University of Alberta

The Study of Kidney Microvascular Injury and Repair

by

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ABSTRACT

Thrombotic microangiopathies are group of diseases that refer to clinical and pathological syndromes where endothelial injury results in the manifestations of capillary thrombosis, microangiopathic hemolytic anemia, schistocytosis, and can lead to renal failure. In this thesis we aimed to study glomerular endothelial cell (GEC) injury and repair. We developed an animal model of GEC-specific injury that is based on the binding specificity of MOA lectin to the carbohydrate epitope expressed in the mouse renal microvasculature. The lectin was conjugated to saporin to enhance the killing activity. The lectin-saporin (L-S) caused a uniform and reproducible GEC injury that captured many features frequently observed in TMA cases. To study renal repair we isolated and characterized human endothelial progenitor cells (hEPC) that were injected into mice treated with a sublethal dose of L-S. The human cells persisted in the kidney microvasculature of injured mice for 4 days but not in the uninjured control group. We also identified a novel function of PI3K catalytic subunit p110β in angiogenesis where *in vitro* analysis showed that p110β knockdown caused a defect in tip cell differentiation and sprouting formation, and decreased cellular migration and proliferation. *in vivo* analysis demonstrated that endothelial-restricted p110β ablation rendered the mice more susceptible to kidney injury and impaired renal microvasculature repair. In addition, we demonstrated an important role for platelet-derived growth factor B (PDGFB) in initiating kidney GEC repair mechanism as

systemic inhibition of PDGFB by soluble PDGF receptor β led to high morbidity in the mice treated with a sublethal dose of L-S. In this thesis we developed an important animal model that can be used to further our understanding about the GEC injury and the potential application of EPC transplantation for renal restoration, and to gain more insights about the endogenous factors that govern the kidney microvascular repair mechanism.

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2-D:	Two dimension
3-D:	Three dimension
Ac-LDL:	Acetylated low density lipoprotein
ADAMTS:	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ADMA:	Asymmetric dimethyl arginine
Ad Fc:	Adenoviral vector expressing IgG Fc portion
Ad sPDGFR/PH:	Adenoviral vector expressing soluble PDGFR
AKI:	Acute kidney injury
Akt:	Protein kinase B
ALK:	Activin receptor-like kinase
AMP:	Adenosine monophosphate
AMR:	Antibody-mediated rejection
ANOVA:	Analysis of variance
BSA:	Bovine serum albumin
bFGF:	Basic fibroblast growth factor
bHLH:	Basic helix-loop-helix
BM:	Basement membrane
BMDC:	Bone marrow-derived cells
BMP:	Bone morphogenetic protein
BP:	Base pair
BUN:	Blood urea nitrogen
CAC:	Circulating angiogenic cells
CAD:	Coronary artery disease
CCM:	Cell conditioned medium
CAM:	Chorioallantoic membrane assay
CCR5:	C-C chemokine receptor type 5
Cdc-42:	Cell division control protein 42 homolog
CFU:	Colony forming unit
CRP:	C-reactive protein
CXCR4:	C-X-C chemokine receptor type 4
DAF-2 DA:	Diaminofluorescein diacetate
DDAH:	Dimethylarginine dimethylaminohydrolase
Dll4:	Delta-like 4
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DNA-PK:	DNA-dependent protein kinase
DSB:	DNA double-strand break
DTT:	Dithiothreitol
EBM2:	Endothelial basal medium
ECD:	Extracellular domain

EC:	Endothelial cells
ECM:	Extra cellular matrix
ECFC:	Endothelial colony forming cells
E. coli:	*Escherichia coli*
EdU:	5-ethynyl-2'-deoxyuridine
EGF:	Epidermal growth factor
ELISA:	Enzyme-linked immunosorbent assay
EndMT:	Endothelial to mesenchymal transition
eNOS(NOS3):	Endothelial nitric oxide synthase
eEPCs:	Embryonic endothelial progenitor cells
EPC:	Endothelial progenitor cells
EPO:	Erythropoietin
ERG:	Ets related gene transcription factor
ERK:	Extracellular signal-regulated kinase
ESAM:	Endothelial cell selective adhesion molecule
ESM1:	Endothelial-specific molecule 1
ESRD:	End-stage renal disease
FAK:	Focal adhesion kinase
FBS:	Fetal bovine serum
FGD5:	Faciogenital dysplasia 5 protein
FGF:	Fibroblast growth factor
FLT1:	FMS-like tyrosine kinase
FOXO:	Forkhead transcription factor
FPLC:	Fast performance liquid chromatography
FSP:	Fibroblast specific protein
G-CSF:	Granulocyte colony-stimulating factor
GEC:	Glomerular endothelial cells
GEFs:	Guanine nucleotide exchange factors
GFP:	Green fluorescent protein
GPCR:	G-protein coupled receptors
H & E:	Hematoxylin & Eosin
HA:	Hyaluronic acid
HAEC:	Human heart aorta endothelial cells
HBSS:	Hank's balanced salt solution
HDL:	High-density lipoprotein
Hes1:	Hairy and enhancer of split-1 transcription factor
Hey1:	Hairy/enhancer-of-split related with YRPW motif protein 1
HGF:	Hepatocyte growth factor
HIF1:	Hypoxia inducible factor 1
HSPG:	Heparan sulphate proteoglycans
HSV:	Habu snake venom
HUS:	Hemolytic uremic syndrome
HUVEC:	Human umbilical vein endothelial cells
ICAM:	Intercellular adhesion molecule
Ids:	DNA-binding protein inhibitors

IGF2:	Insulin-like growth factor 2
IGF2R:	Insulin-like growth factor 2 receptor
IGFBP3:	Insulin-like growth factor binding protein 3
IL-8:	Interleukin-8
ILK:	Integrin-linked kinase
IQGAP1:	Ras GTPase-activating-like protein IQGAP1
ISVs:	Intersegmental vessels
IV:	Intravenous
Jag1:	Jagged 1
JAM:	Junctional adhesion molecule
kDa:	Kilo Dalton
KDR:	Kinase insert domain receptor
KIM-1:	Kidney injury molecule 1
LC/MS/MS:	Liquid chromatography and tandem mass spectrometry
LFA3:	Lymphocyte function antigen 3
LPS:	Lipopolysaccharide
L-S:	MOA Lectin-saporin conjugate
MAPK:	Mitogen activated protein kinase
MAPKAP-2:	Mitogen activated protein kinase activated kinase 2
MCEC:	Mouse cardiovascular endothelial cells
MCP:	Membrane co-factor protein
MCP1:	Monocyte chemotactic protein 1
M-CSF:	Macrophage-colony stimulating factor
M-CSFR:	Macrophage-colony stimulating factor receptor
Met-SO:	Methionine sulfoxide
MHC-II	Major histocompatibility class II
MMD:	Moyamoya disease
MMP-9:	Matrix metalloproteinase-9
MNC:	Mononuclear cells
MOA:	*Marasmius oreades* agglutinin
MPGN:	Mesangioproliferative glomerulonephritis
MRM:	Multiple reaction monitoring
mRNA:	Messenger ribonucleic acid
mTOR:	Mammalian target of rapamycin
mTORC1:	mTOR complex 1
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH:	Nicotinamide adenine dinucleotide phosphate
N-Cadherin:	Neural Cadherin
NF-κB:	Nuclear factor κB
NK:	Natural killer
NRPs:	Neuropilin receptors
NO:	Nitric oxide
NOS:	Nitric oxide synthase
OCT:	Optimal cutting temperature

Ox-LDL:	Oxidatively modified low density lipoprotein
PAEC:	Porcine aorta endothelial cells
PAK:	Serine/threonine-protein kinase
PAR:	Protease activated receptor
PAS:	Periodic acid-Schiff
PBST:	Phosphate buffered saline with tween-20
PECAM:	Platelet endothelial cell adhesion molecule
PCNA:	Proliferating cell nuclear antigen
PCR:	Polymerase chain reaction
PDGF:	Platelet-derived growth factor
PDGFR:	Platelet-derived growth factor receptor
PDK:	phosphoinositide-dependent kinase
PDG2:	Prostaglandin D2
PDGE2:	Prostaglandin E2
PGF2α:	Prostaglandin F2α
PGH2:	Prostaglandin H2
PGI2:	Prostacyclin
PI:	Phosphoinositide
PI3K:	Phosphotidylinositide-3 kinase
PIP2:	Phosphotidylinositol (4,5) bisphosphate
PIP3:	Phosphotidylinositol (3,4,5) triphosphate
PLC-γ	Phospholipase C-γ
PlGF:	Placental growth factor
PKC:	Protein kinase C
PKCbII:	Protein kinase CbII
PtdIns:	Phosphotidylinositol
PTEN:	Phosphatase and tensin homologue deleted on chromosome 10
qPCR:	Quantitative polymerase chain reaction
Rac1:	Ras-related C3 botulinum toxin substrate 1
Rag:	Recombination activating gene
RANTES:	Regulated and normal T cell expressed and secreted
RAS:	Renal artery stenosis
ROBO4:	Roundabout homolog 4
ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinases
S1P:	Sphingosine 1-phosphate
SDF1:	Stromal cell-derived factor 1
SH2:	src homology
SHP:	SH2 protein-tyrosine phosphatases
siRNA:	Small interfering RNA
Smad:	Mothers against decapentaplegic homolog
Stx:	Shiga toxin
Sulfo-LC-SPDP:	Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido) hexanoate

Tcfs:	Transcription factors (E2A immunoglobulin enhancer-binding factors E12/E47)
TGF-β:	Transforming growth factor β
TGF-βR:	Transforming growth factor β receptor
Tie-2:	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TMA:	Thrombotic microangiopathy
TMB:	3,3',5,5'-Tetramethylbenzidine
TNFα:	Tumor necrosis factor α
tPA:	Tissue-type plasminogen activator
TSAd:	T-cell-specific adaptor
TTP:	Thrombotic thrombocytopenic purpura
TUNEL:	Terminal deoxynucleotidyl transferase nick-end labeling
TxA2:	Thromboxane A2
UEA:	*Ulex europaeus* agglutinin
ULvWF:	Ultra large vWF
uPa:	Urokinase type plasminogen activator
uPAR:	Urokinase receptor
VCAM:	Vascular cell adhesion molecule
VE-Cad:	Vascular endothelial cadherin
VEGF:	Vascular endothelial growth factor
VEGFR2:	Vascular endothelial growth factor receptor 2
Vps34:	Vesicular protein-sorting protein 34
vWF:	von Willebrand factor
X-gal:	5-bromo-4-chloro-indolyl-b-galactopyranoside

CHAPTER I

INTRODUCTION

1.0 General Introduction

Kidney disease affects millions of people worldwide with dialysis and kidney transplantation as the main renal replacement therapies. Chronic kidney disease is a condition of endothelial dysfunction, accelerated progression of atherosclerosis and elevated cardiovascular risk. In addition, alterations of the renal microcirculation can contribute to development of acute kidney injury (AKI), a condition that can lead ultimately to chronic kidney disease [1]. Mounting evidence indicates that injury to renal endothelial cells (EC) contributes to the pathogenesis of acute kidney injury. It appears that EC injury plays an important role in the extension and maintenance of AKI through pathways related to vascular tone. Moreover, a decrease in the ultrafiltration units of the kidney is implicated in the progression of AKI to chronic kidney disease [2].

The endothelium stands at the interphase between health and disease [3]. Because of its numerous physiological functions, it is reported that the endothelium is involved in almost all disease conditions as the primary source or as a secondary target [3]. Knowing that tissue survival requires an adequate supply of nutrients and oxygen, and a healthy endothelium [4], which place an enormous burden on developing different

strategies to overcome endothelium dysfunction and most importantly understanding how the endothelium functions in different vascular beds.

Endothelial cell heterogeneity represents a major obstacle in translating bench to bedside treatments targeting EC for a specific organ [3, 5]. Moreover, not all endothelial cells share common ultrastructural features such as Weibel-Palade bodies and fenestrae. Conversely, caveolae are not restricted only to the endothelium [6]. Although the main function of EC is to line the inside of blood vessels, not all EC perform the same functions. For instance, postcapillary venule EC are responsible for leukocyte migration into the surrounding tissues, while EC lining the arteries regulate vascular tone [3].

It is important to make the distinction between activated and dysfunctional endothelium. Generally, the endothelium is active when performing its normal functions in maintaining an anticoagulant, anti-inflammatory, and vasodilator roles, but when activated the endothelium becomes pro-coagulant, pro-inflammatory, and constricts blood vessels [3]. By contrast, the endothelial dysfunction represents a maladaptive state of EC characterized by cell death and/or loss of structural features that lead to disease induction or progression [7].

Since Asahara's [8] seminal report on the isolation of a population of cells termed endothelial progenitor cells (EPC) there has been an overwhelming interest in vascular repair concept in adults. There are

several strategies developed to restore vascular supply to damaged organs to repair and regenerate damaged cells. Some strategies are based on delivering angiogenic factors or conditioned cell media; however this approach has not yielded a significant result so far [9]. Another promising approach relies on transplantation of pluripotent stem cells. Despite the great promise stem cells hold, the results of bone-derived stem cells (BMDC) transplantation are somewhat controversial and without clear evidence of differentiation into the correct phenotype and engraftment at the injured site in the kidney [10-12]. Other stem cells such as hematopoietic stem cells transplantations even resulted in worsening of renal function in the mouse ischemia/reperfusion model [12, 13]. Many reports are focused on transplantation of endothelial progenitor cells to repair injured blood vessels to circumvent BMDC maldifferentiation problems [12]. For example, there is evidence of EPC amelioration of kidney injury after transplantation in Thy1.1 glomerulonephritis animal model [14].

The importance of a healthy renal microvasculature in combating progression of kidney injury is paramount. It becomes essential to understand and identify the factors that govern the endogenous repair mechanism at the site of injury and the proper strategy to introduce EPC to accelerate and to enhance renal microvasculature repair.

1.1 Thesis Objectives

The main hypothesis of this thesis is endothelial progenitor cells can contribute to and accelerate kidney microvascular repair. The main objective of my thesis is to study glomerular endothelial cells injury/repair. The specific aims of my research project are: (a) To develop an animal model of endothelial specific kidney injury; (b) To isolate and characterize EPC at the clonal level; (c) To study the process of angiogenesis *in vitro* and investigate the role of phosphoinositide-3 kinases (PI3K) during this process; (d) To study the role of PI3K catalytic isoform p110β in glomerular endothelial cell injury/repair *in vivo*; and (e) to investigate the role that platelet-derived growth factor B (PDGFB) plays in renal endothelium repair after a glomerular endothelial cell specific (GEC) injury.

1.2 Thesis Outline

In chapter 2, I review the current literature with respect to EPC biology, angiogenesis, and thrombotic microangiopathies. Chapter 3 contains a detailed description of materials and methods used in different experiments that comprise this thesis. Chapter 4 provides a report on the development of a glomerular endothelial cell specific injury animal model. Chapter 5 provides a description of endothelial progenitor cells isolation and characterization. Chapter 6, describes the study on the angiogenic potential of EPC, and investigates the roles of select genes involved in the angiogenesis process. Chapter 7 is based on the introduction of EPC *in*

vivo using the GEC specific injury animal model to study homing and incorporation of EPC in the kidney microvasculature at the site of injury. Chapter 8 explores the role of PI3K catalytic subunit p110β in sprouting angiogenesis as well as in vascular repair *in vivo* using p110β endothelial-restricted knock out mouse. Chapter 9 focuses on the role of platelet-derived growth factor B (PDGFB) in kidney vascular repair using our TMA model. Chapter 10 contains concluding remarks and provides general discussion of the important findings described in this thesis, in addition to highlighting future directions that can complement and advance further our knowledge in the field of regenerative medicine.

CHAPTER II
LITERATURE REVIEW

2.0 Overview

Thrombotic microangiopathies refer to a group of diseases that include hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), antibody-mediated transplant rejection and disseminated intravascular coagulation, which are characterized by loss of endothelium, intravascular thrombi, thrombocytopenia, schistocytosis, and variable degrees of organ ischemia and dysfunction [15]. Renal injury is a predominant feature of HUS, which in some cases can lead to irreversible kidney damage that requires dialysis or kidney transplant [16]. Regenerative medicine strategies have been developed to restore damaged endothelium in various vascular diseases that are based on the utilization of bone marrow-derived stem cells or *in vitro* expanded endothelial progenitor cells. However, those strategies are still in their early stages and require more research before cellular transplantation becomes a treatment for vascular diseases [12].

Endothelial progenitor cells (EPC) are a controversial cell population that came to prominence in 1997 after Asahara's [8] report on the isolation of putative progenitor cells that were capable of forming new blood vessels postnatally. Since the first publication, EPC became the most widely studied cell population, as over 9000 papers exist for this topic if endothelial progenitor cells are keyed in the Web of Science search

engine. The popularity of those cells stems from the important role EPC believed to play in postnatal neovascularization and the mechanisms that govern this process in human health and disease [17]. EPC are mainly isolated from the mononuclear cell fraction of peripheral blood, and from the bone marrow. However, despite their popularity, EPC remain shrouded with controversy for several reasons; (1) lack of uniform definition to describe these cells; (2) lack of specific phenotype marker(s) to distinguish these cells from other cell types; (3) multiple methods have been used for their isolation; and (4) different cell populations are termed EPC [18].

In this review, I provide description and discussion of the current literature that described the various applications for EPC. The primary focus of the review is on the emerging determinants and principles that are important in clarifying the conflicting reports surrounding the method of isolation and characterization, differentiation between EPC and circulating angiogenic cells, and current status of EPC in regenerative medicine. In addition, the topics of thrombotic microangiopathies, endothelial cells, angiogenesis, vascular endothelial growth factors (VEGF) and VEGF receptors, platelet-derived growth factors, and phosphoinositide-3 kinases are covered in this section as they relate to the scope of this thesis.

2.1 Thrombotic Microangiopathy

Thrombotic microangiopathies (TMA) are group of diseases such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura, antibody mediated organ rejection, and disseminated intravascular coagulation. TMA is characterized by the presence of thrombi in the microvasculature of various organs, thrombocytopenia, and schistocytosis [16]. Widespread thrombi formation in the capillaries and arterioles depletes platelets, leading to an increase in the levels of shear stress causing erythrocytes fragmentation. The kidney, heart, and brain are the major organs affected by the thrombosis [16]. TMA is also recognized as a serious condition that causes renal transplant complications that can develop within days or years after transplantation [19].

2.1.1 Hemolytic Uremic Syndrome (HUS)

HUS is a severe acute renal disease that predominantly affects young children under the age of four. It leads to renal damage and death if left untreated [20]. HUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure [21]. The classic form is known as diarrhea-positive (D+ HUS), which is caused by a verotoxin (i.e., Shiga toxin) produced, although not exclusively, by *Escherichia coli* serotype O157:H7 [22]. The verotoxin interferes with the protein synthesis process, leading to microvascular endothelial cell death [23]. The onset of D+ HUS is manifested by the presence of watery diarrhea approximately one week

before hemolysis and thrombocytopenia are detected. Several days later, the patients may experience decreased urine production, which is a condition known as oliguria and/or anuria [21]. D+ HUS is more prevalent in children, about 80 % of childhood cases are D+ HUS, only 5 % of the adult patients [24]. The Shiga toxin associated HUS affects the elderly more severely than children as older patients registered close to 90 % mortality rate, which is 10 to 20 fold higher than in children [25, 26].

The atypical form, known as diarrhea negative (D- HUS), is more severe and marked by proteinuria and hypertension. D- HUS can be triggered by different factors, including viruses, autoimmune diseases, HIV, malignancy, pregnancy, or systemic diseases [22, 27].

The familial and sporadic non-Shiga-toxin HUS are caused by genetic defects involving complement regulatory proteins factor H, factor I, and membrane co-factor protein (MCP, CD64). Factor H is a soluble glycoprotein that binds to glycosaminoglycans expressed on self cells and acts as a cofactor to factor I that mediates C3b cleavage, which is generated by the alternative complement pathway. MCP is a type I membrane-bound protein with cofactor activity to factor I [28, 29]. The deficiency of the complement regulatory proteins lead to uncontrollable C3b activation and the deposition of the C5b9 membrane attack complex on the surface of endothelial cells damaging otherwise healthy endothelium. Factor H has a half-life of about six days and in familial HUS

patients the activity of factor H is less than 1 % of normal [30, 31]. Lack of factor H can cause low C3 levels and frequent HUS bouts [32].

Strong evidence implicates endothelial cell damage in the pathogenesis of HUS, leading to the release of von Willebrand factor, platelet activating factor, and plasminogen activator inhibitor-1, resulting in platelet aggregation and thrombus formation within the injured glomeruli [21].

2.1.2 Thrombotic Thrombocytopenic Purpura

TTP is closely related to HUS but affects mainly older patients and it is manifested in the central nervous system more frequently and involves multiple organs [22]. The clinical hallmarks of TTP are the presence of schistocytes in peripheral blood, thrombocytopenia, increased LDH levels, and hemolytic anemia. Fever may be present in some patients [33, 34]. The pathophysiology of TTP involves a genetic basis. The most common is autoimmune development of antibody against von Willebrand Factor (vWF) cleaving enzyme ADAMTS 13 [16]. vWF is a multimeric glycoprotein produced by endothelial cells and stored within the Weibel-Palade bodies and megakaryocytes. It is involved in platelet aggregation at the site of vascular injury under high shear stress. Under normal conditions ultra large vWF (ULvWF) multimers are cleaved by the zinc metalloprotease ADAMTS 13 into monomers to prevent platelet adhesion [16]. Generally, familial TTP is caused by ADAMTS 13 deficiency, a condition that prevents the enzyme from breaking down the large vWF polymers causing platelet aggregation and thrombi formation in the

microvasculature [35]. The lack of ULvWF cleavage and platelet adhesion mediated by ADAMTS 13 deficiency is dependent on additional factors including the state of shear stress in a given microvasculature, platelet adhesion capacity, and the amount of vWF produced and released by the endothelium. When ADAMTS 13 expression levels drop by 15-20 %, it is sufficient to increase the expression levels of ULvWF [16].

Acquired deficiency of ADAMTS 13 is a result of the presence of anti ADAMTS 13 antibodies in 50-90 % of TTP cases as determined by plasma mixing studies [36]. Plasma exchange is applied to patients who are positive for ADAMTS 13 inhibitory antibodies to control thrombosis [16].

2.1.3 Antibody Mediated Allograft Rejection

Graft rejection is grouped into three categories hyperacute, acute, and chronic. Hyperacute rejection occurs within hours and even minutes after graft transplantation, and is due to pre-sensitization of recipient to donor antigen. Acute rejection can occur few days after transplantation or it may take years and it is mediated by the development of reactive T-cells and/or antibodies to the allograft. Chronic rejection may develop as early as few months or it is manifested years after the transplantation, and is caused by T-cells and/or antibodies developed against the graft [37].

Evidence of antibody-mediated organ rejection was reported as early as the 1970s. But skepticism prevailed until the 1990s when the breakthrough was established largely due to the reliable detection of the

complement cleaved product C4d in allograft microvasculature. In addition, the presence of improved methods to detect donor-specific antibodies [38, 39].

The endothelium appears to be the focal point of antibody-mediated rejection (AMR) as antibodies are generated against antigens expressed on endothelial cells such as AB blood group, and MHC class I and II antigens [40]. The mechanism of AMR involves the activation of the classical complement pathway, which requires antibody deposition and activation of complement C1. This initial event leads to activation of other complement molecules and ultimately to the assembly of C5b-C9 which constitute the membrane attack complex that can cause endothelial cells lysis and graft rejection [37]. Complement activation leads to the recruitment of inflammatory cells macrophages and neutrophils that express receptors for complement split-products C3a and C5a that act as chemoattractant and also activate the immune cells to release pro-inflammatory compounds that exacerbate tissue damage [37]. C3a and C5a can bind to and activate endothelial cells, which lead to an increase in expression of the adhesion molecules E-selectin, intercellular adhesion molecule 1 (ICAM1), and vascular cell-adhesion molecule 1 (VCAM1), in addition to the release of cytokines interleukin-1α (IL-1α) and IL-6, and the chemokines RANTES and IL-8 [41, 42]. *In vitro* studies have demonstrated that endothelial cell activation can occur in the absence of complement. Antibodies against MHC class I can activate NF-κB pathway

and the release of CCL2 and CXCL1, which may lead to arterial intimal proliferation a feature of complement-independent antibody-mediated rejection [37, 43].

2.1.4 Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a disease characterized by widespread intravascular thrombosis and fibrin deposition in the circulation [44]. DIC comprises a wide range of presentations and is believed to be caused by an underlying condition, with the majority of cases are associated with systemic inflammation or infection. In addition, DIC is observed in trauma and burns, malignancy, vascular disorders, toxic reactions, and in obstetric complications [45-49]. Because of constant activation of the coagulation system, excessive consumption of clotting factors and platelets may occur leading to bleeding at multiple sites [50]. There are many factors that contribute to the development of DIC such as dysfunctional endothelial cells, and/or defective procoagulant, anticoagulant, and fibrinolytic factors. In DIC patients there is an excessive generation of thrombin and an improper localization of thrombin production, which is a constant finding in DIC condition [51].

The activation of the coagulation cascade during an inflammatory response in patients with systemic inflammation can lead to microvascular thrombosis and multiple organ failure. Microvascular fibrin deposition in kidneys, lungs, liver and other organs is a major finding in experimental

animal models of bacteremia or endotoxemia. Inhibition of various factors involved in the coagulation cascade reduced fibrin deposition, and improved organ function in aforementioned animal models [52]. For example, the inhibition of tissue factor/factor VIIa pathway resulted in complete abrogation of thrombin generation [44]. It appears that in DIC patients all major anticoagulant pathways (i.e. antithrombin III, tissue factor pathway inhibitor, and protein C/protein S) are impaired [53].

Treatment of the underlying condition of DIC is essential in the management of patients with DIC. There are several strategies that are applied in the treatment of DIC including, administration of antithrombotic agents, plasma and platelet transfusion, restoration of anticoagulants to physiological levels, and use of fibrinolytic inhibitors [44].

2.2 Endothelial Cells

The endothelium lines the blood vessels and provides a selective barrier for the transport and exchange of materials between blood and tissues [54]. It occupies a large surface area that is estimated to be about 4000-7000 m^2 for materials exchange [55]. In addition, EC play essential functions in many physiological processes including regulation of the permeability of blood vessels, maintaining the local balance between pro-inflammatory and anti-inflammatory mediators, generation of new blood vessels, and provision of anti-coagulant and anti-thrombotic environment to prevent platelets and immune cells from adhesion to the endothelium

[56, 57]. For example EC regulate the above mentioned processes by the release of the vasodilators nitric oxide (NO) and prostacyclin, and by the presence of anticoagulant molecules such as thrombomodulin at the cell surface [57, 58]. On the other hand, activated EC become pro-coagulant and pro-inflammatory by expressing tissue factor, releasing von Willebrand factor and coagulation factor V, and by increasing the expression of adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), which facilitate the adherence of circulating immune cells to the endothelium [59, 60].

Endothelial dysfunction is a vague term often used to refer to the decreased synthesis and release of NO (endothelium-derived relaxing factor) leading to formation of atherosclerotic plaques [61], which is believed to be due to the fact that lack of NO contributes to impairment in vascular relaxation, platelet aggregation, increases smooth muscle proliferation, and increases leukocytes adhesion [62-65]. In addition, it is reported that cardiovascular risk factors including hypercholesterolemia, hyperglycemia, hypertension, and smoking are also documented to cause endothelial dysfunction [66]. Consequently, endothelial dysfunction is considered an initial step in the process of atherosclerotic plaque formation [67].

NO production is catalyzed by three isoforms of nitric oxide synthases (NOS): endothelial (eNOS or NOS3), neuronal (nNOS or

NOS1) are constitutively expressed, and inducible (iNOS or NOS2) which is responsible for NO generation during an immune response. Unlike iNOS, eNOS and nNOS are mainly regulated by Ca^{2+} fluxes and the subsequent binding of calmodulin [68]. The NOS isoforms catalyze nitric oxide reaction by converting L-arginine and O_2 to the intermediate molecule N^{w}-hydroxyl-L-arginine that is further oxidized into L-citrulline and NO [69]. The reactive nitrogen intermediates (RNI) include the immediate products NO· radical, NO^-, NO^+, and the secondary products, NO_2, NO_2^-, NO_3^-, N_2O_3, N_2O_4, S-nitrothiols (S-NO), peroxynitrite ($ONOO^-$) and nitrosyl-metal complexes [70]. In addition to modulating the activities of platelet and leukocytes, and vascular smooth muscle contraction, NO is also shown to control the release of endothelial-derived vasoconstrictors such as endothelin-1 (ET-1) and cyclooxygenase- (COX-) derived eicosanoids [71]. There are six primary COX-derived eicosanoids in the endothelium, prostaglandin I2 (PGI2, prostacyclin), prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α), prostaglandin H2 (PGH2), and thromboxane A_2 (TxA_2) [72]. The prostaglandins act in both autocrine and paracrine fashion with number of different biological functions such as, vasodilation, vasoconstriction, and platelet activation. It is accepted that PGI2, PGD2 and PGE2, are vasodilators, while PGH2, PGF2a, and TxA_2 are vasoconstrictors [73].

Interestingly, a decrease in NO production could be an indication of reduced eNOS expression. However, it has been demonstrated that

endothelial dysfunction can lead to increase eNOS expression. The exact mechanism of eNOS increased expression is not well understood but It has been reasoned that the increased levels of reactive oxygen species (ROS), particularly, hydrogen peroxide can increase eNOS expression at the transcriptional and translational levels [71, 74]. Despite the increased eNOS expression however endothelial dysfunction can lead to a reduction of NO generation, which can be attributed to the production of superoxide anions (O2-) by ROS-producing enzymes (e.g. NADPH oxidase, xanthine oxidase). The superoxide anions can interact with NO to generate peroxynitrite that has been shown to uncouple eNOS that can worsen the endothelial dysfunction [74].

Arginine residues in proteins can be methylated and subsequent proteolysis can yield methylarginine compounds such as asymmetric dimethylarginine (ADMA), a natural inhibitor of eNOS. It competes with arginine for binding eNOS. ADMA levels are regulated in the body by dimethylarginine dimethylaminohydrolase (DDAH) but in patients with renal failure ADMA levels are increased and associated with reduction in both NO production and DDAH levels [75]. The consequences of NO inhibition in the kidney are numerous including: reduced glomerular flow and glomerular filtration rate, reduced renin secretion, reduced sodium excretion, increased blood pressure, increased production of ROS and nitric peroxide, and worsened renal function [75].

Endothelial cells also have a role in antigen presentation. In humans, almost all microvascular and small vessel endothelial cells constitutively express major histocompatibility complex class II (MHC-II). Unlike bone marrow-derived antigen presenting cells such as dendritic cells, endothelial cells utilize lymphocyte function antigen 3 (LFA3)/CD2 pathways to activate CD45RO + B7-independent subpopulation T cells [76]. EC antigen presentation has been implicated in acute and chronic organ transplant rejection in humans [76].

Moreover, endothelial cells play a central role in blood vessel formation. The vasculature forms by two separate processes vasculogenesis and angiogenesis. Vasculogenesis refers to the differentiation of endothelial cells from the mesoderm and their assembly into blood vessels during embryonic development, and it results in the formation of the earliest stages of heart and large vessels as well as primary capillary network [77]. On the other hand, angiogenesis refers to the proliferation and migration of endothelial cells from pre-existing blood vessels. It can occur in both embryo and adult during the processes of wound healing, as well as tumor vasculogenesis in adults [78]. The signaling of proangiogenic factor vascular endothelial growth factor (VEGF) is essential in the formation and maintenance of blood vessels. Vascular growth is also dependent on platelet-derived growth factors (PDGF) and angiopoietins along with VEGF to stabilize the vasculature and to provide survival signals [79].

Vascular maintenance and repair can be performed not only by mature endothelial cells that migrate and proliferate to replace injured cells, but also by a cell population termed endothelial progenitor cells [8]. Mounting evidence indicates that bone marrow-derived circulating EPC contribute partly to the regeneration of injured endothelium [80].

2.3 Endothelial Progenitor Cells

2.3.1 Isolation and Characterization of EPC in Adults

Since the first report on the identification of EPC in 1997 by Asahara et al. [8], three methods have emerged describing the isolation of putative EPC from peripheral blood. These methods are known as colony forming unit as modified by Hill (CFU-Hill) [81], circulating angiogenic cells (CAC) [82], and endothelial colony forming cells(ECFC) [82]. The CFU-Hill method is a modified assay that was first described by Asahara et al [8]. Hill et al [81] preplated human mononuclear cells isolated from peripheral blood for 48 hours on fibronectin coated tissue culture dishes. The non-adherent cells were then replated and the colony-forming unit EPC were observed few days later. Hur et al. [82] successfully isolated two different types of EPC from one source and were termed early EPC (CAC) and late EPC (ECFC) based on the time they appeared in culture.

Given the different methods of isolating putative EPC populations, however the identity of these cells remains controversial. Emerging evidence suggests that the cells deriving from the CFU-Hill and the CAC

methods are not “true” EPC. Yoder et al. [83] have demonstrated that plating human mononuclear cells from peripheral blood or umbilical cord blood following the CFU-Hill method resulted in the formation of colonies that are made of small round cells at the center and spindle-shaped cells radiating from the center. Further analyses of CFU-Hill colonies have revealed that they are composed of hematopoietic cells and T lymphocytes [83]. The spindle-shaped cells were macrophages that express many endothelial cells surface markers, however, these cells failed to form blood vessels *in vivo*. Moreover, these cells were capable of ingesting bacteria indicating that these cells are of monocytic lineage [83]. Yoder et al. [83] went on to demonstrate that CFU-Hill EPC are hematopoietic-derived cells, as the clones generated from human peripheral blood from polycythemia vera patients (harboring a genetic mutation in hematopoietic stem cell, HSC) all expressed the HSC specific mutation in the *Janus kinase 2* gene .

Hur et al. [82] reported the isolation of two types of endothelial progenitor cells from human mononuclear cells and were called early EPC (CAC) and late EPC (ECFC). The two cell populations differed greatly. The early EPC had spindle shape and peak growth at 2 to 3 weeks, but died out after 4 weeks. However, the early EPC had lower vascular endothelial growth factor receptor 1 (VEGFR1), and eNOS. Early EPC did not express endothelial cell markers vascular endothelial-cadherin (VE-cadherin), and VEGFR2. In addition, the early EPC rarely formed tubes,

but produced higher concentrations of angiogenic cytokines such as vascular endothelial growth factor (VEGF), and interleukin 8 (IL-8). The late EPC had cobblestone shape and emerged after 2 to 3 weeks in culture and lasted up to 12 weeks. Moreover, the late EPC produced more nitric oxide, were incorporated into human umbilical vein endothelial cells monolayer, and formed tube-like structures on matrigel [82].

There is a reasonable hypothesis that the putative EPC that express endothelial markers but fail to form tube-like structures (CAC) may be monocyte-macrophage subsets that function as regulators of angiogenesis, vascular homeostasis, tissue remodeling, and tumorogenesis without developing into an integral part of the endothelium [84, 85]. It is also being suggested that some macrophages, like the placenta's trophoblast cells may temporarily adhere to the vasculature where endothelial cells are absent and assume endothelial functions as they express a large number of endothelial markers, without ever committing to endothelial lineage [86].

The CFU-Hill and CAC methods that were devised to identify EPC from mononuclear cells were demonstrated, as described above, to be hematopoietic cells. However, the other distinctly different EPC population that emerges later in culture, ECFC, is the cell population that displays properties of EPC, and can give rise to highly proliferative ECFC colonies. These cells can form spontaneously human blood vessels *in vivo* and be incorporated into the vasculature [87]. The ECFC cells represent a minute

fraction of the total mononuclear cells and occur at a frequency of 1 in 60 million [83]. Moreover, Yoder et al. [83] have demonstrated that the ECFC-derived cells are not of a hematopoietic origin and are not clonally related to the EPC derived by the CFU-Hill method.

It appears that in the absence of a unified definition of EPC and with no specific surface marker to positively separate EPC from mature endothelial cells, and hematopoietic cells, it is essential to perform a variety of tests to ensure the putative EPC isolated from peripheral blood are endothelial progenitor cells. Among the various techniques used to identify EPC include 1) expression of endothelial markers such as, VEGFR2, von Willebrand factor (vWF), CD144, eNOS, and CD34, 2) uptake of acetylated low density lipoprotein (Ac-LDL) and binding of *Ulex europaeus* agglutinin (UEA lectin), 3) *in vitro* tube formation, and 4) the integration in the host's vasculature *in vivo*.

2.3.2 Identification of Putative EPC

2.3.2.1 Expression of Endothelial Markers

Since Asahara et al. [8] first reported the isolation of putative endothelial cells from peripheral blood using CD34+ cells as the starting point, this surface antigen appears to become the principal marker to isolate EPC. CD34 is a sialomucin protein and functions as cell-cell adhesion factor expressed on different cell populations including, early hematopoietic, endothelial, fibroblast, and number of epithelial cell

lineages [88-91]. In addition, another stem cell marker, CD133, is used to isolate EPC from peripheral blood, and G-CSF mobilized peripheral blood in the presence of stem cell growth factor and VEGF [92]. Kinase insert domain receptor (KDR, VEGFR2, or CD309) is another surface antigen that is used in the progenitor cells isolation from whole blood. Those cells have been shown to differentiate into EPC and were shown to incorporate into capillaries of mice with ischemic hindlimb [8]. Other endothelial cell markers are frequently used to identify EPC, such as VE-cadherin, eNOS, CD31, and vWF [17].

Interestingly, Case et al. [93] isolated a cell population co-expressing CD34, CD133, and KDR from human peripheral, cord, and G-CSF mobilized peripheral blood in an attempt to identify cell progeny that participates in neoangiogenesis and vasculogenesis. However, this progenitor cell population did not give rise to EPC but instead they developed into hematopoietic progenitor cells that were also positive for expression of the leukocyte common antigen CD45. Case et al. [93] reasoned that circulating CD34+ CD133+ KDR+ and CD34+ CD45+ cells are distinct, early hematopoietic progenitor cells and not EPC. Further comparison between CD34+CD45+ and CD34+CD45- cells showed that only the CD34+CD45- cells were capable of differentiating into EPC but not the CD34+CD45+ cells [93].

2.3.2.2 Uptake of Acetylated LDL and *Ulex* Lectin Binding

The uptake of acetylated LDL by endothelial cells has become an indispensable assay in the identification of EPC. Earlier studies have demonstrated that rats injected with acetylated LDL and oxidatively modified LDL (Ox-LDL) were rapidly cleared from the blood circulation by the liver [94]. Further analyses showed that the endothelial cells were the primary cells responsible for the uptake of Ac-LDL, whereas the Ox-LDL were removed by the liver's Kupffer cells [31]. The uptake of modified LDL is mediated by scavenger receptors expressed on the surface of endothelial cells and in the case of macrophages the uptake of Ox-LDL leads to the formation of foam cells in atherosclerotic lesions [95, 96]. Fluorescently labeled Ac-LDL with the probe 1,1'-dioctadecyl-3, 3,3', 3'-tetramethylindocarbocaynine perchlorate is commonly used in the identification of EPC. However, this property is not unique to endothelial cells, but also shared by other cell types such as monocytes, and macrophages, which are capable of Ac-LDL uptake via the scavenger receptors as well [97]. Thus this assay by itself is insufficient in the identification process of EPC.

Binding of lectin by cell surface glycoproteins is another characteristic of endothelial cells that is employed for positive identification of EPC. Generally, *Ulex europaeus* agglutinin is used since it was found to bind primarily vascular endothelium in various human tissues [98]. However, lectin can also bind to other cell types including epithelial [99],

and hematopoietic cells [100]. Therefore, lectin binding to endothelial cells cannot specifically distinguish endothelial cells from other cell types alone, and it requires additional analyses for positive identification.

2.3.2.3 *in vitro* Sprouting

The development of an *in vitro* three-dimensional (3D) matrix angiogenic model has emerged as a defining assay for the identification of putative EPC population. A number of different methods using a variety of extracellular matrix components are available to test the tube formation of EPC *in vitro*, including matrigel, collagen, and fibrin. Matrigel is prepared from mouse Engelbreth-Holm-Swarm sarcoma and is supplemented with collagen type IV, nidogen, and laminin. EPC plated on Matrigel form cord-like structures. However, the cells only align and do not sprout or proliferate, and most importantly do not make intercellular lumens [101]. In addition, it has been reported that other cell types such as fibroblasts and U87-MG glioblastoma cells can form cord-like structures on matrigel as well [102]. The lumen formation process *in vitro* is studied in 3D extracellular matrix (ECM) environment using type I collagen. Because collagen is a major ECM component, scientists reasoned that endothelial cells in direct contact with collagen type IV simulate *in vivo* condition. Under these conditions, endothelial cells undergo the vacuolization process, which *in vitro* models of lumen formation in 3D ECM have shown that the process is mediated by the formation and coalescence of pinocytosis intracellular vacuoles [103]. This *in vitro* observation is

supported by an *in vivo* study of vascular development in zebrafish. Bayless and Davis [104] have demonstrated that vessel development in zebrafish expressing Cdc-42-GFP fusion protein is also regulated by intracellular vacuolization and coalescence. Alternatively, fibrin is used to study tube formation, branching and anastomosis of endothelial cells. The endothelial cells are coated onto Cytodex beads and embedded in fibrin gels. This method involves the plating of fibroblasts on top of the fibrin gels for sprouting and tube formation to occur [101].

The broad definition of EPC and the lack of a specific marker have resulted in controversies that affected greatly the field of vascular repair. The different types of cells being considered as EPC vary considerably from one study to another. The definition of EPC according to Ingram and Yoder [17] states that the EPC are circulating cells that must produce endothelial progeny that are capable to form endothelial tubes *in vitro* and contribute to endothelium repair and restore the function of the endothelial lining or participate in the *de novo* formation of blood vessels *in vivo*. Based on the extensive work of Ingram and Yoder characterizing the different EPC population, they reasoned that to date the only EPC that display endothelial activity at a clonal level are ECFC [17, 83]. However, they suggested that the other EPC populations participate in neoangiogenesis in some capacity to facilitate, initiate, and regulate the function of ECFC in blood vessels formation, maturation and remodeling [17, 83].

2.3.2.4 EPC Homing and Mobilization

EPC mobilization from their particular niches can be activated by ischemic or mechanical injuries through the activation of hypoxia-inducible factor-1 (HIF-1) and the release of VEGF, stromal derived factor (SDF1), erythropoietin, GM-CSF and G-CSF [105-107]. Cytokines and proteinases play an important role in mobilizing EPC and stem cells in general. Cytokines induce mobilization by dissociating stem and progenitor cells from bone marrow stromal cells permitting these cells to enter the blood stream through the sinusoidal endothelium [108]. One important cytokine that is able to mobilize stem cells is granulocyte colony stimulating factor (G-CSF), which induces mobilization of CD34 + cells, and at the same time G-CSF promote the release of elastase and cathepsin G from neutrophils leading to the detachment of stromal cells from the integrins expressed on hematopoietic stem cells [108]. In addition, elastase and cathepsin G separate SDF-1 released by stromal cells from its receptor CXCR4 expressed on progenitor cells. The SDF-1-CXCR4 dissociation leads to increased levels of circulating SDF-1, which reverses the SDF-1 gradient across the bone marrow barrier, allowing CXCR4+ progenitor cells to exit the bone marrow and enter the bloodstream [108]. Furthermore, matrix metalloproteinase-9 (MMP-9) appears to play a central role in the mobilization of EPC, because the mobilization of EPC by VEGF, SDF-1, and placental growth factor (PlGF) depends on the activity of MMP-9, which releases soluble c-kit ligand thus inducing survival,

proliferation, and mobilization of progenitor cells [109]. The mobilization mediated by MMP-9 is also dependent on eNOS, since eNOS knockout mice have lower activity of pro-MMP-9 and EPC mobilization is greatly reduced [110]. Homing of EPC to the injured endothelium is a fairly rapid process 1-2 days approximately and it is facilitated by the adhesion molecules P and E selectins and integrins, which mediate rolling and adhesion of mobilized EPC to the blood vessel wall [4, 111]. In addition, the homing process requires the activity of SDF-1/CXCR4 signaling, very late antigen 4 (VLA-4)/vascular cell adhesion molecule 1 (VCAM-1), and the interaction between CD44 and hyaluronic acid [112]. CXCR2 also is important in the homing of circulating EPC to the site of injury possibly by increasing the adhesion of EPC to extracellular matrix [113]. A recent study by Maeng et al. [114] reports that insulin-like growth factor 2 (IGF2) and IGF-2 receptor (IGF2R) play an important role in promoting EPC homing to the site of injury. IGF2-induced hypoxia stimulated the mobilization of EPC and their incorporation into newly forming vessels. The activity of IGF2 is dependent on G protein linked to IGF2R and intracellular Ca^{2+} that is stimulated by β2 isoform of phospholipase C [114].

2.4 Evidence of EPC Participation in Vascular Repair

2.4.1 EPC in Cardiovascular Disease

Hill et al. [81] originally demonstrated that the number of circulating EPC inversely correlate with cumulative cardiovascular risk. In addition,

Hill's group showed that using the number of EPC in peripheral blood is far more accurate indication of cardiovascular risk than the applied Framingham risk factor score [81].

The number of circulating EPC is affected by a variety of factors that are linked to cardiovascular diseases. In patients with hypercholesterolemia the capacity of EPC to proliferate and migrate was decreased as it was shown in age-matched patients with increased levels of low density lipoprotein [115]. On the other hand, higher high-density lipoprotein (HDL) correlates with higher numbers of EPC in patients with coronary artery disease (CAD) [116]. In addition, the number of circulating EPC is impacted by smoking and physical inactivity. Chronic smokers appears to have lower levels of EPC, a condition that can be rectified by smoking cessation [117], whereas, physical activity significantly enhanced the number of circulating EPC in healthy and CAD people after standardized physical activity [118]. The cardiovascular risk factor C-reactive protein (CRP) is also associated with decreased number of EPC and the progression of endothelial dysfunction as it serves to impair EPC antioxidant defenses and promote their apoptosis and telomerase inactivation [119]. Moreover, there is circumstantial evidence that the levels and bioactivity of CD34+/KDR+ EPC in patients with atherosclerosis are reduced, and it appears related to the progression and increased risk of cardiovascular events and death [120].

Recent evidence have indicated that EPC contribute to endothelial repair by repopulating areas of damaged endothelial cells as EPC covered implanted Dacron grafts in dogs [121], the ventricular assist device surface as well as the damaged endothelium of the artery in patients undergone angioplasty procedure [122]. However, EPC not only involved in repairing damaged endothelium but may also accelerate the progression of atherosclerotic plaques formation as well as their size and composition in apolipoprotein E knockout mice [123].

Given the controversy surrounding the true identity of EPC since it has been shown that EPC derived through the Hill's and CAC methods are hematopoietic cells not capable of forming secondary endothelial colonies *in vitro* [83], Massa et al. [124] determined the frequency of ECFC in peripheral blood from patients with acute myocardial infarction to be significantly greater (10 times) than ECFC from cord blood or from peripheral blood from healthy individuals.

2.4.2 EPC in Cerebrovascular Disease

Stroke is a cardiovascular disease and involves a complicated chain of events including disrupted blood flow, cerebral ischemia, inflammation, and neuronal necrosis of the blood-brain barrier [125]. However, it is not very clear which of these mechanisms are required for the mobilization of EPC from the bone marrow to the peripheral blood, but vascular trauma and tissue ischemia along with the release of cytokines

and VEGF appear to be involved in the EPC mobilization process [126]. The repair of brain tissue after a stroke is a difficult task, mainly because the lesions are very often large and affects different cell types. However, the identification of EPC may offer a new strategy involving cell therapy, as the transplantation of EPC, derived from donor bone marrow of transgenic mice constitutively expressing β-galactosidase in endothelial cells, in focal cerebral ischemia animal model showed that Tie2-lacZ-positive endothelial cells were incorporated into the site of neovascularization near the infarct region. This process was concomitant with a decrease of the effect of ischemic stroke [127].

In an observational study Ghani et al. [128] have reported a decrease in the number of early adhering cluster-forming EPC obtained from peripheral blood of patients with ischemic stroke as compared to that of healthy volunteers by using the Hill method of EPC isolation. They also reported that elderly and people with cardiovascular diseases generally have lower number of EPC and that the number of circulating EPC did not increase the weeks after recovering from acute or stable stroke [128]. Moreover, Chu et al. [129] found that the number of EPC colonies was reduced in patients with acute stroke compared to that of healthy volunteers, and it was even lower in people with large-artery atherosclerosis compared to patients with cardioembolism.

It is reported that higher number of EPC during the acute stage improves prognosis, angiogenesis, and regeneration of ischemic brain

tissue in human and animal model studies [126]. In a prospective study that included 48 stroke patients it was reported that an increase of EPC during the first week was associated with a neurological improvement and a reduction of infarct growth after 3 months [130].

Circulating endothelial progenitor cells are also used as a pathogenic marker of moyamoya disease (MMD), which is an unusual form of chronic cerebrovascular occlusive disease involving the formation of abnormal blood vessels at the base of the brain [131]. The authors isolated EPC from the peripheral blood of MMD patients using the colony-forming unit method (CFU-Hill). After seven days of culture CFU were counted and found that their number was significantly lower in MMD patients than that found in healthy individuals [132]. However, during long-term culture (2 months) the outgrowth cells from the CFUs were found in only 10% of the control group, but were found in 33% of the MMD patients [132].

2.4.3 EPC in Cancer Therapy

Emerging evidence suggests that changes in EPC numbers could be used to predict the effectiveness of an anticancer drug combinations [133]. Nolan et al. [134] showed that bone marrow-derived EPC play a major role in the neovascularization of newly formed tumors. EPC and their high proliferative capacity may have great potential in systemic cancer gene therapy as was demonstrated by Wei et al. [135] where they

showed that intravenously administered embryonic endothelial progenitor cells (eEPC) into mice homed preferentially to hypoxic lung metastases. The eEPC were genetically modified to express a suicide fusion gene that transforms harmless 5-fluorocytosine to the cytotoxic compound 5-fluorouracil (5-FU) that is used in clinical practice. 5-FU diffuses into the surrounding cells and exerts its strong bystander cytotoxic effects [135]. eEPC carrying the suicide fusion gene were injected into the tail vein and homed mainly into metastases in the lung where the tumor cells were effectively killed by the bystander cytotoxic effects of 5-FU five days post-injection [135]. Interestingly, eEPC were able to evade the immune system as they do not express the major histocompatibility class I and are not eliminated by NK cells [135]. Conversely, It was demonstrated that mature EC injected intravenously did not home preferentially to any organ and were not detected into subcutaneous implanted tumors [136].

Other approaches for cancer treatment using endothelial progenitor cells are being considered. For example, EPC are proposed to deliver antiangiogenic agents by gene transfer, as this approach will provide the advantage of producing high drug concentrations at the site of the tumor, consequently avoiding systemic toxicity [137]. Davidoff et al. [138] demonstrated that when bone marrow-derived cells expressing a truncated version of VEGFR2 and were transplanted into mice, some of the cells were incorporated into post-transplant tumor vasculature. The

truncated VEGR2 was expressed for an extended time and that correlated with reduced tumor growth and vascularity.

2.4.4 EPC in Kidney Disease

Acute kidney injury (AKI) is characterized by retention of urea and other nitrogenous wastes and in the imbalance of extracellular fluid volume as well as electrolyte and acid-base homeostasis. The dysfunction and apoptosis of tubular epithelial cells play a major role in the pathophysiological consequences of AKI [139]. Vascular injury results in modification of renal oxygenation and hemodynamics that may have adverse effects on renal function and may lead to chronic kidney disease [140, 141]. A number of reports suggesting that endothelial progenitor cells may enhance vascular regeneration in different ischemic organs, including the mobilization and homing of EPC after acute renal ischemia to the injured kidney areas which, resulted in improvement of kidney function by repairing the injured microvasculature [139]. Moreover, Li et al. [142] demonstrated that introduction of human CD34+ hematopoietic stem/progenitor cells (HSPC) into a mouse model of ischemia/reperfusion injury resulted in their recruitment to the kidney. The HSPC acquired EPC markers and produced proangiogenic factors that was associated with enhanced renal microvasculature repair. However, the HSPC-derived EC rarely engrafted into the mouse capillary walls demonstrating that human HSPC mediate kidney repair by paracrine mechanism and not by replacing damaged EC.

In a study by Abe-Yoshio et al. [143] reversible glomerulonephritis was induced by habu snake venom (HSV) and the role of EPC in restoring the kidney function in the injured mice was examined. The bone marrow cells from transgenic mice expressing β-galactosidase gene under the control of Tie-2 promoter were transplanted into lethally irradiated FVB/N mice. The snake venom was injected after the bone marrow transplantation. The kidneys from the experimental and control groups were collected at 7, 28, and 56 days post-injection. In the control group a small number of β-galactosidase-expressing cells were identified after X-gal staining. However, in HSV treated mice, there was a significant increase of β-galactosidase-expressing cells in the damaged glomeruli with maximum number achieved at day 28. However, the number of X-gal-positive cells was reduced at day 56 post injection coinciding with glomeruli recovery [143].

Chade et al. [144] demonstrated that the administration of a mixed populations (late and early outgrowth cells) of autologous EPC preserved the architecture and function of kidneys in pigs with chronic renal artery stenosis (RAS) and decreased the microvascular remodeling. The hemodynamics and function of kidneys, in pigs with RAS, were determined by a multidetector computed tomography *in vivo*. After 4 weeks of intrarenal infusion of autologous EPC, which were the late outgrowth cells, there was an increased expression of angiogenic factors in the kidneys. Moreover, it appears that the EPC stimulated the

proliferation and maturation of new blood vessels, decreased renal fibrosis and microvascular remodeling, and EPC restored the renal filtration function [144].

Schlieper et al. [145] studied the number of circulating endothelial progenitor cells in hemodialysis patients. EPC from peripheral blood expressing CD34+KDR+ were isolated and grown for 7 days and the colony-forming units were evaluated in 65 hemodialysis patients. The number of circulating EPC was significantly decreased as compared to 27 healthy controls or to patients with coronary artery disease but with normal renal function. On the other hand, coronary calcifications did not correlate with the numbers of circulating EPC, CD34+KDR+, or isolated acetylated-LDL+ and lectin+ endothelial progenitor cells. Moreover, multivariate analysis showed that the numbers of acetylated-LDL+ and lectin+ EPCs were negative predictors in hematocrit and reticulocytes, whereas the low levels of fetuin-A in the serum was a positive predictor of low circulating endothelial progenitor cells in hemodialysis patients [145].

Endothelial progenitor cells may play an important role in the increased risk of cardiovascular disease in end-stage renal disease (ESRD), which is associated with accelerated atherosclerosis and impaired angiogenesis [146]. Metsyuanim et al. [146] investigated the levels of circulating EPC in children undergoing hemodialysis and after transplantation. The EPC were isolated from peripheral blood and characterized for their expression of endothelial-specific genes including,

VE-cadherin, CD146, CD31, Tie-2, KDR, CD133, in addition to growth factor with known effects on endothelial function such as, VEGF, erythropoietin (EPO), and SDF-1. The mRNA levels of VE-cadherin, CD146, KDR, VEGF, and EPO were decreased in ESRD children. VE-cadherin expression was most significantly reduced in all the patients and was inversely correlated with serum urea and creatinine concentrations. In addition, VE-cadherin expression levels among the ESRD children only was inversely correlated with diastolic blood pressure, interventricular septum thickness, and left ventricular mass index, suggesting that children with ESRD may have an increased cardiovascular morbidity and lower angiogenic potential [146].

2.5 Endothelial Cell Role in Blood Vessels Formation

Formation of new blood vessels can occur by two distinct processes: (1) by vasculogenesis or *de novo* formation of blood vessels from bone marrow mobilized cells, or (2) by angiogenesis or formation of new blood vessels from pre-existing vessels [147].

2.5.1 Vasculogenesis

During embryonic development the colocalization of endothelial and hematopoietic progenitors within the yolk sac gave rise to the hypothesis that both lineages arise from a common precursor called the hemangioblasts [148]. But the definitive isolation and localization of hemangioblasts proved to be difficult. However, recent evidence

demonstrated that cells with hemangioblasts characteristics were present transiently in the posterior segment of the primitive streak during gastrulation and the hemangioblasts were shown to express VEGF receptor 2 and the mesoderm gene brachyury [149]. The initiation process of embryonic blood vessels formation requires the signaling of fibroblast growth factor (FGF) and bone morphogenetic proteins (BMP) 2 and 4 as was demonstrated by gene deletion experiments [150, 151]. The earliest marker available at this time of endothelial differentiation from stem cells is the expression of VEGF-R2, which is also expressed on hematopoietic cells but VEGF-R2 persist only in mature endothelium. VEGF-R2 expression suggests a critical role for VEGF during early embryonic endothelium development as mice lacking VEGF or VEGF-R2 die early in gestation [152].

There are evidence that angioblast or endothelial progenitor cell can differentiate directly from mesoderm [153]. During embryonic vasculogenesis the angioblasts become organized and form cord-like structures that are lumenless and are arranged at different embryonic positions [154]. The angioblasts undergo another differentiation stage and transition from cuboidal to flat cells and arrange their contact junctions to the cords periphery and eventually mature into endothelial cells lining the inside of a newly formed blood vessel in a process known as tubulogenesis [155].

For proper lumen formation during vasculogenesis the endothelial cells must interconnect through adherens, tight junctions and to the extracellular matrix. The cellular junctions proteins are specialized transmembrane and intracellular molecules that allow the adjacent cells to adhere to each other [156]. The adherens junction proteins belong to the cadherin and catenin families. Cadherins form multimeric complexes that mediate transmembrane linkage, whereas catenins anchor cadherins to the cytoskeleton. Vascular endothelial cadherin (VE-cadherin) is the major adherens junctional protein expressed in endothelial cells of all vascular beds. The loss of VE-cadherin in knockout mice is lethal at E9.5 due to cardiovascular failure and defects in lumen formation are noticed by E8.5 of the embryonic development [157]. It has been suggested that VE-cadherin is important in maintaining endothelial cells proper polarity that may play an important role in lumen formation [158]. N-cadherin on the other hand is suggested to anchor endothelial cells to extra cellular matrix and regulates the expression levels of VE-cadherin. In addition, N-cadherin knockout mouse is lethal at the embryonic stage where severe vascular defects are detected in the embryos [159]. Catenins are cytoplasmic proteins where β-catenin interacts with and propagates the extracellular signaling of VE-cadherin and α-catenin links β-catenin-VE-cadherin complex to the actin cytoskeleton. When activated β-catenin translocates to the nucleus and interacts with the transcription factors Tcf [160]. Elimination of the β-catenin gene caused embryonic lethality at the

gastrulation stage, whereas endothelial-restricted knockout of β-catenin resulted in embryo death at E11.5-13.5 due to defects in vascular organization [161, 162].

The tight junctions tether the cells closely together forming an almost impermeable fluid barrier. The tight junction proteins consist of claudins, occludins, junctional adhesion molecules (JAM), and the endothelial-endothelial enriched endothelial cell selective adhesion molecule (ESAM) [154]. However, despite the critical role that tight junctions play in endothelial cells function, they have not been shown to be important in vascular lumen formation [163].

Endothelial cell interaction with extracellular matrix is important for proper lumen formation during vasculogenesis. It was shown that endothelial cells are connected to the endodermal basement membrane and to the matrix produced by the mesenchymal cells during mouse aortic vasculogenesis [164]. It appears fibronectin is the first extracellular matrix component produced by endothelial cells of the aorta, whereas as other components such as laminin is secreted at a later stage to form the basement membrane around the circumference of the aorta [164]. However, conditional knockdown studies of various extracellular matrix proteins including, vitronectin, nidogens, vWF, and perlecan did not result in any adverse effects on vasculogenesis suggesting that vascular defects are likely not observed due to redundancies of related molecules [165].

