<td>11</td>
<td>91</td>
</tr>
<tr>
<td>Flk fl/fl, Nephrin-Cre Tg/+ (6-12 weeks of age)</td>
<td>Normal</td>
<td>0</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Flk fl/GFP, Nephrin-Cre Tg/+ (6-12 weeks of age)</td>
<td>Normal</td>
<td>0</td>
<td>27</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Nephrin-CreTg/+, ROSA26-rtTA Tg/+, tet-O-VEGF-A-164Tg/+, Flk fl/GFP (induced at birth)</td>
<td>Proteinuria and glomerular disease at 3 weeks of age</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 2.5. Urine protein quantification. Values are expressed as urinary protein/creatinine (U-P/C) ratios and represent mean and standard error of mean (SEM) (n = 5 for CON, n = 5 for Pod-VEGF+++, n = 10 for Pod-VEGFR-2, n = 4 for Pod-VEGF+++ VEGFR-2 DM). One-Way Anova, followed by Bonferroni’s Multiple Comparison Test was used for statistical analysis (***: P< 0.001, significantly different from control mice).
Figure 2.6. Podocyte-specific overexpression of VEGF at birth leads to proteinuria and ultrastructural changes. Mice induced to overexpress VEGF at P0 leads to proteinuria after 3 weeks with protein deposition in the tubules and subtle glomerular alterations on light microscopy (B) (H&E stain). Focal foot process effacement (red arrow) develops in the VEGF overexpressors (D) compared to control wild type mice (C) (TEM).
Figure 2.7. Podocyte-specific overexpression of VEGF in adult glomeruli leads to ultrastructural changes depending on stage of induction. Glomeruli from mice induced to overexpress VEGF for 1 week (B) are similar to those of controls (A). In contrast, 4 weeks of induction results in protein deposition in the renal tubules (C) and glomerular structural changes including mesangial expansion, nodular sclerosis (D, E) (H&E stain). 1 week of VEGF overexpression preserves the ultrastructure of the podocyte foot processes, slit diaphragms and endothelial cells (G), comparable to controls (F) (TEM). 4 weeks of continuous VEGF overexpression results in the loss of endothelial cell fenestrations, podocyte foot process fusion and increased and irregular GBM thickness (H, I) (TEM).
2.4.4 Deletion of VEGFR-2 from developing podocytes does not affect glomerulogenesis

Although we were not able to identify expression of VEGFR-2 in podocytes in vivo, we thought that very low levels of expression at the mRNA level might represent meaningful protein expression below the sensitivity of our assays. To definitively determine whether VEGFR-2 is required in the podocyte for glomerular and podocyte development, we deleted VEGFR-2 from podocytes by breeding mice that with a podocyte-specific deletion of one VEGFR-2 allele (VEGFR-2 loxP/+, Nephrin-Cre +/−) with mice homozygous for the VEGFR-2 allele (VEGFR-2 loxP/loxP) (figure 2.3 b). VEGFR-2 loxP/loxP, Nephrin-Cre +/− mice were born at the expected Mendelian ratio. In addition, another breeding strategy was established to generate VEGFR-2loxP/GFP, Nephrin-Cre +/− mice. In the latter model, GFP is knocked into the VEGFR-2 locus and is equivalent to a VEGFR-2 null allele, enhancing the excision. Podocyte-specific VEGFR-2 knockout mice were born and were followed for 12 weeks postnatal and never developed proteinuria, renal insufficiency or glomerular injury. Light micrographs show normal glomeruli and TEM studies reveal an intact GFB (figure 2.8). These data confirm that signaling through VEGFR-2 does not occur in podocytes in vivo. As our Cre line excises at the capillary loop stage of development, we cannot exclude the possibility of an early role in S-shape stage podocyte precursors.
Figure 2.8. Loss of VEGFR-2 from podocytes does not affect glomerular function. Light micrographs of podocyte-specific VEGFR-2 knockout mice appear similar to controls (H&E stain) and electron micrographs show normal ultrastructure of the GFB.
2.4.5 Podocyte-specific loss of VEGFR-2 does not rescue the proteinuric phenotype in mice with upregulated VEGF expression in the glomerulus

To further investigate whether VEGFR-2 serves a functional role in the podocyte, compound transgenic mice were generated by breeding our podocyte-specific VEGFR-2 knockouts with mice with upregulated VEGF164 levels in the podocyte (figure 2.3 c). PCR was used to identify double mutant mice that contained all transgenes (tetOVEGF164, Nephrin Cre, Rosa rtTA, VEGFR-2 floxed allele, VEGFR-2 GFP allele) and appropriate controls. As expected, podocyte-specific VEGF overexpressors developed proteinuria at approximately 3 weeks of age when induced at P0. Although proteinaceous material was observed in the tubules, the majority of glomeruli appeared normal on light microscopy (figure 2.9 b). Double mutants (podocyte-specific VEGFR-2 mutants and podocyte-specific VEGF overexpressors) that were induced at P0 developed proteinuria and glomerular disease at 3-4 weeks of age (figure 2.9 c, d). These data show that genetic deletion of VEGFR-2 from the podocyte does not rescue the glomerular defects caused by upregulation of the VEGF 164 ligand within the glomerulus.
Figure 2.9 Loss of VEGFR-2 from the podocyte does not rescue glomerular phenotype in podocyte-specific VEGF overexpressors. Light micrographs of glomeruli of mice with upregulated VEGF expression in podocytes show proteinaceous casts within the tubules (B) which is not observed in glomeruli of control mice (A). Glomerular disease is not prevented when VEGFR-2 is deleted from podocytes (C, D; double mutants). (H&E stains).
2.5 DISCUSSION

In this study, we demonstrate that increased VEGF production from the podocyte disrupts the integrity of the GFB during development and in the adult kidney. In addition, we were unable to detect the expression of VEGFR-2 using two knock-in reporter lines, lineage tagging and RT-PCR analysis from FACS sorted primary podocytes, indicating that autocrine signaling through the VEGFR-2 may not exist in the glomerulus. The failure of a developmental phenotype in the podocyte-selective VEGFR-2 mutants further provide in vivo data which suggests that VEGFR-2 signaling within the podocyte is disposable for glomerulogenesis. We propose two possible explanations: 1) A reciprocal paracrine signaling loop exists between the glomerular endothelium and podocytes and plays a more prominent role in the formation and maintenance of the GFB or 2) VEGF signals through another VEGF receptor in the podocyte.

Much work has focused on a paracrine signaling pathway, whereby vascular support cells produce VEGF that binds and activates transmembrane VEGFR-2 that is expressed by all endothelial cells. Loss of the VEGFR-2 in gene targeting studies in mice demonstrated that it is essential for formation of all blood vessels [101]. Phosphorylation of VEGFR-2 at Tyr 951, 1054, 1059, 1175 and 1214, results in the activation of various signal transduction molecules including PI3K; PLC-; the Src family of tyrosine kinases; Ras GTPase-activating protein (Ras GAP); Akt/protein kinase B (PKB); protein kinase C (PKC); and p38 mitogen-activated protein kinase (MAPK) which leads to increased migratory, proliferative, survival and differentiation capacity of endothelial cells [99, 117, 119]. By contrast, loss of VEGFR-1 receptor from knockout mice leads to increased endothelial proliferation and death due to abnormal structure of resulting blood vessels.
Although VEGFR-1 can be phosphorylated at multiple positions that include Tyr1169, Y1213, Y1242, Y1309, a kinase dead mutant survives and has normal blood vessels [95, 96, 104, 119]. Taken together, these data suggest that the primary role of VEGFR-1 is to function as a decoy receptor, sequestering VEGF, thereby regulating its availability. The affinity of VEGFR-1 for VEGF is 10 fold higher than the affinity of VEGFR-2, further implicating it as a negative regulator of endothelial cell activation [81]. In fact, increased circulating levels of soluble VEGFR-1 is associated with the glomerular endothelial injury observed in preeclampsia; this glomerular injury is similar to the injury observed in a subset of patients receiving VEGF inhibitory therapy and in mutant mice that have undergone selective deletion of VEGF from their podocytes [2, 188]. Together, these data suggest that VEGFR-1 and VEGFR-2 act in concert to establish and maintain the vasculature.

