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Podocyte repair and recovery in kidney disease

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A thesis submitted to the University of Edinburgh for the degree of PhD

August 2011
Declaration

I hereby declare that, apart from the acknowledged assistance, this thesis is all my own work and no part of it has been submitted for any degree.

Yu Zhou
Abstract

Introduction
Podocytes are terminally differentiated, highly specialized glomerular cells that form the final barrier to protein loss. Podocyte injury is characterised by proteinuria. Proteinuria is an important prognostic marker in kidney diseases, and lowering proteinuria has become a principal clinical goal.

Compelling evidence supports the notion that continuing loss of podocytes plays a major role in the initiation and progression of glomerular diseases. It is my hypothesis that interventions that reduce the disruption by rescuing susceptible podocytes next to injured ones are potential therapies to restore podocyte phenotype and filtration behaviour, thereby protecting the kidney from progressive deterioration. Prevention of this damage, or ways to aid its recovery, could therefore be important to improving the management of human kidney diseases.

Methods
Transgenic mice expressing the human diphtheria toxin receptor on podocytes had been previously generated in our laboratory. Characterization of two lines showed that graded specific podocyte injury could be induced by single intraperitoneal injection of diphtheria toxin. Eight-week intervention studies involved administration of oral drug in water or food from 24h after toxin injection. Two control groups received no drug or were non-transgenic (wild-type) littermates. Primary endpoints were glomerulosclerosis and kidney function (serum creatinine). Other readouts included blood pressure, albuminuria, serum albumin, podocyte quantification and collagen staining of kidney. The angiotensin converting enzyme inhibitor (ACEi) captopril was tested because of its proven protective effect on renal function in patients with proteinuria. Subsequently another proteinuria-reducing drug, the endothelin receptor A antagonist sitaxsentan was tested alone and in combination with captopril.
**Results**

Captopril reduced proteinuria and ameliorated scarring, with matrix accumulation and glomerulosclerosis falling almost to baseline. Podocyte counts were reduced after toxin administration and showed no significant recovery irrespective of captopril treatment.

In the following sitaxsentan and captopril combined intervention study, glomerular scarring was significantly reduced in all drug-treated groups either alone or in combination, but only combination drug treatment reduced glomerular damage to levels comparable to wild-type controls, demonstrating a synergistic effect of the two agents. Similarly, serum creatinine was lowered further in combined but not single drug-treated groups. Blood pressure of all drug treated mice was lowered compared to the placebo group. Surprisingly in this second study there were no significant differences in proteinuria between treated and untreated groups.

**Conclusion**

These results support the hypothesis that continuing podocyte dysfunction is a key abnormality in proteinuric disease, and plays a major role in progressive glomerulosclerosis. Both captopril and sitaxsentan alone or in combination provided protection without substantial preservation or restoration of podocyte numbers at the degree of injury induced in these experiments. Combined therapy showed a synergistic effect in protecting the kidney from progressive damage. These results suggest that protection may be at least partly due to change in podocyte phenotype.

The model is ideal for studying strategies to protect the kidney from progressive damage following specific podocyte injury. Further elucidations on the mechanism of action of the drugs may aid development of superior future therapeutic treatments in the field of renal diseases.
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<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACEi</td>
<td>Angiotensin converting enzyme inhibitor</td>
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<tr>
<td>ACR</td>
<td>Albumin creatinine ratio</td>
</tr>
<tr>
<td>ACTN-4</td>
<td>Alpha-actinin-4 gene</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin-like-4</td>
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<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>APOL1</td>
<td>Apolipoprotein L1</td>
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<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>AT1</td>
<td>Angiotensin II receptor 1</td>
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<tr>
<td>ATRA</td>
<td>All-trans-retinoic acid</td>
</tr>
<tr>
<td>bw</td>
<td>Body weight</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
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<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>CDK2</td>
<td>Cyclin dependent kinase-2</td>
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<td>CD2AP</td>
<td>CD2-associated protein</td>
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<td>DDS</td>
<td>Denys-Drash syndrome</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acids</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
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<td>ESRD</td>
<td>End stage renal disease</td>
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<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor</td>
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<td>FSGS</td>
<td>Focal segmental glomerulosclerosis</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GBM</td>
<td>Glomerular basement membrane</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>GCS</td>
<td>Glomerular cross section</td>
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<td>GEC</td>
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<td>GFB</td>
<td>Glomerular filtration barrier</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GLEPP1</td>
<td>Glomerular epithelial protein 1</td>
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<td>GLUT</td>
<td>Glucose transporters</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IL-2</td>
<td>Interleukin-2</td>
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<td>h</td>
<td>Hour</td>
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<td>hDTR</td>
<td>Human diphtheria toxin receptor</td>
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<tr>
<td>H-ESRD</td>
<td>Hypertension-attributed end-stage renal disease</td>
</tr>
<tr>
<td>hHB-EGF</td>
<td>Human heparin binding epidermal growth factor receptor</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HSPG</td>
<td>Heparan sulphate proteoglycans</td>
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<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
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<td>K⁺</td>
<td>Potassium</td>
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<td>LAMβ2</td>
<td>Laminin β2 protein gene</td>
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<td>MCD</td>
<td>Minimal change disease</td>
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<td>MCGN</td>
<td>Mesangiocapillary glomerulonephritis</td>
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<td>MCP-1</td>
<td>Macrophage chemotactic protein-1</td>
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<td>Min</td>
<td>Minute</td>
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<td>MN</td>
<td>Membranous nephropathy</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MYH9</td>
<td>Non-muscle myosin heavy chain</td>
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<td>Na⁺</td>
<td>Sodium</td>
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<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NOS</td>
<td>Not otherwise specified</td>
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<tr>
<td>NPHS1</td>
<td>Nephrin gene</td>
</tr>
<tr>
<td>NPHS2</td>
<td>Podocin gene</td>
</tr>
<tr>
<td>NPHS3</td>
<td>Phospholipase C epsilon 1 gene</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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PAN  Puromycin aminonucleoside nephrosis
Par  Partitioning defective
PCR  Polymerase chain reaction
PLA2R  Phospholipase A2 receptor
PLCe1  Phospholipase C epsilon 1
Podo-DTR  Podocyte expressing-diphtheria toxin receptor transgenic mice
PPAR-γ  Peroxisome proliferator receptor-gamma
RANTES  Regulated on activation, normal T cell expressed and secreted
ROS  Reactive oxygen species
RT-PCR  Reverse transcriptase polymerase chain reaction
SGK1  Serum/glucocorticoid regulated kinase-1
Tg  Transgenic
TGF-β  Transforming growth factor-β
TRPC6  Transient receptor potential cation channel 6
VEGF  Vascular endothelial growth factor
VEGFR2  Vascular endothelial growth factor receptor 2
WT  Wild-type
WT-1  Wilms’ tumour suppressor gene
ZO-1  Zonula occludens 1
CHAPTER 1: Introduction

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Introduction

1.1 General introduction
This thesis investigates the role of podocyte in proteinuric kidney diseases using a transgenic Podo-DTR mouse model in which specific podocyte injury can be induced by dose-dependent administration of diphtheria toxin. In the following chapter I will give an overview of the importance of the kidney diseases and why proteinuria is an essential prognostic marker of renal diseases.

To support the critical role of podocyte in the glomerular filtration system and handling of protein, I provide a series of existing but fast growing evidence linking podocyte dysfunction and subsequent loss to proteinuria, progressive glomerulosclerosis and glomerular diseases. This leads to the aims of my project justifying the focus of my study on the podocyte and why potential future therapeutic treatments should target more specifically on podocytes.

Overview of kidney diseases
Damage to the kidney can lead to chronic kidney disease (CKD), a long lasting irreversible condition that if left untreated can progress to end stage kidney failure, at which stage dialysis or kidney transplant are the only treatment options.

Approximately 5-10% of the UK population have reduced glomerular filtration rate (GFR) and stage 3-5 CKD (Stevens et al., 2007). The risk of CKD development increases with age and there is higher prevalence of CKD in South Asian and African communities compared to Caucasians (Nicholas et al., 2005).

The presence of excess protein in the urine is a diagnostic marker of renal disease as well as an independent risk factor for cardiovascular disease (Best et al., 2004). In a healthy individual only traces of protein are found in the urine. Measurement of urinary protein can be performed using simple semi-quantitative dipstick test or expressed as protein or albumin:creatinine ratio (ACR) to give a more reliable and accurate result that corrects for dehydration and protein concentration. Ratio more
than 15mg/mmol for protein (or 3mg/mmol for albumin) are considered abnormal levels, while levels above 300mg/mmol are classed as nephrotic range proteinuria.

Glomerular diseases account for a significant proportion of acute and chronic kidney diseases. The cost of end-stage renal disease (ESRD) is growing constantly, and the cumulative ESRD costs are even greater than the direct treatment cost of cancer (Szczech & Lazar, 2004). With a growing population of higher life expectancy in developed countries, a huge economic burden to both patients and health care system and shortage of kidney donors, there could be huge benefits from alternative and better treatment to prevent or slow down kidney disease progression.

**Why is proteinuria such an important prognostic marker?**

There are two controversial hypotheses justifying the importance of proteinuria as a prognostic marker of kidney diseases. A widely accepted hypothesis is that filtered proteins reabsorbed by tubules are directly toxic to renal tubular cells, hence causing tubular damage and progressive injury (Morigi et al., 2005, Wilmer et al., 2003). An alternative hypothesis, not mutually exclusive, is that podocyte injury and loss is the primary problem that causes progressive renal damage, and severity of proteinuria is correlated with severity of podocyte damage.

We favour the latter hypothesis on the basis of lines of evidence that are described later. One important example that challenges the “filtered protein toxicity” hypothesis is that nephrotic range proteinuria of minimal change disease and membranous nephropathy can be present over years without progressing to ESRD.

**Kidney function and components of the nephron**

The main function of the kidney is to maintain normal blood homeostasis by filtering low molecular weight plasma products into the urine while restricting the passage of larger molecules such as albumin and immunoglobulin.

In a healthy 70kg adult, the kidneys filter approximately 180L of blood daily to produce 1.5-2L of urine, which is passed from the kidneys to the bladder via the ureters (Fig.1.1).
Other functions of the kidney include maintenance of sodium (Na\(^+\)) and potassium (K\(^+\)) levels, production of vitamin D, required for absorption of calcium (Ca\(^{2+}\)), production of hormones such as erythropoietin (EPO) for red blood cell generation, regulation of blood pressure and blood pH.

The filtration of the blood occurs in the functional units of the kidneys known as nephrons. There are approximately one million nephrons in a normal adult kidney (Fig. 1.2a), and the number decreases with age. The nephron consists of glomerulus and renal tubules (Fig. 1.2b). The blood enters the glomeruli via the afferent arterioles. Blood flows under pressure into a network of capillaries as part of the glomerular tuft, and each of these capillary loops is enclosed by the Bowman’s capsule. The process of blood filtration occurs at the glomerular filtration barrier and ultrafiltered blood leaves the glomerulus via the efferent arteriole.
Figure 1.1. Schematic representation of the urinary system. In a healthy 70kg adult, the kidneys filter approximately 180L of blood daily to produce 1.5-2L of urine, which is passed from the kidneys to the bladder via the ureters.
**Glomerular filtration function and components**

The glomerular filtration barrier (Fig. 1.2c) is a selective filter that functions to retain most circulating macromolecules while filtering low molecular weight solutes and water. The filtration process is dependent on both size and possibly charge of the molecules.

The 3 main components of the glomerular filtration barrier (GFB) consist of the fenestrated endothelial cells, the glomerular basement membrane (GBM) and the highly specialised visceral epithelial cells known as podocytes. Although each component plays important roles in the filtration barrier, they are interdependent on one another to maintain and deliver normal filtration function. Therefore insult or disruption to the glomerular filtration barrier in the kidney causes leakage of proteins in the urine (proteinuria).

**Glomerular endothelial cells**

Glomerular endothelial cells (GEC) lie on the inner surface of the capillary walls in the glomerulus. The characteristic fenestrations formed between the cells partly play a role in the glomerular filtration barrier. The surface of endothelial cells is covered by a layer of glycocalyx, which is believed to contribute to the selectivity of size and charge of the filtrates. Indirect evidence from many proteinuric animal models showed the correlation between the reduced GEC glycocalyx and proteinuria supporting the role of GEC glycocalyx in the filtration barrier function (Jeansson & Haraldsson, 2006).

**Glomerular basement membrane**

Glomerular basement membrane (GBM) is sandwiched between the layer of glomerular endothelial cells and podocytes. It is derived from fusion of the basement membrane of precursors of these two cell types (Kriz, 2007). In the mature glomerulus, the GBM is approximately 100-200nm thick and contains of α3-5 chains of type 4 collagen and α3, β2 and γ1 chains of laminin. Furthermore, negatively charged proteoglycans such as heparan sulphate proteoglycans (HSPG) may also contribute to the size and charge selectively of the macromolecules.
The filtration of the blood occurs in the functional units of the kidneys known as nephrons, there are approximately one million nephrons in a normal adult kidney. The nephron consists of glomerulus and renal tubules. The blood enters the glomeruli via the afferent arterioles, and ultrafiltered blood leaves via the efferent arteriole. Blood flows under pressure into a network of capillaries as part of the glomerular tuft, and each of these capillary loops is enclosed by the Bowman's capsule. The process of blood filtration occurs at the glomerular filtration barrier, which consist of podocyte with their foot processes, glomerular basement membrane (GBM) and fenestrated glomerular endothelial cell (GEC) covered in a layer of glycocalyx.
Podocytes

Podocytes are highly specialised epithelial cells, which cover the outer surface of the glomerular basement membrane of the capillary and form the final barrier to the passage of macromolecules such as albumin and immunoglobulin during glomerular filtration (Fig. 1.2c-d). Podocytes have a prominent cell body, from which major processes extent out and divide into numerous long thin structures, known as foot processes. The foot processes are intimately wrapped around the capillary and interdigitate with the neighbouring podocytes to form a highly specialised cell-cell junction, termed the slit diaphragm. The size of the slit diaphragm is estimated to be approximately 40nm, and it is believed to provide resistance and restriction of macromolecules passage by size selection.

In proteinuric renal diseases, the retractions of foot processes also known as foot process effacement, which is an indication of “sick” podocyte, are often observed. In addition, discovery of mutations specific to podocyte molecules (such as nephrin, podocin, CD2-associated protein of the slit diaphragm) and structural proteins such as α-actinin 4 are often associated with nephrotic syndrome.

In the fully developed kidney, podocytes are primarily responsible for production and maintenance of the GBM, as well as providing structural support to glomerular capillary loops. In addition, normal function of podocyte is essential for endothelial cells, as normal function of GEC is tightly regulated by levels of vascular endothelial growth factor (VEGF) and angiopoietin-1, which are secreted by podocytes in the glomeruli (Qiu et al., 2010, Satchell et al., 2004). However, it is important to note that glomerular endothelial cells themselves also secrete angiopoietin-1, which contributes to the reciprocal interaction/regulation between the two cell types.