The intracellular signaling molecules that have been shown to regulate vascular tubulogenesis belong to the family of Rho monomeric G protein GTPases. The Rho GTPases are hydrolase enzymes involved in a number of cellular functions including cellular proliferation, migration, adhesion, cytoskeleton rearrangement, and cellular polarity [166]. Rho GTPases are involved in cellular signaling pathways activated by integrin-ECM interactions and interact with phosphoinositide-3 kinases (PI3K) to propagate the cellular signaling [167, 168]. The following members Rac, Cdc42, Rho, Ras, and Rap are ubiquitously expressed and have been shown to regulate both epithelial and endothelial lumen formation process [164, 169]. In a study using 3D collagen matrices it was demonstrated that Cdc42 and Rac1 play an important role in endothelial cells vasculogenesis and lumen formation and their activities is mediated by downstream signaling molecules including the serine/threonine-protein kinases PAK2 or PAK4, which are cytoskeleton signaling regulators, and Par3 and Par6, which are cell-polarity proteins that establish endothelial cells polarity through Cdc42 and the atypical protein kinase C (PKC). The siRNA knockdown of any molecule in the polarity complex Cdc42-Par3-Par6-PKCzeta inhibited the endothelial lumen formation in the *in vitro* 3D collagen system [170].

2.5.2 Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. Angiogenesis is an important process in various

physiological and pathological processes, such as embryogenesis, wound healing, and angiogenic neovascularization supports the growth of solid tumors [171]. Angiogenesis can occur through either two distinct processes known as sprouting angiogenesis and intussusception [147]. Sprouting angiogenesis is a complex process that requires an intricate balance between the endothelial cells and the signals from various growth factors, extracellular matrix proteins and mural cells. Angiogenesis includes a series of distinct and coordinated steps including, endothelial cells activation, degradation of basement membrane, migration, proliferation, lumen formation, alignment, anastomosis, and the recruitment of mural cells [171-173]. Sprouting angiogenesis is initiated when endothelial cells in an already formed blood vessel respond to stimulatory signals from angiogenic factors such as vascular endothelial growth factor (VEGF)-A specifically VEGF-165. The activated endothelial cells differentiate to remodel intercellular and cell-matrix adhesions, and begin secreting basement membrane degrading proteases and start migrating towards the stimulating factor. The sprouting cells arrange themselves into tip cells that extend long filipodia but they lose their ability to proliferate. The tip cells are followed by the stalk cells that are proliferative but do not form long filipodia [174]. The intussusception angiogenesis describes the process of development of new blood vessel from a pre-existing one by the formation of transcapillary pillars of extracellular matrix that split the blood vessel into two [175].

2.5.2.1 Sprouting Angiogenesis

This process begins when quiescent EC responds to proangiogenic signals such as VEGF, FGF, angiopoietins-2 or chemokines that are produced by hypoxic, inflammatory, or tumor cells [174]. The sequential steps of vessel branching begin when pericytes detach from the blood vessel and VEGF increases EC permeability causing plasma proteins to leak and deposit provisional ECM scaffold. In response to integrin signaling, EC migrate towards the ECM surface. To prevent a large number of EC to differentiate into tip cells, there is a selection process of one endothelial to lead the tip in the presence of VEGF receptors and Notch-Dll4 signaling. The cells following the tip cell become the stalk cells that are capable of proliferating and forming lumina [174, 176, 177].

Mismatched oxygen and nutritional requirements are critical components of vascular adaptations in a given tissue that can lead to EC activation and induction of the angiogenesis process [178]. Hypoxia is a strong determinant of new blood vessel growth into hypoxic tissues. For instance hypoxic retinal astrocytes express VEGF in non-vascularized areas. As a result, quiescent phalanx endothelial cells expressing VEGF-R2 respond to the stimulus and develop into tip cells and extend filopodia that detect the hypoxic areas and migrate towards them. [158, 174, 179].

Hypoxia is a main inducer of angiogenesis and it has been shown that EPC in the bone marrow of diabetic patients followed a chemokine

gradient to the hypoxic sites where they contributed to neovascularization. Hypoxia can induce expression of VEGF in endothelial cells in a process that involves the transcription factor hypoxia inducible factor-1α (HIF-1α) [178, 180, 181]. In addition aerobic glycolysis has a regulatory effect on angiogenesis. The endothelial cells are capable of surviving hypoxic conditions and sprout in avascular tissues largely due to their ability to produce sufficient energy from glycolysis under anaerobic conditions. Interestingly, despite the immediate access to O2, endothelial cells generate most of their energy through anaerobic glycolysis. Endothelial cells are reported to metabolize > 90 % of glucose anaerobically *in vitro* [182, 183]. In contrast, under normoxic conditions the endothelial cells are quiescent and rarely divide [178]. The activation of HIF-1α can drive VEGF expression and its signaling through VEGF-R2 leads to the expression of the tip cell marker Delta-like 4 (Dll4) which can play an important role in the arterial versus venous fate. Higher expression of Dll4 can promote an arterial fate, whereas, veins express low levels of Dll4 [184].

2.5.2.1.1 Tip Cell Biology

As the endothelial cells in a blood vessel are activated by VEGF only some will respond by directing the filopodia extensions from the activated cells located at the tips of the budding blood vessel. During retinal vascularization, a model of developmental angiogenesis, EC differentiate into tip cells in response to VEGF-A that guide tip cells migration and induce stalk cells proliferation [174]. Another feedback loop,

based on notch / delta-like-4 (Dll4) signaling, is established between tip and stalk cells. The tip cells express slightly higher levels of Dll4 and less notch activity and thus controlling the fate of the neighboring cells, the stalk cells, which will have increased notch signaling, are prevented from differentiating into tip cells. The activation of the notch pathway inhibits VEGFR2 and Dll4 expression levels, a process that limits the number of cells differentiating into tip cells under VEGF stimulation [185, 186]. The tip cells are also regulated by the Fringe family of glycosyltransferases, which glycosylate notch receptors and enhance notch/Dll4 signaling in the stalk cells [177]. Jagged1, a notch ligand with elevated expression levels in stalk cells, antagonizes Dll4/notch signaling in cells expressing Fringe, promotes angiogenesis by down-regulating Dll4-notch signaling in tip cells allowing more cells to differentiate into tip cells. In addition, Jagged1 acts as a partial agonist to limit Dll4-notch signaling in adjacent stalk cells, which helps to maintain VEGFR expression levels in the newly formed vascular network [177]. Dll4-notch signaling controls the number of cells that will differentiate into tip cell phenotype as the inhibition of this pathway results in a substantial increase in sprouting [185]. Similarly, the inhibition of Dll4-notch signaling by Dll4 morpholinos in a zebrafish intersegmental vessels (ISV) model induces excessive branching of ISVs, whereas, the overexpression of notch signaling reduces the sprouting of the ISV [187]. In addition, Dll4-notch signaling regulates the expression levels, of VEGF recptors (R1, R2, and R3) in tip cells. VEGFR2 is down-regulated, but

VEGFR1 and soluble VEGFR1 are up-regulated which modulates the responsiveness to VEGF stimulation [188, 189]. VEGFR3 is down-regulated in the stalk cells and is highly expressed in the tip cells. Blocking VEGFR3 by genetic targeting or by monoclonal antibodies significantly reduces vascular sprouting and density, and endothelial cells proliferation [179]. In addition, during angiogenesis VEGFR3 forms a heterodimer with VEGFR2 that modulates VEGFR2 kinase activity in the tip cells [190].

The tip cells form cytoplasmic extensions called filipodia that guides the tip cells migration in response to VEGF stimulation. The filipodia are controlled by cytoskeleton remodelling which is dependent on myosin II contractility. The downregulation of myosin II permits the formation of lamellipodia and tip cells branching [191, 192]. The filipodia directionality is dependent on the activity of, a small GTPase of the Rho family, CDC42 and on the spatial cues provided by the heparin-binding of the VEGF isoforms $VEGF^{165}$, and $VEGF^{188}$ in the microenvironment [193, 194].

As endothelial cells are activated by an angiogenic factor such as VEGF and differentiate into tip cells, they undergo genetic changes that distinguish them from quiescent endothelial cells and from stalk cells [195]. Transcriptome analysis of retinal endothelial cells from $Dll4^{+/-}$ heterzygote mice identified three clusters of genes that were significantly up-regulated. These clusters of genes included extra cellular matrix (ECM) degrading enzymes, secreted molecules, and basement memebrane (BM) proteins [195]. The major ECM/BM proteins that are up regulated include;

nidogen-1, urokinase receptor (uPAR), and integrin β1. The secreted molecules consist of apelin, endothelial-specific molecule 1 (ESM1), insulin-like growth factor binding protein 3 (IGFBP-3), and angiopoietin-2. The mRNA expression levels of the microarray results were validated at the protein levels and confirmed the increased expression at the tip cells position [195].

Tip cells play an important role in sprouts anastomosis. The exact mechanism how tip cells meet and make contact with other tip cells is not fully understood [196]. However, it appears that VE-cadherin is involved in the process of connecting the vascular network. VE-cadherin is found not only at cell-cell junctions but also at the tip cells filipodia. The tip-tip localization of VE-cadherin was demonstrated to make contact with adjacent sprouts [197].

2.5.2.1.2 Stalk Cell Biology

Stalk cells are generally regarded as the cells that follow the tip cells and form the main body of the sprouts, capable of proliferation, form a lumen, and responsible for generation of the basement membrane and recruitment of pericytes [174]. The adjacent cells of an emerging sprout produce soluble VEGFR1 (sVEGFR1) to reduce the available VEGF to stalk cells and prevent them from differentiating into tip cells. At the same time, the depletion of VEGF from the stalk cells create a “corridor” of

higher VEGF concentration available to the tip cells that aids in sprout guidance [196, 198].

Proliferation of stalk cells is an important factor that contributes to the growth of newly formed sprouts, as tip cells do not, or rarely, proliferate [174]. However, it has been demonstrated that stalk cells proliferation does not propel the sprouts forward but it is the tip cells' stimulation with angiogenic factors and chemotactic migration that drives sprout extension [199, 200].

The expression of Dll4 in the tip cells regulates the fate of the neighboring stalk cells by activating notch signaling that leads to the regulation of the specific transcription factors Hes/Hey of the basic helix-loop-helix (bHLH) family which, result in the down regulation of VEGFR2 and VEGFR3 and the up regulation of VEGFR1 and thus limiting the activity of the available VEGF in the stalk cells [179, 185, 186].

The suppression of stalk cells differentiation into tip cells by Dll4/Notch signaling also requires the activation of the Smad1/Smad5 pathway by bone morphogenetic proteins as the conditional knockout of Smad1 and Smad5 in endothelial cells resulted in an increase in tip cells numbers and an impaired Dll4/Notch signaling. The deletion of Smad1 and Smad5 in mice is embryonically lethal due to excessive sprouting, loss of vascular remodeling and impaired tip cell polarity [201]. The down regulation of Smad1/Smad5 *in vitro* was shown to reduce the expression

level of a number of notch target genes and other stalk cells enriched genes including VEGFR1, Jagged1, Hes1, Hey1 and DNA-binding protein inhibitors (Id) 1-3. The Id proteins operating downstream of Smad1/5 acts as stalk cells competence factors by heterodimerizing with Hes1 that strengthen Notch signaling and stabilize Hes1 in endothelial cells [201].

2.6 Proangiogenic Factors

2.6.1 The Family of Vascular Endothelial Growth Factors

Vascular endothelial growths factors (VEGF) are important regulators of embryonic vascular development and during angiogenesis process in adults [202]. VEGF belong to a family of proteins that consist of VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and placenta growth factor (PlGF). Alternative splicing generates many variants of VEGF and the different isoforms have specific functions in blood vessels formation and homeostasis [203-205]. There are at least seven VEGFA homodimeric isoforms that are identified by their number of amino acids such as, VEGFA-121, -145, -148, -165, -183, -189, or -206 [205]. The VEGFA monomers are encoded by a single gene that contains eight exons. All isoforms contain the exons, 1, 5, and 8 except VEGFA-148. The alternative splicing takes place in exons 6 and 7, which encodes heparin-binding domains. The presence or absence of the heparin-binding domains determines the isoform solubility and binding to the receptor(s). The isoforms that contain exon 6 (VEGFA-148, -189, and -206) express

the matrix-binding domain and are not diffusible. The heparin-binding domain encoded by exon 7 confers moderate diffusibility. In the case of VEGFA-121, the lack of exons 6 and 7 makes it highly diffusible [206]. In contrast, VEGFA-165 is the most abundant isoform and binds to heparan sulphate proteoglycans (HSPG), and engages the VEGF receptors, and the neuropilins co-receptors. When VEGFA-165 is bound to HSPG expressed on the glycocalyx surface of endothelial cells, the affinity to VEGF-R2 is increased [203]. The HSPG found in the extracellular matrix are necessary to establish a gradient of VEGFA-165 that promotes sprouts guidance during angiogenesis [207]. Interestingly, the highly diffusible isoform VEGFA-121 that lacks the heparan-binding domain is not presented as a gradient and is rendered inefficient in guiding tip cells, but it can induce endothelial cells proliferation in mouse retina [174]. Mice expressing only VEGFA-120 develop unbranched and broad vessels, whereas the expression of only VEGFA-188 in mice causes hyperbranched and thin vessels, since VEGFA-189 has a high affinity for HSPG and therefore is restricted to the extracellular matrix [208, 209].

VEGFB expression is abundant in the skeletal muscle, pancreas and in the adult myocardium [205]. Through alternative splicing of exon 6 two VEGFB isoforms are generated VEGFB-167 and VEGFB-186. VEGFB-167 is around 21 kDa and is bound to cell surface heparan sulfate proteoglycans, whereas VEGB-186 has a molecular weight around 32 kDa and it is secreted. Both VEGFB are produced as homodimers however

they can form heterodimers with VEGFA when coexpressed. VEGFB selectively binds to VEGF receptor1 and can induce the expression of urokinase type plasminogen activator (uPa) and plasminogen activator inhibitor 1 (PAI-1) in endothelial cells suggesting that VEGFB can regulate extracellular matrix degradation and cellular adhesion [206, 210].

VEGFC has a high expression in the heart, placenta, ovary, and small intestine [211]. VEGFC is produced as a preproprotein of around 47 kDa and must be proteolytically cleaved to generate the mature protein that is secreted. Several VEGFC forms are generated by proteolytic processing and show strong binding activity to VEGF receptor 3, whereas only the fully mature VEGFC can bind to VEGFR-2 [211, 212]. VEGFC plays an important role in lymphangiogensis and regulates endothelial cell growth [213].

VEGFD is highly expressed in the lung, skeletal muscle, heart, and small intestine. VEGFD is generated as preproprotein and needs to be proteolytically cleaved in the N- and C-terminal for proper activity [214]. VEGFD forms a homodimer and binds to VEGFR-2 and VEGFR-3. VEGFD can induce lymphangiogenesis, endothelial cell growth, and vascular permeability [215].

VEGFE is encoded by the Orf virus, a parapoxvirus that infects sheep, goats, and humans in some cases. VEGFE has ~25 % amino acid homology with mammalian VEGF. It is about 20 kDa and does not contain

a basic domain and has no affinity to heparin. VEGFE may play a role in pathological angiogenesis in lesions infected by viruses [206, 216].

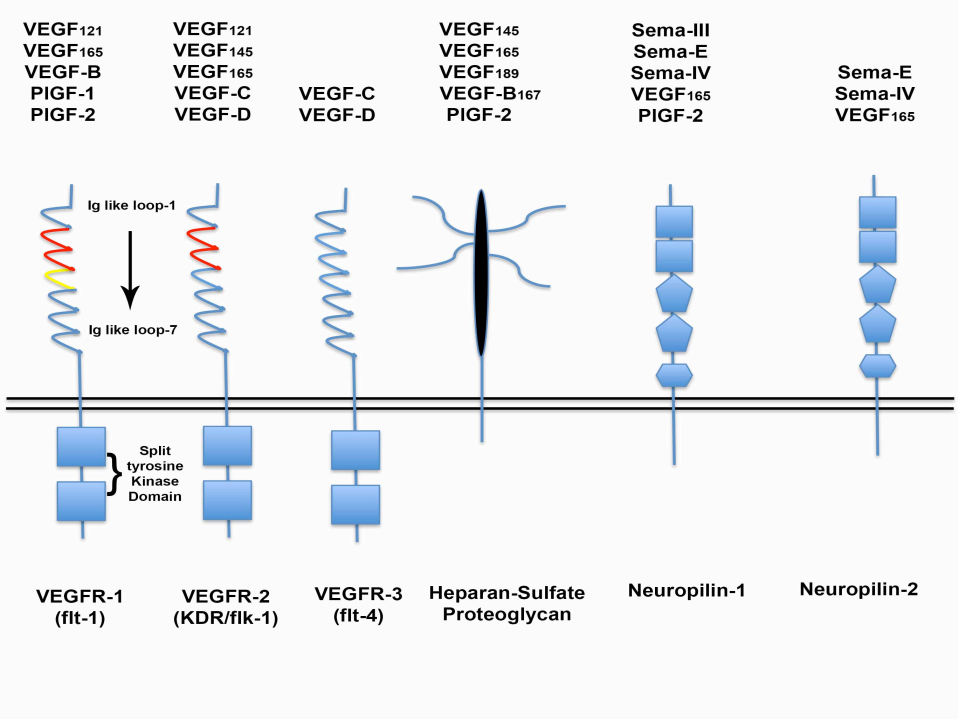

Figure 2.1: Schematic representation of VEGF family of growth factors and their receptors.

VEGF are involved in a large number of biological and cellular processes that include survival, migration, proliferation, differentiation, communication, and vascular permeability [217-219]. VEGF isoforms are produced by different cell types including: endothelial cells, leukocytes, fibroblasts, and tumor cells [220, 221]. VEGF are induced strongly by hypoxia, through the transcription factor hypoxia inducible factor-1 (HIF-1), which is thought to stimulate the angiogenesis process during development [222, 223]. The indispensable role of VEGF in vascular development is shown in knockout mice where the absence of one VEGF

allele was embryonically lethal with severe defects in the vasculature [224, 225].

There are three main VEGF receptors that belong to the family of receptor tyrosine kinase and are denoted VEGF-R1, -R2, and –R3. VEGF-R1 is also known as fms-like tyrosine kinase (Flt-1), VEGF-R2 is known as kinase domain region (KDR in human) or fetal liver kinase (Flk-1 in mouse), whereas VEGF-R3 is referred to as Flt-4. The receptors share structural homology as they have an extracellular domain with seven immunoglobulin-like domains, one transmembrane region, and a cytoplasmic tail with a split tyrosine kinase domain [226].

VEGF-R1 is considered the main receptor for PlGF but can also bind VEGFA and VEGFB [226]. VEGF-R1 is expressed predominantly in endothelial cells but is also found in hematopoietic cells where it acts as a positive regulator of monocytes and macrophages migration [223]. VEGF-R1 is also expressed in trophoblast cells and in mesangial cells of the kidney [223, 227, 228]. The result of VEGF-R1 signaling in endothelial cells is still ambiguous with contrasting reports. It has been shown that tyrosine 1169 in VEGF-R1 kinase domain provides a binding site for phospholipase C-γ (PLC-γ) after VEGF stimulation that also resulted in weak activation of extracellular signal-regulated kinase (ERK) in VEGF-R1 overexpressing NIH3T3 cells [229, 230]. Alternatively, ERK was strongly activated in porcine aortic endothelial cells (PAEC) expressing VEGF-R1 whereas there was no evidence of PLC-γ activation in response to PlGF

[231]. The best-described function of VEGF-R1 is as a negative regulator of VEGF-R2. VEGFA binds VEGF-R1 with higher affinity than VEGF-R2, and with the presence of a soluble form of VEGF-R1, VEGFA is trapped, limiting its availability to VEGF-R2 [232, 233]. Accordingly, the deletion of the VEGF-R1 gene in mice is embryonically lethal at E8.5 with excessive endothelial cells growth and vascular disorganization, in addition to an increase in overall number of endothelial progenitor cells [234, 235]. Interestingly, the deletion of the cytoplasmic domain of VEGF-R1 in mice did not cause an adverse effect to the vasculature, as the animals were fertile and developed normally, however, there was a suppression of macrophage migration in vitro in response to VEGF stimulation [236].

VEGF-R2 is highly expressed during early development in endothelial progenitor cells, but the expression level decreases in quiescent endothelial cells in adults [202]. VEGF-R2 can be found also in hematopoietic stem cells, retinal progenitor cells, and megakaryocytes [237, 238]. The propagation of VEGF-R2 signaling leads to the activation of several cellular responses including survival, proliferation, migration, differentiation, and neovascularization in physiological or pathological conditions [202]. Upon ligand binding VEGF-R2 undergoes dimerization and autophosphorylation of the four tyrosine residues located in the kinase insert region and in the kinase domain [239]. In addition, VEGF signaling through VEGF-R2 can lead to the release of nitric oxide and prostacyclin (PGI_2), Ca^{2+} mobilization, endothelial cells migration and proliferation, and

AKT/PI3K and PLC-γ activation [240-243]. VEGF-R2 mediated cellular survival is provided by the activation of the AKT pathway. The phosphorylation of Tyr 1173/1175 in VEGF-R2 cytoplasmic domain by VEGF leads to the recruitment of the PI3K via p85 regulatory subunit then activation of AKT supplying a survival signal in endothelial cells [244]. However PI3K/AKT signaling is not required for regulation of nitric oxide production that mediates cellular permeability [245]. Other Molecules containing SH2 domain can interact directly with VEGF-R2 such as PLC-γ, which binds phosphorylated Tyr 1175 and mediates the activation of the mitogen-activated protein kinase (MAPK) through PKC, resulting in endothelial cells proliferation [246]. Endothelial cells migration can be mediated through different pathways. VEGF-induced migration is mediated by binding of the adaptor molecule Shb to phosphorylated Tyr 1175 on VEGF-R2 [247]. VEGF-R2 signaling can lead to the activation of the focal adhesion kinase (FAK) via phosphorylated Tyr 1175, and p38MAPK through Tyr 1214 to regulate cellular migration [248]. In addition, migration is regulated by the activation of phosphorylated Tyr 951 and T-cell-specific adaptor (TSAd) [249]. VEGF-R2 delivers the specific differentiation signal of vascular progenitor cells into endothelial cells through phosphorylated Tyr 1175 and PLC-γ1 [250]. The knockout of VEGF-R2 gene in mice is lethal at E8.5. The embryos show severe vascular defects and deficient endothelial cell development, highlighting the importance of VEGF-R2 during blood vessel formation [251]. The

VEGF-induced activity is modulated by the SH2 protein-tyrosine phosphatases SHP-1 and SHP-2 [252].

VEGF-R3 is the receptor for VEGFC and VEGFD and plays a major role in lymphatic development and function [253]. VEGF-R3 is abundant in lymphatic endothelial cells and found in fenestrated capillaries and veins, and on monocytes and macrophages [218]. During development VEGF-R3 is expressed on all endothelial cells but become mostly restricted to lymphatic endothelium in adults [254]. Interestingly, VEGF-R3 is implicated in post-natal angiogenesis as it is highly expressed in tip cells. The inhibition of VEGF-R3 activity by monoclonal antibodies substantially decreased angiogenic sprouts and affected endothelial proliferation, vessel branching, and vascular density in a mouse angiogenesis model [179].

Neuropilins are transmembrane glycoproteins with a short cytoplasmic domain that act as co-receptors for VEGF-165, but do not bind VEGF-121. They are maybe better known for their function in neuron biology as receptors for semaphorins. There are two identified neuropilin receptors NRP1 and NRP2. NRP1 is expressed in arteries, and NRP2 is abundant in venous and lymphatic vessels [202, 255]. NRP1 interaction with VEGF-R2 enhances VEGF-165 binding and endothelial cells had a better migratory response when NRP1 was present as compared to migration response of cells expressing only VEGF-R2 and no NRP1 [256]. The deletion of NRP1 gene is embryonically lethal demonstrating the

importance of this gene in blood vessel development as the knockout embryos had severe cardiovascular defects [223, 257].

2.6.2 The Family of Platelet-Derived Growth Factors

Platelet-derived growth factors (PDGF) play an essential role in many different physiological and pathological conditions. They have mitogenic activity for many cells of mesenchymal origin, are chemoattractant, involved in erythropoiesis, modulate endothelial proliferation, and participate in wound healing and angiogenesis [258-260]. It has been reported that PDGFB induce angiogenesis by up-regulating the expression and production of VEGF [261]. In addition, PDGFB/PDGFRβ signaling is involved in pericytes recruitment to the site of newly formed endothelial tubes [79]. PDGFB is also involved in embryonic vascular development as it stimulates embryonic stem cells to differentiate into EC through a process that requires calcium-mediated generation of reactive oxygen species [262]. PDGFB is widely expressed by the endothelium during embryonic development but expressed in adult in EC in a spatial-temporal pattern, and is up-regulated in sprouting tip cells [263].

PDGF belong to a highly conserved family of growth factors that are related based on the structure and function. PDGF exist as homodimers PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD, and as a heterodimer PDGF-AB. PDGF-CC and –DD are secreted in a latent form that must be

cleaved outside the cell to become active, whereas PDGF-AA and –BB are secreted in the active form [264, 265]. PDGF-CC is cleaved by tissue-type plasminogen activator (tPA) and by plasmin, and urokinase-type plasminogen activator (uPA) acts on PDGF-DD [265].

PDGF isoform deletion is embryonically lethal. PDGF-AA and -CC knockout mice die perinatally with reported defects in the renal cortical interstitium, a phenotype also observed in PDGFR-α deficient mice [266, 267]. Similarly, PDGF-BB knockout mice die perinatally with notable renal defect characterized by an absence of mesangial cells and an abnormal glomerular tuft formation. On the other hand the glomerular endothelial cells, podocytes, and the basement membrane are normal [268].

The expression of PDGF isoforms and their receptors in human and mouse kidney cells is well established. PDGF-AA is expressed by podocytes and epithelial cells of distal tubules and of collecting duct in human, and in the loop of Henle cells in mouse [269, 270]. Low levels of PDGF-BB are expressed in human mesangial cells and in mouse arteries smooth muscle cells [267]. PDGF-CC is found in human glomerular parietal epithelial cells, proximal and distal tubules, and in endothelial cells in arteries. In mouse PDGF-CC is expressed in glomerular endothelial cells and in arterial smooth muscle and endothelial cells [271]. PDGF-DD expression in humans is limited to podocytes and to vascular smooth muscle cells, whereas, in mouse PDGF-DD is constitutively expressed by mesangial cells [272].

All PDGF isoforms are overexpressed in injured kidneys, a finding observed in almost all animal models of kidney disease [273]. PDGFA is overexpressed after injury in the glomerulus by mesangial cells and in the interstitial compartment [274, 275]. PDGFB is up-regulated in the glomerulus and is expressed mainly by mesangial, parietal epithelial cells, by platelets and circulating leukocytes [267, 276-278], and by arterial smooth muscle cells [279]. In addition, tubular and interstitial cells contribute to PDGFB production after a renal injury [280]. PDGFC expression is up- regulated in mesangial cells, podocytes, interstitial cells and macrophages [267, 271]. PDGFD expression after renal injury is limited to mesangial and interstitial cells [272, 281].

The PDGFR-α is up regulated mainly by interstitial cells and macrophages [272], whereas the isoform beta has much wider expression pattern that includes the glomerular and interstitial compartments. The PDGFR-β expression is observed in mesangial cells, podocytes, parietal epithelial cells, and in interstitial cells [282-284].

The PDGF isoforms bind to receptor tyrosine kinase homodimers PDGFR-$\alpha\alpha$, PDGFR-$\beta\beta$, and the heterodimer PDGFR-$\alpha\beta$ with different affinities [264]. PDGF have strong mitogenic activity and the activation of their cognate receptors is important during embryonic development and in wound healing. In addition, PDGF signaling is a mechanism associated with disorders such as cancers, and vascular diseases [285]. PDGF exert

their effect mainly on smooth muscle cells and fibroblasts that can potentially lead to vascular fibrosis and other pathologies [286].

PDGF isoforms are constitutively expressed, or components of the PDGF/PDGFR system can be induced in most kidney cells. It is reported that the majority of experimental models and human kidney diseases have an irregular expression of PDGF isoforms and/or receptors [267].

Overexpression PDGF-BB and -DD in glomeruli can lead to mesangial cell proliferation and to the development of glomerulosclerosis and eventually tubulointerstitial fibrosis [287]. PDGF-DD has been directly implicated in the development of tubulointerstitial fibrosis likely by inducing epithelial to mesenchymal transition. This process is controlled by the microRNA miR-200 family [287, 288].

2.7 Phosphoinositide 3-Kinases as Integrators of Proangiogenic Cues

The phosphoinositide 3-kinases (PI3K) belong to a family of enzymes implicated in various cellular activities such as survival, proliferation, differentiation, trafficking, and chemotaxis [289]. The PI3K act downstream of receptor tyrosine kinases (RTK), and G protein-coupled receptors (GPCR). PI3K phosphorylate phosphotidylinositol (PtdIn) a phospholipid without phosphate group attached to the inositol ring. The PtdIn is called phosphoinositide (PI) once it is phosphorylated. There are

three different classes of PI3K divided upon their structure, and lipid specificity [290].

2.7.1 Class I PI3K

Class IA PI3K form heterodimers and are composed of one catalytic and one regulatory subunit that contains two src homology (SH2) domains. There are three catalytic subunits p110α, p110β, and p110δ that are encoded by separate genes. The major regulatory subunits are p85α, p55α, and p50α, which are splice variants of the same gene whereas p85β, and p55γ are expressed by other genes [291]. The only member of class IB is p110γ and is generally associated with the adapter protein p101. P110γ is highly expressed in leukocytes and acts downstream of GPCRs and plays important roles in inflammation and allergy [292, 293].

Upon ligand binding to a RTK receptor or a GPCR PI3K are recruited to the receptor and become active by relief of allosteric inhibition from the regulatory subunit. Activated PI3K phosphorylate phosphotidylinositol (4,5) bisphosphate (PIP2) to form phosphotidylinositol (3,4,5) triphosphate (PIP3), which is localized at the plasma membrane and acts as binding site for proteins containing a pleckstrin homology (PH) domain [294, 295]. PIP3 recruits Akt (also known as protein kinase B) to the plasma membrane where it is phosphorylated on Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and on Ser473 by PDK2 (mTOR complex2) [296-298]. Akt exerts its major activities via a

serine/threonine kinase known as mammalian target of rapamycin (mTOR) signaling pathway that regulates numerous cellular activities including cellular growth, proliferation, survival, and motility [299, 300].

Akt mediates cell cycle entry by regulating the forkhead transcription factors (FOXO) and cyclin D1. The cellular survival activity of Akt is mediated by phosphorylation of IκB kinase (IκBK), Bad, and caspase 9. The combined actions of activated Akt and guanine nucleotide exchange factors (GEF) lead to the activation of Cdc42 and Rac that promote cellular growth and proliferation, and an increase in cellular motility [291, 301].

PI3K is implicated in cell cycle regulation. When PI3K pathway is activated by growth factors, it facilitates the transitions from G0->G1 and G1->S, as PI3K activity peaks within minutes of growth factor-agonist binding and peaks again in late G1 phase [302, 303]. The enhanced production of PIP3 accelerates the entry into the cell cycle, whereas the reduction in PIP3 concentration has an opposite effect [304]. The differential activity of p110 catalytic isoforms is evident during the cell cycle. p110α is activated first as the cell transits from G0 and into G1 with minor p110β activity. In late G1 phase, p110α activity was detected first but p110β reached maximum activity at this stage of the cell cycle. The inhibition of either p110α or p110β resulted in significant reduction in cell cycle entry [305]. Moreover, p110β activity has been implicated during cell

division. Nuclear p110β was found associated with PKB and the inhibition of p110β activity had an adverse effect on the DNA replication rate, on the proliferating cell nuclear antigen (PCNA) binding to chromatin, and on PCNA binding to polymerase δ. The mechanism by which p110β exerts its activity during DNA replication appears to be related to the regulation of the nuclear activation of PKB and PCNA negative regulator $p21^{Cip}$ phosphorylation during the S phase [306].

The PI3K catalytic isoform p110β is involved in cell repair response to DNA double-strand break (DSB), which is one of the most damaging lesions for genomic integrity. It has been shown that p110β is necessary to sense the DSB as it regulates the DNA damage sensor protein nibrin or Nbs1. In p110β-deficient cells Nbs1 failed to bind to DSB, resulting in a defective DNA damage response, cell cycle arrest at G2, radiation sensitivity, and genomic instability [307].

There are ample evidence suggesting PI3K signaling, especially, class IA catalytic isoforms are essential for growth and survival of cancer cells. PI3K signaling activates Akt, which is considered the major effector molecule of PI3K in many cancers. PI3K-Akt signaling leads to the activation of the effector molecule mTOR complex 1 (mTORC1), which is implicated in numerous cellular functions including growth factor signaling, cellular AMP levels (energy), and the availability of the oxygen and nutrients to the cell [308]. The PI3K-Akt-mTORC1 axis is constitutively

activated in many cancers. The inappropriate activation of PI3K-Akt pathway is largely due to growth factors/receptor tyrosine kinases activation and the accumulation of somatic mutations in some components of PI3K-Akt-mTORC1 signaling pathway [308].

The activities of PI3K are modulated by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN is a multifunctional phosphatase with PIP3 being its major substrate. PTEN is a tumor suppressor gene and is mutated in many cancers [309, 310].

2.7.2 Class II PI3K

Class II PI3Ks are molecules around 170 kDa in size and are comprised of three catalytic isoforms C2α, C2β, and C2γ encoded by three separate genes. PI3Ks class II are defined by their C-terminal C2 domain that can bind to phospholipids in a Ca^{2+}- independent manner, and show strong substrate specificity to phosphotidylinositol (4) monophosphate (PIP) and to PIP2 [311, 312]. However, *in vivo* studies about the exact nature of the lipids produced by class II PI3K have not been conclusive, as overexpression of class II PI3K did not result in an increase in PIP2/PIP3 levels [313]. Class II PI3K can be activated by various growth factors and chemokines including, insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), integrins, and monocyte chemotactic protein 1 (MCP-1) [314-317]. It appears that class II PI3K are constitutively associated with the plasma membrane unlike class I members, which are

found mainly in the cytoplasm. The deletion of the C2 domain did not alter the location of the class II PI3K [312].

2.7.3 Class III PI3K

There is one class III PI3K catalytic subunit that forms a complex with a serine/threonine kinase p150. It has been demonstrated *in vitro* that class III PI3K is capable of generating phosphotidylinositol-3 phosphate from phosphotidylinositol. The catalytic subunit is homologous to the yeast vesicular protein-sorting protein Vps34, which may suggest a role in proteins and vesicles trafficking [290, 318]. The extracellular stimuli that regulate Vps34 activity are not well established, but there is evidence of regulation by amino acids, glucose, and GPCR [319-321]. Vps34 can function as a protein scaffold since not all effectors interact with phosphotidylinositol-3 phosphate [322]. The global deletion of Vps34 is lethal in yeast and flies, but it has not been studied in mice yet [322].

2.7.4 The Role of PI3K in Angiogenesis

Several lines of evidence implicate the PI3K signaling in endothelial cells during vascular development in embryo, tumor growth, and after injury/ischemia [323]. PI3K signal downstream of receptor tyrosine kinases, which are natural receptors to a variety of growth factors such as VEGF, FGF, EGF, and hepatocyte growth factor (HGF) that can also deliver an angiogenic stimulus. Moreover, the inhibition of the PI3K activity impedes the angiogenic process [324, 325].

The exact role of the PI3K class I catalytic isoforms in angiogenesis is not fully understood [326]. The isoforms p110α and p110β are ubiquitously expressed, however p110δ has low expression in endothelial cells but enriched in leukocytes [313, 327]. The genetic deletion of *Pik3ca* that encodes p110α in mice is embryonically lethal at mid-gestation with evidence of vascular abnormalities, whereas the p110β knockout mouse does not exit the blastocyst stage [328, 329]. The conditional knockout of p110α or the expression of a kinase-dead allele in endothelial cells both resulted in embryonic lethality at mid-gestation. The embryos developed normally until E9.5 in the absence of p110α, however, by E10.5 the blood vessels and trunk were poorly developed and all the embryos died by E12.5 likely due to angiogenic remodeling defects [330]. On the contrary, the conditional knockout of p110β or p110δ had no obvious vascular defects, as the mice were viable and fertile [330]. p110α is the predominant isoform that is activated by VEGF-A and affects angiogenesis, whereas, the outgrowth of angiogenic sprouts in aortic rings assay were reduced in p110β-deficient endothelial cells when stimulated by GPCR agonists such as sphingosine 1-phosphate (S1P), SDF1α, and interleukin-8 (IL-8) [330].

PI3K catalytic subunits contain a domain that binds Ras, Rac, and cdc42. Interaction with these small GTP binding proteins may modulate PI3K activity [331]. In a study to identify vascular signaling by H-Ras, two

H-Ras mutants were generated H-RasV12S35 and H-RasV12C40 that constitutively activated MAPK and PI3K pathways respectively. The MAPK pathway was shown to be important for vascular permeability, whereas the PI3K pathway was important for both angiogenesis and vascular permeability. In addition, pharmacological inhibition of the PI3K catalytic isoforms p110α and p110β resulted in disruption of angiogenesis and vascular permeability *in vivo*, whereas the inhibition of p110δ and p110γ affected vascular permeability mediated by H-Ras or VEGF [332].

2.8 Summary

Endothelial injury to the kidney microvasculature is a major pathologic pathway leading to loss of kidney function. This mechanism is observed in a group of diseases called thrombotic microangiopathies. A common feature of typical hemolytic uremic syndrome (HUS), atypical HUS, and alloantibody-mediated rejection is direct endothelial injury and loss from glomerular microvasculature. However, the mechanisms of repair of the renal microvasculature are largely unknown.

The immense interest shown in endothelial progenitor cells because of the tremendous potential they have in regenerative medicine has flooded the literature. With that came controversy and confusion as different cell populations were given the name EPC. Despite the lack of an EPC specific marker, there is a general consensus today that the population which, actually represents endothelial progenitor cells is the late outgrowth cells obtained from the endothelial colony-forming cells method. EPC are

studied extensively in various diseases in the prospect of repairing microvasculatures but so far there is limited but encouraging success. Endothelial cells are responsible for forming new blood vessel in two distinct processes called vasculogenesis and angiogenesis. Angiogenesis is a complex process that requires the coordination of several cellular processes and involves different cell types. One major family that provides an angiogenic stimulus is the vascular endothelial growth factors that signal through VEGR receptors and activates PI3K-Akt and MAPK pathways that lead to cellular survival and proliferation. Activation of endothelial cells during angiogenesis results in the differentiation of tip cells that lead the sprout and control the fate of the following cells or stalk cells as the sprouts eventually mature into a new blood vessel.

CHAPTER III

MATERIALS AND METHODS

3.0 Animals

Older than twelve weeks C57BL/6, Rag1, and Rag2 were used in this thesis. The BL/6 and Rag1 mice were purchased from Jackson Laboratories (Sacramento, CA), Rag2 from Taconic (Hudson, NY). Hartley guinea pigs and Lewis rats six weeks old were purchased from Charles River (Wilmington, MA). The $Tie2^{ERT2}Cre/p110\beta^{flox/flox}$ mice were a kind gift from Dr. Gavin Oudit (University of Alberta) and Dr. Bart van Haesebroeck (Queen Mary, University of London, UK). All the mice were housed at the VAF unit in filter-top cages, and the other animals were kept at the conventional facility of Health Sciences Laboratory Animal Services (HSLAS) at the Department of Medicine. The animals were maintained according to the Canadian Council for Animal Care (CCAC) guidelines.

3.1 Antibodies

The following is a list of the primary antibodies used throughout this thesis. Negative control mouse IgG1 (DAKO, X0931); anti-human CD62, E-selectin (DAKO, M7105); anti-human CD184, CXCR4 (BD Pharmingen, 551510); anti-human eNOS (Chemicon Int, AB16301); anti-human S100A4, FSP1 (Abnova, PAB0010); anti-human smooth muscle actin (R&D Systems, MAB1420); anti-human TGF-βRII (R&D Systems, MAB2411); anti-human TGF-βRI, ALK5 (R&D Systems, AF3025); anti-

human endoglin, CD105 (R&D Systems, AF1097); anti-human SMAD3 (Abnova, H00004088-M05); anti-human phospho-SMAD3 (R&D Systems, AB3226); anti-human PECAM, CD31 (BD Pharmingen, 555445); anti-human VEGFR2, KDR (R&D Systems, FAB357P); anti-human cleaved caspase 3 (Cell Signaling, 9661); anti-human ERG (Sigma-Aldrich, E3158); anti-human ALK1 (R&D Systems, AF370); anti-human DLL4 (R&D Systems, MAB1389); anti-human apelin (abcam, ab59469); anti-human ESM1 (abcam, ab103590); anti-human vWF was a kind donation from Dr. Nadia Jahroudi (University of Alberta).

Anti-mouse CD31 (BD Pharmingen, 550274); anti-mouse podocalyxin (R&D Systems, AF1556), anti-mouse fibrinogen/fibrin (GenWay, GWB-85DBCA); anti-mouse podocin (Santa Cruz, sc-22298).

Anti-saporin (abcam, ab42903; Advanced Targeting Systems, AB-15HRP), DyLight 549 goat anti-rabbit (Jackson ImmunoResearch, 111-506-003); DyLight 488 goat anti-mouse (Jackson ImmunoResearch, 115-485-164); FITC nouse anti-goat (Jackson ImmunoResearch, 205-095-108); HRP rabbit anti-sheep (Jackson ImmunoResearch, 313-035-003); HRP goat anti-mouse (115-035-003); HRP goat anti-rabbit (Jackson ImmunoResearch, 111-035-008); HRP goat anti-rat (Jackson ImmunoResearch, 112-035-143); rabbit anti-biotin (Polysciences, Inc. 23867). Anti- 6X His tag® antibody (abcam, ab18184).

3.2 Isolation of Endothelial Progenitor Cells

The EPC were isolated according to the endothelial colony-forming cells method described by Lin *et al.* [333] with some modifications. Human mononuclear cells (MNC) were isolated from 120 mL peripheral blood from healthy adult donors or from 1-liter leukapheresis blood obtained from the blood bank. The blood was diluted at 1:1 ratio with Hanks balanced salt solution (HBSS) (Lonza, 10508F) and 20 mL of the blood HBSS mixture were layered over 10 mL of Lymphoprep™ (Axis-Shield, 1114544) and centrifuged for 30 min at 1800 rpm. The MNC were removed and washed with HBSS 2X at 1600 rpm for 10 min. The MNC were suspended in 1 mL solution of PBS, 2% FBS, 1 mM EDTA and counted to prepare the cells for CD34+ selection. CD34+ cells were isolated using EasySep human CD34 selection kit (StemCell Technologies, 18956) according to manufacturer's instructions. The resulting CD34+ cells were counted and seeded on fibronectin coated 6-well tissue culture plates in complete endothelial basal medium-2 (EBM-2 supplemented with 10 % FBS and VEGF, bFGF, IGF, EGF, heparin, ascorbic acid, gentamicin) (Lonza, CC-4176). After 24h incubation at 37 ˚C, 5 % CO_2 non-adherent cells were discarded and the adherent cells were fed every other day with complete EBM-2 until EPCs colonies appeared in culture 7 - 21 days after seeding. In all experiments, EPC were used between passages 3-6 exclusively.

3.3 Isolation of Human Macrophages

Peripheral blood monocytes were isolated as described in [334]. The cells were cultured in complete DMEM supplemented with 10 % FBS, 2mM glutamine, 15 mM HEPES, 0.02 % sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 500 U/mL macrophage- colony stimulating factor (M-CSF), and incubated at 37 ˚C, 5 % CO_2. The cells were cultured for seven days and changed medium every other day. The macrophages were characterized for the expression of macrophage markers by real time PCR using CD14, CD45, and M-CSF receptor primers (see Table 1 for sequences).

3.4 Quantitative PCR

Real time PCR was performed using 7500 thermocycler (Applied Biosytems). Total RNA was isolated using RNeasy mini kit (Qiagen, 74104) and one μg of total RNA was reversed transcribed into cDNA using qScript™ cDNA supermix (Quanta, 84034) and the primers were designed using PrimerExpress 3.0 (Applied Biosystems) software and purchased from IDT (Toronto, ON). The complete list of primers used in this thesis is presented in Table 3.1.

3.5 End-point PCR

The presence of human cells in the mouse tissues was evaluated by end-point PCR using the human specific primers for β_2-microglobulin

and/or CD34 genes (see Table 3.1 for the primers sequences). The cycling parameters were as follows 5 min at 95 ˚C, and 35 cycles of 95 ˚C for 15 s, 58 ˚C for 15 s, and 72 ˚C for 15 s, and the PCR reaction was finished with a final step of extension at 72 ˚C for 10 min. The PCR products were resolved on an agarose gel stained with ethidium bromide and visualized using MultiImage 2® and FluorChem software (Alpha Innotech).

3.6 SDS-PAGE and Western Blot

SDS-PAGE was done using 25 μg of cell lysate (cell lysis buffer: 50 mM TRIS-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50mM sodium fluoride, 0.5% Triton X-100, 1mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and a protease inhibitor cocktail) and resolved using 12 % acrylamide/Bis gels under denaturing conditions at 120 Volts for 90 min. Proteins were transferred onto nitrocellulose membrane (BioRad) at 100 Volts for 60 min and the membranes probed using the primary antibodies at the manufacturer's recommended concentrations. The membranes were washed 5 times with TTBS and probed with the appropriate HRP labeled anti-species secondary antibodies (Jackson Laboratories) for 1 hr. The membranes were washed 5 times with PBST and were developed using ECL prime kit (GE Healthcare, 45002401) and visualized using MultiImage 2® and FluorChem software (Alpha Innotech).

3.7 IL-8 ELISA

Ready-Set-Go ELISA (eBioscience; cat. No. 88-8086-22) was used as per manufacturer's directions to measure interleukin-8 (IL-8) secretion by endothelial progenitor cells. Briefly, Corning Costar 9018 96-well ELISA plates were coated using 100 μL/well of capture antibody in coating buffer. The plates were incubated overnight at 4 ºC. After incubation the plates were washed 5 times with 250 μL/well 1X PBST. The plates were blocked with 200 μL/well using 1X assay diluent and incubated at room temperature for 1 hour. The plates were washed 5 times with 250 μL PBST. One hundred μL of the test samples and standards were added to wells and the plates incubated overnight at 4 ºC. After incubation the plates were washed 5 times using 250 μL PBST and 100 μL/well of Avidin-HRP-antibody were added to the wells and the plates were incubated at room temperature for 30 min. The plates were then washed 7 times using 250 μL PBST, followed by the addition of the substrate solution 1X TMB and the plates were incubated for another 15 min at room temperature. The reaction was stopped by the addition of 50 μL of 2 N H_2SO_4. The plates were read at 450 and 570 nm, and then the values of 570 nm were subtracted from those of 450 nm.

3.8 Nitric Oxide Assay

The nitric oxide concentration released by endothelial cells was determined using nitric oxide synthase (NOS) detection kit (Sigma-Aldrich,

FCANOS1) according to the manufacturer's instruction. Briefly, 50 thousands cells were seeded into each well of a black, clear bottom, 96 well plate and incubated overnight 37 ˚C, 5 % CO_2. The next day the wells were washed 2X with PBS and 200 μL of reaction mixture (8 μM 4,5 diaminofluorescein diacetate DAF-2 DA, 2 μM β-NADPH, 100 mM L-arginine) were added to each well in presence or absence of 1 mM of the NOS inhibitor L-NMMA. The plate was incubated for 2 hours at room temperature in the dark and the fluorescence intensity was measured at the wavelengths 485 – 527 nm.

3.9 Flow Cytometery

The EPC were grown in 6-well tissue culture plates coated with 0.1 % gelatin. The cells were collected by centrifugation and resuspended in 500 μL of wash buffer (PBS, 1 % BSA) after the cells were fixed (PBS, 4 % formaldehyde) for 10 min at RT. For extracellular antigens the cells were incubated with the primary antibodies for 1 h at RT after the cells were blocked with blocking buffer (PBS, 2.5 % BSA, 1 % non-specific IgG) for 1 h at RT. The cells were washed 3X with wash buffer and incubated with species-specific fluorescently labeled secondary antibodies for 30 min. The cells were washed 3X and resuspended in 400 μL of wash buffer and analyzed using the FACScalibur at the Faculty of Medicine and Dentistry Flow Cytometery Facility. For intracellular antigens, the same

procedure was applied but before the blocking step the cells were permeabilized with ice-cold 100 % methanol for 30 min on ice.

3.10 MTT assay

The MTT assay was used to evaluate the cell killing activity of the lectin-saporin conjugate and the method used according to [335]. Either porcine aorta endothelial cells (PAEC) or mouse cardiovascular endothelial cells (MCEC) were seeded at 50 thousands cells/well in a 96-well tissue culture plate in triplicate and treated with medium, PBS, 10 ng/mL TNFα and 3 μg/mL cyclohiximide, 10 μg/mL MOA lectin, 10 μg/mL saporin, 10-50 μg/mL Gala1-3Gal (V-Labs, Inc. LA), and a range of concentrations of MOA lectin-saporin conjugate (0.1 – 20 μg/mL). The PAEC cells were incubated overnight at 37 ˚C and 5 % CO_2. The next day the cells were washed 2X with PBS and 100 μL of 0.5 mg/mL MTT were added to each well and the plate was incubated for further 4 h at 37 ˚C and 5 % CO_2. After incubation the plate was centrifuged at 1000g for 10 min and the MTT solution was removed by inverting the plate and blot onto paper towels. The MTT product (Formazan) was solubilized with 100 μL DMSO for 10 min at RT and the plate was read at 570 nm.

3.11 Two-Dimensional Tube Formation Assay

The matrigel basement membrane matrix (BD Biosciences, 356234) was diluted 1:5 with PBS and 250 μL were placed in a 24-well tissue culture plate and incubated at 37 ˚C until the matrigel formed a

semi-solid gel. Twenty thousands EPC or HUVEC were placed on top of the gel and incubated at 37 ˚C and 5 % CO_2 for 24 h.

3.12 Uptake of Dil-Ac-LDL and Ulex Lectin Binding

The ability of EPC to uptake acetylated low density lipoprotein and the binding of *Ulex europaeus* agglutinin was performed according to the protocol described in [336]. The acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate was purchased from Biomedical Technologies Inc. (BT-902). The FITC labeled *U. europaeus* agglutinin was obtained from Sigma-Aldrich (L-9006).

3.13 EdU Proliferation Assay

Cellular proliferation was evaluated using Click-iT® EdU (5-ethynyl-2'-deoxyuridine) flow cytometry assay kit (Invitrogen, C-10420) as per manufacturer's instructions. Briefly, hEPC treated with siRNA were incubated with 10 μM EdU and incubated at 37 ˚C and 5 % CO_2 for 24 h. The cells were washed with wash buffer (PBS+ 1% BSA) and fixed with Click-iT fixative for 15 min at RT in the dark. The cells were later washed and permeabilized with Click-iT Triton X-100 based permeabilization buffer for 30 min at RT in the dark. The EdU was detected by Alexa-488 antibody provided by the kit and the proliferating cells were analyzed by flow cytometer.

3.14 Chemotaxis Assay

The cell migration assay was performed using 24 well Transwell plate with 8 um pores (Corning, 3422). The EPCs (treated with p110β siRNA, control siRNA, or left untreated) were seeded at a density of 10^5 cells in the insert in the presence of incomplete EBM2. Complete EBM2 (with SingleQuots kit and 10% FBS) was placed in the bottom chamber. To eliminate the concentration gradient of the chemotactic factors, in some wells complete EBM2 was placed in the top insert and in the bottom well. The plate was incubated at 37 ˚C and 5 % CO_2 for 24 h. To determine the number of cells that migrated into the bottom well, the insert was washed once and the receiver well 2X with PBS. 350 μL Calcein AM diluted in cell dissociation solution (Sigma-Aldrich, C5914) were added to the bottom well and the insert was placed back in the well and the plate was incubated at 37 ˚C and 5 % CO_2 for 1 h. The inserts were removed and the plate was read at 485-527 nm using fluorescent plate reader (Fluoroskan, Thermo Scientific).

3.15 siRNA Transfection

All siRNA sequences used in this thesis were purchased from Qiagen (see Table 3.3). The gene knockdown procedure was based on Qiagen HiPerFect (301705) Transfection protocol. The hEPC were transfected on two consecutive days with 10-50 nM siRNA at 50-60 %

confluent for 5 hours. The gene knockdown was evaluated by Western blot and/or real time PCR after 72 h.

3. 16 Three-Dimensional Angiogenesis Assay

The study of sprouting angiogenesis of hEPC *in vitro* was determined by using the protocol described by Nakatsu [172] with some modifications. hEPC were mixed with collagen coated Cytodex 3 microcarrier beads (Sigma-Aldrich, C3275) and incubated at 37 ˚C and 5 % CO_2 for 4 h with shaking every 20 min. The coated beads were then transferred to T25 tissue culture flasks and incubated for further 2 hours. The coated beads were washed with PBS 2X and mixed with 2 mg/mL fibrinogen (Sigma-Aldrich, F4753) and 0.15 U/mL aprotinin (Sigma-Aldrich, A1153). Five hundred μL of the hEPC coated beads and fibrinogen solution were mixed with 0.625 U/mL thrombin (Sigma-Aldrich, T4648) in a 24-well tissue culture plate and incubated at 37 ˚C and 5 % CO_2 for ~15 min until the fibrin gel hardened. One mL of EBM2 was added to each well and sprouts formation were analyzed after 18-24 hours. In some experiments 20 thousand fibroblasts/well were added on top of the gel.

3.17 *in vivo* Vasculogenesis by Matrigel Plugs

The *in vivo* study of vasculogenesis was performed according to the method described in [337]. Briefly, growth factor reduced matrigel (BD Biosciences, 356231) was mixed with EPC (treated with 50 nM PI3-kinase catalytic subunit p110β siRNA, control siRNA, or left untreated) and with

human aorta smooth muscle cells (Cell Application, Inc. 311-500) at a ratio 75 EPC : 25 SMC. The matrigel plugs were injected into Rag 2 mice (Taconic) subcutaneously and retrieved after one week. The plugs were placed in OCT and snap frozen in liquid nitrogen. Five μm sections were stained with anti-human and -mouse CD31 and visualized using Leica fluorescent microscope. The capillaries that are formed by human cells were counted in 20 fields of view under 20X/0.45 objective lens.

3.18 MOA Lectin-Saporin Conjugation

Five mg of each pure MOA lectin and saporin were conjugated using sulfo-LC-SPDP as per manufacturer's instructions. Briefly, 5 mg of MOA lectin and 5 mg of saporin were mixed with 25 mM SPDP separately and dissolved in 1 mL of PBS containing 1 mM EDTA. The mixture was incubated at RT for 1 hour. Two desalting columns (Zeba desalt spin columns, Thermo Scientific) were equilibrated with PBS-EDTA. The proteins-SPDP mixtures were loaded onto desalting columns to buffer exchange to remove reaction byproducts and excess nonreacted SPDP. SPDP-modified saporin was reduced with 50 mM DTT to modify the sulhydryl group of SPDP. The sulfhydryl-modified saporin solution was buffer exchanged using Zeba desalting column to remove DTT. Finally, the SPDP modified protein and the sulhydryl-modified protein were mixed together and incubated for 18 hours at 4 ˚C. The resulting reaction was visualized either by silver stain (Sigma-Aldrich, PROTSIL2-1KT) or by anti-saporin antibody (abcam®, ab42903) to determine the presence of lectin-

saporin conjugates. The conjugate soultion was further processed by FPLC and a size-exclusion column (Superdex 75, GE Lifesciences). The resulting fractions were analyzed for killing activity, and the active fractions of pure L-S conjugate were pooled, concentrated, and filter-sterelized using 0.2 micron filters.

3.19 Tail Vein Injection of Biotinylated MOA Lectin

Five C57Bl/6 mice were injected via tail vein with 10 mg/Kg biotinylated MOA lectin. The animals were euthanized by cervical dislocation after 3 h and the following organs were collected: heart, lung, kidney, liver, skeletal muscle, and brain. The organs were placed in optimal cutting temperature (OCT) solution and snapped frozen in liquid nitrogen. The MOA lectin in the tissues was vizualized using Alexa Fluor 594 conjugated streptavidin and either by fluorescence or confocal microscopy.

3.20 Intra-Carotid Artery Delivery of MOA Lectin

The mice were anaesthetized using Isoflurane. A small incision was made on the left side of the neck and the underlying tissues were removed gently to expose the carotid artery. The carotid artery was ligated at the proximal and distal ends. A small cut was made at the distal portion, and the polyethylene catheter (0.011" I.D. x 0.024" O.D. Intramedic) was inserted and secured inside the carotid artery. One hundred microliters of L-S or saline were delivered using 1 ml syringes and 27 gauge needles.

The neck wound was closed with two stitches and the mice were place inside a 37 ˚C incubator for three hours before they were housed at room temperature for the remainder of the experiment.

3.21 Enzymatic Creatinine Assay

The mice sera were evaluated for creatinine concentration by Mouse Creatinine Kit (Crystal Chem, Cat# 80350) as per manufaturer's instructions with minor modifications. Breifly, 10 μL serum was placed into a 96-well tissue culture plate (Costar) and mixed with 270 μL of reagent CC1. The plate was incubated at 37 ˚C for 10 min and read at 550 nm (A1). Then 90 μL reagent CC2 were added and the plate was again incubated for 10 min at 37 ˚C and read at 550 nm (A2). The mean change in absorbance (A2-A1) from samples were interpolated using a calibration standard curve, and creatinine concentration was reported in μM.

3.22 Blood Urea Nitrogen Assay

The urea concentration in animal sera was evaluated by using Urea Assay Kit (QuantiChrom™, Cat # DIUR-500) as per manufacturer's instructions. Briefly, 10 μL serum were added to a 96-well tissue culture plate (Costar) and mixed with 200 μL of equal volumes of combined reagent A and reagent B and the plate was incubated at room temperature for 20 min and the plate was read at 450 nm.

3.23 Urine Total Protein Microassay

The urine samples were assayed for proteinuria using Genzyme (cat # 450-50) total protein microassay as per manufacturer's instructions. Briefly, 2 μL of urine were mixed with 200 μL of pyrogallol red molybdate solution were place in a 96-well ELISA plate and incubated at RT for 10 min. The plate was read at 570 nm.

3.24 Histology and Immunohistochemical Staining

Kidney, heart, lung, liver, spleen, skeletal muscle, and brain were collected from each euthanized mouse and either placed in OCT and snapped frozen in liquid nitrogen or placed in IHC zinc fixative (BD Pharmingen™). The tissues in the zinc fixative were processed at the Hisotlogy Core Facility at the University of Alberta and stained with Hematoxylin & Eosin (H & E) and with periodic acid- Schiff (PAS). For immunofluorescence staining, 5 μm frozen sections were stained for fibrin, CD31, podocalyxin, podocin, or stained with the tomato lectin *Lycopersicon esculentum* to specifically detect glomerular and peritubular capillaries. All tissues were incubated with the primary antibodies overnight and detected with a fluorescently labelled anti-species antibodies. The controls consisted of either no primary antibody or a non-specific IgG.

3.25 Microscopy

Renal tissues ~1 mm^2 from L-S or saline injected mice were fixed using Karnovsky fixative solution (Poly Scientific, New York, NY) and embedded in the low viscosity embedding Spurr's Kit (Electron Microscopy Sciences, Hatfield, PA) according to manufacturer's instructions. Ultra-thin sections were stined with uranyl acetate and lead citrate (Reynold's stain) and viewed using Philips 410 transmission electron microscope (Department of Cell Biology, University of Alberta) under different magnification settings. Confocal microscopy was performed at the Department of Medicine Cell Imaging Centre using Quorum Wave FX-X1 spinning disk confocal. Immunofluorescence imaging were taken using Leica DM IRB or Zeiss Axioplan-2.

3.26 Schistocytes Staining

Mouse blood samples were collected by cardiac puncture and 20 μL were mixed with equal volume of 0.3 % (w/v) Brilliant Cresyl blue solution. The mixture was incubated for 10 min at R.T. and then the blood was spread over a microscope slide.

3.27 Human bFGF ELISA

Human mesangial cells (Lonza, cc-2559) were grown to confluence in MsBM™ (Lonza, cc-3147) supplemented with 10 % FBS. The mesangial cells were seeded into 6-well tissue culture plate and serum starved in the presence of 2 % FBS overnight. The next day the cells were

washed 2X with HBSS and fresh DMEM supplemented with 2 % FBS and treated with 20 ng/mL recombinant human PDGFBB (eBioscience®, 14-8501) overnight, or left untreated and served as control. The supernatant from PDGFB treated cells or from control cells were used to assay for bFGF production using RayBiotech® Huamn bFGF ELISA Kit (ELH-bFGF-001) as per manufacturer's instructions. Briefly, 100 μL of standards and samples were added to a 96-well bFGF microplate and incubated overnight at 4˚C. The plate was then washed 4X with wash buffer and 100 μL of biotinylated antibody were added and the plate was incubated for 1 hour at R.T. After, 4 more washes with wash buffer, 100 μL of strepavidin conjugate was added and the plate was incubated for further 45 min at R.T. The plate was washed again 4X with wash buffer and 100 μL of Tetramethylbenzidine (TMB) substrate was added and the plate was incubated for 30 min in the dark at R. T. The reaction was stopped with 50 μl of 0.2 M sulfuric acid and the plate was read at 450 nm.

