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EXPRESSION PROFILING OF BLOOD VESSELS IN PERICYTE DEFICIENCY AND DIABETES

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Stockholm 2010

Published by Karolinska Institutet. Printed by Vasastadens Bokbinderi AB

ISBN 978-91-7457-121-9

Be proactive
Begin with the end in mind
Put first things first

Stephen R. Covey

To Emil, my love

ABSTRACT

The kidney glomerulus is essential for the filtration of waste products from the blood into the urine and in controlling the volume and composition of body fluids. Injury to the glomerulus can lead to altered glomerular filtration rate, proteinuria, thickening of the glomerular basement membrane (GBM) and accumulation of mesangial cell matrix. The injuries can occur due to primary kidney diseases or as a secondary effect of other diseases such as diabetes mellitus. The blood-brain barrier (BBB) differs in many ways from of the glomerular filtration barrier. While glomerular endothelial cells are fenestrated and highly permeable, the endothelial cells of the blood-brain barrier are continuous and held together by tight junctions. Passage across the BBB is tightly controlled and serves to protect the neuronal tissue of the central nervous system from fluctuations in hormones, nutrients, metabolites and other substances in the blood.

The targeted deletion of either platelet-derived growth factor-B (PDGF-B) or PDGFR- β is embryonic lethal due to cardiovascular complications and lead to a failure in recruiting pericytes to the developing vasculature and mesangial cells into the glomerulus. In paper I we investigated the role of endothelium-derived PDGF-B in the recruitment of pericytes and mesangial cells using a mouse model with a targeted conditional deletion of PDGF-B in endothelial cells. We found that the phenotype mimics that of the null mutation, but is much milder. Thus the endothelium appears to be the main source of PDGF-B in the vasculature.

In the kidney, the initial deficiency of glomerular mesangial cells normalized soon after birth and later led to a very light albuminuria and enlargement of the glomeruli. To increase our knowledge of the glomerular transcriptome we constructed a cDNA array from isolated mouse glomeruli.

In paper II we used it to identify a number of novel glomerular transcripts. In a series of experiments these transcripts were assigned to specific cell types and characterized as podocyte or mesangial cell/juxtaglomerular markers. Further study on podocyte marker Foxc2 revealed a role in podocyte differentiation and glomerular development.

In paper III we used several pericyte deficient mouse models to elucidate the role of pericytes in the integrity of the BBB. We found a correlation between the degree of pericyte deficiency and the extravasation of injected tracers across the BBB via macromolecular trancytosis, for the first time *in vivo* demonstrating the importance of pericytes in maintaining the BBB function.

To gain insight into the transcriptional changes behind diabetes-related glomerular injury, we analyzed kidney function and gene expression in the db/db mouse, a model of type 2 diabetes. We found increased expression of genes relating to the infiltration of monocytes/macrophages into the glomerulus, changes in expression of genes involved in the composition of the GBM and the extracellular matrix, as well as in signal transduction and growth factor expression. We also noted strong and consistent upregulation of Ym-1, a marker for alternatively activated macrophages (aaMac). Further analysis of our data set revealed that expression of several markers for aaMac were upregulated, while markers for classically activated macrophages remained unaltered.

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IV. **Glomerular transcriptome analysis and the presence of alternatively activated macrophages in diabetic nephropathy.**
Norlin J, He L, Tryggvason K, Betsholtz C.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

aaMac	alternatively activated macrophage
ACE	angiotensin-converting enzyme
AER	albuminuria excretion rate
Agrp	agouti-related protein
Akr1b7	aldo-keto reductase family 1 member B7
AJ	adherence junction
AMDCC	Animal Models of Diabetes Complications Consortium
Ang-1	angiopoietin-1
Ankrd1	ankyrin repeat domain 1
ANOVA	analysis of variation
APC	antigen presenting cell
Aqp-4	aquaporin-4
ARB	angiotensin receptor blocker
ASMA	alpha smooth muscle actin
BB rat	bio breeding rat
bp	base pair
caMac	classically activated macrophage
Calca	calcitonin/calcitonin-related polypeptide alpha
Chi3l3	chitinase 3-like 3
Cited2	Cbp/p300-interacting transactivator 2
CKD	chronic kidney disease
CNS	central nervous system
Col5a1	collagen 5 alpha 1
Csf1r	colony stimulating factor 1 receptor
E18.5	embryonic day 18.5
EAAT1-3	excitatory amino acid transporters 1-3
ECM	extracellular matrix
EST	expressed sequence tag
Fcgr3	Fc gamma receptor III
FDR	false discovery rate
Fn1	fibronectin
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GLEPP1	protein tyrosine phosphatase receptor type O
GLUT1	glucose transporter-1
Grp	gastrin releasing peptide
HLA	human leukocyte antigen
HSPG	heparin sulphate proteoglycan
Igfbp5	insulin-like growth factor binding protein 5
IHC	immunohistochemistry
INF-gamma	interferon gamma
Ins2Akita	insulin-2 Akita
ISH	*in situ* hybridization
JAM	junctional adhesion molecule

Lama5	laminin alpha 5
Lcp2	lymphocyte cytosolic protein 2
LAT1	L-system for large neutral amino acids
LIF	leukaemia inhibitory factor
Lmo7	lim domain only protein 7
LPS	lipopolysaccaride
Mc3r	melanocortin receptor-3
MIF	macrophage inhibition factor
NIH	National Institutes of Health
Nphs1	nephrin
Nphs2	podocin
NO	nitrixoxide
NOD	non-obese diabetic
OAP	orthogonal array of particles
PAI-1	plasminogen activatior inhibitor 1
PAS	periodic acid-Schiff
Pdgf	platelet derived growth factor
Pgp	P-glycoprotein
Pi15	protease inhibitor 15
Podxl	podocalyxin
PPAR-γ	peroxisome proliferator-activated receptor- γ
Ptpro	protein tyrosine phosphatase receptor type O
qPCR	quantitative polymerase chain reaction
RAS	renin-angiotensin system
Ren1	renin
Rhpn1	rhophilin1
RIN	RNA integrity number
ROS	reactive oxygen species
SAM	significance analysis of microarray
SD	slit diaphragm
Sem2	semaphorin sem2
Sfrp2	secreted frizzled-related protein 2
Synpo	synaptopodin
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TEM	transmission electron microscopy
TJ	tight junction
UAE	urinary albumin excretion
Vegf	vascular endothelial growth factor
Wt1	Wilm´s tumor protein
1m	1 month of age

INTRODUCTION

1.1 THE GLOMERULUS

1.1.1 The function of the kidney

The most important function of the kidney is to rid the body of waste materials, either ingested or produced by metabolism. A second important function is to control the volume and composition of the body fluids. The regulation of water and electrolytes by the kidneys is what maintains a stable environment for the cells in our bodies, so that they can perform their various tasks. Other important features of the kidneys also include:

- Regulating acid-base balance by excreting acids and regulating the body fluid buffer stores
- Regulate arterial blood pressure in the long term by regulating sodium and water excretion and in the short term by secreting vasoactive factors such as renin
- Produce, secrete and regulate hormones involved in the production of red blood cells and the active form of vitamin D
- Regulate blood glucose levels during prolonged fasting by gluconeogenesis [1]

The filtration unit of the kidney is the *nephron*, where a large amount of plasma is filtered in a tuft of capillary in the *glomerulus* and is converted into urine in the long *tubule* on its way to the renal pelvis. Each human kidney contains about one million nephrons. The kidney can't regenerate nephrons lost due to renal injury, disease or normal aging. The glomerulus is a network of branching and anastomosing capillaries with high hydrostatic pressures, leading to effective filtration. In a healthy kidney only water, various solutes and small proteins can pass through the glomerular filtration barrier. If the barrier is disrupted, as is the case in various nephropathies, macromolecules such as albumin leak to the primary urine. The tubular system is very effective in concentrating the primary urine by reabsorbing substances such as ions and small proteins, but fails to absorb larger proteins, causing them to remain in the final urine. This is called *proteinuria*, or more specifically *albuminuria*, referring to the leakage of the plasma protein albumin.

1.1.2 The glomerular filtration barrier

The glomerular filtration barrier consists of three layers: fenestrated glomerular endothelial cells, the glomerular basement membrane (GBM) and the highly specialized epithelial podocytes. Data shows that all three layers need to be intact for functional filtration [2, 3].

The *glomerular endothelial cells* are highly fenestrated to allow the passage of large volumes of water and solvents across the endothelial barrier [4]. The fenestrations in the glomerulus lack diaphragms, making them too large to effectively filter large macromolecules. However, the endothelial cells have a coating of glycocalyx composed of negatively charged glycoproteins, aminoglycans and protoglycans [5]. This layer probably provides a charge-selective function to the glomerular barrier, since

it has been shown that degradation of the endothelial glycocalyx layer results in an increased urinary output of albumin [6].

The *glomerular basement membrane* (GBM) is a fibrous network of type IV collagen, nidogen/entactin, laminin, nidogen/entactin together with proteoglycans such as agrin and perlecan and glycoproteins. The collagen IV network can be described as the backbone of the membrane and mutations in collagen IV chains give rise to severe pathlogical conditions such as Alport syndrome (hereditary glomerulonephritis) [7, 8]. The large amount of negatively charged heparin sulphate proteoglycans in the GBM has lead to the hypothesis that the negative charge of the barrier would work as a charge-selective barrier by repelling negatively charged plasma proteins and thus preventing them from passing the GBM [9, 10]. Lately this theory has been challenged since it has been shown that transgenic mice lacking agrin, the major heparin sulphate proteoglycan of the GBM, do not develop proteinuria [11, 12]. In another experiment it has also been shown that there was no difference in the sieving curves for negatively charged Ficoll versus neutral Ficoll in isolated GBMs [13]. These experiments suggest that the majority of the charge-selective barrier lies in the endothelial or epithelial layers.

The visceral epithelial cells known as *podocytes* line the outside of the GBM and wrap around the glomerular capillaries. They are highly specialized cells with large cell bodies and multiple, long foot processes. The foot processes are separated by a filtration slit that is 25-60 nm wide and covered by a diaphragm. The slit diaphragm consists of a complex of proteins and forms a zipper-like structure that bridges the foot processes, making it permeable to water and solvents but relatively impermeable to plasma proteins [14, 15]. The proteins in the slit diaphragm have been extensively studied [16-20] and some of them, such as nephrin have been shown to be vital for the maintenance of normal glomerular filtering selectivity. Mutations in nephrin cause massive proteinuria in patients suffering from congenital syndrome of the Finnish type [21-23].

1.1.3 Assessing kidney function

Urine formation results from *glomerular filtration*, *tubular reabsorption* and *tubular secretion*. The blood is first filtered in the glomerulus where the high hydrostatic pressure pushes fluid from the blood across the filtration barrier and into the Bowman's capsule. Since most low molecular-weight substances of the plasma are freely filtered their concentrations in primary urine are almost the same as in the plasma. The filtered fluid then passes through the capsule into the tubule and its composition is modified by tubular reabsorption and secretion until the final urine leaves the nephron, passes through the collecting ducts into the renal pelvis and leaves the kidney through the ureter. Examples of tubular reabsorption are water and many electrolytes such as sodium, which are partially reabsorbed. Nutrients such as amino acids and glucose are filtered but not excreted into the urine, because in a healthy individual all of the filtered nutrients are reabsorbed in the tubuli. Some substances, such as potassium and hydrogen ions, are filtered by the glomerulus and also secreted by the tubule, thus causing the substance to be rapidly cleared from the blood. The rate at which a

substance is cleared from the blood and excreted in the urine depends on the relative rate of filtration, absorption and excretion for that particular substance [1].

In the classification of diabetic kidney disease, two main methods are used. The first is the classification system by Mogensen based on data from patients with type 1 diabetes [24, 25] ; the other system is the more general Kidney Disease Quality Outcomes Initiative (KDQOI) for classification of chronic kidney disease (CKD) [26]. While their classification systems into five stages of CKD are similar, the diabetes-specific classification is based on albuminuria while the more general classification system uses glomerular filtration rate as a measurement of kidney disease severity.

Clinically, *glomerular filtration rate* (GFR) is generally accepted as the best over all measure of kidney function [27]. GFR is measured as the clearance of an inert filtration marker that is filtered by the glomeruli but neither absorbed nor excreted by the tubuli. Common exogenous markers to be used are inulin or EDTA. However, measuring GFR using exogenous markers is too expensive and too complex for routine clinical practice. Urinary clearance of an endogenous marker can be calculated from blood samples and timed urine collection. Collecting urine over 24-hour periods is tedious, and since there is a relationship between the serum level of an endogenous marker and the level of GFR, GFR is normally estimated without urine collection. One of the most commonly used endogenous markers is creatinine, an amino acid derivative released by muscle cells. Many studies support the similarity of creatinine clearance to GFR and its reciprocal relationship with the serum creatinine level [28, 29]. Creatinine is secreted by tubular cells, so the measured creatinine clearance will exceed GFR. There is also some inter and intra-individual variation, especially when there is a mild to moderate reduction in GFR [30]. Different equations used to estimate GFR may cause analytical bias [31]. Another possible endogenous marker under investigation is Cystatin C, a small protein that is freely filtered and later reabsorbed and catabolised by tubular epithelial cells [32]. In some hospitals a combination of creatinine and cystatin C is used to get a more reliable estimation of GFR [33].