Despite the critical role for VEGF signaling in the endothelium, there has been a growing amount of interest in understanding whether an autocrine signaling pathway exists for this growth factor in the glomerulus. Cell culture work from two groups has provided evidence for a role of VEGF in podocyte survival. However, whether this occurs through VEGFR-2 or not remains to be an area of controversy. Saleem and colleagues identified the presence of Np-1, Np-2, VEGFR-1 and VEGFR-3, but not VEGFR-2 in immortalized human podocytes and found that VEGF exerts an anti-apoptotic effect upon serum starvation that is mediated by VEGFR-1 [4]. Ziyadeh et al. found similar expression patterns and detected VEGFR-1 mRNA and protein by real-time PCR, immunoblotting and immunohistochemistry in cultured mouse podocytes [3]. In addition, they demonstrated that VEGF induces phosphorylation of VEGFR-1 in a dose
and time-dependent manner. Using the same mouse podocyte cell line, Guan et al. reported the presence of Np-1, Np-2, VEGFR-1 and VEGFR-2 mRNA in undifferentiated and differentiated states; however, upon differentiation, VEGFR-2 levels increase [5]. They also extend Foster et al.’s previous findings that VEGF is a survival factor for podocytes. However, they suggest that VEGF’s anti-apoptotic activity is mediated by VEGFR-2, not VEGFR-1. The discrepancy in these findings may be due to inherent differences in the cell lines used: podocyte-selective genes such as nephrin and podocin are expressed by the Saleem podocytes but not in the mouse cell lines used. These differences likely arise from clonal effects.

In this study, we report that increased VEGF production from the podocyte results in increased protein leakage from the glomerulus. VEGF upregulation at a developmental stage results in focal foot process effacement whereas continuous upregulation in the adult disrupts the podocyte as well as the endothelial cell compartment. Since podocyte cell defects were observed in both inductions, we postulated whether an aberrant VEGF autocrine signaling through VEGFR-2 is responsible for the phenotype. We performed genetic studies to delete the VEGFR-2 gene from podocytes and analysed mice that carried 2 floxed VEGFR-2 alleles or 1 global null allele and 1 floxed allele (enhances any phenotype). In both models, glomerular development was unaffected and mice did not exhibit any signs of glomerular defect and proteinuria was not observed.

Our results demonstrate that VEGF upregulation has physiologic consequences at every stage of glomerular development or life, emphasizing the importance of balanced VEGF levels in the glomerulus. Furthermore, autocrine loop VEGF signaling through VEGFR-2 is not required for glomerular development and VEGFR-2, if expressed by
podocytes, is expressed at very low levels. Because the glomerular phenotype resulting from VEGF overexpression is not prevented by deletion of VEGFR-2 from the podocyte, we speculate that VEGF may exert its effects on the podocytes through other receptors. It is also likely that main target of VEGF in the glomerulus is the endothelial cell compartment. Future work will determine the precise role of additional VEGF receptors and interplay among different cell types within the glomerulus.
Chapter 3

Loss of VEGFR-1 from podocytes disrupts glomerular function in mice

A version of this chapter is in preparation for publication.

Sison, K, Li, C, Eremina, V, Rossant, J, Gerber, HP, Quaggin, S. Loss of VEGFR-1 from podocytes leads to glomerular disease in mice.
3.1 Abstract

The glomerulus is a special vascular bed that is the main filtration unit in the kidney. One of the key signaling pathways important in the physiologic function of the glomerulus is VEGF acting through its receptors, VEGFR-1 and VEGFR-2. Of the two receptors, VEGFR-2 is believed to transduce many of VEGF’s activity, while the signaling capacities of VEGFR-1 are still not clear. Although VEGF is primarily an endothelial-specific growth factor, the identification of both VEGF receptors in cultured podocytes has suggested that additional signaling pathways for VEGF exists within the glomerulus. To address this question in vivo, we measured the mRNA levels of various VEGF receptors in primary podocytes. We also studied the functional relevance of VEGFR-1 and VEGFR-2 by genetically deleting each receptor specifically within the podocyte using the Cre-loxP system. We report for the first time that podocytes express the soluble form of VEGFR-1, sVEGFR-1, in vivo and mice that lack the VEGFR-1 gene from the podocyte developed proteinuria and glomerular disease at 6 weeks of age with profound foot process effacement. As the disease progressed, changes in the glomerular endothelium were observed. The lack of any glomerular phenotype in VEGFR-1 (TK) /- suggest that VEGFR-1 signaling does not play a role in podocyte biology. Previous data show that upregulation of VEGF in podocytes results in some similarities to VEGFR-1 podocyte-selective knockout mice, suggesting that VEGFR-1 serves a ‘decoy’ function, titrating the amount of VEGF available to the surrounding renal tissue. Surprisingly, we also observed increased cell adhesiveness of podocytes treated with sVEGFR-1 in vitro, suggesting that sVEGFR-1 plays multiple roles in the glomerulus. We propose that
sVEGFR-1 is involved in maintaining the integrity of the GFB by acting as a VEGF ligand trap as well as a mediator for podocyte cell attachment.

3.2 INTRODUCTION

The glomerulus is a specialized vascular structure that functions in the filtration of blood, allowing the passage of water and small molecules while preventing that of albumin and other larger molecules. Filtration occurs across the glomerular filtration barrier (GFB) that consists of three layers: podocytes on the urinary side, an inner layer of fenestrated endothelial cells and an intervening glomerular basement membrane (GBM). The effective filtration of the glomerulus is largely dependent on the combined properties of the three layers and damage to any one layer can lead to the leakage of protein into the urine and renal disease.

In mouse development, the definitive, metanephric kidney begins to form at embryonic day 10.5 when the ureteric bud extends into the surrounding metanephric mesenchyme [137, 138]. Inductive signals from the mesenchyme results in the repeated branching and growth of the ureteric bud. In turn, a subset of mesenchymal cells condense and convert to a polarized epithelial vesicle which subsequently develops a cleft, generating a comma-shaped figure, followed by the formation of a second cleft, leading to the s-shaped body. At this stage, podocyte precursors begin to express vascular endothelial growth factor (VEGF) which attracts VEGFR-2-positive endothelial cells to migrate into the cleft. Together with mesangial progenitors, these cells give rise to the glomerular capillaries, which characterizes the capillary loop stage of glomerular development.
Interactions between endothelial cells and the differentiating podocytes lead to the formation of the GBM.

VEGF production by the podocyte is critical for the development of the glomerulus. Gene targeting studies in mice demonstrated that a tight regulation of VEGF gene dosage is critical during glomerulogenesis. Mice that lacked both VEGF alleles in the podocyte died at birth due to renal failure [1]. These mice did not develop a functional GFB as a result of defects in endothelial cell migration, proliferation and survival. Loss of only one VEGF allele leads to endotheliosis, a characteristic lesion seen in patients with pre-eclampsia. In contrast, upregulation of the 164 isoform of VEGF within the podocyte during development led to a collapsing–glomerulopathy that resembles the lesion seen in HIV-associated nephropathy.

VEGF is a potent inducer of angiogenesis and regulates many aspects of endothelial cell biology. It belongs to the VEGF/PDGF (platelet-derived growth factor) gene superfamily that includes PIGF, VEGF-B, VEGF-C, VEGF-D and VEGF-E [225]. Alternative splicing of the VEGF gene results in at least 9 different isoforms, including VEGF 121, 165, 189 and the anti-angiogenic VEGFxxxb isoforms [14, 225]. In mice, VEGF isoforms lack a glycine residue of the mature protein and are therefore one amino acid shorter than their corresponding human isoforms. VEGF binds to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) [80, 99, 117]. A majority of VEGF signaling is mediated through VEGFR-2, whereas the signaling capacities of VEGFR-1 remain to be fully determined. VEGFR-1, however, has been suggested to be a decoy receptor, binding to and sequestering VEGF, preventing its association with VEGFR-2. The soluble form of VEGFR-1 (sVEGFR-1) also performs this function.
Similar to other fenestrated vascular beds in the body, podocytes continue to express high levels of VEGF in the healthy, mature glomerulus. The lack of persistent angiogenesis; however, suggest that mechanisms that regulate VEGF action and/or availability must exist. The expression of endogenous inhibitors of VEGF may offer a potential explanation. Angiopoietin-1 is another endothelial-specific growth factor that has been detected in podocytes [148]. As opposed to VEGF, it reduces endothelial permeability in vitro and in vivo [147]. Furthermore, inhibitory isoforms of VEGF such as VEGF165b have been identified in normal kidney and isolated human glomeruli [13]. VEGF165b prevents VEGF165-mediated endothelial cell migration and proliferation in culture [251]. The presence of sVEGFR-1 may also explain why the high concentrations of VEGF in adult glomeruli do not cause angiogenesis [151].

sVEGFR-1 and VEGFR-1 expression has been detected in cultured mouse and human podocytes; however, its role still remains to be established [3-5]. In endothelial cells, the kinase activity of VEGFR-1 is one-magnitude lower than VEGFR-2 but its affinity to VEGF is 10-fold higher [80, 81]. VEGFR-1 is thus primarily believed to function in a negative manner, similar to the decoy function attributed to sVEGFR-1. Work from Ziyadeh et al., however, suggests that VEGFR-1 may transduce signals in podocytes [3]. They demonstrated that VEGF induces VEGFR-1 phosphorylation in a dose and time-dependent manner and influences collagen IV synthesis. Foster and colleagues also found VEGFR-1 in immortalized human podocytes and implicates VEGFR-1 in mediating VEGF’s anti-apoptotic effect [4]. In contrast, Guan et al. suggest that VEGF prevents podocyte apoptosis through VEGFR-2 not VEGFR-1 [5].