3.28 Combined Direct Flow Injection and LC-MS/MS Compound Identification and Quantification

We have applied a targeted quantitative metabolomics approach to analyze the mouse serum samples using a combination of direct injection mass spectrometry (Absolute*IDQ*™ Kit) with a reverse-phase LC-MS/MS Kit. The Kit is a commercially available assay from BIOCRATES Life Sciences AG (Austria). This kit, in combination with an ABI 4000 Q-Trap

(Applied Biosystems/MDS Sciex) mass spectrometer, can be used for the targeted identification and quantification of up to 180 different endogenous metabolites including amino acids, acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids and sugars. The method used combines the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards and other internal standards are integrated in Kit plate filter for metabolite quantification. The Absolute*IDQ* kit contains a 96 deep-well plate with a filter plate attached with sealing tape, and reagents and solvents used to prepare the plate assay. First 14 wells in the Kit were used for one blank, three zero samples, seven standards and three quality control samples provided with each Kit. All the serum samples were analyzed with the Absolute*IDQ* kit using the protocol described in the Absolute*IDQ* user manual. Briefly, serum samples were thawed on ice and were vortexed and centrifuged at 13,000x g. 10 µL of each serum sample was loaded onto the center of the filter on the upper 96-well kit plate and dried in a stream of nitrogen. Subsequently, 20 µL of a 5% solution of phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Extraction of the metabolites was then achieved by adding 300 µL methanol containing 5 mM ammonium acetate. The extracts were obtained by centrifugation into the lower 96-deep well plate, followed by a dilution step with kit MS running solvent. Mass spectrometric analysis was

performed on an API4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with a solvent delivery system. The samples were delivered to the mass spectrometer by a LC method followed by a direct injection (DI) method. The Biocrates MetIQ software was used to control the entire assay workflow, from sample registration to automated calculation of metabolite concentrations to the export of data into other data analysis programs. A targeted profiling scheme was used to quantitatively screen for known small molecule metabolites using multiple reaction monitoring, neutral loss and precursor ion scans. The metabolomics analysis was performed at Dr. David Wishart's lab and this section was written by Dr. Ruparsi Mandal (The Metabolomics Innovation Centre, University of Alberta).

3.29 Semi-Quantitative Analysis of the Renal Injury

The H&E and PAS slides were evaluated by the University of Alberta Hospital pathologist Dr. D. Rayner. The kidney sections were scored using the scale 0-4 (0 = no thrombosis, 1 ≤ 25 % thrombosed capillary loops, 2 ≤ 50 %, 3 ≤ 75 %, and 4 ≥ 75 % thrombi filled capillary loops). Capillary loops density were scored using a grid system where each square that contained an open capillary loop was counted. Thirty glomeruli were scored per each animal. The apototic cells were reported as the total number present per glomerulus.

3.30 Statistical Analysis

The statistical analyses were performed using GraphPad PRISM® (La Jolla, CA).The data were expressed as mean ± SEM, and analyzed using one-way ANOVA followed by Tukey's post hoc analysis, or 2-way ANOVA where appilcable. The non-parametric data were analyzed using Mann-Whitney *U*-test. A p value < 0.05 was considered significant.

Table 3.1: List of human primers

Gene	Forward primer	Reverse primer	Acession #
CD14	5'-GACCTAAAGATAACCGGCACCAT-3'	5'-GTAGGCGCAAGCTGGAAAGT-3'	NM_000591
MCSF	5'-GGCTGATTGACAGTCAGATGGA-3'	5'-GGATCTTTCAACTGTTCCTGGTCTA-3'	NM_000757
iNOS	5'-AAGCCCAGGTTCTACTCCATCA-3'	5'-AGGTGACCACGGCCACAGT-3'	AB022318
KDR	5'-CGGCAAATGTGTCAGCTTTG-3'	5'-GGTCACGTGGAAGGAGATCAC-3'	NM_002019
CD133	5'-AGCGATCAAGGAGACCAAAGAG-3'	5'-CTGTGTACTTTGTTGGTGCAAGCT-3'	NM_006017
GAPDH	5'-GGTGGTCTCCTCTGACTTCAACA-3'	5'-GTGGTCGTTGAGGGCAATG-3'	NM_002046
CD31	5'-AGTGGAGTCCAGCCGCATAT-3'	5'-CAGTTCGGGCTTGGAAAATAGT-3'	NM_000442
CD144	5'-TCACGATAACACGGCCAACA-3'	5'-GGGTAGGAAGTGGACCTTGGTA-3'	NM_001795
MCSFR	5'-GCCTTCCTCGCTTCCAAGA-3'	5'-CCACATGACCATTGGTCAACA-3'	NG_012303
vWF	5'-CAGTGTTCCCTATTGGAATTGGA-3'	5'-GTCTTCGATTCGCTGGAGCTT-3'	NM_000552
CD34	5'-CCTCTGTAATCAGCACAGTGTTCA-3'	5'-GACAGGCTAGGCTTCAAGGTTGT-3'	NM_001773
18S rRNA	5'-TTCGTATTGCGCCGCTAGA-3'	5'-CATTCTTGGCAAATGCTTTCG-3'	NR_003286
FSP1	5'-CCAGAAGCTGATGAGCAACTTG-3'	5'-GCAGGACAGGAAGACACAGTACTC-3'	NM_002961
ALG9	5'-GGGTTGCACGTGAGTCGAAT-3'	5'-GGAATGCTGATGATGAGCAAAA-3'	NM_024740
eNOS	5'-GCCAACGCCGTGAAGATCT-3'	5'-CATACAGGATTGTCGCCTTCACT-3'	NM_000603
aSMA	5'-CTGCCTTGGTGTGTGACAATG-3'	5'-AAAACAGCCCTGGGAGCAT-3'	X13839
ALK1	5'-ACGGCTCCCTCTACGACTTTCT-3'	5'-ATGCCGCGGACACAGCTA-3'	NM_001077401
ERG	5'-CCTGTCGGACAGCTCCAACT-3'	5'-CGTCGGGATCCGTCATCTT-3'	NM_001136154
VEGF R1	5'-AAAGGCCGTGTCATCGTTTC-3'	5'-CCATATGCGGTACAAGTCAGGAT-3'	NM_001160031
PTEN	5'-TCAGTGGCGGAACTTGCAA-3'	5'-CGTCGTGTGGGTCCTGAATT-3'	NM_000314
p110a	5'-AGAGGTTTGGCCTGCTTTTG-3'	5'-TGCCTCGACTTGCCTATTCA-3'	NM_006218
p110b	5'-CCTCGGGAAGCTACCATTTCT-3'	5'-GGAGGTTGAACATTGGGTAATTG-3'	NM_006219
p110d	5'-AGACTTCAGCTTCCCCGATTG-3'	5'-CAGCTCATCGTCCGTCAGTTT-3'	NM_005026
ESM1	5'-GGTGGACTGCCCTCAACACT-3'	5'-GTCGTCGAGCACTGTCCTCTT-3'	NM_007036
DLL4	5'-TGGGTCAGAACTGGTTATTGGA-3'	5'-CTGCAGATGACCCGGTAAGAG-3'	AB043894
Apelin	5'-CCCATGCCCACATATTGCA-3'	5'-TCAGTTTGAGGCCAGTTGACCTA-3'	NM_017413
Angiopoietin 2	5'-CGCTGCCATTCTGACTCACA-3'	5'-CCGGTTATATCTTCTCCCACTGTT-3'	NM_001147
Claudin 5	5'-GCCTTCCTGGACCACAACA-3'	5'-GTCGTACACTTTGCACTGCATGT-3'	AK222466
Rac1	5'-AAGGAGATTGGTGCTGTAAAATAC-3'	5'-CGCTTCGTCAAACACTGTCTTG-3'	NM_006908
B2M (RT PCR)	5'-CTGGCGGGCATTCCTGAAGCTGA-3'	5'-GCCAGCCCTCCTAGAGCTACCTGT-3'	NM_004048
CD34 (RT PCR)	5'-CTCAAGTTAGTAGCAGCCAAGGAGAG-3'	5'-CCGTTGTTGTCAAGACTCATGAACC-3'	NM_001773

Table 3.2: List of mouse primers

Gene	Forward primer	Reverse primer	Acession #
CD31	5'-AGGACGATGCGATGGTGTATAA-3'	5'-AAGACCCGAGCCTGAGGAA-3'	NM_008816
Lipocalin2/NGAL	5'-CCCAGGACTCAACTCAGAACTTG-3'	5'-AACTGATCGCTCCGGAAGTCT-3'	NM_008491
KIM-1	5'-CCGCAGAAAAACCCTACTAAGG-3'	5'-TGCTCACAAGGAGCAGTAGCA-3'	NM_134248
VEGFA	5'-GCAGGCTGCTGTAACGATGA-3'	5'-TCCGCATGATCTGCATGGT-3'	NM_001025250
Angiopoietin 2	5'-ACAGGGCCAATCTGTGTCAAC-3'	5'-CCAGGTCCATCCTGGTGATC-3'	NM_028333
Nephrin	5'-GCGAGGCACTTCGTGAAAC-3'	5'-CACTTGCTCTCCCAGGAACTCT-3'	AF168466
SDF1/CXCL12	5'-CCAGAGCCAACGTCAAGCAT-3'	5'-TTCTTCAGCCGTGCAACAATC-3'	NM_021704
CD144/VE CAD	5'-ATGAGCCCCCTGTCTTCCA-3'	5'-GTGCCGATCAGCGGTTTCT-3'	NM_009868
TIE 2	5'-GGGCGAAAAAAGTTGTTTGG-3'	5'-CGAACTCGACCTTCACAGAAATAA-3'	NM_013690
eNOS	5'-TGTCTGCGGCGATGTCACTA-3'	5'-CATGCCGCCCTCTGTTG-3'	NM_008713
PDGFB	5'-CCCTCGGCCTGTGACTAGAA-3'	5'-AATGGTCACCCGAGCTTGAG-3'	NM_011057
HGF	5'-CTGACACCCCTTGGGAGTATTG-3'	5'-TCCATAGGGACATCAGTCTCATTC-3'	NM_010427
bFGF	5'-GAGCGACCCACACGTCAAA-3'	5'-GTTGGCACACACTCCCTTGAT-3'	M30644
cMet	5'-ACAGTCGAGAGACGACGCAATA-3'	5'-GGGCCGTACCTCGGAGAA-3'	NM_008591
FLK1	5'-GATGCCCGACTCCCTTTGA-3'	5'-ACCACACATCGCTCTGAATTGT-3'	NM_010612
Apelin	5'-TAGCCCCTGACACTGGTTGTC-3'	5'-TTCTCCATCCCCCAAAAGC-3'	NM_013912.3
DLL4	5'-CAGCTCAAAAACACAAACCAGAA-3'	5'-GCAGTTTGCCACAATTGGACTT-3'	NM_019454.3
ESM1	5'-AGCGAGGAGGATGATTTTGGT-3'	5'-TGCATTCCATCCCGAAGGT-3'	NM_023612.3
CXCR4	5'-AGGATCTTCCTGCCCACCAT-3'	5'-TGACCAGGATCACCAATCCA-3'	NM_009911.3
PDGFbR ECD	5'-GTGCTGACACTGACCAATGTCACTG-3'	5'-CAAGGTACGGTTGTCCTTGAGCC-3'	NM_001146268.1

Table 3.3 List of siRNA used in this thesis.

Gene	Accession no.	Cat. No.
ALK1	NM_000020	SI02758392
Apelin (1)	NM_017413	SI00118013
Apelin (2)		SI02642570
ERG	NM_001136154	SI00063903
FGD5	NM_152536	SI00386680
ILK	NM_001014794	SI00288176
IQGAP1	NM_003870	SI02655268
KDR	NM_002253	SI1027400
p110α	NM_006218	SI02665369
p110β (1)	NM_006219	SI04436327
p110β (2)		SI02622214
p110β (3)		SI00085862
p110δ	NM_005026	SI02223809
PTEN	NM_000314	SI00301504
RAC1	NM_006908	SI02655051
siRNA C		1027281

CHAPTER IV
EXPERIMENTAL GLOMERULAR MICROVASCULAR ENDOTHELIAL INJURY AND REPAIR

4.0 INTRODUCTION

Thrombotic microangiopathies (TMA) are initiated by diverse processes. Among them are uncontrolled complement protein activity caused by inherited or acquired defects in complement regulatory proteins, epidemic Shiga-toxigenic (Stx) bacterial infection, or immune responses to allogeneic endothelium in the context of allogeneic bone marrow or solid organ transplantation. In the recent German epidemic of Shiga-toxigenic *E. coli* infection, the kidney glomerular microvascular endothelium was most commonly affected, however the brain microvasculature was also targeted to contribute to the high death rate observed in the epidemic [338, 339]. Similarly, transplant-associated TMA is a significant cause of morbidity, mortality, and kidney graft loss in the transplant setting [340, 341].

Acute microvascular thrombosis of the kidney glomerulus, with compromised kidney function, is a presenting feature in most cases of fulminant TMA as seen in epidemic hemolytic uremic syndrome. *In vitro* studies indicate Shiga toxin binds human microvascular endothelial cells (EC) to induce apoptosis [342, 343], but a variety of subtle effects on endothelial gene transcription induced by sublethal toxin concentrations

may also contribute to the kidney pathology [344, 345]. Study of the mechanisms of glomerular injury and repair following Shiga toxin administration to rodents, however, are confounded by predominant injury of the kidney tubular epithelial cells *in vivo* [346, 347].

In transplantation, both classical T cell-mediated and antibody-mediated (AMR) allograft rejection target the microvasculature, recognized in clinical biopsy specimens by features such as subendothelial accumulation of lymphocytes in the allograft arterial intima, glomerulitis, and complement C4d labeling of the endothelium [348]. Endothelial injury in rejection can be fulminant, typically associated with acute alloantibody-mediated attack, resulting in widespread loss of the endothelium, microvascular thrombosis, and parenchymal cell injury as a consequence of the disturbed microcirculation [349-353]. In addition, these features dominate immune responses mounted in cross-species (xeno) transplantation [354, 355], and remain a risk in transplantation into a blood group or allo-sensitized recipient [356-358].

More indolent glomerular endothelial cell (GEC) injury is also seen in transplanted organs. This may be accompanied by direct evidence of antibody-dependent cell or complement-mediated alloantibody attack, such as C4d labeling. However, chronic injury in the kidney allograft is also mediated by structural changes to the microvasculature, including transplant glomerulopathy and/or multiple lamination of the peritubular capillary basement membranes, linked to altered EC gene transcription

reflecting the EC stress response [359-362]. These observations in clinical specimens are supported by experimental evidence [363] to imply that the microvasculature endures repetitive cycles of injury and repair, which ultimately result in graft failure [364, 365]. Nevertheless, the full spectrum of features of alloantibody-mediated injury has been difficult to reproduce in animal models, hampering efforts to understand the responses of the endothelium to injury and mechanisms of microvascular repair.

We describe a mouse model of acute microvascular endothelial injury isolated to kidney glomerular endothelium with synchronized vascular damage and repair. Selective delivery of a toxin to the glomerular endothelium induces a wave of injury characterized by microvascular thrombosis and fulminant kidney failure. At sublethal doses, microvascular fibrin deposition, glomerular microvascular cell apoptosis and EC loss are evident.

4.1 Results

4.1.1 Characterization of Lectin-Saporin Conjugate

To generate a potent compound to injure glomerular microvascular endothelial cells, we conjugated the toxin, saporin, to *M oreades* (MOA) lectin (Figure 4.1b). We next verified that the lectin does not bind human cells but can bind to mouse cardiovascular endothelial cells (MCEC) (4.1 a). The lectin saporin conjugate was very effective in killing MCEC in a dose-dependent manner (Figure 4.2 a), and this killing activity was reduced by pre-incubating the L-S with the carbohydrate epitope

Galα(1,3)Gal (Figure 4.2 b). Earlier work identified glomerular endothelial binding specificity of this lectin in the mouse [30]. Preliminary testing *in vitro* determined that the lectin-saporin (L-S) conjugate bound cultured MCEC and induced apoptosis, indicated by activated caspase-3 staining after 6 hours of treatment by flow cytometer (Figure 4.3 a & b) and by immunofluorescence (Figure 4.3 c & d). This result indicates that the lectin efficiently delivered the toxin to the EC to induce endothelial apoptosis in vitro in the absence of an additional pro-apoptotic stimulus.

4.1.2 Binding Characteristics of MOA Lectin in Bl6 Mice

Next, we determined the binding characteristics of MOA lectin in vivo. Following iv injection into Bl/6 mice, we observed that biotinylated MOA lectin selectively labeled heart and kidney glomerular microvascular EC (Figure 4.4) but MOA lectin was not detected in liver, lung, muscle, and in brain (Figure 4.4). In contrast, when the biotinylated lectin was used as a staining reagent on frozen sections of normal kidney, both glomerular and peritubular capillary EC were uniformly labeled (data not shown), suggesting that the circulating lectin was largely adsorbed from the blood during transit through the glomerulus. Since we wished to avoid injury to the heart microcirculation, we injected the biotinylated lectin intra-arterially retrograde via the carotid artery (Figure 4.5). We found glomerular but no heart EC labeling using this approach (Figure 4.6 a, b). Within the glomerulus we found that lectin selectively bound the EC, but not the mesangial cells or podocytes (Figure 4.6 c).

4.1.3 Results of Intra-Carotid Artery Injection of Lectin-Saporin

Bl/6 mice were then treated with saporin, unconjugated MOA lectin, L-S (200 μg/Kg or 500 μg/Kg), LPS (50 μg/Kg), LPS + L-S, or saline. Tissues were harvested at 12h and examined by a blinded observer (DCR) for evidence of thrombotic microvascular injury. No injury was observed among animals treated with saporin, MOA lectin, or LPS alone. As shown in Figure 4.7 b,c using H & E and PAS staining, we observed diffuse glomerular capillary thrombosis involving 72 ± 14% of glomeruli in animals treated with LPS and the L-S conjugate, but no injury in the other groups (control groups that included LPS, MOA lectin, saporin, and saline) (Figure, 4.7 a , c). Glomerular capillaries stained for fibrin by immunohistochemistry, and fibrin clot was identified in glomerular capillary loops on transmission electron microscopy of LPS/L-S-treated mice (Figure 4.8 b, c). The glomerular ultrastructure revealed loss of fenestrated endothelium in the glomerular capillaries, but intact podocyte foot process distribution (Figure 4.8 b). No injury to the tubular epithelial cells was evident at this timepoint. The presence of intraluminal apoptotic cells was observed under the light microscope and by cleaved caspase-3 staining (Figure 4.9 a, b). In addition, the presence of schistocytes was evident in the L-S injured animals (Figure 4.9 c). The low concentration of LPS was included in with the L-S treatment to enhance the killing activity. LPS can stimulate immune cells, such as macrophages, to release tumor necrosis

factor alpha (TNFα) [366] that can provide an additional apoptotic signal, which can synergize with L-S to kill the glomerular endothelial cells. This data demonstrates that LPS/ L-S treatment induced rapid, selective injury of the glomerular endothelium resulting in thrombotic microangiopathy.

4.1.4 Functional Characterization of Kidney Injury

Mice were treated with LPS/L-S to characterize the functional consequence of the toxin-induced glomerular endothelial injury. We observed that animals did not tolerate treatment with L-S 500 μg/kg or higher doses, and morbid mice were euthanized before Day 4 (Figure 4.10). However, lower doses of LPS/L-S were tolerated, and tissues were examined in mice given L-S 200 μg/kg. Sublethal injury to the microvasculature was seen at Day 4 by loss of microvascular EC and regenerative changes evident in the glomerular endothelial and tubular epithelial cell compartments of the kidney. As shown in Figure 4.11, within the glomerulus we identified intralumenal apoptotic cells, frequently adherent to the capillary wall (Figure 4.11 a) and inside the tubules (Figure 4.11 b). We observed around 35 % of the glomeruli with signs of injury (figure 4.11 c) such as thrombosed capillary, apoptotic cells, and capillary loss. Mononuclear cells were more commonly seen in the glomerular capillaries of LPS/L-S treated mice, but were infrequent, and PMN were not seen. Immunostaining for EC was discontinuous in glomerular capillaries of mice treated with a sublethal dose of L-S (Figure 4.12 b). Fibrin deposition was evident around the margin of the glomerular capillary

loops in immunofluorescence microscopy (Figure 4.13 b, c). Injury in the tubular cell compartment was prominent at Day 4 as a consequence of the disordered microcirculation in the sublethally injured animals (Figure 4.14 a). Similarly, KIM-1, a marker of tubular injury was elevated in both L-S treatment groups at Day 4 after injury. At Day 7 after L-S injury, pathologic features of tubular injury were resolving, and KIM-1 expression is normalized (Figure 4.14 b, c). This data indicates that sublethal doses of L-S induce microvascular endothelial loss that is followed by evidence of tubular epithelial injury and regeneration.

Repair of the injured vascular endothelium was evident on Day 7 in tissue sections of the sublethal L-S injured group, reflected by mitotic figures within the glomerular capillaries and in tubules (Figure 4.15). Tubular repair became evident at day 4 with signs of tubular regeneration (Figure 4.15 top panel). By day 7 post sublethal L-S treatment both GEC and tubular repair were also evident by the presence of intraluminal mitotic figures (Figure 4.15 lower left panel) and tubular regenerative changes including epithelial denudement and simplification, as well as enlarged tubular nuclei (Figure 4.15 lower right panel).

The abundance of constitutively expressed EC-specific transcripts, CD31, TIE2, and eNOS, in the kidney cortex was reduced ~40% in sublethal L-S-treated vs control mice at day 4 (Figure 4.16). However, CD31 and Tie 2 gene expression level was normalized by day 7 in the sublethal injured group, but eNOS mRNA expression remained relatively

low at day 7. The podocyte-specific transcript nephrin was unchanged during the acute phase of injury between Day 0-4 in LPS/L-S-treated animals vs controls, but it was significantly up regulated by day 7 post injury (Figure 4.16).

We next assessed certain candidate growth factors that may play a role in tissue repair such as VEGFA, SDF1α, and HGF [107, 205, 367] were down-regulated at the mRNA level in the high dose, lethally injured, group at day 4, whereas HGF receptor cMet expression did not change. In contrast, PDGFB and bFGF were up-regulated in the high dose group at day 4 (Figure 4.17). To characterize microvascular EC repair we evaluated expression of characteristic angiogenic tip genes Dll4 and apelin [195]. The mRNA expression level of both Dll4 and apelin was down-regulated in the injured animals with either low dose or high dose at day 4, but the expression level of both genes was normalized by day 7 in the low dose group. On the other hand, the tip cell marker endothelial-specific molecule 1 (ESM1) mRNA expression level did not change in either low dose or high dose groups (Figure 4.18).

4.1.5 Metabolomic Analysis

We characterized the metabolic consequences of selective glomerular EC injury using LC/MS/MS of serum sampled at baseline, Day 4, and Day 7 from animals subjected to L-S-mediated endothelial injury. A panel of metabolites was used to classify the animals in an unsupervised cluster analysis (Figure 4.19 a). The Day 4 samples demonstrated clear

metabolic derangement elicited by low dose L-S treatment vs control animals, similar to lethal doses of L-S. In contrast, the Day 7 samples showed marked regression to the baseline metabolomic profile. Serum creatinine, as a measure of kidney function, was increased in the high L-S dose group, consistent with acute renal failure. The creatinine showed an intermediate rise at each timepoint in the low dose group, indicating normalization of impaired renal function was not complete by Day 7 (Figure 4.19 b). In addition, the presence of proteinuria was evident in the low dose group as compared to control (Figure 4.19 c).

Nitric oxide (NO) generated by the endothelium is a critical physiologic regulator of vascular tone that is impaired in vascular disease, and is an important mediator of angiogenesis. Metabolic derangement in the NO synthesis pathway is identified by MetaboAnalyst software [368] in mice following glomerular EC injury (Figure 4.20). We observed a decrease in the NOS3 substrate, L-arginine, and an increase in the NOS3 antagonist, asymmetric dimethyl L-arginine (ADMA), in the serum of LPS/L-S-treated mice. Further, citrulline, the metabolic byproduct of NO synthesis by NOS3, was reduced in acutely injured mice. These findings suggest marked impairment of endothelial-dependent vascular regulation occur after glomerular endothelial injury. The abnormalities resolved in the Day 7 samples harvested from sublethal LPS/L-S-treated mice, consistent with vascular repair. In contrast, a metabolic marker for oxidative stress, Met-SO, was not increased after L-S treatment.

The metabolomic study of injured and control mice sera resulted in the identification of 142 metabolites with the majority belonging to lipids (Table 4.1). The analysis of the compounds that positively correlated with serum creatinine, as a marker of renal insufficiency, are shown in Figure 4.21. Those metabolites include many glycerophospholipid molecules, ADMA and some amino acids. The concentration of many lipid molecules was increased in injured mice with high dose L-S treatment and a modest increase in the low dose treatment group as compared to control mice (Figure 4.22).

4.1.6 Discordant Transplantation

Finally, we investigated discordant transplantation in order to compare the nature of the injury caused by anti-endothelial antibodies with our lectin-saporin injury model. We transplanted guinea pig kidneys and hearts into Lewis rats, and then collected the transplanted organs at 5 and 15 min. The transplanted guinea pig hearts ceased beating completely 15 min after transplantation. The guinea pig hearts and kidneys were perfused with the rat blood for 15 min, enhanced by visible platelet clots in capillary lumens when examined by electron microscopy (Figure 4.23).

4.2 Discussion

Endothelial injury of the kidney glomerular microvasculature is a key feature of diverse diseases resulting in thrombotic microangiopathy. The consequences of these disorders on organ function represent a considerable health burden to affected individuals. In particular, Stx-

mediated GEC injury in epidemic toxigenic *E. coli* infections contributes to morbidity and mortality of both affected children and adults [338, 339]. Moreover, among transplant recipients, cell-mediated allo-immune responses, and the more recently recognized antibody-mediated rejection target the EC in kidney allografts [350]. Although low-grade injury is tolerated for a time, the cumulative burden of damage to the microvasculature ultimately limits kidney allograft survival [364, 365, 369, 370]. Animal models of this disease are needed to develop an understanding of the consequences of injury and importantly, the key repair mechanisms that might be exploited to minimize organ damage or prolong graft survival.

We describe a new model of temporally coordinated glomerular EC damage in the mouse, and characterize the immediate consequences of glomerular microvascular compromise on the kidney. With massive injury, widespread thrombosis of the glomerular capillaries is seen, followed by features of tubular injury and functional compromise of the kidney precipitating death in a few days. These features emulate the early phase of microvascular injury observed in clinical specimens with Stx-or alloantibody-mediated damage [350, 351, 353, 361, 371-373]. More limited endothelial injury, on the other hand, provokes moderate functional compromise associated with features of repair in the vascular and tubular cell compartments. We describe disturbance in the metabolome following

the glomerular microvascular endothelial injury that progressively normalizes in association with repair.

Other approaches to model thrombotic microangiopathy in the mouse have been developed. Mutations in complement regulatory proteins [374], or vascular endothelial growth factor [375] elicit chronic progressive microvascular thrombosis and ultimately death. Administration of Shiga-like toxins causes acute kidney injury in the mouse [345, 376], but appear to directly damage kidney tubular epithelial cells [346, 347]. Several lectins have been identified that broadly label the endothelial glycocalyx in rodents after intravenous injection [5, 377]. Concanavalin A, for example, binds endothelium from diverse microvascular beds in rodents, but selective injection into a renal artery followed by anti-concanavalin A antibody induces widespread microvascular injury associated with inflammatory changes in the glomerulus and peritubular capillaries [378]. In a model of allo-immune EC injury, adoptive transfer of high-titer antibody from CCR5-deficient mice sensitized to donor alloantigen has been shown to elicit microvascular complement deposition and heart allograft rejection in B-cell-deficient recipient mice [379]. These valuable models support investigations of the role of complement, alloantibody, and innate immune cell actions on endothelium, but often induce asynchronous endothelial injury. Deficient expression of one or more components of the complement system in many inbred laboratory mouse strains may confound these approaches [380]. The current model

has the strength of widespread, synchronized, and selective GEC injury resulting in TMA and compromised kidney function.

The MOA lectin has been characterized to specifically bind to the Gal-α(1,3) Gal epitope on glycoproteins expressed on the glycocalyx of glomerular endothelium [381]. In previous work, injection of the MOA lectin alone at high doses was reported to elicit proteinuria, but not features of diffuse TMA [381]. In contrast to this previous report, we observed the lectin binding to the heart microvascular EC after iv injection, but selective kidney injury could be achieved by systemic intraarterial injection. Injury of the heart microvasculature is under study to model the effects of microvascular injury and repair in that organ.

Injury of the glomerular endothelium was evident in both the high dose and low dose L-S treatment groups. We observed focal loss of the glomerular endothelium and EC injury reflected by loss of fenestration on transmission electron microscopy, in homogeneous glomerular EC immunostaining, and focal fibrin deposition in glomerular capillaries after L-S treatment. This morphological data is supported by decreased transcripts of the characteristic constitutively- expressed endothelial genes CD31, TIE2, and NOS3, but EC remain able to increase PDGFB and bFGF expressions as a stress response. In contrast, injury to the podocyte and tubular cell compartment is evident later. Taken together this data establishes primary GEC injury induced by L-S treatment, resulting in features of TMA.

Structural damage to the glomerular microvasculature is accompanied by impairment of kidney function. The serum creatinine is found to be elevated early after L-S treatment in both treatment groups indicating impaired glomerular filtration rate. Similarly we identify widespread changes in the metabolome after glomerular EC damage. Although precipitated by glomerular TMA, these effects are likely to reflect changes in kidney function.

Strikingly, we found evidence for profound metabolic disturbance of eNOS activity, occurring within hours of glomerular microvascular damage. First, we observe markedly reduced plasma concentrations of the eNOS substrate, L-arginine. Second, the endogenous eNOS inhibitor, asymmetric dimethyl-arginine, is increased in the systemic circulation early after injury. Third, eNOS within the kidney is reduced in keeping with the other endothelial markers in renal cortical mRNAs, but unlike CD31 and TIE2, eNOS does not recover by Day 7. In line with this observation, the by-product of NO production, citrulline, is reduced.

Endothelial NO production plays a central role in defending the vasculature against injury. Renal disease has been associated with dysfunction of endothelial NO production [382], while deficient eNOS activity strongly potentiates renal injury [383-386]. For example, the effect of ischemia reperfusion-induced oxidative stress on tissue is exacerbated by eNOS deficiency [387, 388]. Loss of endothelial nitric oxide synthase

expression and NO production is linked to human chronic allograft microvascular injury in the kidney glomerulus and heart [389, 390].

Interestingly, we did not observe evidence for oxidative stress that could transform NO to noxious reactive intermediates. Taken together, our data suggests an early reduction in eNOS activity within the kidney and more broadly within the vasculature occurring after selective damage to the kidney endothelium. Further, it suggests a deleterious positive feedback loop to promote renal injury may be at play.

Metabolomic analysis identified an array of glycerophospholipid molecules that correlated positively with creatinine. Given that creatinine is a marker of kidney injury, disturbed phospholipid profile may be a marker of kidney failure or vascular injury. This needs to be verified further. Interestingly, the excretion of phospholipids in the urine was previously shown to be a sensitive marker of renal injury, where the excretion of phospholipids significantly increased in contrast to other molecules such as proteins, acetyl-beta-glucosaminidase (NAG), and neutrophil gelatinase-associated lipocalin (NGAL) [391]. It is also suggested that kidney injury may alter arachidonic acid metabolites and glycerophospholipids species were present in injured rat kidney samples [392].

In summary, we describe a mouse model of selective glomerular endothelial cell injury that elicits the pathological features of TMA similar to

those seen in Stx-mediated acute kidney injury, and acute and chronic allograft rejection. Endothelial injury is associated with kidney dysfunction and pronounced metabolic changes. These include dysregulation of NO production during the injury and early repair phase that may blunt re-endothelialization of the microvasculature.

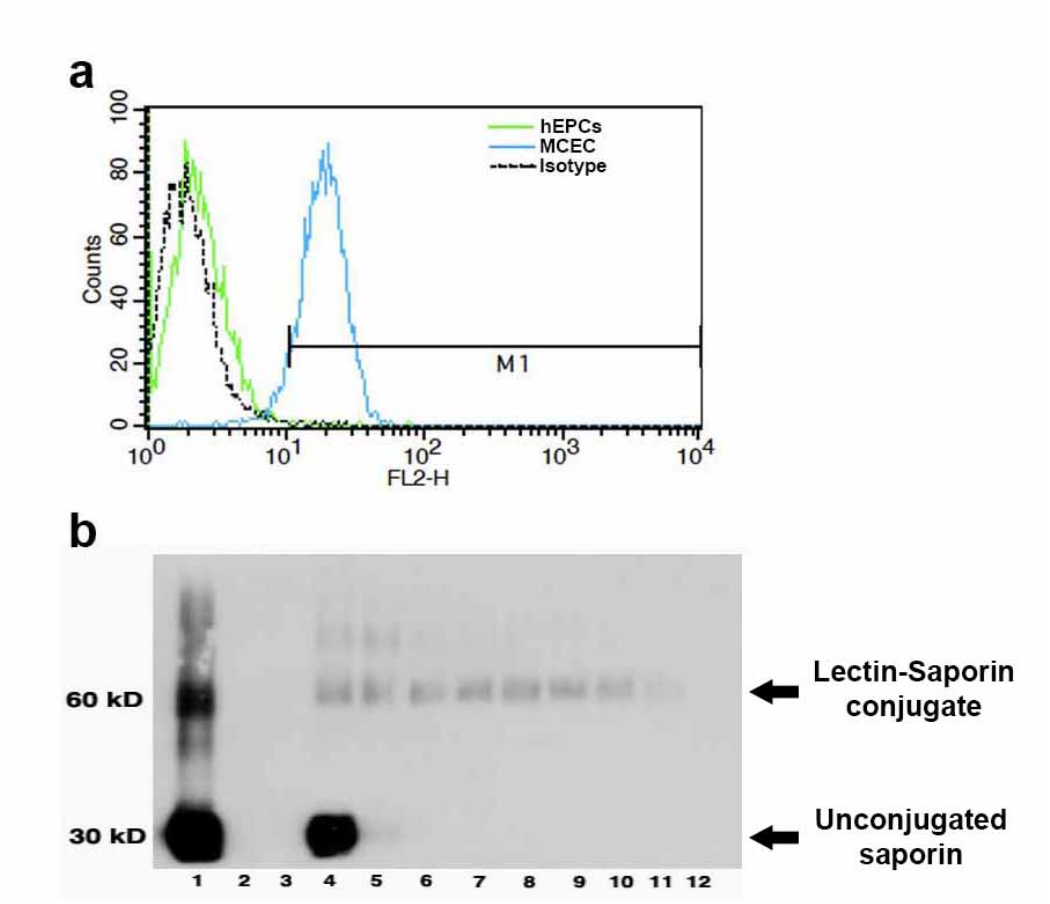

Figure 4.1: MOA lectin binding to MCEC and conjugation of MOA lectin and saporin. The binding characteristics of MOA lectin to hEPC and to MCEC were determined by flow cytometery using Biotinylated MOA lectin and an Alexa 647 conjugated streptavidin, where it shows that MOA lectin does not bind human cells (a). The conjugation of MOA lectin with saporin was achieved using the Sulfo-LC-SPDP crosslinker. The L-S conjugate is detected by SDS-PAGE and Western blot using anti-saporin antibody (saporin 30 kDa, MOA lectin 30 kDa, and L-S conjugate 60 kDa) of FPLC fractions of Superdex 75 size exclusion column (lane 1 unfractionated lectin saporin conjugate, 2-3 empty, 4-12 selected FPLC fractions) (b).

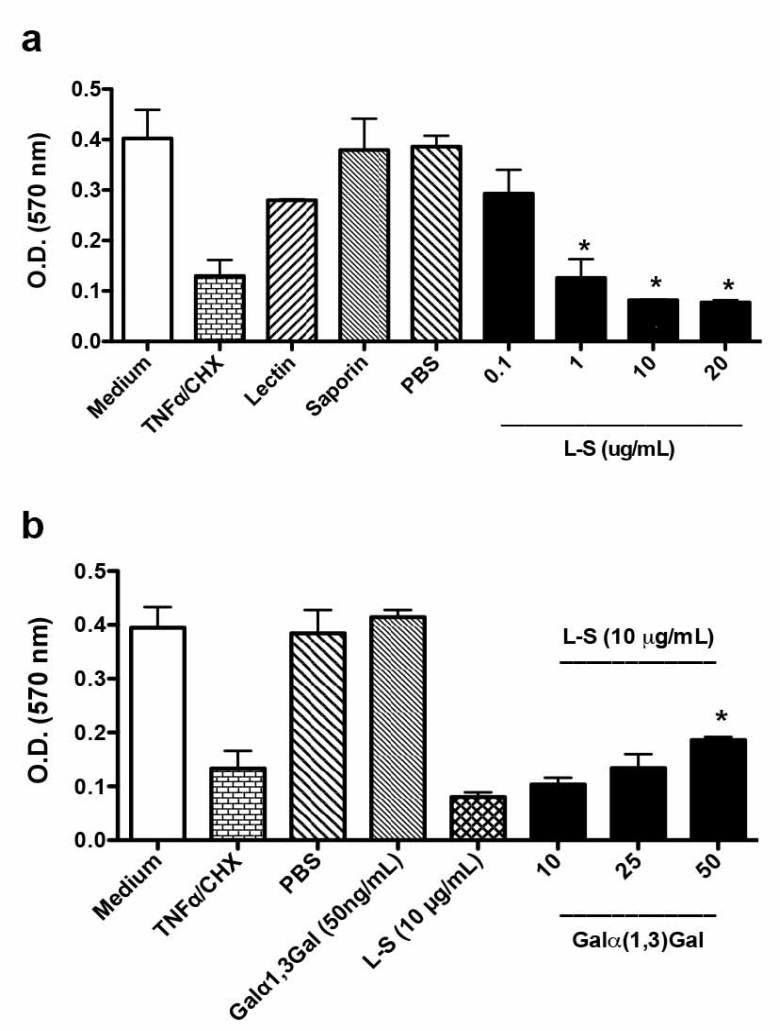

Figure 4.2: Killing activity of L-S conjugate as determined by MTT assay. Dose-dependent response of pooled L-S (a) and dose-dependent inhibition of L-S activity by the carbohydrate epitope Galα(1,3)Gal (b). MCEC cells were seeded in a 96-well tissue culture plate and treated with various concentrations of L-S conjugate, medium, 20ng/mL TNFα + 3 μg/mL cycloheximide, 20 μg/mL pure lectin, 20 μg/ mL saporin, or PBS and incubated overnight at 37˚C and 5 % CO2. The next day the cells were washed and incubated with MTT for 4 h and the formazan products were solubilized with DMSO and the plate was read at 570 nm. Each bar represents mean ± SEM; n = 3 independent experiments; $p<0.05$ vs medium control one-way ANOVA.

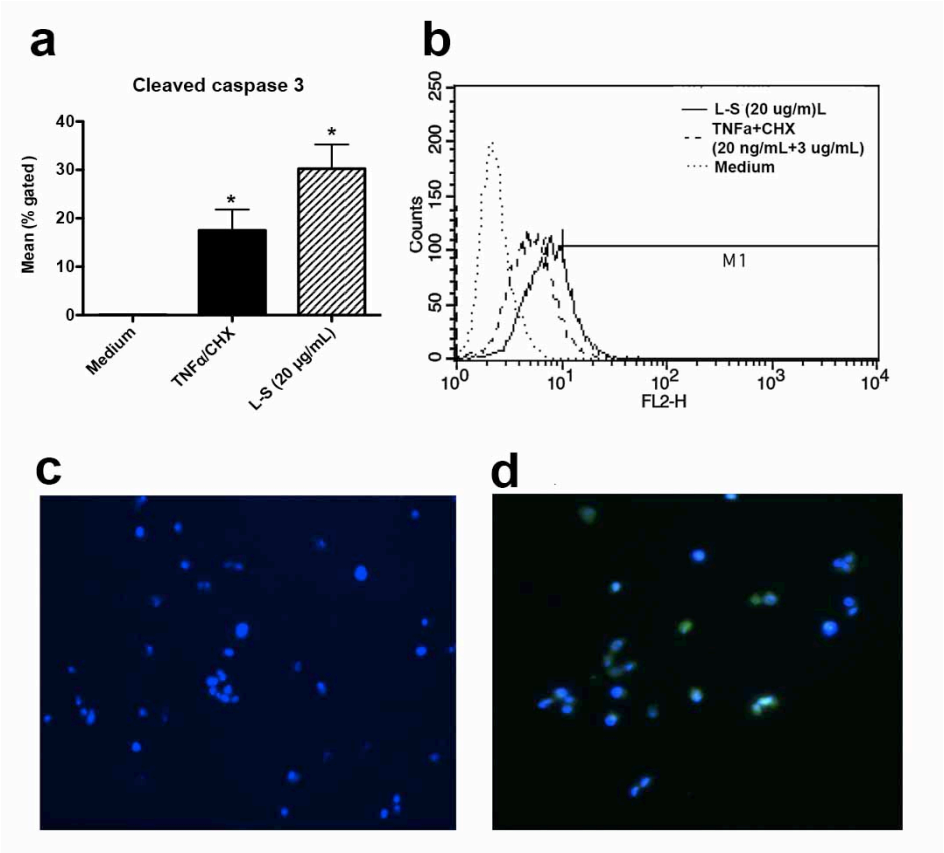

Figure 4.3: L-S induces cell death of MCEC by apoptosis as determined by cleaved caspase 3 detection. MCEC were cultured in 35 mm tissue culture dishes and treated with 20 ng/mL TNFα + 3 μg/mL cycloheximide, 20 μg/mL lectin-saporin conjugate for 6 hours or left in untreated. FITC-labeled Cleaved caspase 3 was added to the cells and incubated for 1 hour. The cells were analyzed by flow cytometer. (a) Shows the percentage of cells that expressed cleaved caspase 3. (b) A representative overlay of the flow cytometer results demonstrating the shift of fluorescence in the cells that expressed cleaved caspase 3. The cleaved caspase 3 activity was detected by immunofluorescence (c) shows a representative image from medium control cells with DAPI (blue) nuclei staining and no signal for cleaved caspase 3, whereas (d) shows a representative image from MCEC cells treated with 20 μg/mL L-S conjugate (green: cleaved caspase 3). Each bar represents mean ± SEM; n = 3 independent experiments; $p < 0.05$ vs medium control one-way ANOVA.

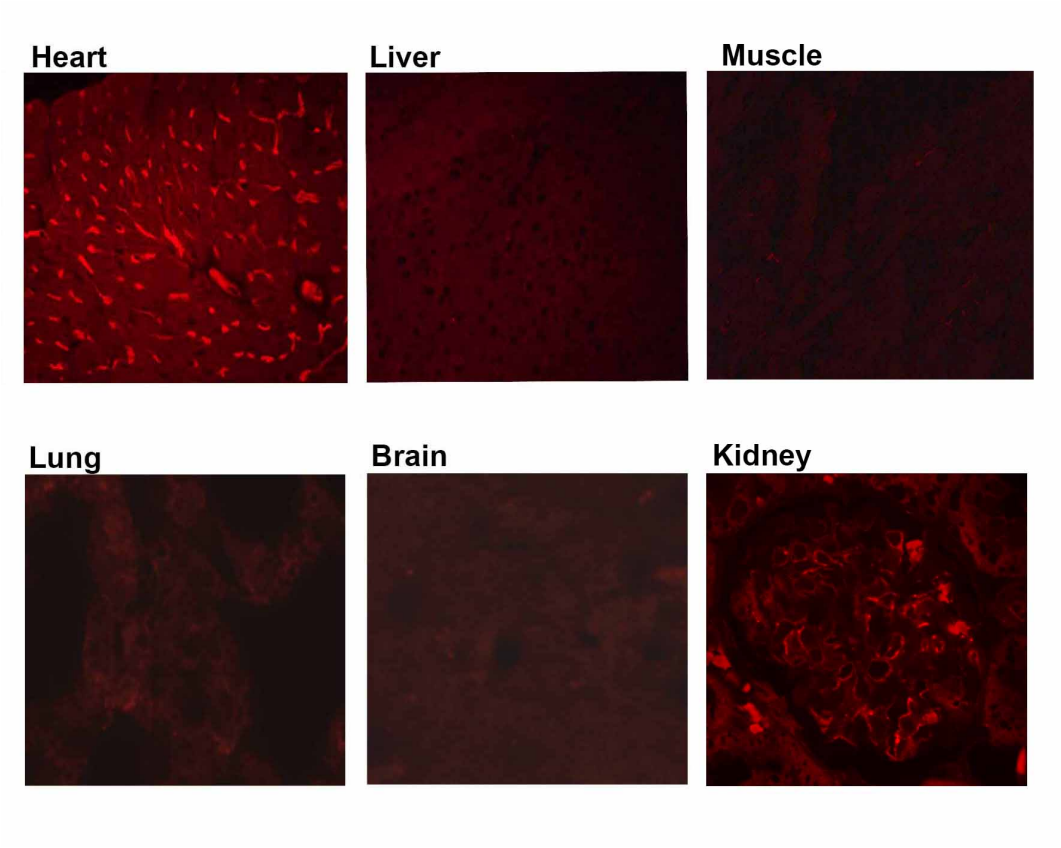

Figure 4.4: MOA lectin binding characteristics after intravenous delivery. Biotinylated MOA lectin (10 mg/mouse) was delivered via tail vein injection of C57Bl/6 mice. The animals were euthanized after 3 hours and various tissues were removed and placed in OCT. Five μm tissue sections were stained with Alexa 647 conjugated streptavidin. Shown are representative images from heart, liver, skeletal muscle, lung, brain, and kidney sections showing specific binding of MOA lectin to heart microvasculature. n=5.

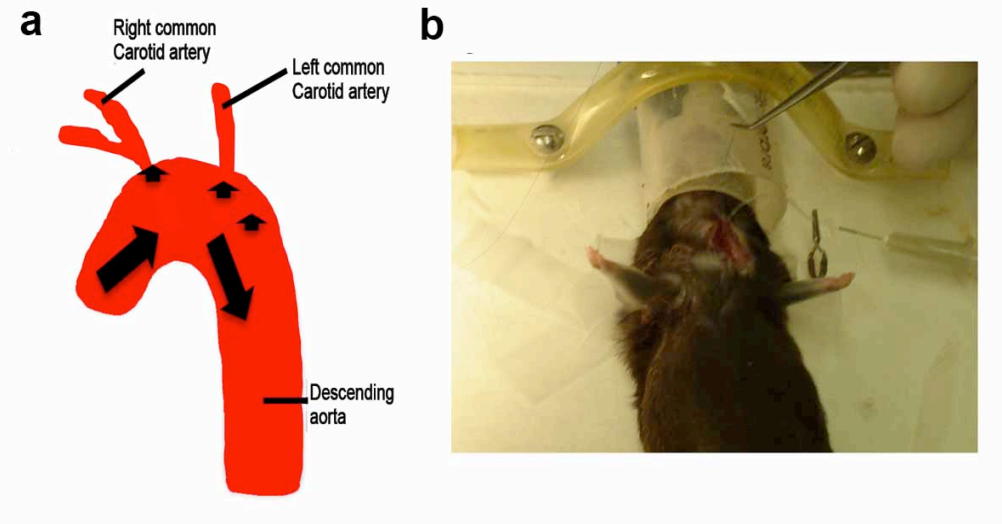

Figure 4.5: The development of the intra-carotid artery surgical procedure. The left carotid artery was cannulated to bypass the heart microcirculation and the lectin was injected through the carotid artery and down the descending aortic arch (a). (b) A representative image of a mouse with cannulated carotid artery.

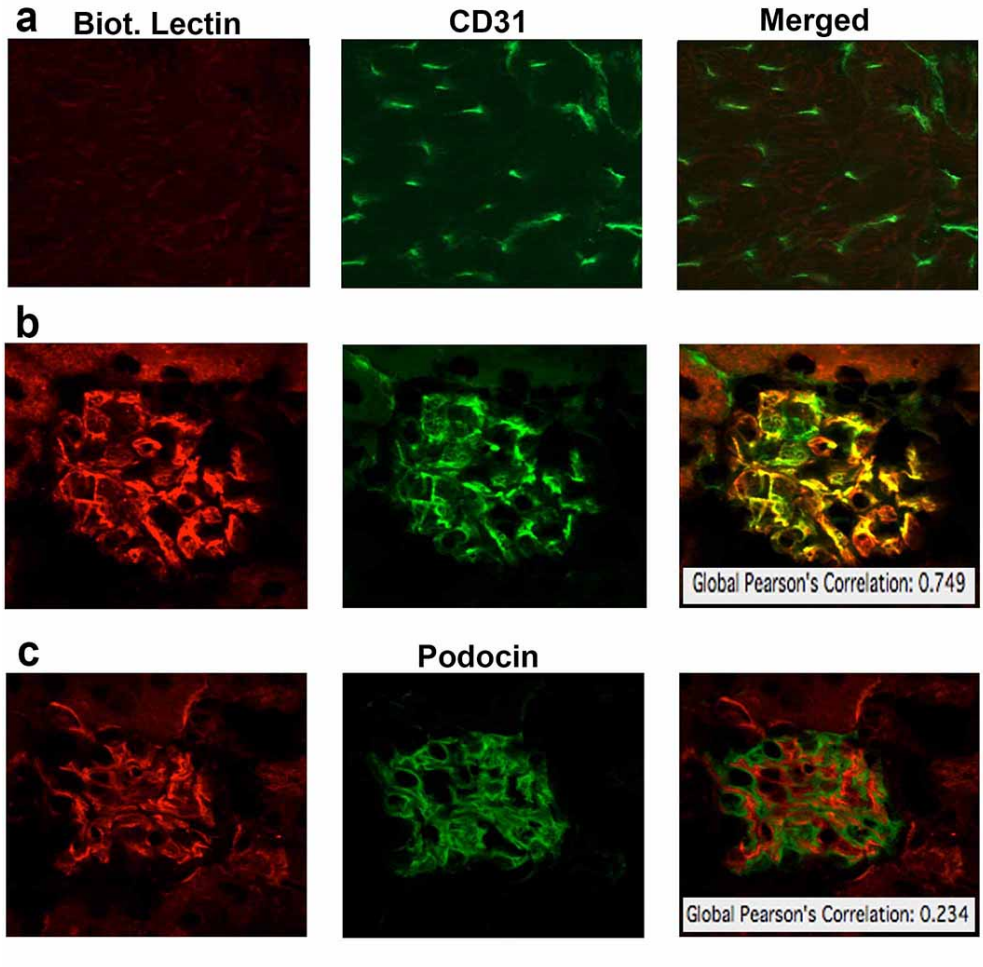

Figure 4.6: MOA lectin binding after intra-carotid artery delivery. Biotinylated MOA lectin (10 mg/mouse) was injected down the carotid artery and the mice were euthanized after 3 hours and various tissues were removed and placed in OCT. Five μm tissue section were stained with Alexa 647 conjugated streptavidin. As shown in (a) the heart tissue section reveals the lack of biotinylated MOA lectin to the heart blood vessels where the presence of such vessels is demonstrated by the endothelial marker CD31. (b) Shows the binding of the lectin to kidney capillary walls and verified by the overlapping signals from MOA lectin and CD31. (c) Demonstrates the specific binding of MOA lectin to glomerular endothelial cells as the podocytes marker podocin signal does not overlap with MOA lectin signal.

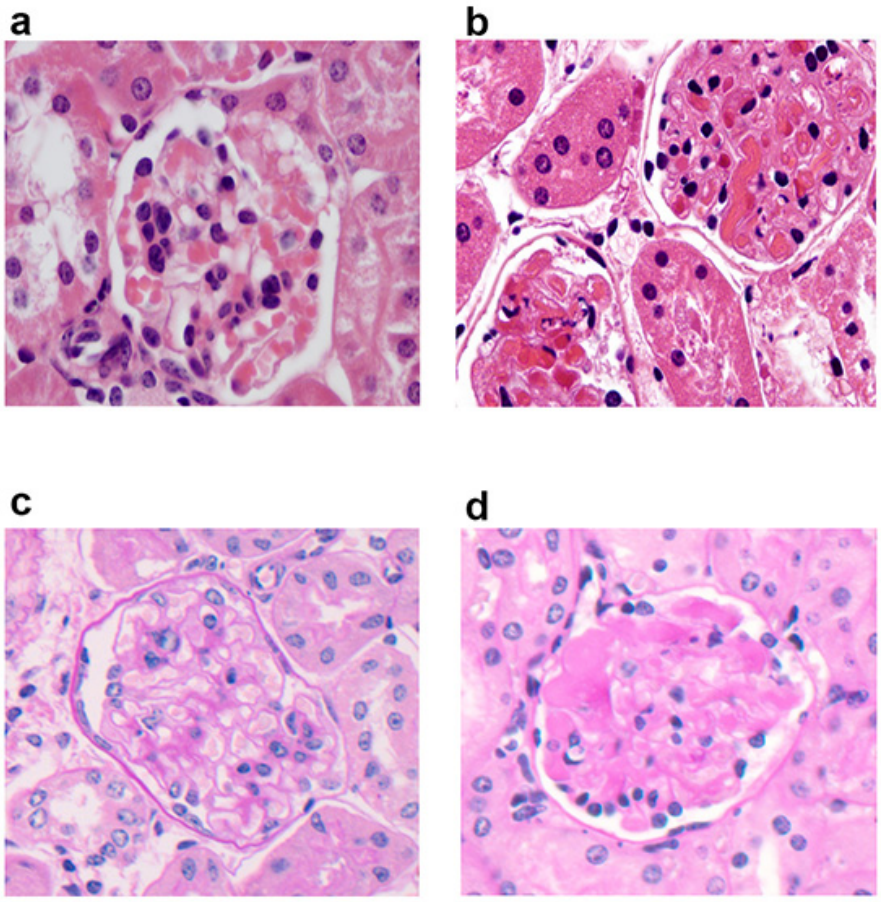

Figure 4.7: Glomerular thrombotic microangiopathy after L-S/LPS treatment. Kidneys were harvested 12h after saline (a, c) or LS (500 μg/kg + 50 μg/kg LPS) (b, d). Hematoxylin & eosin (a, b), and periodic acid-Schiff (c, d) stains reveal widespread amorphous eosinophilic glomerular capillary thrombi (b, d), whereas red blood cells are seen in patent capillaries in (a, c).

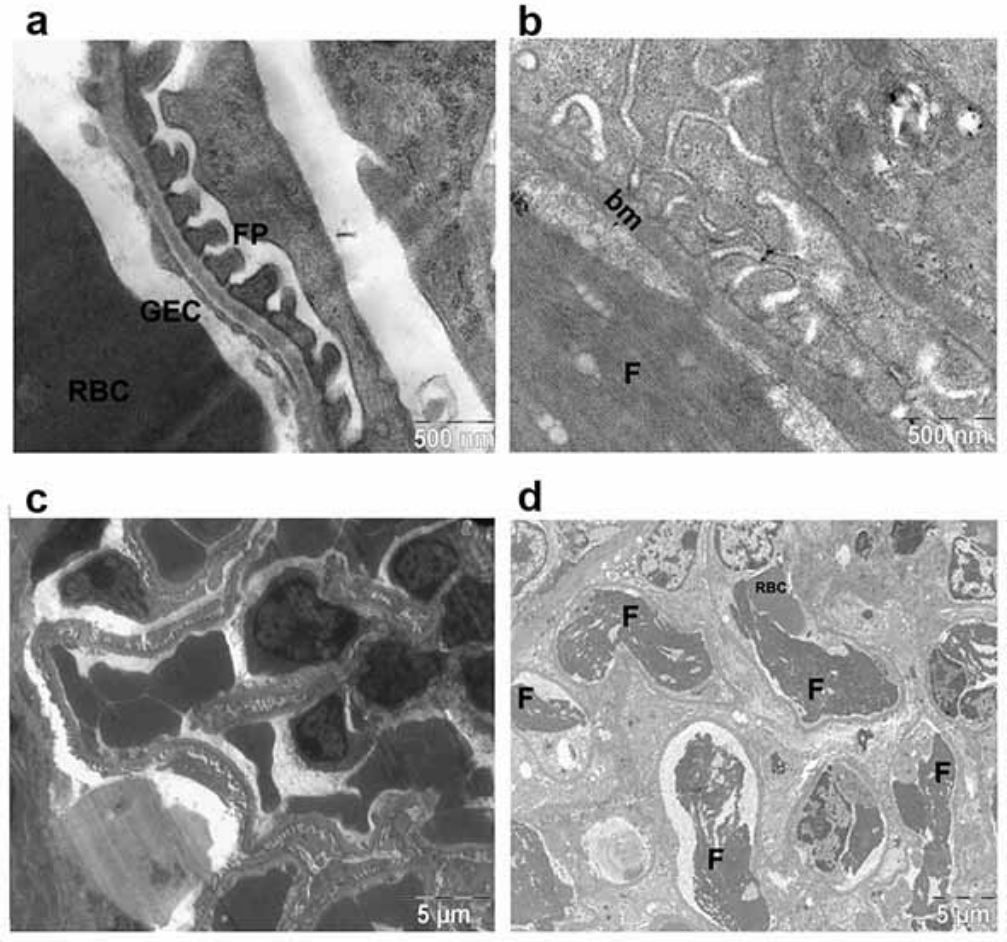

Figure 4.8: Transmission electron microscopy shows focal loss of glomerular endothelium (b), and capillary thrombosis (b, d), but preserved podocyte foot processes similar to the control (a, c). Magnification of (a, b) is 34K, whereas (c, d) are at 4.3K. (bm: basement membrane, GEC: glomerular endothelial cell, F: fibrin, FP: podocyte foot processes, RBC: red blood cell).

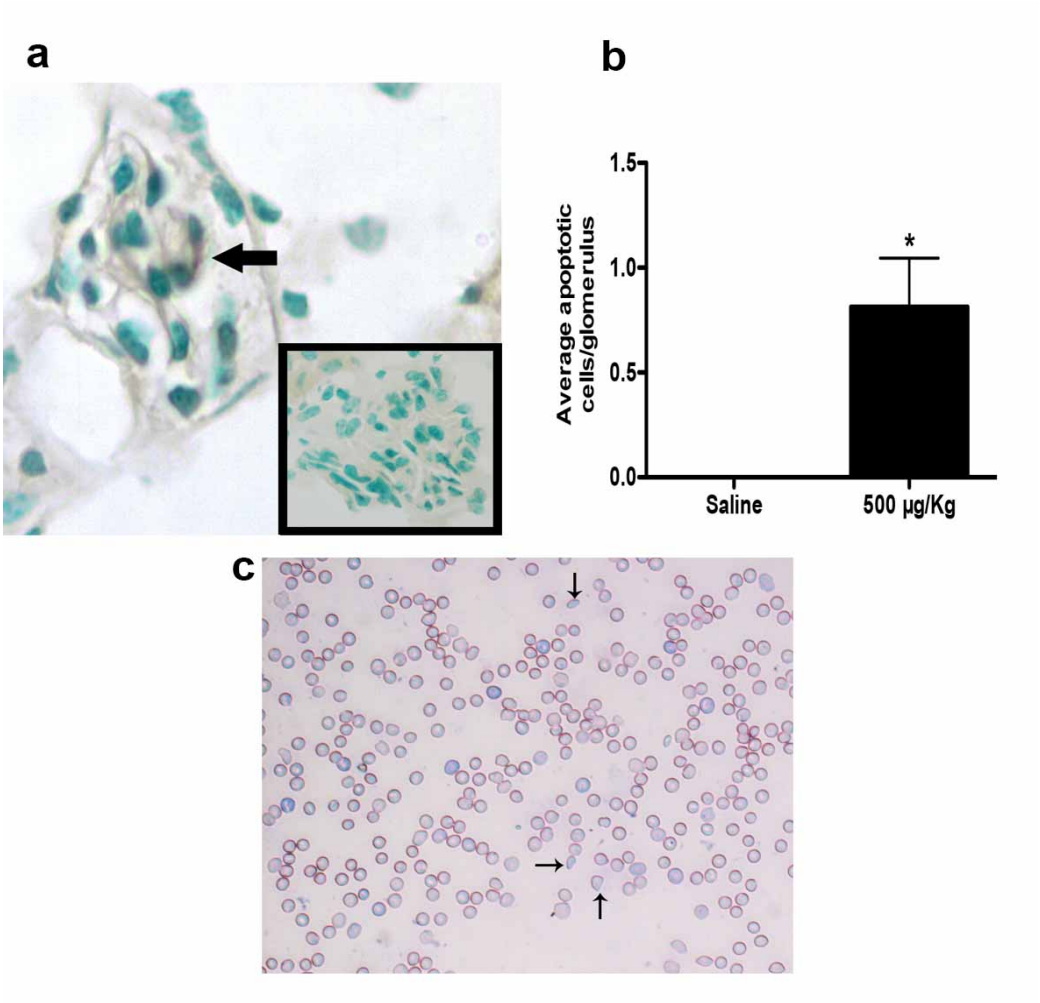

Figure 4.9: L-S conjugate induces cell death by apoptosis *in vivo*. (a) A representative glomerulus with positive cleaved caspase-3 staining (arrow), x1000 original magnification, and (b) quantitation of the average apoptotic cell/glomerulus. (c) A representative image of a blood smear indicating the presence of schistocytes (arrows), x400 original magnification. Each bar represents mean ± SEM; $n \geq 5$, $p < 0.05$ vs saline control, Mann-Whitney *U* test.