Another way to asses kidney function is to look for *albuminuria*, the leakage of albumin into the urine. This is sometimes referred to as Albumin Excretion Rate (AER) and is usually done quite easily with a dip stick. Microalbuminuria is defined as passage of 30-300 mg of albumin per 24 hours and macroalbuminuria as >300 mg/24h [34]. There is a strong link between micoalbuminuria and early diabetic renal disease where microalbuminuria usually indicates the beginning of diabetic nephropathy [35-37]. Albuminuria has also been shown to predict mortality in both type 1 diabetes [38] and type 2 diabetes [39] because it is linked to cardiovascular disease. Traditionally, increased in AER to above 300 mg/24h has been considered to indicative of the onset of a decreased GFR. But when exogenous markers for GFR have been used rather than formulas to calculate GFR from serum creatinine, a decrease in GFR has been seen already at microalbuminuric levels. It has been suggested that both GFR and AER should be assessed in the staging of diabetic nephropathy [34].

Transgenic mouse models or rodents with experimentally induced diabetes are frequently used to study kidney function, and the models most commonly used to study diabetic complications will be discussed in more detail below. However, assessing

kidney functions in rodents require some methodologies that are different from the ones used on human patients in the clinic.
Blood sampling to test for *blood glucose* in mice should ideally be done in the fasting state to rule out fluctuations due to recent feeding. Since mice and rats are nocturnal eaters, removing the food during night would result in a prolonged fast of almost 24 hours. A standard protocol developed by the National Institutes of Health (NIH) suggests removing the chow at 7 am, fasting the animals between 7 am and 1 pm, and sampling the blood at 1 pm [40, 41]. The relationship between HbA1c (glycosylated haemoglobin) and long term glucose control is valid in mice, as well as in humans [42].
GFR can be estimated by measuring plasma creatinine, but it has been reported that creatinine levels in plasma in mice were greatly overestimated when the Jaffé alkaline picrate method was used as opposed to when in the use of HPLC [43]. The assay-dependent variability in the accuracy of creatinine as a measure of GFR in rodents has been noted in other studies using enzymatic tests as well [44]. GFR estimation by inulin clearance, the old golden standard, has been adapted for use in rodents using FITC-labeled inulin [45, 46].
Albuminuria measurement is the method most commonly used in rodents to grade the severity of DN. Dip stick analysis should be avoided since the method is inaccurate in mice, probably due to other proteins present in mouse urine [47, 48]. The Animal models of Diabetic Complications Consortium (AMDCC), a NIH consortium, recommend to use an ELISA with antibodies raised against mouse albumin and to collect urine in metabolic cages for 24 hours [40]. That is the method I have used in paper I and IV. Another method to estimate kidney function is to measure the ratio between albumin and creatinine in spot urine. In most cases this ratio correlates significantly with 24 hour urinary excretion rate (UAE), but not in all genetic backgrounds [49]. It should also be noted that the creatinine level should be measured with HPLC [43].
Histopathology is usually done to assess mesangial matrix expansion, general glomerular morphology and tubulointerstitial pathology. Hematoxylin and eosin does not highlight basement membranes well and does not clearly distinguish between cytoplasm and matrix components in mesangial regions. Periodic acid-Schiff (PAS) provides crisp basement membranes and capillary loops and easily distinguishable mesangial regions. Another, more difficult method is to use silver methenamine staining or immunohistochemical staining for collagen IV to visualise basement membranes and mesangial matrix [50]. In paper I, I used PAS to visualise mesangial matrix and alpha smooth muscle actin (ASMA) to visualise the absence of mesangial cells.

Protocols and other resources for phenotyping of mouse models with diabetic nephropathy can be found on the AMDCC homepage: http://www.amdcc.org

1.2 PERICYTES AND PLATELET-DERIVED GROWTH FACTORS

1.2.1 Pdgf and Pdgf-receptor biology

Platelet-derived growth factor (Pdgf) proteins were discovered already in 1974, as a platelet-dependent serum factor that promotes the growth of smooth muscle cells and fibroblasts *in vitro* [51, 52]. In 1979 the protein was purified from platelets,

characterized and named Pdgf [53, 54]. Pdgf-a, -b, -c and –d are the four ligands that form five possible combinations of disulphide-linked dimers, Pdgf-aa, -ab, -bb, -cc and –dd. They bind to the Pdgf receptors-α and –β. Pdgf-a and –b are secreted in their active form, while Pdgf-c and –d require proteolytic cleavage of their N-terminal CUB domain in order to become biologically active [55]. Although a number of ligand-receptor combinations signal *in vitro*, only a few appear to have physiological significance *in vivo* namely those of Pdgfaa and –cc over Pdgfrα and Pdgfbb over Pdgfrβ [56]. During the embryonic development of the vascular system, Pdgfb is mostly expressed by the developing vascular endothelium [57] while Pdgfrβ is expressed by vascular smooth muscle cells/pericytes [58].

Targeted deletion of either Pdgfb or Pdgfrβ leads to a failure in recruiting pericytes to the developing vasculature and the embryos die at late gestation due to edema, dilation of the heart and large blood vessels, capillary haemorrhage and subsequent heart failure [57-60]. In targeted conditional deletion of Pdgfb in endothelial cells the phenotype mimics that of the null mutation, but is much milder, and the offspring is viable and fertile (See Paper II and [61]). Pdgf-a and –b both contain a basic amino acid stretch at the c-teminal end of the protein, often called a retention motif. This sequence causes the protein to be retained in close proximity to the cell surface and the extra cellular matrix instead of diffusing away [62]. The recruitment of pericytes to vessels depends largely on the expression of Pdgfb by the endothelial cells and its binding to the Pdgfrβ receptor on pericytes. The proper location of Pdgfb is crucial for the pericytes to adhere closely to the vessel wall. The positive charges in the retention motif binds to negatively charged sulphate groups in the heparin sulphate proteoglycans (HSPGs) of the matrix, thus keeping the majority of the secreted protein in close contact with the vessels. A gradient is created and this is required for proper investment of pericytes into the vessel wall. The role of Pdgfb retention motif was addressed *in vivo* by deleting the motif by targeted mutagenesis [63]. In the Pdgfb retention motif knock-out animals (Pdgfb ret/ret), the Pdgfb is unable to bind to the HSPG on the surface of blood vessels and is instead freely diffusible, and thus the gradient of Pdgfb surrounding the vasculature is disrupted. This leads to a severe reduction in the number of pericytes in the vasculature (discussed further in paper III) causing disorganized retinal vasculature, retinopathy and in the kidney abnormal, ballooning glomeruli lacking mesangial cells and later glomerulosclerosis. Like the endothelial-specific knock-outs, the Pdgfb ret/ret mice also survive into adulthood and are valuable in studies of the physiological functions of pericytes in postnatal animals.

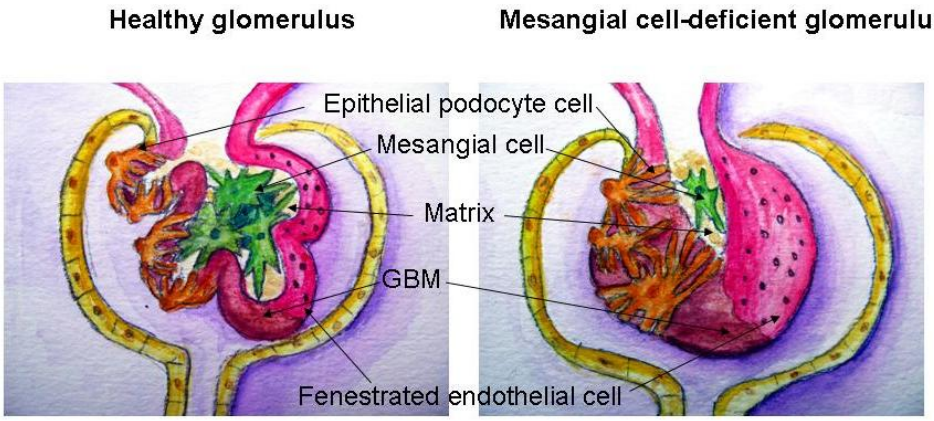
1.2.2 Pdgf pathology

In the adult individual Pdgfs are often expressed at low levels, which can be increased during stressful situations such as arthrosclerosis and wound healing. Arthrosclerosis is viewed as a local chronic inflammation and Pdgf is one of many factors secreted by infiltrating immune cells as well as by smooth muscle cells in the vessel wall [64]. ApoE-/- mice lacking Pdgfb in circulating monocytes and platelets show a delayed formation of smooth muscle cell accumulation in atherosclerotic lesions and pharmacological blockade of Pdgf receptors have the same effect [65]. A follow up study showed a shift towards a selective pro-inflammatory phenotype in macrophages in the sclerotic lesions of ApoE-/- mice lacking Pdgfb in hematopoietic cells [66]. Pdgf-

a and -b have also been implicated in several fibrotic conditions in the lung (pulmonary fibrosis) [67, 68], liver (liver sclerosis) [69, 70], heart (cardiac fibrosis and allograft rejection) [71, 72], skin (scleroderma) [73, 74] and kidney (glomerulosclerosis) [63] by driving proliferation of myofibroblast-like cells and excessive deposition of extracellular matrix (ECM) [56, 75].

1.2.3 Mesangial cells and Pdgfb in the glomerulus

As mentioned above, the glomerular filtration barrier has three components; endothelial cells, GBM and podocytes. There is, however, another important cell type in the glomerulus; the mesangial cells. Mesangial cells are the pericytes of the glomerulus. The mesangial cells are situated at the centre of the glomerulus and have several important functions including serving as a scaffold for the glomerular vascular tuft, generate and control the turnover of mesangial matrix, secrete and act as a target for growth factors, provide housekeeping functions and regulation of glomerular capillaries as well as play a role in the pathophysiology of many glomerular diseases [76]. Mesangial cells can be viewed as the central stalk of the glomerulus and they are in continuity with the extraglomerular mesangium and the juxtaglomerular apparatus. Like smooth muscle cells, they have contractile abilities and can contract or relax in response to vasoactive substances such as Angiotensin II, and thus modify glomerular filtration locally [77]. Inside the glomerulus the mesangial cells are in direct cell-cell contact with the endothelial cells, with no basement membrane separating the two. The mesangial cells generate mesangial matrix, which provides structural support for the mesangial cells as well as functions in growth factor sequestration [78]. Mesangial cells express Pdgfrβ and need Pdgfb expressed from the glomerular endothelial cells to be properly recruited into the glomerulus. In the Pdgfb null mutants the mesangial cells are absent from the glomerular core and the complexity of the glomerular tuft is replaced by a single capillary loop [79]. This makes the glomeruli appear as a balloon shaped structure filled with erythrocytes.



As is discussed in paper I, the endothelium appears to be the main source of Pdgfb in the vasculature. When Pdgfb is knocked out from endothelial cells by targeted mutagenesis, the phenotype resembles that of the null mutant but is milder [80]. During the first days and weeks postnatally, the number on mesangial cells in the glomeruli rapidly increased and at 1 month of age the number of mesangial cells did not differ from controls. There was, however, still a slight dilation of both the

glomerular cross section and the individual capillary loop diameter. (Paper I, fig 4D-G) There was also a small but statistically significant increase in AER in mutants older than 12 months. (Paper I, fig 4I)

Not only the presence of Pdgfb, but also the correct spatial localization of Pdgfb is crucial for the proliferation and survival of the glomerular mesangial cells. When glomeruli from the Pdgfb ret/ret were analysed at late gestation they phenocopied to a major extent the characteristic ballooning capillaries of the Pdgfb/Pdgfrβ null mutants. The glomeruli lacked all, or almost all, mesangial cells. However, the viability of the Pdgfb ret/ret mice offered a unique opportunity to study the postnatal effects of mesangial cell loss. As in the conditional endothelial knock-out of Pdgfb (Paper I), by postnatal day 5 the number of mesangial cells had increased dramatically in the Pdgfb ret/ret and by day 30 the glomeruli were undistinguishable from wild type controls. Later, at 6 months of age, the glomeruli showed signs of sclerosis, with fewer mesangial cells in the glomerular tuft and excessive mesangial matrix deposition. The mice also showed microalbuminuria [63].

Taken together, these data suggest that Pdgfb/Pdgfrβ is essential for the proper development of kidney glomeruli and that the system is sensitive to alterations in expression levels as well as spatial localization of the Pdgfb protein.

1.3 THE BLOOD-BRAIN BARRIER

1.3.1 Structure and function of the blood-brain barrier

The blood-brain barrier (BBB) separates substances in the circulating blood from the sensitive cells of the brain. The blood-brain barrier (BBB) differs in many ways from of the glomerular filtration barrier. While glomerular endothelial cells are fenestrated and highly permeable, the endothelial cells of the BBB are continuous and held together by tight junctions. There is still passage over the BBB, but it is tightly controlled and aims to maintain a precisely regulated microenvironment that is optimal for neuronal signaling. The purpose is to protect the neuronal tissue of the central nervous system (CNS) from fluctuations in hormones, nutrients, metabolites and other substances in the blood. Adjacent endothelial cells of the BBB are held together by tight junctions (TJs) and adherence junctions (AJs), severely restricting the flow of polar solutes in the paracellular space and thus separating the blood from the brain extra cellular fluid. [81-83]. The junctional complexes also confer apical and basal polarity to the cells. One other important aspect of the BBB worth mentioning is to separate the neurotransmitters and neuroactive substances into two pools – one for the peripheral circulation and one for the central nervous system [83]. Only a small number of drugs and neuroactive substances are allowed to freely penetrate the BBB and access the CNS. Examples of substances allowed free access are small gaseous molecules such as oxygen and carbon dioxide and small lipophilic substances including ethanol [82].