Angiopoietin-1 acts via the endothelial Tier2 receptor and maintain the endothelium in the vascular bed, regulating some actions of VEGF. In addition, it has the property of reducing endothelial cell permeability, stabilizing the endothelium and resisting inflammation and angiogenesis in the mature endothelium (Thurston et al., 2000, Thurston et al., 1999). In adult, continued expression of angiopoietin-1 even in
quiescent vessels, plays a role in vascular maintenance (Wong et al., 1997). On the other hand, VEGF-A is a family of isoforms with contrasting properties. However, the VEGF_{165} isoform is the predominant pro-angiogenic, pro-permeability isoform and acts as paracrine regulators via biding of the endothelial VEGF receptor 2 (VEGFR2) (Petrova et al., 1999). Therefore, angiopoietin-1 in combination with VEGF-A play essential roles in modulating GEC barrier properties by regulating vascular permeability, angiogenesis and are critical for development and maintenance of intact glomerular function (Baelde et al., 2004, Satchell et al., 2004).

In summary, the multifunctional roles of podocyte acting as a major structural barrier of the glomerular filtration as well as playing essential roles in regulating and maintaining the two other main components of the filtration barrier namely GBM and endothelial cells, suggest an important link between podocyte damage and filtration malfunction leading to protein leakage. Therefore, the relationship between podocyte injury and proteinuric diseases deserves further attention.
Figure 1.2. (d) Scanning electron micrograph of human podocytes (illustrated in green) wrapped around the glomerular capillaries (purple) in the kidney with their interdigitating foot processes. (Image: Dennis Kunkel Microscopy Inc. Google image accessed on 25.07.11 http://www.google.co.uk/imgs?imgurl=http://2.bp.blogspot.com/).
1.2 What causes proteinuria?

1.2.1 Key link between proteinuria and podocyte injury/dysfunction

The clinical signature of podocyte injury and dysfunction is the presence of proteinuria, which is often but not always associated with reduced renal function. The pathological leakage of proteins from the blood into the urine can occur without any detectable morphological changes under light microscopy in certain diseases. The level of proteinuria can range from mild (<3g/day) to nephrotic range (>3g/day). The predominant causes of nephrotic range proteinuria due to podocyte damage include, membranous nephropathy, minimal change disease and focal segmental glomerulosclerosis (FSGS), which will be described more in details below. Other diseases, which are not covered here in this section, also include mesangiocapillary glomerulonephritis (MCGN), amyloid, diabetic nephropathy and IgA nephropathy (Fig. 1.3). These diseases are named based on the histologic descriptions rather than the primary causes or mechanism involved in the disease development.

Membranous nephropathy

Disease & histology of membranous nephropathy

Membranous nephropathy is one of the leading causes of idiopathic nephrotic syndrome in adults, and is an organ-specific, cell-specific autoimmune disease of the kidney, where circulating autoantibodies bind to target antigens on the podocyte foot processes initiating the disease with pronounced direct and subsequent secondary effects.

The deposition of the antibody-antigen complexes and the additional matrix accumulation at the foot processes of podocytes give rise to the characteristic feature of thickened glomerular basement membrane (GBM) under the light microscopy. This is more evidently observed at later stages of the disease.

The antibody binding to the cell is responsible for podocyte injury and subsequent proteinuria. This is supported by data from immunofluorescent staining where
deposits for total immunoglobulin G (IgG) or IgG4 are shown as fine granular staining in a capillary loop pattern. While the electron microscopy data shows more detailed amorphous, electron-dense deposits in the subepithelial and the intramembranous compartments with additional foot process effacement as characteristic sign of podocyte injury.

**Pathogenesis of membranous nephropathy**

Heymann nephritis is a rat model of human membranous nephropathy that was first developed in the late 1950s by Walter Heymann. Megalin, a transmembrane protein was located and identified within coated pits of podocyte foot processes and proximal tubular borders in this rat model as the primary antigen target. Later, a human counterpart of megalin was identified as neutral endopeptidase (NEP) protein as the target antigen in a small subset of patients with antenatal membranous nephropathy (Debiec et al., 2002).

In a more recent study Beck LH et al. (2009) identified M-type phospholipase A2 receptor (PLA2R) to be the human membranous nephropathy antigen (Beck et al., 2009). This protein is also expressed in the lung and on neutrophils (Granata et al., 2005, Silliman et al., 2002), but high expression seems to be largely restricted to the podocyte in the kidney.

Autoantibodies to PLA2R are mostly IgG4, which is the predominant subclass in idiopathic membranous nephropathy immune deposits. Anti-PLA2R IgG was localized in biopsy samples of patients with idiopathic membranous nephropathy, but not lupus-associated membranous nephropathy or IgA nephropathy. To date the role and function of PLA2R in podocyte is still unknown and elucidation is required to define the precise pathogenetic of anti-PLA2R.

**Forms of membranous nephropathy**

Idiopathic membranous nephropathy accounts for the majority of the cases in developed countries, while secondary membranous nephropathy can be related to a number of factors such as chronic infections of hepatitis B, malaria, syphilis and
schistosomiasis. A link is also drawn between some autoimmune diseases and membranous nephropathy, these include systemic lupus erythematosus and rheumatoid arthritis. In addition solid tumours as well as some medications for treatment of rheumatoid arthritis e.g. gold salts, penicillamine and some non-steroidal anti-inflammatory drug (NSAID) are also associated with membranous nephropathy.

Although clinically and histologically the two forms of membranous nephropathy are similar, some subtle histopathological clues can be used to differentiate the secondary from the idiopathic membranous nephropathy form, however this is not always the case. Secondary membranous nephropathy, in particular membranous lupus nephritis, often has mesangial and subendothelial deposits as well as subepithelial and intramembranous deposits that are seen in idiopathic membranous nephropathy. Another distinct difference lies within the subclasses of IgG within the glomerular deposits. While IgG4 is predominantly found in the idiopathic membranous nephropathy, IgG2 and IgG3 are typically more abundant in secondary (lupus- and malignancy-associated) forms of membranous nephropathy (Haas, 1994, Kuroki et al., 2002, Ohtani et al., 2004).

Therefore, measurement of anti-PLA2R autoantibodies (which are mostly IgG4) proposed by Beck LH and Salant D may be valuable in future serologic test to diagnose idiopathic membranous nephropathy, and to distinguish idiopathic membranous nephropathy from secondary membranous nephropathy forms (Beck & Salant, 2010).
### Spectrum of glomerular diseases

**Figure 1.3.** At one end proteinuric/nephrotic glomerular diseases caused by specific injury to podocytes, or structural change of the glomerulus affecting podocyte function. At the opposite end of the spectrum, inflammation leads to cell damage and proliferation causing damage of glomerular basement membrane (GBM) and subsequent leak of blood into urine (Diagram adapted from ANT 2011)

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<td>Mechanism</td>
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<td>• Injury to podocytes</td>
<td>• Inflammation</td>
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<td>• <em>Changed architecture</em></td>
<td>• Reactive cell proliferation</td>
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<td>- Scarring</td>
<td>• Breaks in GBM</td>
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<td>- Deposition of matrix or other elements</td>
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**Mechanism**

- Injury to podocytes
- *Changed architecture*
- Scarring
- Deposition of matrix or other elements

**Mechanism**

- Inflammation
- Reactive cell proliferation
- Breaks in GBM
- Crescent formation

Proteinuria | Haematuria
---|---
Minimal change disease | Diabetic nephropathy
FSGS | MCGN
Membranous nephropathy | Post-streptococcal glomerulonephritis
Amyloid | Anti-GBM disease
Small vessel vasculitis | IgA nephropathy
Systemic lupus erythematos (SLE) |
Minimal change disease

Disease and histology of minimal change disease
The disease mainly affects children with a prevalence of approximately 90% of cases of nephrotic syndrome in the UK. In black African origin children it is less common and it can also occur in adults at lower rate (15-20%) (Mathieson, 2007).

Histologically the glomeruli in minimal change disease appear normal under light microscopy, however EM images can reveal the pathological change of podocyte morphology with presence of foot process effacement, but absence of electro-dense immune-deposits seen in membranous nephropathy. Typically immunofluorescence microscopy is also negative.

Clinical features of minimal change disease
Minimal Change Disease typically presents with heavy proteinuria leading to nephrotic syndrome. The main symptom of patients is oedema affecting the face and limbs. Patients are often lethargic and have reduced exercise tolerance, but excretory renal function is typically normal.

Onset is often abrupt, triggers may include infection, other illnesses, a recent vaccination or atopy (a predisposition towards allergy development). In rare cases, minimal change disease is precipitated by drug treatment (e.g. NSAID), however, this can be resolved once the offending drug is withdrawn. There is also a well-described association with lymphoma, particularly Hodgkin’s disease, in which treatment of the lymphoma leads to resolution of the nephrotic syndrome and relapse of proteinuria may indicate recurrence of the underlying lymphoma (Audard et al., 2006). However, in the majority of cases, no underlying causes can be identified.

In adults, in order to exclude other causes whose management may differ from minimal change disease, the diagnosis is made by renal biopsy. By contrast, in children, treatment for minimal change disease is often initiated without the
supporting evidence of renal biopsy as steroid-responsiveness is almost pathognomonic, and minimal change disease is the most likely diagnosis.

**Pathogenesis of minimal change disease**

The exact causative mechanism of minimal change disease is still not defined. The disease was originally thought to be immune-mediated, possibly due to a disorder of T cell function in which a circulating factor is thought to play a role. For decades minimal change disease has been thought to be caused by an immunological disturbance, however, the evidence for immune mechanisms are all indirect. There is no inflammation in the glomerulus, no infiltration with lymphocytes or other immune cells, and no deposition of immunoglobulin or complement in the kidney. As a result, studies have concentrated on possible soluble mediators.

Although the literature is conflicting, there is suggestion that a type two cytokine bias might be important (Mathieson, 2003). Podocytes express receptors for interleukin (IL)-4, 10, and 13, and these cytokines have been shown in vitro to have direct effects on podocyte mediator production and barrier function (Coers et al., 1995, Parry et al., 2001, Van Den Berg et al., 2000). Podocytes also express functional CCR and CXCR chemokines (Huber et al., 2002). Whether these known cytokines and/or chemokines are the elusive soluble mediators in minimal change disease remains unproven, but podocytes clearly have the potential to respond to various soluble products of the immune system.

Lai KW et al. (2007) have shown that over-expression of the cytokine IL-13 in rats, induces minimal change disease-like nephropathy, with changes in podocyte structure and gene expression similar to those seen in the human disease (Lai et al., 2007). However, transgenic mice overexpressing IL-13 do not develop nephrotic syndrome as seen in rats. This may be due to mice strains being resistant to the induction of glomerular disease. A possible mechanism by which excess IL-13 might induce nephrotic syndrome is shown by changes in podocyte protein trafficking and proteolytic enzyme secretion seen when these cells are incubated in vitro with IL-4 or IL-13.
Other evidence for an immune/autoimmune pathogenesis in minimal change disease includes immunogenetic factors (Gong et al., 2000), especially association with products for the major histocompatibility complex (Clark et al., 1990), and the response to drugs which have effects on the immune system. The relative specificity of mode of action of cyclosporin for T lymphocytes led to suggestions that its beneficial effects in minimal change disease strongly support the importance of T cells in the pathogenesis (Meyrier, 1989). The opposing argument to this hypothesis is that cyclosporin has numerous other effects such as hemodynamic influences and actions on numerous other cell types including direct effect on podocyte, therefore the immune interpretation may be overly simplistic.

In a recent study, Clement LC et al (2011) suggested a role for angiopoietin-like-4 (ANGPTL4) in glucocorticoid-sensitive nephrotic syndrome (Clement et al., 2011). Evidence in both experimental models as well as human minimal change disease showed an upregulation of ANGPTL4 in the serum and in podocytes. Overexpression of ANGPTNL4 in podocyte in a rat model lead to development of heavy albuminuria, loss of GBM charge and development of foot process effacement, while mice without endogenous ANGPTNL4 were protected from lipopolysaccharide induced proteinuria, suggesting a causative role of ANGPTL4 in minimal change disease (Clement et al., 2011). The authors suggest that ANGPTL4 in minimal change disease might represent a podocyte-derived factor that may act in an autocrine or paracrine manner.

Hemopexin, which is an abundant plasma protein that scavenges heme has also been suggested to play a role in glomerular diseases. Evidence supporting this includes induction of reversible proteinuria and minimal change like glomerular lesions in a rat model after intra-renal infusion of hemopexin and increased levels of activated hemopexin in children with minimal change nephrotic syndrome (Bakker et al., 2005a, Bakker et al., 2005b). A group from Bristol later demonstrated that active hemopexin causes nephrin dependent remodelling of podocytes and increases permeability of the glomerular filtration barrier by possibly degrading the glycocalyx.
(Lennon et al., 2008). Both in vivo and in vitro effects of the active isoform of hemopexin (80kD) could be attributed to protease activity of this molecule. However, whether activation of hemopexin is a cause or effect in minimal change disease is uncertain, as the precise circumstances in which hemopexin is activated are not known.

Although advances are being made in the past decade in the attempt of identifying the causative agent of minimal change disease, the exact potential soluble circulating factor still need to be defined. Future studies on podocytes may elucidate the causative agent of minimal change disease and its mechanism of action.
Focal segmental disease (FSGS)

Disease and histology of FSGS

Focal segmental glomerulosclerosis (FSGS) refers to a histopathological description of the renal biopsy. As the name itself implies, FSGS describes scarring (sclerosis) in some parts (segmental) of some glomeruli (focal). FSGS is often described together with minimal change disease, as they share many clinical features. There is strong debate in the past as to whether primary minimal change disease and FSGS are variants of the same disease, or whether they represent separate pathogenetic entities. More recent findings support the latter notion, which is discussed in a review by Mathieson (Mathieson, 2007). However, similarities and differences of the two diseases will be covered briefly below.

To date, the mechanisms of injury of FSGS as that of minimal change disease are still poorly understood, but the involvement of immunologically mediated processes such as the effects of soluble mediators produced by lymphocytes is a likely possibility (Mathieson, 2007).

While in minimal change disease, the podocyte injury is reversible, thus progression to ESRD is rare, in FSGS, the podocyte injury tends to be more prominent, therefore may be irreversible, hence there is a higher risk of progressive loss of renal function. FSGS together with minimal change disease constitute the main causes of idiopathic nephrotic syndrome. Both minimal change disease and FSGS are classed under glomerular diseases where podocyte pathology is the common denominator. Electron microscopy images of both diseases show disruption and effacement of foot processes of podocytes. In addition, the two diseases are characterised by diffuse capillary wall defect in the absence of immune deposits.

In both conditions, immunosuppressive therapies are widely used due to the belief that the diseases are immunologically mediated. However, patients with minimal change disease tend to respond more effectively to immunosuppressive drugs than FSGS. Some of the routine treatment drugs include corticosteroids,
cyclophosphamide, and cyclosporine or tacrolimus. In clinical practice, approximately 95% minimal change disease patients respond to corticosteroids, while most FSGS patients are resistant to steroids therapy (Wei & Reiser, 2011).