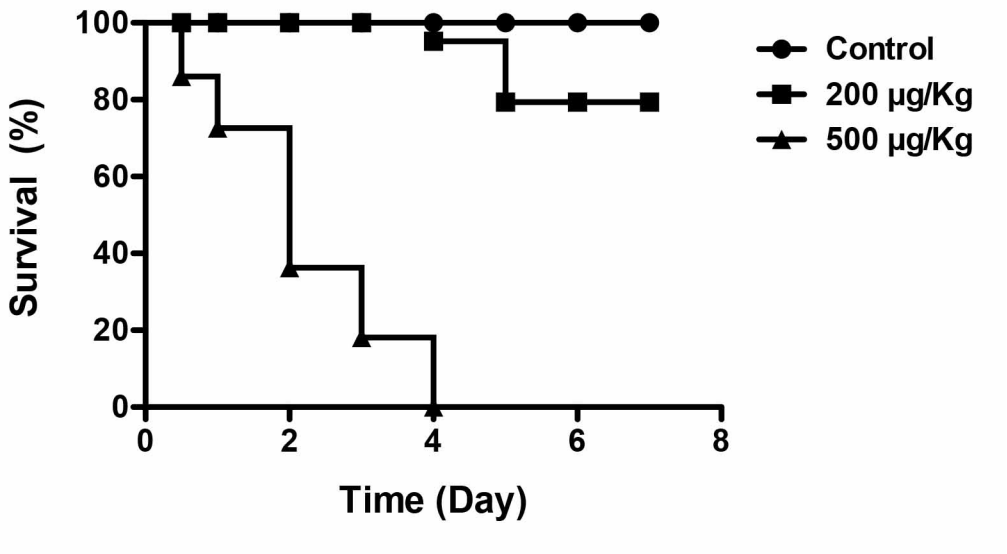

Figure 4.10: Dose-response effect of LS/LPS treatment in mice. Kaplan-Meier survival graph of animals treated with 500 μg/kg, or 200 μg/kg LS, and control (includes: LPS, saporin, MOA lectin, and saline) ($n \geq 5$ / group, $p < 0.0001$ Log-rank test).

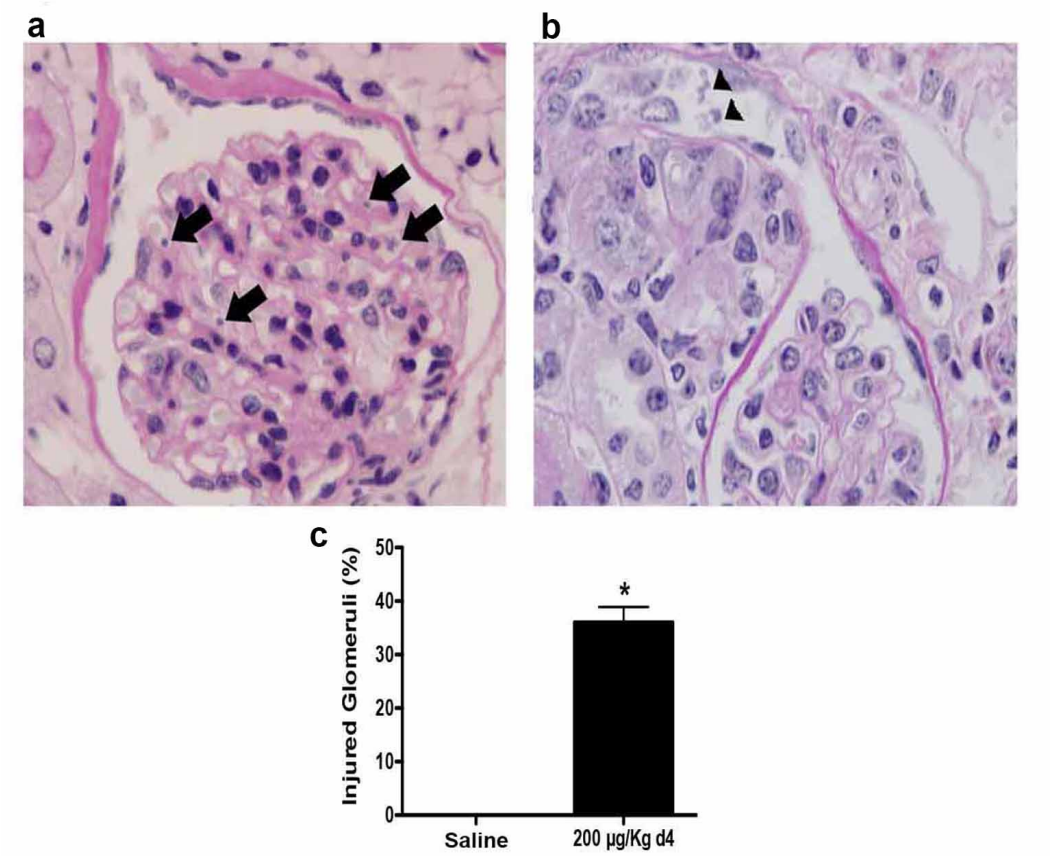

Figure 4.11: Glomerular microvascular injury after sublethal L-S treatment. Mice were treated with 200 μg/kg L-S/LPS via intra-carotid injection and were euthanized after 4 days. Kidney sections were stained with periodic acid-Schiff and apoptotic cells are seen in the glomerular capillaries (a) and tubules (b), x1000 original magnification. Injury in glomeruli (thrombosed capillaries, apoptotic cells, capillary loss) was quantitated in 30 consecutive glomeruli. Each bar represents mean ± SEM, $n \geq 5$, $p < 0.05$ vs control, Mann-Whitney *U* test.

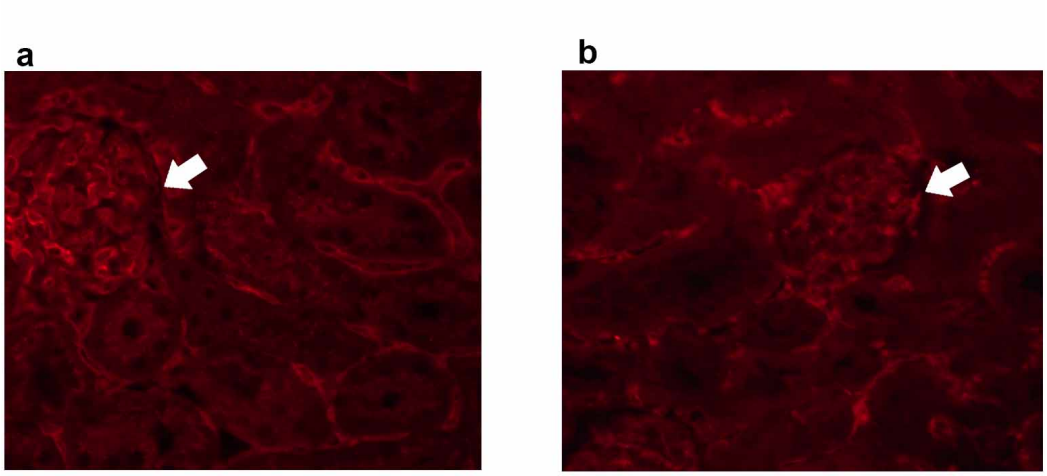

Figure 4.12: Glomerular and peritubular capillaries injury after sublethal LS treatment. Kidney sections from control (a) and mice treated with 200 μg/kg L-S (b) were stained with the endothelial cells specific tomato lectin (*L. esculentum*), x400 original magnification. The arrows indicate the location of the glomeruli. There is a noticeable decrease of the lectin staining in the injured mouse (b) as compared to control (a) indicating the loss of endothelial cells. (n = 5).

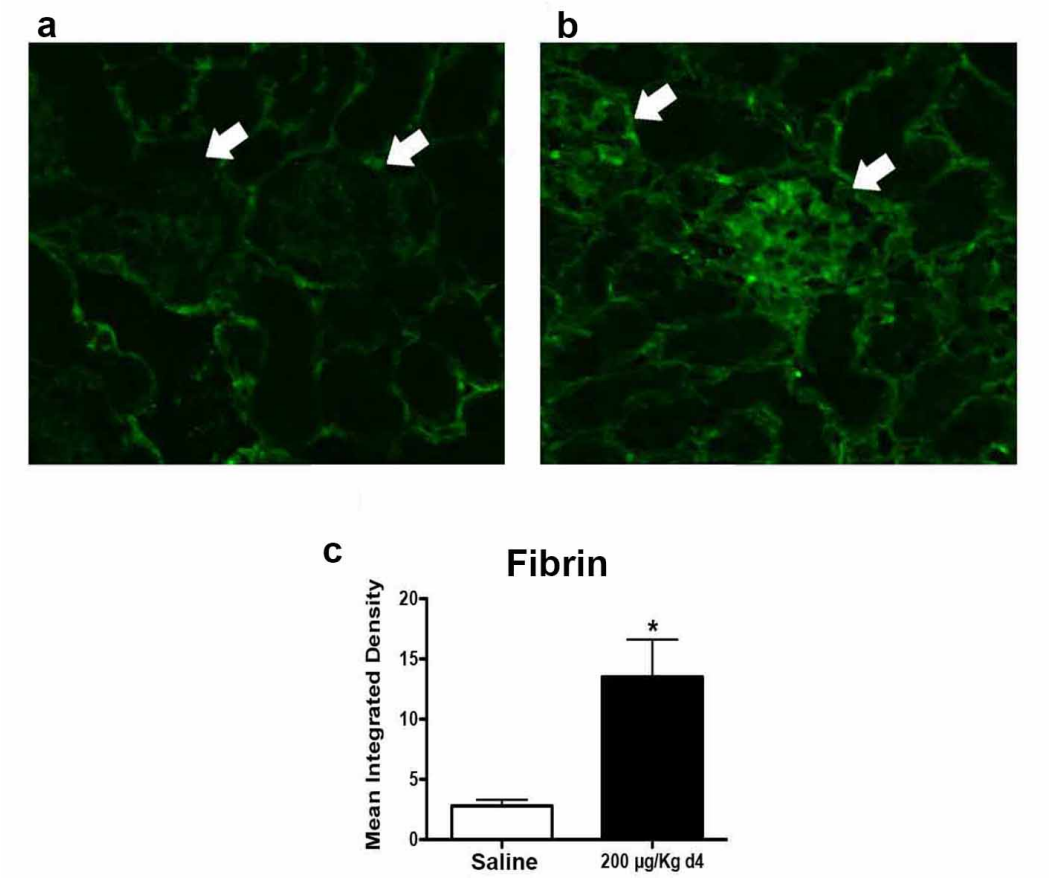

Figure 4.13: Fibrin is still present in the glomeruli of sublethally injured mice after 4 days. Direct immunofluorescence shows fibrin deposition in glomeruli (arrows) in injured mice (b) as compared to control (a), x400 original magnification. (c) Shows the quantitation of glomerular fibrin as measured by the mean integrated density of the fluorescence using Image J. Thirty glomeruli were analyzed and each bar represents mean ± SEM, $n \geq 5$, $p < 0.05$ vs saline control, one-way ANOVA.

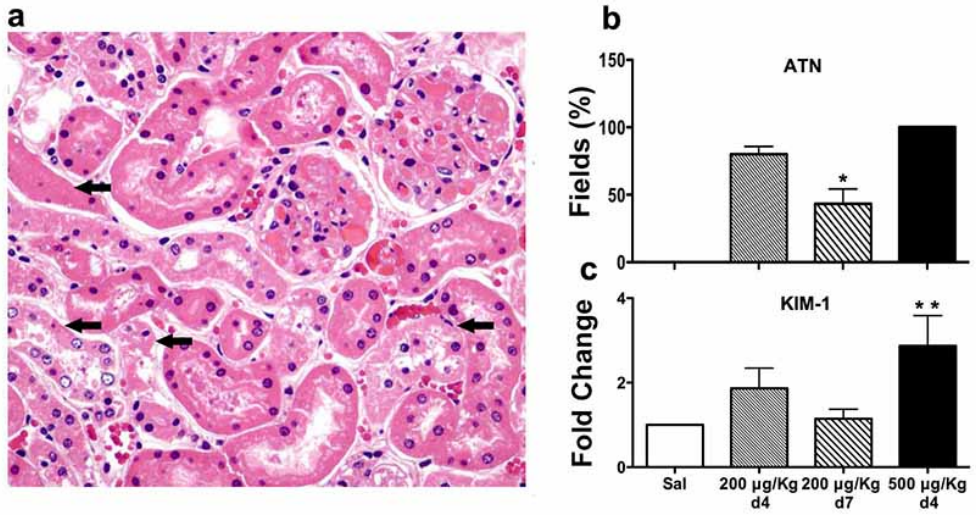

Figure 4.14: Tubular epithelial cell damage occurs after glomerular endothelial injury. Tubular epithelial cell effacement and regeneration is evident at day 4 after L-S/LPS treatment (left panel PAS x400 original magnification). Features of tubular injury and repair are quantitated in (upper right panel) as described in Methods. Expression of the tubular epithelial cell stress gene KIM-1 is quantitated by qRT-PCR in (lower right panel). (n ≥ 5 mice / group; *-$p < 0.05$ vs 200 μg/kg L-S/LPS D4 group; **-$p < 0.05$ vs. saline group). ATN: acute tubular necrosis; KIM-1: kidney injury molecule-1.

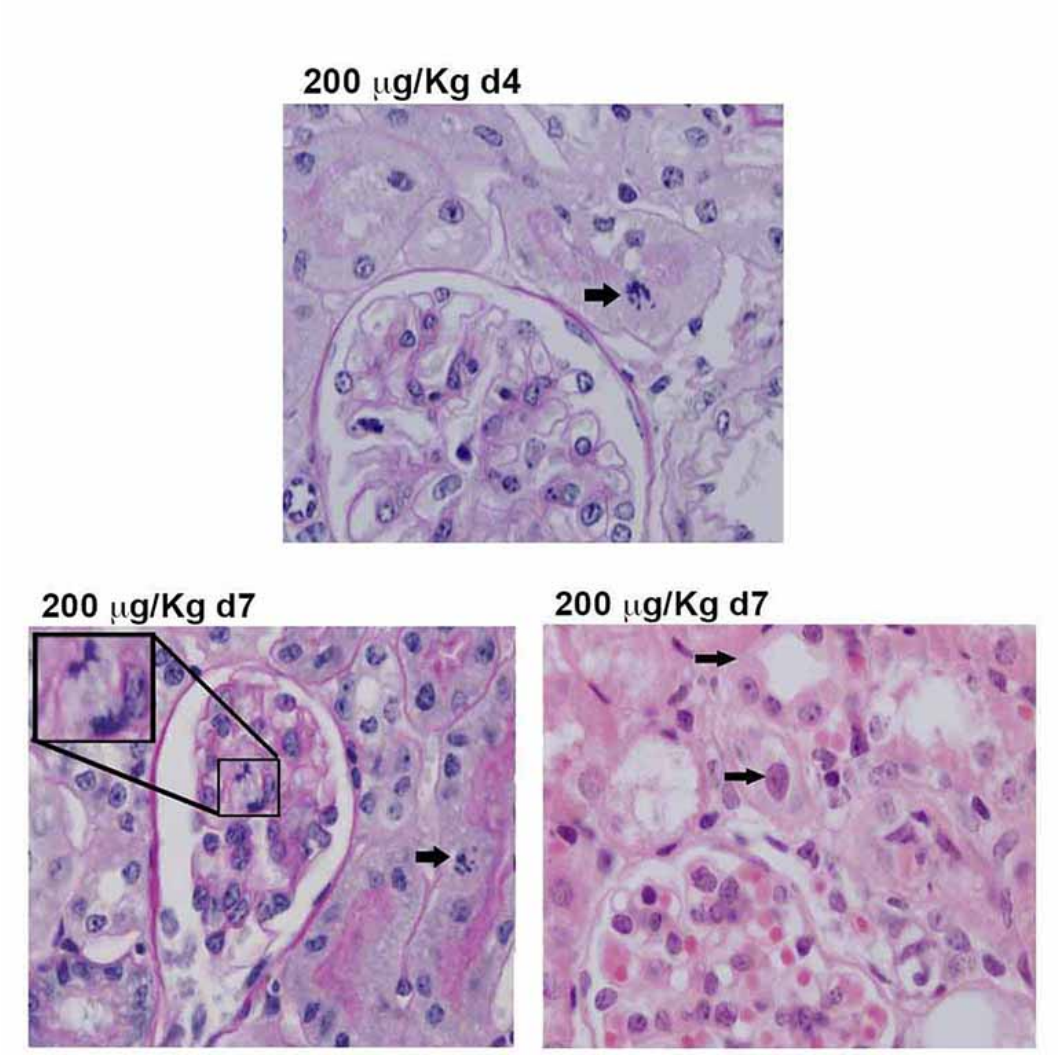

Figure 4.15: Evidence of glomerular and tubular injury and repair after sublethal dose of L-S. Left panel: A tubular cell undergoing mitosis (arrow, top panel), consistent with regenerative activity after injury, PAS, x1000 original magnification. Lower left panel: endothelial mitotic figure (inset). A tubular cell shows features of apoptosis (arrow), PAS, x1000 original magnification. Lower right panel: multiple tubular profiles show features of acute injury, including epithelial denudement and simplification. Enlarged tubular nuclei are consistent with a regenerative change (arrow), hematoxylin-eosin x 1000 original magnification.

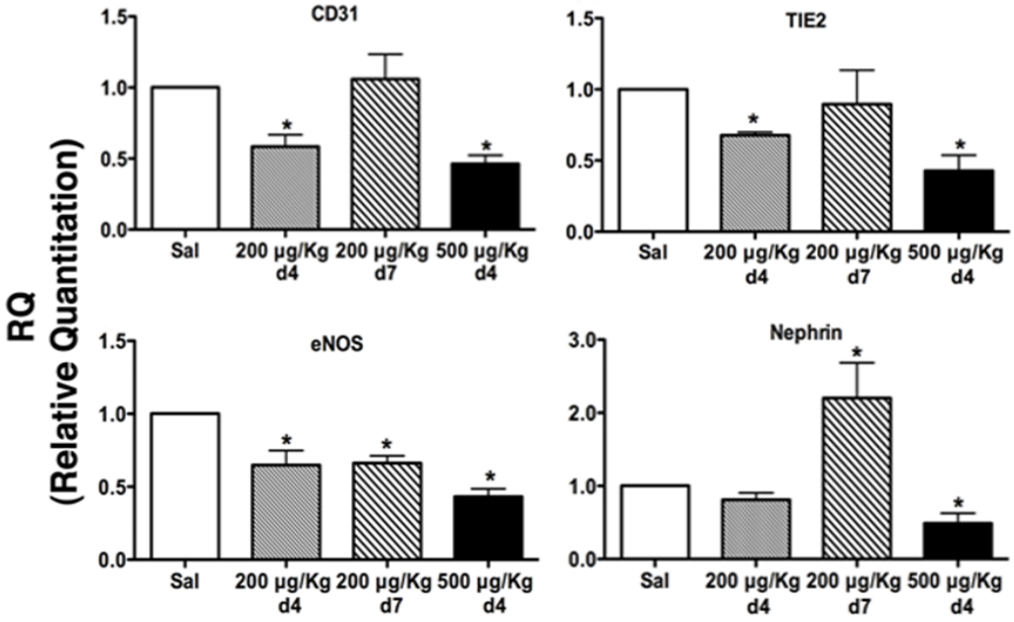

Figure 4.16: Endothelial gene expression after L-S injury. Mice were treated with saline or L-S/LPS to induce glomerular EC damage, then kidney cortex mRNA was isolated at day 4 or day 7 after injury. Constitutively expressed endothelial genes CD31, TIE2, and eNOS or the podocyte-specific gene nephrin were assessed by qRT-PCR as described in Methods ($n \geq 5$ mice / group; * -$p < 0.05$ vs saline control one-way ANOVA).

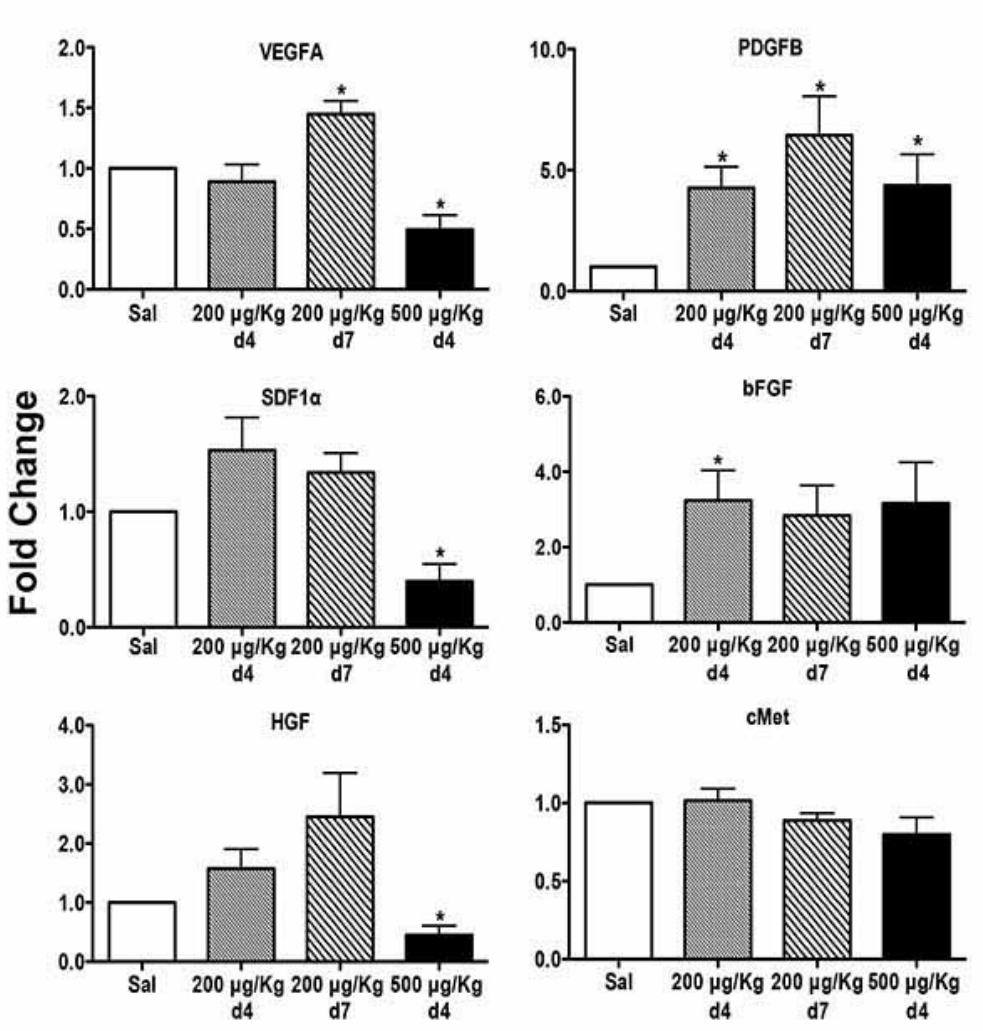

Figure 4.17: Growth factor gene expression after L-S injury. Mice were treated with saline or L-S/LPS to induce glomerular EC damage, then kidney cortex mRNA was isolated at day 4 or day 7 after injury. VEGFA, PDGFB, SDF1a, bFGF, HGF, and cMet were assessed by qRT-PCR (n ≥ 5 mice / group; * -$p < 0.05$ vs saline control one-way ANOVA).

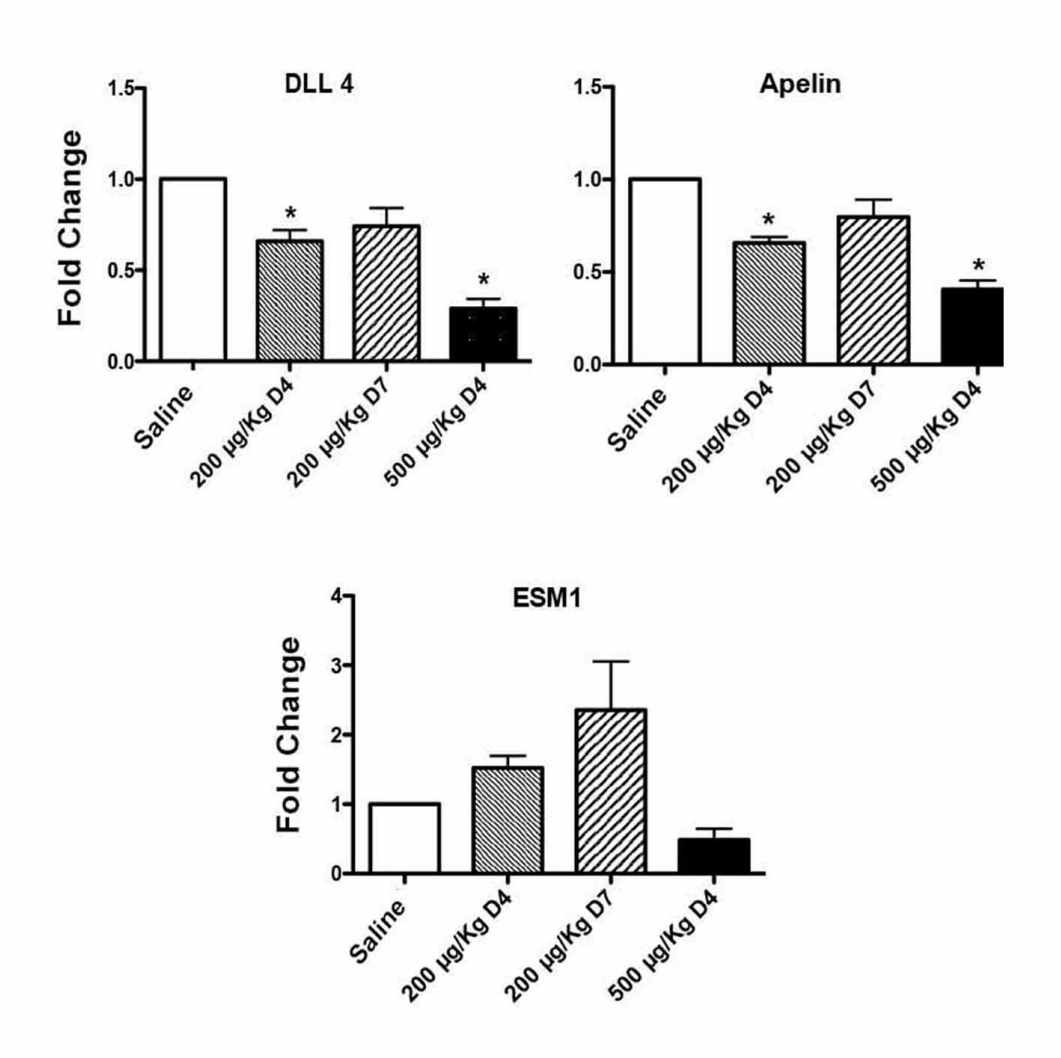

Figure 4.18: Tip cells marker gene expression after L-S/LPS injury. Mice were treated with saline or L-S/LPS to induce glomerular EC damage, then kidney cortex mRNA was isolated at day 4 or day 7 after injury. DLL4, Apelin, and ESM1 were assessed by qRT-PCR ($n \geq 5$ mice / group; * -$p < 0.05$ vs control, one-way ANOVA).

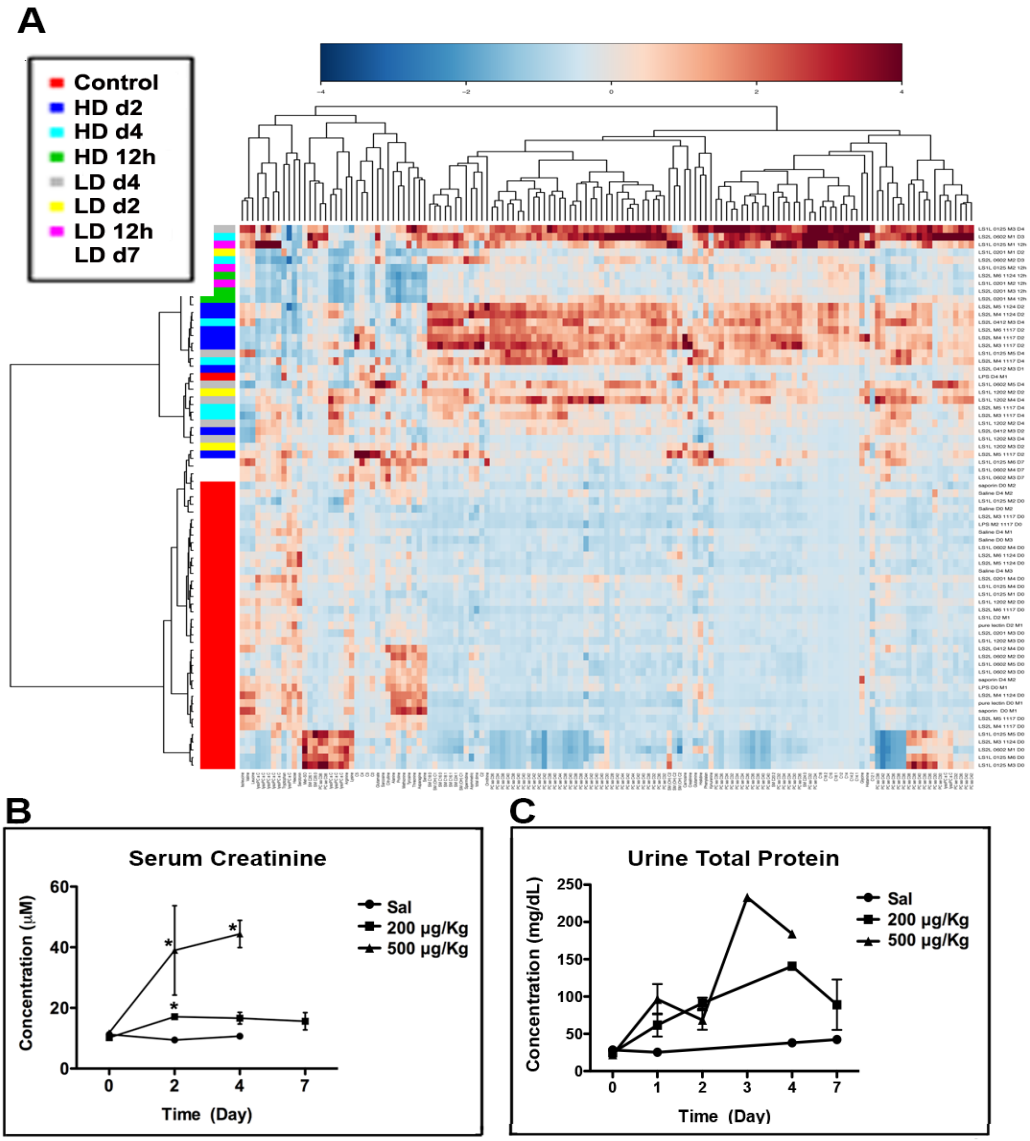

Figure 4.19: Metabolic derangement after glomerular endothelial injury. (A) Unsupervised cluster analysis of sera from mice treated with 500 μg/kg L-S/LPS (High Dose, HD), or 200 μg/kg L-S/LPS (Low Dose, LD) harvested at 12 hours, 4 days, or 7 days after L-S challenge using the Metaboanalyst software and Pearson statistical analysis and Ward clustering algorithm. Mice treated with saline, MOA lectin, or saporin alone are grouped together as controls. The panel of metabolites was analyzed by LC/MS/MS as described in Methods. (B) Kidney function is impaired in L-S-treated mice. Serum creatinine was assayed by LC/MS/MS and urine total protein was determined by pyrogallol red-molybdate method (c). (n ≥ 5 mice / group; * -p < 0.05 vs saline control, one-way ANOVA).

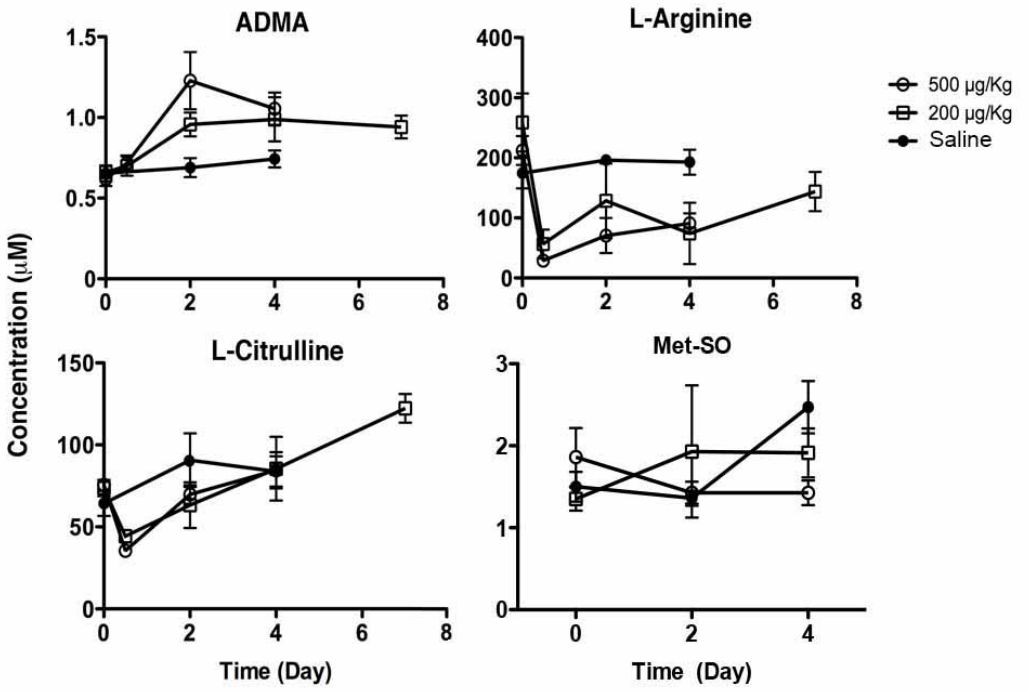

Figure 4.20: Derangement in the nitric oxide metabolic pathway after glomerular endothelial injury. Sera from mice treated with 500 μg/kg or 200 μg/kg L-S/LPS were assayed by LC/MS/MS for the endogenous eNOS inhibitor, asymmetric dimethyl L-arginine (ADMA), the eNOS substrate, L-arginine, or the by-product of NO generation, L-citrulline, and the oxidative stress marker Met-SO. n ≥ 5 mice / group; ADMA differed in both high dose and low dose vs saline control, L-arginine differed between high dose and saline control; L-citrulline differed between high dose and saline control, Met-SO was not different, $p < 0.01$ two-way ANOVA.

Table 4.1: A list of the metabolites identified in the sera samples from control and injured mice

Metabolites	Detected
Glycerophospholipids	82
Aminoacids	20
Acylcarnitines	15
Sphingolipids	14
Biogenic amines	9
Hexose	1

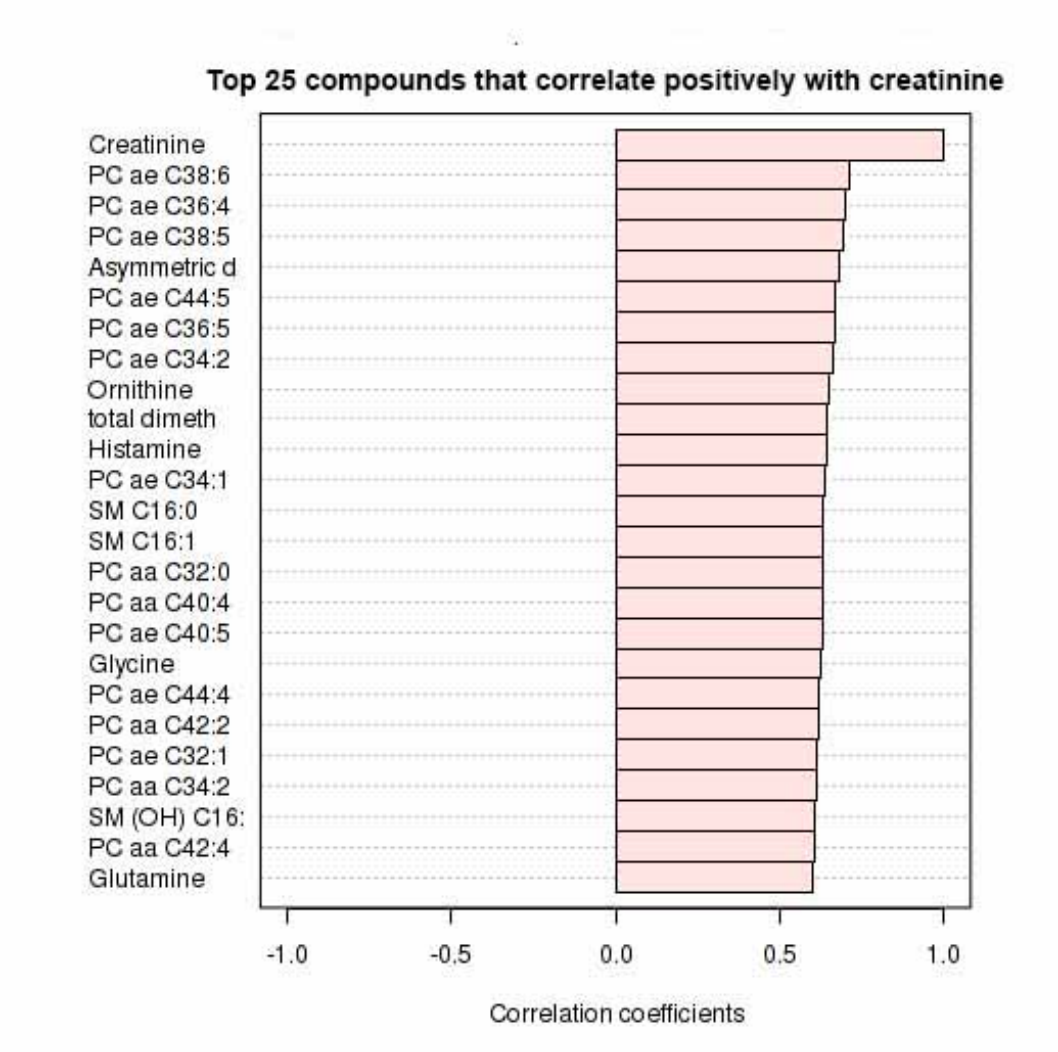

Figure 4.21: List of the top 25 metabolites that correlated positively with creatinine. The metabolites were analyzed using Metaboanalyst software and Spearman rank correlation statistical analysis.

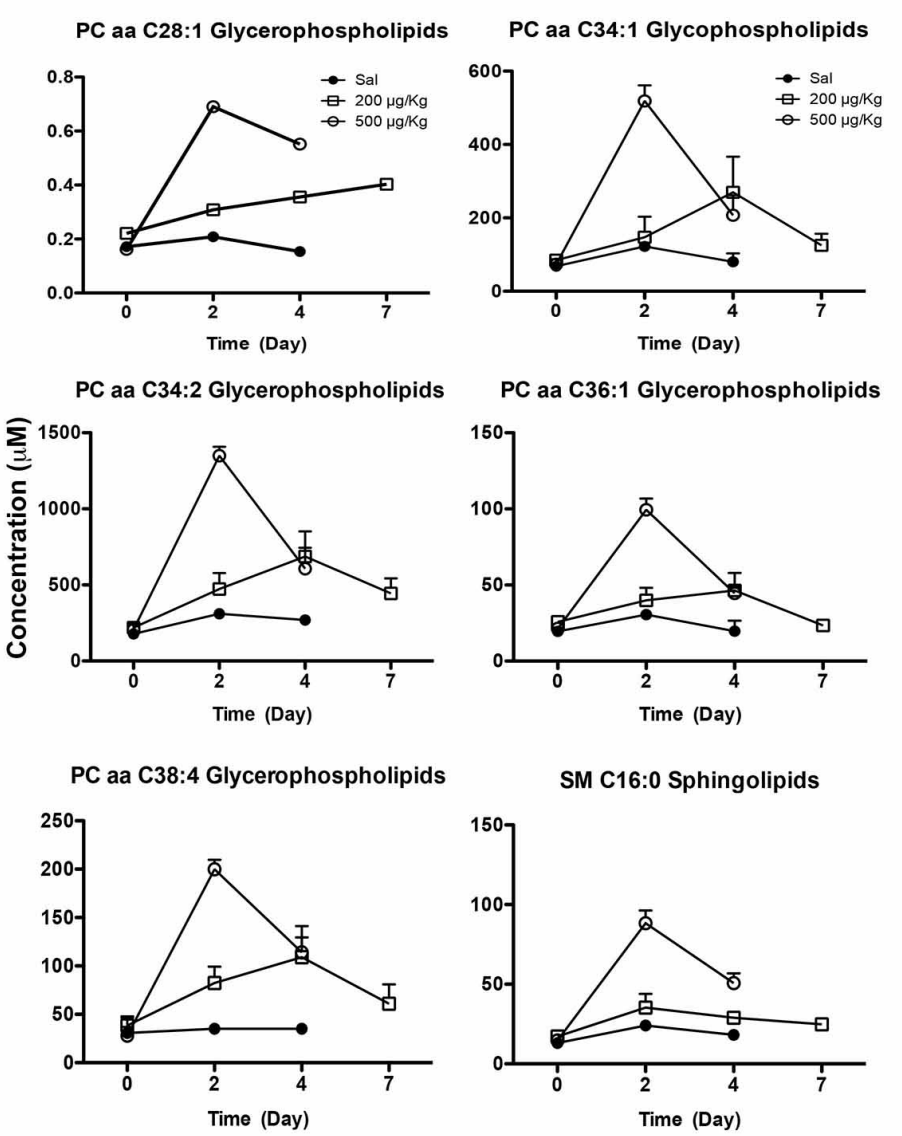

Figure 4.22: The concentration of phospholipids in the injured animals. Glycerophospholipids concentration was determined by liquid chromatography and tandem mass spectrometry LC/MS/MS. n ≥ 5 mice/ group, $p < 0.001$ vs saline control, two-way ANOVA.

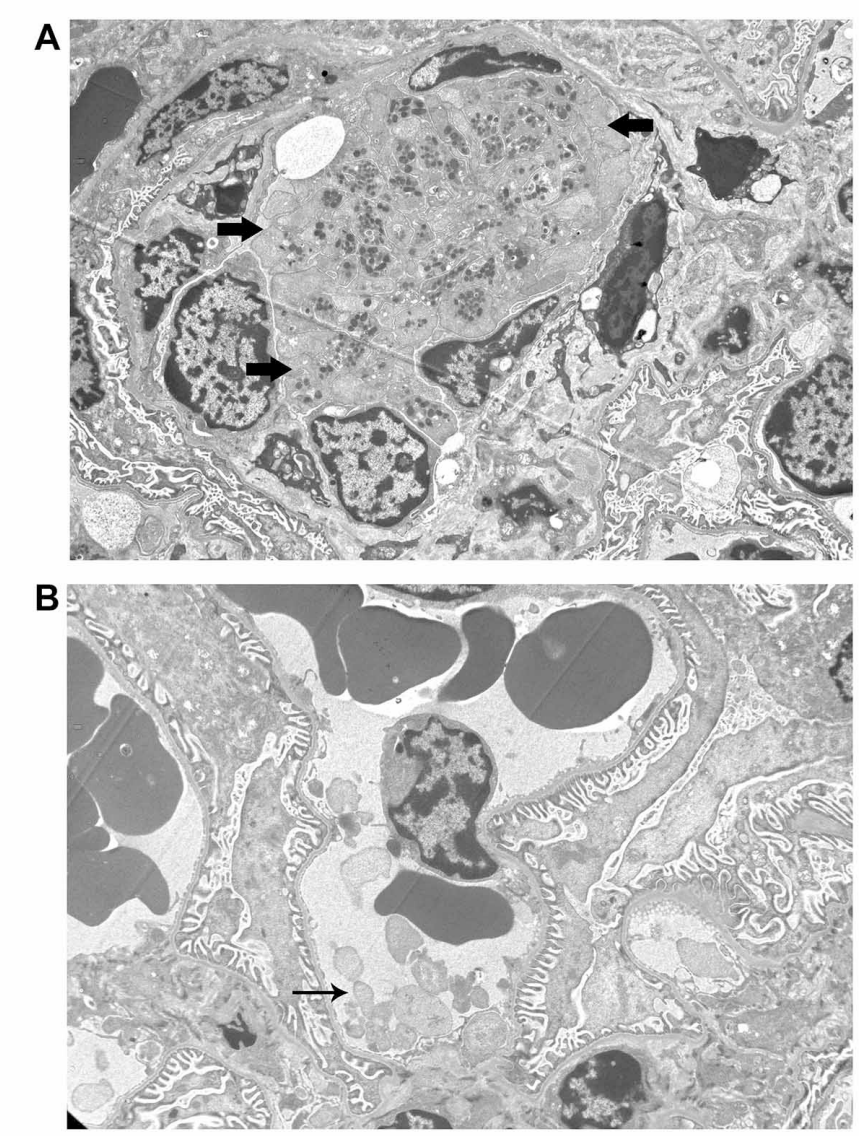

Figure 4.23: Electron microscope images from discordant transplantation. Guinea pig kidneys were transplanted into Lewis rats for 15 minutes. (A) Shows a platelet clot (large arrows), and (B) shows accumulation of unidentified vesicles in the capillary lumen (small arrow), x2000 original magnification.

CHAPTER V

ISOLATION AND CHARACTERIZATION OF HUMAN CD34+ DERIVED ENDOTHELIAL PROGENITOR CELLS

5.0 INTRODUCTION

Early reports of endothelial progenitor cells (EPC) challenged the existing dogma that vasculogenesis process does not occur postnatally. However, many studies, mainly in mouse models have demonstrated that bone marrow- derived cells are incorporated into the blood vessels during wound healing, re-endothelization of denuded vessels, and restored blood flow in ischemic hindlimbs and in cardiac muscle [393-396].

EPC were first described by Asahara et al. [8] in 1997 and since then, interest in primitive endothelial lineage cell populations has exploded. With this came the controversy and the bewilderment, as the literature is flooded by reports on the isolation methods of various cell types collectively called EPC. Yet there is no one uniform definition or consensus on a surface marker to positively identify this cell population with some degree of certainty. However, out of the three reported methods: colony-forming unit (CFU)-Hill [397], circulating angiogenic cells (CAC) [121], and endothelial colony forming cells (ECFC) [333], only the latter yield cells that are currently considered genuine endothelial progenitor cells [17].

In this chapter, I report the isolation and characterization of EPC generated from CD34+ cells derived from peripheral blood of healthy volunteers and/or from G-CSF-mobilized leukapheresis blood obtained from the blood bank, using the ECFC method.

5.1 RESULTS

5.1.1 EPC Isolation by ECFC Method

The isolation of human EPC was carried out using the endothelial cells colony-forming method since it is the method that has been demonstrated to yield EPC that express all endothelial markers, none of the leukocytes markers, and demonstrate the ability to stably incorporate into the vasculature [17]. The mononuclear layer was isolated from peripheral blood from healthy volunteers ranged in age from 23 – 50 years old or from leukapheresis blood obtained from the blood bank and processed as described in (Chapter 3, section 3.2) and summarized in Figure 5.1. The colonies generally appeared in culture after three weeks of incubation with only one exception. A colony appeared after one week in culture, and incidentally, this colony derived from the blood of a 23-year-old donor whose cells produced 3 EPC clones. When a colony appeared in culture it was transferred to a 6-well fibronectin-coated tissue culture dish until the cells became confluent. Thereafter the cells were transferred to a T-25 tissue culture flask, which at this stage the cells were highly proliferative (Figure 5.2). In general, peripheral blood cells produced on

average one EPC clone per 120 mL of peripheral blood (Table 5.1). Out of the twelve individual clones four colonies were passaged 12 times, 3 of them were derived from a young male, and one clone was derived from a 40-year old man. The clones derived from female donors (2) and the older male donors (2) were not subcultured beyond passage 4. The remaining 4 EPC clones were passaged 8 times. Interestingly, freezing down individual clones in liquid nitrogen caused the cells to lose their high proliferative ability and were only passaged once after being thawed.

Alternatively, we opted to generate EPC from leukapheresis product because we were able to obtain considerably more EPC clones. On average we obtained 80 clones from ~ 80 mL of leukapheresis product. Interestingly, the overall percentage of clones derived from CD34+ cells isolated from leukapheresis product was identical to that generated from peripheral blood ~ 0.0002 % or one in 510^5 (Table 5.1).

5.1.2 Characterization of EPC Clones

In the absence of a specific marker to positively identify EPC a number of different tests are considered as standard identification procedure, including uptake of acetylated low-density lipoprotein (Ac-LDL) and *Ulex europeaus* agglutinin binding, expression of endothelial markers, and the lack of expression of leukocytes markers.

The EPC clones that we obtained in the lab were rigorously subjected to the identification tests to ensure that clones were of

endothelial lineage. Endothelial cells, among other cell types, are known for their ability to take-up fluorescently labeled Ac-LDL (red) and the binding of *U. europeaus* lectin (green) as shown in Figure 5.3.

The EPC clones were evaluated for the expression of endothelial-specific genes by real time PCR and compared to the mature endothelial cell line of HUVEC. As Figure 5.4 demonstrates EPC express all of the endothelial markers similar to HUVEC albeit at a slightly lower levels particularly the two genes, endothelial nitric oxide synthase (eNOS, NOS3) and von Willebrand factor (vWF). It appears that EPC like HUVEC do not express the pluripotent stem cells marker CD133, and maintained expression of CD34. The gene expression levels of EPC and HUVEC were calibrated against CD34+ cells.

Moreover, the real time PCR evaluation of mRNA expression was validated by flow cytometery and by Western blotting. The EPC clones expressed endothelial cells markers such as CD31, kinase-insert domain receptor (KDR, CD309), vascular-endothelial cadherin (VE cad, CD144), eNOS, and vWF, as demonstrated by flow cytometer analysis (Figure 5.5 a), or by Western blot (Figure 5.5 b). Interestingly, the protein expression levels of eNOS and vWF in EPC were comparable to that observed in HUVEC.

To ensure EPC clones do not express the pan leukocyte marker CD45, which is reported to be expressed on EPC isolated by CFU-Hill

method. We isolated human macrophages from peripheral blood of healthy volunteers and assessed the expression CD45 and the macrophage marker CD14 by real time PCR. HUVEC were used as a negative control (Figure 5.6). The results demonstrated the lack of CD45 and CD14 expression in the EPC clones as well as in HUVEC further supporting the endothelial lineage of our EPC.

We assessed the production of nitric oxide (NO) and interleukin-8 (IL-8) to address the functional characteristics of the EPC clones. The nitric oxide assay was based on the membrane permeable compound 4,5-diaminofluorescein-2 diacetate (DAF-2 DA) that is cleaved inside the cell and become fluorescent when it interacts with NO. As Figure 5.7 shows a significant increase of intracellular NO production in response to 20 ng/mL VEGF in both EPC and HUVEC as compared to cells cultured in the absence of VEGF. The VEGF-stimulated DAF-2 fluorescence was blocked in the presence of the nitric oxide inhibitor L-NAME.

Endothelial cells are major producers of interleukin-8 where it is stored in the Weibel-Palade bodies and secreted upon a pro-inflammatory stimulus such as TNF-α [398]. IL-8 production in EPC clones was assessed using a commercially available ELISA kit. As Figure 5.8 shows a significant IL-8 increase after 50 ng/mL TNF-α treatment in both EPC and HUVEC as compared to medium control treated cells.

5.1.3 EPC Clones Lineage Commitment

The term “progenitor” indicates that the cells have differentiated along a certain lineage, to the extent that they are committed to become a mature cell of a particular cell type. However, it has been reported that progenitor/stem cells in many instances maldifferentiate when transplanted *in vivo* [399, 400]. We sought to investigate the potential of EPC clones to differentiate into another cell type under in vitro conditions. We cultured EPC in media that are formulated to support the growth of cells other than endothelial cells such as, chondrocytes, adipocytes, and macrophages under conditions reported to support pluripotent stem cells differentiation. Initially, the EPC were cultured in the presence of complete endothelial medium (EBM2) until they reached confluency, thereafter the cells were washed with PBS and one specific growth medium was added into a separate EPC dish. Very early in culture it became evident that chondrocyte and adipocyte media do not support the growth or survival of EPC, as by day 3 there were many floating cells. Virtually half of the cells died out by day seven and almost all the cells were dead by day 14 as demonstrated by MTT cell viability assay shown in Figure 5.9, whereas Figure 5.10 shows representative images of cells in different culture media at day 7. The macrophage medium supported the EPC survival better than chondrogenic and adipogenic media with noticeable decrease in cell density observed by day 14. By day 28 almost all the cells were gone in sharp contrast to the EPC grown in EBM2 where the cells survived in the

same culture dishes until the experiment was terminated 28 days later. However in prolonged culture a fraction of EPC cells cultured in EBM2 expanded in size suggesting senescence.

Since EPC survived over two weeks in macrophage medium, we examined whether those cells gained macrophage specific markers such CD14, CD45, and MCSFR. Quantitative PCR was performed using cDNA generated from RNA extracted from day 14 cells. Figure 5.11 shows EPC grown in macrophage medium did not gain expression of any macrophage specific genes as compared to EPC grown in EBM2 and to macrophages grown in their own medium. However, EPC in macrophage medium showed reduction in mRNA expression of vWF and endothelin 1 when cultured in macrophage medium.

5.1.4 Investigation of Endothelial to Mesenchymal Transition

Endothelial to mesenchymal transition (EndMT) is an important embryonic developmental process by which the endothelial cells respond to signals from the underlying myocardium to transition into mesenchymal cells that ultimately form the atrioventicular cushion, the septa, and the primodia of the valves of the adult heart [401]. Recent evidence attributed cardiac fibrosis in adult tissue to the EndMT process in response to signals from TGF-β1, whereas BMP7 had a protective effect preserving the endothelial phenotype [402]. The EPC clones are intended for *in vivo* studies, and a change of endothelial phenotype into fibroblast would be

detrimental for investigation of vascular repair by EPC. We sought to examine the effect of TGFβ isoforms β1, and β2 on the lineage commitment of the EPC clones.

EPC clones were cultured in complete EBM2 and/or in EBM2 minus the growth factors VEGF and bFGF in the presence of 2 ng/mL of either TGFβ1 or TGFβ2 for one week at 37 ˚C and 5 % CO_2. Cell lysates and total RNA were collected from the EPCs cultured in the presence of TGFβ isoforms 1 and 2 or grown in complete EBM2. The EPC maintained expression of the endothelial-specific genes such as CD144, CD31, KDR, and vWF as demonstrated by real time PCR, and flow cytometery shown in Figure 5.12 (a & c). In addition, there was low expression of α-smooth muscle actin in EPC at mRNA and protein levels Figure 5.12 (a & b). However, the TGFβ treatment did not result in EPC gaining the fibroblast marker FSP1 as both real time PCR and Western blot results were negative. FSP1 was highly expressed in the human fibroblast cell line HFF1 as shown in Figure 5.12 (a & b).

To ensure the TGFβ isoforms were functional, we probed for phospho-Smad-3 (p-Smad3) a signaling molecule activated by TGFβs binding to their receptors. As shown in Figure 5.13 (b) phosphorylated Smad-3 was detected in the TGFβ isoforms 1 & 2 -treated EPC but not in the control cells (starved EPC in incomplete EBM2 + 2% FBS). Total Smad-3 was detected in all cell lysates and tubulin expression

was used as a loading control. In addition, we assessed the expression of TGFβ receptors ALK1, TGFβrII, ALK5 (TGFβrI), and endoglin (Figure 5.13 a) to ensure that EPC express the receptors and can propagate TGFβ isoforms signaling.

5.2 DISCUSSION

EPC are considered to derive from multipotent stem cells called hemangioblast and are generally identified by the expression of CD34, VEGFR2, and CD133 on their surface [403]. The hemangioblasts give rise to multipotent hematopoietic stem cells and to angioblasts that differentiate into all blood cells and into endothelial cells respectively [404]. Endothelial progenitor cells are bone marrow derived cells that were first isolated from peripheral blood from CD34+ pool of cells [8]. Many studies later isolated putative EPC cells based on the expression of various extracellular markers. One specific subset of mononuclear cells expressing CD34+VEGFR-2+CD133+ was widely accepted as “true” EPCs however later studies demonstrated that this specific subset represent a population of CD45+ hematopoietic progenitor cells (HPC) that did not form endothelial cells in vitro [405]. A subset of HPC cells CD34+CD45+ did not differentiate into endothelial cells in vitro whereas another subset of HPCs cells CD34+CD45- formed EPC demonstrating that EPCs can be separated from HPCs. Thus not all EPC preparations yield pure population and may be contaminated with HPC, hence a

validation process of the putative EPC is required when performing endothelial differentiation studies [18, 405].

There are large numbers of measures that can be used to isolate and quantify EPC. One approach deals with adhesion and growth *in vitro*, and the second approach relies on cell selection based on surface markers by flow cytometery with the aid of fluorescently labeled antibodies [17]. In the absence of a specific marker unique to EPC, many tests are required to positively identify putative EPC that can differentiate into mature endothelial cells. Some of the tests performed include cobblestone morphology in 2-dimensional culture, the uptake of Dil-Ac-LDL, the binding of *Ulex europaeus* agglutinin, *in vitro* tube formation assay, and the expression of endothelial specific markers such as KDR, CD31, eNOS, vWF, and CD144 [17]. It is important to note that isolation of EPC based on the expression of endothelial markers alone is inadequate as macrophages cultured in endothelial medium supplemented with fetal calf serum may express the endothelial specific proteins [406, 407]. Additionally, EPC are assayed for nitric oxide (NO) and interleukin-8 (IL-8) production to confirm the endothelial lineage and functionality of EPC in vitro [17, 408].

It has been suggested that EPC may only give rise to endothelial cells in the presence of exact combination of growth factors [18]. Therefore, we sought to analyze EPC lineage commitment in the presence of different growth factors that favour the growth of other cell types to

provide us with an indication of how the EPC may perform when transplanted *in vivo*. Our results suggest that late outgrowth EPC are capable of surviving for extended time in the absence of the endothelial specific growth factors *in vitro* (two weeks in macrophage medium without loss of endothelial markers). In addition, treatment with TGFβ did not transition EPC into fibroblast phenotype. Taken together, our results suggest that EPC isolated by ECFC method are committed to the endothelial lineage *in vitro*. Nevertheless, we have not been able to systematically verify lineage commitment *in vivo* (see chapter VII on hEPC transplantation).

In this chapter, I studied the characteristics of EPC expanded in vitro. I routinely isolated EPCs from peripheral and leukapheresis blood and systematically characterized the cells for expression of endothelial markers at the mRNA and protein levels. I have performed the battery of tests to positively identify our EPC clones *in vitro*. The CD34+CD45- cells differentiated into late outgrowth EPC clones, had the cobblestone morphology, the high proliferative capacity, expressed all the endothelial markers and had the functional characteristics of mature endothelial cells. Functionally, EPC produced NO and IL-8, formed tube-like structures in 2-D and 3-D gels. In addition, the data show that late outgrowth EPC are committed to endothelial lineage *in vitro*, which make them a suitable EPC population to study *in vivo* for vascular repair in an appropriate animal model.