Tight junctions are composed of occludins, claudins and junctional adhesion molecules (JAMs) which span the intercellular space and link to the cytoskeleton via regulatory

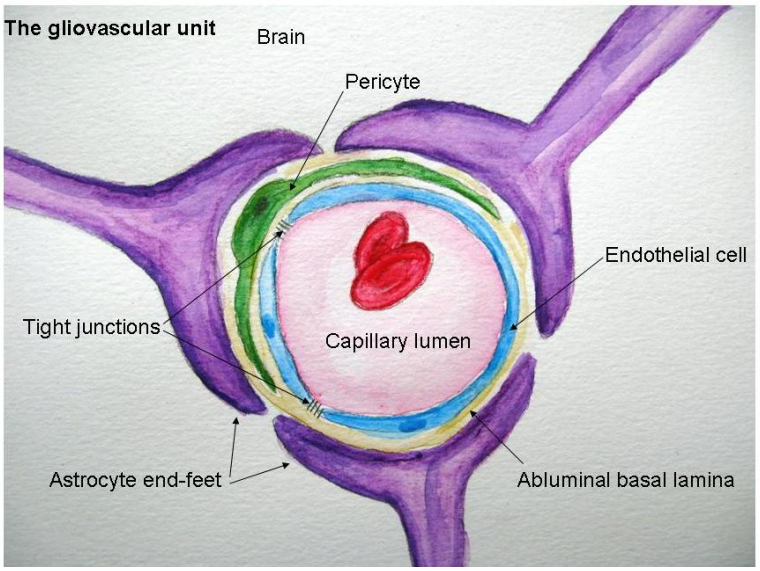
proteins as ZO-1, -2 and -3 and cingulin [84, 85]. Adherence junctions consist of cadherin proteins that span the intracellular space and link to the cytoskeleton via α-, β- and γ-catenin [84, 85].

1.3.2 BBB pathology

The BBB is not a fixed structure. The system can be manipulated and the barrier can tighten or open up during different conditions. Many CNS illnesses cause dysfunction of the BBB. Multiple sclerosis is characterized by demyelinisation, increased permeability of the BBB and the infiltration of inflammatory cells into the CNS. In the experimental autoimmune encephalomyelitis model of the illness both ZO-1 and occludin had been dysregulated in areas of the brain that show signs of inflammation [86-88]. In Alzheimer's disease the neurotoxic amyliod beta-peptide accumulates in the CNS, possibly through deregulated LRP-1/RAGE transport, and causes neurovascular inflammation leading to BBB compromise and disturbances in the CNS milieu [89-93]. Other illnesses affecting the BBB include Parkinson's disease [94, 95], epilepsy [96], brain tumors [97], inflammation and meningitis [98-100].

1.3.3 The gliovascular unit and Pdgfb in the BBB

The specific characteristics of the BBB are formed from interactions between endothelial cells, the astrocytic perivascular end-feet and pericytes. Sometimes this group of cells is referred to as a *gliovascular unit*, meaning that no cell is an island, but rather the cells interact in developing and maintaining the barrier function of the BBB.



Brain *endothelial cells* are characterized by the presence of tight junctions, a lack of fenestrae and pinocytic vacuoles. They are selectively permeable to molecules with suitable mass and lipophilicity. The tight junctions restrict the movement of most substances across the intercellular space, and this means that transport from the blood to the interstitial fluid surrounding the brain must take the intracellular route and pass through the endothelial cells rather than between them. Transport across the endothelial membrane uses for example glucose transporter-1 (GLUT1) and several amino acid transporters such as L-system for large neutral amino acids (LAT1) to supply nutrients to the brain. Some transporters such as GLUT1 and LAT1 can be found on both the luminal and abluminal side of the endothelial cell [101]. Others, like P-glycoprotein (Pgp) (on the luminal side) or the Na+-dependent transporters like excitatory amino acid transporters 1-3 (EAAT1-3) (on the abluminal side) are only found on either side of the cell and thus maintain the clear polarization of

these cells [102-104]. The endothelial cells secrete a substance called endothelium-derived leukaemia inhibitory factor (LIF) that induce astrocyte precursor cells to differentiate into mature astrocytes [105].

The *astrocyte end feet* are in close contact with the endothelial cells and the pericytes of the BBB, separated only by the basal lamina. The astrocyte end feet provide the cellular link to the neurons. They also secrete a number of chemical agents such as transforming growth factor-β, glial-derived neurotropic factor and angiopoetin-1 that induce BBB-like characteristics by enhancing the barrier function of tight junctions in endothelial cells *in vitro* [106, 107]. The perivascular part of the astrocytic end feet expresses aquaporin-4 (Aqp-4) and Kir4.1K+ channels which are important for fluid and ion balance in the brain. The Aqp-4 and Kir4.1 channel proteins are arranged in a specific pattern called orthogonal arrays of particles (OAPs) at the side of the end feet that faces the micro vessel wall and the basal lamina [84, 108]. The localization of OAPs and Aqp-4 to the end foot is thought to be dependent on a heparin sulphate proteoglycan called agrin in the basal lamina, and agrin has been shown to be important for maintaining the barrier function. During pathological conditions when the BBB is disrupted, agrin is lost directly indicating the need for agrin in the functional polarization of astrocyte end feet [97, 109]. Agrin binds to α-dystroglycan [110]. α-dystroglycan is a member of a complex called the dystrophin/dystroglycan complex and it too localizes to the astrocyte end feet membrane [82]. Another member in the dystrophin/dystroglycan complex, α-syntrophin, has been shown to be important for the correct localization of Aqp-4 in the perivascular membrane of the end feet [111]. α-syntrophin also binds Kir 4.1, which explains the co-localization of Kir 4.1 and Aqp-4 [82].

The *pericytes* are located in very close proximity to the endothelial cells and the astrocyte end feet, sometimes even sandwiched between the two and only separated by a thin basal lamina. Little notion has been given to pericytes when the BBB is discussed, despite their central location at the gliovascular unit. Not a lot is known about their role in maintenance of the BBB function. Studies on pericyte deficient mouse models have shown that the absence of pericytes correlates with brain edema and brain vascular leakage [112]. In co-culture experiments it has been shown that pericytes and endothelial cells, in the presence of astrocytes, are capable of arranging themselves into linear capillary-like structures. In the same study the presences of pericytes also appear to stabilize the capillary-like structures and prevent endothelial cells from going into apoptosis [113]. They may also have a role in regulating TJ properties, as brain endothelial cells upregulate the TJ protein occludin in response to conditioning with pericyte media. The effect was mediated by pericyte-derived Angiopoietin-1 (Ang-1) on the Tie-2 receptor of the endothelial cells [114]. In studies where pericytes were co-cultured with endothelial cells, it was shown that pericytes significantly reduced the paracellular permeability in cell monolayers [115, 116]. The upregulation of the barrier function of the endothelial cells was concluded to be due to TGF-β1 expression from the pericytes [115]. Sine PDGF-B is essential for the recruitment of pericytes to the vasculature; it seems that mouse models that are hypomorphic for PDGF-B would make great tools for studying the effect of pericyte-deficiency on the BBB. In paper 4 we used two of these models to show that there was an increased permeability for tracers across the BBB in mice with low pericyte density.

1.4 DIABETES

1.4.1 Primary and secondary effects of diabetes

Diabetes Mellitus (DM) is one of the growing illnesses in the western world. It was estimated that in the year of 2000 the world wide prevalence of diabetes in all age groups was 2,8%, and that by the year of 2030 this will have increased to 4,4% [117]. Cardiovascular and renal complications of diabetes accounts for most of the mortality and are thought to be due to a complex interplay between metabolic factors, vascular constriction and fibrosis as well as inflammation [118, 119].

Type I Diabetes Mellitus (T1DM) also called Insulin Dependent Diabetes Mellitus (IDDM) is an autoimmune disorder where the pancreatic β-cells are destroyed, leading to a rapid loss of the glucose regulating hormone insulin. It was a lethal illness until Banting identified insulin and started experimental treatments with pancreatic extracts in the early 1920s [120, 121]. Several immune cells have been found to be involved in the β-cell death. In NOD mice, both CD4+ and CD8+ T-cells have an important role in the destruction of the β-cells [122], but cells from the innate immune system has also been found to play a role in the development of the illness [123-125]. T1DM is considered to be under polygenic control, with most of the susceptibility being accounted for by variations in the haplotype for human leukocyte antigen (HLA) alleles [126]. But there is also an environmental factor, highlighted by the concordance rate of 40-60% in monozygotic twins and also by the dramatic increase in T1DM incidence in the last decades [127, 128]. Currently the incidence of T1DM is rising faster than can be accounted for by genetic change. It has been suggested that the common challenges that our immune system used to face historically, such as microbes, worm infections and viruses have a role in down modulating certain autoimmune or allergenic responses [129-131].

Type II Diabetes Mellitus (T2DM) also previously called Non-Insulin Dependent Diabetes Mellitus (NIDDM) is insulin insensitivity, meaning that the β-cells are producing insulin at normal or increased levels but the peripheral tissue insulin receptors are not sensitive to the hormone resulting in increased blood glucose levels. When the β-cells no longer can compensate for the peripheral insensitivity, diabetes occurs [132]. Even before the onset of T2DM there are metabolic abnormalities present, usually called the Pre-diabetic state, such as high blood pressure, abdominal obesity, lipid disorders and insulin resistance [133]. T2DM is becoming more common as the rates of childhood obesity increases. Reports of incidence rates of T2DM range between 17 and 49 cases per 100,000, with most new paediatric diagnoses documented among 15-to 19-year-old minority groups [134]. T2DM now accounts for 45% of the new cases of diabetes in the paediatric population [135]. Although the obesity epidemic is largely to blame for the rapid increase in T2DM, there is also a genetic component. The risk of T2DM is 40% if one parent has the illness, while in the general population it is 7%. Also, the sibling of a person with T2DM has 3 times higher risk of developing T2DM than the general population [136]. Several susceptibility genes have been identified. For example, the common Pro12Ala polymorphism in peroxisome

proliferator-activated receptor-γ (PPAR- γ) where a proline residue increase the relative risk of T2DM 1.25 fold compared to the alanine residue [137]. In the same pro12ala mutation in PPAR- γ, albuminuric persons carrying the proline residue had excess risk of worsening of their microalbuminuria compared to those with the alanine allele [138]. Another example is the KCNJ11 gene, coding for islet ATP-sensitive inward rectifier postassium channel 11, also known as Kir 6.2. A glutamic acid to lysine mutation at codon 23 is associated with an odds ratio of 1.18 for T2DM [139]. The mutation also causes decreased insulin secretion in non-diabetic subjects [140].

Adults with diabetes have a 5-10 years shorter life expectancy than non-diabetics due to vascular complications [141]. This increase in death rate is mostly due to a higher incidence of cardiovascular disease (CVD) in diabetic subjects. Macro-vascular injuries include stroke, ischemic heart disease and peripheral vascular disease. Micro-vascular injuries include retinopathy, neuropathy and nephropathy [118, 141]. The prolonged hyperglycaemia and dyslipidemia associated with diabetes promotes increased oxidative stress, inflammatory molecules and results in vascular damage [142].

1.4.2 Diabetic nephropathy

Diabetes is the most common cause of end stage renal disease (ESRD), and the prevalence of diabetes is rapidly increasing [117]. The vascular complications from diabetes cause chronic kidney disease (CKD), which can in turn develop into ESRD where dialysis or kidney transplant surgery are the only treatment options [143]. Once a patient has had to start dialysis the future is bleak, since about 2/3 of dialysis patients die within 5 years after starting the dialysis treatment [144]. The early detection of CKD is usually through microalbuminuria, sometimes just a few years after diabetes has been diagnosed. After another 5-10 years macroalbuminuria develops in those patients that are on the track to ESRD. During the next 5-10 years the kidney function starts to decline further and the glomerular filtration rate falls [145-147]. Usually the degree of proteinuria correlates well with tubulointerstitial fibrosis and glomerulosclerosis [148]. There is also an increased risk for cardiovascular disease in patients with DN [149]. The diabetic milieu causes activation of a number of metabolic pathways by glycated proteins, hyperglycemia, hemodynamic changes and the generation of reactive oxygen species (ROS) [150-153]. This in turn leads to a cascade of inflammatory cytokines and chemokines causing injury to podocytes, tubular fibrosis, thickening of the GBM and the accumulation of mesangial matrix. One important downstream mediator of renal fibrosis and glomerulosclerosis is transforming growth factor-β (TGF- β), which acts by stimulating the production of collagen and vascular endothelial growth factor (VEGF) [154-156].

To prevent the patient from heading down the path to ESRD, it is important to start treatment early in the pathogenesis. The first concern is treating the diabetes with oral hypoglycaemic agents or insulin injections to obtain normalized blood glucose levels and normalized Hb1Ac, a long time measure of blood glucose levels [157]. Good glycemic control is essential in halting the progression of DN [158]. Other treatments are directed at the renin-angiotensin system (RAS) as a major pathway in the development of DN [159]. RAS blockade has been shown to reduce damage to kidney [160], brain [161] and heart and to reduce cardiovascular mortality [162, 163]. The

hormonal cascade is initiated by circulatory or kidney produced renin catalyzing the conversion of angiotensinogen to angiotensin I. Angiotensin I is in turn converted to angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II can induce proliferation [164, 165], stimulate mesangial matrix accumulation and fibrosis through TGF- β induction [166, 167], generate ROS [168, 169] and affect renal blood flow through aldosterone [170]. RAS blockade can be obtained by ACE inhibitors or with angiotensin receptor blockers (ARBs) [171-173]. Statins (HMG Co-A reductase inhibitors) are used to treat dyslipidemia and work by inhibiting HMG Co-A reductase, the rate limiting enzyme in cholesterol synthesis. Statin therapy was hypothesised to be beneficial in DN because hyperlipidemia has been linked to glomerulosclerosis and tubulointerstitial fibrosis [174-176]. The effect of statin therapy on DN progression is not as clear cut as for the RAS blockade. Statins have only been showed to have a modest beneficial effect in cases of mild to moderate CKD [177], although they did have a good effect on cardiovascular disease [178, 179] and should therefore be considered for all diabetic patients who are at risk for vascular complications [179].