Clearly,
there are discrepancies between which receptor, VEGFR-1 or VEGFR-2, is expressed by podocytes.

In this study, we examined the expression levels of various VEGF receptors in podocytes in vivo. We also sought to determine whether podocyte-expressed VEGF receptors play a role in the GFB by using the Cre-loxP system to genetically delete each VEGF receptor specifically in the podocyte. Our results show that sVEGFR-1 is the primary VEGF receptor that is expressed by the podocyte in vivo. Although loss of the VEGFR-1 gene does not affect glomerular development, it leads to increased glomerular permeability and glomerular disease by 6 weeks of age and is accompanied by podocyte foot process effacement which may be due to disruptions in podocyte-GBM interactions. With disease progression, defects in the glomerular endothelium were observed. The onset of the glomerular phenotype is accelerated when VEGF is upregulated, suggesting that VEGFR-1 serves a decoy function, regulating the amount of VEGF available to surrounding cells. Together, these data suggest that VEGFR-1 expression by the podocyte maintains the integrity of the GFB by regulating VEGF bioactivity and mediating adhesive interactions between the podocyte and GBM extracellular matrix.
3.3 MATERIALS AND METHODS

3.3.1 Generation of Transgenic Mice
Floxed VEGFR-1 mice contains loxP sites that flanks the first exon (H.P Gerber, formerly Genentech, San Francisco, CA, USA). Podocyte-specific VEGFR-1 knockout mice were generated by breeding bitransgenic mice, which carry one floxed VEGFR-1 allele and the Nephrin-Cre transgene to homozygous floxed VEGFR-1 mice. Site-specific recombination between the loxP sites of the VEGFR-1 gene results in a VEGFR-1 null allele only in podocytes.

3.3.2 Genotyping
Isolation of genomic DNA from mouse tails have been previously described [248]. Presence of the floxed VEGFR-1 gene was detected by PCR using the oligonucleotide primers: FltL0X1335F, 5’CCTGCATGATTCTGATTGGA3’ and FltL0X3207R, 5’GCCTAAGCTCACCTGCGG 3’ [252].

3.3.3 Urinalysis
Urine was collected from 0, 2, 4, 6, 7 and 8 week-old mice. A urine dipstick (Chemstrip 5L; Roche Diagnostics Corp., Indianapolis, Indiana, USA) was used to detect the presence or absence of protein in the urine and the standard colorimetric assay was performed according to the manufacturer’s instructions. In addition, the urine protein and creatinine concentration were measured by using the Bradford microassay (BioRad, Toronto, ON, Canada) and the Jaffe method respectively [249]. Results are expressed as total protein/creatinine ratios (mg/mg).
3.3.4 Histologic and Ultrastructural Analysis

Freshly dissected kidneys were fixed in 10% formalin, embedded in paraffin and sectioned at 4 um. Sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Sections were examined and photographed with a DC200 Leica camera and Leica DMLB microscope (Leica Microsystems Inc., Deerfield, IL). For ultrastructural analysis using transmission electron microscopy, kidney tissue was fixed in 1.5% glutaraldehyde, embedded in Spurr (Canemco Inc., Saint-Laurent, Quebec, Canada), and sectioned.

3.3.5 Immunohistochemistry and in situ hybridization

Kidneys were dissected from mice of various ages and were fixed in 4% RNase-free paraformaldehyde overnight. Tissues were transferred into 30% sucrose for 12-24 hours, embedded in Tissue-TEK OCT and snap frozen. 10µm tissue sections were cut and stored at -20°C until needed. DIG-labeled probes used for in situ analysis include nephrin and WT1. Immunostaining with antibodies to CD-31, PDGFR-β and KI67 were performed.

3.3.6 FACS sorting of primary podocytes

Glomeruli were isolated from 1, 3 and 6 week-old Nephrin–CFP transgenic mice using the sieving method. Nephrin-CFP mice express the CFP reporter gene that is under the control of the podocyte-specific nephrin promoter. Following enzymatic digestion with collagenase, trypsin and EDTA in PBS, dissociated cells were washed in PBS 3X. Glomerular cells were sorted using a BD FACSaria flow cytometer (BD Biosciences). CFP-expressing (CFP+) primary podocytes were separated from other glomerular cells based on CFP fluorescence.
3.3.7 RT-PCR

Total RNA was extracted from FACS sorted primary podocytes with Trizol reagent (Invitrogen) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Fermentas) and random hexamer primers (Fermentas) according to the manufacturers’ protocol. cDNA samples and standards were amplified in qPCR MasterMix Plus for SYBR Green I (BioRad) with primers specific for VEGF (sense, 5'-TCACCAAGCCCAGCACATAG-3', antisense, 5'-AATGCTTTCTCCGCTCTGAA-3'), Nephrin (sense, 5'-ACCCTCCAGTTAACTTGTCTTTGG-3', antisense, 5'-ATGCAGCGGAGCCTTTGA-3'), sVEGFR-1 (sense, 5'-GG AAG ACA TCC TTC GGA AGA-3', antisense–5' TCC GAG AGA AAA TGG CCT TTT-3'), VEGFR-1 (sense, 5'-TTC GGA AGA CAG AAG TTC TCG TT-3', antisense, 5'-GAC CTC GTA GTC ACT GAG GTT TTG-3') and VEGFR-2 (sense, 5'-GCCGTTGGTGACAGTATCTT-3'; antisense, 5'-GTCACTGACAGGCGATGA-3') or hypoxanthine phosphoribosyltransferase (HPRT) (sense, 5'-GGCTATAAGTTCTTGCTGACCTG-3'; antisense, 5'-AACCTTTATGTCCCCCGTTGA-3'). Sample mRNA quantity was analysed in triplicate. Relative quantification of target gene expression was evaluated using the comparative C_T method [250]. The ΔC_T value was determined by subtracting the HPRT C_T value of each sample from its respective target C_T. Fold-changes in gene expression of the target gene were equivalent to 2^-ΔACT. The values obtained were then entered into a Student's t test.
3.3.8 Western Blot Analysis

Sieved glomeruli were isolated from control and mutant and were lysed in RIPA lysis buffer. Protein concentration was determined by the Biorad Assay according to the manufacturer’s instructions. Samples were resolved on an 8% SDS-PAGE gel and were transferred to a PVDF membrane. Membranes were blocked in 5% non-fat dried milk for 1hr. Membranes were incubated with the appropriate primary antibodies diluted in blocking solution at 4ºC overnight, followed by a series of three washes in 0.05% Tween 20 in PBS rinse buffer. Membranes were incubated with HRP-conjugated secondary antibodies for 1hr at room temperature, washed and developed with enhanced chemiluminescence (ECL, Amersham). The following primary antibodies were used for immunoblotting: rabbit anti-VEGFR-1, rabbit anti-VEGFR-2, rabbit anti-PY1054/1059-VEGFR-2 (all used at 1:1000; provided by Dr. Wang Min, Yale University) and mouse anti-β-actin (1:5000, Santa Cruz). Secondary antibodies were goat-anti-rabbit IgG-HRP and goat-anti-mouse-IgG-HRP (1:10000; Santa Cruz).