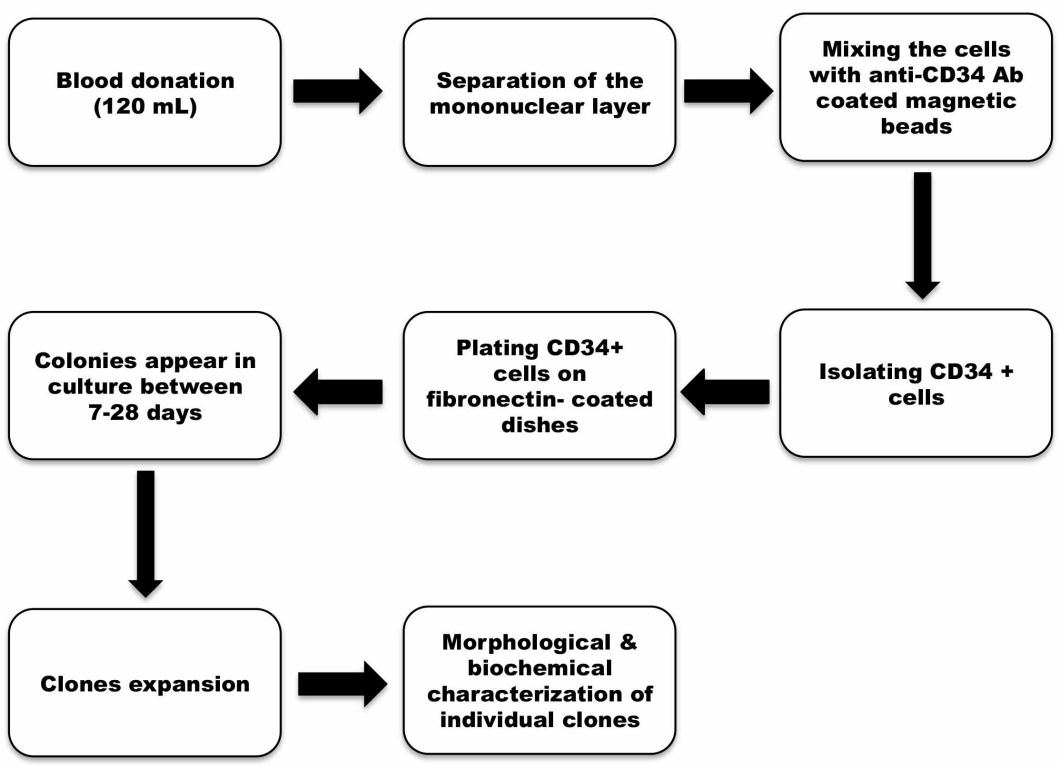

Figure 5.1: Flow chart demonstrating the strategy applied to isolate human EPC from peripheral blood.

Table 5.1: Comparison between peripheral and leukapheresis blood for generation of EPC clones.

Peripheral Blood							
# of donors	Avg blood volume (mL)	Avg # of colonies	Max Col/dnr	Min col/dnr	Avg total MNC	Avg CD34+	% EPC clones from CD34+
18	100	1	3	0	1.67 x 10^8	4.9 x 10^5	0.0002
Leukapheresis Blood (averages are from each bag of blood)							
3	83	80	98	58	3.15 x 10^9	4.85 x 10^7	0.0002

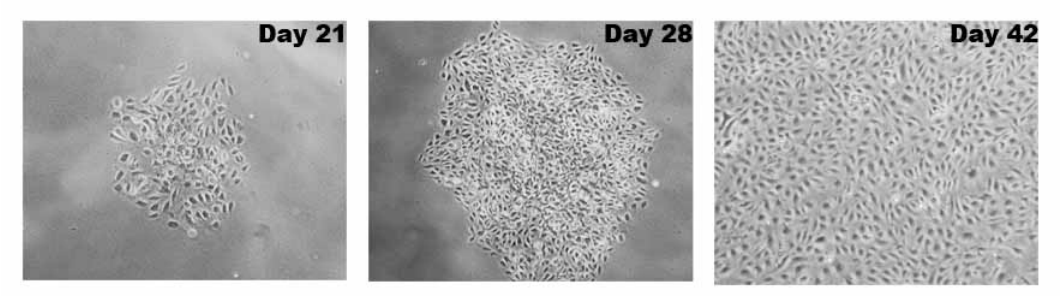

Figure 5.2: Representative images of EPC clones. Colonies generally appear in culture approximately three weeks after plating. On average one or two clones of EPC are generated from 120 mL of blood. The individual colonies are expanded to ~ 2.10^6 cells by day 40 of culture, at which time the EPC are highly proliferative and are passaged a further 8-12 times before they reach the senescence stage, x100 original magnification.

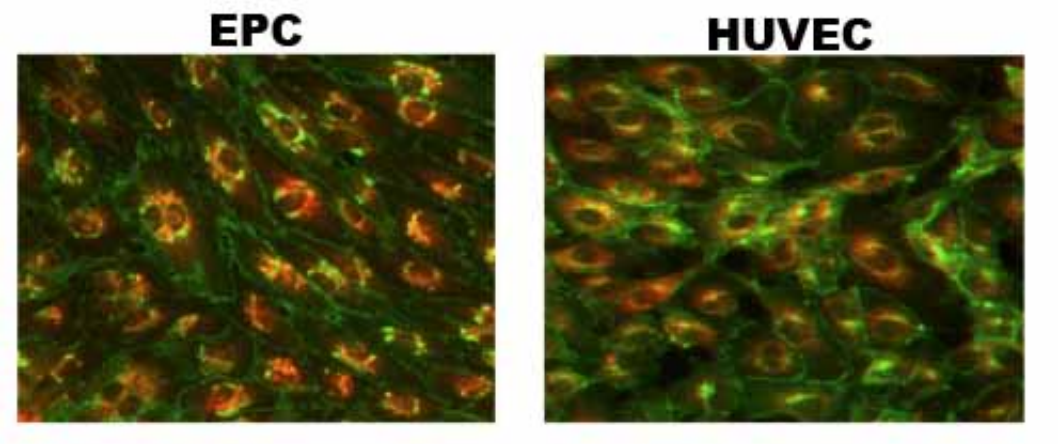

Figure 5.3: Uptake of Dil-Ac-LDL and Ulex binding. Representative images of the uptake of acetylated low-density lipoprotein (Dil-Ac-LDL) (red) and the binding of *Ulex europeaus* lectin (green) are characteristic features of endothelial cells. The images are representatives of six independently isolated clones, x400 original magnification.

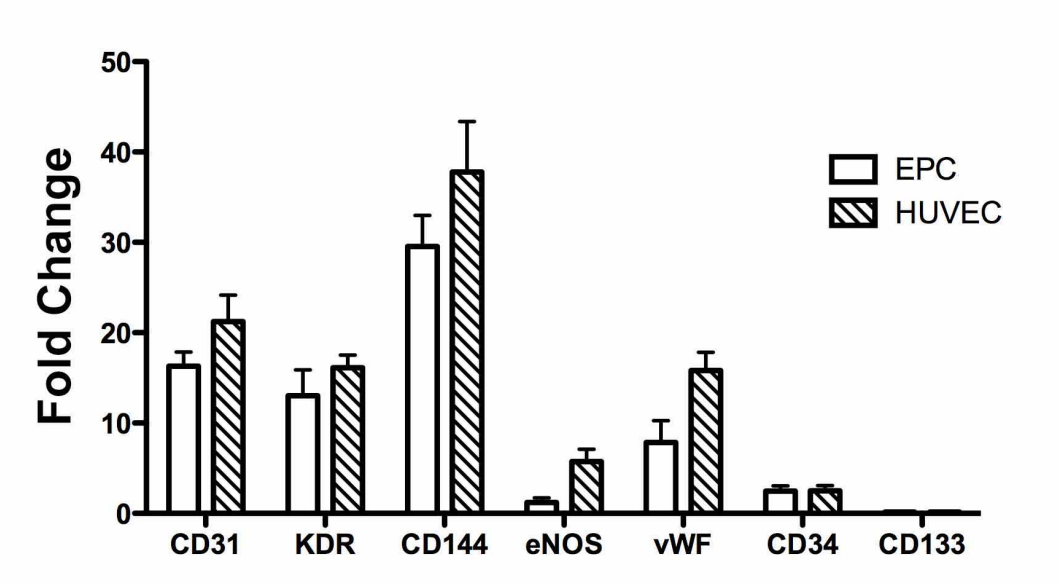

Figure 5.4: mRNA expression levels of endothelial genes. The expression levels of endothelial specific genes: CD31, KDR, CD144, eNOS, and vWF, and that of stem cells markers: CD34, and CD133 as determined by real time PCR. The data were calibrated against the expression levels of CD34+ cells. The bars represent the mean ± SEM of three independently isolated clones (n = 3, p = ns).

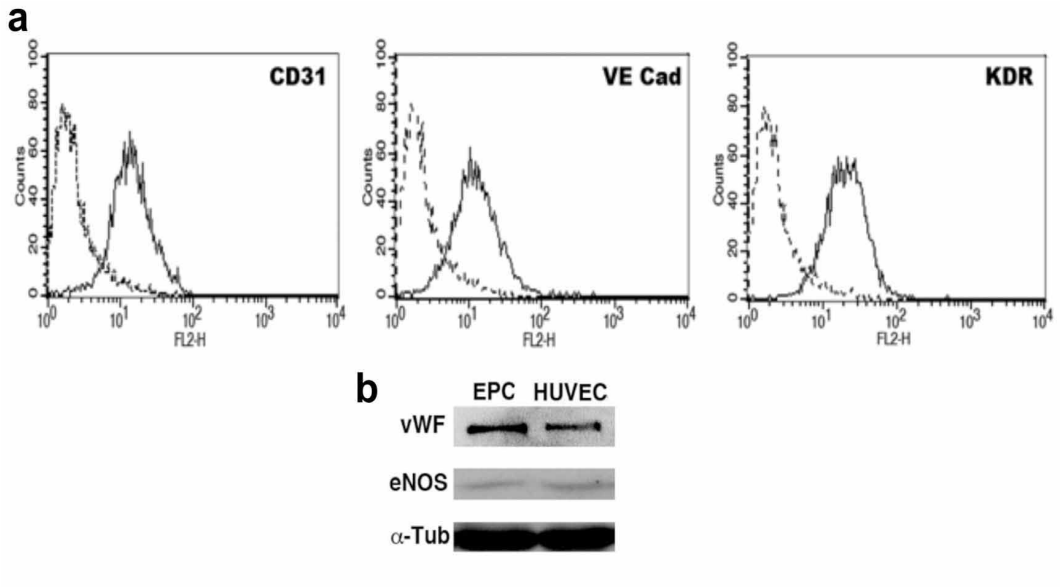

Figure 5.5: The expression of endothelial markers as determined by flow cytometer and by Western blot. The expression of CD31, VE cadherin and VEGFR2 in EPC clones was evaluated by flow cytometery (A). The EPC were labeled with antigen specific antibodies that are fluorescently tagged. The peaks represent the expression level of the cell surface marker (solid line) and compared to isotype control (dashed line). (B) shows a representative Western blot of von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS) demonstrating the expression of endothelial markers in EPC as compared to that in HUVEC. The flow cytometer and the Western blot were performed at least on three different EPC clones.

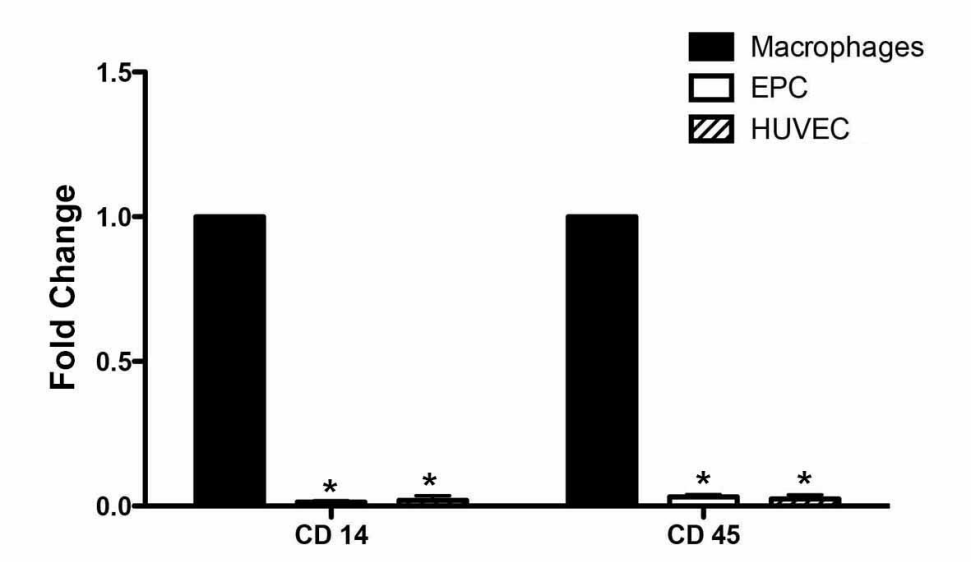

Figure 5.6: Lack of expression of macrophage markers in EPC. The mRNA expression levels of macrophage specific gene CD14 and the pan leukocyte marker CD45 in EPC and HUVEC were evaluated by real time PCR and demonstrate the lack of expression of macrophage markers in endothelial cells. The bars represent the mean ± SEM of three independent EPC clones ($p < 0.05$ vs macrophages one-way ANOVA).

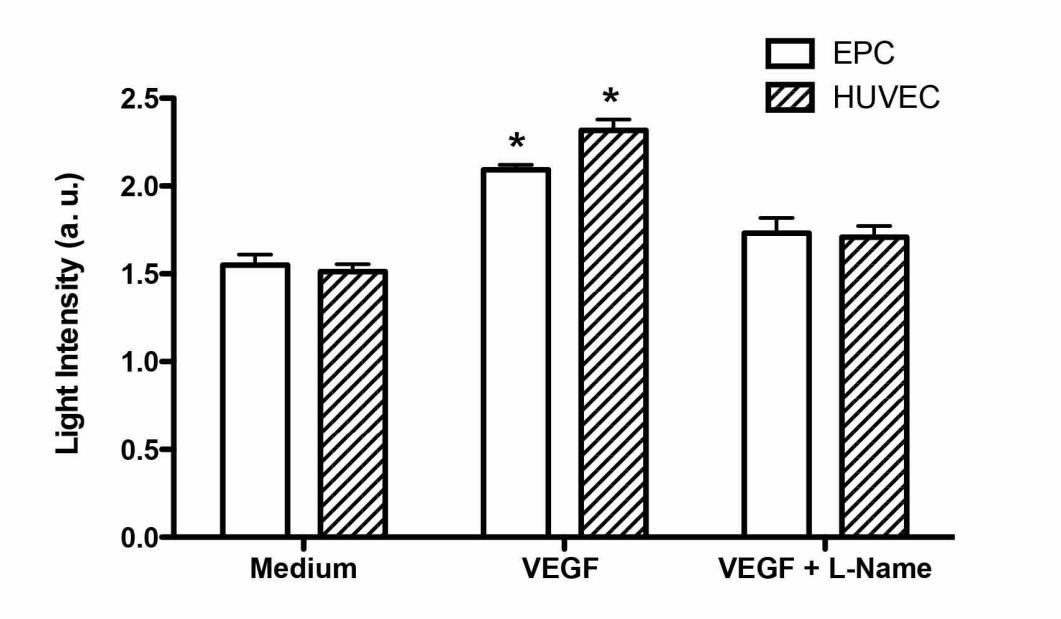

Figure 5.7: The production of nitric oxide (NO) by hEPC and HUVEC. NO was evaluated using the nitric oxide method based on diaminofluroscein-2 compound. The NO activity increased after treatment with VEGF (20 ng/mL) as compared to cells in medium control. The VEGF-mediated increase of NO was inhibited by NO-specific inhibitor L-NAME (1 mM). The bars represents the mean ± SEM of three independently isolated clones. $p < 0.05$ as determined by one-way ANOVA and compared to medium control. VEGF+ LNAME treatment was not statistically significant when compared to medium control.

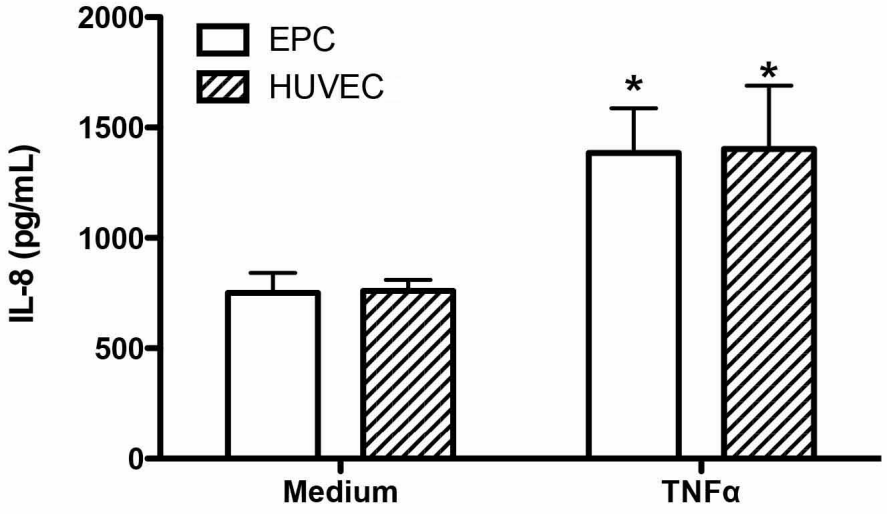

Figure 5.8: Interleukin-8 (IL-8) production by EPC and HUVEC. IL-8 release was evaluated using the Ready-Set-Go IL-8 ELISA kit. IL-8 production by EPC was comparable to that of HUVEC at basal conditions. Similarly, the treatment with the pro-inflammatory cytokine TNFα (50 ng/mL) overnight significantly increased IL-8 levels as compared to medium control. However, there was no difference in IL-8 production between EPC and HUVEC in response to TNFα treatment. The bars represent the mean ± SEM of three independently isolated clones. $p < 0.05$ as determined by one-way ANOVA and compared to medium control.

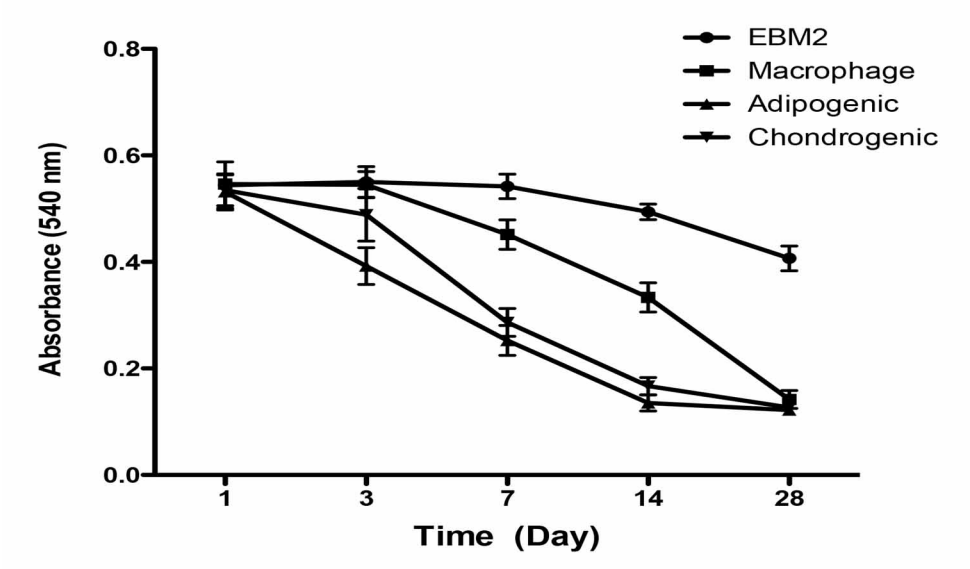

Figure 5.9: Cell viability of EPC in various culture conditions. EPC were in culture media that support the growth of different types of cells and followed for viability over 28 days by MTT assay. EPC grown in endothelial basal medium (EBM2) survived best over the course of experiment without noticeable cell death until day 28 and by that time many senescent cells appeared in the tissue culture dishes. EPC cultured in macrophage supporting medium survived well for almost a week thereafter there were very noticeable cell death that almost all cells disappeared by day 28. Adipogenic and chondrogenic media did not support the growth of EPC as most of the cells died out within the first week of the experiment. The bars represents the mean ± SEM of three independent experiments using three independently isolated clones ($p < 0.005$ for treatment and time 2-way ANOVA).

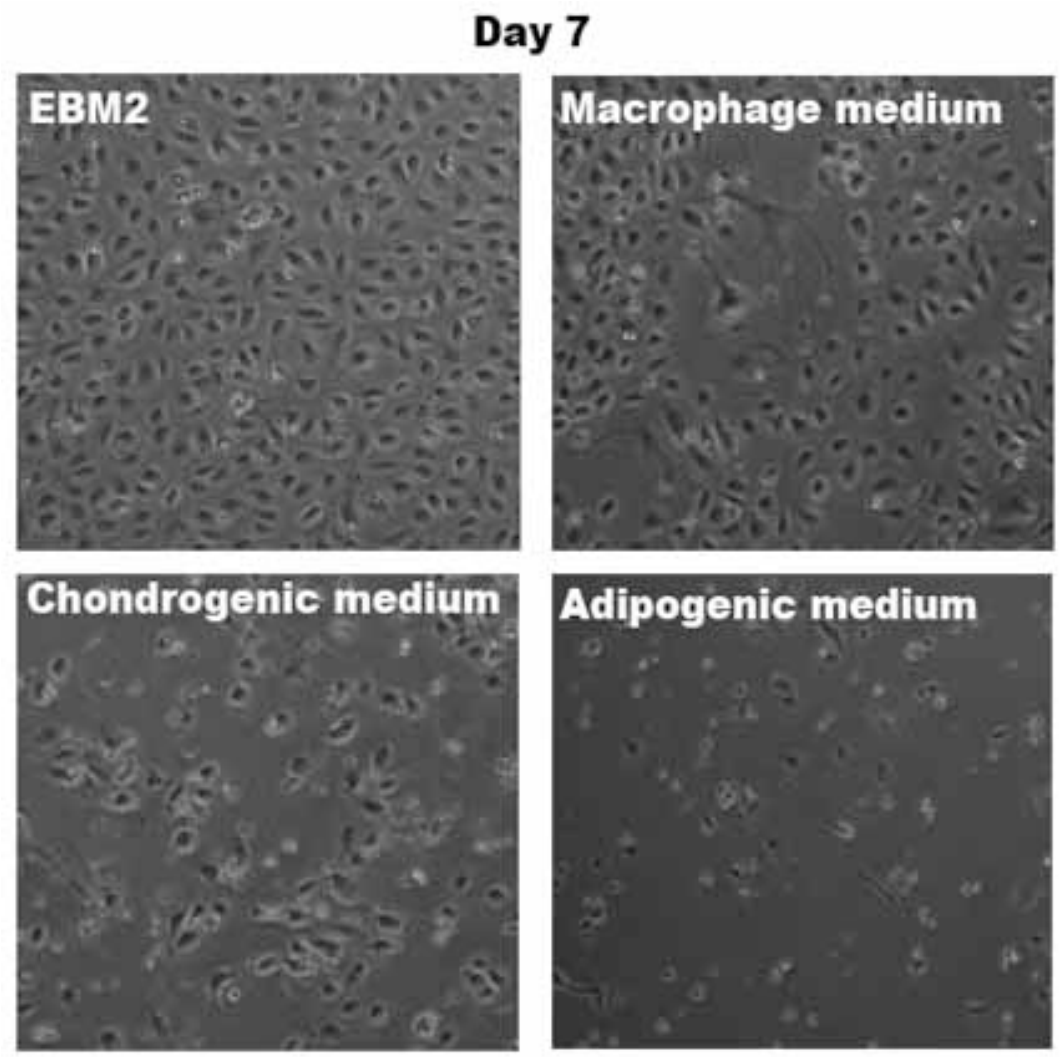

Figure 5.10: Representative images from day 7 cultured EPC under different growth conditions that demonstrate the preference of EPC to endothelial basal medium (EBM2), x100 original magnification.

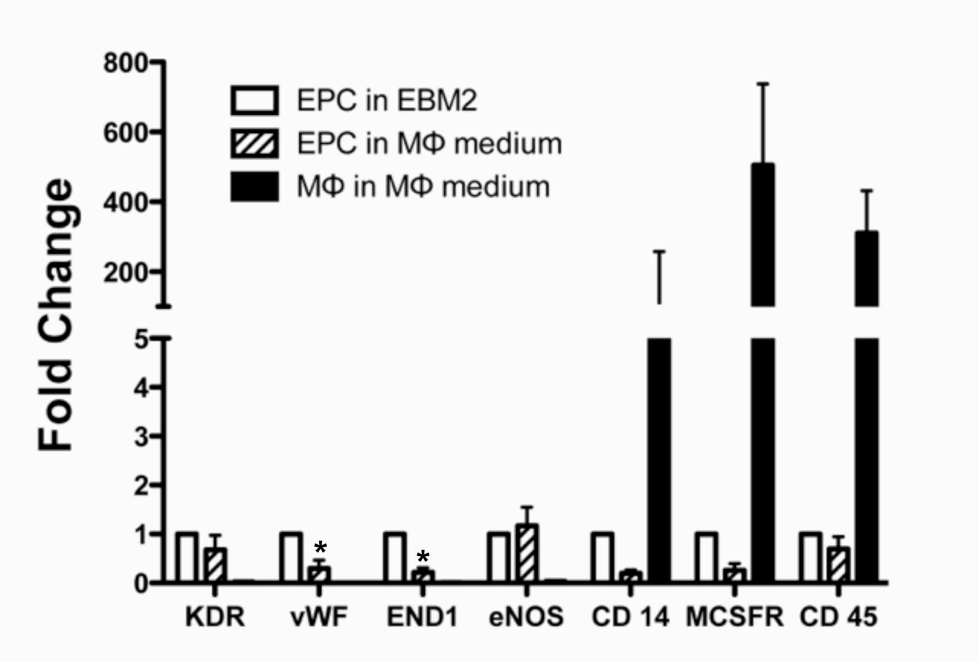

Figure 5.11: The mRNA expression levels of endothelial and macrophage genes by real time PCR. EPC were cultured in EBM2 or in macrophage (MΦ) medium and macrophages were grown in macrophage medium. Total RNA was extracted from the cells after 14 days in culture. The gene expression level was calibrated against EPC grown in EBM2. The results indicate that the EPC grown in macrophage medium maintain their expression of endothelial genes with some decrease in vWF, End1 and a slight increase in eNOS expression (statistically insignificant). On the hand, EPC grown in macrophage medium did not gain the expression of macrophage specific genes, CD14, MCSFR, or CD45. The bars represent the mean ± SEM of three independently isolated clones $p < 0.005$ EPC in MΦ medium vs EPC in EBM2, one-way ANOVA.

a

Average Ct

	CD144	CD31	KDR	vWF	αSMA	FSP1
EPC in EBM2	21.982 ± 0.79	20.051 ± 0.89	22.689 ± 1.27	20.915 ± 0.46	26.558 ± 0.61	Not detected
EPC + TGFβ1	22.735 ± 0.37	21.265 ± 0.47	21.054 ± 0.73	20.615 ± 0.76	26.945 ± 0.84	Not detected
EPC + TGFβ2	22.170 ± 0.74	19.868 ± 1.16	22.572 ± 0.59	20.251 ± 0.71	26.272 ± 0.77	Not detected
HFF1(fibroblast)	31.655 ± 0.46	Not detected	30.611 ± 0.55	30.117 ± 1.02	23.249 ± 0.81	18.865 ± 0.24

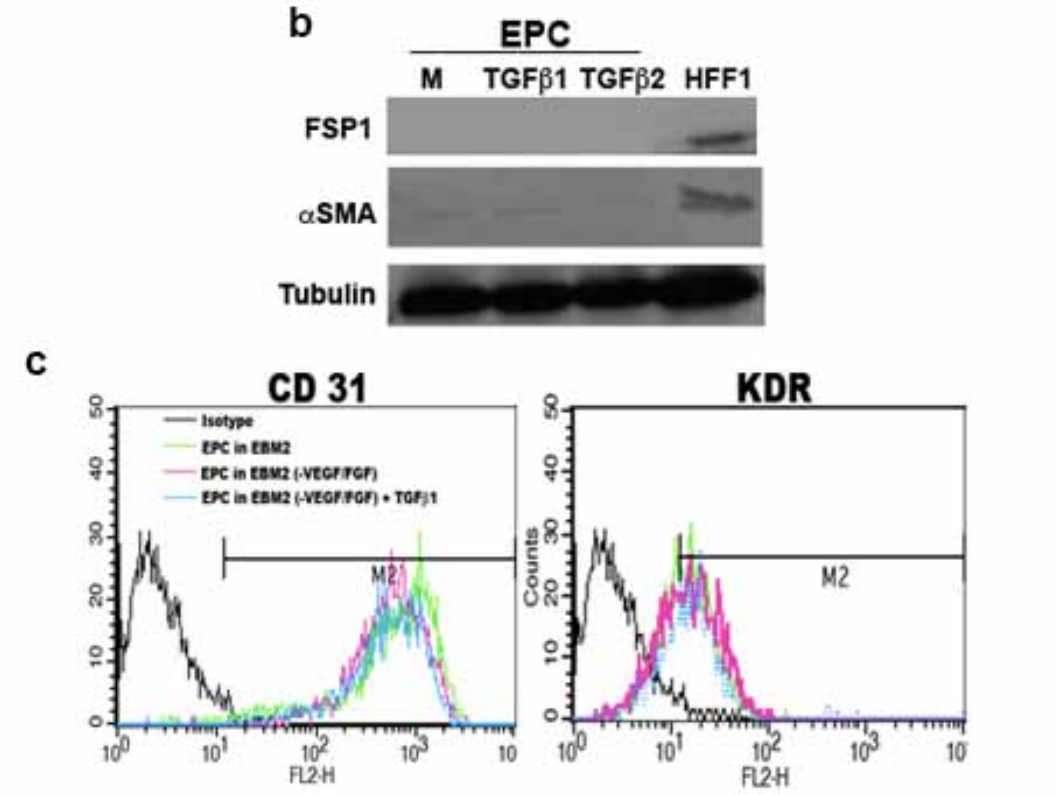

Figure 5.12: The effect of TGFβ isoforms on endothelial to mesenchymal transition. EPC were grown in EBM2 and/or –VEGF/FGF and in the presence of 2 ng/mL TGFβ1 or TGFβ2 for one week. EPC were then analyzed for the expression of fibroblast specific markers FSP1 (S100A4) and αSMA by qPCR (a), and Western blot (b). TGFβ treatment did not drive the EPC into a fibroblast phenotype. There was no gain of fibroblast markers or loss of the endothelial markers. EPC maintained expression of endothelial marker CD31 and KDR as demonstrated by flow cytometer analysis (c). The results shown are representatives of three independent experiments performed. Table (A) shows the mean ± SD.

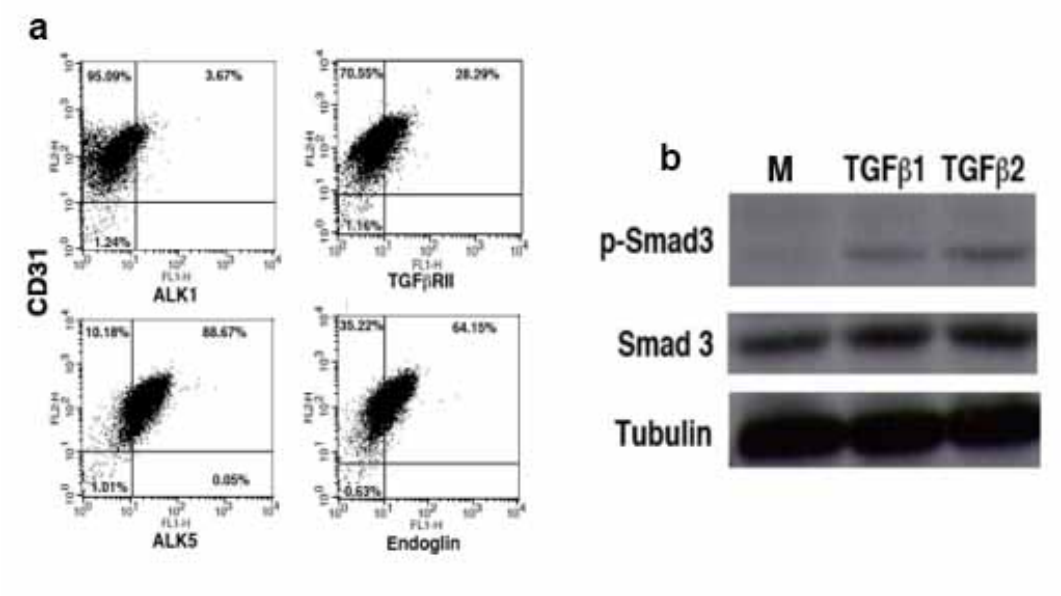

Figure 5.13: TGFβ receptors expression in EPC. (A) flow cytometer evaluation of ALK1, TGFβRII, ALK5, and endoglin demonstrate that EPC are capable of receiving TGFβ stimulus. (B) Smad3 phosphorylation in EPC lysates pre-treated with TGFβ1, and TGFβ2. EPC were starved in low serum concentration (2 %) overnight and stimulated the next day with 2 ng/mL TGFβ1 or TGFβ2 for 1 hour and the cell lysates were assayed for Smad proteins. The results shown are representatives of three independently isolated clones.

CHAPTER VI

THE STUDY OF ENDOTHELIAL PROGENITOR CELLS ANGIOGENIC POTENTIAL

6.0 INTRODUCTION

The formation of new blood vessels can occur by two distinct processes (1) by vasculogenesis or *de novo* formation of blood vessels from bone marrow mobilized cells and (2) by angiogenesis or formation of new blood vessels from pre-existing vessels [147].

During embryonic development, hemangioblasts, the precursor cells that give rise to hematopoietic stem cells and angioblasts (also called endothelial progenitor cells), differentiate from mesodermal stem cells of the bone marrow [409, 410]. The endothelial-committed angioblasts, during vasculogenesis, aggregate into clusters called blood islands to form the primary vascular plexus that eventually develop into the complex microcirculation [147, 411]. In adults, new blood vessels are generated mainly through angiogenesis where new capillaries develop from pre-existing mature endothelial cells within the blood vessel [412]. However, circulating EPC can contribute to adult angiogenesis and can get incorporated into the site of neovascularization in both physiological and pathological conditions [413-415]

Angiogenesis is a complex processes that requires the endothelial cells to integrate the signals from various growth factors, extracellular matrix proteins and mural cells. Angiogenesis includes a series of distinct and coordinated steps including, endothelial cell activation, degradation of basement membrane, migration, proliferation, lumen formation, alignment, anastomosis, and the recruitment of mural cells [171-173].

During the angiogenesis process EC respond to chemical stimuli such as VEGF, bFGF, PDGF, EGF, S1P, and angiopoietin 2, which can be secreted by the surrounding tissues within the microenvironment or be produced by the endothelial cells [147, 174, 416-418]. Once activated by angiogenic stimuli ECs differentiate into specialized cells called tip cells that spearhead the growing sprout. The tip cell function in guiding the growing capillary toward a concentration gradient of an angiogenic factor, and in preventing the trailing stalk cells from differentiating into tip cells in the same outgrowing capillary. Tip cells are characterized by the formation of large number of filipodia, rarely proliferate, and express high levels of Dll4, apelin, ESM1, and CXCR4. In contrast, stalk cells are highly proliferative, form lumen, and express high levels of Robo4, Jag1, and Flt1 [195, 199, 200, 419].

In this chapter, I study the competence of human late outgrowth EPC to form tube-like structures *in vitro*, utilizing a three-dimensional angiogenesis assay. The fibrin bead angiogenesis assay captures many features of the *in vivo* angiogenesis process [172]. The endothelial cells

are surrounded by a matrix and provided with growth factors and over few days the cells undergo sprouting and can form visible lumens by day ~7 of culture. The fibrin bead assay provides a setting that closely resembles the microenvironment *in vivo* and also permits manipulation of gene expression, study sprouts and lumen formation and the factors that govern this process.

6.1 RESULTS

6.1.1 Comparison of Angiogenic Activity of Different Endothelial Cell Types

The angiogenesis assay was performed in the presence of fibroblast cells seeded on top of the fibrin gel to provide additional angiogenic growth factors.

To determine whether different endothelial cell types have differential angiogenic activities, we compared EPC, human umbilical vein endothelial cell (HUVEC), and human aorta endothelial cells (HAEC) sprouting potential using 3-D fibrin bead angiogenesis assay in vitro. EPC and HAEC formed more tip cells per bead (Figure 6.1 a) and migrated the farthest distance away from the bead (Figure 6.1 b) as compared to HUVEC after 24 h of incubation. The tip cells generally (depending on the cell line) appeared in culture after ~ 16 h of incubation at 37 ˚C. At this time point the budding sprouts consisted for the most part of tip cells only based on the morphology (cells with multiple filipodia) (Figure 6.1), and

actual sprouts, composed of both tip cells and stalk cells, did not appear until day 3-4 of culture (Figure 6.1), to eventually form lumens by day 7-10 of culture (Figure 6.1).

To further identify differences between EPC and HUVEC we coated the beads with equal number of both cell types in an attempt to analyze if a particular cell type has a preference to occupy the tip cell or stalk position. The HUVEC when mixed with EPC had an increase in tip cells per bead (Figure 6.2 a) but both cell types did not migrate very far away from the bead (Figure 6.2 b). However, EPC cell-conditioned medium did not have the same effect (Figure 6.3 a, b). Moreover, by day 4 of culture there was a preference for EPC to occupy the tip position whereas the HUVEC preferentially occupied the stalk position (Figure 6.4 a, b).

To understand the contribution of the factors released by fibroblasts, we cultured EPC in the absence of human fibroblasts (HFF1). For the first day there was no observable differences between EPC grown in the presence or absence of fibroblast cells. However, by day 4 of culture EPC grown in the absence of fibroblast died out (Figure 6.5 a, b) despite the fact that the cells were grown in complete EBM2 that contains VEGF, bFGF, IGF, EGF and 10 % FBS. This demonstrate the importance of the factors released by the fibroblast to prevent cellular death and maintain “healthy” sprouts that eventually develop lumens by day 7-10 of culture. However the initial sprouting event is independent of co-culture with fibroblast.

6.1.2 The Effect of Cellular Proliferation and Migration on Sprouting Angiogenesis

Cellular proliferation and migration are important processes during angiogenesis [172]. To study the effect of inhibition of cellular proliferation and migration on sprouting angiogenesis we treated the EPC with mitomycin C and/or nocodazole, a cell cycle inhibitor and a microtubule inhibitor respectively. The beads were analyzed 24 h after embedding in fibrin gel. Treatment with mitomycin C was effective in inhibiting tip cells formation and migration in a dose dependent manner (Figure 6.6, a, b). Similarly, treatment with the microtubule inhibitor nocodazole inhibited tips cells formation and migration also in a dose dependent manner (Figure 6.7 a, b).

6.1.3 The Effect of siRNA Knockdown of Various Genes on Sprouting Angiogenesis

New blood vessel formation is a complex process that requires the activity of multiple cellular processes [172]. To understand the role of various genes during angiogenesis in vitro, siRNA specific to genes listed in Table 6.1 were used and the results of each gene on sprouting angiogenesis is summarized in Table 6.1. SiRNA knockdown of several genes including ALK1, apelin, ERG, FGD5, ILK, KDR, PI3K catalytic subunits p110α, p110β, and p110δ, and rac1 significantly reduced cellular sprouting *in vitro*. On the other hand, the siRNA knockdown of Dll4, and

PTEN had the opposite effect as sprouting angiogenesis increased. The knockdown of IQGAP1 had no detectable effect on the angiogenesis process *in vitro*.

6.1.4 The Study of Phosphatidylinositide-3 Kinases Role on Sprouting Angiogenesis *in vitro*

To study whether all PI3K catalytic isoforms are involved in the angiogenesis process or if they have differential functions, a gene knockdown strategy by siRNA was applied to specifically eliminate one single isoform at a time. P110α knockdown resulted in a decrease in tip cells formation and reduced the distance the tip cells migrated away from the bead (Figure 6.8 a, b). The siRNA knockdown was confirmed by real time PCR (Figure 6.8 c). Similarly, the knockdown of p110β isoform resulted in a decrease of sprouting angiogenesis in vitro (discussed in more details in Chapter VIII). Interestingly, the siRNA knockdown of p110δ caused more pronounced inhibition of sprouting angiogenesis (Figure 6.9 a, b) in *vitro*. The specific knockdown of p110δ by siRNA was confirmed by real time PCR. The involvement of PI3K in angiogenesis was confirmed by using the pan PI3K inhibitor wortmannin (Figure 6.10) as few tip cells formed and did not migrate too far away from the beads. Consistent with these data the knockdown of the PI3K counter-regulatory phosphatase PTEN had the opposite effect; we observed a significant increase in the number of tip cells per bead. The most pronounced effect was that these

sprouts extended longer (Figure 6.11 a, b). The knockdown efficiency of PTEN was evaluated by real time PCR (Figure 6.11 c).

6.1.5 Down Regulation of Tip Cells Markers Apelin and Dll4 Correlates with Reduced Angiogenesis

To understand the genetic regulation of sprouting angiogenesis, total RNA was extracted from sprouting beads embedded in fibrin gel and the mRNA expression levels of tip cell markers apelin, Dll4, and ESM1 were determined. There was a down-regulation of apelin and Dll4 in the siRNA treatments that resulted in reduction of tip cell formation (Figure 6.12) including wortmannin, the PI3K catalytic isoforms p110 alpha, beta, and delta, FGD5, and Rac1 as compared to medium control. However, the expression levels of the tip cell markers did not change in the cells treated with either non-silencing or PTEN siRNA. In contrast, the mRNA expression level of ESM1 was unchanged across the various siRNA treatments (Figure 6.12).

The EPC-coated beads in fibrin gel were cultured in the presence of complete EBM2 (Includes, SingleQuots supplemented with 10 % FBS), or in incomplete EBM2 (supplemented with 2 % FBS) overnight at 37 ˚ C and 5 % CO_2. Total mRNA was extracted from the cells cultured in fibrin gel and reverse transcribed into cDNA as described in materials and methods. The expression of the mRNA levels of ESM1, Dll4, and apelin was determined by real time PCR. There was an up regulation of ESM1, Dll4,

and apelin in the cells grown in the presence of complete EBM2 when compared to the expression levels of those genes in the cells grown in incomplete EBM2 (Figure 6.13).

6.1.6 Apelin and Dll4 Differentially Regulate Sprouting Angiogenesis

Since the two cell markers apelin and Dll4 mRNA expression levels were down-regulated when angiogenesis was inhibited, the effect they may have on sprouting angiogenesis after gene knockdown was investigated. As a result, apelin knockdown significantly reduced tip cells formation and also affected their migration distance away from the beads (Figure 6.14 a, b). In contrast, Dll4 knockdown resulted in an increase in sprouting with many cells migrating farther distances in the gel as compared to control cells (Figure 6.15 a, b).

6.1.7 FGD5 siRNA Knockdown Reduces Sprouting Angiogenesis *in vitro*[1]

Facio-genital dysplasia-5 (FGD5) was identified by gene expression profiling, and it is enriched in endothelial cells. Investigation in the lab placed the molecule as a novel regulator of PI3K function. Therefore, siRNA specific to FGD5 was applied to knockdown this gene and study its effect on sprouting angiogenesis. Consistent with the observed effect on PI3K signaling, FGD5 siRNA-treated EPC produced fewer tip cells and

[1] The FGD5 results are published as part of a manuscript by Nakhaei-Nejad et al. 2012. *ATVB* 33: 2694-2701

reduced cell migration distance when compared to control cells (Figure 6.16 a, b).

6.2 DISCUSSION

Formation of blood vessels is a critical function of EC in health and in disease. To study EPC *in vivo* for rescue of glomerular endothelial cell injury in our animal model (described in Chapter 4). We first needed to learn the angiogenic capabilities of EPC *in vitro*. Further we sought to identify what genes/proteins that are involved in the angiogenesis process that can be modified to enhance or disrupt angiogenesis depending on the conditions.

Although, there are many models to study angiogenesis, such as corneal neovascularization assay, *in vivo* / *in vitro* chick chorioallantoic membrane (CAM) assay, *in vivo* matrigel plug, and aorta ring assay, we selected the 3-D fibrin bead angiogenesis assay because this assay captures many stages observed during blood vessels formation *in vivo*. Angiogenesis is a multistep process where activated endothelial cells must digest the basement membrane, migrate, proliferate, differentiate into tip/stalk cells sprout from the pre-existing vessel, align, form lumen, and eventually anastomose with other vessels [172, 420]. The fibrin bead assay reproduces each of these stages, which renders this assay an invaluable tool to study angiogenesis under the controlled environment in

vitro. Further the technique is amenable to short term gene inactivation using siRNA or small molecule inhibitors.

We and others [83, 421] have observed that EPC are in many ways indistinguishable from HUVEC. However, when we compared the angiogenic potential, EPC were by far the more angiogenic cells. They produced more tip cells and migrated farther in fibrin gel after 24 h of seeding. These observations contrast to what Finkenzeller et al. [422] has previously reported that putative EPC and HUVEC demonstrated almost identical angiogenic potential as determined by the 3-D spheroid sprouting assay and by 2-D matrigel assay. A few discrepancies must be highlighted between Finkenzeller study and the results reported in this chapter. First, the EPC in Finkenzeller's study were obtained by a different method. They did not select CD34+ cells and kept the adherent peripheral blood mononuclear cells after they removed the non-adherent cells at day 4 of seeding. This indicates that the authors followed a similar method of obtaining circulating angiogenic cells, which are the early outgrowth cells and do not show "true" endothelial phenotype [17]. Second, they do not specify if EPC colonies appeared in culture or not. Third, the 2-D matrigel assay has many inherent problems and it is not suitable to assess angiogenic potential. Specifically, in 2-D culture on matrigel EC do not form intercellular lumens, do not sprout or proliferate, and no mRNA or protein synthesis changes occur. In addition, other cell types such as fibroblast and U87-MG glioblastoma cells, can align and form similar cords

on matrigel hence the assay does not reflect EC-specific behavior [172, 420]. Nevertheless, despite the fact that late outgrowth EPC in our studies displayed higher angiogenic potential at early time points (~ 24h), by day 10 after embedding in fibrin gel there was no difference in the number of sprouts with lumens formed by EPC and HUVEC. There was one sprout with lumen or none per bead.

The co-culture of EPC with HUVEC showed an enhanced angiogenic response by HUVEC with more tip cells formed but shorter migration distance. Interestingly, the EPC showed a remarkable selection for tip cell position whereas HUVEC occupied the stalk cell position. It is likely that EPC with their higher proliferative potential [421] respond faster to the angiogenic stimuli and differentiate into more tip cells to lead the growing sprouts. The development of more tip cells exert control over the fate of the following cells to differentiate into stalk cells through the established mechanism that involves Dll4-notch signaling [185]. Interestingly, Sieveking et al. [423] reported that late outgrowth EPC do not exert paracrine angiogenic effect in contrast to our results. However, the authors demonstrated this effect by using a transwell assay to assess paracrine angiogenic effect. The assay accounts for only secreted angiogenic factors if they are produced. It is likely that the enhanced angiogenic activity of HUVEC when co-cultured with EPC is due to cell-cell contact rather than to the release of soluble angiogenic factors. In accordance with Sieveking et al., EPC cell conditioned medium (CCM)

had no effect on HUVEC sprouting unlike when the EPC and HUVEC were mixed together.

The role of PI3K signaling in blood vessel formation is not well understood. It is established that the catalytic isoform p110α function is important during embryonic vascular development [330]. However, little is known about the activity of the other class IA catalytic isoforms p110β (discussed in detail in Chapter 8) and p110δ during angiogenesis process. Surprisingly, siRNA knockdown of p110δ resulted in a severe impairment of sprouts formation *in vitro*, given that p110δ is expressed minimally in endothelial cells and it is not thought that this isoform contributes much to PI3K activity [330]. Further, p110δ germline knockout mouse is not defective in embryonic angiogenesis [424]. However, Geng *et al*. [425] have reported that the antagonist IC486068 of p110δ enhanced tumor vascular destruction by radiation *in vivo*, reduced endothelial migration and tubule formation *in vitro,* and attenuated radiation-induced AKT phosphorylation.

The role of PI3K during angiogenesis is extended to class 1B as well. The only catalytic subunit of class 1B is p110γ that functions mainly downstream of G protein-coupled receptors (GPCR) [313]. The treatment of HUVEC with p110γ specific siRNA resulted in a strong impairment of endothelial cells angiogenic potential due to the suppression of AKT and MAPK pathways *in vitro*. In addition, p110γ inhibitor AS605240 or PI3K

gamma knockout resulted in defective reparative neovascularization in a myocardial infarction animal model. Both treatments resulted in an activation of apoptosis in both EC and cardiomyocytes and inhibition of endothelial cell proliferation [426].

Apelin, Dll4, and ESM1 are genes found in different microarray studies to be overexpressed in tip cells over stalk cells and are considered as tip cell markers [195, 419, 427].

Apelin is a preproprotein that is cleaved into functional peptides (13, 17, and 36 kDa) with diverse physiological activities including angiogenesis [428]. Apelin knockout mice exhibit impaired retinal vascularization in the early postnatal period. Corneal pocket assay demonstrated that angiogenic response to proangiogenic factors VEGF and FGF2 was significantly decreased in apelin knockout mice [429]. Apelin and its G protein-coupled receptor APJ have been shown to regulate angiogenesis by induction of endothelial cells migration, proliferation, tube formation, maturation, and increasing the new blood vessel caliber. In addition, apelin plays a role in tip cell-stalk cell communications and modulates stalk cells proliferation [195, 430]. Apelin-APJ axis has been reported to be highly expressed in tumour endothelium. The co-expression of apelin and its receptor in endothelial cells of tumour vasculature suggests that apelin can exert autocrine and paracrine activities on endothelial cells [431]. Apelin-APJ has been shown to be overexpressed in the vasculature of diabetic retinopathy disease. Retinal

angiogenesis was inhibited in the apelin deficient mouse model of diabetic retinopathy although temporal expression of VEGF did not change between wild type and apelin deficient mice [432]. Our results also suggest that apelin is critical for proper tip cell differentiation *in vitro*.

Dll4-Notch1 signaling regulates angiogenesis by controlling the number of differentiated endothelial tip cells and by suppressing stalk cells from becoming tip cells [185]. The inhibition of Dll4 signaling can lead to increase in tip cells formation, however, intact Notch1 signaling via Dll4 antagonist jagged 1 can lead to reduction in tip cell numbers [185]. It is also suggested that Dll4 expression can bring to an end the proliferative stage of angiogenesis and begin the stage of maturation and stabilization [188]. Similarly, we observed a modest increase in tip cell formation but significantly longer migration distance from the bead in EPC treated with Dll4 specific siRNA *in vitro*.

Interestingly, the mRNA expression levels of Dll4 and apelin were markedly decreased when PI3K was down-regulated and where we observed inhibition of sprouting angiogenesis, suggesting that the activity of apelin and Dll4 together is important for the differentiation of activated EC into tip cells and proper sprout formation. However, the mRNA expression level of the other tip cell marker ESM1 did not change across the various treatments. This result suggests a complex regulation of the tip cell differentiation program. It should be noted that total RNA was extracted from whole fibrin gels of sprouting beads and not from pure tip

cells only, however, we believe that after 24 h of seeding the coated beads, there is an enrichment of tip cells at that time point. Regardless, the data need to be validated by mechanically excising tip cells for example by laser capture microdissection.

In this Chapter, I studied the angiogenic potential of EPC and compared it with other types of endothelial cells. I showed that EPC have higher angiogenic potential than HUVEC. I studied several genes by siRNA knockdown that may play a role during the sprouting angiogenesis process *in vitro*. I also confirmed the role of p110α in angiogenesis, as well as, demonstrating a role for the other class IA PI3K catalytic isoforms p110β and p110δ in blood vessel formation *in vitro*. In addition, my data also suggest that Dll4 and apelin are necessary for proper tip cell differentiation.

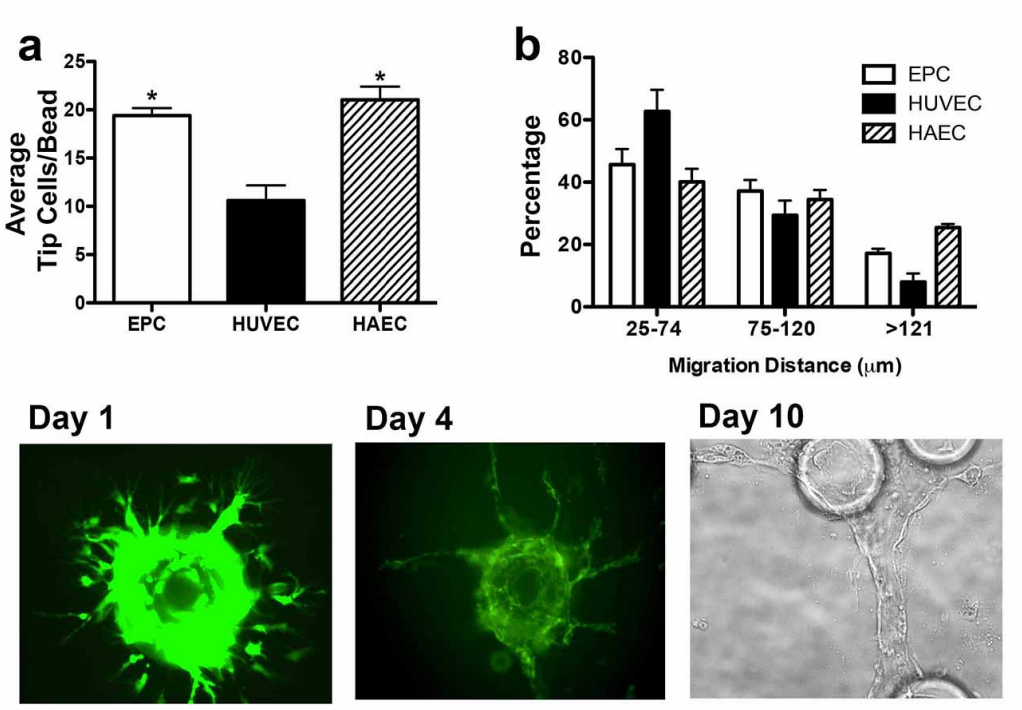

Figure 6.1: Comparison of the angiogenic activity of various types of endothelial cells. Endothelial progenitor cells, human umbilical vein endothelial cells, and human aorta endothelial cells were coated on Cytodex beads and embedded in fibrin gel. The cells were evaluated after 24 hours and 30 beads per treatment were scored for number of tip cells/bead (A), and for the distance the cells migrated away from the bead (B). Representative images of tip cells formation after 24 h (x200 original magnification), sprouts (tip cells + stalk cells) formation by day 4 (x200 original magnification), eventually lumens are formed by day 10 (x100 original magnification). (n = 5, the bars represent mean ± SEM, *p < 0.05 EPC vs HUVEC, HAEC vs HUVEC, one-way ANOVA).

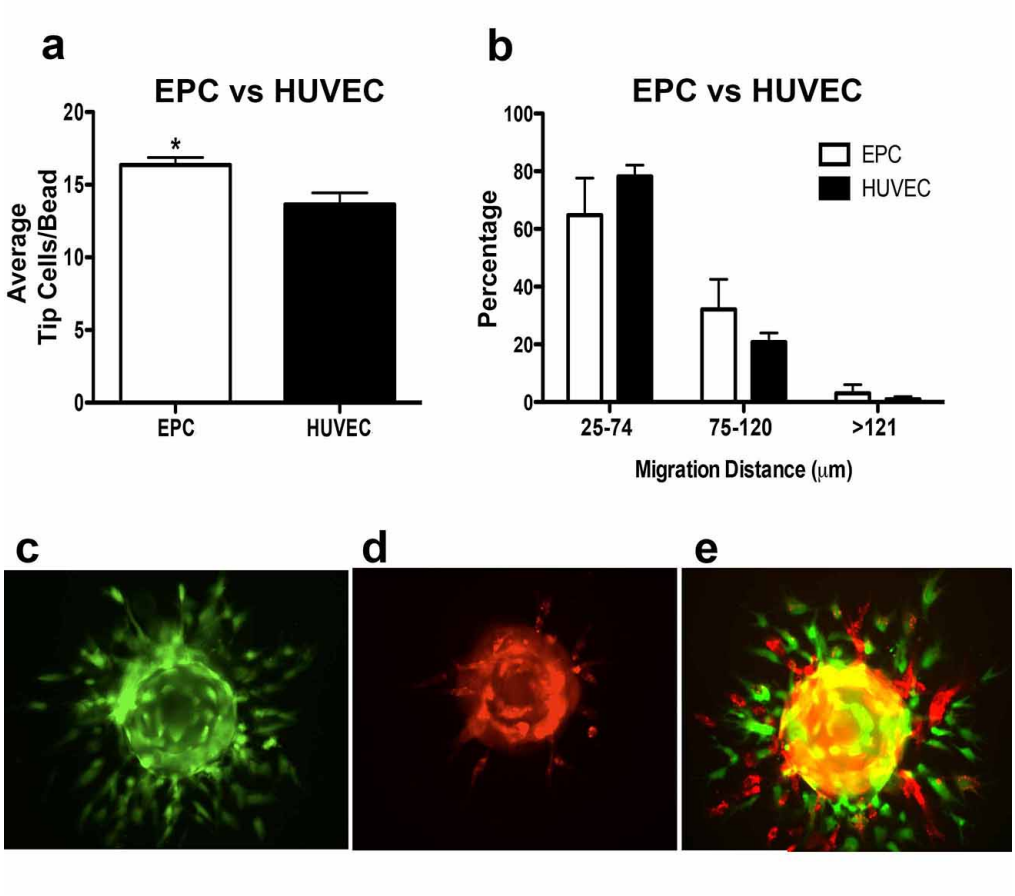

Figure 6.2: Competition assay between EPC and HUVEC. EPC (green) and HUVECs (red) were coated on the Cytodex beads at equal number and embedded in fibrin gel with fibroblast placed on top of the gel. The beads were analyzed after 24 h of culturing at 37 ˚C and 5 % CO_2. The number of tip cells per bead was evaluated (a) and the migration distance away from the beads (b). The image in (c) represents a bead coated with EPC after 24 of culture, (d) shows a bead coated with HUVEC after 24 h. (e) shows a representative bead with mixed EPC and HUVEC at the same time point as above, x200 original magnification. (n = 5, the bars represent mean ± SEM, *$p < 0.05$ EPC vs HUVEC one-way ANOVA).

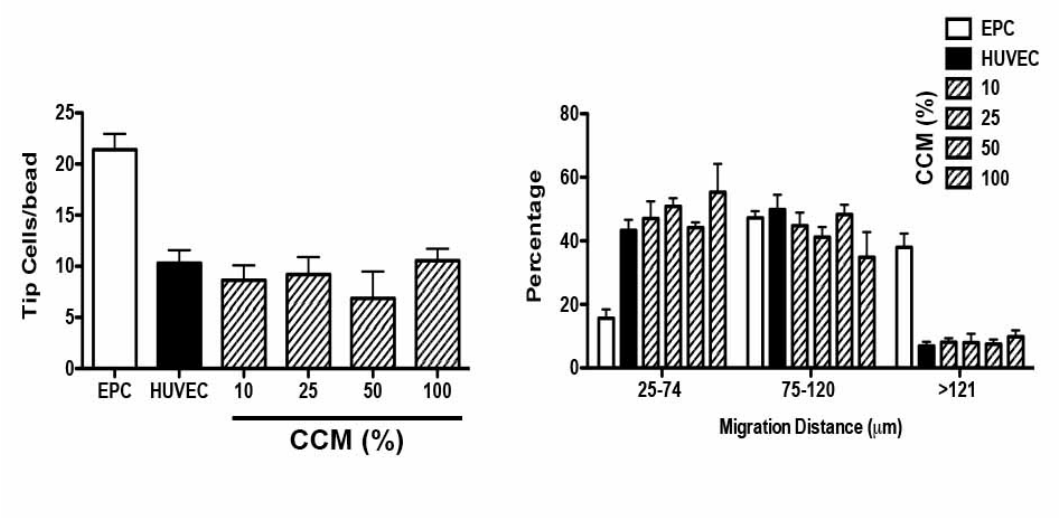

Figure 6.3: The effect of EPC cell-conditioned medium (CCM) on HUVEC sprouting. HUVEC embedded in fibrin gel were cultured in the presence of an increased concentration of EPC CCM (10, 25, 50, 100 %). The beads were analyzed 24 h after plating. (n = 3, p = NS).