1.4.3 Animal models of diabetic nephropathy

T1DM models:

Several models are used to study diabetic nephropathy in T1DM. One model is the destruction of pancreatic β-cells by *streptozotocin (STZ).* Induction of diabetes occurs after injection of STZ, a chemical composed of two deoxyglucose molecules linked to nitrosourea. The compound is taken up by the β-cells and the nitrosourea reacts within the cells to form a nitrixoxide-based toxin that damages the DNA of the cell and causes necrosis [180]. Induction can be done by two methods; one high dose injection or several low dose injections. The high dose method requires about 150-200 mg/kg of STZ to induce chronic hyperglycemia. With this method substantial tissue toxicity may occur, including the nephrons [181, 182]. The low dose method uses multiple low dose injections of STZ leading to low grade β-cell damage, and lymphocyte infiltration to induce diabetes. Usually daily intra peritoneal injections of 35-50 mg/kg STZ are given for 5-6 days [183, 184]. The advantage of STZ is that it can be used to induce T1DM in transgenic models to study the effect of targeted genes on diabetes. A drawback of the model is that nephrotoxicity that has been reported from STZ treatment in mice [181, 182, 185] as well as patients with islet cell carcinoma [186, 187]. Strict metabolic control has been shown to greatly influence renal function after STZ induction of diabetes [188], and the model is still widely used. I used isolated glomeruli from low dose injected STZ mice in paper IV.

The *insulin-2 Akita (Ins2Akita)* mouse is a mutant mouse model of T1DM [189-191]. The mutation is in the insulin-2 gene, causing misfolding of the protein and gradual β-cell death. It is an autosomal dominant mutation where homozygosity for Ins2Akita leads to failure to thrive and death at 1-2 months of age. Heterozygot mice develop hyperglycemia, hypoinsulinemia, polydipsia, polyuria beginning at about 3-4 weeks of age. The hyperglycemia is worse in male mice than in females. Albuminuria is mild on the C57BL6 background and DN is described as “not robust” [40]. On a different genetic background, the KK background, the nephropathy was much more prominent,

with significant albuminuria, renal hypertrophy and a reduction in GFR at 20 weeks of age compared to the mutation on C57BL6 background [192].

One of the most well studied models of T1DM is the *non-obese diabetic (NOD)* mouse [193], which develop spontaneous autoimmune destruction of the β-cells due to abnormalities in the immune system at around 5 months of age [40]. The NOD mouse has a mutation in the major histocompability complex that causes diabetes to develop in 60-80% of females and about 20% of males [194-196]. Symptoms are hyperglycemia, polyuria, polydipsia, glucosuria and hypercholesterolemia [193]. The NOD mouse is mostly used to study the development of diabetes, and is not commonly used to study secondary effects of diabetes such as diabetic nephropathy due to the late onset of diabetes and the need for daily insulin after onset [40].

FVB-OVE26 is a model of T1DM with early onset. The mutation is an insulin promoter-driven calmodulin gene that results in β-cell toxicity. The model develops significant albuminuria, an increase in GFR at the onset of diabetes and later a decrease as the DN progresses. Histopathology show thickened GBM, enlarged glomeruli and increased mesangial matrix [197]. The model is available from The Jackson Laboratory.

A rat model is the *bio-breeding (BB) rat*. The model develops spontaneous T1DM due to abnormalities in the immune system. In the BB rat, 40% of the pups develop diabetes due to cell mediated autoimmunity [198-200]. Like the NOD mouse it is rarely used to study secondary effects of diabetes.

T2DM models

The *db/db* mouse has a mutation in the leptin receptor, leading to abnormal splicing and defective signalling [201]. The result is hyperphagia, high insulin and leptin levels and severe obesity. The mutation was initially found in the C57BLKS/J strain and has been backcrossed to pure C57BL/6J background as well. In the BLKS background hyperinsulinemia develops at 10 days of age, blood glucose levels are slightly elevated at 4 weeks of age and with frank hyperglycemia at 8 weeks. The hyperglycemia increases gradually until bodyweight and blood glucose levels begin to fall at 5-6 months due to β-cell destruction [202, 203]. After 4 months of age there is a 3-fold increase in mesangial matrix expansion [204]. Albuminuria is reportedly between 68-303 ug/24h in the male db/db and 4-21 ug/24h in controls [205, 206]. (In paper IV I report similar results with mean AER of 250, 208 and 215 ug/24 hours for db/db and 13, 9 and 4 ug/24 for controls at 2, 4 and 6 months.) The susceptibility for diabetes is in the KS strain background, as the db/db mutation on the C57BL/6J background develops only mild hyperglycemia [204]. Although the DN may not be as robust on the C57BL/6J as it is on the C57BLKS/J background, the C57BL6/J background is easier to cleanly intercross with transgenic and knockout strains, since they are commonly available on this background. I used the db/db model to do the expression analysis in paper IV.

The *ob/ob* mouse is a mutation in the leptin gene, the ligand for the leptin receptor [207]. The model is commonly available on the C57BL/6J and renal lesions are reported to be very mild [40, 204].

The *agouti* or *Ay* mouse has a mutation in agouti, a protein produced in the hair follicle. Aside from being a paracrine regulator of pigmentation of the fur [208] it also resembles agouti-related protein (Agrp), a potent antagonist for melanocortin receptors-3 and -4 (Mc3r and Mc4r) expressed in the hypothalamus and involved in weight regulation and energy expenditure [209]. The Ay mouse expresses agouti protein ubiquitously from an alternative transcriptional promoter [210, 211]. The ectopically expressed agouti protein interacts with the Mc3r and Mc4r and leads to obesity, insulin resistance and albuminuria on the KK genetic background [212, 213].

Strain dependence
As implicated several times above, the sensitivity to diabetes and DN is dependent on the genetic background in the mouse strain. The most widely used *C57BL6/J* mice are prone to develop diet-induced obesity, hyperglycemia and hyperinsulinimea, they are relatively resistant to DN with low levels of albuminuria and glomerulosclerosis [49, 214, 215]. *DBA/2J* mice in contrast are prone to develop albuminuria and glomerulosclerosis [49, 216], as are mice on a *KK* background [49, 217]. Non-diabetic mice on the BALB/c background are usually used as controls for KK mice [213]. Further studies are needed to broaden our knowledge of the genetics that make certain strains more susceptible to DN than others.

1.5 MICROARRAY

1.5.1 Transcriptional profiling

With microarray technique it is possible to gauge patterns of gene expression by sampling thousands of genes in a single experiment. Most microarray experiments are designed to investigate the patterns of expression in related samples and to look for genes that are differentially expressed. Comparisons can be done between cells or issues that are treated/untreated, sick/healthy, fed/starving etc to provide information about which genes, pathways, cell types etc are involved in the biological process. The technique is based on the hybridization of a nucleic acid strand to another strand with a complimentary sequence of base pairs (bp). Complementary sequences of DNA, called probes, are attached to the surface on a slide (called a chip). The probes can be cDNA probes or oligonucleotide probes, based on the system used [218, 219]. Gene expression profiling can be used to find "finger prints" unique to a specific biological processes or illnesses, to classify and to predict the outcome in for example acute lymphoblastic leukemia[220].

1.5.2 cDNA and oligonuclucleotide microarrays

In *cDNA microarrays* the probes are made up of amplified cDNA fragments of about 500 bp in length. The cDNA is dissolved in a printing buffer and printed on glass slides as an array of spots. Each spot represents one gene. For cDNA arrays "two colour hybridization" is usually used, meaning that two RNA samples are labeled with

different fluorescent dyes and hybridized on the same slide. The ratio of fluorescence between the different dyes that hybridize to a spot then indicate the relative mRNA abundance between the two samples. The advantage of cDNA microarrays is that they can be manufactured and produced in-house and hybridization costs can be kept relatively low, compared to oligoarrays. The disadvantage is that there is an increased tendency for false positives from cross-hybridization due to the length of the probes, compared to the shorter probes on oligoarrays [221]. There is also a lack of control for sequence homologues. In paper II we constructed a cDNA microarray from isolated mouse glomeruli and used it to analyse gene expression in healthy glomeruli and in glomeruli from mice with a knockout of the gene Foxc2. In our hybridizations we used Cy-3 and Cy-5 flourescent dyes and performed two colour hybridization with one sample of interest hybridized against a reference sample of pooled mRNAs. The reference sample was used as a background to calculate different abundance of expression; similar to what is done for oligomicroarrays, to facilitate comparisons between all relevant samples. The reference design is described in Kerr and Churchill 2001[222].

The most well known commercially produced *oligomicroarrays* are made by Affymetrix, GeneChip®. They are made by light directed chemical synthesis of nucleotide probes on the slide [223]. The Affymetrix oligoarrays consists of pairs of 25-bp probes called probe pairs. Each probe pair consists of one probe that is a perfect match (PM) to the gene, and one probe that has a mismatch nucleotide in the middle of the probe is called a mismatch (MM) probe. The PM probe is designed to hybridize only with transcripts from the intended gene, giving specific hybridization. The MM probe is designed to measure the level of unspecific hybridization. Each gene is represented by 11-20 pairs of probes, called a probe set. Oligomicroarrays use "one colour hybridization" with only one biotin labelled sample being hybridized to each chip [224]. Oligomicroarrays are more expensive than cDNA arrays, but the density (number of genes tested) is usually much higher. For paper IV I used Affymetrix GeneChip® Mouse 430 2.0. The array has 45 000 probe sets to analyze over 39 000 transcripts from more than 34 000 mouse genes.

1.5.3 The experimental process

The key to a successful expression analysis is good experimental design and attention to detail. There are many steps between sample preparation and data analysis, and each step has the potential to introduce errors and bias that would affect data analysis and interpretation of the results. To minimize this and have confidence in the results it is necessary to have the correct number of both technical and biological replicates in the experiment [225]. In general a minimum of 5 biological replicates is recommended, based on inbred animals. Outbred animals will increase the variance and the number of replicates should reflect this [224].

The experimental process typically starts with the isolation of RNA from the biological samples, for example glomeruli isolated from diabetic and non-diabetic control mice. The quality and concentration of the RNA is tested. A method for testing is using the Agilent BioAnalyzer for standardization of RNA quality control, using an algorithm to calculate an RNA integrity number (RIN) [226]. If the quality of the RNA is high

enough, the RNA is amplified to cDNA, labeled and hybridized to probes on the arrays in a manner that allows for gene expression to be measured and compared between samples. This can be done either by using a single dye and individual arrays for each sample, or by using two differently dyed samples to be hybridized against each other on the same array. Data quality is essentially the same using either one colour or a ratio of two colours for hybridization [227].

After hybridization the arrays are scanned and an image file is generated for each sample. The images are analyzed to identify the relative fluorescence intensities in each sample on an array. The hypothesis is that the fluorescence intensity for each arrayed gene is representative of its expression level. In the data preprocessing differences due to non-specific signal is corrected, questionable and low-quality measurements are removed and the measured intensities are adjusted in a processed called normalization to facilitate the comparisons in and between different hybridisations. The standard Affymetrix software MAS5 [228] uses the difference in signal between the PM probe and the MM probe in background adjustments, but in some cases the MM probe detects signal as well as non-specific binding, resulting in negative signal values and false-negatives [229]. To adjust for this an alternative model called RMA was developed that ignored the MM probes in background adjustment. RMA is more sensitive in detecting differential expression, but the model tends to underestimate unspecific binding. To improve accuracy another algorithm was suggested, GCRMA, that adjust background based on probe sequence information [230]. In paper IV we used the GCRMA method. After normalization a number of genes that differentially expressed between samples can be identified. Modified *t*-tests have been developed for microarray analysis to test for statistical significance. A widely used test is Significance Analysis of Microarray, (SAM) [231]. If samples from many conditions are being compared Analysis of Variance, (ANOVA) can be used [232]. The statistics for microarray studies require compensation for the large numbers of genes being tested to avoid false positives. The array means that thousands of tests are performed simultaneously, and to set the threshold for statistical significance at $p \leq 0.05$ in an array with 10 000 genes would mean on average 500 false positive genes being selected as statistically significant by chance. To select the genes that are truly differentially expressed out of these 500, the p-values need to be adjusted to compensate for multiple testing. To compensate for multiple test errors and remove false positives multiple test correction a method called False Discovery Rate (FDR) is commonly used [233].

To be able to discover patterns and groups of genes in the data set, the significantly differentially expressed genes are then classified into categories depending on their function, cellular location or biological process, to mention a few examples. KEGG is a database focused on pathways. It is available at www.genome.jp/kegg/ GOminer is another database that organizes genes into hierarchical categories based on biological process, molecular function or cellular component. The database is available at www.geneontology.org A very good tool for annotation is the Database for Annotation, Visualization and Integrated Discovery (DAVID) [234], available at http://david.abcc.ncifcrf.gov In paper IV we used the David tool to analyze patterns in gene expression.