**Clinical features of FSGS**

In children and some adults, FSGS presents as nephrotic syndrome, which is characterized by oedema (fluid retention associated with weight gain), hypoalbuminemia (low serum albumin in the blood), hyperlipidemia and hypertension. In adults it may also present as kidney failure and proteinuria without reaching nephrotic range levels.

The initial clinical presentation of FSGS may be identical to that of minimal change disease, however, the rate of developing hypertension and/or renal function impairment is higher in FSGS. In children the diagnosis of FSGS may be suspected when the condition is steroid-resistant. In adults, with unexplained nephrotic syndrome, FSGS is diagnosed by renal biopsy. Prevalence of FSGS continues to rise and is increasingly important as a cause of end-stage renal disease (Kitiyakara et al., 2004).

FSGS consists of a number of histological subtypes: cellular variant, collapsing glomerulopathy and glomerular tip lesions, which will be discussed later on. Immunochemistry is either negative or with presence of IgM and/or C3 in sclerosed segments.

Sclerosis and more specifically abnormalities of podocyte structure can be observed by EM. These include fusion of podocyte foot processes which may overlie otherwise normal glomerular capillaries, focal detachments from the GBM, and lipid droplet accumulation.

The podocyte as a target of injury in FSGS is supported by EM observation, but more strongly evidence comes from study of rare inherited forms of the disease.
(described later in genetically inherited glomerular diseases section), supporting the notion that damage to podocyte is indeed the cause of FSGS.

**Forms and classification of FSGS**

Depending on the cause, FSGS can be broadly classed as primary and secondary FSGS. Primary or idiopathic FSGS typically presents with the acute onset of nephrotic syndrome with no evidence of pre-existing renal disease. On the other hand, secondary FSGS typically presents with non-nephrotic proteinuria and often renal insufficiency caused by other underlying renal diseases.

A non-specific pattern of secondary FSGS can result from renal scarring due to previous injury. This can be observed with a number of disorders including focal proliferative glomerulonephritis, vasculitis, and lupus nephritis. Additional causes include toxins and drugs, such as heroin, interferon, cyclosporine, and pamidronate; genetic abnormalities from familial forms of FSGS described later in the podocyte-specific protein mutations; infections (e.g. HIV-associated nephroapathy); hyperfiltration (e.g. chronic pyelonephritis and reflux) and diabetes mellitus (Lu et al., 2007).

**Histologic variants of FSGS**

Based on histology of renal biopsy, five mutually exclusive variants of focal segmental glomerulosclerosis may be distinguished: 1) collapsing variant, 2) glomerular tip lesion variant, 3) cellular variant, 4) perihilar variant and 5) not otherwise specified (NOS) variant. Recognition of these variants may have prognostic value in individuals with idiopathic FSGS (i.e. where no underlying cause is identified).

The collapsing variant is associated with higher rate of progression to end-stage renal disease (ESRD), whereas glomerular tip lesion variant has low rate of progression to ESRD in most patients (Thomas et al., 2006). Cellular variant shows similar clinical presentation to collapsing and glomerular tip variant, but it has intermediate outcomes between these two variants. The prognostic significance of perihilar and
NOS variants has not yet been determined. The NOS variant is the most common subtype (Thomas et al., 2006).

**Collapsing variant**

The collapsing variant is considered the most rapidly progressive form of FSGS. The distinction of collapsing glomerulopathy from other cellular forms of FSGS is the lack of endocapillary proliferation. The clinical features of idiopathic collapsing variant include heavy proteinuria of nephrotic range, high serum creatinine levels (234-657µmol/L) at diagnosis and a rapid course to ESRD (15 months rather than 38 months) (Haas et al., 1995). The incidence is much higher in black races and it does not typically respond to therapy. The scarring quickly affects the entire glomerulus, causing it to collapse. Most patients that are diagnosed with the collapsing variant will require dialysis or a kidney transplant within one to two years despite treatment.

Collapsing FSGS has also been associated with certain drugs, such as pamidronate (Barri et al., 2004). Other causes of collapsing glomerulopathy may be due to environmental or infectious factors. The main cause of secondary collapsing glomerulopathy is HIV-associated nephropathy reviewed by Herman and Klotman (Herman & Klotman, 2003). However, other viruses have also been found in the kidneys of patients with collapsing glomerulopathy including parvovirus B19, simian virus 40, cytomegalovirus, hepatitis C virus and Epstein-Barr virus. Whether these viruses are indeed the cause of induction of FSGS is not defined, therefore the possibility of the infection simply being a coincidence cannot be ruled out.

**Cellular variant**

The cellular variant implies an overabundance and hypertrophy of podocytes (within the glomeruli). The presence of the excess cells causes the glomerular blood vessels to narrow and eventually close off completely. In its extreme form of collapsing glomerulopathy, classic features of the podocytopathy characterize the initial derangement, followed by subsequent matrix deposition, capillary collapse and
fibrosis. Podocyte distribution within the tuft is markedly modified and their phenotype altered (Meyrier, 2005a).

**Pathogenesis of FSGS**

The earliest histological features seen in transplanted kidney biopsy in recurrent FSGS patients are changes in the cellular phenotype of podocytes. These include acquisition of macrophage markers and podocyte proliferation associated with changes in the cell cycle inhibitors that are believed to limit the proliferation capacity of podocyte under normal circumstances (Bariety et al., 2001).

In FSGS patients, the risk of recurrence of the disease and proteinuria is high after the kidney transplant, this can occur within minutes of the revascularization after organ transplant in some patients suggesting that the cause lies in the recipient bloodstream. Various assays have been developed to analyse the putative permeability factor(s), either in *in vitro* experiments by incubating glomeruli in plasma from patients with FSGS and measure the swelling of glomeruli (Savin et al., 1996) or infuse plasma directly into rodents and measure the resultant proteinuria (Sharma et al., 2002). However, the reliability and utility of the method used in vivo in rodents, as well as the specificity for FSGS is debatable (Esnault et al., 1999, Le Berre et al., 2000).

An alternative hypothesis for which there is increasing evidence is that the cause may be due to a missing signal/component from the nephrotic plasma rather than the presence of something abnormal compared to healthy/normal plasma. A number of lines of evidence support this notion. Firstly, proteinuria-inducing effects of nephrotic plasma are lost when the plasma is pre-incubated with the urine of the same patient (Carraro et al., 2003); secondly the effect on glomerular swelling is abrogated by mixing with normal plasma (Sharma et al., 2000); thirdly the effects of nephrotic plasma on human podocytes *in vitro* are reversed when the plasma is mixed one to one ratio with normal plasma (Coward et al., 2005). These evidences favour an imbalance of a protease-antiprotease pair, presumably due to loss of the
inhibitor in the urine and/or the increased synthesis of the active protease but not its inhibitor (Carraro et al., 2004).

Plasmapheresis (a protein absorption strategy used to remove potential circulating factors responsible for FSGS from the bloodstream) has achieved success in treating patients with recurrent FSGS to a certain degree, as the treatment is only short-lived. The beneficial effect of this method may be due to removal of an abnormal substance and/or by the replacement of normal plasma.

In addition immunoadsorption by protein A, which predominantly removes IgG, has also been reported to be successful, where no replacement plasma is given. However, there is evidence that the active component of the immunoabsorbed material is not IgG, and that the effect of immunoadsorption is not specific to FSGS (Esnault et al., 1999).

In summary membranous nephropathy, minimal change disease and FSGS described above are three typical examples of proteinuric podocyte-injury-induced glomerular diseases, where the evidence supports the strong link between podocyte dysfunction and proteinuria.
1.2.2 Podocyte injury and related glomerular diseases

All glomerular diseases including membranous nephropathy, minimal change disease and FSGS covered previously arise from damage and disruption of one or more components of the glomerular filtration barrier described earlier. Causes of podocyte injury can be classed under two main categories: genetic and acquired (Ronco & Debiec, 2007, Shankland, 2006).

Genetically inherited glomerular diseases

Genetic causes of podocyte damage leading to proteinuria include mutations that affect directly or indirectly phenotype and functions of podocytes and podocyte-specific proteins. In particular, these include proteins of the specialised cell-cell junction of the slit diaphragm, such as nephrin and its binding partners CD2-associated protein (CD2AP), podocin and transient receptor potential cation channel 6 (TRPC6) (Kim et al., 2003, Tryggvason et al., 1999) (Fig. 1.4).

Congenital nephrotic syndrome of the Finnish type from NPHS1 mutation

Congenital nephrotic syndrome of the Finnish type is caused by mutation of the nephrin gene NPHS1. This gene encodes a transmembrane protein, which is believed to form the main building block of the slit diaphragm filter structure. Therefore, defects in nephrin lead to loss of normal podocyte function due to the abnormal or absent slit diaphragm, which results in the onset of severe foetal/neonatal proteinuria (Tryggvason et al., 1999).

Classic FSGS from CD2AP mutation

Another example of congenital nephrotic syndrome is caused by mutation of CD2-associated protein (CD2AP) gene. CD2AP is one of nephrin’s binding partners and is a component of the filtration complex in the kidney (Kim et al., 2003). Studies showed that mice with CD2AP haploinsufficiency developed glomerular changes and had increased susceptibility to glomerular injury by nephrotoxic antibodies or immune complexes. Electron microscopic analysis of podocytes also revealed defects in the formation of multivesicular bodies, suggesting an impairment of the intracellular degradation pathway (Kim et al., 2003).
Denys-Drash and Frasier’s syndrome from WT-1 mutation
Wilms’ tumour gene (WT-1) encodes a transcription factor that regulates key podocyte genes including nephrin. In an experimental murine model, reduced expression of WT-1 associated with downregulation of nephrin and development of mesangial sclerosis. Mutation of WT-1 can also cause Wilms’ tumour, alone or with mesangial sclerosis in Denys-Drash syndrome (DDS), and gonadoblastomas in Frasier’s syndrome (FS) (Patek et al., 2003). Both of these are characterised by male pseudohermaphrodism and progressive glomerular disease, which is often resistant to pharmacological treatment (McTaggart et al., 2001, Menke et al., 2003, Patek et al., 2003).

Steroid resistant nephrotic syndrome and FSGS from NPHS2 mutation
Mutations in the podocin gene (NPHS2), which encodes for slit diaphragm protein podocin causes autosomal recessive steroid-resistant nephrotic syndrome and FSGS in children (Boute et al., 2000). Podocin interacts with the intracellular domains of nephrin, Neph1 and with CD2AP. The disease is characterised by onset of nephrotic syndrome in early childhood, which is resistant to treatment with steroids. Podocin-knockout mice develop severe proteinuria and they die within a few days after birth (Roselli et al., 2004).

FSGS from ACTN-4 mutation
Alpha-actinin-4 (ACTN-4) gene mutation leads to autosomal recessive focal segmental glomerulosclerosis (FSGS) (Kaplan et al., 2000). ACTN4 encodes the crosslinking protein, alpha-actinin-4, which plays an important role in the podocyte structure and its dynamic movement together with other structural components. Mutations of this gene in man lead to proteinuria and podocyte foot process effacement (Kaplan et al., 2000), and similar outcome occurs in experimental models when α-actin-4 is overexpressed or knocked out (Kos et al., 2003, Michaud et al., 2003).
**FSGS from NPHS3 mutation (PLCε1)**

The NPHS3 gene encodes for phospholipase C epsilon 1 (PLCε1), mutation of which is associated with either diffuse mesangial sclerosis or FSGS on renal biopsy (Boyer et al., 2010, Gbadegesin et al., 2008). Polymorphism of this gene is likely to cause genetic predisposition and increased susceptibility to development of early onset nephrotic syndrome after a secondary insult such as infection. This possibly explains the reason why some patients are partially responsive to steroid treatment or calcineurin inhibitor, as the drug is effective in treating the secondary cause.

**FSGS from TRPC6 mutation**

The TRPC6 gene encodes a calcium channel known as the transient receptor potential cation channel 6 (TRPC6). This protein belongs to a family of non-selective cation channels that regulate the intracellular calcium concentration after the activation of G-protein-coupled receptors and receptors tyrosine kinases. In podocytes, TRPC6 appears to be associated with the slit pores, where it is likely to be involved in slit diaphragm signalling. Mutation of this gene leads to autosomal dominant FSGS2 (Reiser et al., 2005, Winn et al., 2006).

**FSGS, HIV-associated nephropathy and hypertension-attributed ESRD from APOL1 mutation**

A recent study (Genovese et al., 2010) showed that genetic variants within Apolipoprotein L1 (APOL1) gene are associated with increased susceptibility of FSGS, HIV-associated nephropathy and hypertension-attributed end-stage renal disease (H-ESRD) in African-American populations but not in Caucasian Americans. This increased risk was previously wrongly attributed to mutations of a neighbouring non-muscle myosin heavy chain (MYH9) gene (Kao et al., 2008, Kopp et al., 2008), where the authors justified disruption caused by MYH9 variation in actin-myosin filaments to be responsible for maintenance of podocyte structure and associated increased risk of developing FSGS and HIV-associated nephropathy.

However, it is now known that APOL1 mutations, which is positively selected in certain African regions to protect against the parasitic infection of trypanosomiasis...
*Trypanosoma brucei* is responsible for the increased susceptibility to non-diabetic CKD in African descendents. An interesting postulation by Hartleben and colleagues from nephrological perspective is that APOL1 may be involved in autophagic pathways (Hartleben et al., 2010, Zhaorigetu et al., 2008), a major potential protective mechanism against podocyte aging and glomerular injury. However, whether APOL1 variants have indeed influence on homeostatic balance of cells in the glomeruli still need to be further elucidated (Kronenberg, 2011).

**Nail-Patella syndrome from LMX1B mutation**

The LMX1B gene encodes for the transcription factor that is believed to regulate the expression of key podocyte proteins including nephrin, podocin and CD2AP (Lemley, 2009). Mutation of this gene cause Nail Patella syndrome (Bongers & Knoers, 2003, Lemley, 2009, Witzgall, 2008), an autosomal-dominant disorder that can result in variable renal phenotype, ranging from normal renal function to nephrotic syndrome or early renal insufficiency. However, the defect is not limited to kidneys, as LMX1B mutations are also associated with nail, bone and eye abnormalities.

**Pierson syndrome from LAMβ2 mutation in GBM**

The LAMβ2 gene encodes for the laminin β2 protein, which is part of an integral component of the glomerular basement membrane and functions to anchor cells, and importantly plays a role in podocyte differentiation. Mutation of LAMβ2 gene causes the rare Pierson syndrome (Zenker et al., 2004), which is characterised by heavy proteinuria from birth and early progression to renal failure. Aside from renal impairment, mutation of the gene can also cause eye abnormality. Therefore, although laminin β2 protein is not a podocyte specific protein, it is essential for normal podocyte differentiation and function.