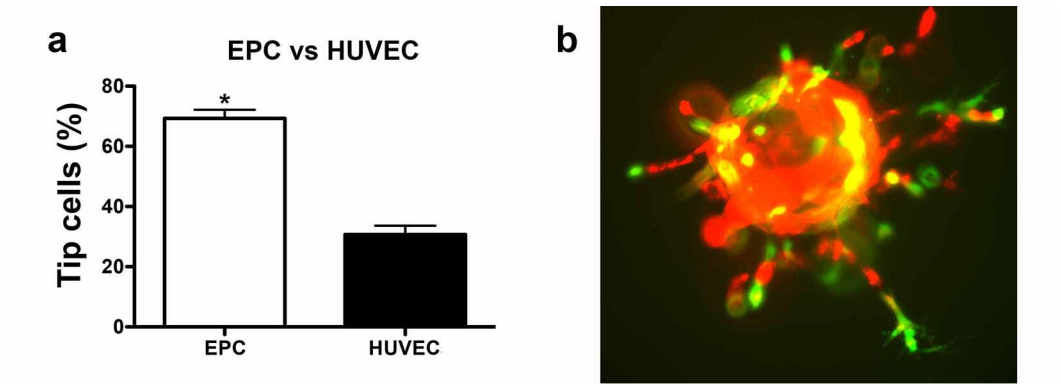

Figure 6.4: EPC preferentially occupy tip cell position. EPC (green) and HUVEC (red) were coated on the Cytodex beads at equal number and embedded in fibrin gel with fibroblast placed on top of the gel. After 4 days in culture the beads were analyzed for the type of cell that occupied the tip cell position of a growing sprouts. EPC preferentially occupied the tip cell position in the majority of the sprouts (a). (b) A representative bead showing chimeric sprouts with EPC occupying the tip position whereas the HUVEC locating mostly in the stalk position, x200 original magnification. (n = 3, the bars represent mean ± SEM, * $p < 0.05$ EPC vs HUVEC, one-way ANOVA).

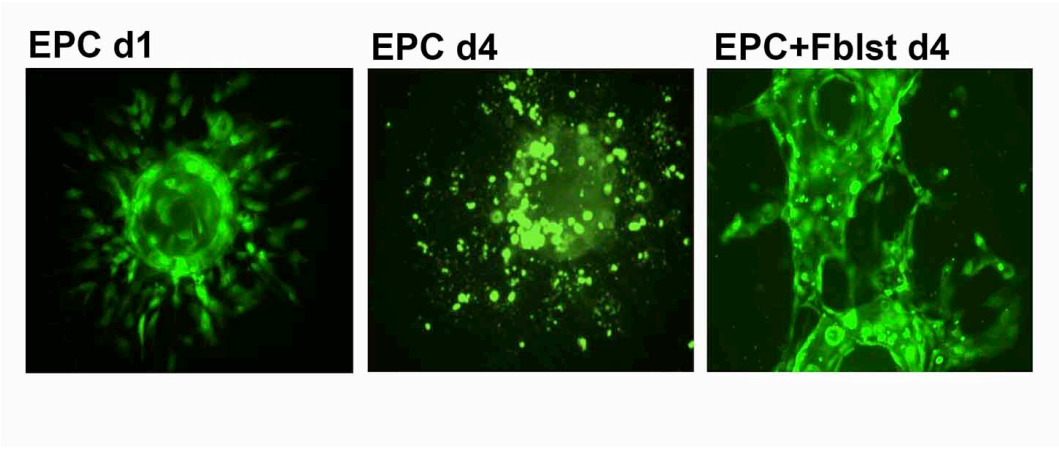

Figure 6.5: The role of fibroblasts in maintaining cellular survival and sprout development. EPC cultured in the absence of fibroblasts exhibit fast growth in the first day of culture but the cells undergo cell death by day 4, x200 original magnification. However in the presence of fibroblast, EPC survive past day 4 and start to form sprouts that eventually form lumens.

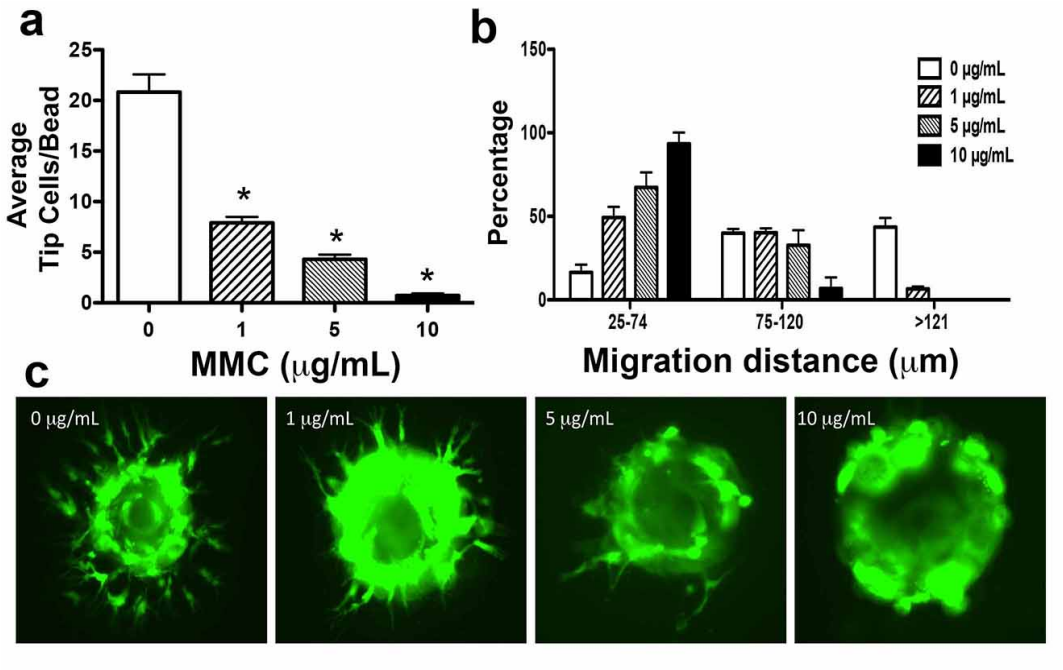

Figure 6.6: The effect of cell cycle inhibitor mitomycin C on sprouting angiogenesis. The EPC were treated with various concentrations (0-10 μg/mL) overnight. The next day the cells were coated on Cytodex beads. The tip cells formation and the cells migration distance were analyzed the next day. Images x200 original magnification. (n = 3, the bars represent mean ± SEM, * $p < 0.05$ vs untreated cells one-way ANOVA).

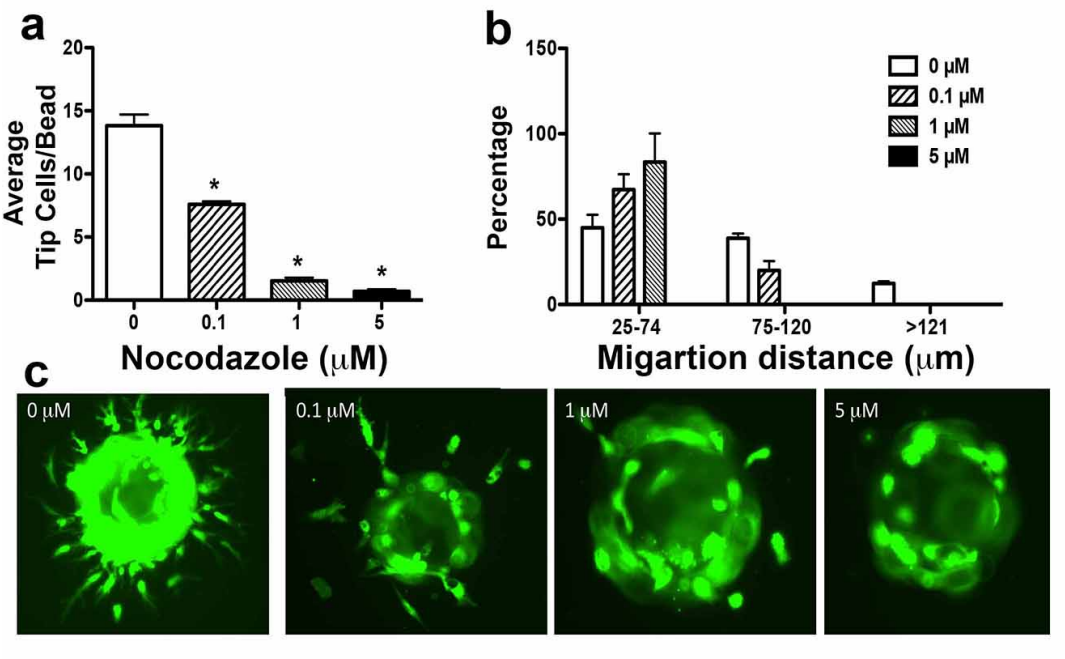

Figure 6.7: The effect of microtubule inhibitor nocodazole on sprouting angiogenesis. The EPC were treated with various concentrations (0-5 μM) overnight. The next the cells were trypsinized and coated on Cytodex beads. The tip cell formation and the cells migration distance were analyzed the next day. Images x200 original magnification. (n = 3, the bars represent mean ± SEM, * $p < 0.05$ vs untreated cells one-way ANOVA).

Table 6.1: Summary of the effect of siRNA knockdowns of various genes on sprouting angiogenesis. Downward pointing arrows indicate reduction, upward pointing arrows indicate increase, and right pointing arrow indicate no change in sprouting angiogenesis.

SiRNA for	Effect on sprouting
ALK1	↓
Apelin	↓
Dll4	↑
ERG	↓
FGD5	↓
ILK	↓
IQGAP1	→
KDR	↓
p110α	↓
p110β	↓
p110δ	↓
PTEN	↑
Rac1	↓

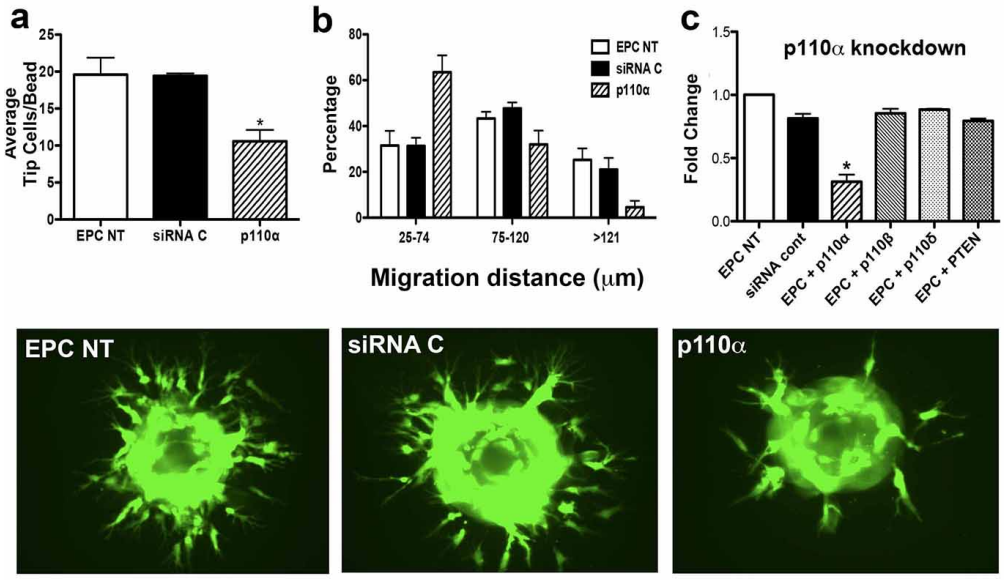

Figure 6.8: The effect of siRNA knockdown of p110α on angiogenesis. EPC were transfected with 50 nM of p110α siRNA twice. After 72 h of transfection the cells were coated on Cytodex beads and embedded in fibrin gel and incubated at 37 ˚C, 5 % CO2 overnight. The next day the beads were analyzed for tip cell formation and for migration distance away from the beads (a, b). The siRNA knockdown was verified after 72 h of transfection by real time PCR (c). Images x200 original magnification. (n = 5, the bars represent mean ± SEM, *p < 0.05 p110α vs siRNA C, one-way ANOVA).

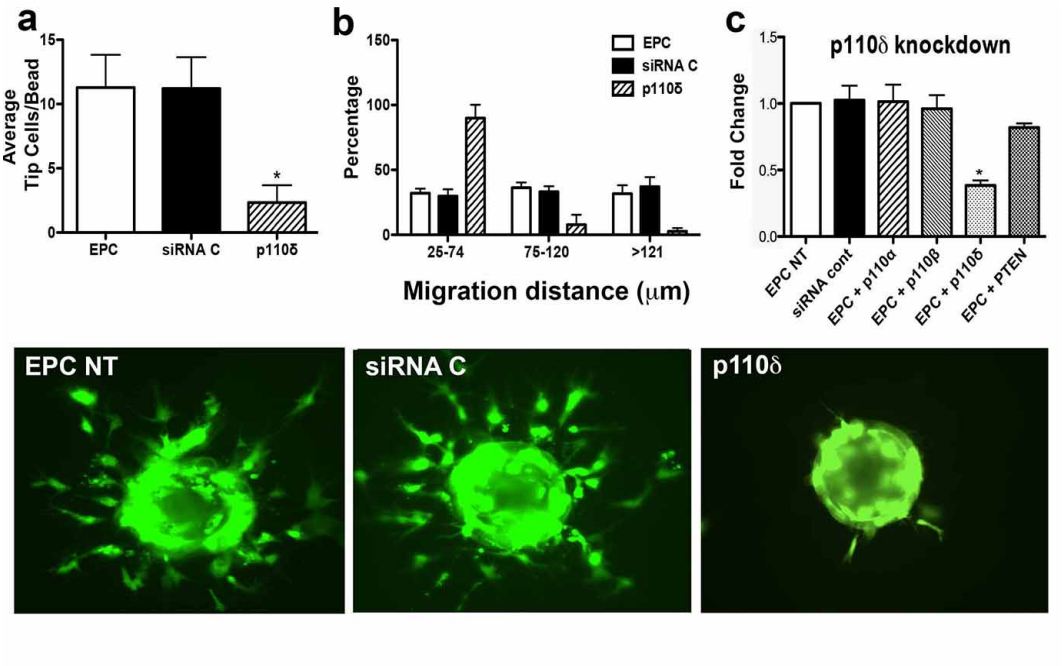

Figure 6.9: The effect of siRNA knockdown of p110δ on angiogenesis. EPC were transfected with 50 nM of p110δ siRNA twice. After 72 h of transfection the cells were coated on Cytodex beads and embedded in fibrin gel and incubated at 37 ˚C, 5 % CO2 overnight. The next day the beads were analyzed for tip cell formation and for migration distance away from the beads (a, b). The siRNA knockdown was verified after 72 h of transfection by real time PCR (c). Images x200 original magnification. (n = 6, the bars represent mean ± SEM, * $p < 0.05$ p110δ vs siRNA C,one-way ANOVA).

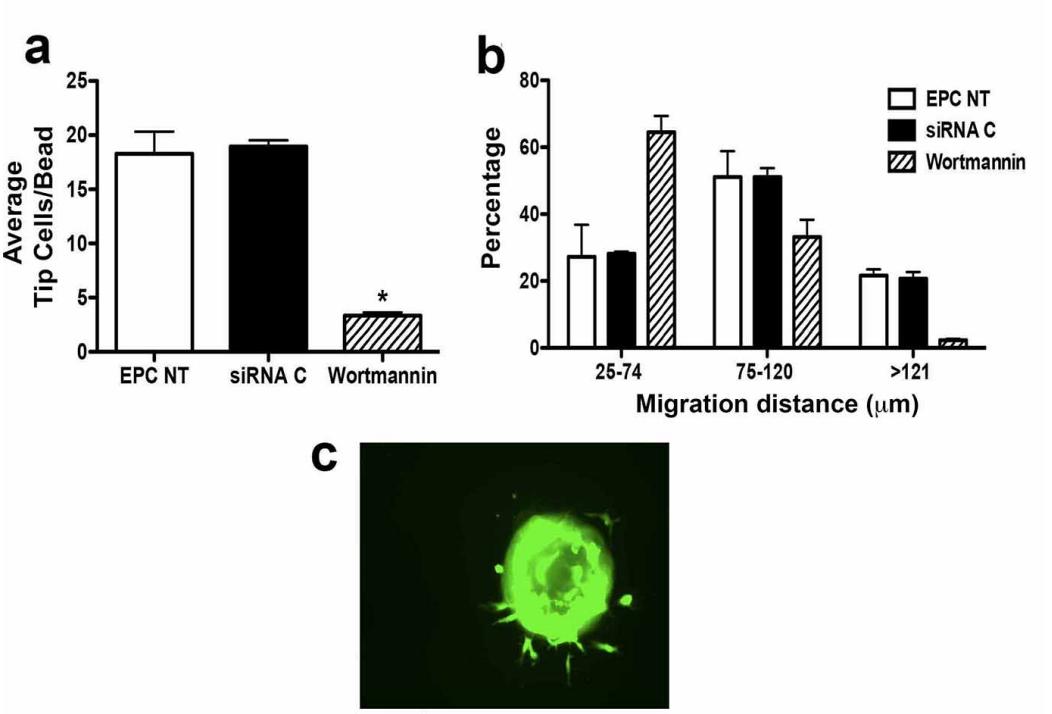

Figure 6.10: Inhibition of sprouting angiogenesis by wortmannin. EPC were treated with 10 μg/mL of wortmannin overnight. Next the cells were coated on Cytodex beads and incubated at 37 ˚C, 5 % CO2 overnight. The next day the cells were analyzed for tip cells formation (a) and the distance they travelled away from the beads (b). (c) Shows a representative image of a bead coated with EPCs treated with wortmannin. Image x200 original magnification. (n = 3, the bars represent mean ± SEM, * $p < 0.05$ vs siRNA C, one-way ANOVA). NT: no treatment; c: control.

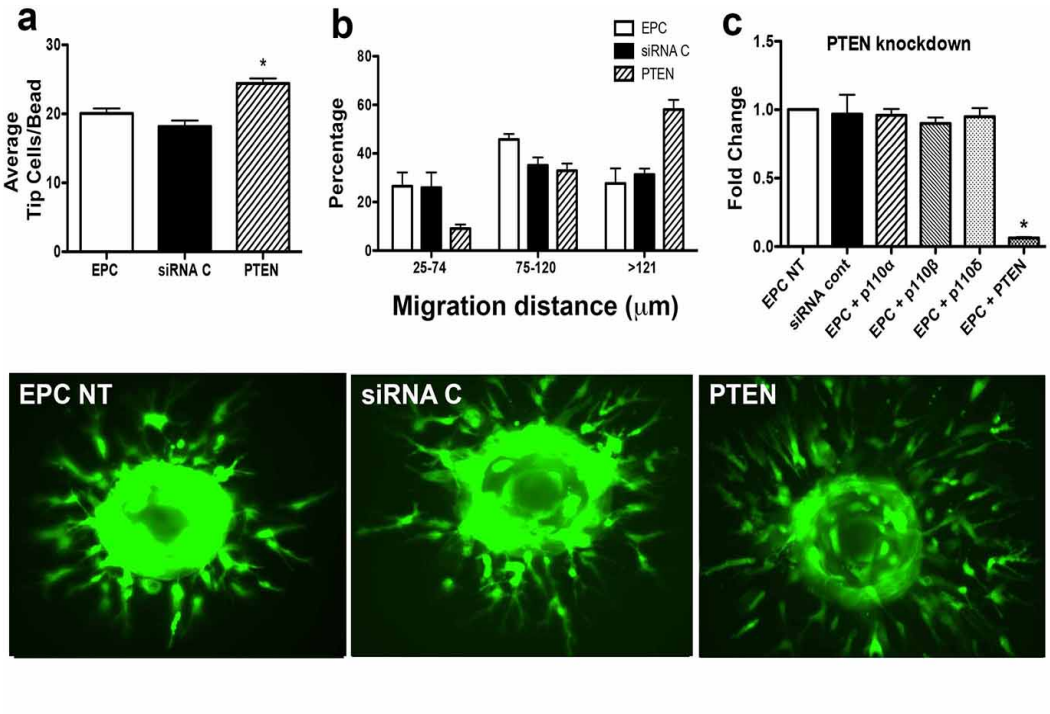

Figure 6.11: The effect of siRNA knockdown of PTEN on angiogenesis. EPC were transfected with 50 nM of PTEN siRNA twice. After 72 h of transfection the cells were coated on Cytodex beads and embedded in fibrin gel and incubated at 37 ˚C, 5 % CO2 overnight. The next day the beads were analyzed for tip cell formation and for migration distance away from the beads (a, b). The siRNA knockdown was verified after 72 h of transfection by real time PCR (c). Images x200 original magnification. (n = 5, the bars represent mean ± SEM, * $p < 0.05$ PTEN vs siRNA C, one-way ANOVA).

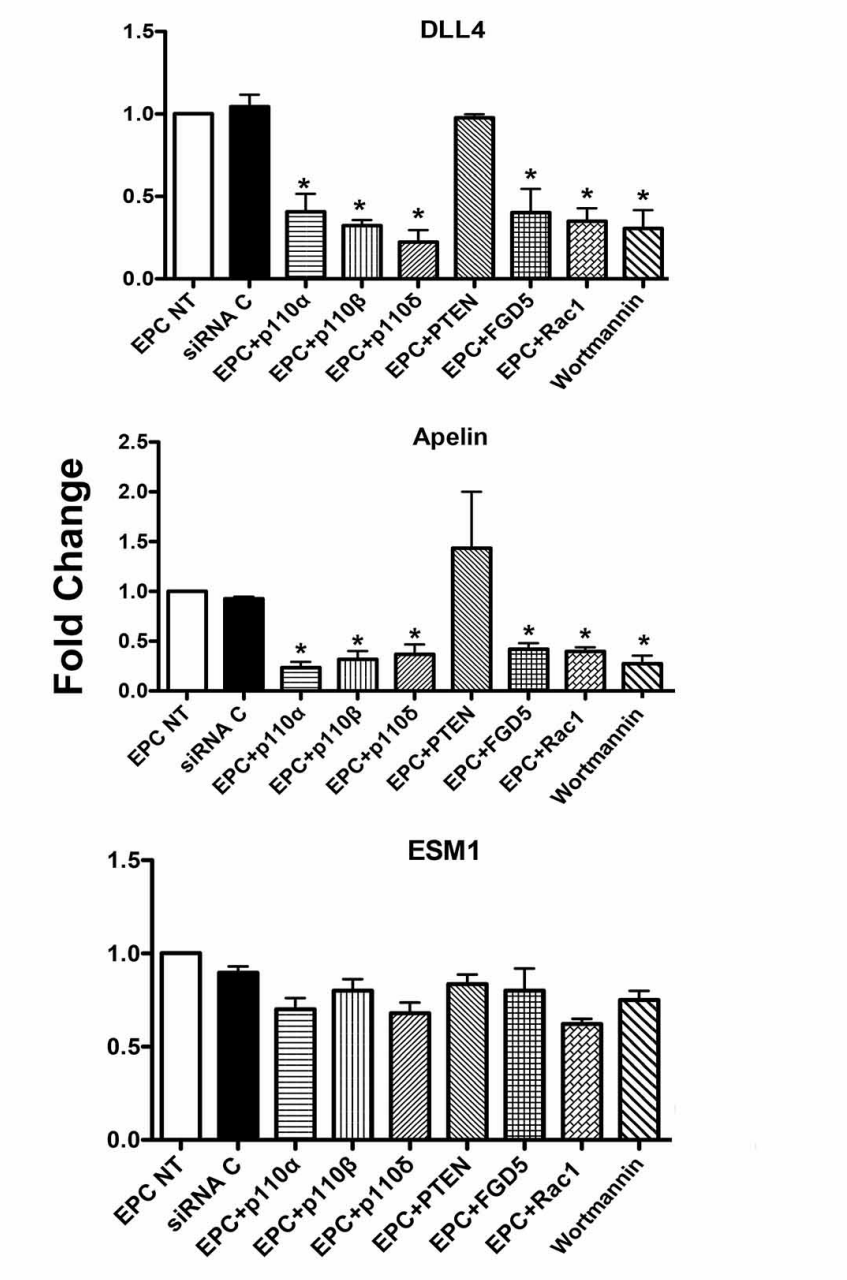

Figure 6.12: Real time expression of tip cells markers apelin, Dll4, and ESM1. Total mRNA was extracted using TRIzol/chloroform method from sprouting beads 24 h after seeding. One microgram of total RNA was reverse transcribed into cDNA. SYBR Green and Delta Delta Ct method was used to quantify mRNA expression levels. (n = 3, the bars represent mean ± SEM, * $p < 0.05$ vs siRNA C, one-way ANOVA).

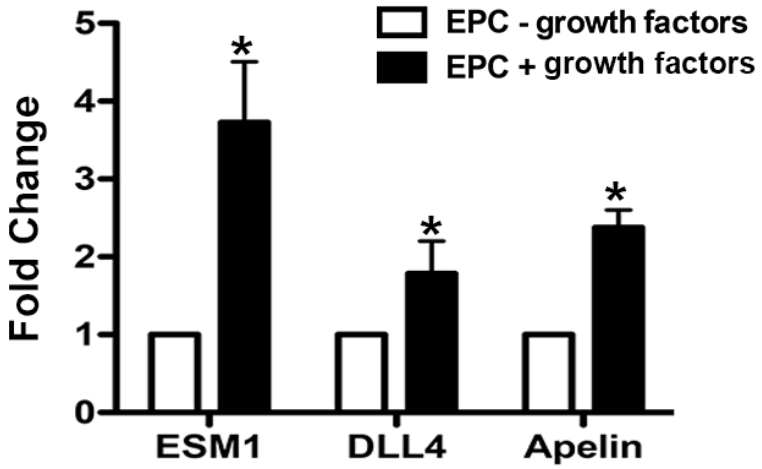

Figure 6.13: Induction of tip cell markers ESM1, Dll4, and apelin. The mRNA was extracted from beads coated with EPC and embedded in fibrin gel and cultured overnight in the presence of complete EBM2 (includes all growth factors and 10 % FBS), or in incomplete EBM2 (do not include the growth factors and supplemented with 2 % FBS). One microgram of total RNA was reverse transcribed into cDNA. SYBR Green and Delta Delta Ct method was used to quantify mRNA expression levels. (n = 3, the bars represent mean ± SEM, * $p < 0.05$ one-way ANOVA).

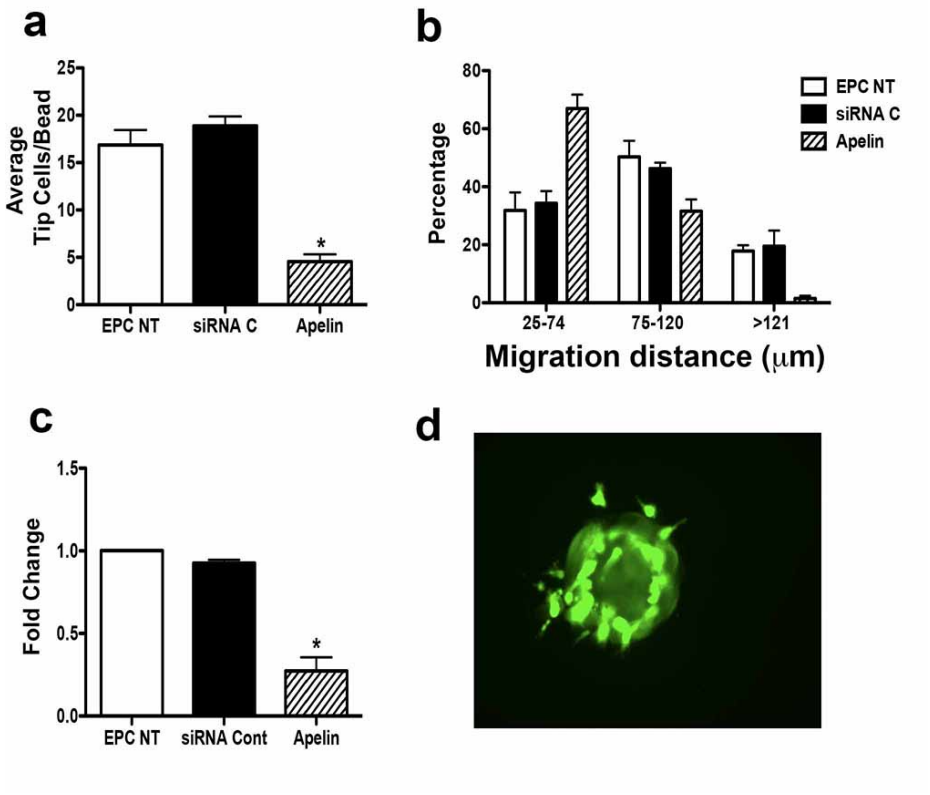

Figure 6.14: The effect of siRNA knockdown of apelin on tip cell formation. EPC were transfected with 50 nM of apelin siRNA twice. After 72 h of transfection the cells were coated on Cytodex beads and embedded in fibrin gel and incubated at 37 ˚C, 5 % CO2 overnight. The next day the beads were analyzed for tip cell formation and for migration distance away from the beads (a, b). The siRNA knockdown was verified after 72 h of transfection by real time PCR (c). (a) A representative sprouting of EPC treated with apelin siRNA. Image x200 original magnification. (n = 6, the bars represent mean ± SEM, * $p < 0.05$ vs siRNA C, one-way ANOVA). NT: no treatment; c: control.

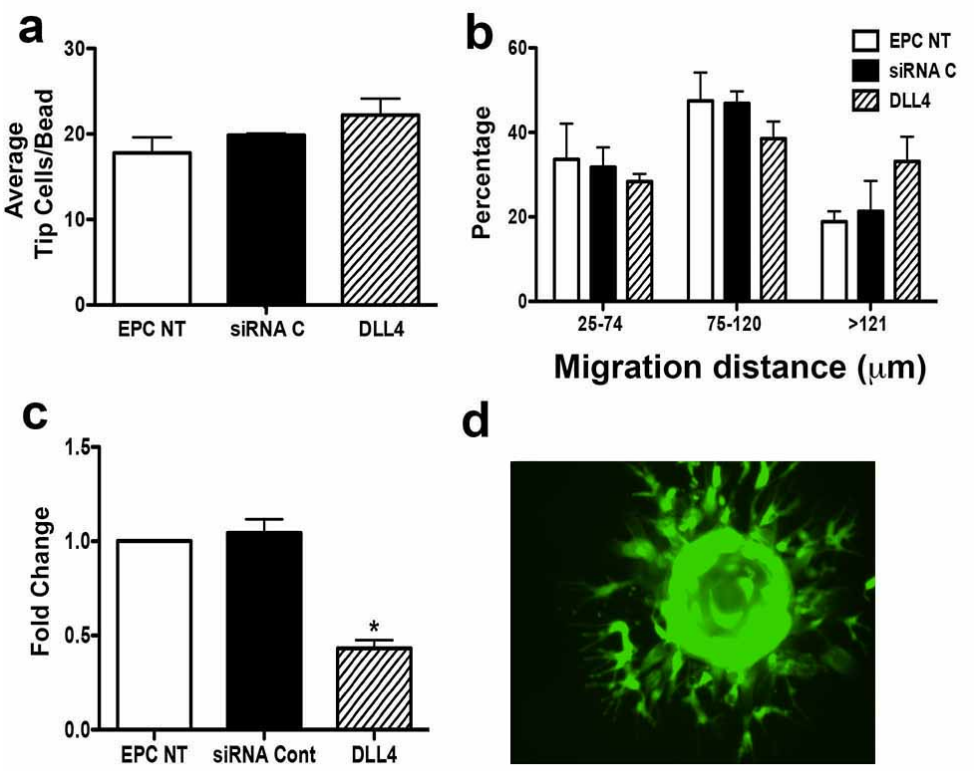

Figure 6.15: The effect of siRNA knockdown of Dll4 on angiogenesis. EPC were transfected with 50 nM of Dll4 siRNA twice. After 72 h of transfection the cells were coated on Cytodex beads and embedded in fibrin gel and incubated at 37 ˚C, 5 % CO2 overnight. The next day the beads were analyzed for tip cell formation and for migration distance away from the beads (a, b). The siRNA knockdown was verified after 72 h of transfection by real time PCR (c). (d) A representative sprouting of EPC treated with Dll4 siRNA. Image x200 original magnification. (n = 5, the bars represent mean ± SEM, * $p < 0.05$ vs siRNA C, one-way ANOVA). NT: no treatment; c: control.

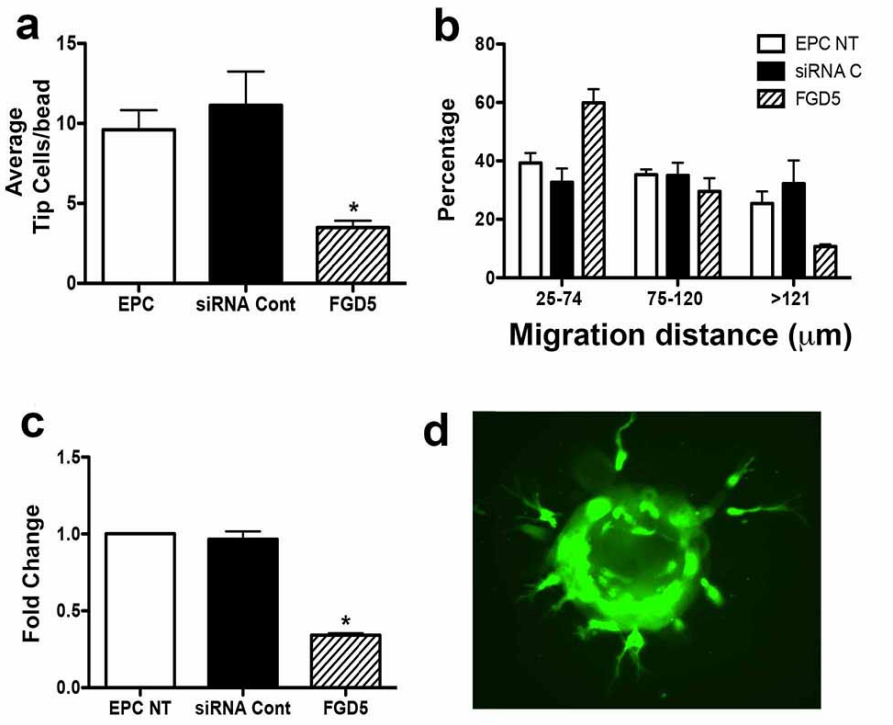

Figure 6.16: The effect of siRNA knockdown of FGD5 on angiogenesis. EPC were transfected with 50 nM of Dll4 siRNA twice. After 72 h of transfection the cells were coated on Cytodex beads and embedded in fibrin gel and incubated at 37 ˚C, 5 % CO2 overnight. The next day the beads were analyzed for tip cell formation and for migration distance away from the beads (a, b). The siRNA knockdown was verified after 72 h of transfection by real time PCR (c). (d) A representative sprouting of EPCs treated with FGD5 siRNA. Image x200 original magnification. (n = 5, the bars represent mean ± SEM, * $p < 0.05$ vs siRNA C, one-way ANOVA). NT: no treatment; c: control.

CHAPTER VII

THE STUDY OF KIDNEY MICROVASCULAR REPAIR BY HUMAN ENDOTHELIAL PROGENITOR CELLS

7.0 INTRODUCTION

Acute kidney injury in the setting of existing damage is a leading cause of end stage renal disease [433]. Despite the medical advancements made in the past 30 years in intensive care medicine and dialysis modalities, there is still high mortality rate in hospitalized patients with acute renal failure [141, 433, 434]. Chronic endothelial dysfunction is considered to be the initial step in the development of vascular diseases that leads to accelerated arteriosclerosis and impaired angiogenesis [435]. Renal hypo-perfusion caused by various pathologies adversely affect the function of kidney tubular epithelial cells, a condition that has been recognized as a secondary effect of the microvascular endothelial cells dysfunction that impedes post-ischemic kidney reperfusion and interrupts the repair process [140, 436].

The vascular repair concept came to prominence when Asahara et al. [8] reported on the isolation of bone marrow-derived endothelial progenitor cells. Subsequently, many researchers have described studies on the participation of EPC in vasculogenesis or *de novo* blood vessels formation from circulating endothelial precursors cells [80, 395, 437].

There has been an explosion in the field of regenerative medicine because of the tremendous potential EPC hold as a therapeutic strategy for vascular diseases [17]. Due to this great interest in EPC and in the absence of a uniform definition and a specific marker, there has been a well-documented controversy stemming from the various cell populations called EPC. Recently it appears that there is a general consensus that the EPC population known as late outgrowth cells, that are derived from the endothelial colony-forming cells (ECFC), are the cells that "truly" differentiate into mature endothelial cells. Whereas the other EPC types are known to generate angiogenic factors but express hematopoietic markers [17, 18], also reviewed in Chapter 2 section 2.2.

There are a few reports indicating that patients with chronic renal disease have dysfunctional "EPC", insignificantly lower numbers as compared to control patients [12, 399, 435, 438]. The causes of the EPC incompetence are broad and encompass many cellular processes such as mobilization, impaired endothelial nitric oxide synthase (eNOS), viability, reduced engraftment, and impaired differentiation into mature endothelial cells (reviewed by [399]).

Encouraging evidence points to a role of EPC transplantation in neovascularization and blood vessel reendothelization in cardiovascular diseases [439]. For example, *ex vivo* generated EPC introduced into an animal model of myocardial infarction reduced ventricular scarring and improved cardiac function [440, 441]. Similarly, introduction of EPC

ameliorated blood flow in hind limb ischemia models in mice and rats [442, 443]. However, there are fewer published reports on endothelial regeneration by EPC of damaged kidney microvasculature [12]. Early report by Rookmaker et al. [444] showed a fourfold increase of transplanted bone marrow-derived glomerular endothelial cells after 7 days in Thy-1.1 glomerulonephritis animal model. Using the same Thy-1.1 animal model, Uchimura et al. [14] showed that bone marrow-derived EPC administered into the renal artery reduced glomerular endothelial cell injury and mesangial cell activation.

Other studies have attempted to genetically modify EPC to enhance their activity *in vivo*. Some strategies involved the overexpression of eNOS and heme oxygenase-1 to inhibit neointimal hyperplasia [445]. Murasawa et al. [446] have overexpressed telomerase reverse transcriptase to increase the mitogenic, migratory, and cell survival activity of EPC. Using a different approach, Yamamizu et al. [447] constitutively overexpressed protein kinase A in order to increase EPC differentiation into mature endothelial cells.

The vast majority of studies that focus on EPC reparative potential are based on chronic kidney disease with the rat Thy-1.1 animal model being the most widely used, and the early outgrowth EPC and/or circulating EPC being the transplanted cells utilized in the majority of the early reported studies. Late outgrowth EPC may represent the cells with “true” endothelial phenotype these cells have not been functionally studied

extensively *in vivo* and in the clinical situation [12, 448]. In this chapter, I show the results of the late outgrowth EPC transplantation study using our lectin-saporin animal model of acute kidney injury.

7.1 RESULTS

7.1.1 Introduction of Human EPC into Rag Knockout Mice

The initial studies of human EPC transplantation into lectin-saporin injured animals were carried out using recombination activating gene 1 (Rag 1, lack functional T and B cells) knockout mice where the human cells (2.10^6 cells/mouse) were not detected in the mouse organs including kidney, Lung, liver, heart, and spleen after 24 h of injecting the cells. In addition, the Rag1 mice were very sensitive to a high dose of lectin-saporin (L-S 500 μg/Kg) and the majority of the animals died within two days. In further pilot studies we used Rag $2^{-/-}$ (lack functional T, B, and NK cells) mice and a higher concentration of cells. In this protocol we first inject the L-S and 24 h later we delivered the hEPC. This strategy resulted in a positive identification of human EPC in mouse organs within 24 h of cell delivery; however the majority of the animals did not survive past day 1 of cell injection. The mice were subjected to intraarterial surgery procedures in two consecutive days. The best results were obtained by delivering low-dose L-S (200 μg/Kg) conjugate together with the hEPC at 4.10^6 cells/mouse concentration in one intraarterial injection but the

majority of the animals were morbid before day 4 post injection (Figure 7.1). All the strategies applied in this study are summarized in Table 7.1.

7.1.2 Detection of Human EPC in Mouse Tissues

Human cells in mouse tissues were detected by means of end-point PCR, quantitative PCR and by immunohistochemistry and immunofluorescence. The hEPC were detected in mouse tissues using a primer set designed to specifically amplify a 650 bp of human beta-2-microglobulin (B2M) that was validated not to amplify the mouse B2M gene. We were able to detect the hEPC in the mouse organs up to 24h post cells injection in both PBS control animals and lectin-saporin injured animals (Figure 7.2 a). At later time points, for example at day 2, we noticed that the hEPC injected into PBS control mice were not detectable in any mouse organ, whereas the cells persisted in the injured animals at least in the lung and in the kidney (Figure 7.2 b). Interestingly, the hEPC persisted in the kidneys of the injured mice up to day 4 (Figure 7.2 c). However it was unfortunate that no animal survived past day 4 post injection, thus confounding the long term study of hEPC engraftment into the kidney microvasculature.

The hEPC engraftment into the mouse kidney glomerular capillaries was confirmed by immunohistochemistry staining by using species-specific anti-human CD31 antibody that was raised in mouse. The hEPC were detected in the glomeruli of the injured animals but not in the

peritubular capillaries. The cells were not detected in the PBS control mice at day 2 post injection (Figure 7.3 a,b). It was more difficult to detect the hEPC at day 4 but few positive glomeruli were observed by immunofluorescence (Figure 7.3 c).

In a pilot analysis, the expression of endothelial markers CD31 and Tie2 was evaluated by quantitative PCR using mouse specific primers. To our surprise the expressions of both genes were up regulated in injured mice (Figure 7.4) that received hEPC. This was contrary to what we observed previously in injured animals that did not receiving hEPC where CD31 and Tie2 mRNA transcripts were down regulated (Chapter 4, Figure 4.16). Most likely the mouse primers were amplifying the human transcripts in the mouse tissues. The mouse CD31 and Tie2 primers binding to human CD31 and Tie2 cDNA was confirmed by amplifying the two transcripts from human cells cDNA. Human specific CD31 and Tie2 primers were used to detect the hEPC mRNA transcripts in the mouse kidney cDNA by qPCR. Those showed specific detection of human CD31 and Tie2 transcripts in the injured animals at day 1 and day 2 post injection but they were only detected in day 1 cDNA from mice treated with PBS (Table 7.2). The qPCR results confirmed indirectly the persistence of hEPC in mouse kidney up until day 4 post injection in the mice that were treated with lectin-saporin, whereas no there was no signal of hEPC in the mice that received hEPC and PBS at that time point.

Renal injury was evaluated with qPCR, using the detection of kidney injury markers KIM1 and Lipocalin2. Both genes were significantly up-regulated in the injured animals but not in the animals that received PBS and hEPC (Figure 7.4). The kidney function was evaluated by serum creatinine and blood urea nitrogen (BUN) concentrations. The serum creatinine and BUN concentrations were significantly higher in the injured mice as compared to PBS control and PBS + hEPC group (Figure 7.5).

The expression of the growth factors PDGFB, bFGF, SDF1α, and VEGFA in the kidney were evaluated by qPCR. There was a significant up-regulation of PDGFB and bFGF transcripts at day 4 in the group of mice that received low dose of lectin-saporin and not the control groups. On the other hand, the expression of SDF1α, and VEGFA was significantly down regulated in the injured mice as compared to control (Figure 7.4).

7.2 DISCUSSION

We sought to investigate the potential of repairing injured glomerular capillaries by introducing human CD34+ derived EPC expanded in vitro. The lectin-saporin animal model (described in Chapter 4) caused an endothelial specific glomerular injury. The high dose (500 μg/kg L-S/LPS) caused severe irreparable renal damage and no animal survived past day 4 post L-S/LPS injection. On the other hand, the low dose was well tolerated by the mice. Injury was associated with a mild

kidney dysfunction that the animals' endogenous repair mechanism was sufficient to overcome the injury.

In an attempt to rescue the mice with severe renal injury, we injected high dose L-S conjugate intraarterially at day 1 and the following day we introduced 2 millions hEPC also intraarterially in $Rag1^{-/-}$ immunodeficient mice. Unlike the wild type C57Bl6 mice, the Rag1 mice were more sensitive to the surgery procedure and required longer time to awaken from the anesthetic and no mouse survived for longer than 12 hours after the second surgical procedure. The administration of a low dose of L-S was well tolerated despite surgical procedures in two consecutive days. Unfortunately, we were not able to detect the hEPC in the mouse tissues even 24 h after transplantation. Similarly, higher concentration of hEPC was not successful as well. Although, the $Rag1^{-/-}$ immunodeficient mice lack functional T and B cells [449] but they still generate functional NK cells [450]. It is possible that NK cells are responsible for eliminating hEPC.

For this reason we opted to study hEPC engraftment *in vivo* by using another strain of immunodeficient mouse. The $Rag2^{-/-}$ mouse strain is a double knockout mutant (both the Rag2 and the X-linked IL2rγ genes are targeted) that lacks functional T, B, and NK cells [451]. Thus the Rag $2^{-/-}$ (i.e. $Rag2^{-/-}$ $\gamma c^{-/-}$) mouse is a better-suited animal strain for xenotransplantation studies. The double surgery strategy was not suitable for this strain as all the animals died within few hours after the second

surgery. We modified our procedure to include only one surgery to deliver both lectin-saporin conjugate and the hEPC. This strategy suited the animals better but the majority of the mice died or were very morbid within two days after receiving low dose L-S+EPC or L-S only. Only 3 mice treated with L-S+EPC survived to day 4 post injection, but those animals were very morbid. We were able to detect the hEPC in the mouse kidneys of both control and injured mice up to 24 h after the delivery of the cells, and only in the kidney glomeruli of the injured animals past 24 h of transplantation. It is very interesting that the hEPC were located in the kidney glomerular capillaries of the injured mice suggesting an engraftment of the cells in the capillary walls by day 2 and up to day 4 post cells injection. The same was not true for the PBS control mice where the hEPC were not detected after 24 h post cells injection, suggesting that hEPC engraftment requires likely denuded areas in the microvasculature for the cells to take up residence. Alternatively, perhaps the presence of growth factors that are released at the site of injury aid the transplanted cells to persist and survive longer in that microenvironment. It is likely that the presence of hEPC in the injured mice increased the expression of growth factors such as PDGFB and bFGF that have mitogenic and angiogenic activities [260, 452] and aided the survival of hEPC *in vivo*. Indeed, Chade et al. [453] reported that autologous EPC transplantation increased the expression of angiogenic factors such as VEGF, HIF1α and angiopoietin-1 in injured kidneys.

Our results suggest that hEPC are suitable for transplantation studies as they can survive in a foreign host up to 4 days and were capable of homing to the site of injury in the mouse renal microvasculature, but the Rag 1 & 2 mouse strains are not suitable for this type of investigation.

The beneficial contribution of EPC treatment of renal injury is the reversal of vascular damage that occurs during acute kidney injury leading to progressive renal dysfunction [399, 454, 455]. Regardless of the origin and phenotype of the EPC there are mounting evidence that these cells have a renoprotective potential and most importantly are without significant side effects [456]. Treatments with EPC have improved renal function by reducing inflammation, and repairing vascular endothelium and improving tubular damage [454, 455, 457, 458]. EPC niches have been identified in the bone marrow and also in the kidney papilla, cortex and in the renal capsule that can be mobilized to repair damaged endothelium if those cells are healthy and intact [459-461]. However, renal injury can lead to the destruction of the endogenous EPC as was demonstrated in an adriamycin model of nephropathy [458].

There are different clinical settings designed to study EPC repair and regeneration potential of ishaemic and damaged microvasculatures. These clinical trials are aimed at evaluating EPC expanded *ex vivo* that includes studying autologous EPC transplantation, drug treatment, and physical exercise (reviewed by [462]).

Despite the current advancements in EPC transplantation there are still major obstacles that must be overcome. One of the major problems is the method of EPC delivery. It is reported that intravenous (IV) injections result in only ~ 3 % of the cells homing to the site of microvascular injury in the kidney and the rest of the cells undergo programmed cell death by anoikis (induced by detachment from the extracellular matrix) or get trapped in the pulmonary vasculature [463].

To overcome the IV injection problems researchers have engineered scaffolds designed to deliver EPC for treatment of acute kidney injury. The bioengineered scaffolds provide protection and a favorable microenvironment for their viability in order to maximize their therapeutic potential. The hyaluronic acid (HA) hydrogels have shown a remarkable benefit in delivering EPC embedded in them. In experimental adriamycin induced nephropathy, EPC implanted in HA-hydrogels transplanted in the renal capsule reduced serum creatinine concentration by 50-60 % and proteinuria by 60-75 %, a greater improvement from the IV delivered EPC [456, 458].

Despite the current obstacles in therapeutic regenerative angiogenesis of EPC and the lack of consistent data, there are still 19 published and 31 registered and ongoing clinical trials [462] that can improve and further our understanding of EPC biology. In the near future EPC can be used as a therapeutic agent.

Other alternative strategies using different cell types are also being evaluated. Several lines of evidence indicate that administration of mesenchymal stem cells (MSC) reduce kidney injury accelerate repair [464]. Bone marrow fraction that contains MSC injected intravenously into ischemia/reperfusion animal model of kidney injury resulted in a decrease in BUN concentration, whereas whole bone marrow had no effect [11, 465]. Similarly, injected MSC protected against renal injury in a glycerol-induced nephropathy model [466]. Interestingly, injected MSC are not shown to incorporate into the injured tubules but it is suggested that MSC modulate renal injury by paracrine/endocrine mechanism. Several studies have shown that MSC can secrete VEGF, bFGF, MCP1, HGF, and IGF-1 [464, 467]. These authors suggest that MSC can home to the site of microvascular injury and inhibit apoptosis thus blunting the renal injury. Other studies have demonstrated that MSC cell-conditioned medium could protect against renal injury [468].

In this chapter, I studied the possibility of using hEPC to repair damaged renal microvasculature in a mouse model of thrombotic microangiopathy. The encouraging result from this study is that the hEPC persisted in the mouse kidney in particularly in the glomeruli of injured mice but not in the PBS control animals. However, long term study of hEPC homing and engraftment was not feasible, as the immunodeficient mouse strain $Rag2^{-/-}$ did not tolerate the surgical procedure.

Table 7.1: Strategies utilized in the study of hEPC reparative potential in lectin-saporin mouse model of TMA. n ≥ 5 animals/group.

Strategy	mouse strain	L-S dose	# of EPCs	Outcome
2 surgeries	Rag1	High	2.10^6	Died > 12 h
2 surgeries	Rag1	Intermediate	2.10^6	Died > 24 h
2 surgeries	Rag1	Low	2.10^6	Survived but EPCs were NOT detected
2 surgeries	Rag1	Low	4.10^6	Survived but EPCs were NOT detected
2 surgeries	Rag2	Intermediate	2.10^6	Died > 12 h EPCs were detected
2 surgeries	Rag2	Intermediate	4.10^6	Died > 12 h EPCs were detected
1 surgery	Rag2	Low	4.10^6	Died 1-4 days EPCs were detected
1 surgery	Rag2	Low	6.10^6	Died > 12 h EPCs were detected

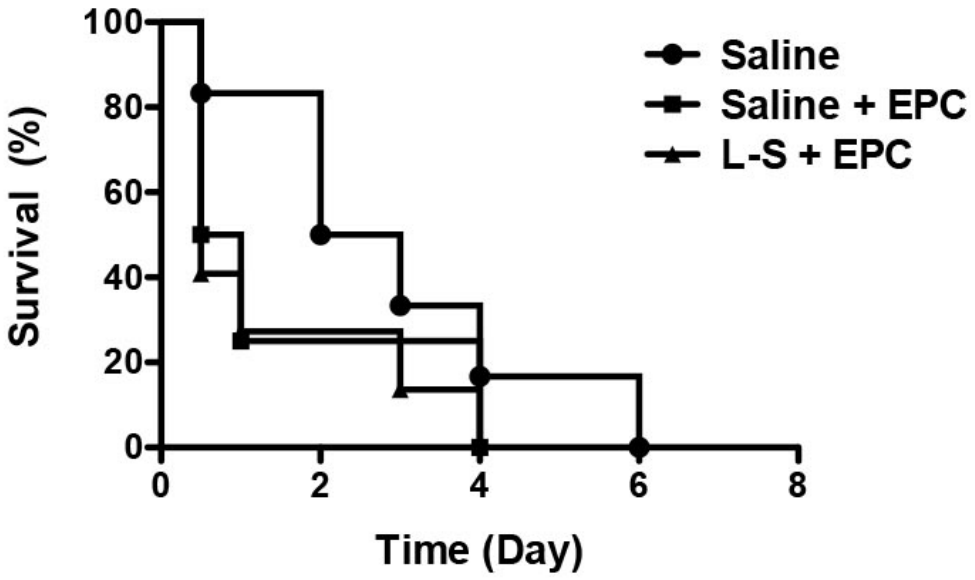

Figure 7.1: Kaplan-Meier survival graph for Rag2$^{-/-}\gamma^{-/-}$. The mice were injected with either saline or L-S conjugate (200 μg/Kg) along with human EPC via the carotid artery. ($n \geq 5$, p = NS Log-rank test)

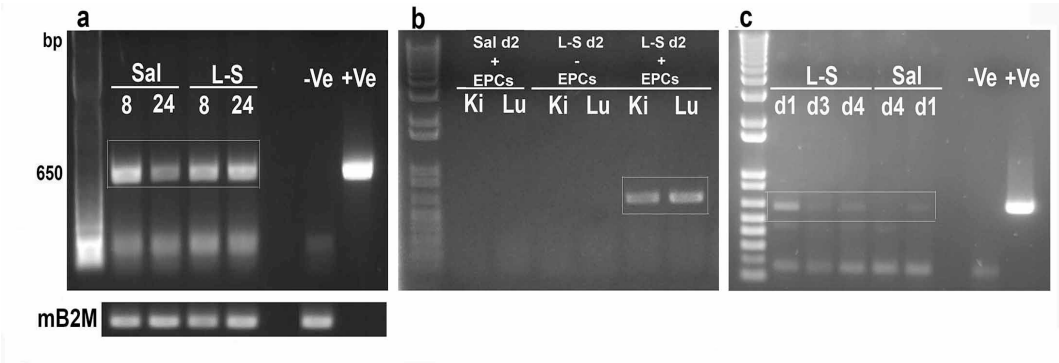

Figure 7.2: Detection of human EPCs in Rag $2^{-/-}$ tissues by end-point PCR. The immunodeficient mice were injected with 200 μg/Kg L-S/LPS along with 4.10^6 hEPC, injected with PBS and hEPC, treated with L-S only. The mouse tissues were aseptically removed and placed in TRIzol reagent to extract total RNA by phenol/chloroform method. Five μg of total RNA were reversed transcribed into cDNA. Human cells were detected using human specific B2M primers that amplify 650 bp of the mRNA transcript. n ≥ 3 animals/group.

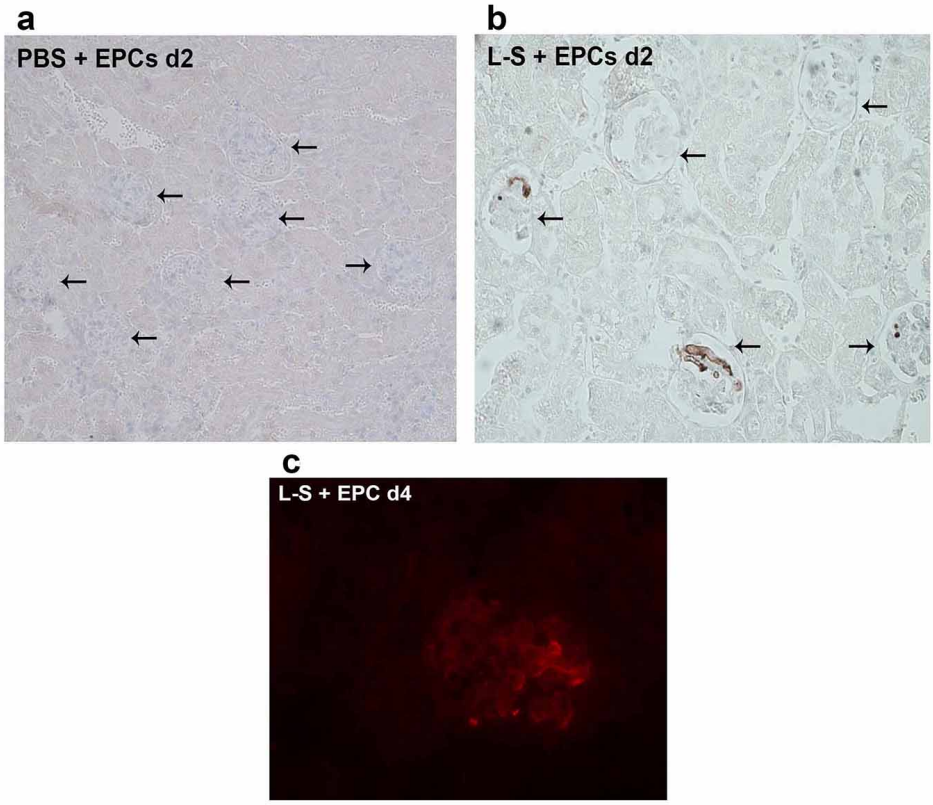

Figure 7.3: Detection of human EPC by immunocytochemistry and immunofluorescence in Rag $2^{-/-}$ mice. The human cells were detected in the mouse kidney sections using anti-human CD31 antibody. Five μm of OCT frozen kidney sections were stained with anti-hCD31 and an anti species HRP-conjugated secondary antibody and 3,3'-diaminobenzidine (DAB) substrate or an anti-species fluorescently labeled secondary antibody, IHC images x200, and IF image x400 original magnification. n ≥ 3 animals/group.

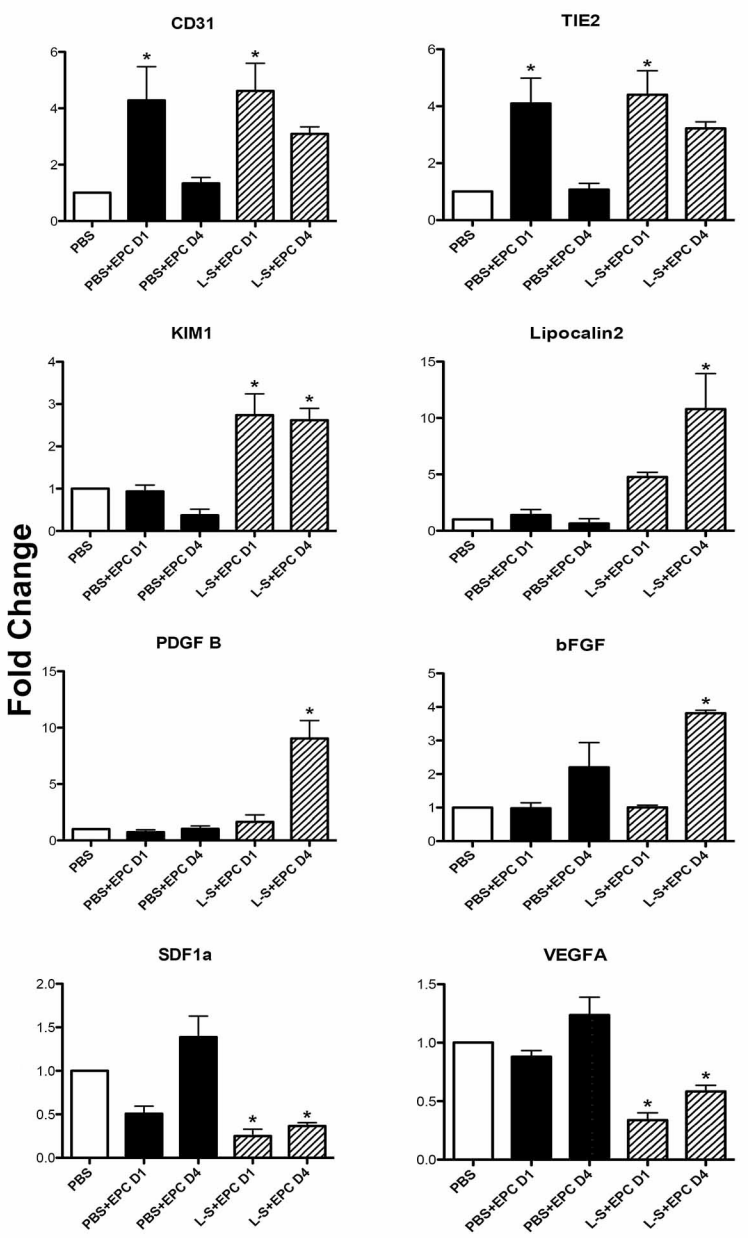

Figure 7.4: Real time PCR analysis of mRNA expression in Rag $2^{-/-}$ kidneys. Endothelial-specific genes CD31 and Tie2, kidney injury specific markers KIM1 and lipocalin2, in addition to the growth factors PDGFB, bFGF, SDF1α, and VEGFA. ($n \geq 3$ animals/group, * $p < 0.05$ one-way ANOVA).