3.3.9 Cell adhesion assay

Immunological 96-multiwell plates (Nunc, cat. CA62409-024 ) were coated with 50 µl of 10 µg/ml of Recombinant Human VEGFR-1/Flt-1/Fc Chimera (R&D, cat. No. 321-FL), Recombinant Human VEGFR-2/KDR/Flk-1/Fc Chimera (R&D, cat. No. 357-KD ), Recombinant Human PDGFR-β/Fc Chimera (R&D, cat. No. 385-PR ), Human Fibronectin (Sigma, cat. F0895), or Human IgG (Calbiochem, cat. No. 400120), solubilized in PBS with 0.1% BSA (Invitrogen) for 2 hrs at 33ºC. Coating solution was removed and the well surface was blocked with 100 µl of 3% BSA in PBS at 33ºC for 18 hours. 3 x 10³ human podocytes in 100 ul of serum-free RPMI 1640 medium supplied
with ITS were plated into each well of 96-well plates and were incubated at 33°C for 1 hr. Following incubation, the wells were washed with PBS three times. Attached cells were fixed with 3% formaldehyde for 10 min at room temperature, then washed with PBS three times. Fixed cells were stained with 50 µl of 0.5% crystal violet (Sigma, cat. No. HT90132), diluted in 10% ethanol for 20 minutes at room temperature. Plates were rinsed with tap water and 100 µl of 1% SDS were added to each well for dye extraction. Spectrophotometric measurement of the absorbance at 540 nm was performed using Microplate reader 3550-UV (Bio-Rad). Three independent experiments were performed in triplicate.
3.4 RESULTS

3.4.1 Loss of VEGFR-1 from the podocyte leads to glomerular abnormalities

We have previously shown that VEGFR-1 is the main VEGF receptor expressed by primary FACS-sorted podocytes (figure 2.1). To determine whether podocyte-derived VEGFR-1 plays a functional role in the glomerulus, we generated mice that lacked VEGFR-1 specifically in podocytes using the Cre-loxP gene targeting system (figure 3.1 a). To delete VEGFR-1 from the podocyte, we bred mice that carried two floxed VEGFR-1 alleles with heterozygous VEGFR-1 floxed, Nephrin-Cre transgenic mice. Mice homozygous for both the VEGFR-1 floxed allele and carrying one copy of the Nephrin-Cre transgene (Nephrin-Cre+/−; VEGFR-1 fl/fl) were born at the expected Mendelian frequency. To confirm the deletion of VEGFR-1, VEGFR-1 mRNA and protein levels were examined in isolated glomeruli of control and mutant littermates. RT-PCR and western blotting revealed the deficiency of VEGFR-1 in glomeruli of mice that carry one floxed, one deleted VEGFR-1 allele, and the Nephrin-Cre transgene (figure 3.1 b, c). VEGFR-1 fl/fl, Nephrin-Cre mice also showed decreased levels of VEGFR-1.

No abnormalities were observed in control mice which include VEGFR-1 fl/fl mice and Nephrin-Cre transgenic mice. Podocyte-specific VEGFR-1 knockout mice (VEGFR-1 fl/fl, Nephrin-Cre) developed marked proteinuria by dipstick analysis at 6 weeks of age (table 3.1). As an additional assessment for protein excretion, total urine protein-creatinine ratio was determined and was increased in the VEGFR-1 mutant mice at 4, 6, 7 and 8 weeks of age compared with age-matched littermate controls (figure 3.2). Histologic analysis of kidneys at the 6-week timepoint revealed protein deposition in the tubules while the majority of glomeruli appeared normal (figure 3.3 a). Electron
microscopy (EM) studies showed extensive podocyte foot process effacement and subtle changes in the endothelium while the GBM appeared intact (figure 3.3 a). However, at 7 weeks of age, abnormalities in the endothelial cell compartment were apparent. In addition, peri-glomerular and interstitial infiltrates that stained for activated monocytes/macrophages were observed (figure 3.3 b). To determine the progression of disease, kidneys were analyzed at earlier time points including P0, 2 weeks and 4 weeks. Glomeruli of podocyte-specific VEGFR-1 knockouts were indistinguishable from control mice by light microscopy (figure 3.4). Ultrastructural examination by EM showed no overt abnormalities in the GFB at P0 and 2 weeks of age but displayed slight alterations in the foot processes at 4 week of age (figure 3.4).
Figure 3.1. Generation of a podocyte-specific VEGFR-1 knockout mice. A) Breeding scheme to delete VEGFR-1 in podocytes. Breeding scheme to delete VEGF receptors in the podocyte. Podocyte-specific Nephrin Cre mice were bred to floxed VEGFR-1 mice. Cre expression leads to the excision of the floxed gene specifically in podocytes. B) mRNA expression of full length VEGFR-1 and sVEGFR-1 is reduced in glomeruli of podocyte-specific VEGFR-1 mutants (n=5 for CON, n=12 for VEGFR-1 fl/fl, Neph Cre). c) Western blot (WB) analysis show reduction of VEGFR-1 protein levels in lysates of isolated glomeruli from mutant and control mice.
Table 3.1. Numbers of affected vs. unaffected mice. Affected mice develop glomerular disease and proteinuria.
Figure 3.2. Urine protein quantification by protein-creatinine ratio (U-P/C Ratio) over time in VEGFR-1 fl/fl, Neph Cre and control mice. Values represent mean and standard error of mean (SEM) (n = 5 for CON, n = 5-10 for VEGFR-1 fl/fl, Neph Cre). One-Way ANOVA was performed for statistical analysis followed by Bonferroni’s test to compare groups of means (***: $P<0.001$, significantly different from control mice).
A

Control

VEGFR-1 fl/fl, Neph Cre 6 weeks

VEGFR-1 fl/fl, Neph Cre 7 weeks

B

Control

VEGFR-1 fl/fl, Neph Cre

VEGFR-1 fl/fl, Neph Cre
Figure 3.3. Loss of VEGFR-1 from podocytes leads to glomerular disease. A) Light and electron micrographs of glomeruli from podocyte-specific VEGFR-1 knockouts and controls. At 6 weeks of age, protein deposition is present in the renal tubules (H&E stain) and is accompanied by podocyte foot process effacement (red arrow, TEM). Glomerular lesions worsen by 7 weeks of age. B) F480 (macrophage marker) immunostaining is markedly increased in VEGFR-1 knockouts compared to controls.
Figure 3.4. Course of disease in podocyte-specific VEGFR-1 knockout mice. Glomeruli of podocyte-specific VEGFR-1 mutant mice appear normal at P0, 2 and 4 weeks of age by light microscopy (H&E stain). Transmission electron micrographs show normal components of the GFB at P0 and 2 weeks of age. Slight foot process effacement is observed at 4 weeks of age (red arrow).
3.4.2 Molecular analysis of glomeruli of podocyte-specific VEGFR-1 mutant mice

VEGFR-1 loss from the podocyte leads to clinical GFB dysfunction at 6 weeks of age and is first accompanied by morphologic changes in the foot processes and slight alterations in the endothelium. The question; however, remains whether a deficiency in sVEGFR-1 or full length VEGFR-1 directly affects the podocyte or whether the endothelium is the main target of damage leading to secondary effects on the podocyte. To determine whether an increased autocrine or paracrine loop is responsible for the reported phenotype, we analyzed each glomerular cell compartment using immunohistochemical methods and in situ hybridization. Since VEGFR-1 has been shown to mediate podocyte cell survival in cultured human podocytes, we speculated that the primary defect may occur within the podocyte cell [4]. In situ hybridization revealed a reduction in nephrin mRNA expression in glomeruli of 12 week-old VEGFR-1 mutant mice (figure 3.5), indicating either a loss of podocytes or podocyte dedifferentiation. Kidney sections of 6, 8 and 12-week control and VEGFR-1 mutant mice were stained for TUNEL and were observed to be negative, indicating that podocytes were not undergoing apoptosis (data not shown).