Therefore, apart from LAMβ2, all of the above are podocyte genes, emphasizing the critical role of this highly specialized cell in the glomerular and protein filtration setting.
Figure 1.4. Simplified schematic representation of podocyte foot process and various structural molecular components. The slit diaphragms (indicated by red arrowheads) of the podocyte foot processes form the final barrier to filtration. Components of the slit diaphragm complex (nephrin, CD2AP, podocin, TRPC6), structural proteins (actin, α-actinin-4, synaptopodin) and the underlying glomerular basement membrane (GBM) composed of laminin11, α3-α4-α5 collagen and the anchoring α3-β1 integrin are shown. (Diagram obtained from ANT 2008)
Other acquired glomerular diseases

Although classed as acquired glomerular diseases, subtle polymorphism of genes may well contribute towards increased genetic susceptibility in some of the following diseases.

Infection

Examples of acquired glomerular diseases include mesangiocapillary glomerulonephritis (MCGN) caused by hepatitis C and HIV-associated nephropathy due to the direct infection of podocytes by the human immunodeficiency virus (HIV) (Ross & Klotman, 2002).

Diabetes

Numerically the most important cause of podocyte injury is diabetes. Although diabetic nephropathy has been considered a mesangial disease in the past, it is also associated with significant podocyte injury and marked proteinuria (Pagtalunan et al., 1997). Diabetic glomerulopathy is an important cause of morbidity and mortality in developed countries as the disease is among the most common causes of ESRD. Microvascular and macrovascular complications are often associated with the disease, which makes it more difficult to treat and manage. Pathologically, the first changes coincide with the onset of microalbuminuria and include thickening of the GBM and accumulation of matrix material in the mesangium. Subsequently, nodular deposits are characteristic, and glomerulosclerosis worsens as heavy proteinuria develops, until glomeruli are progressively lost and renal function deteriorates (Diez-Sampedro et al., 2011).

Amyloid

Amyloidoses are a group of acquired (and hereditary) disorders characterized by extracellular deposition of insoluble proteins and fibrils. Accumulation of proteins may be localised or systemic, and the clinical manifestations depend upon the organ(s) affected. Clinical and histological features of different types of amyloidosis can vary substantially. Generally, higher levels of proteinuria are seen in the primary light chain amyloidosis (AL) type, whereas microscopic haematuria is more
prominent in the secondary (AA) type as a result of other illnesses (e.g. multiple myeloma, chronic infections or chronic inflammatory diseases). Histologically, amyloid deposition of primary type tends to deposit more frequently and strongly to GBM than mesangium, and spicule formation is more frequently observed. On the other hand, the secondary type has a higher affinity to tubular basement membrane and interstitial area (Nishi et al., 2008). Other types of amyloidosis not detailed in the thesis also include inherited familial amyloidosis and beta-2 microglobulin amyloidosis developed after long-term dialysis.

In summary, whether genetically inherited or acquired, damage or injury to podocyte causes proteinuric glomerular diseases as detailed above with the exception of Pierson syndrome that involved LAMβ2 mutation in the GBM.
1.2.3 Podocyte damage/abnormality: central role that drives glomerulosclerosis

So far I have presented evidence of podocyte dysfunction, resulting from genetic as well as acquired causes, strongly linked to proteinuria. In addition, data from experimental studies demonstrates how injured podocyte may drive progressive glomerulosclerosis (Ichikawa et al., 2005, Kriz et al., 1998, Wharram et al., 2005).

How could podocyte injury drive progressive glomerulosclerosis?

More than a decade ago Kriz W et al (1998) described a potential mechanism by which progressive podocyte injury may lead to chronic renal failure in a number of renal diseases (Fig. 1.5). The onset and magnitude of glomerulosclerosis is correlated with podocyte loss (Kriz et al., 1998, Kriz & LeHir, 2005). Due to limited potential of podocyte to replicate or replace, the loss of these highly specialised cells through apoptosis or detachment leads to areas of “denuded” GBM. As a consequence of reduced podocyte number to counteract the outward forces of glomerular pressure, the capillary loop shape, which is normally maintained by podocytes is no longer intact. This leads to outward bulging of the GBM in denuded areas and subsequent formation of synachiae, which consists of attachment of bare GBM to the Bowman’s capsule. Kriz described this as the first “committed step” for the formation of FSGS (Kriz et al., 1998). It is this first trigger that sets off the vicious circle of “podocyte damage damages podocyte”, which if left untreated, can potentially lead to further injury and subsequent progression of glomerular diseases (Ichikawa et al., 2005, Kriz et al., 1998).

Decrease in podocyte number predicts progression

Additional evidence of podocyte loss leading to progressive deterioration comes from both experimental and clinical data. Studies have shown that podocyte number is reduced in the majority of diabetic and non-diabetic glomerular diseases (Lemley et al., 2002, Steffes et al., 2001). Wiggins and colleagues have also shown that a reduction in podocyte number is associated with aging in a rat model (Sanden et al., 2003). The consequences of reduced podocyte number include proteinuria and glomerulosclerosis. Studies in IgA nephropathy (Lemley et al., 2002) and diabetic
diseases (Pagtalunan et al., 1997, Steffes et al., 2001) have shown that proteinuria increases as podocyte number decreases.

However, it is important to note that although in the majority of progressive glomerular diseases the podocyte number is reduced, there are a number of exceptions such as HIV-associated nephropathy, cellular/collapsing FSGS and crescentic glomerulonephritis, where the podocyte number is actually increased. Nevertheless, in all glomerular diseases described here regardless of podocyte number alteration, the affected podocytes have a pathological phenotype.

To investigate this further, animal models have been used to test the hypotheses and further elucidate the mechanism of proteinuric disease progression. These will be covered in the following experimental models section.
Figure 1.5. Schematic to show the development of segmental glomerulosclerosis.

(a) Normal glomerulus with vascular and urinary poles. Smooth muscles, extra glomerular mesangial, and mesangial cells proper are hatched; podocytes are shown in blue, parietal epithelial cells in red. The GBM is shown in black, the parietal basement membrane in yellow, tubular epithelia are shown in white. (b) A dilated and podocyte-denuded capillary is attached to Bowman's capsule. The attachment is accomplished by the affixation of parietal cells to the naked GBM. Thereby a gap in the parietal epithelium has come into existence, permitting filtration/exudation towards the cortical interstitium (arrow). (c) The adhesion has spread to neighboring capillaries resulting in either the collapse or in hyalinosis (shown in a dark grey pattern) of the involved capillaries. Fluid leakage from perfused capillaries inside the adhesion has created a paraglomerular space (shown in yellow) that contains the sclerotic tuft remnants (that is, collapsed or hyalinized GBM formations). (d) Via the vascular pole the sclerotic process has reached a further lobule. A small “intact” tuft remnant protrudes into the urinary space still covered by the parietal epithelium. The sclerotic tuft remnants are located outside the parietal epithelium in the paraglomerular space that is separated from the cortical interstitium by a complete layer of cortical fibroblasts. In later stages, fibroblasts will invade the sclerotic area, resulting in fibrous organization. (Kriz W et al 1998)
Experimental models are needed to test hypotheses and therapies

Animal models, in particular rat and mice models have played essential roles in the advancement of podocyte biology and understanding of mechanism of proteinuric disease induction and progression. Genetically modified podocyte-specific rodents as well as toxin/drug induced experimental models have been used to study various podocyte diseases (D'Agati, 2008, Pippin et al., 2009b). Examples of diseases in which podocyte is known to be a major participant include membranous nephropathy, diabetic nephropathy and crescentic glomerulonephritis. However, for the purpose of this thesis I will mainly cover acquired podocyte disease models where podocyte has been specifically targeted to mimic human glomerular diseases, namely minimal change disease and FSGS.

Historically, the major animal models of FSGS involved direct podocyte injury by toxin administration (e.g. puromycin aminonucleoside, adriamycin) and indirect podocyte injury due to adaptive responses by renal ablation (i.e. 5/6 nephrectomy). Increasing evidence indicates that podocyte depletion is a major pathomechanism mediating proteinuria and glomerulosclerosis. Podocyte-specific toxin models suggest that podocyte loss is sufficient to cause FSGS in a dose-dependent manner. Knockout and transgenic models have provided proof of concept that mutations in specific podocyte proteins mediate genetic forms of FSGS (Kaplan et al., 2000, Kos et al., 2003, Michaud et al., 2003, Reiser et al., 2005).

Two well established toxin models that represent experimental model of human minimal change disease and FSGS are rat puromycin aminonucleoside (PAN) and mice adriamycin nephropathy. The primary target of PAN and adriamycin is believed to be podocytes.

1. Rat model of puromycin aminonucleoside (PAN)

While most of rat strains tend to be susceptible to PAN, historically mice have been resistant to PAN. A number of factors can influence the onset of proteinuria and extent of injury. These include rat strain, PAN dose, and route of administration. However, cumulative exposure of PAN is the determining factor of progression of
glomerular lesions from podocyte effacement to glomerular scarring development of histological features of minimal change disease or FSGS (Diamond & Karnovsky, 1986).

A study by Shiiki et al (1998) showed that rats given subcutaneous PAN for 5 days developed lesions consistent with minimal change disease that eventually regressed. However, an additional 5 days treatment with PAN lead to development of irreversible sclerotic lesions (Shiiki et al., 1998). In a different study, Kim YH et al (2001) showed that progressive podocyte depletion induced by increasing dose of PAN correlated directly with degree of glomerulosclerosis at 3 months (Kim et al., 2001). Glomerulosclerosis was initiated when 10-20% of podocytes were lost.

**Mechanism and relevance of PAN model**

The mechanism of injury of PAN is direct DNA damage via production of reactive oxygen species (Diamond et al., 1986). Evidence supporting this is that rats pre-treated with oxygen radical scavengers before receiving PAN have less proteinuria and podocyte injury (Diamond et al., 1986, Thakur et al., 1988).

Ultrastructural changes seen in the PAN model are similar to those described in human nephrosis with presence of podocyte foot processes disorganization a few days after the toxin treatment progressing to typical flattening and loss of processes at later timepoint (Caulfield et al., 1976, Messina et al., 1987). Podocyte detachment and proteinuria occur simultaneously and subsequent apoptosis of podocytes is associated with the development of FSGS (Caulfield et al., 1976, Shiiki et al., 1998).

Change in podocyte cytoskeleton and subsequent slit diaphragm alteration/loss is accompanied by altered mRNA and protein levels leading to loss of size and charge selectivity, which is related to proteinuria development. In addition, detachment of podocyte leaves denuded areas of GBM, which closely correlated onset of massive proteinuria (Whiteside et al., 1989, Whiteside et al., 1993).
One major advantage of using rat PAN model is that it offers insights into how podocyte lesions evolve in the pathological processes of minimal change disease and FSGS. Unlike in the human setting where serial biopsy is limited, the PAN rat model permits induction of disease in dose-dependent manner and gives the opportunity to evaluate serial changes in the podocytes. Therefore, this model has proven useful in providing information for some of the effective diagnosis and treatment of disease entities of minimal change disease and FSGS.

2. Mouse model of adriamycin nephrosis
Most mouse strains tend to be resistant to adriamycin, but BALB/c and 129/SvJ mice are susceptible. Administration of adriamycin is thought to cause selective injury to podocytes resulting in severe proteinuria and progressive renal failure with subsequent development of experimental model of FSGS in mice (Chen et al., 1995, Cheng & Kopan, 2005, Zheng et al., 2005). However, whether the agent can induce injury to other cell types apart from podocytes is uncertain.

Wang Y et al. showed that a single intravenous injection of adriamycin at 10-11mg/kg body weight produced a stable and reproducible murine model of chronic progressive nephropathy with significant and persistent proteinuria (Wang et al., 2000), while other studies have employed the use of a second dose two to four weeks following the initial dose without an increase in animal mortality rates.

Mice develop severe proteinuria within days after administration of the toxin, histologically glomerular hypertrophy, hyaline deposits, reabsorption droplets, and intratubular casts are seen by week two. At later timepoints, glomerular vacuolization, tuft collapse, and severe interstitial fibrosis and inflammation are observed by week four with development of some global sclerosis by week six (Wang et al., 2000). In addition, extensive podocyte foot process effacement is observed by EM analysis (Wang et al., 2000).
Potential mechanisms and relevance of adriamycin mouse model

The exact pathophysiological mechanisms underlying the initial cytotoxicity and the delayed progression remain to be determined. However, studies have shown that several potential pathways of injury may be responsible (Gewirtz, 1999, Park et al., 2005). Acute cytotoxicity is thought to be secondary to DNA damage or lipid peroxidation, direct cell membrane effects, cell death via necrosis or apoptosis (Gewirtz, 1999, Park et al., 2005), and or promotion of senescence-like growth arrest (Rebbaa et al., 2003).

It has been suggested that delayed progression may be secondary to generation of reactive oxygen species (ROS) (Yilmaz et al., 2006), leading to mitochondrial DNA damage (Lebrecht et al., 2004), alteration of GBM (Kramer et al., 2006), chemokine release by injured resident kidney cells, including monocyte chemotactant protein-1 (MCP-1), RANTES with subsequent renal infiltration by macrophages and T cells (Wu et al., 2005), and/or apoptosis secondary to reactive free radical formation or activation of Fas/Fas ligand signalling pathway (Song et al., 2000).

In mice, the adriamycin nephropathy model has proved to be a robust experimental model of human FSGS. Because FSGS is the final common pathway for loss of functioning glomeruli, and because podocyte loss is a critical event in the initiation of FSGS, the murine adriamycin model is ideal to elucidate the underlying mechanisms that control the response of the podocyte to injury. Establishing adriamycin nephropathy in mice carries the special advantages of ease of handling, economy, and the potential for application of genetic and monoclonal antibody manipulation to study pathogenesis (Wu et al., 2005).

Therefore both rat PAN- and mouse adriamycin- models have essentially been used to investigate the mechanism by which the toxins induce podocyte injury, characterize ultrastructural change in podocyte after toxin exposure and study the correlation of the time course and extent of podocyte injury with onset of proteinuria. However, one of the weaknesses of these models is the uncertainty of the toxin specificity, as it is not known whether both agents can induce injury to other cell types apart from podocytes.
Podocyte specific ablation models

While puromycin and adriamycin toxins are believed to have specific toxic effects on podocytes via oxidative stress, the exact mechanism of the induction of the injury is still uncertain. Whether the deleterious effects of the toxins can be exerted on other neighbouring cells is also debatable. In order to overcome this shortfall, models of targeted podocyte cell ablation have been developed (Asano et al., 2005, Matsusaka et al., 2005, Wharram et al., 2005) to investigate whether specific podocyte depletion is sufficient to cause glomerulosclerosis and FSGS.