Table 7.2: Expression of human CD31 gene in mouse kidney after receiving hEPC as determined by quantitative PCR. n ≥ 3 animals/group.

CD31	Avg ddCt
Sal	Undet.
PBS d1 + EPCs	33 ± 1
PBS d4 + EPCs	Undet.
L-S d1 + EPCs	33 ± 1
L-S d4 + EPCs	35 ± 2

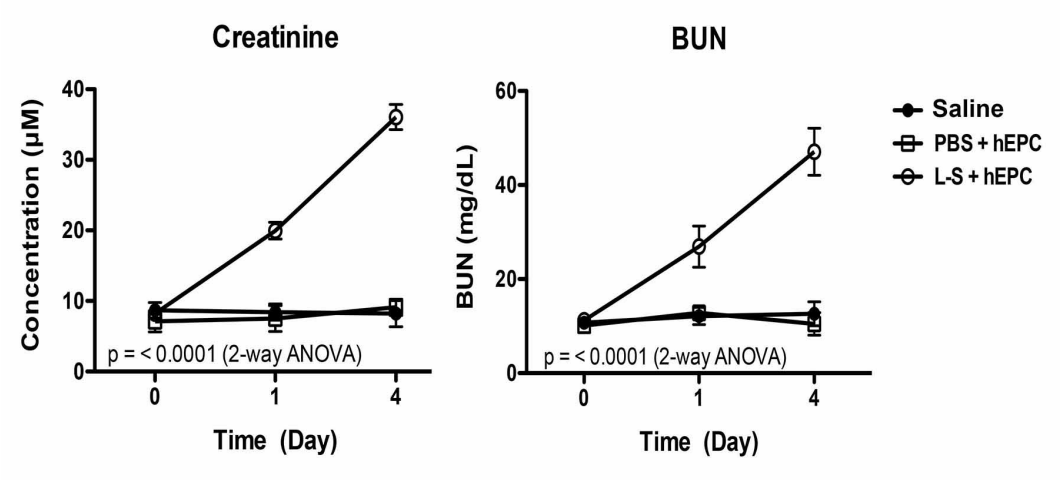

Figure 7.5: Evaluation of kidney function after hEPC introduction. The kidney function was determined by determining serum concentrations of creatinine and blood urea nitrogen in control and injured mice (200 μg/Kg L-S/LPS). n ≥ 3 animals/group, $p < 0.05$ two-way vs saline control ANOVA.

CHAPTER VIII

ENDOTHELIAL EXPRESSION OF THE PI 3-KINASE CATALYTIC ISOFORM P110 BETA IS REQUIRED FOR SPROUTING ANGIOGENESIS AND KIDNEY MICROVASCULAR REPAIR

8.0 INTRODUCTION

Acute and chronic endothelial injury and loss from the microvasculature underlies a group of disorders termed thrombotic microangiopathies (TMA) [469]. Common causes of such endothelial damage include exposure to Shiga-like toxin associated with epidemic *E. coli* infections, drug toxicity, and acute and chronic immune responses against allogeneic vascular endothelium in the context of bone marrow or solid-organ transplantation. These diseases often follow a fulminant course leading to rapid failure of affected organs [470]. In particular, thrombosis involving the kidney glomerular capillaries, a particularly susceptible microcirculation, results in acute kidney failure and is a major cause of morbidity in Shiga-like toxigenic infection [471-473].

Despite catastrophic kidney glomerular endothelial damage requiring renal replacement therapy, many affected individuals recover kidney function [473]. This observation points to the regenerative capacity of the microvascular endothelium to repair injury. Yet the mediators that cue repair responses, and the signal transduction pathways triggered in

the endothelium, are poorly defined. Candidate growth factors inferred from vascular development in the embryo or neoangiogenesis into a tumour include vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2), or thrombin and sphingosine-1-phosphate signaling to the endothelial cells through receptor tyrosine kinases (RTK) or G-protein coupled receptors (GPCR) respectively [176]. Nevertheless, the cues used to repair a mature endothelium in the adult may differ from these well-modeled scenarios.

Downstream of both endothelial RTK and GPCR, the phosphatidylinositide-3 kinases (PI3K) act to integrate survival and growth signals [474]. PI3 kinases are a family of lipid kinases that include 4 catalytic isoforms expressed in EC, p110 -α, -β, -δ, -γ that associate with a regulatory subunit to couple PI3 kinase activity to receptor activation [475]. PI3 kinases that associate with the p85 regulatory subunit are grouped into class IA, whereas the single class IB, γ, isoform is regulated via p101 and coupled to GPCR. The class I PI3 kinase-β is the only isoform coupled to both RTK and GPCR [476, 477]. In embryonic development it is established that the PI3 kinase-α isoform is critically required, since both global and EC-restricted knockout of *Pik3ca*, the α catalytic subunit, results in death of the embryo with features of disordered vascular development [330, 478]. Conversely, global loss of PI3 kinase-β expression is lethal in early embryogenesis, but EC-restricted loss is

tolerated in development [329, 330]. The function of PI3 kinase-β remains largely unknown.

To address the question of PI3 kinase-β function in the adult microcirculation, we examined the effect of selective endothelial loss of PI3 kinase-β expression in TMA injury. We observe a dramatic phenotype of lethal kidney failure among mice lacking endothelial expression of the PI3 kinase-β isoform, indicating a critical role of the enzyme in the adult microvasculature. *In vitro* studies using human late blood outgrowth progenitor endothelial cells (hEPC) demonstrate defects in proliferation, cytoskeletal reorganization, and gene transcription associated with a failure to extend sprouts into 3D fibrin gels that model the environment in a thrombosed microvessel. Further, we observe that hEPC deficient in *Pik3cb* expression fail to assemble into microvessels *in vivo*.

8.1 RESULTS

8.1.1 Endothelial-Specific p110β Knockout Renders Mice More Susceptible to Injury and Reduces Survival

To determine the role of p110β in the mature microvasculature, adult Tie2 ^{ERT2}Cre/p110β$^{flox/flox}$ mice received 2mg of tamoxifen or vehicle (oil) daily for 5 days via an intraperitoneal injection to induce the Cre recombinase activity selectively in vascular EC to delete exons 21 and 22 that constitute the kinase domain (Figure 8.1). Mice were then rested for a week before 200 μg/Kg lectin-saporin conjugate (L-S) was injected intra-

arterially to selectively injure the glomerular capillary microvasculature as described in Chapter V. Vehicle-treated mice tolerated the sublethal dose of the lectin-saporin (L-S) toxin conjugate incurring 25% death at 7 days after injury (Figure 8.2). In contrast, the tamoxifen-treated group, lacking endothelial p110β, showed signs of morbidity as early as 24 hours after receiving the L-S dose and none survived more than 3 days after L-S injection.

To understand the nature of the renal injury incurred by the p110β EC- knockout mice, kidney histology was examined. We observed features of persistent glomerular injury of microvascular TMA of glomerular capillaries. Figure 8.3 a, illustrates a thrombus and an apoptotic cell in an arteriole adjacent to a glomerulus with thrombosed capillary lumens. In contrast Figure 8.3 b, shows a healthy glomerulus with patent glomerular capillary lumens. The degree of glomerular injury quantitated by a blinded observer is shown in Figure 8.3 c. Glomerular injury was more frequent among p110β-deficient mice compared to controls. Consistent with the glomerular damage, features of acute tubular necrosis (ATN) were widespread in both p110β-deficient and L-S treated control groups (Figure 8.3 d). The kidney function was indistinguishable among the uninjured p110β-deficient and p110β-wild type mice. However, the kidney function of the EC p110β-deficient mice after TMA injury is markedly worse vs controls as assessed by creatinine (Figure 8.3 e) and BUN (Figure 8.3 f).

Fibrin deposition in the kidney glomerulus was evaluated using immunofluorescence staining. Fibrin was present in glomeruli of injured p110β-deficient mice vs controls (Figure 8.4).

EC loss was evaluated by qRT-PCR of endothelial-specific transcripts in kidney cortex. As shown in Figure 8.5, we observed a significant decrease in expression of CD31 and Tie 2 among L-S/LPS-treated animals. Similarly, tubular injury is also evident by the significant increase in mRNA expression of tubular cell injury markers KIM1 and lipocalin 2 (or NGAL). Taken together these data indicate that endothelial p110β loss in quiescent mature microvessels is tolerated. However, EC p110β is critically important to maintain organ function and survival of the mouse after glomerular TMA injury.

8.1.2 P110β Knockdown Reduces Sprouting Angiogenesis *in Vitro*

We next evaluated the role of endothelial p110β in repair of microvascular injury by studying ECs *in vitro*. To evaluate angiogenic sprouting, the hEPC treated with p110β specific siRNA were coated on Cytodex beads and analyzed for sprout formation after 18 h of stimulation with growth factors. The p110β siRNA knockdown was verified by Western blot and real time PCR (Figure 8.6 a, b). p110β-deficient hEPC consistently produced fewer cell protrusions, and shorter extensions from the beads compared to EPC treated with non-specific siRNA, or EPC grown in complete EBM2 without siRNA transfection, even when the beads (control and p110β knockdown) were cultured in the same fibrin gel

(Figure 8.7 a, b, c). Similarly, the treatment with p110β specific inhibitor TGX-221 resulted in a modest decrease of sprouting and resulted in shorter migration distance (Figure 8.7 d, e). To determine if p110β loss caused a cell autonomous defect in endothelial sprouting, we co-cultured p110β-deficient and p110β-wild type hEPC on the Cytodex beads, then evaluated the sprouts to determine which cell type led the protruding sprout (Figure 8.8 a, b). We observed that p110β wild type cells preferentially occupied the tip cell position, but that p110β-deficient hEPC could contribute to either tip or stalk position. Similar to p110β-deficient hEPC cultures, p110β-deficient hEPC initiated few sprouts in co-culture with wt cells, hence, p110β-sufficient EC were unable to rescue the sprouting defect of p110β knockdown. This data suggests that endothelial p110β loss impairs EC responses to reparative cues.

8.1.3 P110β Knockdown Reduces Cellular Proliferation and Migration

Next we sought to determine whether p110β knockdown affects hEPC proliferation and migration. We observed a modest reduction in the fraction of proliferating EC after p110β knockdown (Figure 8.9). In contrast, inhibition of cell division using mitomycin C treatment completely suppressed cell proliferation.

Cell migration was assessed using a modified Boyden chamber assay. As shown in Figure 8.10 a, p110β-deficient EPC showed

decreased migration vs the control cells. Similarly, the treatment of EPC with a known p110β specific inhibitor TGX-221 decreased migration by ~ 30 %. To evaluate a defect in chemokinesis, we eliminated the concentration gradient of the chemotactic agents by adding complete EBM2 in the top and bottom chambers. We observed a similar reduction in chemokinetic cell migration among p110β-deficient or p110β-inhibited hEPC vs controls (Figure 8.10 b). These data indicate loss of p110β function reduces hEPC movement.

8.1.4 P110β Loss Inhibits Differentiation of hEPC into Tip Cells

To evaluate the effect of loss of p110β function on EC differentiation that enables invasion of the fibrin matrix, we determined the expression of the tip cell markers DLL4, apelin, and ESM1 [479]. We extracted total RNA from hEPC cultured in the fibrin gel after 18 h of growth factor stimulation. As shown in Figure 8.11 a, we observed a significant decrease in Dll4 and apelin mRNA expression in the cells pre-treated with p110β siRNA versus controls. In contrast, the expression of a third tip cell gene, ESM1, did not change after p110β knockdown. These results were confirmed by Western blot (Figure 8.11 b) to demonstrate that the expression Dll4 and apelin protein was decreased in hEPC treated with p110β siRNA versus the cells pre-treated with non-specific siRNA. Taken together with the defective invasion of fibrin matrices, this data suggests loss of p110β impairs full differentiation of EC to acquire the invasive tip cell phenotype.

8.1.5 PTEN knockdown rescues sprouting in p110β knockdown cells

To investigate whether the loss of p110β activity can be overcome during *in vitro* sprouting angiogenesis. We evaluated the combined knockdown of p110β and PTEN on sprouting *in vitro*. As Figure 8.12 a shows that the double knockdown of p110β and PTEN significantly increased sprouting as compared to p110β-treated cells. It appears that PTEN knockdown can reverse the effect of p110β loss and restores sprouting to normal levels as observed in siRNA control-treated cells.

8.1.6 P110β Loss Reduces Microvessel-Like Formation *in vivo*

To evaluate the impact of p110β knockdown in hEPC function *in vivo*, we evaluated *in vivo* vasculogenesis. hEPC transfected with non-silencing or p110β siRNA were suspended in matrigel and injected subcutaneously into immunodeficient $Rag2^{-/-}$ mice. After one week, control siRNA-treated hEPC in the matrigel plugs were observed to form microvessel-like structures, and to interact with the invading host vasculature to form chimeric blood vessels (Figure 8.13 a). Some of these were perfused with mouse blood. However, the hEPC treated with p110β siRNA were generally restricted to the center of the matrigel plug, formed fewer microvessel-like structures, and no chimeric blood vessels were observed (Figure 8.13 b & c). Taken together, these results demonstrate

that p110β-deficient hEPC are defective in remodeling associated with microvascular repair.

8.2 DISCUSSION

Thrombotic microangiopathy involving the kidney glomerulus is a devastating injury that often results in rapid loss of kidney function. Early observations in clinical specimens identified that endothelial loss and apoptosis [480] was accompanied by proliferative reparative changes in the endothelium [371, 481]. Yet the mechanisms underlying glomerular endothelial defense and repair are largely unknown. PI3 kinase activity can be generated by several isoforms expressed in the endothelial cell to integrate survival and growth signals from a variety of extracellular cues, hence is of interest for development of therapies to enhance repair.

We adopted a genetic approach to examine the role of the PI3 kinase–β isoform in the adult vasculature, and observe that mice are markedly more susceptible to microvascular injury after post-natal, EC-selective, loss of *Pik3cb*. A normally sublethal injury to the glomerular endothelium results in fulminant kidney failure and death. Although, genetic deletion of *Pik3cb* in mice leads to early embryonic death before formation of the primitive vasculature [329], later deletion at the time of vasculogenesis is well tolerated [330]. Consistent with this observation, postnatal EC *Pik3cb* deletion alone did not alter kidney structure or function.

We observe no effect of PI3 kinase-β deficiency on endothelial susceptibility to pro-apoptotic stress. Signal transduction to Akt, a major pathway associated with defense against apoptosis, is mediated via PI3 kinase-α in EC as in other cell types in response to receptor tyrosine kinase agonists such as VEGF [245], but is defective in response to several GPCR agonists among PI3 kinase-β-deficient EC [330, 482]. These observations are consistent with the finding that any activity on the pathway is sufficient to confer protection against death [483]. Taken together, the selective loss of PI3 kinase-β in the endothelium subjected to cytotoxic TMA injury appears to predominantly affect the repair response.

We investigated the effect of PI3 kinase-β loss on fibrin gel invasion *in vitro* to model repair of the injured glomerular capillary endothelium along fibrin-lined basement membrane sheaths [484]. Since PI3 kinase-β in the mouse appears functionally downstream of agonists coupled to both RTK and GPCR [330, 485-487], we anticipated that repair in the glomerular microcirculation involved many of the growth factors, notably VEGF, constitutively expressed by the adjacent glomerular podocytes or mesangial cells [488, 489]. Strikingly, primary human EC, derived from either late blood outgrowth progenitors or mature umbilical veins, required PI3 kinase-β signaling to extend angiogenic sprouts in response to a potent combination of these proangiogenic growth factors. This indicates that PI3 kinase-β in the adult vasculature is functionally coupled to integrate signals from a variety of receptor tyrosine kinases, such as the

VEGF and FGF2 receptors, in vascular repair, and reflects distinctly different requirements for EC PI3 kinase-β expression in the adult versus vascular development in the embryo [330].

The downstream events controlled by PI3 kinase-β are largely unknown. A subtle defect in ex vivo angiogenesis in response to SDF-1α GPCR agonist, but not VEGF, could be demonstrated in aorta from $Tie2^{ERT2}Cre/p110\beta^{flox/flox}$ mice, but was not linked to a cellular phenotype [330]. Interestingly, defective regulation of integrin adhesion is recognized to contribute to poor platelet aggregation, aggregate stability, and attenuation of thrombosis in vivo after pharmacologic inhibition of PI3 kinase-β in wild-type animals [490, 491]. A similar defect in adhesion complex remodeling of PI3 kinase-β knockdown in EPC may explain the modestly impaired EC migration we observed *in vitro* and account, in part, for impaired endothelial invasion *in vitro*, and vascular assembly by human EC *in vivo*.

Matrix invasion involves the characteristic differentiation of EC leading sprout extension, and the trailing stalk cells [176]. For example, the endothelial notch ligand, Dll4, is expressed by the leading cell and plays a central role in directing specification of the stalk cell [185, 492]. We observed defective induction of both Dll4 and apelin, but not ESM1, among PI3 kinase-β-deficient sprouts. Moreover the lack of PI3 kinase-β could not be complemented by wild-type EC to normalize tip cell sprouting.

This suggests that a fundamental cell-autonomous defect in tip cell differentiation contributes to the impaired repair of PI3 kinase-β-deficient endothelium.

However, currently only indirect regulation of gene expression is attributed to PI3 kinase-β [493]. Further work is required to define this pathway.

Although acute injury to the endothelium, as in Shiga-like toxin exposure, is generally repaired by a healthy host vasculature, repetitive injury may overcome or exhaust the reparative potential. For example, genetic deficiency of the complement regulatory proteins that defend the endothelium, or sustained immune attack on the allogeneic microvasculature in a transplant recipient, lead inexorably to organ failure. In diabetic individuals, retinopathy and nephropathy are linked to endothelial apoptosis and the progressive dropout of the microcirculation. Indeed, progressive microcirculation rarefaction may be a common mechanism of kidney failure initiated by diverse causes. Identification of the checkpoints that guard the endothelium and sustain repair may suggest novel treatment strategies for further development.

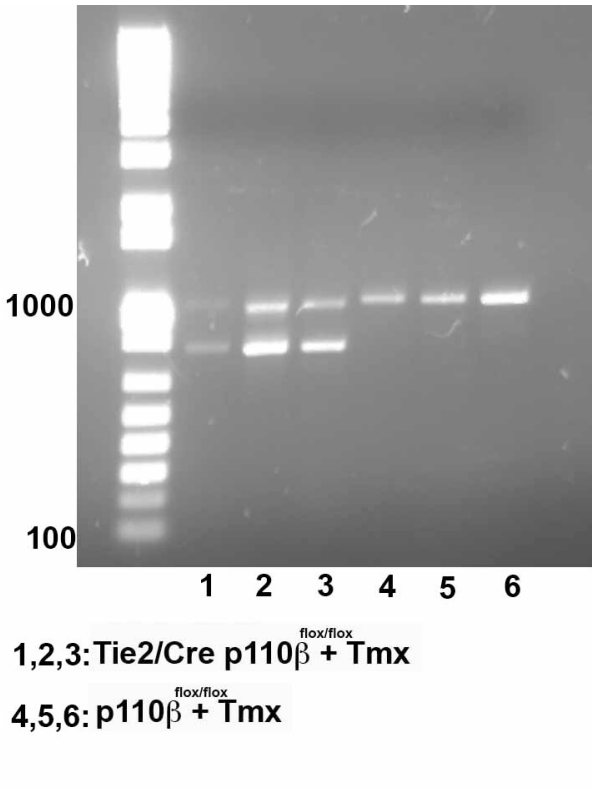

Figure 8.1: Detection of exons 21 and 22 deletion of *Pik3cb* gene by PCR. Tie2 ^{ERT2}Cre/p110$\beta^{flox/flox}$ or p110$\beta^{flox/flox}$ mice received 2mg of tamoxifen or vehicle (oil) daily for 5 days via an intraperitoneal injection. The deletion of exons 21 and 22 was detected by RT-PCR. Primers were designed to amplify a region between exons 16 and exon 24. In the Cre recombinase induced mice the PCR product is expected around 850 bp and 1000 bp in Cre un-induced mice.

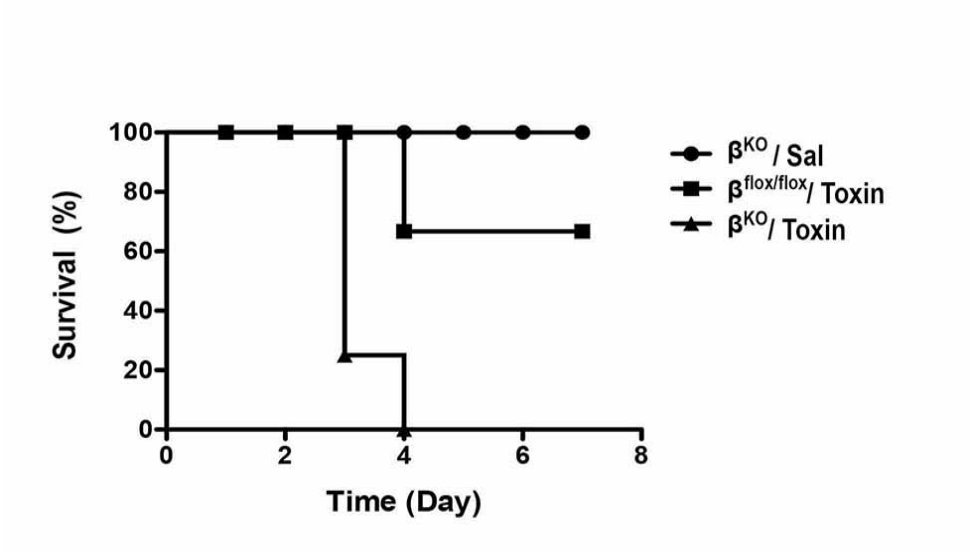

Figure 8.2: Kaplan-Meier survival graph of Tie ^{ERT2}Cre/p110$\beta^{flox/flox}$ mice. Either Cre recombinase was induced and the animals received saline (β^{KO}/sal) and/or 200 μg/Kg Lectin-saporin conjugate (β^{KO}/Toxin), or not induced and the animals received 200 μg/Kg Lectin-saporin ($\beta^{flox/flox}$/ Toxin) ($n \geq 5$ mice/group, $p = 0.0006$).

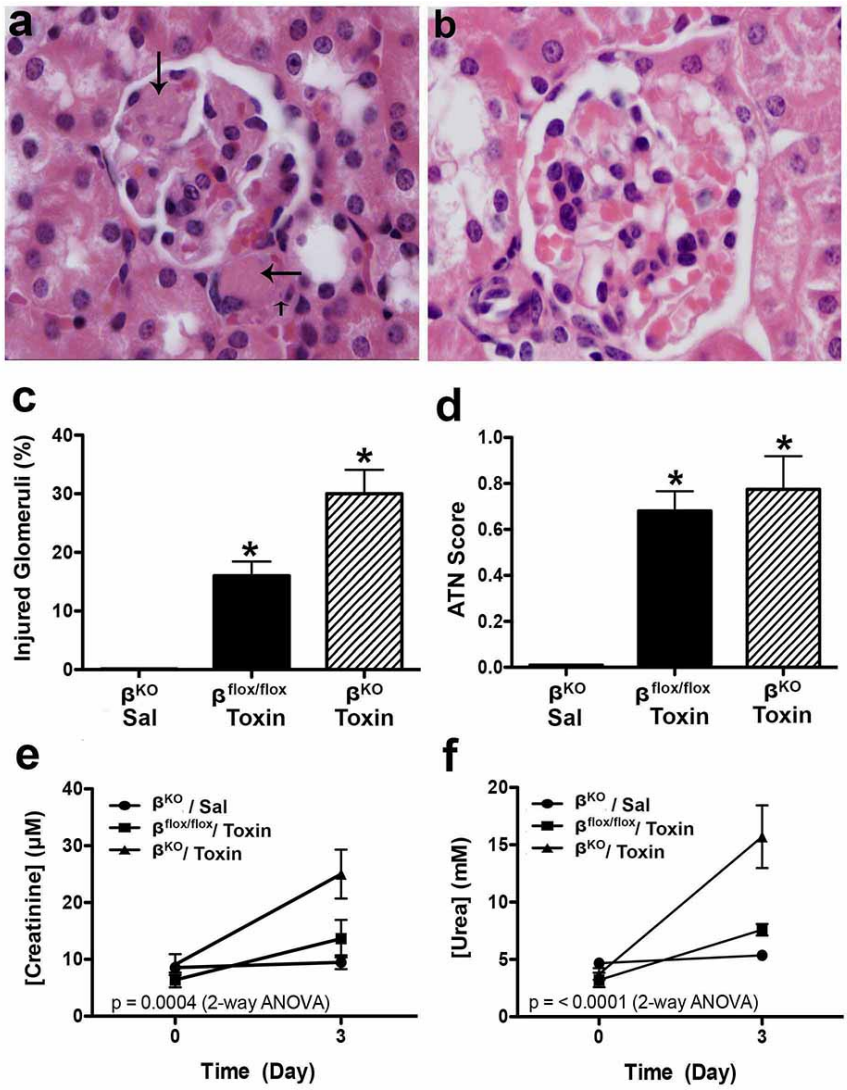

Figure 8.3: Histological evaluation and functional analyses of the renal injury. (a) A representative glomerulus from the Cre recombinase induced group (β^{KO} / toxin) show thrombosed arteriole and glomerular capillary lumens (large arrows), and an apoptotic nucleus in the arteriole wall (small arrow), x1000 original magnification. (b) A representative image showing a normal healthy glomerulus from the β^{KO}/ saline group, . (c) a comparison of the percentage injured (thrombosis, apoptosis, loss of capillary loop density) glomeruli among the various groups (β^{KO}/ saline, $\beta^{flox/flox}$/ Toxin, and β^{KO}/ toxin). (d) Semi-quantitative evaluation of acute tubular necrosis (1> 25 %, 2 > 50 %, 3, > 75 %, and 4< 75 % of the field of view). (e) Creatinine concentration in the serum as determined by enzymatic creatinine assay. (f) Determination of blood urea nitrogen levels. N ≥ 5 animals per group. Each bar represents mean ± SEM.* $p < 0.05$ Mann-Whitney *U* test or two-way ANOVA analysis where applicable.

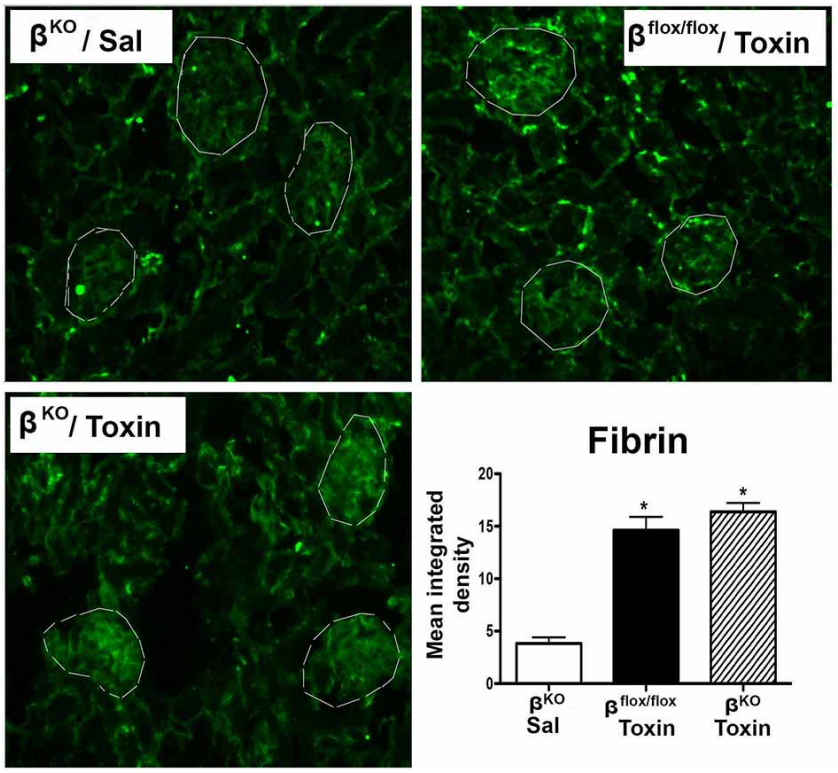

Figure 8.4: Detection of fibrin deposition in glomeruli by immunofluorescence. At day 3 post L-S injection, the mice were euthanized and kidneys were placed in OCT and snap frozen in liquid nitrogen. Five um sections were stained with anti-mouse fibrin antibody overnight and an anti-species FITC-labeled secondary antibody. Shown representative images from the groups (β^{KO}/saline, $\beta^{flox/flox}$/ Toxin, and β^{KO}/ toxin), x200 original magnification. The graph shows quantitation of the mean integrated density of light inside 10 glomeruli of 5 mice/group, n = 5, * $p < 0.05$ vs saline control, one-way ANOVA.

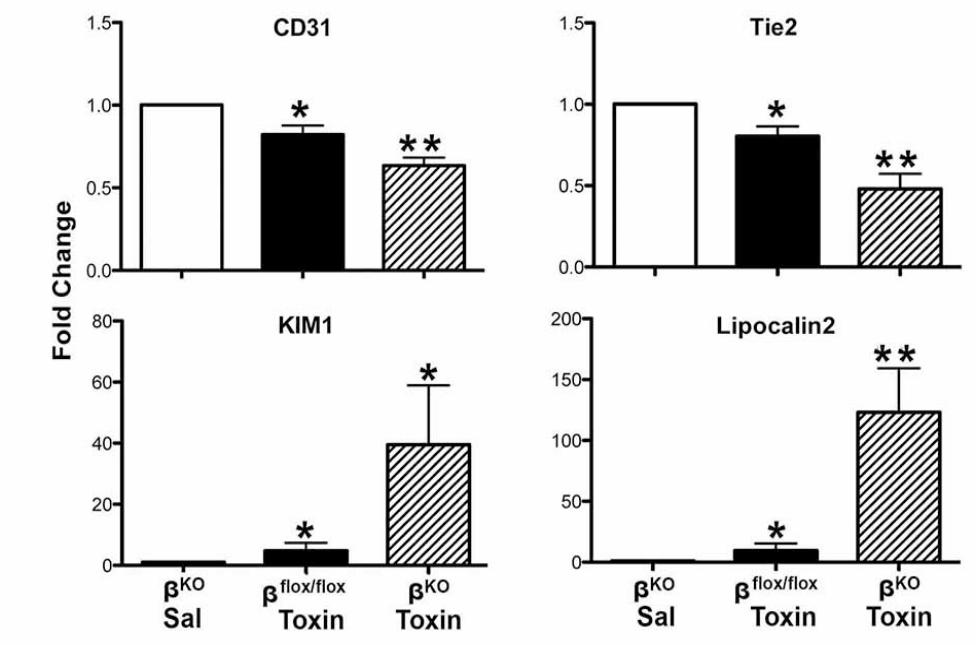

Figure 8.5: Real time PCR expression of endothelial markers and kidney injury markers. Total RNA was isolated from kidney cortices at day 3 after L-S/LPS treatment and reverse transcribed into cDNA as described in Methods. Constitutively expressed endothelial genes CD31, TIE2, and kidney injury markers KIM1 and lipocalin2 were assessed by qRT-PCR (n ≥ 5 mice/ group; * -$p < 0.05$ vs control, one-way ANOVA).

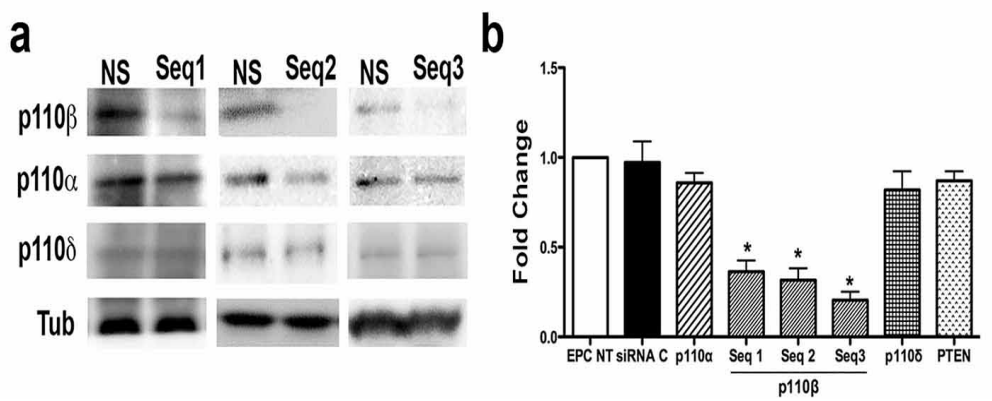

Figure 8.6: Quality control of p110β knockdown by Western blot and qPCR. (a & b) show the selective knockdown of p110β without affecting the other PI3K catalytic isoform alpha and delta by Western blot and qPCR. ($n \geq 5$; * -$p < 0.05$ vs control, one-way ANOVA).

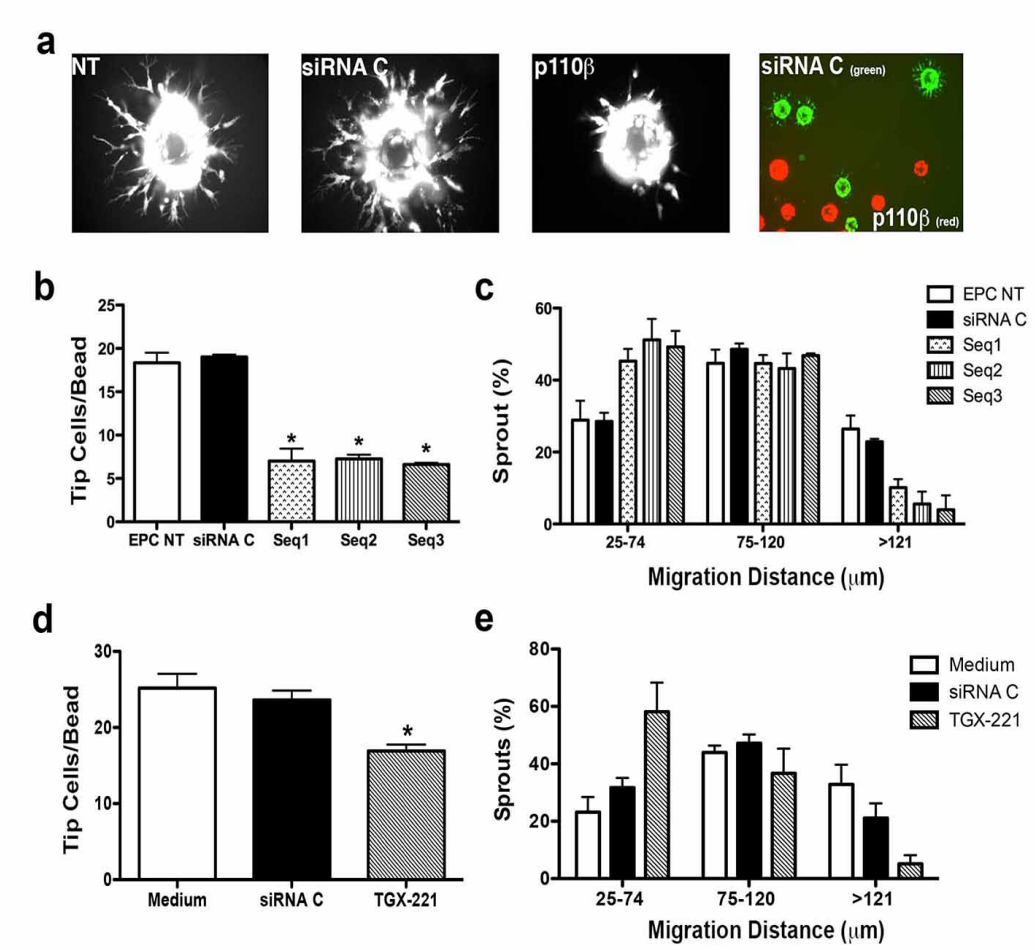

Figure 8.7: Analysis of sprouting angiogenesis in three-dimension fibrin gel. The EPC were coated on Cytodex beads and embedded in fibrin gel in complete EBM2 and incubated overnight at 37°C, 5% CO_2. hEPC were treated with 50 nM of p110β siRNA using three different sequences. (a) Representative images showing consecutively sprouting beads from control (medium or no treatment NT), siRNA control, p110β (x200 original magnification), and control and p110β beads cultured in the same fibrin gel x50 original magnification. (b & c) Demonstrate the defect of sprouting by p110β knockdown cells as significantly less cells formed tip cells and migrated shorter distances in the fibrin gel. The results were validated using p110β specific inhibitor TGX-221 (50 ng/mL) (d, e).($n \geq 5$; * $p < 0.05$ vs control, one-way ANOVA).

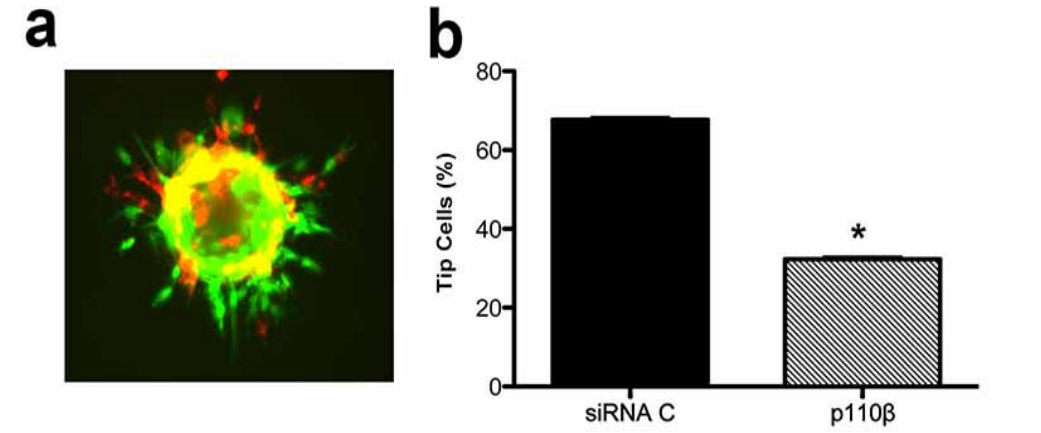

Figure 8.8: Competition assay between control and p110β knockdown cells. (a) Represents a bead (x200 original magnification) from day 4 of 2 cell populations non-silencing treated EPC and p110β knockdown showing a preference of the control cells to occupy the tip position (b). (n = 5; * -p < 0.05 vs control, one-way ANOVA).

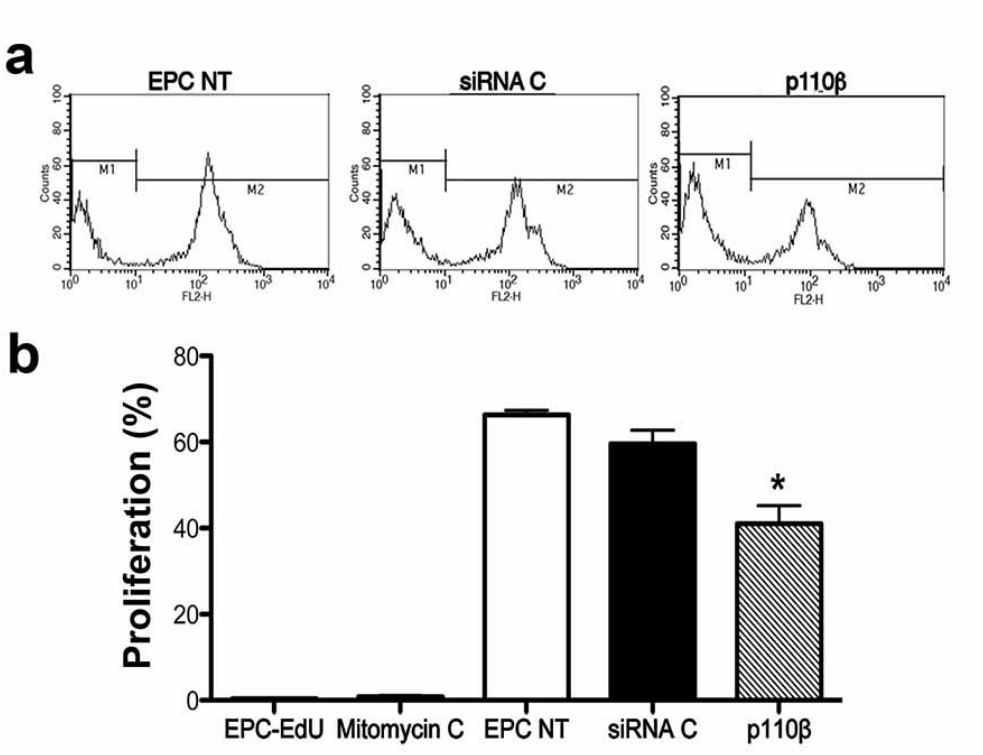

Figure 8.9: The effect of p110β knockdown on cellular proliferation as measured by EdU incorporation. The cells were incubated in the presence of 10 mM EdU overnight and assayed for EdU incorporation by flow cytometery. (A) EPC treated with p110β siRNA significantly reduced the proportion of proliferating cells as compared to EPC cultured in complete EBM2 and received EdU (EPC NT), and to EPC treated with non-specific siRNA sequence (siRNA C). EPC not treated with EdU and EPC treated with proliferation inhibitor mitomycin C (10 μg/mL) serve as negative controls. (B) Representative graphs of flow cytometer results. The bars represent the mean ± SEM of three independent experiments. n=3; * $p < 0.05$ p110β vs EPC NT and siRNA C.

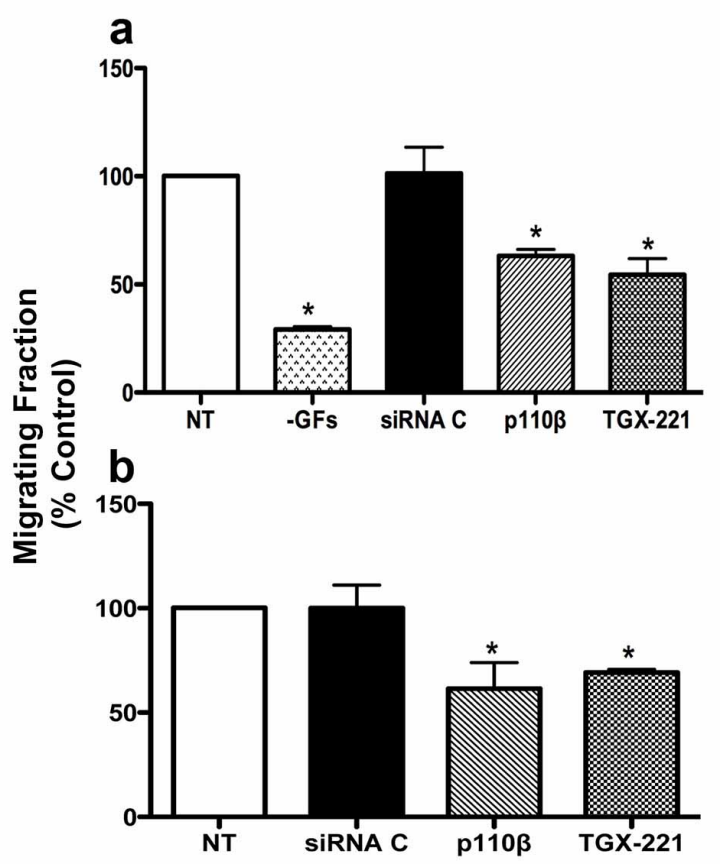

Figure 8.10: The effect of p110β knockdown on cellular migration. The chemotaxis assay was performed using modified Boyden chamber. (a) The siRNA knockdown and p110β specific inhibitor significantly reduced cellular migration. To eliminate the concentration gradient, or chemokinesis, of the chemotactic compounds, complete EBM2 was added in the top and bottom chambers (b) resulting in decreased random migration by the cells treated with p110β siRNA or by the inhibitor as compared to control. The bars represent the mean ± SEM of three independent experiments. * $p < 0.05$ vs NT one-way ANOVA. NT: no treatment, -GFs: no growth factors.

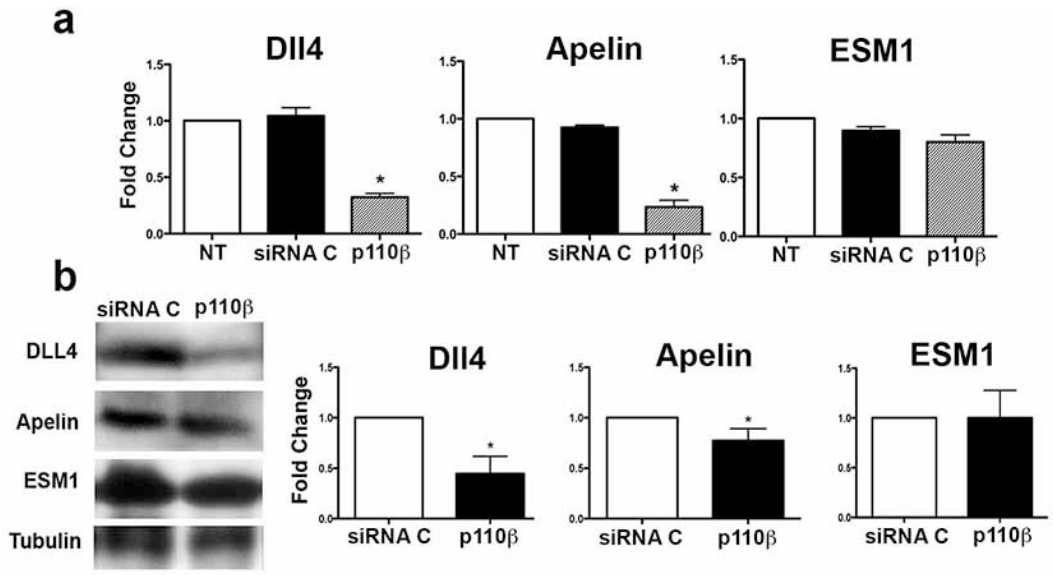

Figure 8.11: The expression of tip cell markers, DLL4, apelin, and ESM1 by qPCR and by Western blot. Total RNA/ cell lysates ware extracted from fibrin gels. The mRNA expression level of ESM1 did not change among the different treatments, however, DLL4 and apelin mRNA expression levels were significantly reduced in EPCs treated with p110β siRNA (a). Similarly, Dll4, and apelin at the protein level were down regulated. The bars represent the mean ± SEM of four independent experiments. * $p < 0.05$ analyzed by one-way ANOVA p110β vs EPC NT and siRNA C.

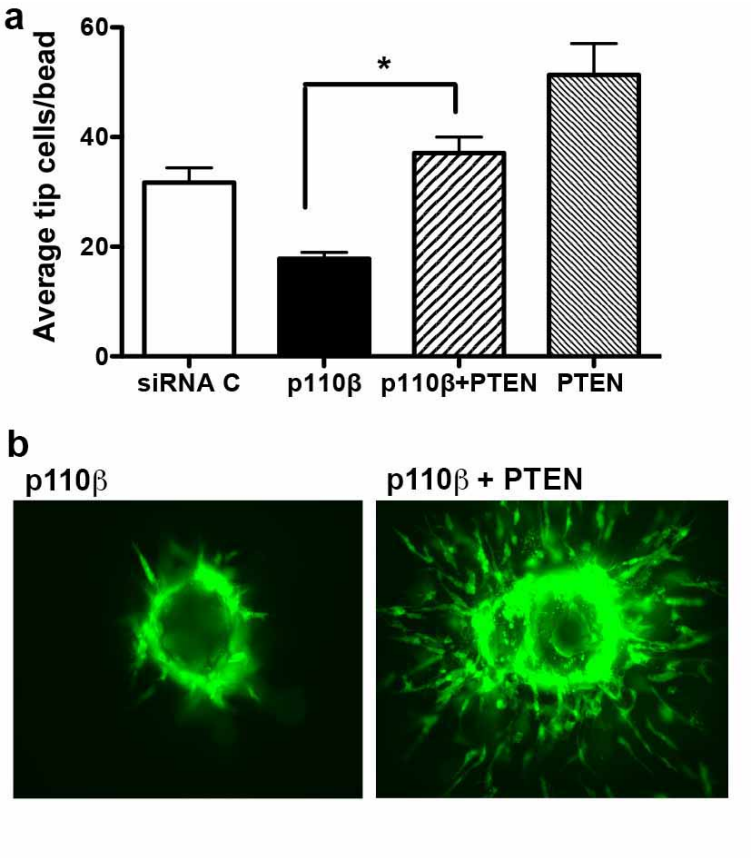

Figure 8.12: PTEN knockdown overrides p110β knockdown and restores normal sprouting. EPC were transfected with non-silencing, p110β, PTEN, or both p110β/PTEN siRNA. (a) Shows the combined knockdown of p110β and PTEN rescues the sprouting activity of EPC. (b) Shows representative sprouting beads from p110β k/d and p110β/PTEN k/d groups. k/d: knockdown

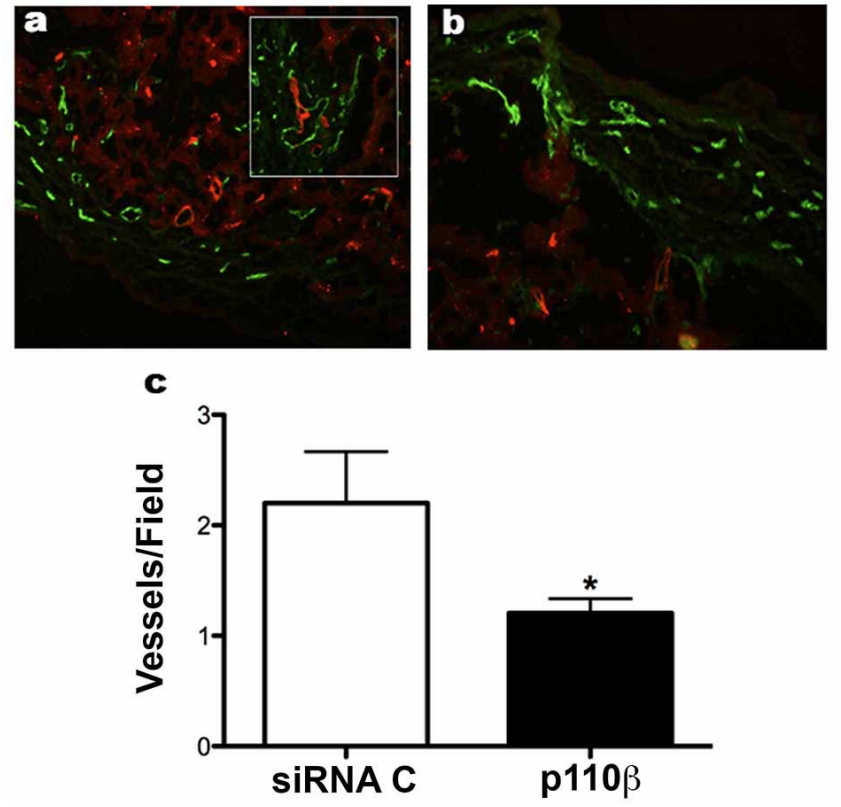

Figure 8.13: *in vivo* vasculogenesis assay using siRNA control and p110β siRNA treated EPC. The matrigel plugs were removed after 7 days and embedded in OCT. Five um sections were stained with anti-human and anti-mouse CD31. Twenty fields of view under 200X magnification were counted and analyzed for the presence of microvessels in a double-blinded fashion. Image (a) shows capillaries formed by the human EPC (red) treated with siRNA control, inset, shows a chimera vessel made of control EPCs and mouse endothelial cells. (b) Shows a representative image from the human EPCs treated with p110β siRNA, x200 original magnification. (d) Quantitation of the number of microvessels per field of view. The bars represent the mean ± SEM of four independent experiments. * $p < 0.05$ analyzed by one-way ANOVA, p110β vs siRNA C.

CHAPTER IX

INHIBITION OF PDGFB IMPAIRS KIDNEY MICROVASCULAR REPAIR

9.0 INTRODUCTION

Platelet-derived growth factors (PDGF) belong to a highly conserved family of growth factors that are related based on structure and function. PDGF exist as homodimers PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD and as heterodimer PDGF-AB. PDGF-AA and –BB are secreted in the active form, whereas PDGF-CC and –DD are secreted in a latent form that must be cleaved outside the cell to become active [264, 265]. PDGF-CC is cleaved by tissue-type plasminogen activator (tPA) and by plasmin, whereas urokinase-type plasminogen activator (uPA) acts on PDGF-DD [265].

The PDGF isoforms bind to receptor tyrosine kinase homodimers PDGFR-$\alpha\alpha$, PDGFR-$\beta\beta$, and the heterodimer PDGFR-$\alpha\beta$ with different affinities [264]. PDGFAA binds to PDGFRα chain exclusively, PDGFBB is a ligand to the three receptors, PDGFCC appears to be specific ligand to the alpha chain of either homo- or heterodimer form, whereas PDGFDD binds PDGFRβ [265, 494, 495]. PDGF have strong mitogenic activity and the activation of their cognate receptors is important during embryonic development and in wound healing. In addition, PDGF signaling has been associated in disorders such as cancers, and vascular diseases [285].

PDGF exert their effect mainly on smooth muscle cells and fibroblasts that can potentially lead to vascular fibrosis and other pathologies [286].

The expression of PDGF isoforms and their receptors in human and mouse kidney cells is well established. PDGF-AA is expressed by podocytes and epithelial cells of distal tubules and of collecting duct in human, and in the loop of Henle cells in mouse [269, 270]. PDGFBB is expressed in the glomerulus of a mature mouse by either podocytes or glomerular endothelial cells. However during embryonic development PDGFBB transcripts are highly expressed by vascular endothelial cells of the developing kidney [270]. During embryonic development, endothelium specific deletion of PDGFBB results in failure of glomerular maturity due to impaired recruitment of mesangial cells [496]. Similarly, early post-natal blockade of PDGFBB signaling reduces mesangial cell recruitment and disrupts glomerular development [497]. Inhibition of PDGFBB signaling impaired renal regeneration in a rat model of acute tubular necrosis (ATN) [498]. In addition, low levels of PDGF-BB are expressed in human mesangial cells and in mouse arteries smooth muscle cells [267].

PDGF-CC is found in human glomerular parietal epithelial cells, proximal and distal tubules, and in endothelial cells in arteries. In mouse, PDGF-CC is expressed in glomerular endothelial cells and in arterial smooth muscle and endothelial cells [271]. PDGF-DD expression in humans is limited to podocytes and to vascular smooth muscle cells, whereas, in mouse PDGF-DD is constitutively expressed by mesangial

cells [272]. PDGFR-α is localized to kidney interstitial and mesangial cells. PDGFR-β is found in parietal epithelial, mesangial, and interstitial cells [499].

PDGF isoforms are constitutively expressed, or components of the PDGF/PDGFR system can be induced in most kidney cells. It is reported that the majority of experimental models and human kidney diseases have an irregular expression of PDGF isoforms and/or receptors [267]. Overexpression PDGF-BB and –DD, which both drive activation of PDGFRβ in glomeruli can lead to mesangial cell proliferation and to the development of glomerulosclerosis and eventually tubulointerstitial fibrosis [287]. PDGF-DD has been directly implicated in the development of tubular interstitium fibrosis likely by inducing epithelial to mesenchymal transition that is controlled by the microRNA miR-200 family [287, 288].

Since we identified early up-regulation of PDGFB in sublethally injured mice challenged with the L-S toxin to induce TMA. We sought to investigate the impact of inhibiting PDGFB on renal microvascular injury and repair. We infected Bl/6 mice with an adenovirus vector expressing the extracellular domain of PDGFRβ and/or the Fc portion of IgG (control) and induced glomerular endothelial cells specific injury with a sublethal dose of lectin-saporin (L-S) conjugate (described in Chapter 4). Remarkably, the injured animals expressing the soluble PDGFRβ did not survive past day 2 post L-S/LPS injection, whereas the control animals

survived to day 7 when they were euthanized as planned. Our results demonstrate that PDGFB is an essential growth factor that plays an important role to initiate the repair mechanism in the kidney.

9.1 RESULTS

9.1.1 Cloning Soluble PDGFRβ and Adenovirus Production

The adnenovirus vector expressing the extra-cellular domain (ECD) of PDGFRβ (Ad sPDGFRb/PH) was generated in Dr. Kuo's lab [500] and sent to us on a Whatman filter paper. We received two plasmids one control that encodes the Fc portion of IgG (Ad Fc) and the second encodes the sPDGFRβ. The plasmids were eluted off the filter papers with water and cloned into DH5α competent *E. coli*. Seven colonies from sPDGFRβ and one colony from the Fc transformation reations were grown overnight to isolate the plamids. The plasmid mini-prep reaction was resolved on 0.8 % agarose gel (Figure 9.1 a). To ensure the plasmids from the sPDGFRβ transformation reaction encodes the ECD of PDGFRβ, a PCR reaction was performed, using ECD specific primers that amplify 650 bp of the ECD, and resolved on 1.2 % agarose gel (Figure 9.1 b).

The Ad sPDGFRβ and Ad Fc plasmids were sent to the Molecular Biology Core at the Cardiovascular Research Center at the University of Alberta to generate the adenovirus. Viral DNA was isolated from HEK 293 cells infected with the adenovirus and the expression of sPDGFRβ ECD was detected by PCR to confirm the adenoviral vector is carrying the sequence of interest (Figure 9.2 a). To ensure the adenoviruses are

infective and can generate the recombinanat proteins. EPC and HEK 293 cells were infected with 50 μL of adenovirus cell lysates and protein expression in the supernatants of the infected cells was detected by Western blot using anti His-tag antibody. Both adenoviruses Ad sPDGFRβ and Ad Fc were infective and the protein products were detected as shown in Figure 9.2 b.

9.1.2 sPDGFRβ Inhibits Mesangial Cells bFGF Production After PDGFB Stimulation

We tested the functionality of the soluble PDGFRβ receptor by blocking PDGFBB stimulation of mesangial cells and inhibition of bFGF production. Human mesangial cells stimulated with recombinant human PDGFBB released significant amount of bFGF as compared to unstimulated cells. However, pre-incubation of the cells with supernatants from adenovirus (Ad sPDGFRβ) infected cells significantly reduced bFGF release by mesangial cells after PDGFBB stimulation. On the other hand, pre-incubation of mesangial cells with the supernatants from Ad Fc infected cells had no effect on bFGF production after PDGFBB stimulation (Figure 9.3). This data indicates effective blockade by sPDGFRβ of PDGFBB signaling. In addition, it demonstrates PDGFBB stimulation of mesangial cells induces bFGF expression. This result suggests a cascade of reparative growth factors in the glomerulus with early PDGFBB production, signaling, and subsequent bFGF secretion.

9.1.3 *in vivo* Study of the Effect of PDGFB Inhibition After Renal Injury

C57Bl/6 mice were infected with the optimized virus concentration of 10^8 pfu per mouse intravenously. Three days later the mice were challenged with 200 μg/Kg (low dose) lectin-saporin. The timeline of the experimental procedure is shown in Figure 9.4. The animals appeared in good conditions 24 h after the delivery of the L-S/LPS in both groups (control Ad Fc and the Ad sPDGFRβ). However, the mice that received the sPDGFRβ adenovirus became morbid 48 h after they received the L-S/LPS injection and had to be euthanized (Figure 9.5). In contrast, the animals that received the control Ad Fc adenovirus did not show any signs of distress and survived to day 7 as expected after treatment with the low dose of L-S/LPS toxin.