Light micrographs of 12-week old VEGFR-1 mutants revealed a striking histology with cells being present within the Bowman’s space (figure 3.6 a). We also detected proliferating cells within the VEGFR-1 mutant glomeruli compared to controls (figure 3.6 b). To determine the cell type observed, we stained kidney sections with antibodies to CD-31/PECAM (endothelial cell surface marker) and PDGFR-β (mesangial cell marker) and interestingly, these cells stained positive for CD-31, indicating endothelial cell activation and/or proliferation (figure 3.5). These results led us to
speculate that podocytes highly express VEGFR-1 and/or sVEGFR-1 as an endogenous modulator for VEGF and the removal VEGFR-1 results in increased amounts VEGF that is available for endothelial cell effects. To test this hypothesis, we examined the developmental expression profile of sVEGFR-1 mRNA in FACS-sorted primary podocytes from 1, 3 and 6-week old Nephrin-CFP transgenic mice. Interestingly, we observed increasing sVEGFR-1 mRNA levels with time and correlates with VEGF mRNA expression (figure 3.7).
Figure 3.5. Molecular analysis of podocyte and endothelial cells of podocyte-specific VEGFR-1 knockouts. In situ hybridization for WT-1 reveal no significant difference between control (A) and VEGFR-1 fl/fl Neph Cre (B) mice at 6 weeks of age. In situ hybridization for nephrin at 12 weeks of age appear shows reduced expression in VEGFR-1 fl/fl Neph Cre mice (black arrowhead) (D) compared to control (C). Immunofluorescence staining shows CD-31 positive endothelial cells within the Bowman’s space in the VEGFR-1 fl/fl Neph Cre mice (white arrowhead) (F) which are absent in controls (E) (CD-31=red, PDGFR-B=green, blue=DAPI).
**Figure 3.6.** Glomeruli from 12 week-old podocyte-specific VEGFR-1 knockouts contain cells within the Bowman’s space (H&E) (A) and exhibits cell proliferation by Ki67 staining (B).
**Figure 3.7.** mRNA expression levels VEGF (A) and sVEGFR-1 (B) in FACS-sorted primary podocytes by RT-PCR. Values are expressed as fold increase over CFP- cell fraction and represent mean (CFP+=podocytes, CFP-=endothelial cells and mesangial cells).
3.4.3 VEGFR-1 serves to sequester VEGF in the mature glomerulus

3.4.3.1 Increased VEGF expression accelerates glomerular disease in podocyte-specific VEGFR-1 knockout mice

We have previously generated a mouse model with inducible and podocyte-specific overexpression of VEGF164. Induction with doxycycline (DOX) at birth and in the adult resulted in proteinuria and glomerular lesions that resemble the podocyte-specific VEGFR-1 mutants. In order to further investigate whether podocyte-derived VEGFR-1 is primarily acting as a decoy receptor to sequester the VEGF ligand, we sought to determine whether a preliminary increase in VEGF would enhance the glomerular phenotype. Thus, we generated compound transgenic mice by breeding the inducible podocyte-specific VEGF overexpressor mouse model with the podocyte-specific VEGFR-1 knockout mice. These mice carry the Nephrin-Cre transgene, RosartTA, tet(O)-VEGF164 and two floxed VEGFR-1 alleles. Interestingly, mice that lack VEGFR-1 from the podocyte but upregualted VEGF expression developed proteinuria by dipstick analysis and urine protein:creatinine measurements at an earlier time of 5 weeks of age (Pod-VEGF++, VEGFR-1 DM n=6) (figure 3.8 b). Histologic examination show protein deposition in the tubules and EM studies show podocyte foot process effacement (figure 3.8 a). These data further suggest that VEGFR-1 serves a decoy function and the deletion of podocyte VEGFR-1 may increase the amount of VEGF bioavailability in the glomerulus and the additional increase in VEGF accelerates the onset of the proteinuric phenotype.
Figure 3.8. Double mutant mice (podocyte-specific VEGF overexpressors and VEGFR-1 knockouts) develop glomerular disease. Protein deposition is observed in the tubules at 5 weeks of age (H&E stain) and is accompanied by podocyte foot process effacement (red arrow, TEM) (A). Urine protein:creatinine (U-P/C) ratios increase at an earlier timepoint in double mutants (n=2-3 for all groups) (B).
3.4.3.2 VEGFR-1 TK-/- display normal renal function

Although, VEGFR-1 has been shown to contain a number of phosphorylation sites, its signaling capacity remains elusive. In fact, VEGFR-1 TK-/- mice which lack the cytoplasmic tyrosine kinase region due to the replacement of exon 17 with a neo cassette that carries its own polyA termination signal develop normally and do not show overt phenotypic abnormalities, suggesting that signaling through VEGFR-1 is not required for development [104]. To determine whether VEGFR-1 signaling plays a functional role in the podocyte, we examined the renal function of the VEGFR-1 TK-/- at various stages. Mice were tested weekly for proteinuria beginning at 3 weeks of age. None of the mice tested positive by dipstick analysis and was confirmed by total protein:creatinine measurements (n=10) (figure 3.9). Light microscopy demonstrated normal glomeruli at 10 weeks of age (figure 3.10 b). From these data, we conclude that VEGFR-1 signaling in the podocyte does not appear to play a major role in the maintenance of GFB function. These observations also provide additional evidence for our hypothesis that VEGFR-1 is primarily a regulator of VEGF availability and the presence of the extracellular domain of VEGFR-1 is necessary and sufficient to absorb the amount of VEGF in the glomerulus, regulating its activity.

To further determine whether it is the lack of sVEGFR-1 and its VEGF trapping activity is the cause of glomerular disease in our podocyte-specific VEGFR-1 knockouts rather than VEGFR-1-mediated signaling defects, we bred the VEGFR-1 TK (-/-) mice with mice that carry one floxed VEGFR-1 allele and the Nephrin-Cre transgene. Since VEGFR-1 TK-/- mice only express sVEGFR-1 but do not express the full length VEGFR-1 tyrosine kinase receptors, we expect the restoration of sVEGFR-1 levels in the
VEGFR-1 fl/TK-, Nephrin-Cre mice and predict the rescue of the glomerular phenotype. These mice (n=8) were monitored weekly and still developed proteinuria (figure 3.9) at 6 weeks of age, displaying similar glomerular defects on light (figure 3.10 c) and electron microscopy (figure 3.10 d) as the VEGFR-1 fl/fl, Nephrin Cre mice. To understand the lack of rescue, we measured sVEGFR-1 and VEGFR-1 mRNA in isolated glomeruli and found similar levels between the VEGFR-1 fl/TK-, Neph-Cre and VEGFR-1 fl/fl, Neph-Cre mice, indicating that sVEGFR-1 levels were not restored to normal levels (figure 3.10 e).
Figure 3.9. Quantification of urine protein:creatinine (U-P/C) ratio. VEGFR-1 TK-/- display normal U-P/C and is comparable with control mice. Pod-VEGFR-1 fl/TK mice develop proteinuria at 6 weeks of age (n=4 for CON, n=6 for VEGFR-1 TK-/-, n=4 for Pod-VEGFR-1 fl/TK).
Figure 3.10 Light micrographs of mutant and control glomeruli. Glomeruli from 10 week-old VEGFR-1 (TK) -/- mice (B) appear similar to controls (A). Glomeruli of VEGFR-1 fl/TK, Neph-Cre mice show similar lesions as the podocyte-specific VEGFR-1 knockouts by light (H&E stain) (C) and transmission electron microscopy (red arrow) (D). (E) sVEGFR-1 mRNA levels are not restored to normal in VEGFR-1 fl/TK Neph-Cre mice (n=3) as assessed by RT-PCR.
3.4.4 sVEGFR-1 mediates podocyte cell adhesion in vitro

Podocyte foot process effacement may occur due to the decrease in podocyte adhesion to the GBM. As sVEGFR-1 has been shown to act as a ligand for integrin-mediated cell-matrix interactions in cultured endothelial cells, we hypothesized that sVEGFR-1 plays a similar role in podocytes. To examine the effects of sVEGFR-1 on podocyte adherence, we compared the adhesive/attachment properties of human podocyte cells to recombinant sVEGFR-1 (sFlt-1/Fc) and recombinant sVEGFR-2 (sFlk-1/Fc). Gross observations reveal an increased cell adhesion to sVEGFR-1-treated cells after 1 hr of incubation (data not shown). Cell adhesion assays revealed a significant and dose-dependent increase to sVEGFR-1 compared to recombinant sVEGFR-2, although not to the same extent to fibronectin, a known initiator or cell adhesion (figure 3.11 a, b). These data show that sVEGFR-1 enhances the attachment of podocytes to plastic and influences cytoskeletal dynamics which may explain the foot process effacement phenotype observed in the podocyte-specific VEGFR-1 knockout. Further studies are required to investigate the pathways involved.
Figure 3.11. Podocyte cell adhesion. Cultured human podocytes were plated on sVEGFR-1 (sFlt-1 Fc), sVEGFR-2 (sFlk-1/Fc), PDGFR-β/Fc, fibronectin and Human IgG and bovine serum albumin (BSA) as negative controls. Podocyte adherence to sVEGFR-1 (sFlt/Fc) is increased (A) and is dose-dependent (B).
3.4.5 Genetic deletion of VEGFR-2 does not rescue the glomerular phenotype in podocyte-specific VEGFR-1 mutants