3. Inducible rat diphtheria toxin model

Wharram BL et al. (2005) used a transgenic rat model in which specific expression of human diphtheria toxin receptor on podocytes is driven by podocin promoter. In this model, the authors are able to produce different stages of glomerular injury depending on the percentage of podocytes depleted after administration of diphtheria toxin, correlating to a dose-response (Wharram et al., 2005). A high dose of diphtheria toxin causes massive proteinuria by day 2, followed by FSGS lesions by day 28. Up to 20% loss of podocytes produce transient proteinuria with mild mesangial expansion and no effect on renal function. A greater loss of podocyte number (21-40%) lead to synechiae and FSGS lesions with sustained mild proteinuria and normal renal function. Depletion of podocytes over 40% causes focal segmental and global glomerulosclerosis with severe proteinuria and reduced renal function. Therefore, in this model, the degree of podocyte depletion directly correlates with structural and functional measures of glomerular injury. Importantly, there is a threshold effect above which FSGS lesions are produced.

4. Inducible mouse Pseudomonas endotoxin model

In a different podocyte toxin model, where podocytes are specifically targeted, a Japanese group led by Ichikawa et al. (Asano et al., 2005, Matsusaka et al., 2005) engineered a transgenic mouse model of glomerulosclerosis by selectively expressing human CD25 (human IL-2 receptor light chain) in podocytes under control of the nephrin promoter. Injection of anti-Tac (Fv)-PE38 (LMB2) immunotoxin induced progressive proteinuria and glomerulosclerosis in a dose-
dependent manner. The toxin consists of a fusion of pseudomonas exotoxin A with the variable region of anti-CD25 antibody, directing toxin specifically to the podocytes expressing CD25. Like diphtheria toxin, the pseudomonas exotoxin A causes cell death by ribosylation of elongation factor 2 and inhibition of protein synthesis. By permanently labelling the podocyte lineage with lacZ, the investigators could follow their fate over time.

This lineage-specific tag is retained despite severe dysregulation of the podocyte and loss of mature markers such as synaptopodin. The number of lacZ-stained podocytes progressively declined, epithelial cells proliferated to cover the denuded tuft, resembling collapsing FSGS. These studies therefore demonstrate the critical role of podocyte depletion in the process of glomerular sclerosis. The authors also suggest that the mass of glomerular epithelial cells in collapsing FSGS is comprised largely of proliferating parietal epithelial cells.

Next, the investigators produced a chimeric model in which only some of the podocytes expressed the CD25 receptors (Ichikawa et al., 2005). Surprisingly, administration of immunotoxin resulted in the same degree of glomerulosclerosis as in the transgenic model where every podocyte expresses the receptor.

This study showed that if the initial insult is of sufficient impact, there may be spreading of injury to adjacent podocytes that have escaped the original insult, producing a kind of domino effect. Injury may spread from one podocyte to its neighbour until the entire glomerular lobule is captured.

Small losses of podocytes may be contained by synechiae to Bowman’s capsule. However, critical losses of a large number of podocytes may spread to involve an entire segment, as suggested by the threshold demonstrated in the diphtheria toxin model by Wharram and colleagues (Wharram et al., 2005).

It seems that damage to podocyte can be inflicted not only via direct cell injury but it can also spread through to its interdigitating partner. This could be due to toxic
substances secreted in a paracrine or autocrine manner (such as TGF-β or Ang II) or via reduction in survival factors (such as VEGF or nephrin signalling) (Ichikawa et al., 2005). This shows that an intact slit diaphragm with unaffected cell signalling is likely to be a critical process in the survival of podocyte.

In summary, the experimental models of podocyte-specific injury have aided huge and important advances in understanding the role of podocyte in the development of proteinuria and experimental glomerular diseases. The critical role of these cells makes them an ideal potential target of treatment of proteinuria in clinical practice. Therefore, further elucidation and knowledge of podocyte biology might give insight to new therapeutic opportunities. These will be discussed in the next section.
1.2.4. Study of podocyte biology may give therapeutic opportunities

*Origin and biology*
Podocyte originate from the metanephric mesenchyme during glomerulogenesis (Saxen & Sariola, 1987). The proliferative capacity of the podocyte is altered during the various stages of glomerular development. In early stage of glomerulogenesis, podocyte precursors in comma and S-shaped bodies are able to engage and progress through the cell cycle. However, at later stage of capillary loop development, podocytes exit the cell cycle, cease to proliferate and acquire a terminally differentiated and quiescent phenotype.

Podocytes are susceptible to various pathological stimuli such as toxins, infection and immune-mediated insults. These cause podocytes to lose the characteristic complex structures known as foot processes required for the barrier to protein filtration. Because podocytes have limited capacity to replicate, it was previously believed that once these cells are injured or lost, the numbers cannot be recovered. Although more recent studies have provided evidence of podocyte replacement from intrinsic renal progenitor cells (Appel et al., 2009, Ronconi et al., 2009).

*Podocyte structure*
Each mature podocyte consists of distinct anatomical parts; it has a prominent large cell body that lies in the urinary space, from which major processes extend out and divide into numerous long thin structures, termed foot processes. These small but abundant foot processes cover large surface area by intimately wrapping around the glomerular capillaries and interdigitate with the neighbouring podocytes to form highly specialised cell-cell junction, known as slit diaphragms. These structures play key role in the glomerular filtration, and will be covered more in details below.

*Molecular components of podocyte*

* i. Actin cytoskeleton
Podocytes are highly dynamic cells and are capable of altering their shape. The complex shape and dynamic properties of the podocytes are due to the presence of
abundant actin cytoskeleton, which serves as the backbone of the cells (Mundel et al., 1997). Three distinct ultrastructural elements are found in the cytoskeleton, which include microfilaments, intermediate filaments and microtubules. Microfilaments are the main cytoskeletal constituents of the foot processes, and contain a dense network of F-actin and myosin. Several actin-binding proteins including synaptopodin and alpha-actinin-4 are probably important in maintaining podocyte shape (Fig. 1.4).

The actin cytoskeleton is linked with other proteins, based on the location in the podocytes, they can be divided into apical, basal and junctional cell membrane domains (Kerjaschki, 2001).

ii. Slit diaphragm
Slit diaphragms are specialised cell-cell junctions formed between foot processes of adjacent podocytes. These gap structures measure approximately 40nm wide, and seem to play the essential role of limiting protein leakage through size and charge barrier possibly achieved via phosphorylation of certain proteins. The junctional domain includes proteins of the slit diaphragm such as nephrin, podocin, CD2AP, FAT1, Neph1-3, ZO-1, and densin. Rodewald and Karnovsky first described the slit diaphragm in 1974 as a porous and zipper-like structure (Rodewald & Karnovsky, 1974). However, it is only in the last decade or so that the molecular composition and architecture of the slit diaphragm has started to be unravelled. Unlike other cell-cell junctions, the slit diaphragm is not only composed of commonly found proteins cadherins and catenins but also specific podocyte proteins that are essential for normal signalling and function of these specialised cells. Some of these proteins will be described more in details below.

Nephrin
Nephrin, encoded by NPHS1 gene was the first transmembrane protein identified in the slit diaphragm (Tryggvason et al., 1999). The crucial role of nephrin for the filtration barrier is supported by mutation of the gene in human and absence of nephrin in the knockout mice, both of which result in lack of slit diaphragm with
massive proteinuria at perinatal stage and subsequent neonatal death. Nephrin has a short intracellular domain, a transmembrane domain and an extracellular domain with eight IgG-like motifs and a fibronectin motif. Nephrin molecules is likely to interact in the slit diaphragm with one another in a homophilic fashion (Putaala et al., 2001) to give rise to the porous zipper-like structure of the slit diaphragm previously described.

Intracellularly, nephrin and the slit diaphragm are connected to the actin cytoskeleton through linker proteins CD2AP and Nck proteins, which are covered in more details below.

*CD2AP and Nck proteins*

CD2-associated protein (CD2AP) binds directly to nephrin and actin, and serves as a direct link between the slit diaphragm and the actin cytoskeleton. This link is essential for the normal function of glomerular filtration, as CD2AP deficient mice die due to massive proteinuria and exhibit foot process effacement (Shih et al., 1999).

The Nck proteins (Nck1 and Nck2) are composed of an SH2 domain, which can interact with phosphotyrosines, and of SH3 domains, which can recruit several other proteins involved in the regulation of actin assembly. In podocytes, Nck has been shown to interact with tyrosine phosphorylated nephrin (Jones et al., 2006, Verma et al., 2006). This interaction is required for the development of normal filtration barrier as mice lacking both Nck proteins in podocytes develop massive proteinuria (Jones et al., 2006).

Furthermore, Nck proteins are required in the maintenance of the mature filtration barrier, as inactivation of Nck proteins in adult mouse podocytes result in proteinuria and foot process effacement (Jones et al., 2006). Therefore evidence supports the notion that both CD2AP and Nck proteins are essential for linking the slit diaphragm to actin. These interactions mediate the actin polymerization and the cytoskeletal reorganization in foot processes that is required for normal functional barrier.
**Neph1-3**

Neph1, Neph2 and Neph 3 are also slit diaphragm proteins, and they are structurally related to nephrin. All Neph-proteins have 5 extracellular IgG-like motifs and interact with nephrin. Neph1 seems to assist with nephrin in actin recruitment via Nck proteins (Garg et al., 2007). Neph1 deficient mice result in massive proteinuria and perinatal death, highlighting its important role in the filtration.

**Podocin**

Podocin is another main component of the slit diaphragm that was discovered through studies on hereditary proteinuric syndromes, where mutations in podocin coding gene (NPHS2) were identified (Boute et al., 2000). Podocin is a haipin-shaped integral membrane protein with both ends directed into the intracellular space. Podocin interacts directly with nephrin, Neph1 and CD2AP (Schwarz et al., 2001, Sellin et al., 2003), and seems to be crucial for the recruitment of nephrin to the slit diaphragm (Schwarz et al., 2001). The critical role of podocin in the glomerular filtration barrier has been demonstrated in podocin deficient mice that lack slit diaphragms and die perinatally due to severe proteinuria (Roselli et al., 2004).

**TRPC6**

Transient receptor potential cation channel 6 (TRPC6) is a member of a family of non-selective cation channels that are involved in the regulation of intracellular calcium concentration in response to the activation of G-protein-coupled receptors and receptor tyrosine kinases. TRPC6 is found in the slit diaphragm, dominant mutations in the TRPC6 gene have been identified in families with a progressive proteinuric renal disease (Reiser et al., 2005, Winn et al., 2006), while some mutations found in patients lead to increased amplitude and duration of calcium influx after stimulation. TRPC6 knockout mice do not show any obvious renal phenotype, suggesting that the protein is not essential for the normal function of the filtration barrier (Patrakka & Tryggvason, 2009). Taken together, these data suggest
that activating mutations that cause increased calcium influx lead to the development of TRPC6 nephropathy.

_Cadherin proteins (proteocadherin Fat1, P-cadherin, VE-cadherin)_

Three cadherin proteins have been localized in the slit diaphragm, proteocadherin Fat1, P-cadherin, and vascular endothelial cadherin (VE-cadherin). Fat1 is a large protein with 34 tandem cadherin-like repeats, and plays an essential role in the filtration barrier, as Fat1 knockout mice lack slit diaphragms and develop proteinuria (Ciani et al., 2003). Unlike Fat1, P-cadherin does not seem to be essential for the functional renal filtration barrier, whereas the role of VE-cadherin in the podocyte is still not known. Also their roles in relation to other components of the slit diaphragm still need to be elucidated.

_Par3, Par6 and aPKC_

Partitioning defective (Par) 3, Par6 and atypical protein kinase c (aPKC) constitute the cell polarity complex and is located in the cytoplasmic side of the slit diaphragm (Simons et al., 2009). Par3 can bind directly to nephrin and Neph1, and recruit Par6/aPKC to the slit diaphragm area. The deletion of an aPKC isoform in podocytes leads to proteinuria suggesting that the cell polarity complex is critical for the glomerular filtration barrier.

_Dendrin_

Dendrin is a cytosolic protein originally identified in telencephalic dendrites, but is also localized at the cytoplasmic face of the slit diaphragm where it interacts directly with nephrin and CD2AP (Asanuma et al., 2007, Patrakka et al., 2007). In experimental proteinuric model, dendrin relocates to nucleus and enhances TGF-β1 mediated apoptotic signalling suggesting that changes in the slit diaphragm can affect the fate of podocyte survival, and this link maybe mediated via dendrin. However, dendrin knockout mice have normal functional glomerular filtration barrier indicating that this protein is not critical for normal podocyte biology.
Others slit diaphragm proteins

Other proteins such as zona occludens 1 (ZO-1), junctional adhesion molecule 4 (Jam4), densin have also been reported to be associated with the slit diaphragm (Tryggvason et al., 2006), however, their roles in podocytes remain to be elucidated.

iii. Apical domain

The apical domain of podocytes is negatively charged due to presence of the surface anionic proteins podocalyxin (Kerjaschki et al., 1984), podoplanin (Matsui et al., 1999) and podoendin. This may play a role in the limitation of the passage of albumin that is also negatively charged and possibly also maintaining adjacent podocyte separated by anionic charge.

*Podocalyxin* is the main molecular component of the apical plasma membrane of podocyte foot processes (Nielsen & McNagny, 2009). It is an extensively O-glycosylated and sialylated transmembrane protein that is responsible for the negative charge of the apical membrane domain. Intracellularly, podocalyxin is connected to the actin cytoskeleton and may be involved in the regulation of podocyte morphology in some disease processes (Takeda et al., 2001).

*Glomerular epithelial protein 1* (GLEPP1) is a receptor tyrosine phosphatase found in the kidney exclusively on the apical cell surface of the podocyte (Thomas et al., 1994). GLEPP1 is believed to play critical role in the regulation of glomerular filtration rate and normal podocyte structure, as mice deficient of this protein exhibit abnormal podocyte morphology, have reduced glomerular filtration rate and are prone to develop high blood pressure, but with no proteinuria. However, the mechanism of interaction with other components of the foot processes is still unknown.

iv. Basal domain

The basal domain serves the purpose of anchoring podocyte to the underlying GBM mainly via α3β1 integrin (Kreidberg et al., 1996) and αβ-dystroglycans. In addition, they also connect the podocyte cell body to certain matrix proteins within the GBM.
Podocytes, as all epithelial cells are attached to the underlying basement membrane through transmembrane cell receptors, such as integrins, tetraspanins and dystroglycans. The $\alpha_3\beta_1$ integrin is the most abundant isoform found in podocytes and is needed for the development of the glomerular capillary tuft (Kreidberg et al., 1996). Defects in the $\alpha_3\beta_1$ complex in podocytes also result in proteinuria and foot process effacement (Kanasaki et al., 2008, Pozzi et al., 2008, Sachs et al., 2006). $\alpha_3\beta_1$ integrin is a major receptor for the GBM component laminin-521 (Doi et al., 2002), leading to the speculation that disruption of the integrin-laminin complex may well result in a weakened podocyte-GBM interaction leading to detachment of podocytes from the GBM and subsequent loss. As a result proteins are filtered through the denuded area.

The importance of integrins has been further highlighted in studies on mice lacking integrin-linked kinase (ILK), specifically in podocytes as these mice develop proteinuria and progressive proteinuric renal disease (Dai et al., 2006, El-Aouni et al., 2006).