The production of the recombinant proteins in the mice was evaluated at day 5 post virus injection or at day 2 post L-S/LPS challenge. The sPDGFRβ and Fc portion of IgG were expressed in the animals sera as shown in Figure 9.6.

The semi-quantitative histological evaluation of the renal injury revealed a greater susceptibility to injury in the Ad sPDGFRβ + L-S group when compared to the group that received Ad Fc control virus + L-S. There were more injured glomeruli (Figure 9.7 a & c) and a greater percentage of fields of view exhibiting signs of acute tubular necrosis (Figure 9.7 b & d) in the Ad sPDGFRβ + L-S/LPS group.

The kidney function was evaluated by the measurement of serum creatinine, BUN, and the presence of proteinuria. The Ad sPDGFRβ group had significantly higher serum creatinine and BUN concentrations as compared to Ad Fc and to saline groups at day 2 post L-S injection (Figure 9.8 a & b). The presence of protein in the urine was higher in the Ad Fc group (Figure 9.8 c) when compared to saline control group.

The presence of fibrin clot in the glomeruli was evaluated by immunofluorescence. Fibrin positive glomeruli were detected in both Ad sPDGFRβ and Ad Fc groups (Figure 9.9). Quantitation of the fluorescence intensity in the glomeruli showed a slight increase in the Ad sPDGFRβ group when compared to Ad Fc group. No glomeruli with positive fibrin staining were detected in the saline control group (Figure 9.9).

The kidney injury was also evaluated by real time PCR. The mRNA expression levels of endothelial markers CD31 and Tie 2 were markedly down-regulated in the sPDGFRβ group. However, only Tie2 was significantly down regulated in the Ad Fc group as compared to saline control group (Figure 9.10). The markers of renal injury KIM1 and lipocalin 2 were up regulated in the sPDGFRβ group but modestly up regulated in the Ad Fc group as compared to control (Figure 9.10).

The expression levels of bFGF and VEGF were down regulated in the group of mice that was treated with Ad sPDGFRβ + 200 μg/Kg L-S + LPS as compared to control mice. Interestingly, VEGF was also down

regulated in the group of mice that received Ad Fc and L-S + LPS at day 7 post L-S/LPS injection (Figure 9.11)

The mRNA expression levels of PDGF isoforms B, A, C, and D had differential expression levels among the different groups. PDGFB was significantly up regulated in the injured mice that received sPDGFRβ and/or Ad Fc at day 2, then expression was normalized in the surviving Ad Fc group by day 7. PDGFA mRNA expression was found significantly up-regulated in the Ad sPDGFRβ group at day 2 and Ad Fc group at day 7. PDGFC mRNA expression levels did not change among the various groups and at the different time points. Lastly, PDGFD mRNA expression level was only up regulated in the mice that received Ad Fc + L-S/LPS albeit at day 7 post L-S/LPS injection (Figure 9.12).

We examined expression of EC tip cell genes to evaluate endothelial repair. The mRNA expression levels of tip cell markers apelin Dll4, ESM1, and CXCR4 were determined by real time PCR. Interestingly, the expression of apelin and Dll4 were mostly up regulated in the mice that received the Ad Fc adenovirus and L-S/LPS at day 7. ESM1 expression did not change, but CXCR4 mRNA expxression was up regulated in the Ad Fc + L-S/LPS group at day 2 post L-S injection (Figure 9.13).

9.2 DISCUSSION

In the present chapter, we investigated the role of PDGF-B in primary glomerular endothelial cells damage. In the L-S TMA mouse model of kidney injury, the inhibition of PDGF-B by sPDGFRβ resulted in

decreased kidney function, worsened glomerular injury, and excacerbated tubular damage, when compared to mice that received low dose L-S+LPS only. The most striking observation was the high mortality rate of the mice that received low dose L-S+LPS and sPDGFRβ inhibition as no animal survived past day 2 after induction of kidney injury. In contrast, there was one death in the control groups receiving L-S+LPS+ Ad Fc group. The adminstration of adenovirus expressing sPDGFRβ or Ad Fc without L-S treatment did not result in any mortality or caused glomerular injury or proteinuria.

The PDGF family is the best-characterized growth factors in healthy and diseased kidneys [285]. All components of the PDGF family, ligands and receptors, are either constitutively expressed or induced in almost all kidney cells [285].

Platelet-derived growth factors were first thought to mediate inflammation during renal injury, based on the temporal expression of PDGF and their receptors [501]. Indeed, mounting evidence implicate PDGF in kidney pathologies ranging from induction of mesangial proliferation, development of kidney fibrosis, to glomerular angiogenesis [267]. The main PDGF isoforms linked to mesangial proliferation and the development of glomerulscelrosis are PDGF-B and PDGF-D. Eventually, the autocrine and paracrine signaling of PDGF-B and -D in the glomeruli lead to development of tubulointerstitial fibrosis [287]. Despite the overwhelming evidence of PDGF role in renal disease pathogenesis we

should not neglect the beneficial effect of PDGF during tissue remodeling and repair [502]. In a Thy1.1 mesangioproliferative glomerulonephritis (MPGN) rat animal model that is accompanied by extensive glomerular endothelial cell (GEC) damage [503], the infusion of PDGF-C resulted in reduction of mesangiolysis and microaneurysm as well as increased GEC proliferation. In addition, PDGF-C infusion increased bFGF expression in the glomeruli. Similarly, inhibition of PDGF-C in a mouse model of thrombotic microangiopathy exacerbated the renal injury and reduced GEC area [503]. GEC can also participation in capillary repair and restoration of glomerular architecture after mesangial injury that occurs in the Thy1.1 model of MPGN. Although mesangial proliferation is a principal response to the injury, GEC proliferation was also observed from day 2 to day 14 that was accompanied by capillary repair and up-regulation of VEGF. The GEC proliferation was reduced by 40 % with neutralizing bFGF antibodies [504]. The inhibition of PDGF-B and PDGFR with either trapidil or Ki6896, which inhibit PDGF-B binding to its receptor and selectively inhibit PDGFRβ phosphorylation respectively, adversely affected tubular repair after an ischemia injury in rats. The inhibition of PDGF-B/PDGFRβ axis leads to increase serum creatinine concentration, inhibition of tubular epithelial cells proliferation and most importantly, higher mortality rate [498].

It appears that systemic inhibition of PDGF-B in the presence of a kidney injury is lethal. In our TMA model of glomerular endothelial cells

injury a dose of 200 μg/Kg L-S/LPS resulted in a mild renal damage such that the overwhelming majority of the mice overcame the injury and showed signs of repair by day 7 that were almost indistinguishable from normal uninjured mice (described in Chpater 4). However, despite the mild renal injury the inhibition of PDGF-B exacerbated the damage to overwhelm repair and the mice succumbed to the injury. Cross talk between the different glomerular cell types is of a great importance in health and in disease. PDGF-B does not exert its activity directly on glomerular endothelial cells as the PDGF receptors have not been detected in adult mouse GEC, and inhibition of PDGFB by neutralizing antibodies did not affect GEC proliferation in Thy1.1 animal model [267, 504]. It has been shown that PDGF-B is up regulated in the glomeruli after an injury and it is produced by mesangial cells, podocytes, parietal epithelial cells and by circulating leukocytes [276-278, 505]. Through autocrine and paracrine stimulation PDGF-B can activate mesangial cells and podocytes to release bFGF and VEGF that can activate GEC and induce vascular repair [267, 282, 504]. We demonstrated that sPDGFRβ both blocked PDGFB-stimulated bFGF expression by mesangial cells *in vitro*, and bFGF transcripts expression in the kidney cortex of injured animals. Endothelial recovery is a crucial factor in the resolution of the injury in our TMA animal model. Growth factors such as VEGF and bFGF are very important elements in endothelial survival and glomerualr capillary repair [504, 506]. Thus, the inhibiton of PDGF-B deprives GEC

from the mitogenic and angiogenic effects of bFGF and VEGF [205, 416] likely resulting in impairement of vascular repair mechanism and leading to more severe renal damage.

Inhibition of PDGFRβ can lead to glomerular endothelial cell apoptosis in neo-natal mice. The administration of a PDGFRβ neutralizing antibody daily resulted in disturbance of glomerular structure that was apparent by noticeable reduction in mesangial cells. Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) showed that GEC in the outer kidney cortex region underwent apoptosis. The tubular cells did not appear to sustain any damage [497].

PDGF-B is one of the 4 isoforms present in injured kidney, but is up-regulated after injury and is more widely expressed, along with PDGFRβ, in animal models of renal diseases than any other isoform [267]. PDGFB binds to all PDGFR isoforms αα, αβ, and ββ [494, 495]. PDGFB is a strong inducer of mesangial cells proliferation and migration [507]. PDGF-A binds only to PDGFRαα, which is expressed on mesangial and tubular cells [267, 272], and exerts a weak effect on mesangial cells proliferation and migration [507]. PDGF-D binds predominantly to PDGFRββ [265]. PDGF-C is largely a ligand for PDGFRαα and can bind PDGFRαβ [265]. Boor et al. [503] reported that inhibtion of PDGF-C by neutralizing antibodies in a conA-TMA mouse model resulted in a significant increase in glomerular injury, but BUN and proteinuria were not different from controls at day 4 after induction of injury. Although, the

conA-TMA model is different from our L-S TMA model, the results largely support the premise that PDGF have an important function in jumpstarting vascular repair mechanism after a renal injury. Our systemic evaluation of PDGF isoforms expression in kidney cortex after injury indicates PDGFB is both markedly elevated vs baseline, and is up-regulated earlier than other PDGF isoforms after injury. Our observation that blockade of PDGFRβ signaling results in collapse of the repairative response after TMA injury, emphasizes the central role that PDGFBB plays in preserving kidney function after vascular stress.

In this chapter, I studied the role of PDGF-B in glomerular capillary repair. The inhibition of PDGF-B by adenovirus vector expressing soluble PDGFRβ followed by induction of GEC specific injury by L-S resulted in high mortality as no mouse survived past day 2 after the injection of a sublethal dose of L-S. The kidney injury was characterized by increased glomerular damage, high serum creatinine concentration and BUN, and an increased glomerular fibrin staining. At the molecular level, the endothelial markers CD31 and Tie2 mRNA expression were down regulated, and the kidney injury markers KIM1 and lipocalin2 were significantly up regulated.

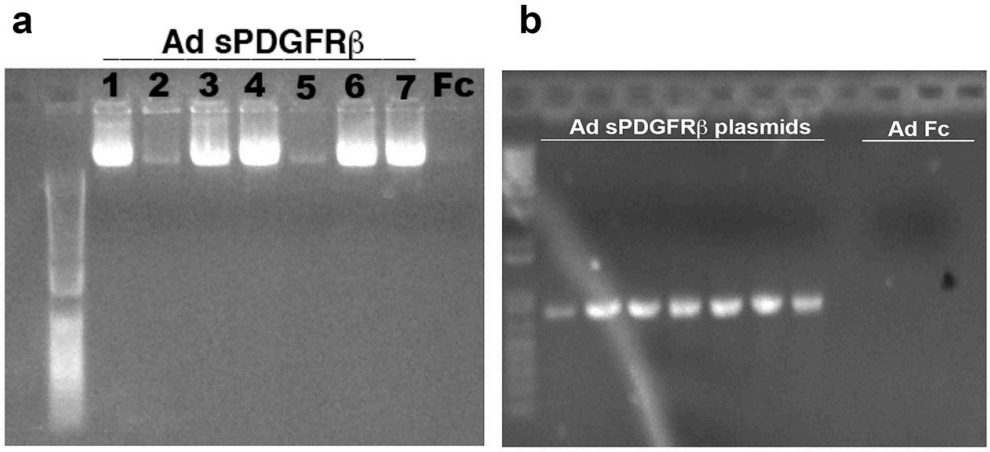

Figure 9.1: Cloning of sPDGFRβ and Fc vectors. The Ad sPDGFRβ and Ad Fc plasmids were cloned into DH5α competent *E. coli* strain and the resulting colonies were grown overnight. The plasmids mini-prep reactions were resolved on 0.8 % agarose gel (a). The plasmids were used in a PCR reaction to amplify 650 bp portion of the sPDGFRβ ECD (b) and the reaction was analyzed on 1.2 % agarose gel. Primers are listed in Chapter 3, Table 2.

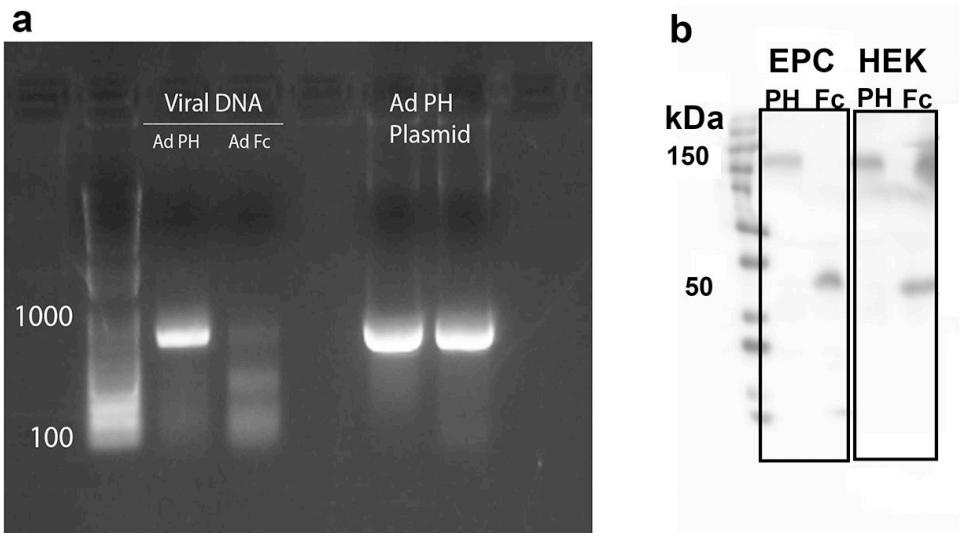

Figure 9.2: Production of adenoviruses and protein detection. Viral DNA was isolated from cell lysates of adenovirus-infected cells that express the E1 region of the adenovirus geneome. The ECD of sPDGFRβ was detected by PCR (a). EPC and HEK 293 cells were infected with 50 μL of adenovirus cell lysates. The sPDGFRβ (Ad PH) and IgG Fc were detected in the infected cell supernatant by Western blot, using anti 6-His tag antibody, 24 h after infection (b).

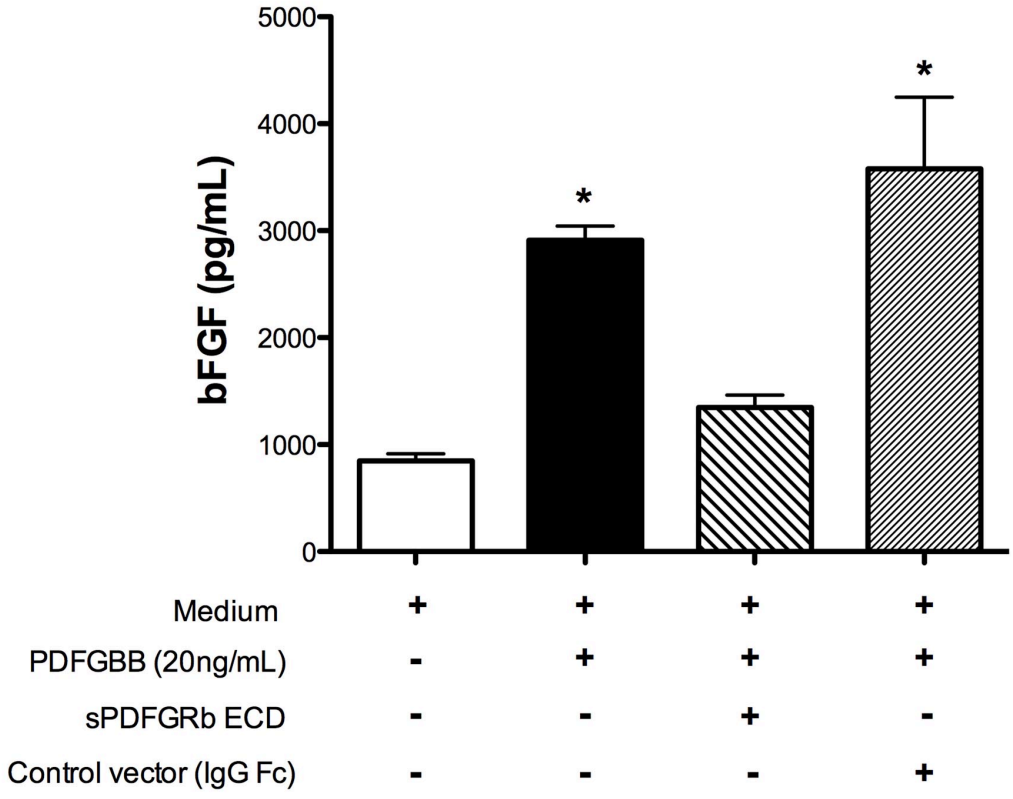

Figure 9.3: Inhibtion of mesangial cell bFGF production by PDGFBB stimulation using sPDGFRb. Human mesangial cells were treated with 20 ng/mL recombinant human PDGFBB, pre-incubated with 25 μL supernatant from adenovirus (Ad sPDGFRβ, Ad Fc) infected cells, or left untreated. One hundred uL of the supernantants from the mesangial cells were assayed for bFGF using human bFGF ELISA kit. (n=3. The bars represent mean ± SEM, $p < 0.05$ vs medium control one way ANOVA).

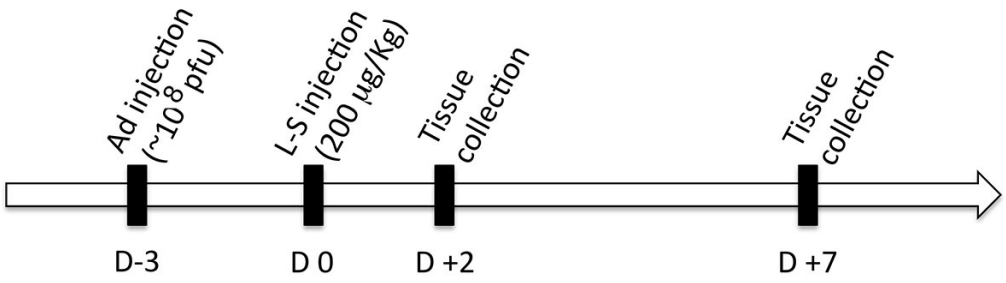

Figure 9.4: Timeline of the experimental procedure. C57Bl/6 mice were injected with 10^8 pfu/mouse Ad sPDGFRβ and/or Ad Fc intravenously 3 days before the mice were challenged with 200 μg/Kg lectin-saporin conjugate and LPS. Tissues were collected at day 2 post L-S challenge as all the Ad sPDGFRβ infected mice were euthanized at that day and the animals infected with control virus (Ad Fc) were euthanized at day 2 and day 7 post challenge.

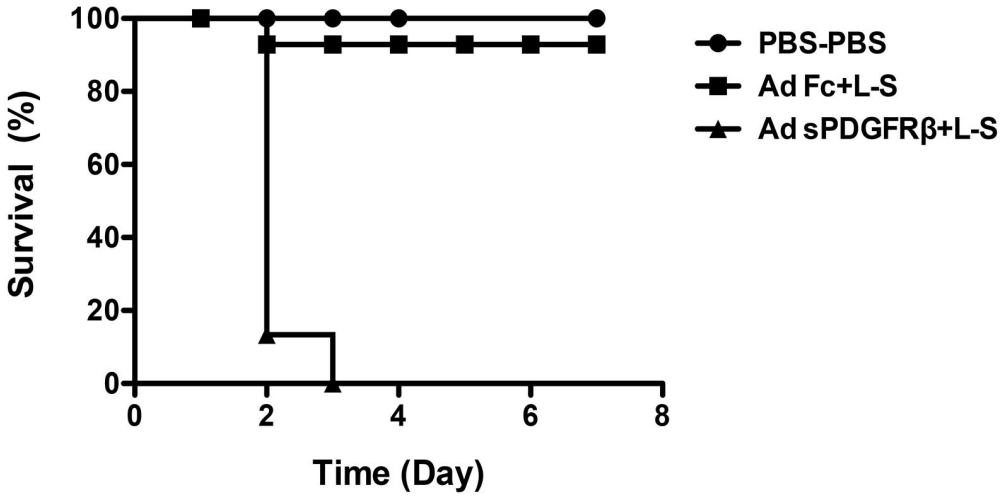

Figure 9.5: Effect of PDGFBB inhibition on the response to L-S challenge in mice. Kaplan-Meier survival graph of animals treated with PBS, Ad Fc + 200 μg/kg L-S/LPS, or Ad sPDGFRβ + 200 μg/kg L-S/LPS (n ≥ 5 / group, p = 0.0004 Log-rank test).

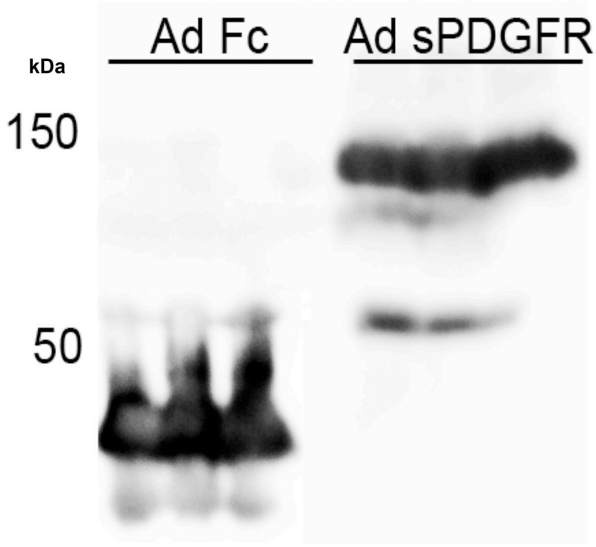

Figure 9.6: Detection of recombinant protein expression in mice sera. C57Bl/6 mice were injected with 10^8 pfu/mouse intravenously of Ad sPDGFRβ and/or Ad Fc adenoviruses. Blood samples were collected 5 days later and assayed by SDS/PAGE and Western blot for the expression of proteins of interest using anti His-tag antibody.

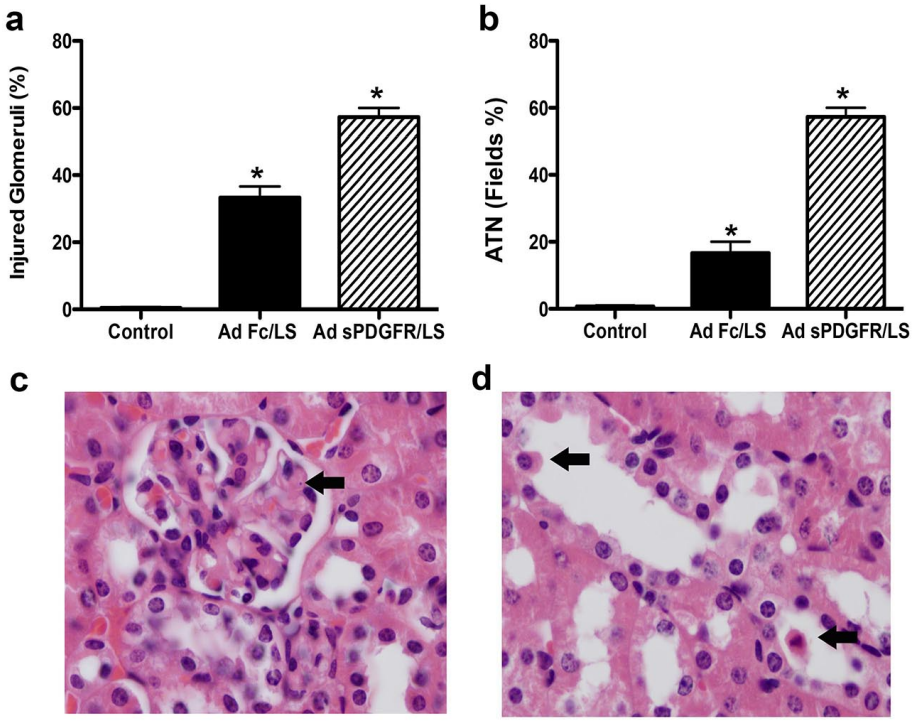

Figure 9.7: Semi-quantitative evaluation of the kidney injury. C57Bl/6 were injected with 10^8 pfu/mouse of either Ad Fc (control virus) or Ad sPFGFRβ virus or received saline (control). Three days later the animals received 200 μg/Kg L-S+LPS or received only saline. Glomerular and tubular injuries were analyzed by pathologist (DCR) blinded to treatment groupds (30 different glomeruli and 30 different fields of view respectively). The inhibition of PDGFB resulted in an increase in injured glomeruli and in ATN (a & b) as compared to saline (control). The mice that received the Ad sPDGFRβ adenovirus + L-S showed more susceptibility to the L-S injury than their Ad Fc + L-S mice. (c) shows a representative image of an injured glomerulus from Ad sPDGFRβ group with an apoptotic cell (arrow) in the capillary wall. (d) shows injured tubular cells from the Ad sPDGFRβ group as well. (x1000 original magnification). (n = 5, The bars represent mean ± SEM, $p < 0.05$ vs control Mann-whitney *U* test).

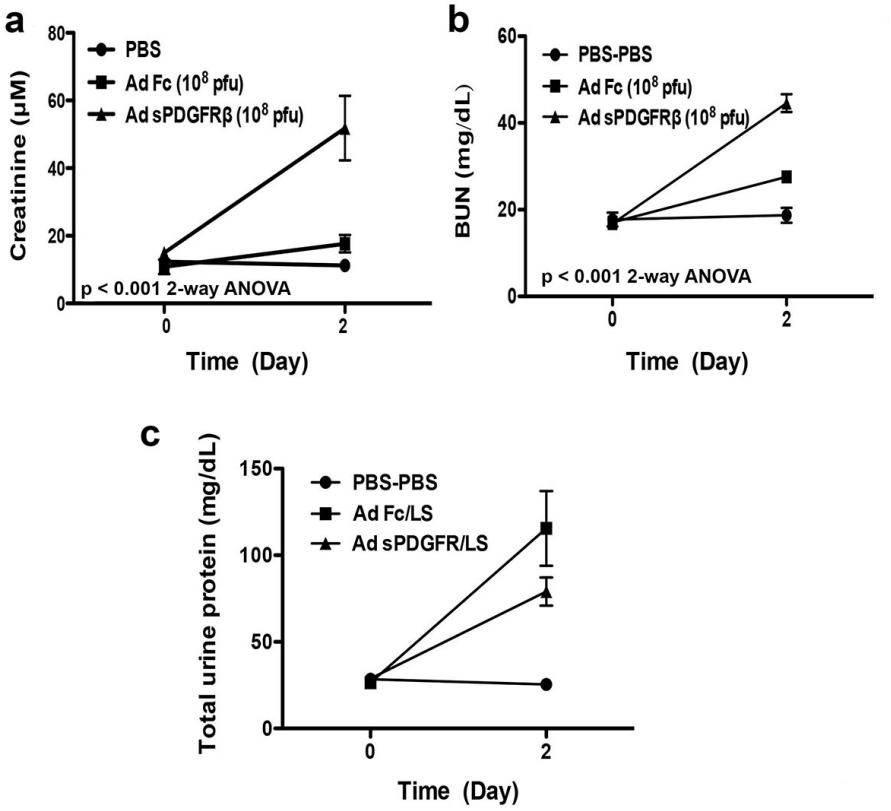

Figure 9.8: Evaluation of renal function as measured by serum creatinine blood urea nitrogen (BUN) and proteinuria. The mice that received Ad sPDGFRβ + L-S showed a significant increase in serum creatinine concentration and BUN (a & b) as determined by enzymatic cretinine assay and BUN kit respectively. The total protein presence in urine was significantly increased in both Ad sPDGFRβ and Ad Fc groups as compared to contol mice. (n = 5, the bars represent mean ± SEM, $p < 0.05$ vs control).

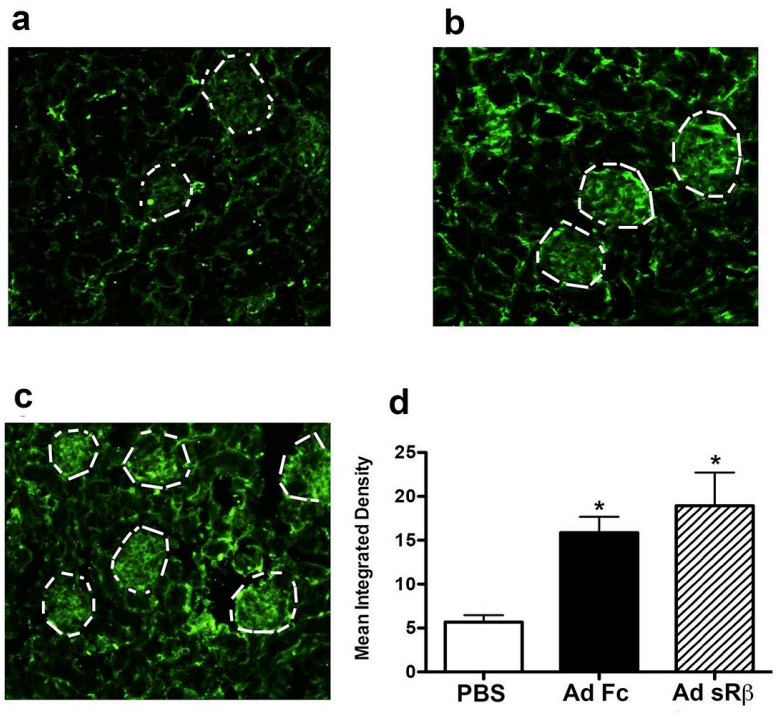

Figure 9.9: Detection of fibrin deposition in glomeruli by immunofluorescence. At day 2 post L-S injection the mice were euthanized and kidneys were placed in OCT and snap frozen in liquid nitrogen. Five μm sections were stained with anti-mouse fibrin antibody overnight and an anti-species FITC-labeled secondary antibody. (a) A representative image from saline group, (b) an image from Ad Fc+L-S group, and (c) from Ad sPDGFRβ+L-S group (Ad sRβ), x200 original magnification. (d) Quantitation of the mean integrated density of light inside 10 consecutive glomeruli (n = 5. The bars represent mean ± SEM, $p < 0.05$ one way ANOVA).

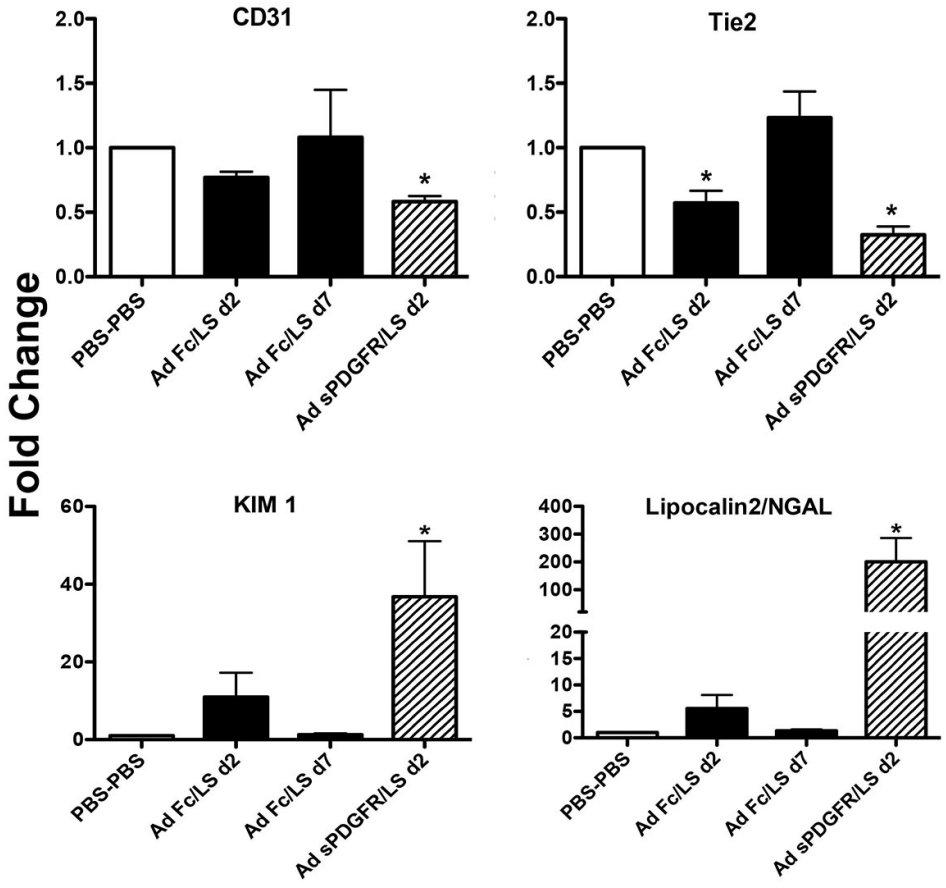

Figure 9.10: Expression of endothelial and kidney injury markers. Total RNA was isolated from kidney cortices reversed transcribed into cDNA as described in Methods. Constitutively expressed endothelial genes CD31, TIE2, and kidney injury markers KIM1 and lipocalin2 were assessed by qRT-PCR (n = 5. The bars represent mean ± SEM, $p < 0.05$ vs control PBS-PBS, one way ANOVA).

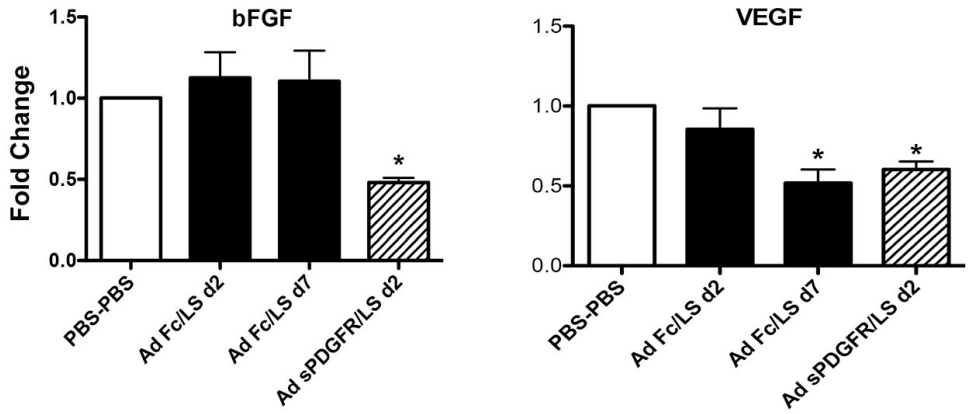

Figure 9.11: The effect of PDGFBB inhibition on expression of bFGF and VEGF among injured mice. bfgf expression was decreased among mice treated with sPDGFRβ and 200 μg/Kg L-S+LPS. VEGF was down-regulated in the mice that were treated with the control Ad Fc virus or sPDGFRβ and 200 μg/Kg L-S + LPS. (n = 5. The bars represent mean ± SEM, $p < 0.05$ vs control PBS-PBS, one way ANOVA).

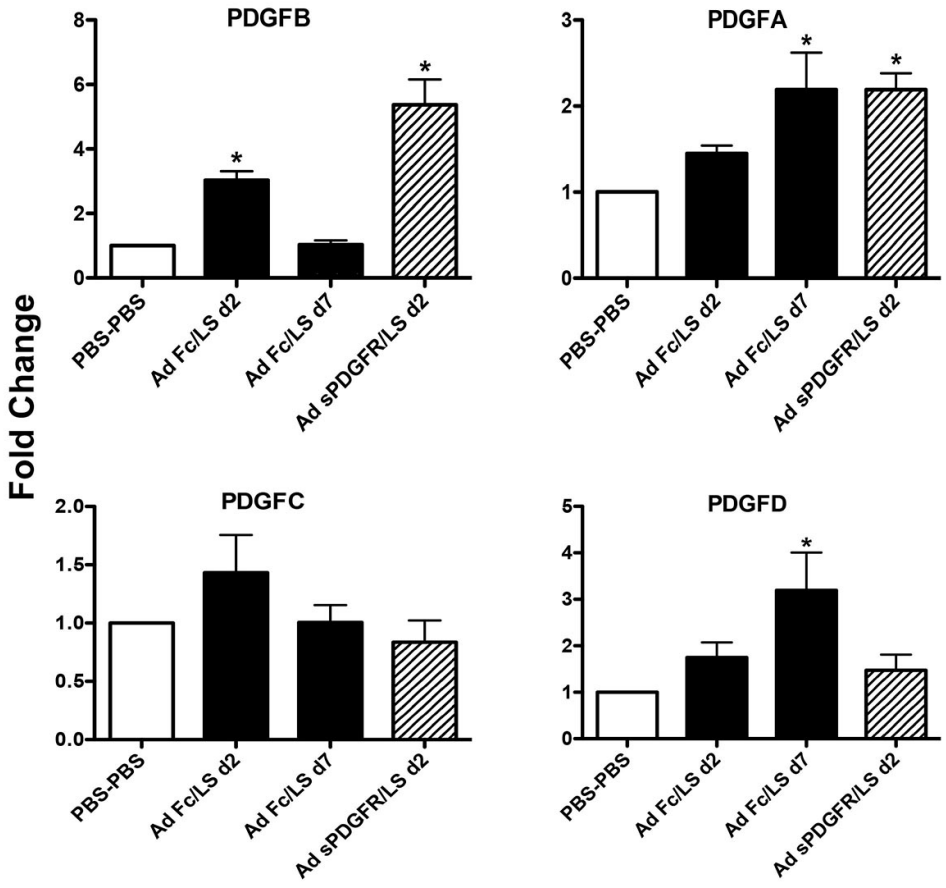

Figure 9.12: The mRNA expression levels of PDGF isoforms after injury. C57Bl/6 were injected with 10^8 pfu/mouse of either Ad Fc (control virus), Ad sPFGFRβ virus, or saline (control). Three days later the animals received 200 μg/Kg L-S+LPS or saline. Total RNA was isolated from kidney cortices. PDGF-B, -A, C, and –D mRNA expression was assessed by qRT-PCR (n = 5. The bars represent mean ± SEM, $p < 0.05$ vs control PBS-PBS one way ANOVA).

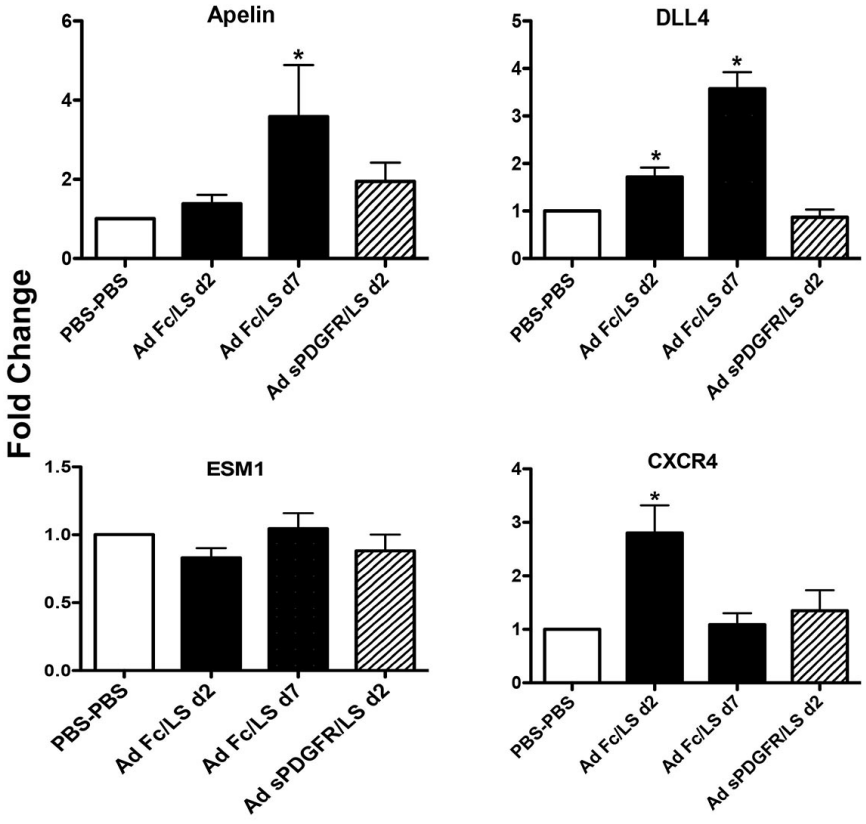

Figure 9.13: Tip cell marker gene expression after adenovirus and L-S injection. C57Bl/6 were injected with 10^8 pfu/mouse of either Ad Fc (control virus), Ad sPFGFRβ virus, or saline (control). Three days later the animals received 200 μg/Kg L-S+LPS or saline. Total RNA was isolated from kidney cortices. DLL4, Apelin, and ESM1 mRNA expression was assessed by qRT-PCR (n = 5. The bars represent mean ± SEM, $p< 0.05$ vs control PBS-PBS, one way ANOVA).

CHAPTER X

GENERAL DISCUSSION AND FUTURE DIRECTIONS

10.0 Overview of Findings

Functional and structural damage to the kidney microvasculature are major contributors to the development and progression of acute kidney injury (AKI). AKI can lead to acute renal failure, which is prevalent among hospitalized patients and is estimated between 1-35 % and is associated with high mortality rate [508, 509]. Renal endothelial injury is a hallmark of diseases termed thrombotic microangiopathies (TMA) that include hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), vasculitis, and certain modes of renal transplant rejection [21, 24, 34, 378]. Aside from dialysis and renal transplantation, clinicians do not have many options at their disposal for renal replacement therapy. Many investigators are studying alternative therapeutic strategies to repair the renal microvasculature and restore kidney function. The majority of the new strategies are based on the delivery of angiogenic factors and on endothelial progenitor cells (EPC) transplantation [12, 462, 510].

The concept of vascular restoration has gained enormous attention since Asahara's report on the isolation of peripheral blood CD34+ cells that differentiated into endothelial-like cells *in vitro* termed EPC [8, 12]. For over a decade of research on the identity of EPC, we still do not have

good understanding of this cell population. To date EPC lack a specific marker and are only identified by the time they appear in culture. The cell populations are termed early or late outgrowth cells. The cells are recognized as two distinct cell types. The early outgrowth cells are known to produce angiogenic factors but do not incorporate into the blood vessel wall. In contrast, the late outgrowth cells do not produce angiogenic factors but are involved in neovascularization [12, 17, 397, 421]. Regardless of the controversy, EPC still represent a great potential for treatment of vascular diseases with many ongoing clinical trials aimed at studying means of increasing EPC numbers and mobilization, as well as autologous EPC transplantation [462]. However, the results of the clinical trials have not reached satisfactory levels to use EPC in clinical practice, and more research is still needed to enhance our understanding, and to devise new strategies to improve the efficacy of EPC as a treatment for vascular diseases [462, 511].

This thesis work is focused on studying renal microvascular injury and repair. We investigated repair by EPC transplantation into the GEC-specific TMA model that we developed in the lab. We studied the role of PI3K catalytic subunit p110β in angiogenesis and in GEC repair. In addition, we examined the role of platelet-derived growth factor- (PDGF)-B in glomerular capillary repair.

In order to study kidney microvascular repair we needed to develop a glomerular endothelial cell specific injury model. Despite the availability

of numerous animal models of kidney injury, however, none met the criteria of injury that we were seeking. We initially adopted the Shiga toxin animal model of kidney injury. The verocytotoxin is the leading cause of non-familial diarrhea + HUS in the human [512, 513]. However, after experimentation we noticed that the injury affected mostly tubular and did not suit our goal of eliciting EC-specific damage. The development of GEC-specific mouse model of renal injury was critical for the progression of the work presented in this thesis. The animal model was based on the binding properties of MOA lectin to Galα(1,3)Gal carbohydrate epitope that was previously shown to bind specifically to glomerular capillary cells in CD1 mice [381]. Warner et al. also demonstrated that a concentration of 23 mg/Kg can induce GEC injury, but the renal damage was not uniform, and the authors presented very shallow description of the renal injury without including descriptions of the hallmarks of TMA. Nevertheless, the carbohydrate binding properties of MOA lectin were taken into consideration to form the basis of our TMA animal model. We used the MOA lectin as a vector to deliver the potent toxin, saporin to enhance the killing activity of MOA lectin. Interestingly, saporin lacks a binding domain and cannot enter the cell on its own but once internalized it is highly toxic [514]. The MOA lectin and saporin were conjugated using the long-chain disulfide crosslinker LC-SPDP. The stoichiometry of the reaction resulted in mainly 1:1 lectin-saporin conjugates. In our experiments with C57Bl/6 strain of mice, we detected binding to the heart microvessels of the

biotinylated MOA lectin in addition the GEC. This is a striking difference from what Warner et al. have reported. It is likely reflecting that the carbohydrate epitope Galα(1,3)Gal is differentially expressed in different mouse strains. In order to avoid the heart microcirculation, we developed a surgical procedure to deliver the lectin-saporin conjugate away from the heart by cannulating the left carotid artery. The toxin was injected retrograde of the blood flow and down the descending aortic arch. The surgical procedure was successful in delivering the toxin molecules to the GEC without affecting the heart microvessels. The lectin-saporin conjugate was very effective in killing GEC at low concentrations since even 500 μg/Kg (compared to the mild injury incurred with 23 mg/Kg of MOA lectin alone reported by Warner et al.) caused severe renal capillary thrombosis within 12 h after injection. The lectin-saporin animal model captures many features of TMA, including thrombosis, loss of GEC, proteinuria, schistocytosis, and increase in serum creatinine and BUN [515], which provides a method to study the consequences of endothelial injury and the subsequent downstream effects leading up to activation of the repair mechanism and injury resolution.

There are few reports on the subject of kidney endothelial cell regeneration by EPC [12]. We sought to study GEC repair, using our TMA animal model, by *ex vivo* expanded human EPC. We selected the late outgrowth EPC because they represent the progenitor population with the "true" endothelial lineage [17, 83]. We comprehensively characterized the

EPC according to the reported functional and biochemical analysis that are required to positively identify this cell population. In addition, we added two more *in vitro* tests to ensure the endothelial lineage commitment of EPC. We cultured the hEPC in different media formulated to drive differentiation of embryonic stem cells to other cell types such as, chondrocytes, adipocytes, and macrophages. This test did not result in change of the endothelial phenotype. Moreover, the treatment of hEPC with TGFβ isoforms 1 and 2 did not mediate endothelial to mesenchymal transition *in vitro*. Taken together, our analyses of hEPC indicate that this progenitor cell population is committed to the endothelial lineage at least under *in vitro* culturing conditions. This makes EPC suitable candidate progenitor cells to study vascular repair *in vivo* and possibly circumvent the reported maldifferentiation problems encountered with stem cells transplantations [400]. The study of GEC repair by hEPC using our TMA animal model was very challenging as the $Rag2^{-/-}\gamma^{-/-}$ mouse strain was sensitive to the surgery procedure despite the treatment with low sublethal dose of L-S conjugate. Nonetheless, the hEPC persisted in the injured animals to day 4 post L-S injection, whereas we did not detect a signal of hEPC in the control uninjured animals after 24h. Interestingly, it appears the hEPC were engrafted only in the glomerular capillaries and maintained expression of endothelial markers such as CD31, and Tie2 as demonstrated by real time PCR. This suggests the approach may well work after the technical challenges are overcome.

Major functions of endothelial cells include forming blood vessels during embryonic development, mediating repair of vascular damage, and contributing to tumor neovascularization [415, 516, 517]. To study the angiogenic competence of EPC, we selected a 3-D *in vitro* angiogenesis assay that captures many features of the angiogenesis process *in vivo* [172]. The EPC proved to be more angiogenic than HUVEC but not more than HAEC. Co-culture of EPC and HUVEC enhanced the latter their angiogenic capacity in a process that is dependent on cell-cell contact and not on the release of pro-angiogenic factors. Cell conditioned-medium (CCM) from EPC did not confer the same effect on HUVEC cultured alone. We also demonstrated that EPC occupy more often the tip cell position of a given sprout, whereas HUVEC were found mostly in the stalk of the angiogenic sprouts. In addition, we demonstrated the involvement of the PI3K catalytic isoforms p110β and p110δ, and FGD5 in sprouting angiogenesis *in vitro*. The selective siRNA knockdown of the aforementioned genes significantly inhibited the sprouting of EPC in fibrin gels.

It was previously reported that PI3K catalytic isoform p110α is required for angiogenesis during development in an endothelial-restricted conditional knockout mouse study. The authors also reported that p110β deletion in endothelial cells had no bearing on the embryonic development of blood vessels [330]. In light of our *in vitro* results where p110β knockdown in EPC resulted in significant inhibition of sprout formation in

fibrin gel, we sought to investigate whether p110β signaling is required during tissue injury and repair using the same conditional EC-restricted p110β knockout mouse generated by Graupera et al. [330] and our TMA model of glomerular injury. Post-natal deletion of endothelial P*ik3cb* was done, and then mice were given a sublethal dose of L-S conjugate. Within the first 24 h the p110β knockout mice did not exhibit signs of morbidity. However, by day 2 post L-S injection the mice began to show signs of distress so that by day 3 all the mice were morbid. In contrast, the vehicle-treated mice plus low dose of L-S conjugate remained relatively healthy (one exception) and survived to day 7. Histological evaluation of kidney sections demonstrated an increase of glomerular and tubular injury in the p110β knockout group as compared to control (no tamoxifen plus 200 μg/Kg L-S/LPS). In addition, the p110β knockout group had higher serum creatinine and BUN levels indicative of acute renal failure. It should be noted that the animals that received the tamoxifen treatment but left uninjured survived over one month, until the experiment was terminated, without exhibiting any sign of morbidity. Our *in vitro* and *in vivo* results indicate that p110β signaling or scaffolding functions are important in responding to repair cues released at the site of microvascular injury.

To understand further the cross talk among the glomerular tuft cells and the type of cues that are released at the site of injury. We sought to investigate the effect of inhibition of PDGFB on glomerular repair. We obtained an adenoviral vector expressing the soluble ectodomain of

PDGFRβ that has been reported to efficiently inhibit PDGFB activity *in vivo* [500]. The adenovirus expression system has the distinct advantage over small molecule kinase inhibitors, which have a wide range of substrate specificities, in that the target is specific and the expression is long lasting (> 30 days). The adenovirus infection remains epichromosomal, lacks E1 region of its genome and cannot replicate, and most importantly is well tolerated [500, 518, 519].

We challenged the mice expressing PDGFB blocker with a low dose (200 μg/Kg + LPS) L-S conjugate to induce GEC injury. In the first 24h after L-S injection the mice appeared healthy however by day 2, post L-S injection, the health of the mice had deteriorated so that they were morbid. On the other hand, the mice that were challenged with low dose L-S but received the control vector survived normally. Histological evaluation of kidney sections revealed higher number of injured glomeruli and more injured tubules in the sPDGFRβ group as compared to control. Functionally, the kidneys from the sPDGFRβ group fared worse than the injured control mice as measured by higher serum creatinine and BUN concentrations as well as higher proteinuria indicative of acute renal failure.

PDGFB activity is important for mesangial activation and production of bFGF and VEGF [488, 520]. In addition, injured glomeruli podocytes gain expression of PDGFRβ where PDGFB likely activates podocytes to increase VEGF production [282, 521]. The complex and close interaction

among the glomerular endothelial cells, mesangial cells, and podocytes is essential for proper glomerular function. Any disturbance in one cell type can generate adverse changes in the others [521]. The inhibition of PDGFB signaling during GEC injury likely deprives the endothelial cells from the proangiogenic effects of bFGF [452] and a concentration gradient of VEGF [174] that are necessary to jumpstart the angiogenesis process and vascular repair by endothelial cells. Our results indicate that inhibition of PDGFB signaling exacerbates renal microvascular injury and impedes the initiation of the repair mechanism.

The major contributions of my thesis work are: 1) the development of a glomerular endothelial cell-specific injury model that can be utilized to further our understanding of thrombotic microangiopathy diseases and the role of endothelial cells in the development and the resolution of the injury, 2) provision of evidence of EPC location and possible engraftment in the glomerular capillary walls and their persistence in the injured animals, which render them suitable to study long-term engraftment and vascular repair, 3) the identification of novel roles of the PI3K signal transduction pathway in the endothelium, specifically, the catalytic subunits p110β and p110δ, and FGD5 are critical for angiogenesis, 4) the demonstration that p110β activity is required for proper GEC repair, as the endothelial-restricted deletion of p110β rendered the mice more susceptible to renal injury that resulted in acute renal failure, and 5) the provision of evidence

on the importance of PDGFB signaling in initiating the repair mechanism after GEC-specific injury.

In summary, my thesis work provides endothelial cell researchers with a GEC-specific animal model suitable to study various aspects of endothelial injury and repair. The data presented in this thesis also advance our understanding of the endogenous repair mechanism and the adverse effect caused by the absence of one growth factor (PDGFB) or the disruption of one signaling pathway. Thus, we identified two checkpoints 1) the up-regulation of PDGFB and 2) intact PI3 Kinase-β signaling that mediate renal microvascular repair. Failure of either exacerbates otherwise reparable injury and can lead to higher morbidity and mortality

10.1 Future Directions

The field of regenerative medicine, in particular, vascular repair has garnered tremendous attention ever since the identification of a putative cell population termed endothelial progenitor cells by Asahara and co-workers almost two decades ago [8]. My thesis work focused on studying glomerular endothelial cells repair by introduction of EPC into GEC-injured mice, and also focused on the factors involved in the mouse endogenous repair mechanism. The data presented in this thesis laid the framework for many future studies that will provide further insights into the repair of renal microvasculature and potentially restoration of kidney function. I believe there are three main areas of future research that could be pursued to expand and complement the findings of this thesis, 1) to study EPC homing in and engraftment at the site of glomerular capillaries injury, 2) to study the endogenous factors that are important in initiating the vascular repair mechanism in the kidney, and 3) to further elucidate the roles of p110β and FGD5 in the angiogenesis process.

(1) To study EPC homing in and engraftment at the site of glomerular capillary injury:

Based on the results presented in Chapter VII of this thesis there is a necessity to overcome the problems presented by the survival or lack thereof of $Rag2^{-/-}$ mice. To study human EPC engraftment specifically it is

essential to utilize an immunodeficient mouse strain to take advantage of all the available knockout mice. Therefore, the current surgical procedure of intra-carotid artery (ICA) delivery of lectin-saporin conjugate could be modified. One option is to cannulate the renal artery of one kidney, clamp the renal vein to inject the toxin directly into one kidney and sparing the other kidney to serve as an internal control. The hEPC can be delivered intravenously to decrease the stress level on the mice. The renal artery procedure has been described previously by [378] and the micro- surgeon at the Surgical Medical Research Institute must first acquire that skill. Alternatively, the injured animals that had undergone the ICA surgical procedure can receive a kidney transplant a day after the first surgery to provide life support. However, this option requires the mice to undergo an invasive surgical procedure and may not survive two surgical procedures in two consecutive days as reported in Chapter VII.

Consideration should be given to establish the lectin-saporin glomerular endothelial cell-specific injury in larger animals such as Rag $2^{-/-}$ rat (from Sage Labs, Product number: TGRS4410) where the lectin-saporin and hEPC can be delivered through a direct injection into the renal artery of one kidney, sparing the other kidney to serve as an internal control. However it must be first validated that the rat kidney microvasculature expresses Galα(1,3)Gal epitope.

Another possibility can be considered but it does not involve hEPC. This strategy involves autologous transplantation of bone marrow from a

transgenic mouse strain that expresses GFP in bone marrow cells such as C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ (Jax mice stock: 006567), since there is no method exists to date to isolate mouse EPC in sufficient numbers. This study requires sub-lethal irradiation of the recipient mouse strain of the same background as the donor strain to eliminate bone marrow cells. The wild type mouse will undergo the ICA surgery to deliver the L-S conjugate and the bone marrow from the donor strain can be delivered by an intravenous injection. The advantage of this study is that it provides insight into the source of endothelial cells responsible for GEC repair, whether the bone marrow-derived EPC are mobilized and homed in to the site of renal injury, whether the repair is achieved from the pre-existing endothelial cells, or it is a combination of both. However, the disadvantage of this approach lies in the side effects of irradiation that can cause high mortality and can also cause kidney damage [522, 523].

(2) To study the endogenous factors that are important in initiating the vascular repair mechanism:

To further our understanding of the genetic components of the repair cues that are released at the site of the glomerular injury using the lectin-saporin animal model. A gene microarray study can be performed on isolated glomeruli from high dose injured animals at 12h and day 4 post L-S injection. Also, from glomeruli 12h, day 4 and 7 after low dose L-S injection, and from glomeruli isolated from saline control mice at the same timepoints. It is critical that the glomeruli isolation procedure yields pure samples. We have tried the glomeruli isolation using Dynabeads® M-450

tosylactivated magnetic beads perfused through the heart and the bead-filled glomeruli were separated from tubules by a magnet. It is a high throughput and fast preparation of glomeruli. However the glomeruli from the injured animals fell apart during the perfusion step and the glomeruli separation step was contaminated with tubules. To circumvent this problem pure glomeruli can be isolated using a micromanipulator tool that can physically capture glomeruli individually without a perfusion step.

We have reported in this thesis (Chapter IX) that systemic inhibition of PDGFB exacerbates the renal injury and leads to high morbidity and mortality rates. PDGFB is reported to be up regulated in the glomeruli after injury. PDGFB acts in an autocrine/paracrine fashion to stimulate mesangial cells to produce bFGF, which is an angiogenic factor that contributes to kidney vascular reendothelization and repair [267, 416, 524]. To gain more insights into the role of bFGF during the repair mechanism after GEC-specific injury using the L-S animal model, bFGF knockout mice (Jax mice stock number, 010698, 010720) can be used for this study. However, there are two types of bFGF knockout mice. One type lacks the low molecular weight (18 kDa) but expresses the high molecular weight (20.5 and 21 kDa), while the other knockout mouse is the opposite. Therefore, both knockout mouse strains are needed to determine which bFGF isoform is more important during GEC repair.

Whether PDGFB treatment after GEC injury can accelerate vascular repair can be considered. However, there are few obstacles that must be overcome: 1) method of delivery as systemic administration of

PDGFB is not effective since PDGFB is eliminated from systemic circulation by α2 macroglobulin [525], 2) PDGFB concentration delivered is critical as excessive PDGFB signaling can cause mesangioproliferative glomerulonephritis [501].

3) To further elucidate the roles of p110β and FGD5 in the angiogenesis process:

The role of PI3K catalytic isoform p110β role in angiogenesis and glomerular injury/repair has been studied extensively in this thesis with *in vitro* and *in vivo* experiments. However, phenotype rescue experiments for *in vitro* angiogenesis are still lacking. P110β coding domains can be inserted into a lentiviral or a retroviral vector that can be used to infect hEPC after siRNA knockdown of p110β to rescue the cells and their ability to form sprouts in the fibrin gel. Moreover, PTEN inhibition can be considered to rescue p110β phenotype using the specific inhibitor VO-OHpic [526] to determine whether other PI3K catalytic subunits can compensate for the lack of p110β activity. The phenotype rescue also allows the study of mutated p110β in order to evaluate the function of individual domains of the molecule e.g. Ras-binding domain (RBD).

Another possible research interest in the role of p110β during kidney injury and repair is to investigate whether *Pik3cb* gene is mutated in the patients that develop HUS but never recover after treatment. In the German Shiga-toxin outbreak in 2011, 20% of the adult patients

developed HUS, and generally dialysis-dependent HUS patients fail to recover kidney function [16, 472].

The role of FGD5 in mammalian angiogenesis is not fully understood and to date there is no FGD5 knockout mouse. Since FGD5 is enriched in endothelial cells it would be of great interest to develop a FGD5 knockout mouse to study blood vessels formation during embryonic development. In the event that FGD5 ablation is embryonically lethal, a conditional endothelial-restricted FGD5 knockout mouse can be considered instead.

The potential future studies as a follow up to the data already presented in this thesis would further enhance our understanding about glomerular endothelial cell injury and the factors produced in the microenvironment of the damaged glomerular capillaries that can lead to initiate the repair mechanism.

CHAPTER XI

FOOTNOTES

[1] The FGD5 results are published as part of a manuscript by Nakhaei-Nejad et al. 2012. *ATVB* 33: 2694-2701

CHAPTER XII

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