We have previously observed that mice deficient in VEGFR-2 in the podocyte do not develop any overt glomerular phenotype under normal conditions. To further confirm that VEGFR-2 does not play a role in the podocyte, we sought to determine whether the glomerulopathy seen in the podocyte-specific VEGFR-1 mutant mice could be prevented by deleting VEGFR-2 in podocytes. Double mutants contain five transgenes (2 floxed VEGFR-1 alleles, 1 floxed VEGFR-2 allele, VEGFR-2-GFP and Neph-Cre transgene, n=7; 2 floxed VEGFR-1 alleles, 2 floxed VEGFR-2 allele and Neph-Cre transgene, n=8). These mice tested positive for proteinuria by dipstick analysis at 6 weeks of age and showed similar glomerular lesions by light microscopy as the VEGFR-1 single mutants (figure 3.12). EM studies demonstrated effacement of foot processes in podocyte-specific VEGFR-1 mutants and were not improved by VEGFR-2 deletion from the podocyte. These data further suggest that an autocrine VEGF signaling loop through VEGFR-2 is not required and podocytes do not depend on VEGFR-2 mediated signaling in vivo in normal and diseased setting.
Figure 3.12. Light and electron micrographs of glomeruli from 7 week-old podocyte-specific VEGFR-1 knockouts (Pod-VEGFR-1) and podocyte-specific VEGFR-1 and VEGFR-2 double mutants (Pod-VEGFR-1, VEGFR-2 DM). Deletion of VEGFR-1 from the podocyte leads to glomerular disease and is not rescued by genetic loss of VEGFR-2 from the podocyte. (H&E stain, TEM).
3.5 DISCUSSION

Our data show, for the first time, that genetic deletion of the VEGFR-1 gene from the podocyte results in loss of the permeselective properties of the GFB and glomerular disease. Since sVEGFR-1 is the predominant transcript found in podocytes and podocyte-specific VEGFR-1 knockouts display similar features as another mouse model with upregulated VEGF expression within the podocyte, we suggest that sVEGFR-1 (instead of the full length tyrosine kinase VEGFR-1 receptor) plays an important role in the maintenance of integrity of the glomerulus. We posit two possible functions for sVEGFR-1: 1) sVEGFR-1 regulates the amount of VEGF ligand available for paracrine and/or autocrine signaling and 2) sVEGFR-1 mediates podocyte-GBM matrix interactions.

Our finding is in keeping with the work of Foster et al. who identified the expression of VEGFR-1 in cultured human podocytes [4]. A number of studies have also reported that full-length VEGFR-1 and sVEGFR-1 is the most abundant VEGF receptor in glomeruli, offering a potential mechanism for VEGF autoregulation [151, 253]. The observation that VEGF and sVEGFR-1 mRNA expression are temporally related further supports this theory. During glomerular development, there is an initial increase in VEGF expression by presumptive podocytes and VEGFR-2 expression by endothelial cells. Although vascular development ceases in the mature glomerulus, podocytes continue to produce high levels of VEGF. VEGF mRNA is also constitutively expressed in other organs including the choroid plexus epithelium and retinal pigment epithelium (RPE) in mice, rats, and humans [116]. Interestingly, VEGFR-1 but not VEGFR-2, shows constitutive expression in the microvessels of the brain and retina [254]. More recently,
Ambati and colleagues demonstrated that sVEGFR-1 is responsible for maintaining the avascularity of the cornea despite the presence of high levels of VEGF [252]. A similar setting may exist in the adult glomerulus whereby VEGF is counterbalanced by podocyte-derived sVEGFR-1.

We have previously generated mice with inducible overexpression of the 164 isoform of VEGF specifically in podocytes. When induced in the adult, proteinuria and the glomerular dysfunction only developed in half of the mice examined, with the remaining mice displaying normal kidney function. In this current study, we report that sVEGFR-1 expression by podocytes increased with age and this finding may explain the inconsistent occurrence of glomerular disease observed in the VEGF overexpressors. The lack of a phenotype may be due sVEGFR-1 counterbalancing the increased VEGF levels, preventing the initiation of VEGF-driven effects. Interestingly, when the VEGFR-1 gene is deleted in the adult VEGF overexpressors, the glomerular disease is accelerated and develops within 5 weeks of age, further suggesting the decoy function of podocyte-derived sVEGFR-1.

Furthermore, we detected the presence of CD-31-positive endothelial cells in the Bowman’s space in 12-week old podocyte-VEGFR-1 mutant mice. The podocyte-VEGFR-1 mutants resemble some features of the VEGF overexpressors, implying that the phenotype may be attributed to the loss of sVEGFR-1’s VEGF trapping function. Consequently, the amount of VEGF available to the endothelium and to surrounding renal cells including the podocyte is increased. Enhanced VEGF may lead to endothelial cell proliferation which may explain the accumulation of endothelial cells in the Bowman’s capsule which is normally not seen. To support this hypothesis, we analyzed
VEGFR-2 phosphorylation in isolated glomeruli from the podocyte-VEGFR-1 knockouts at 4, 6 and 7 weeks of age. We, however, found inconsistent results, observing both an increase and decrease in phosphorylation (data not shown). The degradation and desensitization of VEGFR-2 in response to a continued increase in VEGF levels may explain, in part, why we did not detect increased VEGFR-2 activation [255].

The lack of increased VEGFR-2 phosphorylation may also indicate that VEGF trapping may not be a major contributor to the glomerular phenotype observed, suggesting other functions for sVEGFR-1. In addition to VEGF sequestration, sVEGFR-1 mediates cell-matrix contacts in vitro. In cultured endothelial cells, sVEGFR-1 has been shown to be deposited in the extracellular matrix and associates with α5β1 integrin, demonstrating that sVEGFR-1 also plays a role in cell adhesion through cell-matrix interactions [107]. The predominant in vivo finding of podocyte foot process effacement and actin cytoskeletal rearrangement in the podocyte-specific VEGFR-1 knockouts suggested that this might also be true in vivo in the glomerulus. One of the most surprising findings in this study was the increased podocyte cell attachment in the presence of a recombinant sVEGFR-1. This increase was comparable to the effects of fibronectin, a known integrin ligand. β1 integrin expression by podocytes has been shown to be important in the maintenance of the GFB. Loss of β1 from podocytes leads to endstage renal failure at 3-5 weeks of age and is characterized by podocyte loss and foot process effacement [256]. Deletion of the α3 subunit specifically in the podocyte also results in massive proteinuria and renal disease by 6 weeks of age with ultrastructural defects in the podocyte foot processes and GBM [257]. These results suggest that sVEGFR-1 may also function as a ligand for integrins and the loss of this ligand may lead
to disturbances in the cell-matrix interactions as well as changes in the podocyte cytoskeletal backbone, leading to foot process effacement. Further studies are required to determine whether integrins mediate the sVEGFR-1 effects on podocyte cell adhesion.

While it has been shown that VEGFR-1 elicits a weak phosphorylation in response to VEGF in endothelial cells, there is evidence VEGFR-1 can be phosphorylated in podocytes in culture [3]. We examined the kidney function of the VEGFR-1 (TK) -/- mice which lack the tyrosine kinase domain of VEGFR-1 in all cells. No gross differences in glomerular function were observed even upto 10 weeks of age, suggesting that VEGFR-1 signaling in the podocyte is not a major contributor to glomerular function.

In conclusion, the results presented in this study demonstrate the expression of sVEGFR-1 by podocytes in vivo and loss of this soluble receptor disrupts glomerular function in mice, demonstrating that podocyte-derived sVEGFR-1 plays a role in the maintenance of the integrity of the GFB. The temporal correlation of sVEGFR-1 and VEGF levels implicates that sVEGFR-1 acts as a ligand trap, thereby regulating its activity on endothelial as well as other renal cells. Since alterations in both podocyte and endothelial cells were observed when sVEGFR-1 is deleted, further studies are required to fully understand which glomerular cell compartment is the main target of increased VEGF activity. Strikingly, our results also show that sVEGFR-1 increases the adhesive properties of podocytes in vitro, providing another novel and intriguing mechanism through which sVEGFR-1 affects glomerular biology. In depth studies are required to further delineate the underlying pathway through which sVEGFR-1 mediates cell-matrix interactions.
Chapter 4

Summary and General Discussion
The thesis had two major aims: 1) To further address the role of VEGF in the glomerulus and 2) To determine whether VEGF receptors are expressed by podocytes in vivo and investigate whether a functional autocrine VEGF signaling loop is involved in glomerular development and/or function. These goals were achieved by utilizing a number of genetic tools that allowed for the overexpression of the VEGF ligand and the deletion of each receptor selectively in podocytes. Our data show that 1) an upregulated VEGF expression by the podocyte during development and in the adult mouse leads to increased glomerular permeability, 2) sVEGFR-1 mRNA is highly expressed by podocyte in vivo whereas VEGFR-2 levels are undetectable, 3) Genetic loss of VEGFR-1 in the podocyte leads to proteinuria and glomerular disease while loss of VEGFR-2 does not affect glomerulogenesis and glomerular function. Together, the results presented in this thesis show that a critical balance in VEGF production by the podocyte must be maintained in the mature glomerulus. Furthermore, for the first time, we demonstrate that podocyte-derived sVEGFR-1 plays a role in the maintenance of the GFB.