2 AIMS

- Paper I: To analyze the role of endothelium-derived Pdgfb in a conditional knock-out model
- Paper II: To identify genes involved in glomerulus development and function
- Paper III: To analyze the role of pericytes in the blood-brain barrier
- Paper IV: To analyze glomerular gene expression and markers of inflammation in diabetic mouse models

3 PRESENT INVESTIGATION

3.1 PAPER I: ENDOTHELIUM-SPECIFIC ABLATION OF PDFGB LEADS TO PERICYTE LOSS AND GLOMERULAR, CARDIAC AND PLACENTAL ABNORMALITIES.

Introduction:
Platelet derived growth factor-B (Pdgfb) is instrumental in the development of a healthy, functional cardiovascular system. In this study we set out to study the importance of Pdgfb signalling in the vasculature using a Cre-lox system ablating Pdgfb expression specifically in endothelial cells [61]. Pdgfb and Pdgfrβ null mutants die perinatally due to microvascular problems manifested as edemas and hemorrages. Pdgfb -/- and Pdgfrβ -/- mutant vessels show frequent microaneurysms, abnormal endothelial ultrastructure, variable vessel diameter and signs of increased vascular permeability [59, 112]. Organs that appear to be extra sensitive and show specific defects include the kidney, heart and placenta [59, 60, 235]. In the kidney, mesangial cells express Pdgfrβ and need Pdgfb expressed from the endothelial cells to be properly recruited into the glomerulus. In the null mutants the mesangial cells are missing and the complexity of the glomerular tuft is replaced by a single capillary loop [79].

A conditionally inactive pdgfb gene was generated by incorporation of loxP sites on either side of exon 4. This resulted in a functionally intact pdgfb/flox allele. Mice homozygous for the pdgfb/flox allele were then crossed with pdgfb+/- mice expressing Cre under the endothelial cell promoter Tie1. This crossing generates Cre+Pdgfb-/lox endothelium restricted k.o mice (abbreviation: lox/-) as well as number of controls: Cre-Pdgfb+/flox (flox/+), Cre-Pdgfb-/flox (flox/-) and Cre+Pdgfb+lox (lox/+). Previous analysis by Enge et al. showed that deletion of Pdgf expression was not 100% complete and subject to individual variation with an average of 70% recombination in the endothelial cell population [61]. The mice were born at the expected frequency, viable and fertile and lived into adulthood.

Results and discussion:
It has been reported that the Tie1 promoter drives expression of Cre in the glomerular tuft, and we confirmed expression in glomerular endothelial cells and tubular interstitium using the Rosa 26 reporter (R26R) strain and β-gal staining [79, 236]. By crossing Tie1Cre and R26R reporter mice and staining for β-gal the expression of Cre and the efficiency of the recombinase could be studied. About 70% of the cells were β-gal positive, a ratio that is comparable to the ratio between recombined and uncombined floxed alleles in the brain capillary reported previously [61], suggesting that recombination in the presence of Cre takes place to the same extent in the R26 and *pdgfb* alleles. This also confirmed glomerular endothelial and peritubular expression as well as expression in the placental vessels. In the heart Tie1 expression was seen in the endocardium, cardiac valves and the endothelium. The large vessels such as the aortic arch showed chimeric expression.
A reduction in Pdgfb protein levels was confirmed using Pdgfb-binding aptamers, confirming the fact that recombination at the *pdgfb* site leads to attenuated protein

levels in isolated glomerular fractions. Glomerulus isolation was done using the magnetic bead method developed by Takemoto et al. [237].

Glomeruli in the endothelial specific Pdgfb k.o at embryonic day 18.5 (E18.5) resemble the Pdgfb null k.o and the PDGF-B retention motif k.o, with some glomeruli showing distension of the capillary loops from lack of mesangial cells, but the phenotype is milder and with more individual variation. The variation in the degree of severity coincided with the number of mesangial cells present in the glomerulus, which was confirmed by ASMA staining. ASMA staining showed the complete absence of mesangial cells in some glomeruli and the presence of a reduced cluster of ASMA positive mesangial cells in lox/- mice, leading to less complexity in the glomerular tuft and a dilation of the few remaining capillary loops. The individual variation in the phenotype was also apparent from the analysis of the glomerular capillary diameter. The diameter of the lox/- capillary was significantly larger than in all the control groups. At 3 weeks postnatally the mesangial cell deficiency was largely corrected but there was still a significant glomerular dilation both measured as increased diameter of the individual capillary and as an overall increase in glomerular diameter. By 6 months of age and older, no morphological signs of pathology could be detected. In mutants of PDGFR-β signaling and in the PDGF-B retention motif k.o accumulation of glomerular ECM have been noted [63, 238] but this was not seen in the endothelial cell specific k.o neither at 6 months nor at >12 months old when sections were stained by periodic acid-Schiff's (PAS) reagent.

To investigate albuminuria, urine was collected over 24 hours in metabolic cages and analysed on a mouse-specific ELISA plate (Albuwell-M). In lox/- mice there was a small but statistically significant increase in urinary albumin compared to controls at >12 months but not at 6 months. This would suggest that despite the postnatal normalization of glomerular histology, some disturbances in the glomerular filtration barrier occur at an older age. Albuminuria, to our knowledge, is not mediated by mesangial cells primarily. Podocytes, endothelial cells or the composition of the GBM is probably influenced by the mesangial cells to cause the albuminuria. The level of the albuminuria, although statistically significant, is very low and probably not of pathological significance.

The endothelial cell specific k.o also show cardiac abnormalities similar to what has been reported in the complete Pdgfb k.o, including dilation, myocardial hypnotherapy with thinning of the myocardial wall, myocardial hypertrabeculation and septal abnormalities [58, 59] at E 18.5. Like the glomerular phenotype, the hearts of postnatal ages 1 month, 6 months and >12 months appeared histologically normal and the myocardium had reached normal thickness. Also the placental abnormalities typical of Pdgfb -/- and Pdgfrβ -/- embryos were reproduced in the lox/- embryos with a dilated embryonic blood vessels and reduced numbers of pericytes and trophoblasts in the labyrinthine layer [235]. As was the case with the glomerular situation, the cardiac and placental malformations showed interindividual variation in the lox/- mice, in contrast to the Pdgfb and Pdgfrβ null mutants. The reason for this is probably in the chimeric expression of Tie1 Cre in the tissue resulting in variations in the deletion of Pdgfb.

The recruitment of pericytes to microvessels in the brain is also dependent on functional Pdgfb. To quantify brain pericyte content in the lox/- mouse, the Tie1 Cre/Pdgf-b/flox alleles were crossed into the XlacZ transgenic background, in which LacZ is expressed in vascular smooth muscle cells and pericytes from late gestation and onwards. Quantification was done on midbrains after whole-mount β-gal staining in pdgf-b -/-. Lox/- and control animals. In the pdgf-b -/- the number of brain pericytes was reduced by more than 95% compared to controls, where as in the lox/- showed an intermediate phenotype with reduction of about 70-90%, with individual variation. Even in cases where the overall reduction was 90%, individual capillaries with normal pericyte coverage were found next to neighboring naked capillaries. This again reflects the chimeric situations where most capillaries are composed of Pgfb-negative endothelial cells where as some capillaries are composed of mostly unrecombined, Pdgfb-expressing endothelial cells capable of recruiting pericytes.

Conclusions and future perspectives:

The critical importance of Pdgfb in the recruitment of pericytes and vascular smooth muscle cells to blood vessels and in recruitment of mesangial cells to the glomerulus has been demonstrated previously. This study identifies endothelial cells to be the essential source of Pdgfb in these cases, as well as a role in proper heart and placental development. The defects in heart and placenta could be secondary to the microvascular injuries resulting from the lack of Pdgfb during embryonic development, or be due to some direct effect of Pdgfb on for example placental trophoblasts or cardiomyocytes.

The reasons behind the apparent glomerular recovery are unclear. One possibility is that Pdgfb is not the only factor regulating the mesangial content of glomeruli, and the lack of endothelial Pdgfb causes a delay rather than an irreversible block in mesangial cell recruitment. Another possibility is that non-endothelial sources of PDGF-B such as hematopoietic cells could substitute for lack of endothelial protein. In addition to the endothelium specific Pdgfb k.o, Enge et al. also generated a macrophage specific Pdgfb k.o and saw no vascular effects (unpublished data). This leads us to believe that the contribution of macrophages in providing a source of Pdgfb for recruitment of mesangial cells and pericytes to is probably limited. Two other hematopoietic cell types capable of produce high levels of Pdgfb, monocytes and platelets, probably play a role in recruitment of vascular smooth muscle cells in atherosclerotic lesions and may be important in maintaining immune cell homeostasis in the vascular system [65, 66]. Their impact on mesangial cells and pericyte recruitment to endothelial cells seems limited, however. Another possible option is the compensation by more recently discovered members of the Pdgf family, such as Pdgfb or Pdgfc that could substitute for Pdgfb. However, injections with an inhibitory antibody directed at Pdgfd in the Pdgfb retention motif k.o at postnatal day 1 showed no effect on glomerular size, cellularity, mesangial cell proliferation or ECM content at termination on postnatal day 7, indicating that the possible compensatory role of Pdgfd is limited, at least in the early postnatal development (unpublished data). Yet another option would be that the chimeric expression of Cre from the Tie1 promoter leaves a number of unrecombined cells still capable of production of Pdgfb, and that these cells “rescue” the glomerular phenotype.

If the endothelium is the critical source of Pdgfb, how come the lox/- mice are viable in contrast to the Pdgfb null mice? Again, this probably has to do with the chimeric expression of Tie1 Cre. Quantification of recombination frequency in microvascular endothelium fragments in the lox/- mice showed individual variation but a recombination of between 70-90% in most cases [61]. This correlates with the reduction in the number of brain pericytes seen in whole-mount β-gal staining in midbrain in this study, and is a marked contrast to reductions of >95% in the Pdgfb null mice. It appears from these observations that mice can tolerate up to 90% loss of pericytes without severe organ dysfunction, but when the loss is more extensive the mice die perinatally. A small number of lox/- mice actually do die perinatally, and these may represent the individuals where recombination takes place at a high enough efficiency to lead to loss of more than 90% of pericytes.

In an attempt to overcome the variation caused by the chimeric expression of Tie1 Cre we instead crossed the mice to another endothelial cell specific Cre line, the Tie2 Cre. By doing this we hoped to find out if the Tie2 promoter would give less chimeric expression of Cre protein and thus causing a more severe phenotype. Unfortunately the expression of Tie2 Cre was chimeric as well and we did not observe any significant differences in phenotype between the two Cre models in an initial study (unpublished data). If other Cre models develop that show high endothelium specificity and high rate of recombination, it would be interesting to do further experiments to see if the phenotype in the endothelium specific k.o would mimic that of the null mutant, or if it would still be less severe due to expression from non-endothelial sources.

3.2 PAPER II: LARGE SCALE IDENTIFICATION OF GENES IMPLICATED IN KIDNEY GLOMERULUS DEVELOPMENT AND FUNCTION.

Introduction:
Knowledge of the glomerular transcriptome is limited and the identification of glomerulus specific and glomerulus enriched transcripts would provide a better understanding of the glomerular development and function as well as of the mechanisms involved in glomerular diseases. In this study we constructed a cDNA library from isolated mouse glomeruli, classified the contents and constructed the Glomchip, an in-house printed glomerulus-specific cDNA microarray chip. The microarray chip was used to identify a number of novel glomerular transcripts. In a series of experiments we performed comparative analysis on these transcripts and they were assigned to specific cell types and characterized as podocyte or mesangial cell/juxtaglomerular markers. Further study on podocyte marker Foxc2 revealed a role in podocyte differentiation and glomerular development.

Results and discussion:
RNA from isolated glomeruli from adult and newborn mice was used to produce a cDNA library. After sequencing, filtering out vector parts and short transcripts of less than 100 bps we blasted the sequences against the ENSEMBL mouse gene data base. We received 12 309 high quality hits representing 6053 different genes and 941 EST sequences. The libraries were PCR amplified, purified and printed, resulting the Glomchip. This would provide a very valuable tool in analysing glomerular gene expression.

Our first experiment was to compare isolated glomeruli from 5-day old mice against non-glomerular kidney tissue. This experiment resulted in 357 genes and 63 ESTs that were significantly upregulated more than 2-fold in the glomerular tissue. 48 of these genes are known glomerular genes like podocyte markers nephrin (Nphs1), podocin (Nphs2), podocalyxin (Podxl), synaptopodin (Synpo), protein-tyrosine phosphatase receptor o (Ptpro, GLEPP1) and Wilm´s tumor protein (Wt1) as well as several genes known to be vascular endothelial markers.

To subtract vascular markers we profiled isolated brain capillary and subtracted genes not significantly 2-fold upregulated in glomeruli. This left us with 143 genes and 34 ESTs, containing most known podocyte markers as well as candidate glomerular markers. Most known endothelial cell markers were either enriched in the brain capillary tissue or not differentially expressed between glomeruli and brain capillary.

To further assign the glomerulus enriched transcripts to cell type we FACs sorted GFP positive podocytes from 8-day old mice. In these mice GFP expression was activated under the Z/EG transgene by Cre recombinase expressed under the control of the podocin promoter [239]. Transcripts 2-fold or more upregulated in the GFP positive podocytes revealed 49 genes and 10 ESTs. In the non-podocyte (GFP negative) cells 18 genes were upregulated. The remaining 76 genes and 24 ESTs were not significantly

differentially expressed more than 2-fold between podocytes and non-podocyte glomerular cells.