Tetraspanins are transmembrane proteins expressed in all cell types. CD151 is a member of the tetraspanin family that has been localized to the base of the foot processes and is associated with cell-matrix adhesion complexes such as $\alpha_3\beta_1$ integrin. CD151-deficient mice develop proteinuria and change in GBM structure suggesting the critical role of this protein in the glomerular filtration barrier (Sachs et al., 2006) and a possible role in the maturation and/or maintenance of the GBM structure.

The $\alpha\beta$ dystroglycan is another heterodimeric transmembrane protein found in the basal surface of the podocyte foot processes (Mundel & Reiser, 2010, Regele et al., 2000). Extracellularly dystroglycans bind to laminin and agrin, while intracellularly the complex binds to the actin cytoskeleton of foot processes via utrophin (Mundel & Reiser, 2010, Regele et al., 2000). However, the importance of dystroglycans in the adhesion of podocytes to the GBM still needs to be elucidated.
Podocyte function
The complex structure of podocyte including abundant actin cytoskeleton, slit diaphragm, various receptors and components on the apical and basal domains all serve the requirement of the highly specialized function of podocytes. These include: acting as a size barrier to proteins; maintenance of the capillary loop shape; counteracting the intraglomerular pressure, synthesis and maintenance of the GBM; production and secretion of growth factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1 that are required for glomerular endothelial cells integrity (Jefferson et al., 2011). Therefore disruption in one or more of these functions following progressive podocyte damage or injury cause the clinical signature of marked proteinuria, often with decreased renal function and elevated creatinine.

Podocyte in renal diseases
As described earlier in the section (1.2.2), podocyte injury can be induced by various factors including hereditary and acquired causes. These include mutations of podocyte specific proteins, metabolic syndrome, toxin-, infection-, immune-induced injury. Podocyte pathology is characterised by at least two or more of the three prognostic changes: proteinuria, change in podocyte phenotype (e.g. foot processes effacement) and/or podocyte number alteration.

Although non-invasive tests, such as urinary and serological analysis are informative in glomerular diseases, the definitive diagnosis in most nephrotic syndromes is a renal biopsy, where histological description on light microscopy defines the disease type. However, regardless of the initial cause of podocyte damage, characteristic abnormality in podocyte phenotype is best described by EM images. Typical changes are actin cytoskeleton reorganization of the foot processes, which leads to foot processes effacement and slit diaphragm disruption, characterized by flattening and simplification of the processes and eventual loss of the normal interdigitating pattern with the neighbouring podocytes.
At molecular level, causes of foot processes effacement and subsequent proteinuria include a number of factors. These are: changes in slit diaphragm structure or function, alteration in GBM and/or podocyte-GBM interaction, defects in structure or function of podocyte actin cytoskeleton, disruption of apical podocyte surface charge and modulation of various signalling pathways and transcription factors.

It is important to note that effacement is not specific to one disease, instead is a common characteristic of podocyte injury of many forms. While the early structural changes in foot processes effacement and slit diaphragm disruption in podocyte morphology are reversible, persistent injury leads alteration of podocyte number and high risk of developing severe and progressive glomerular damage (Kriz et al., 1998).

Glomerular diseases in which there is a reduction in podocyte number include membranous nephropathy, diabetic nephropathy, classic FSGS, amyloid and aging (Shankland S 2006). While diseases associated with dysregulated podocyte proliferation and an increase in number include HIV-associated nephropathy and collapsing glomerulopathy (Barisoni et al., 2000, Shankland et al., 2000). On the other hand, minimal change disease tend to have normal podocyte number.

In the majority of cases, the persistence of podocyte injury can cause detachment of the cells from the GBM, cell death via either apoptosis and/or necrosis. The denuded GBM from podocyte loss results in proteinuria, in order to rescue and compensate the damage, the bare GBM comes into contact with Bowman’s capsule to form synachiae (adhesion). However, severe damage results in subsequent development of sclerotic lesion which eventually leads to glomerulosclerosis and ESRD (Kriz & LeHir, 2005).
1.3 Treatment of proteinuric glomerular diseases and outcome

Many kidney diseases that ultimately lead to ESRD originate within the glomerulus and are associated with proteinuria. Current treatments of glomerulopathies are limited and offer partial cures, which often cause adverse side effects. Regardless of the cause of various renal diseases, treatments often aim to lower blood pressure, which has been shown to be beneficial. However, more importantly one of the clinical goals is to reduce proteinuria, as lowering proteinuria to achieve complete or partial remission in nephrotic syndrome is associated with prolonged renal survival and slower rate of kidney disease progression and these benefits were demonstrated even in patients who are not hypertensive (Lewis et al., 1993, Ruggenenti et al., 1999, Ruggenenti et al., 1998, Sarafidis et al., 2007).

Various reasoning has been applied to justify the additive blood-pressure-independent benefits. These include the likely contribution of reduced/normalised intraglomerular (not systemic) pressure via removal of the tonic constrictor effect of angiotensin II on efferent arterioles; the controversial argument about decreased pathological effect of proteinuria itself; and the likely direct improvement in the permselective properties on the glomeruli (independent of changes in glomerular haemodynamics) (Gansevoort et al., 1993, Heeg et al., 1991, Hoffmann et al., 2004).

I hypothesise that the beneficial effects are mainly mediated directly via the podocytes, as the cells play key roles in the permselectivity of the glomerular filtration barrier described in earlier sections. Below I will cover a number of established therapies used in chronic nephropathies that have been shown to have direct effects on podocytes.
1.3.1 Podocyte effects of established therapies

Angiotensin blocked by ACEi and ARB

Angiotensin II (Ang II) blockade is one of the mainstays treatments of proteinuric diseases. On the basis of large randomized clinical trials, ACEi and ARB therapy have developed into the most important antiproteinuric and renoprotective interventions (Reiser & Mundel, 2007). Although their blood pressure-lowering effect is likely to be beneficial, clinical trials demonstrated additional beneficial effects of ACEi and ARB that were blood-pressure independent (Bakris et al., 2004, Lewis et al., 1993).

In a study of diabetic nephropathy, Lewis showed that despite achieving similar blood pressure control, the ACEi captopril was more effective in reducing protein excretion than other hypertensive agents, and that this was correlated with reduced renal disease progression (Lewis et al., 1993). The additional benefit of ACE inhibition was also shown in non-diabetic chronic kidney disease by others (Ruggenenti et al., 1999, Ruggenenti et al., 1998, Sarafidis et al., 2007), as well as in a meta-analysis (2008) of randomized trials (Kunz et al., 2008).

Evidence supporting the notion that ACEi and ARB have direct effects on the local renin-angiotensin system in the glomeruli on podocytes is substantial (Durvasula et al., 2004, Reiser & Mundel, 2007). It is known that increased levels of angiotensin II in podocyte for example by exposure to mechanical stretch and hyperglycemia, have a number of deleterious effects. These include increased podocyte apoptosis (Ding et al., 2002, Durvasula et al., 2004), actin rearrangement, increased VEGF synthesis, increased TGF-β (Chen et al., 2005), reduction of ZO-1 and nephrin levels leading to proteinuria (Blanco et al., 2005) and alteration in calcium and cyclic adenosine monophosphate (cAMP) signalling. In addition, overexpression of angiotensin II type 1 (AT1) receptor in podocytes have been demonstrated to cause marked glomerulosclerosis (Hoffmann et al., 2004). Thus, inhibiting angiotensin II production or its receptor with angiotensin converting enzyme inhibitor (ACEi) or
angiotensin II type 1 receptor blocker (ARB) respectively, can potentially abrogate many deleterious effects on podocytes, thereby reducing the response to injury.

In addition, the inhibition of renin-angiotensin system has been associated with restoration of nephrin expression in both experimental and human diabetic nephropathy (Wolf et al., 2005). The expression of nephrin was retained at the slit diaphragm when rats with experimental membranous nephropathy were treated with an ACEi or with an ARB. Proteinuria was reduced in animal models of progressive nephropathy, suggesting that Ang II antagonism may preserve podocyte function (Benigni et al., 2001).

In a rat model of spontaneous proteinuria, the development of proteinuria was associated with the relocation of zonula occludens-1 (ZO-1) from the slit diaphragm to the podocyte cell body (Macconi et al., 2000). Following treatment with ACEi lisinopril, not only proteinuria was prevented but ZO-1 was also redistributed to the slit diaphragm.

Most direct support for a role of Ang signalling in podocytes as a cause of proteinuria and FSGS comes from a study with transgenic rats overexpressing an AT1 receptor selectively in podocytes (Hoffmann et al., 2004). Thus, these studies support the association between renoprotective effects of ACEi and ARBs and preservation of podocyte function.

However, controversially a recent study by Matsusaka T et al showed that protection from ARB or ACEi in a podocyte-induced glomerulosclerosis mouse model was podocyte AT1 receptor-independent (Matsusaka et al., 2010). First the authors showed that the urinary albuminuria was not attenuated in transgenic mice (*agtr1a* knockout/NEP25) not expressing AT1 receptors on podocyte when compared to the respective controls that did express the receptors after the immunotoxin administration. However, after treatment with ACEi or ARB but not hydralazine, amelioration in proteinuria and sclerosis was seen. In addition, continuous infusion of angiotensin II induced similar levels of microalbuminuria in both *agtr1a* knockout
and wild-type mice, suggesting that angiotensin inhibition can protect podocytes and prevent glomerulosclerosis development independent of podocyte AT1. Possible mechanism suggested by the authors include inhibitory effects on AT1 of other cells or possibly through mechanisms independent of AT1 that still need to be elucidated.

**Aldosterone antagonists**

Aldosterone is a major mineralcorticoid produced by the adrenal gland. The main function of aldosterone is to regulate the balance of potassium, sodium and water by influencing their absorption in the kidney as well as controlling the blood pressure. Aldosterone antagonists are diuretic drugs that antagonise the action of aldosterone at mineralcorticoid receptors. In the subtotal nephrectomy model and the salt-loaded spontaneously hypertensive rat model, the antiproteinuric effects of ACEi and ARB treatment were negated by exogenous aldosterone administration. In addition, Nemeth Z et al showed that mineralocorticoid receptor blockade using spironolactone provided additional nephroprotection over ACE inhibition monotherapy in a subtotal nephrectomy rat model (Nemeth et al., 2009). Although this beneficial effect is likely partly due to glomerular haemodynamics, direct effects mediated via podocyte are likely, as mineralcorticoid receptor is expressed in these cells (Shibata et al., 2007). Also evidence of increased oxidative stress and upregulated podocyte serum/glucocorticoid regulated kinase-1 (SGK1) have been demonstrated after exposure to aldosterone (Shibata et al., 2007), suggesting that antagonizing the receptor of this hormone may have direct and beneficial effects on podocytes (Berl, 2009, Brown, 2005).

**Corticosteroids**

Corticosteroids are the main classes of drugs used in some proteinuric diseases (e.g. minimal change disease and FSGS). On top of the immune-modulating effects, recent studies have shown that corticosteroids may have direct effects on podocytes. Glucocorticoid receptors have been demonstrated on podocytes and shown to translocate to the podocyte nucleus upon dexamethasone treatment (Ransom et al., 2005). Dexamethasone induced “kidney protective proteins” such as ciliary neurotrophic factor, αβ-crystallin and heat shock 27 in podocytes (Ransom et al.,
2005). In addition, glucocorticoids can also increase actin polymerization, and activate cytoskeleton-associated kinases in podocyte that are beneficial to the stabilization of the cell cytoskeleton.

Furthermore, dexamethasone has been shown to reduce podocyte apoptosis induced by puromycin aminonucleoside (PAN) and TGF-β (Wada et al., 2005). The authors showed that dexamethasone altered the subcellular localization of apoptotic inducing factor, suggesting that corticosteroids may reduce proteinuria and glomerulosclerosis by preventing a decrease in podocyte number.

These findings suggest that the antiproteinuric effect of glucocorticoids could at least in part be mediated by direct effects on the podocytes, independent of immunosuppression. Thus, corticosteroids may directly improve proteinuria and effacement through a variety of factors, and also reduce glomerulosclerosis by reducing podocyte apoptosis (Ransom et al., 2005).

**Calcineurin inhibitor**

Calcineurin is a serine/threonine phosphatase that is ubiquitously expressed. One of the main functions of calcineurin is to regulate the nuclear factor of activated T cells (NFAT) signalling. The immunosuppressive action of the calcineurin inhibitor cyclosporine stems from the inhibition of NFAT signalling in T cells (Crabtree & Olson, 2002).

Cyclosporine can induce remission of proteinuric glomerular diseases such as minimal change disease and FSGS (Meyrier, 2005b). However, the fact that cyclosporine can also reduce proteinuria in human (Charbit et al., 2007) and experimental Alport’s syndrome (Chen et al., 2003), which is a non-immunological disease, raised doubts about the earlier T cell immunity hypothesis. Furthermore, LPS-induced proteinuria can develop independent of T cells (Reiser et al., 2004), and mice lacking synaptopodin display impaired recovery from LPS-induced proteinuria (Asanuma et al., 2005).
One reason could be that podocytes are a direct target of cyclosporine, independent of NFAT inhibition in T cells. In fact, cyclosporine blocks the calcineurin-mediated dephosphorylation of synaptopodin, thereby preserving the phosphorylation-dependent synaptopodin-14-3-3β interaction (Faul et al., 2008). Preservation of this interaction, in turn, protects synaptopodin from CatL-mediated degradation and preserves a stable filtration barrier.

The inducible expression of CatL-resistant synaptopodin in podocytes can prevent not only LPS-induced degradation of synaptopodin and proteinuria but also the degradation of the other CatL target dynamin and ZO-1, a vital component of the slit diaphragm (Macconi et al., 2000). In addition, the inducible expression of dominant active calcineurin in podocytes is sufficient to cause the degradation of synaptopodin, thereby inducing proteinuria (Faul et al., 2008). These data revealed a calcineurin signalling pathway in podocytes and contributes to the maintenance of kidney filter function.

In contract to most other calcineurin-controlled signalling events (Aramburu et al., 2004, Crabtree & Olson, 2002, Horsley et al., 2008), the antiproteinuric effect of cyclosporine does not result from the inhibition of NFAT signalling. Taken together, the antiproteinuric effect of cyclosporin results, at least in part, from the maintenance of synaptopodin protein levels in podocytes, which safeguard against proteinuria by maintaining a stationary podocyte phenotype (Faul et al., 2008).

**All-trans-retinoic acid**

Podocytes express receptors for all-trans-retinoic acid (ATRA). Several studies have shown a role for ATRA in diabetic and non-diabetic proteinuric diseases (Oseto et al., 2003). Vaughan et al demonstrated that giving ATRA to rats with experimental podocyte disease significantly improved proteinuria, and that this was likely due to the prevention of decreasing nephrin and podocin expression (Vaughan et al., 2005). However, further studies are required to delineate the protective effects of ATRA on podocytes.
**Cyclin dependent kinase-2 inhibitors**

Cyclin dependent kinase-2 (CDK2) have been targeted by small molecule inhibitors such as roscovitine in mice with experimental anti-GBM disease characterized by podocyte proliferation (Griffin et al., 2003). The study showed that the CDK2 inhibitor roscovitine improved proteinuria and blood urea nitrogen compared to animals receiving vehicle. This was associated with a decline in podocyte proliferation. In a transgenic mouse model of HIV, (Nelson et al., 2003) showed a drastic improvement in renal function after treatment with the CDK2 inhibitor cyclacel. Of note, cyclacel is a small-molecule inhibitor of cyclin dependent kinases that can suppress HIV-1 gene expression by inhibiting RNA polymerase II activity on the HIV-1 promotor. Similar improvement in renal function was also observed in a rat model of experimental membranous nephropathy post cyclacel treatment.