In chapter 2, we investigated the effects of VEGF upregulation by podocytes in developing and adult mice by generating a mouse model with inducible overexpression of the VEGF164 isoform specifically in the podocyte. A developmental increase in VEGF resulted in alterations in glomerular permeability 3 weeks post-induction. Although most glomeruli appeared normal by light microscopy, ultrastructural analysis by TEM revealed focal foot process effacement. Increased VEGF levels in the mature glomerulus also resulted in proteinuria and ultrastructural defects depending on the length of induction. From these results, we conclude that increased VEGF production by the podocyte disrupts the permeselective properties of the GFB. These findings are in keeping with Liu
and colleagues who demonstrated that increased VEGF165 expression in rabbit glomeruli leads to proteinuria and progressive glomerular disease [258]. In 2008, Eremina et al. also utilized an inducible system to genetically delete VEGF in the adult mouse [2]. Similarly, loss of VEGF resulted in proteinuria, indicating that a sensitive threshold for VEGF exists in the glomerulus where too much and too little VEGF is detrimental. It should, however, be noted that the proteinuric phenotype developed in only 50% of VEGF transgenic mice that were induced in the adult stage. At first, we reasoned that the variability in penetrance may be explained in part by Cre-mediated deletion efficiency. Two different podocyte-specific Cre lines were used. Podocin-Cre was used in adult VEGF overexpressors while Nephrin-Cre was used in the VEGF transgenic mice induced at birth. Differences in excision efficiency and frequency may exist, leading to the incomplete deletion of the floxed ‘stop’ codon that precedes rtTA that consequently results in inconsistent and variable VEGF expression levels. The results we report in chapter 3 provided us with another explanation for the variable phenotype. Using RT-PCR, we found that sVEGFR-1, a naturally occurring VEGF inhibitor, is expressed by podocytes and its expression increases with time. Hence, although the VEGF levels are increased in the VEGF overexpressors, this concentration may not initiate any VEGF-driven effects due to the presence of sVEGFR-1.

With regards to human disease, VEGF upregulation has been implicated in the development of diabetic nephropathy (DN). In particular, increased VEGF levels have been proposed to be one of the major mediators of the early features of the disease such as glomerular hypertrophy and proteinuria [199, 202, 203, 259]. Other morphologic features that occur include GBM thickening and mesangial sclerosis. Interestingly, the
adult podocyte-specific VEGF overexpressor mouse model generated and characterized in chapter 2 show some features of DN including proteinuria, GBM thickening and expansion of the mesangial matrix, further implicating VEGF in disease pathogenesis.

Another aim of the project was to determine which receptors are expressed by podocytes in vivo and which receptor, if any, plays a functional role in glomerular biology. In vitro studies have shown that VEGF is a survival factor for podocytes but whether VEGFR-1 or VEGFR-2 are responsible for this effect is still a matter of strong debate. Chapter 2 describes the pattern of mRNA expression of the various VEGF receptors in FACS-sorted primary podocytes. Using RT-PCR, we found high levels of VEGFR-1 but did not observe detectable amounts of VEGFR-2. It is possible that VEGFR-2 mRNA falls below the detection limit of our assay and hence, we generated a mouse model in which we genetically deleted the receptor in the podocyte. Loss of VEGFR-2 did not result in proteinuria or glomerular disease even up to 12 weeks of age, suggesting that an autocrine signaling loop through VEGFR-2 does not play a role in the glomerulus. We further assessed the role of VEGFR-2 by determining whether a lack of VEGFR-2 from the podocyte could rescue the glomerulopathy seen in our podocyte-specific VEGF overexpressors. Double mutants still developed glomerular disease at the same time point as the VEGF overexpressors, providing further evidence that VEGFR-2 is dispensable in glomerular function.

The high expression levels of VEGFR-1 by podocytes prompted us towards further investigation of the role of this receptor in the glomerulus. In chapter 3, we show that loss of the VEGFR-1 gene from the podocyte leads to proteinuria at 6 weeks after birth and is accompanied by focal effacement of foot processes. At this time point, no
gross abnormalities were seen in other glomerular compartments. Kidney function and histology were examined at earlier stages and show normal formation of the GFB, indicating that lack of VEGFR-1 does not affect developmental processes. By 7 weeks of age, further defects were observed in the glomerular endothelium and as the disease progressed, glomerular lesions worsened with many glomeruli appearing sclerosed and surrounded by F480-positive macrophages. One of the interesting findings is the presence of CD-31-positive endothelial cells surrounding the Bowman's space in 12 week-old mutant mice. Since the podocyte-VEGFR-1 mutants and the VEGF overexpressors show some similarities, both of which exhibit proteinuria and focal foot process effacement at early stages, we speculated whether the decoy function of VEGFR-1/sVEGFR-1 is responsible for the phenotype. If so, the glomerular phenotype observed in the podocyte-selective VEGFR-1 knockouts can be attributed to the loss of this VEGF trapping mechanism, thus increasing VEGF availability to the endothelium and to surrounding renal cells including the podocyte. Increased VEGF may lead to endothelial cell activation which may explain the accumulation of endothelial cells in the Bowman’s capsule which is normally not seen. To further study this hypothesis, we analyzed VEGFR-2 phosphorylation in isolated glomeruli from the podocyte-VEGFR-1 knockouts at 4, 6 and 7 weeks of age. We, however, observed both an increase and decrease in phosphorylation. It has been reported that VEGFR-2 is rapidly ubiquitinated and down-regulated following sustained VEGF stimulation in cultured endothelial cells [255]. The desensitization of VEGFR-2 in response to a continued increase in VEGF levels may explain, in part, why we did not detect increased VEGFR-2 activation.
We then sought to determine the underlying mechanism that leads to the podocyte defects observed. VEGFR-1 has been proposed to mediate cell survival in cultured human podocytes [4]. We, however, did not detect podocyte apoptosis in the VEGFR-1 mutants as assessed by TUNEL stains at 6, 8 and 12 weeks of age. To further study whether VEGFR-1 signaling plays a role in the podocyte, we examined the kidneys of mice that lack the tyrosine kinase domain of VEGFR-1 (VEGFR-1 (TK) -/-). These mice exhibited normal kidney function and did not develop proteinuria even up to 10 weeks of age. Light microscopy also showed normal glomeruli, indicating that an autocrine signaling through VEGFR-1 may not play a major role in maintaining glomerular function.

What can then explain the effacement of foot processes in the podocyte-VEGFR-1 mutants? Among the causes of foot process effacement and proteinuria is the disruption of podocyte-GBM interactions and interference with the actin-based podocyte cytoskeleton [260]. Interestingly, sVEGFR-1 is deposited in the ECM and is a ligand for α5β1 integrins in endothelial cells in vitro, promoting cell adhesion [107]. This led us to speculate whether the sVEGFR-1 secreted by podocytes also functions in a similar manner, acting as a ligand for the podocyte α3β1 integrin. Using cultured human podocytes, we found that treatment with a recombinant sVEGFR-1 significantly increased cell adhesion to plastic at 1, 2 and 3 hrs although not to the same extent as fibronectin, a known integrin ligand. Treatment with recombinant sVEGFR-2 and recombinant PDGFR-β as well as human IgG did not result in cell adhesion, suggesting that this is not due to Fc or IgG. Thus, we provide a novel mechanism by which VEGFR-
1 regulates podocyte cell behaviour. Further studies are, however, required to ensure the specificity of the effect.