A number of podocyte and non-podocyte specific glomerular transcripts were validated by In Situ hybridization (ISH). The podocyte specific genes picked for validation was semaphorin sem2 (Sem2), rhophilin1 (Rhpn1), cbp/p300-interacting transactivator 2 (Cited2), protease inhibitor 15 (Pi15) and gene X in comparison with three known podocyte markers (Nphs2, Podxl and Foxc2) [240-242]. The selected novel mesangial cell markers selected were secreted frizzled-related protein 2 (Sfrp2), aldo-keto reductase family 1 member B7 (Akr1b7 and lim domain only protein 7 (Lmo7) in comparison with known mesangial, juxtaglomerular and endothelial transcripts insulin-like growth factor binding protein 5 (Igfbp5), renin (Ren1) and endomucin (Emcn) respectively [243-245]. From the In Situ hybridization we could study temporal expression of our markers, since the kidneys of E18.5 mice contain glomeruli in several different developmental stages (comma shaped, s-shaped, capillary loop stage as well as mature). Foxc2 was the only podocyte marker expressed as early as during the comma shaped phase, where it is still too early to make morphological distinctions between prospective podocytes and tubular epithelial cells. Sem2, Pi15, Rhpn1, Gene X as well as known markers Nphs2 and Podxl appeared in developing podocytes in the s-shaped stage. In the capillary loop stage and the mature glomeruli all markers were expressed. Both novel and previously characterized mesangial markers were expressed first during the capillary loop and mature stages with the exception of Sfrp2, which was expressed in epithelium at the comma and s-shaped stages and later switched to being expressed in mesangial cells in the capillary loop and mature stages. Expression of these markers was also seen in the smooth muscle cells at the juxtaglomerular arterioles.

To examine the functional relevance of podocyte marker Foxc2 during nephron development we obtained Foxc2 -/- kidneys. At E18.5 the kidneys were smaller and had reduced number of glomeruli with fewer than normal capillary loops which were dilated and blood filled. In fact, the mutant glomeruli resembles the mesangial cell deficient glomeruli seen in Pdgf-B and receptor Beta deficient mice [59, 79]. To evaluate the mesangial cell content we performed immunohistochemistry (IHC) staining for mesangial markers NG2 (Cspg4), Pdgfrb and desmin in comparison with endothelial marker pecam and podocyte marker Nphs1. The IHC revealed that mesangial cells were present and expressing the markers at normal levels, but instead of forming a branched mesangial core connecting to the capillary loop nest, the mesangial cells remained in a compact cluster at the centre of the glomerulus. Transmission electron microscopy (TEM) confirmed the abnormal distribution of mesangial cells and revealed that the mesangial cells failed to make normal focal contact with the GBM. Podocyte foot processes and SD were not properly formed but podocytes were instead connected by adherence junction-like structures normally only seen in immature podocytes at the s- and cup-shaped stages. Endothelial cells were thick and lacking the fenestrations which normally develops at the capillary loop stage. The TEM findings suggest that the differentiation of podocytes and endothelial cells is arrested before the capillary loop stage in the Foxc2 -/-. Since Foxc2 is expressed early in glomerular development it might play a role in podocyte differentiation. To further investigate the transcriptional changes we hybridized Foxc2-/- glomeruli to Glomchip. This revealed

upregulation of 501 transcripts and downregulation of 232 transcripts by 2-fold or more. Some podocyte markers (Nphs2, Mabf, Cdkn1c and novel marker Rhpn1) were downregulated while others (Nphs1, Wt1, Cd2ap and novel markers Sem2, Pi15 and Gene X) remained unchanged. The selective downregulation of some podocyte transcripts argues against a general block in podocyte differentiation, considering fact that our ISH data showed that most podocyte transcripts were turned on simultaneously. Podocytes play an important role in creating and maintaining the GBM, so we wanted to also look at the deposition of matrix proteins and integrins in the Foxc2-/- mice using IHC. Expression levels of laminins, protoglycans as well as the podocyte expressed Integrin α3 chain were all normal. The collagen IV chains deposited during early glomerular development, Col4a1 and Col4a2 also showed normal deposition but there was a significant reduction in deposition of the collagens present in the GBM of a mature glomerulus, Col4a3, Col4a4 and Col4a5. This would suggest that the GBM fails to mature properly in the Foxc2-/- glomeruli.

Conclusions and future perspectives:

The construction of a glomerulus-specific cDNA chip enabled us to detect rare transcripts from highly specialized cells like podocytes, fenestrated endothelial cells and mesangial cells, transcripts that would otherwise drown in the noise of transcription from non-glomerular kidney cells. Since albuminuria as a result of injury to the glomerular filtration barrier leads to CKD, it is important to be able to analyze glomerular gene expression. The advantage of using isolated glomeruli instead of whole kidney allows us to monitor transcription in glomerular cell types making up only a fraction of the kidney mass. By this approach we characterized a number of new podocyte and mesangial/juxtaglomerular markers that could be potentially relevant in glomerulopathy. The disadvantages to our approach include the fact that the libraries were not sequenced to saturation, and thus there are glomerular transcripts missing in our libraries and on our cDNA chip. Also, transcripts that are not expressed by glomerular cells in the normal, healthy situation, but that are initiated when the filtration barrier is injured are not present on Glomchip. The same goes for transcripts from migrating cells from for example the immune system, which are not picked up by our chip for the same reason.

In an attempt to investigate the functional relevance of genes picked up by Glomchip we analyzed transcription factor Foxc2-/- glomeruli using the chip and IHC. Foxc2-/- mice die embryonically and perinatally due to anomalies in the aortic arch and collapsed lungs. [246] The glomeruli from E18.5 Foxc2-/- mice displayed a failure to form multi lobulated glomerular tufts, lack of podocyte foot processes and SD, thick and unfenestrated endothelial cells and mesangial cells who failed to make proper contacts with the GBM and aid in splitting of the glomerular tuft vasculature. Since Foxc2 expression within the glomerulus is restricted to podocytes and we didn't see differential expression of mesangial cell transcripts, we believe that the mesangial and endothelial cell defects are likely to be secondary to the podocyte defect and highlights the importance of signalling and communication between the glomerular cell types. The fact that expression of Col4a3, Col4a4 and Col4a5 are reduced in the Foxc2 is interesting, since mutations in these genes are responsible for Alport's syndrome. It does not explain the podocyte differentiation defects in the Foxc2-/- glomeruli however, since experimental inactivation of Col4a3 and Col4a4 as well as Alport's

syndrome results in delayed onset renal disease and glomeruli are morphologically normal during development [247-249]. The fact that Foxc2 is expressed already at the s-shaped stage of glomerular development, earlier than most podocyte markers, suggest that it may play a role in podocyte differentiation. The selective differential expression of podocyte transcripts in Foxc2-/- indicates that the mutation causes complex transcriptional changes and it is not possible to explain the podocyte differentiation defect by differential expression of a single known podocyte protein. Instead the defect is most likely caused by changes in a combination of genes that are directly and/or indirectly orchestrated by Foxc2. The complexity of the change in the transcriptional symphony is illustrated by the fact that more than 700 different genes in the glomeruli are affected by the mutation. To put this in context, transcriptional profiling of isolated glomeruli from Pod1-/-, using the Affymetrix Mouse Genome 430 2.0 chip, showed differential expression of 18 genes and thus have a very small effect on gene regulation during glomerular development [250]. It would be interesting to compare transcriptional profiles of other podocyte transcription factor mutants to look for overlap in podocyte target genes.

3.3 PAPER III: PERICYTES REGULATE THE BLOOD-BRAIN BARRIER

Introduction

In this study we constructed a mouse model where a conditionally silent human Pdgfb (hPdgfb) gene was inserted into the ubiquitously expressed Rosa 26 locus [251]. The vector construct was a Splice Acceptor (SA) followed by loxP flanked intervening cassette containing the selection marker PGK Neo and four Poly-A sequences to terminate transcription. After the floxed selection/termination cassette is a cDNA copy of the human Pdgfb gene. When a mouse bearing the vector in the R26 locus is crossed with a mouse expressing Cre protein the Cre causes excision of the floxed termination cassette, leading to expression of hPdgfb in this specific cell type in the offspring. This mouse can be used to study the over expression of Pdgfb in selected tissues, or it can be used to create a hypomorphic Pdgfb model by rescuing the Pdgfb null mutant. Since the Pdgfb null mutant is perinatally lethal, a hypomorphic model is very useful in studying the effect of Pdgfb on pericytes in postnatal processes. A Pdgfb null mutant expressing one copy of the R26 hPdgfb gene turned on by endothelium specific Cre is here referred to as a hemizygous R26P+/0, while a null mutant expressing two copies of the hPdgfb is called a homozygous R26P+/+. Another example of a hypomorphic Pdgfb model is the Pdgfb ret/ret, where the cellular retention motif binding the Pdgfb protein to the ECM is deleted. This leads to changes in the extracellular distribution of Pdgfb protein and, as a result, to hypoplasia and partial detachment of pericytes from the vessel wall. We used both of these pericyte-deficient models to study the role of pericytes in maintaining the blood-brain barrier integrity *in vivo.*

Results and discussion:

Quantification of pericyte coverage in different regions of the brain using CD13 or Pdgfrβ staining revealed lower pericyte density of 26% in the *pdgf-b ret/ret* and of 40% in the R26P+/0. The corresponding figure for the R26P+/+ was 72%. Absolute quantification with the XlacZ4 reporter strain (a transgenic marker for migrating limb muscle precursor cells and vascular smooth muscle cells, see [252]) confirmed the reduction of pericyte numbers in the Pdgfb ret/ret and the R26P +/-. There was also a reduced vessel density and dilation of the existing vessels in the pericyte deficient mice. The apical-basal polarity remained normal, as shown by staining for the luminal endothelial marker podycalyxin and the abluminal marker collagen IV.

The brains of Pdgfb ret/ret and R26P +/- mice appear enlarged upon gross anatomical inspection and this was confirmed by measuring the wet/dry weight ratios of brains. This led us to suspect edema and impaired integrity of the blood-brain barrier (BBB). The BBB integrity was tested using a variety of injectable tracers, the first being the azo dye Evan's Blue (EB), which binds to plasma proteins and is often used to trace the extravasation of albumin. EB was retained in the brains of the mutants to a degree corresponding to pericyte loss: the mutants with a more severe loss of pericytes retained more dye in the brain than animals with a more normal pericyte coverage. The *ret/ret* mice retained more EB than the R26P +/0 followed by the R26P +/+. To study possible size-selectivity in the leakage across the BBB a series of tracers with different molecular weight was used: 950kDa cadaverine Alexa Fluor-555, fluorescently labelled

66 kDa albumin, 70 kDa dextran and 200 kDa IgG. All of the markers crossed the BBB in the pericyte-deficient animals. A small amount of leakage was seen in the brain parenchyma of the R26P +/+ animals, but no leakage could be detected in the controls. These results show that there is a close correlation between pericyte coverage and the leakage of a range of tracers of different molecular weight across the BBB.
To study mechanism behind the extravasation we analyzed the inter-endothelial junctional complexes in the pericyte-deficient mutants. By using markers for endothelial adherence junctions (VE-cadherin) and tight junctions (ZO-1 and Claudin-5) we could see that the distribution and protein levels for these markers were quite similar in mutants and controls, with only subtle abnormalities. This was confirmed with TEM, which revealed no apparent abnormalities in the ultrastructure of the endothelial junctions in the mutants except that longer, irregular stretches of endothelial overlap was commonly found in the pericyte-deficient mutants.

The endothelial accumulations of all the high molecular weight tracers were speckled, causing us to suspect that they were retained in intracellular vesicles in the endothelial cells. To further study this, we injected another high molecular marker, horseradish peroxidase (HRP) of 44 kDa and studied the distribution in TEM in *ret/ret* mice. We found increased localization of HRP to macro-vesicles in the mutants, while the uptake to micro-vesicles was similar in mutants and control animals. Our observations seem to indicate that high molecular weight molecules cross the BBB in pericyte-deficient mutants via macro-vesicular trancytosis.

During stroke the BBB commonly opens and causes edema in the CNS, Using an experimental mouse model of stroke, this edema could be counteracted by administration of the tyrosine kinase inhibitor imatinib [253]. When we treated with imatinib, we could see almost completely reversed leakage of all tested tracers in our pericyte-deficient mutants in a dose-dependent manner. Interestingly, imatinib treatment seemed to result in retained uptake and enhanced punctuates distribution of tracer, implying that imatinib inhibits the release of tracer from endothelial cells. This suggests that imatinib works by inhibiting a mechanism involved in the exocytosis step of trancytosis.

To analyze if pericytes regulate the endothelial expression of BBB markers, we used Affymetrix gene expression arrays to analyze transcription profiles of microvascular fragments from controls and pericyte-deficient mutants. The results showed that most BBB endothelial markers were unaffected by pericyte-deficiency, like most other endothelial specific mRNA transcripts. Thus pericytes do not seem to regulate BBB endothelial expression at a general level, but some markers showed altered expression levels. One example is transferrin receptor (CD 71), which was slightly downregulated in pericyte-deficient vessels, and this was confirmed on the protein level by staining brain tissue with an anti-CD 71 antibody. The unaltered expression of Glut 1 was confirmed using the same method. These results show that pericyte-deficiency can, at least in some select cases, influence the expression profile of BBB endothelial cells.