**VEGF regulation**

The critical role of vascular endothelial growth factor (VEGF) in podocyte and glomerular endothelial cell biology has become evident in recent years. VEGF is a proangiogenic factor produced by podocytes in the glomeruli. Although it may function as an autocrine-like signal within the podocyte, the growth factor has major role in stimulating glomerular endothelial cells by promoting their survival and enhancing glomerular endothelial permeability. Increased VEGF activity has been associated with collapsing glomerulopathy, but inhibition of VEGF in cancer patients with anti-VEGF antibody (bevacizumab) or VEGFR antagonist has also been associated with the adverse effects of proteinuria, hypertension as well as development of thrombotic microangiopathy. In addition, a study in transgenic mice showed that only podocyte-derived (but not circulating) VEGF leads to the development of thrombotic microangiopathy and hypertension (Eremina et al., 2008). These data suggest that a tight regulation of VEGF levels is required for normal function of podocyte and endothelial cell that constitute two of three essential components of the glomerular filtration barrier. Therefore interventions to balance/normalise the levels of VEGF may be useful in certain glomerular diseases in the future.
**PPAR-gamma signalling**

Insulin sensitising agents such as peroxisome proliferator-activated receptor gamma (PPARγ) (e.g. pioglitazone) have been shown to enhance insulin sensitivity and may have the beneficial effects of reducing proteinuria and slowing progression in type two diabetic kidney diseases, independent of glucose control (Yang et al., 2009). Since insulin receptor has been demonstrated on podocyte, and glucose transporters (GLUT1, GLUT4) were expressed upon insulin signalling, the treatment with PPARγ agonist is likely to have direct effects on podocyte. Welsh GI et al (2010) further demonstrated that transgenic mice with insulin receptor specifically depleted on podocytes develop proteinuria, foot process effacement, GBM thickening and glomerulosclerosis with aging resembling features seen in diabetic glomerulopathy (Welsh et al., 2010), suggesting that cellular insulin resistance seen in type 2 diabetes may contribute to podocyte injury.

**Summary**

Research in the past 20 years to elucidate both molecular and cellular biology of the podocyte has greatly increased insights into many aspects of podocytes’ response to injury. The response to podocyte injury is complex and involves numerous processes, some of which overlap in several podocyte diseases, while others are specific. Therefore identification of specific mechanisms of podocyte injury in specific diseases will help future treatment to target the podocyte directly.
1.4 Purpose and design of the project

Unlike previous models of podocyte injury that involved use of toxin of uncertain specificity, such as adriamycin in mice (Guo et al., 2008, Zheng et al., 2006), puromycin in rats (Inokuchi et al., 1996), and possibly pamidronate toxicity and HIV infection in humans (Dijkman et al., 2006), our group developed a toxin receptor-mediated conditional podocyte knockout model. The principal behind this model is to allow and induce a graded specific podocyte injury in a dose-dependent manner after a single injection of diphtheria toxin.

We believe that disruption of the dynamic interactions between the podocytes sets off the vicious cycle of “podocyte-damage-damaging-podocytes”. It is my hypothesis that interventions that reduce the disruption by rescuing susceptible podocytes next to injured ones are potential therapies to restore podocyte phenotype and filtration behaviour, thereby protecting the kidney from progressive deterioration. Prevention of this damage, or ways to aid its recovery, could therefore be important to improving the management of human kidney diseases.

To address these questions, transgenic Podo-DTR mice expressing the human diphtheria toxin receptor (hDTR) on podocytes previously generated in our laboratory is an ideal model to test therapeutic interventions following targeted podocyte injury.

The project can broadly be divided into three parts:

1. Characterize the transgenic Podo-DTR mouse model and study the process of podocyte recovery in detail following injury by diphtheria toxin.

2. Test the model with known therapeutic agents such as ACE inhibitors, which have a proven protective effect on renal function in patients with proteinuria. However, not all patients respond fully, therefore additional agents are required. This leads to the third part of the project.
3. Carry out intervention studies to test potential podocyte protective drugs.

If successful, the model would permit a graded podocyte injury, which can be combined with other forms of pathology in the future and permit testing of measures to protect or repair podocyte injury. The ultimate objective of this project is to identify new compounds or combination therapy to reduce podocyte damage and preserve renal function.
CHAPTER 2: Materials and Methods

2.1 Transgenic Podo-DTR line
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- Genomic DNA extraction
- pIN PCR reaction
- Agarose gel electrophoresis

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- RNA extraction
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2.4 Urine analysis
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- 24h urinary protein:creatinine ratio
- Improved 24h urinary albumin:creatinine ratio (ACR)

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2.6 Blood pressure: tail cuff plethysmography

2.7 Drug administration
- ACEi captopril and control drinking water
- ETaRA sitaxsentan and control food
Materials and Methods

2.1 Transgenic Podo-DTR (pIN) line

Generation of Podo-DTR (pIN) mice
Transgenic mice with podocytes fully susceptible to diphtheria toxin were made by expressing the human diphtheria toxin receptor (hDTR), also known as human heparin binding epidermal growth factor receptor, (hHB-EGFR) under the control of a fragment of the nephrin promoter. This murine 1.25kb nephrin gene fragment (Moeller et al., 2002) was generated from murine genomic DNA.

Oligonucleotide polymerase chain reaction (PCR) primers for mouse nephrin gene with added NotI and BamHI restriction sites were used to clone the gene fragment (ATGGCCCAGGGATTCAGGTGC' and GCTTGGACCCAGTGTGAACTC). This nephrin fragment was used to replace the albumin promoter in the pMS7 plasmid, which was kindly donated by Dr Saito (Saito et al., 2001).

Transgenic mice were generated by male pronuclear microinjection of murine fertilized ova with the linearised pIN plasmid (see chapter 3 Fig. 3.1) (Ihmoda Ihmnoda PhD thesis 2006, University of Edinburgh). The resulting plasmid consisted of the murine nephrin promoter-fragment and the human HB-EGFR cDNA (Moeller et al., 2002). Expression driven by this nephrin promoter fragment was shown by Moeller et al (2002) to achieve podocyte specific expression in glomeruli without detectable expression outside the kidney by chemiluminescence assay.

PCR primers for Podo-DTR mice detection
Four transgenic founders (Podo-DTR or pIN 21, 47, 57, 65) showing positive PCR results for the hDTR transgene were generated by a previous research fellow in our laboratory.
The following PCR oligonucleotide primers were purchased from MWG Oligo Synthesis and used to probe for the expression of the following transgenes in mice (Fig. 2.1).

1. Murine nephrin gene/rabbit ß-globin intron genotyping (band size: 243bp)
   - pINPCR-For1: 5’-GGA AGA GAG AAG GGC GAG TT-3’
   - pINPCR-Rev1: 5’-GGG TCC ATG GTG ATA CAA GG-3’

2. hDTR gene genotyping (band size: 228bp)
   - pINPCR-For2: 5’-GGT GGT GCT GAA GCT CTT TC-3’
   - pINPCR-Rev2: 5’-GCT TGT GGC TTG GAG GAT AA-3’

The stock concentration of primers was at 100µM. These were diluted at 1 in 10 dilution to give a working solution concentration of 10µM.

**Genomic DNA extraction**

Mice ear notches from individual animal were incubated with 200µl lysis buffer (see lysis buffer reagents for details below) and 5µl proteinase K (Roche Diagnostics, Ref. No: 03115828001) at 55°C in a hybridization incubator (Techne Hybridiser HB-1) overnight. The samples were spun at 13,000 RPM for 10 minutes (min) in a microfuge. The supernatants were transferred to new 1.5ml eppendorfs and 200µl isopropanol was added per tube. The samples were mixed by inversion and left at room temperature for 30-60 min, then spun at 13,000 RPM for 10 min. The supernatants were discarded, leaving the pellets behind in the eppendorfs. At this point, 200µl ice-cold 70% ethanol was added and spun at 13,000 RPM for 5 min to wash the DNA. The supernatant containing ethanol was removed leaving the pellet to air dry for 5-10min. 200µl 10mM Tris was added to each DNA pellet and incubated at 37°C for 2h or 50°C for 20 min to 1 hour (h) to help the DNA to dissolve fully.

**Lysis buffer reagents:**

0.2% SDS (10ml 20% stock)
0.1M Tris pH 8.5 (100ml 1M stock)
5mM EDTA (100ml 0.5M stock)
200mM NaCl (25ml 5M stock)
**pIN PCR reaction**

PCR reactions were performed using puRe Taq Ready-To-Go PCR Beads (Amersham Biosciences, Ref. No: 27-9559-01). These premixed 0.2ml thin-walled tubes contained buffers, dNTPs, Taq DNA polymerase enzyme (2.5 units), stabilizers and bovine serum albumin (BSA). The PCR master mix reaction plus DNA (25µl per reaction) was prepared as detailed below for pINPCR1 (Murine nephrin gene/rabbit β-globin intron genotyping) and pINPCR2 (hDTR gene genotyping). Once mixed and dissolved, each tube should contain 200µM in 10mM Tris-HCl (pH 9 at room temperature) of each dNTPs, 50mM KCl and 2.75mM MgCl₂.

<table>
<thead>
<tr>
<th>PCR for pINPCR₁ Product</th>
<th>PCR for pINPCR₂ Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>2µl</td>
</tr>
<tr>
<td>pINPCR-For1</td>
<td>pINPCR-For2</td>
</tr>
<tr>
<td></td>
<td>1µl</td>
</tr>
<tr>
<td>pINPCR-Rev1</td>
<td>pINPCR-Rev2</td>
</tr>
<tr>
<td></td>
<td>1µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>dH₂O</td>
</tr>
<tr>
<td></td>
<td>21µl</td>
</tr>
<tr>
<td></td>
<td>25µl total</td>
</tr>
<tr>
<td></td>
<td>25µl total</td>
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The following PCR program was used for the reaction:

- **Hot start:** 110°C
- **Initial denaturation:** 75°C 10min
- **3 Step cycling (30 cycles):**
  - **Denaturation:** 95°C 1min
  - **Annealing:** 50°C 1min
  - **Extension:** 72°C 1min 30sec
- **Final extension:** 72°C 10min
- **Hold** 4°C 10min or forever
Figure 2.1. Map of mice genotyping primers.

1F = pINPCR-For1, 1R = pINPCR-Rev 1, 2F = pINPCR-For2, 2R = pINPCR-Rev2

Schematic diagram adapted from Dr Ihmoda PhD thesis 2006 (University of Edinburgh). Diagram not in scale.
**Agarose gel electrophoresis**

Agarose powder (Molecular biology grade agarose, Cambio) (0.75g) was weighed in a 250ml conical flask, to which 50ml Tri-borate-EDTA (TBE) buffer (0.5M) was added (see TBE reagents details below) and melted by heat in the microwave at maximum power for 1 minute 30 seconds to obtain 1.5% agarose gel. After cooling down the melted gel to approximately 40°C, 5µl ethidium bromide was added (DNA chelating agent) in order for the DNA to be visualised by ultraviolet (UV) transluminator. The gel was poured into the small gel electrophoresis apparatus and left to set and polymerise at room temperature for 20-30 minutes. Once set, 50ml of 0.5M TBE buffer was added to the gel tank. Appropriate ØX174 DNA Hae III ladder (NBL Gene Sciences, Ref. No: 031201) and DNA samples (mixed with loading dye at 5 to 1 ratio i.e. 10µl sample plus 2µl loading dye) were loaded in the wells and run for 35 minutes at 90v; 25w; 75mA settings. The gel was visualised by UV transluminator to look for the appropriate band size and photographed to record the results (see Fig. 3.2 of chapter 3).

**5M stock TBE buffer (500ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Tris base</td>
<td>27g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>13.8g</td>
</tr>
<tr>
<td>0.5M Na&lt;sub&gt;2&lt;/sub&gt;EDTA pH8</td>
<td>10ml</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>449.2ml</td>
</tr>
<tr>
<td>Final volume</td>
<td>500ml (adjust final volume to 500ml with dH&lt;sub&gt;2&lt;/sub&gt;O)</td>
</tr>
</tbody>
</table>

Working TBE concentration (0.5M) was obtained by diluting 1 in 10 with distilled water from the stock TBE (5M) buffer.
2.2 Histology and microscopy

Sample collection
Kidneys were decapsulated and cut sagitally in half to allow better perfusion of various fixatives: 10% neutral formalin, methyl Carnoy’s fixative (or methacarn), 4% paraformaldehyde (PFA). In addition 1mm cubed kidney cortex were fixed in 700mOsm Karnovsky’s glutaraldehy fixative for future electron microscopy examination. Kidneys were also snap frozen in liquid nitrogen for cryostat sections. Samples were fixed overnight at 4ºC and subsequently paraffin blocked for long-term storage.

Sections of 2-4µm formalin (10%) fixed tissues were cut and stained with Periodic acid Schiff (PAS), Haematoxylin and Eosin (H&E) and Silver Jones’ staining for histology examination.

Glomerulosclerosis score
Glomerulosclerosis score were carried out on 2µm thick PAS-stained sections using light microscopy at a magnification of x400 (Olympus CX40). 100 glomeruli per animal were scored for glomerulosclerosis adopting the semi-quantitative scoring system proposed by (el Nahas et al., 1991). The scoring system used for earlier experiment (ACEi captopril experiment) was as follows: 0, normal glomerulus or no lesion; 1, <50% sclerosis; 2, 50-100 sclerosis of glomerular tuft area. For later experiments (i.e. ETaRA sitaxsentan), a more detailed scoring system was adopted ranging from score 0-5: 0= 0%, 1= <25%, 2= 25-50%, 3=>50-75%, 4= >75-95%, 5=100% sclerosis. This system also allowed scoring groups to be added up together when analysing data if necessary. Sclerosis index score for individual animal was obtained using the following formula:
Sclerosis index (SI) =: [(0 x n)+ (1x n)+ (2 x n) + (3 x n) + (4 x n) + (5 x n)] / 100)
**Immunohistochemistry (IHC) staining**

Methacarn fixed paraffin-embedded kidney tissues at 3µm were used for collagen (Col) I, IV and podoplanin immunochemical staining. While formalin fixed kidney tissues were used for anti-Wilms’ tumour 1 (WT1) and anti-HB-EGF staining. After deparaffinizing the sections in Histo-clear™ (National Diagnostics), the slides were hydrated through a series of graded ethanol concentration (100%, 90%, 75%, 50%) to distilled water. Only the anti-WT1 and anti-HB-EGF slides were treated with the antigen retrieval solution Borg Decloaker RTU (Ref. BD1000 Biocare Medical) for 2 minutes after reaching pressure in the microwave.