Results reported in chapter 3 show that sVEGFR-1 is highly expressed in the mature glomerulus and loss of this receptor affects glomerular function, with the first most apparent change occurring in the podocyte, with extensive foot process effacement. In human minimal change renal disease, patients develop proteinuria with diffuse loss of the podocyte foot processes despite normal appearance of glomeruli on light microscopy. It would be of great interest to test whether sVEGFR-1 or VEGFR-1 levels are altered in renal disease, including minimal change nephropathy.

In conclusion, the results presented in this thesis provide further evidence that a tight control of VEGF production by the podocyte is critical for the maintenance of the permeselective properties of the GFB even in the adult organism. The data also show, for the first time, that podocytes express high levels of sVEGFR-1 in vivo while VEGFR-2 is expressed at relatively low levels if any. Also, the genetic loss of VEGFR-2 from the podocyte does not affect glomerular development or function, suggesting that an autocrine signaling loop through VEGFR-2 does not play a significant role. In contrast, deletion of VEGFR-1 from the podocyte leads to proteinuria and glomerular disease. The mechanism through which VEGFR-1 influences glomerular biology appears complex. It is likely that similar to other vascular beds which constitutively express VEGF, sVEGFR-1 is co-expressed to sequester the ligand, thereby regulating its activity on surrounding endothelial cells. sVEGFR-1 has also been shown to play a role in mediating endothelial cell-matrix contacts. Strikingly, preliminary data using the podocyte cell culture system suggests that the same phenomenon may occur between the podocyte and the ECM.
Taken together, these data show that sVEGFR-1 is the primary VEGF receptor expressed by the podocytes in vivo and plays a role in the maintenance of the integrity of the GFB. These findings not only further establish the importance of VEGF regulation in glomerular function but provide novel insight regarding the function of podocyte-derived VEGF receptors.
Chapter 5

Future Directions
Major Aim: To further investigate the significance of sVEGFR-1 in the podocyte, as a decoy receptor and/or as a ligand for integrins.

5.1 Demonstration of sVEGFR-1 protein expression on podocytes.

To substantiate the finding that sVEGFR-1 is the predominant receptor found in podocytes in vivo, it would be of interest to visualize protein expression on podocytes. A major caveat is the lack of working and reliable reagents. Furthermore, a majority of VEGFR-1 antibodies cannot distinguish between the full length and soluble forms. With time, tools for detecting the presence and/or localization of sVEGFR-1 will be more available.

5.2 Generation of a podocyte-specific sVEGFR-1 knockout.

One of the major aspects that remains to be delineated is to determine whether the sVEGFR-1 is the major player in the podocyte instead of its full length form. The current VEGFR-1 mouse models include a standard VEGFR-1 knockout whereby VEGFR-1 is deleted in all cells of the body, a VEGFR-1 (TK) -/- mouse model which only expresses the sVEGFR-1 and a truncated transmembrane form of VEGFR-1 but not the full length tyrosine kinase receptor, and sVEGFR-1 (TM-TK) -/- whereby both the transmembrane and tyrosine kinase domains are eliminated [100, 104, 261, 262]. The generation of a conditional sVEGFR-1 mouse model will provide additional proof that sVEGFR-1 plays a key role in maintaining the integrity of glomerular function. As mentioned previously, sVEGFR-1 shares the first 13 exons with the full length VEGFR-1 and possesses a unique 31 amino acid carboxy terminal tail. This truncated transcript is made by utilizing
alternative polyA termination signals within intron 13 of the VEGFR-1 gene [83]. By flanking these polyA sequences with loxP sites and ensuring that the transcription and translation of the full length receptor is not disrupted, sVEGFR-1 may be deleted in any cell type. We are currently in the process of creating the DNA construct that will be used for the generation of this conditional sVEGFR-1 mouse. We predict that a podocyte-specific sVEGFR-1 knockout will mimic the podocyte-specific VEGFR-1 knockout generated in this thesis. Since sVEGFR-1 expression by the podocytes increases with age, we expect normal development and glomerulogenesis; however, proteinuria and glomerular dysfunction will be disrupted at approximately 6 weeks of age.

5.3 Generation of double mutant mice that lack VEGFR-1 from the podocyte and VEGFR-2 from the glomerular endothelium.

It would be interesting to further dissect which compartment is the main target of injury in the podocyte-specific VEGFR-1 knockouts. From our results, we suggest that one of the functions of podocyte-derived sVEGFR-1 is to acts as a VEGF sink, regulating its availability for paracrine signaling towards the VEGFR-2-expressing adjacent glomerular endothelial cells. In 12 week-old podocyte-specific VEGFR-1 knockouts, endothelial cells were found in cells surrounding the Bowman’s space, suggesting an increase in endothelial cell number. The generation of a glomerular endothelial-specific VEGFR-2 loss of function mutant may answer this question. Although there is no available glomerular endothelial-specific Cre line to date, a candidate gene has been suggested. In 2007, a large-scale screen comparing glomerular and kidney-specific genes have led to the discovery of genes that are highly expressed in the glomerulus.
Glomerular specificity was confirmed by analyzing mRNA expression by in situ hybridization and RT-PCR analysis [263]. Patrakka et al. further characterized the expression profiles of 5 glomerular-upregulated transcripts and proteins and found that Ehd3 is exclusively expressed by the glomerular endothelium and not in other endothelial cells in the kidney [263, 264]. We are currently in the process of generating an Ehd3-Cre mouse line whereby cre expression is driven by the endogenous Ehd3 promoter. We predict that the loss of VEGFR-2 from the glomerular endothelium should mimic mice deficient in podocyte VEGF production. If VEGFR-2 signaling is activated in the podocyte-specific VEGFR-1 knockouts, we expect the restoration of glomerular function in double mutant mice which lack VEGFR-1 from the podocytes and VEGFR-2 from the endothelium.
Figure 5.3.1. Breeding scheme to generate double mutant mice that lack VEGFR-1 from the podocytes and VEGFR-2 from the glomerular endothelial cells (ECs). Heterozygous VEGFR-1 fl/+, VEGFR-2 fl/+, Neph-Cre mice will be bred to heterozygous VEGFR-1 fl/+, VEGFR-2 fl/+, EHD-Cre mice to generate offspring positive for all transgenes (Neph-Cre, EHD-Cre, floxed VEGFR-1 and floxed VEGFR-2).
5.4 Generation of double mutant mice that lack both VEGFR-1 and VEGF production from the podocyte.

We have previously generated and validated mice with a genetic deletion of VEGF from podocytes. From the results of our studies, we suggest that the sequestration of the VEGF ligand by podocyte-derived sVEGFR-1 is important for the integrity of the GFB. To provide further evidence, we have established another breeding strategy to generate mice that lack VEGF and VEGFR-1 selectively in podocytes. These mice will carry 4 transgenes: one VEGF floxed allele, two VEGFR-1 floxed alleles and one podocyte-specific Cre transgene. It is necessary to ensure that only one floxed VEGF allele is present since VEGF homozygous mice die soon after birth. We predict normal glomerular function in the double mutants.
Figure 5.4.1. Breeding scheme to generate double mutant mice that lack VEGFR-1 and VEGF from podocytes. Heterozygous VEGFR-1 fl/+, VEGF fl/+, Neph-Cre mice will be bred homozygous VEGFR-1 fl/fl mice. VEGFR-1 fl/fl, VEGF fl/+ Neph-Cre mice will be identified for phenotypic characterization.
5.5 Further investigation of the effects of sVEGFR-1 on podocyte adhesion.

The results presented in this thesis also demonstrate that podocyte-derived sVEGFR-1 plays a complex, multi-functional role. In addition to its decoy function, we believe that it mediates adhesive interactions between the podocyte cell and the GBM matrix, acting as a ligand for integrins. Using a cell culture model, we observed a surprising increase in podocyte cell attachment in the presence of sVEGFR-1. To ensure the specificity of the effect, we are currently repeating the cell adhesion assays in the presence of sFlt-1 and α3β1 integrin blocking antibodies. It is also intriguing to speculate whether the mutation of amino acids responsible for integrin binding will diminish the adhesive effect of sVEGFR-1. Sore et al. identified a domain region of sVEGFR-1 that is involved in integrin binding which they referred to as peptide 12 [265]. Within peptide 12, they found 4 residues, Try 220, Leu 221, His 223 and Arg 223, that directly associated with α5β1 integrin. Mutation of these residues reduced the ability of peptide 12 to mediate cell adhesion. This mutated protein can be tested in cultured podocytes and we predict the attenuation of the adhesive properties when compared to treatment with normal sVEGFR-1.


Kendall, R.L., G. Wang, and K.A. Thomas, Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its