Astrocytes are in constant contact with BBB blood vessels through cap-like cytoplastic processes called end-feet. Since pericytes are partially sandwiched between endothelial cells and the astrocyte end-feet, and since several astrocyte markers were

downregulated in pericyte-deficient mutants in our Affymetrix study, we wanted to investigate whether the lack of pericytes also lead to changes in the astrocyte end-feet distribution or polarization. We used three different astrocyte markers which all localize to the part of the astrocyte end-feet that contacts the endothelium: Aqp-4, α-syntropin and laminin α2 (lama2). The vessel staining for all three markers was weaker in pericyte-deficient mutants than in controls. Interestingly, expression of Aqp-4 and α-syntropin was associated with areas where pericytes had detached from the vessels or were extending between two different vessel branches. The staining pattern of staining for Aqp-4, α-syntrophin and lama2 is affected, indicating that the polarization of the astrocyte end-feet also is affected by the lack of pericytes. It would therefore seem like pericytes could be expressing cues that mediate the attachment of astrocyte end-feet to brain microvessels as well as aid in polarization.

Conclusions and future perspectives:

We successfully constructed a transgenic mouse with inducible expression of human Pdgfb from the Rosa-26 promoter. In this study the transgenic was used to create a hypomorphic Pdgfb model by crossing it with the Pdgfb null mutant. The Rosa 26 transgenic can also be used to study the overexpression of Pdgfb by crossing it with a mouse expressing Cre protein. When crossed with the Tie2-Cre mouse, no significant differences in pericyte number were observed in adults. To see if there was an initial peak in pericyte number during development we looked in retinas at p7. There was a 13% increase in LacZ positive pericytes around veins but no significant difference around arteries or in the capillary network between artery and vein. It seems that it is not possible to significantly increase pericyte numbers by just overexpressing Pdgfb at moderately high levels. (Armulik et al. unpublished data)

Pericytes have previously not been thought to play a role in the formation of the BBB, but here we show for the first time that lack of pericytes causes leakage across the barrier as well as changes in expression of endothelial and astrocyte specific proteins in the BBB.

We demonstrated that the integrity of the BBB is impaired in pericyte deficient mice, as injected tracers extravasate into the brain parenchyma. The extent of leakage correlates with the degree of pericyte deficiency. The route of extravasation appears to be through macromolecular trancytosis since markers for tight junctions and adherence junctions were largely normal. The speckled appearance of the high molecular weight tracers in endothelial cells suggested macromolecular trancytosis and this was confirmed by the distribution of HRP using electron microscopy. The tyrosine kinase inhibitor imatinib inhibits the exocytosis step of the trancytosis in a dose-dependent manner, and thus stop the leakage across the BBB.

In our microarray study certain endothelial markers of the BBB was affected by the lack of pericytes, as well as some astrocyte markers affecting attachment of astrocyte end-feet to the vasculature.

Despite the increase in endothelial trancytosis that causes brain edema and leakage of plasma proteins into the brain, these mice have a normal life span. This makes them an interesting model to further study the functional effects of an impaired BBB, both on

cognitive behaviour and learning and in studies on progression of brain diseases. The fact that immunoglobulins are able to cross the BBB in pericyte-deficient models is pharmacologically interesting. Further studies of the role of pericytes in maintaining the BBB should be of great interest.

3.4 PAPER IV: GLOMERULAR TRANSCRIPTOME ANALYSIS AND THE PRESENCE OF ALTERNATIVELY ACTIVATED MACROPHAGES IN DIABETIC NEPHROPATHY

Introduction:
The prevalence of diabetes is rapidly growing from 2.8% to 4.4% of the population worldwide [117]. The growing numbers have resulted in diabetic nephropathy (DN) being the number one cause of kidney disease in the world. In an attempt to broaden our understanding of the mechanisms behind DN, we used a model for T2DM, the db/db mouse, to perform expression analysis of isolated glomeruli from four different time points during the development and progression of albuminuria and glomerulosclerosis. The time points chosen were at 1 month, 2 months, 4 months and 6 months of age. We used the DAVID tool to find KEGG pathways to gain insight into the transcriptional changes occurring during the development of diabetic nephropathy with albuminuria and glomerulosclerosis. We found a dramatic differential expression of genes involved in the in pathways relating to activation of the immune system, cellular adhesion and transendothelial migration of leukocytes. We also noted differential expression of pathways involved in the composition of the GBM and the extracellular matrix, such as collagens, laminins and fibronectin. At the top of the list of most upregulated genes was Chitinase 3-like 3 (Chi3l3), also called Ym-1, a marker for alternatively activated macrophages [254, 255].

Results and discussion:
Blood glucose levels were tested in age groups of 1 month, 2 months, 4 months and 6 months (1m, 2m, 4m and 6m) and a statistically significant increase was found in the db/db from 2m. Statistically significant albuminuria in the db/db group was found at 2m, 4m and 6m of age using 24h urine collection cages and ELISA analysis for mouse albumin. The levels correlate well with previous findings in this model [205, 206]. RNA was isolated from glomeruli of db/db and control litter mates at all four time points and analyzed on Affymetrix GeneChip 430 2.0 arrays.

Pathway analysis was performed to identify significantly different patterns of expression and several pathways were found to be significantly altered at each stage in the disease progression. For this analysis we used the DAVID tool to perform KEGG analysis [234].

Fourteen genes were significantly already before the onset of albuminuria and remained so at all time points in our study, starting already at 1m. Among these were the upregulated transcripts for Ym-1, ankyrin repeat domain 1 (Ankrd1), gastrin releasing peptide (Grp) and the downregulated calcitonin/calcitonin-related polypeptide alpha (Calca) transcript. The highest fold changes of all were found for Ym-1, where expression was upregulated 16-, 20-, 38- and 56-fold at 1-, 2-, 4- and 6-months of age. Ym-1 has been co-localized with Mac-1 and scavenger receptor, indicating macrophages as the main producers [256]. Ankrd1 is described in cardiac- and skeletal muscle cells where it acts as a messenger in mediating stress response [257, 258].

Ankrd1 is also a regulator of gene expression in the nucleus, interacting with tumor suppressor gene p53. Ankrd1 acts as a co-activator of p53 and can upregulate the p53 activity [259]. Ankrd1 has also been hypothesized as being involved in podocyte injury, as Ankrd1 protein expression in podocytes correlated positively with severe proteinuria in several different glomerulopathologies including DN [260]. In our study Ankrd1 was expressed at the mRNA level before the onset of albuminuria, suggesting that expression may be initiated even before the proteinuria.
Grp is implicated as a morphogen in the progression of colorectal cancer [261]. The protein has been found in synovial fluid and serum of rheumatoid arthritis (RA) patients and the protein levels in synovial fluid and serum correlates with cytokine levels [262, 263]. Another neuropeptide, Calca, was significantly downregulated in all age groups. Calca protein has also been reported in higher levels in synovial fluid in RA patients than in control subjects [264, 265] but there was no correlation to plasma levels. Local expression of neuropeptides at sites of inflammation may play a role in regulating the recruitment of leukocytes into the tissue [266]. The differential expression of these neuropeptides in the db/db glomerulus suggests that neuropeptides may be involved in the regulation of leukocyte recruitment into the glomerular tissue before the onset of albuminuria and sclerosis.

At 2-4 months the KEGG analysis shows changes in pathway involved in cell adhesion molecules and transendothelial migration. There is an upregulation of CD99, a protein present on blood monocytes and required for transendothelial migration [267], changes in expression of cellular adhesion molecules Claudin1, -15 and -16 and upregulation of filamin B, a molecular linker in the Icam1-driven transmigration of leukocytes [268], as well as Icam1 itself. There is also an increased expression of P-selectin, E-selectin and L-selectin and of Mac-1, the ligand for Icam1. CD45, a modulator of antigen signalling [269] is upregulated, as is Itga9 and Itb2, which together forms an integrin receptor expressed on leukocytes, implicated in cell migration and invasion [270]. Two potential ligands for the Itga9/Itb2 receptors, tenascin and fibronectin (Fn1) [271, 272], are upregulated at this time point as well. Together this suggests an increased trans-migration of leukocytes into the glomerulus.

Both vascular endothelial growth factor c (Vegfc) and Vegfd are upregulated and could be involved in focal adhesion and woundhealing [273]. Expression of Flt1, the receptor for another Vegf isoform, is increased as well. Flt1 is expressed on macrophages and acts as a positive regulator of inflammation by promoting macrophage migration towards its ligand, Vegfa [274]. Increased levels of Flt1 in the urine of diabetic patients correlate positively with increased albuminuria [275], suggesting a possible role for Flt1 in the recruitment of macrophages into the glomerular lesions.

There was an increase in cytokine-cytokine receptor expression and compliment activation at 2-4 months as well, pointing towards active inflammation and the recruitment of leukocytes and antigen presenting cells (APCs) into the glomeruli. Both cytokine receptor Ccr1 and Ccr2 are upregulated. Ccr1 is the receptor for macrophage inflammatory protein-1 alpha (MIP-1alpha), RANTES and myeloid progenitor inhibitory factor-1 (MPIF-1) [276-278] on monocytes and APCs. Blockade of Ccr1 reduces the infiltration of leukocytes and reduces inflammation and fibrosis [279, 280]. Ccr2 is the receptor for Ccl, formerly known as monocyte chemotactic protein-1 (MCP-

1) [281]. APC such as monocytes and immature tissue macrophages express Ccr2 and blockade of the receptor has been effective in preventing macrophage recruitment in diabetic renal injury [282-284]. The upregulation of Ccr1 and Ccr2 again suggests macrophage infiltration. Csf and IL-4 in combination induces differentiation of monocytes into tissue macrophages [285]. Monocytes differienting into dendritic tissue macrophages are characterized by the expression of CD16, also known as Fc gamma receptor III (Fcgr3) [286, 287], a low affinity receptor for IgG. The IL-4 receptor alpha (IL4Ra), colony stimulating factor 1 receptor (Csf1r) and Fcgr3 are all upregulated at 4m and 6m in our study, probably indicating increased differentiation of invading monocytes to tissue macrophages. IL-4Ra is the receptor for Il-4 and IL-13 [288-290], a cytokines known to induce expression of markers for alternative activation in monocytes and tissue macrophages. Two such markers are Ym-1 [291, 292] and coagulation factor XIIIA1 [293], both of which are strongly upregulated in our study.

At 2 and 4 months the cell surface protein CD44 is upregulated along with P, E and L selectin. CD44 together with P and E selectin is involved in the intitiation of leukocyte trans-endothelial migration in inflamed vessels [294]. CD74 was also upregulated at this time. CD44 and CD74 form the CD44/CD74 receptor complex [295] on leukocytes. It binds macrophage inhibiton factor (MIF), an inflammatory cytokine, and signals to activate the downstream ERK 1/2 MAP kinase, leading to increased influx of macrophages and T-cells into the kidney [296]. The signal also causes downstream modulations in arachidonic acid metabolism and prostaglandin synthesis [296], and consistent with this we noted upregulation of leukotriene C4 synthase and prostaglandin I2 synthase in our data set. Increased expression of macrophage receptor complex CD44/CD74 and increased ERK 1/2 MAP kinase signalling in suggests increased macrophage density in the tissue.
We also observed upregulation of plasminogen activator inhibitor 1 (PAI-1), previously linked to many complications in diabetes including glomerulosclerosis [297, 298], probably due to decreased degradation of plasmin in the ECM [299] and dysregulation of Fn1 expression [297].

Also at 2-4 months, we noted changes relating to the composition of the extracellular matrix (ECM) and GBM including the upregulation of Fn1 and collagen5 alpha 1 (Col5a1) and laminin alpha 5 (Lama5). Lama5 is expressed by glomerular podocytes and is a necessary component f a functional glomerular filtration barrier [300]. Col1a2 and Lamc2 were downregulated. Col1a2 deficiency in the mouse model for osteogenesis imperfecta leads to accumulation of collagen fibrils in the mesangium and albuminuria [301]. Lamc2 is known t be a component in the GBM, but its role in the mature GBM is not known [302]. The changes we observed in the GBM and ECM may lead to the accumulation of mesangial matrix as well as disturb glomerular function.

At 6 months, late in the progression of diabetes several components of the complement system were upregulated, including C1qa, C1qb and C1qc components, their receptor C1r and C3. Activated C1q can bind to pathogen-associated molecular patterns (PAMPs) as part of the innate immune system or the complement system can be recruited by the adaptive immune system. All three C1q subcomponents were highly upregulated, as was C1r, indicating that the complement system was active and bridging the innate immunity and acquired immunity. The upregulated transcript for

lymphocyte cytosolic protein 2 (Lcp2) is a partner of another upregulated transcript, Vav1. Together they are part of a "signalosome" that signals over the T-cell receptor and its costimulatory receptor CD28 to induce proliferation, IL-2/IL-4 production and Ca(2+) flux [303, 304]. Also upregulated Tyrobp (also called DAP12) is a trans-membrane receptor expressed on several lymphoid and myeloid cells including macrophages, B-cells and T-cells under inflammatory conditions [305]. There was upregulation of leukocyte immunoglobulin-like receptor B3 (Lilrb3). It is expressed on cells of monocytic lineage, T-cells, B-cells and NKT-cells and is a negative regulator of inflammation [306]. CD79b, a protein necessary for expression and function of the B-cell antigen receptor [307] was downregulated at the same time, possibly suggesting a reduction in B-cell receptor activity in the diabetic kidney. Another indication of this is the fact that the B-cell marker CD20 was downregulated as well. When analysed together, the upregulation of transcripts involved in signalling in lymphocytes seem to indicate that at this late point in diabetes (6 months) the cells involved in innate immunity has likely activated both T- and NKT-cells of the acquired immunity, possibly through complement, and there is an influx of T-lymphocytes in addition to the myeloid cells. There is also a possible reduction in B-cell activity and differentiation.

The most strongly and consistently upregulated gene in our time course study was Ym-1, with 16-fold, 20-fold, 38-fold and 56-fold upregulation at 1, 2, 4, and 6 months of age. The upregulation on an mRNA level was confirmed using quantitative PCR (qPCR) in 2m and 4m old animals. Immunohistological staining with a rabbit polyclonal anti-mouse Ym-1 antibody in kidneys from db/db animals and controls showed strong fluorescent staining of solitary cells in the glomerulus but also a more diffuse staining in proximal tubuli. The glomerular staining for Ym-1 was consistent with infiltrating monocytes/macrophages. The staining in proximal tubuli prompted us to investigate if the Ym-1 staining in tubuli reflected expression of Ym-1 protein in tubular cells or uptake in tubular cells of protein expressed by glomerular macrophages and released into the urine. To address this question we performed qPCR comparing the expression in isolated glomeruli and remaining kidney tissue in db/db and control animals at 1 month of age. Expression in glomeruli was 23.6-fold higher in glomeruli than in remaining kidney tissue in db/db, indicating that the expression was from macrophages in glomeruli and that staining in proximal tubuli is most likely a result of tubular uptake of the Ym-1 protein. Also, there was no statistically significant difference in tubular Ym-1 mRNA expression between db/db and controls. To investigate whether Ym-1 positive cells were generally found in other organs in the db/db model we also performed immunohistological staining on muscle, liver, heart, lung and spleen and we observed no differences in expression between db/db and control animals, indicating that the infiltration of Ym-1 expressing cells in the db/db is glomerulus specific. To evaluate whether the presence of Ym-1 expressing macrophages in the db/db was related to diabetes or to other confounding factors such as obesity or genetic background, we investigated the mRNA expression of Ym-1 in isolated glomeruli from 4 and 6 month old STZ diabetic mice. We found 268-fold upregulation in STZ-diabetics compared to control animals and at 6 months the difference was 10.5 fold. The dramatic change in fold change between 4 months and 6 months is in fact due mostly to that expression of Ym-1 was almost undetectable at 4 months in the control animals, but detectable at low levels in control animals at 6 months. In an attempt to see if Ym-1 protein would be detectable in urine, we performed Western Blot on fresh urine. A distinct band of approximately 44.3 kDa was observed in urine from 6 month old db/db animals but not in controls, suggesting that

Ym-1 can be used as a non-invasive urinary marker of infiltrating alternatively activated macrophages in the kidney.

Ym-1 is a single chain secreted polypeptide of 398 amino acids [308]. Co-expression with macrophage markers Mac-1 and Scavenger Receptor defines macrophages as its main producer [256]. Ym-1 is considered a marker for alternatively activated macrophages [291, 292, 309].
Classically activated macrophages (caMac) are characterized as being stimulated by interferon-gamma (INF-gamma) followed by a microbial trigger such as lipopolysaccaride (LPS), resulting in the release of pro-inflammatory cytokines IL-6, IL-1, an increase of TNF and a nitrixoxide (NO) burst [310]. These events in turn lead to an enhanced ability to kill and degrade intracellular microorganisms but also to tissue damage of the host itself [254, 311]. Classically activated macrophages have been postulated as having a pathogenic role in DN by secreting proinflammatory cytokines such as IL-1, IL-6, IL-12, TNF-alpha, CCL-2, CCL-3, CCL-4, Reactive Oxygen Species (ROS) and NO leading to microbicidal tissue inflammation, scarring and fibrosis [282, 312].
Alternatively activated macrophges (aaMac) are characterized by as being activated by IL-4 and IL-13 and their secretion of Ym-1, mannose receptor and scavenger receptor-1. Secretory products and potential biomarkers include cytokines induced by IL-4 such as Retnla (Relm-alpha) also called FIZZ1, Factor XIIIA and Amac-1 [291-293, 313, 314] (Reveiwed by Mosser et al in [315] and Gordon in [254]). Alternatively activated macrophages are thought to have anti-inflammatory effects and lead to cellular regeneration and tissue remodeling [316] and may even contribute to the repopulation of damaged glomerular and tubular cells and have a protective effect on the kidney [317, 318].
The strong and consistent upregulation of Ym-1 mRNA in our study prompted us to look at the expression of other macrophage expressed genes in an attempt to understand whether we could distinguish between different suptypes of macrophage populations in the diabetic glomerulous. We did not observe differential expression of genes in the cluster for classical activation such as iNos (Nos2), Ifn-gamma, Il-6, Il-1 or Tnf. The ratio of iNos/Arginase is often used to distinguish between caMac and aaMac. We did not observe significant changes in expression of these components, but instead was found significant upregulation of Ym-1, coagulation factor XIIIA, IL-33, Il-1R and IL-4R, indicating the presence of aaMac [315, 319, 320] in the diabetic glomeruli.

Conclusions and future perspectives:
The differentially expressed genes in our study can be divided into three, albeit overlapping categories: changes in GBM/ECM , inflammation/innate immunity, and signal transduction/growth factors/transcription factors. The KEGG pathway analysis highlights immune response factors such as cytokines, monocyte and leukocyte receptors and adhesion molecules involved in leukocyte migration into the glomerulus, stressing the role of the immune system in DN. Other differentially expressed transcripts in the db/db mouse glomeruli include genes involved in the composition of the GBM and the extracellular matrix, e.g. collagens, laminins and fibronectin. Changes in the basement membrane synthesis and composition and the accumulation of mesangial matrix may disturb glomerular function and cause albuminuria. The combination of these three events is likely linked together in the development of DN. One obvious hypothesis coming out of our analysis is that networks of genes/proteins are causally related to the pathogenesis of DN.

Macrophage infiltration and inflammation have been implicated in the development of DN in several publications. Previous studies have not attempted subtyping of macrophages in diabetic kidneys into alternatively activated (aaMac) or classically activated (caMac), but studied expression of universal macrophage markers like CD68 [282, 321] and F4/80 [280]. It is important to increase our knowledge of the detailed immunological components and processes involved in DN. In current therapies the immunological aspects of the illness are not treated and if macrophage-mediated injury is an important component in the development of DN, it is crucial that alternative therapeutic approaches are developed.

4 FINAL CONCLUSIONS

Paper I:
Endothelial cells are an essential source of Pdgfb in the recruitment of pericytes and vascular smooth muscle cells to blood vessels and in the recruitment of mesangial cells into the kidney glomerulus.

Paper II:
The construction of a glomerulus-specific cDNA microarray chip enabled us to detect rare glomerular transcripts and provided us with a better understanding of glomerular development as well as of the mechanisms involved in glomerular disease.

Paper III:
Injected tracer molecules cross the BBB via macromolecular trancytosis in pericyte-deficient mice, demonstrating a role for pericytes in maintaining the integrity of the BBB.

Paper IV:
Expression analysis of glomeruli from diabetic db/db mice demonstrated dramatic upregulation of genes involved in activation of the immune system as well as in the composition of the GBM and ECM, stressing a role for the immune system in the development of DN.

5 ACKNOWLEDGEMENTS

First of all I want to express my deep gratitude to **Christer Betsholtz**, my supervisor. Your enthusiasm for science, your genuine curiosity for how things *really* work and your positive outlook meant a lot to me during these years. The freedom you gave me with research was scary at first, but it enabled me to become independent and to find my own interests as a scientist. When I look back I wouldn't want to have it any other way. Thank you also for making the weekly commutes between Stockholm and Göteborg manageable, and for meeting up for coffee and science in Göteborg in the past year. You have a big heart!
I also want to thank **Minoru Takemoto** who taught me everything about cDNA microarray, glomerulus isolation and fed me fantastic Japanese food. All the best to you and your family!
Per Lindblom, who took me on as a summer student and let me wreck havoc on his (mouse) kidneys, his vector constructs and his cell cultures. Thank you!

Thank you, Betsholtz members at KI: **Ying** for the tip about using Retrive-All for epitope retrieval and for sharing a love for glomeruli. **Annika** for taking over the R26 mice and doing a very thorough and successful analysis of them. Also, thank you for the Raclette parties. I have never been so full of cheese! **Johanna** for organisational skills and social skills. **Konstantin** for sharing your chocolate. **Sara** for help with the ordering system. **Radiosa** for nice chats and a quick car ride to the central station, Italian style! **Guillem** for always being so helpful and nice and for teaching me (accidentally) to swear in Catalàn! **Lwaki,** being around you makes me happy! **Kazuhiro**, **Maarja**, **Bàrbara** and **Miyuki** keep up the hard work! **Colin**, good luck with the macrophages. I look forward to more discussions!

Former KI members: **Liqun**, for making magic happen with bioinformatics and for always being super helpful and knowledgeable. **Maya**, for always brightening up the lab. Also, thank you for taking me and my family on a real Norwegian "hytte" vacation. I wish you the best in your new medical career! **Elisabet**, for keeping the lab full of good music. **Mattias** & **Mats**, nice to have you in the group at KI as well as at GU! **Johan**, the bringer of bulle and homegrown plums. **Desiree**, for knowing how to make the most of the time outside the lab.

The **Tryggvason** group, thank you for welcoming us to KI and being so helpful. A special thank you to **Karl** for your never-ending enthusiasm for kidney and for taking us out for oysters and champagne in Philadelphia. **Timo**, for sharing the running bug. **Stefania**, **Elisabeth R**, **Jaakko** and **Masa** for company during the ASN meetings. **Ljubica**, for also struggling with the db/db genotypings! **Mark, Juha, Anne-May, Àsi, Laleh, Bing, Berit, Ann-Sofie, Ann-Charlotte, Anna, Sergey, Kan, Susan, Yi, Yunying, Chen, Dadi, Xiaojun, Jianhua, Eyrùn, Marko, Ulla, Sajila, Gunor** and **Ari** for all the nice chats in the corridors and for being part of a great work environment.
Arindam, for telling me the truth about Boston winters.
Karin, for scientific enthusiasm and crazy stories about dating.

Elisabeth Åhman, Alessandra Nanni and Sarah Löwhagen, thank you for your help with all the paper work. **Chad Tunell**, for saving me from computer chaos. **Åke Rökaeus**, for guidance on how to teach carbohydrate chemistry to medical students without boring them to death. **Teresa and her crew** at the animal house for taking great care of my mice and always being very helpful and nice.

Thank you, former Betsholtz members in Göteborg: **Alexandra**, for running inspiration, "pärlpyssel", cocktail parties in the sauna and being a very good friend. **Simin**, we shared the experience of weekly commuting. Thank you for being great company during all the time we spent on trains. Sharing the long distance commute with you made it a lot more fun! **Mattias B**, for a fun collaboration and a shared love for Orust. **Hans**, my first office mate, for giving the best shoulder massages. Oh how I wished for one while writing this thesis! **Maria**, for always being so helpful and kind and making "the boys" do their share of cleaning in the lab too. **Paula and Mikael**, for mastering xls and strange jokes. **Cicci** for being brave and honest and also for organizing a million things in the lab that nobody ever noticed but that kept lab work running smoothly. **Holger**, for telling me I could do this. **Andrea,** for making me want to run again. **Julia**, thank you for showing me that those of us without housewives can be scientists, too. **Patrik**, for immediately assimilating to the pasta-atmosphere. I hope lunch is more varied at Astra! **Monica**, for keeping me company during the long hours in the microarray room and for helping with the mice.

Mattias and **Fredrik**, my fellow summer students, thank you for great company in and out of the lab. You two made being a summer student even *more* fun. Soon we'll all be PhDs!

Other groups at Göteborg University: the **Lindahl** group, **Semb** group, **Collander** group, **Bergström** group, **Pekny** and **Pekna** groups and **Uv** group for great parties, meetings and coffee breaks! I also wish to thank **Esbjörn Telemo** for an excellent course in immunology.

Thank you **Stefan**, **Marina**, **Kristin** and **Lisbeth** for the fun, coffee-fuelled and darn effective study sessions at the KTB. I missed you when I returned now, years later, to write this thesis!
Charlotte, **Parker**, **Freja** and **Siri** + **Niklas** and **Åsa** for scientific discussions, nice dinners and outings. I think we have the right combination of science/non-science conversations!
Thank you, **Helen** and **Gita** for great company during the immunology course. Helen, thank you also for long runs with baby strollers and even longer "fika" breaks at Con Leche.
Kim, (and **Malin** and **Smilla)**, thank you for tips on how to combine thesis writing with family life.
Thank you, all **running friends**. I have found that I weed through my ideas and projects and organize my days best during early morning runs.
Thank you, **Sara** and **Sarah** for distraction, dinner clubs and laughter. More of that!

Thank you to the **Egecioglu family** for wonderful trips to Turkey, Småland, Oslo and (hopefully soon!) Helsingfors. A special thank you to **Gürel** for being a great "dede" and picking Ingrid up at day-care.

Thank you to my beloved sisters, **Ebba** and **Lotten**: I am so proud of you both and so blessed to have you in my life. I treasure our time together. **Astrid**, you are the coolest, cutest little cousin Ninni could ever ask for! **Gillis**, for being great to Lotten, and for keeping her in check (brandmansgreppet!) and **Fredric** for being there for Ebba.

To my dad **Stig**, thank you for letting me live in your apartment in Stockholm all these years. Also, thank you for always pushing me to be the best I can be.

Thank you mamma **Marie**, without your support and unconditional love I wouldn't be anything. Thank you for all the help with Ninni during thesis writing, and the love I see between you and her.

Emil, my one and only. You make all my dreams come through. I am so, so, so happy to be with you and for the wonderful family we have become. I love you, always!

Ingrid (Ninni), my daughter. I love you more than words could ever describe, more than life itself. Min lilla råtta, you make me wake up happy (and early) every day!

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