The sections were blocked with 2-3% hydrogen peroxide, Avidin/biotin blocking kit (Vector laboratories, Ref. No: SP-2001) and protein block serum-free ready-to-use (Dako Ref. No: X0909) for non-specific endogenous bindings according to the manufacturer’s instructions. The antibodies were diluted in phosphate buffered saline (PBS) alone or containing 0.1% bovine serum albumin (BSA) (Sigma Life Sciences, Ref. No: A2153-1003). The primary antibodies (Abs) were incubated at room temperature (RT) for 1h or overnight at 4ºC, while the secondary biotinylated antibodies were incubated for 30-60 min as detailed below.

**Anti-Collagen I**
Primary goat anti-collagen I UNLB antibody (SouthernBiotech, Ref. No: 1310-01, 0.4mg/ml) at 1:100 dilution incubated for 30-60 min at RT.
Secondary biotinylated rabbit anti-goat IgG (H&L) affinity purified (Vector Laboratories, Ref No: BA-5000, 1.5mg/ml) at 1:300 dilution incubated for 30min at RT.

**Anti-Collagen IV**
Primary rabbit anti-collagen IV antibody (Millipore, Ref. No: AB756P, 1mg/ml) at 1:100 dilution incubated for 30-60 min at RT.
Secondary biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories, Ref. No: BA-1000) at 1:300 dilution incubated for 30min at RT.
Anti-podoplanin
Primary hamster monoclonal anti-podoplanin/gp 36 mouse antibody (Abcam, Ref.: No: ab11936) at 1:3500 dilution incubated for 30-60 min at RT.
Secondary biotinylated goat anti-hamster IgG (H+L) (Vector Laboratories Ref. No: BA-9100) at 1:300 incubated for 30 min at RT.

Anti-WT1
Primary rabbit polyclonal anti-WT1 (C-19): sc-192 (Santa Cruz Biotechnology, 200µg/ml) at 1:50 dilution for 1h at room temperature.
Secondary biotinylated swine anti-rabbit antibody (Dako, Ref. No: E0353) at 1:400 dilution incubated for 30 min at RT.

Anti-HB-EGF
Primary goat anti-human HB-EGF IgG (R&D Systems Ref. No: AF-259-NA, 0.25mg/ml) at 1:50 dilution for 1h at room temperature
Secondary biotinylated rabbit anti-goat IgG (H&L) antibody (Vector Ref. No: BA-5000) at 1:400 dilution incubated for 30 min at RT.

Immunoperoxidase staining
Immunoperoxidase (brown) staining was performed according to the RTU Vectastain Elite ABC kit (Vector laboratories Ref. No: PK-7100) and developed with peroxidase detection system using the substrate diaminobenzidine (DAB) (ImmPACT™ DAB substrate Vector Laboratories Ltd Ref. No: SK-4105) for all the immunostainings.

Quantification of Col I, IV, PSR using CS3/CS5 Photoshop
Photos of kidney cortex of Col I, IV and picrosirius red (PSR) staining were taken using the program QCapture Pro at x200 magnification. An initial running mean of the PSR and Col IV staining was carried out using 44-50 photos per animal (n=3) (Fig. 2.2a-b), and 30 photos/slide (each slide representing one animal) was judged suitable to give optimal and representative result for analysis.
On average 30 (or at least >25) photos of the kidney cortex per animal were taken, and the staining analysed using Photoshop CS3/CS5. The specific brown staining for Col I, IV and red staining for PSR were calibrated at the start for the program to pick up the selected staining and allocated pixels number for quantification. These calibrated tester files were applied to the remaining slides for quantification. Percentage pixels of each photo were calculated against total pixels value of photo (196608 pixels), and mean for each animal calculated.
Figure 2.2. Running mean of (a) picrosirius red (PSR) (b) collagen IV staining quantification using Photoshop CS3. At 30 photos/slide (each slide representing one animal) was judged suitable to give optimal and representative result for analysis.
Quantification of podocyte number using anti-WT1 staining

Podocyte numbers were quantified by counting WT-1 positively stained cells of 30 (sitaxsentan experiment 8) or 50 (captopril experiment 3) consecutive glomerular cross-section (GCS) per animal at x400 magnification (Olympus CX40). Examination of a minimum of 30 glomeruli per slide was judged sufficient and ideal for podocyte number quantification based on the running mean that was carried out previously (Fig. 2.3). The mean podocyte per glomerulus was calculated for each animal. Glomeruli were sampled by moving the stage of view at x400 magnification and following a path from cortex to cortex and back again, moving 1.5-2.0 fields laterally each traverse evaluating all glomeruli that intersected the path by at least one third of their cross section area. Glomeruli were only considered if their entire profile was in the field of view.

Glomerular area measurement

Areas of glomeruli were measured by using ImageJ 1.4r software. The glomerular tuft of 30 or 50 consecutive GCS per animal were manually traced using the computer mouse and measured on pictures taken at x100 magnification using QCapturePro (QImaging Micropublisher 3.3 RTV, Zeiss Axioskop, Germany). The mean glomerular area was calculated to represent each animal.
Figure 2.3. Running mean of WT-1 stained podocyte of control and experimental Podo-DTR mice injected with diphtheria toxin at 1ng/g body weigh. GCS, glomerular cross section; mo, month; Tg, transgenic; wk, week; wt, wild-type; WT-1, Wilm’s tumour-1.
2.3 Nucleic acids methods

**Tissue collection and process for RNA extraction**

Fresh tissues of mouse (Tg 47.1276, WT 57.1283, C57/BL6) organs (kidney, liver, spleen, pancreas, stomach, lung, heart, brain) were collected at necropsy in PBS for immediate RNA extraction or RNAlater (Sigma, Ref. No R0901-10ml) for storage and delayed extraction (up to 1 week in the fridge). Approximately 50-100g tissue was added to 1ml Trizol (Sigma 99%+) in individual 2.0ml eppendorf containing a metal ball for tissue homogenization using tissue lyser. The samples were pulsed for 2-3min at 25Hz (repeating the process if necessary) until the tissue was evenly homogenized. Samples could be used directly for the next step or stored at -80°C for later RNA extraction (see details below).

**RNA extraction**

Homogenized (thawed) tissue samples were mixed with 200µl chloroform (per 1ml Trizol) and centrifuged for 20min at 13,000 RPM at 4°C. The top clear phase solution containing the RNA was transferred to the respective autoclaved clean tubes and 500µl isopropanol (IPA) was added to each tube. The samples were vortexed briefly and stored in the fridge for 15min at 4°C, then centrifuged at 13,000 RPM for 25 min at 4°C. Excess IPA were discarded carefully using the pipette, leaving the RNA pellet at the bottom of the tube. 1ml ice-cold 75% ethanol was added to each sample to wash and placed at -80°C for 1h, then centrifuged for 15min at 13,000 RPM at 4°C. Excess ethanol was discarded and the tubes were left to air dry for 5-10min. Diethylpyrocarbonate (DEPC)-treated water (20µl or 50-100µl if big pellet was present) (Sigma Ref. No 95284 for molecular biology) was added to each tube and samples were placed on a heat block at 55°C for 10min to dissolve the RNA. The concentration of RNA samples was measured using Nanodrops software (Program ND-100 V3.6.0).

**Reverse transcriptase PCR: cDNA synthesis from mRNA**

A final volume of 20µl per reaction should contain: 6.5µl master mix (see details below for reverse transcriptase (RT) master mix, 8.5µl DEPC distilled water (dH₂O),
5μl (containing 1μg) RNA. After mixing the reagents, the samples were run using
the reverse transcriptase PCR machine (BioRad), selecting the reverse transcriptase
reaction program detailed below.

**Reverse transcriptase PCR program**

1. 10 min at 20ºC
2. 20 min at 37ºC
3. 50 min at 99ºC

<table>
<thead>
<tr>
<th>Reverse transcriptase master mix</th>
<th>Reverse transcriptase PCR reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 2μl</td>
<td>Master mix 6.5μl</td>
</tr>
<tr>
<td>dNTPs 2μl</td>
<td>dH2O 8.5μl</td>
</tr>
<tr>
<td>RNase inhibitor 0.5</td>
<td>RNA (1μg) 5μl</td>
</tr>
<tr>
<td>RT enzyme 1μl</td>
<td></td>
</tr>
<tr>
<td>Oligo DT 1μl</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>6.5μl</td>
<td>20μl</td>
</tr>
</tbody>
</table>

**DNaseI treatment using TURBO DNA-free™ Kit (Applied Biosystems)**

An additional step using DNaseI treatment (TURBO DNA-free™ Kit, Applied
Biosystems) was included to remove any DNA contamination from RNA
preparations.

Briefly, a mixture of DNAase (1μl), 10x buffer (2μl) and water (17μl) were added
to the RNA pellet (after removal of ice-cold ethanol) and mixed gently making a final
volume of 20μl per sample. The samples were incubated at 37ºC for 20-30 min.
DNase inactivation reagent (2μl/sample) was added and mixed and incubated for 5
min at RT. The samples were then centrifuged at 10,000 RPM for 1.5min,
supernatants containing RNA were transferred to fresh tubes (leaving the white pellet
behind).

**cDNA oligonucleotide primers**

Complementary (c)DNA oligonucleotide primers of hHB-EGF (or hDTR) for qPCR
were designed using Serial clone 1.3 program: For 1 5’-
TGGGGCTTCTCATGTTTAGG-3’, Rev1 5’-CATGCCCAACTTCACTTTCTC-3’. Working solution concentration of primers was at 10µM (diluted 1 in 10 in diethylpyrocarbonate (DEPC) water from 100µM original stock concentration.

**Quantitative PCR (qPCR) or real-time PCR (RT-PCR)**

Two detection systems were tried (SYBRGreen and TaqMan) to measure hDTR gene expression, however only TaqMan gene expression assay was successfully applied as detailed below. qPCR plates (MicroAmp Fast optical 96-well reaction 0.1ml, Applied Bioscience ABI Ref. No. 4346906) were prepared with appropriate positive (human placenta) and negative (unrelated C57BL/6 mice organs, master mix with water or water alone) controls including housekeeping eukaryotic 18S or glycerldehyde-3-phosphate dehydrogenae (GAPDH) genes.

Quantification of the relative hHB-EGF mRNA abundance was performed using the 7500 Fast Real-time PCR system (Applied Biosystems) using TaqMan Fast Universal PCR Master mix (ABI Ref. No 4352042), with sample cDNA in a final volume of 10µl per reaction. TaqMan probes (Applied Biosystems) used were as follows: human HB-EGF spanned exons three to four (Ref. No. Hs00181813_m1); mouse GAPDH was within exon 1 (Ref. No Mm99999915_g1); human eukaryotic 18S rRNA single exon assay (Ref. No Hs99999901_s1). All data were from 50ng/µl sample cDNA with the exception of the human placenta (10ng/µl) that was obtained from Edinburgh Reproductive Tissue BioBank Ref. No. ERTBB-038). For these experiments, RNA was extracted as described previously, then reverse-transcribed to cDNA.
hDTR and 18S reactions were prepared as detailed below with a final volume of 10µl (1µl cDNA and 9µl master mix (MM) per sample per well).

- **a) hDTR target gene reaction**
  - TaqMan fast buffer: 5.0µl
  - 5µl DEPC H2O: 3.5µl
  - TaqMan assay mix: 0.5µl
  - (primers & probes): 0.5µl
  - cDNA: 1.0µl

- **b) 18S housekeeping gene reaction**
  - TaqMan fast buffer: 5.0µl
  - DEPC H2O: 3.5µl
  - TaqMan assay mix: 0.5µl
  - (primers & probes): 0.5µl
  - cDNA: 1.0µl

The plate was analysed using Applied Biosystems 7500 Fast Real-time PCR System using the following thermal cycler program.

**Thermal Cycler Profile**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Repetitions</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 x</td>
<td>95°C</td>
<td>0:20min</td>
</tr>
<tr>
<td>2</td>
<td>40 x</td>
<td>95°C</td>
<td>0:03min</td>
</tr>
<tr>
<td>3</td>
<td>1 x</td>
<td>60°C</td>
<td>0:30 min</td>
</tr>
</tbody>
</table>
2.3 Urine analysis

*Dipsticks analysis*

Fresh urine samples from mice naturally voiding their bladder were collected and tested with dipsticks (Multistix® 10 SG) for protein (albumin) and blood on a scale from zero (trace) to ++++ (see chapter 3 Table 3.1 for reference). Although the test is very sensitive, it only provided semiquantitative measurements, as it did not take into account the volume of the sample, thus although quick and easy to use, the method has its limitation.

**24h urinary protein:creatinine ratio**

Urine samples were collected in metabolic cages over a period of 24h. Samples were stored at 4°C or -20°C (if longer storage required). Prior to analysis, samples were spun at 13,000 RPM for 1 minute, diluted 1 in 20 with PBS with final volume of 200µl. Urine protein concentrations were measured by Pyrogallol red molybdenum complex reaction. Earlier analysis (including captopril experiment 3), urine and serum creatinine concentrations were measured by the Jaffe reaction. This method involved kits purchased from Alpha Laboratories (Poole UK), and all assays were adapted for use on a Cobas Fara Centrifugal Analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK) according to manufacturer’s instructions.

*Improved 24h urinary albumin:creatinine ratio (ACR)*

To overcome the sex differences between male and female mice producing different amount of protein in their urine (i.e. male mice tend to have higher baseline total protein than female), an immunoturbidimetric assay was developed to measure urinary mouse albumin (instead of protein) concentration using a commercial diagnostic Microalbumin Kit (Olympus Diagnostic Ltd, Watford, UK). Also Jaffe reaction was substituted with creatinase reaction to obtain more accurate measurement of urine and serum creatinine levels. Cobas Fara centrifugal analyser was adapted and used for individual assays as described above.
2.5 Blood analysis: serum creatinine, urea, albumin

Blood samples were collected from terminally anaesthetised mice prior to culling, centrifuged at 10,000 RPM for 10 min and the respective serum samples were stored at -20°C until analyzed. Serum urea, creatinine and albumin were measured by urease, creatinase reaction (Alpha Laboratory Ltd, Poole, UK), while albumin was measured by immunoturbidimetric assay using a commercial diagnostic Microalbumin kit (Olympus Diagnostic Ltd) as described earlier for urine albumin. All assays were adapted for use on a Cobas Fara Centrifugal Analyser (Roche Diagnostics Ltd) according to manufacturer’s instructions.

2.6 Blood pressure: tail cuff plethysmography

Mice blood pressure was taken by using the non-invasive tail cuff plethysmography (Fig. 2.4). Conscious mice were first trained to be handled and restrained in the mouse restrainer prior to experimental data collection. A cuff was used on the tail of the mouse to detect the pulse and measure the blood pressure. At least 3 or 4 systolic blood pressure measurements were taken at any one setting per mouse and mean systolic blood pressure was calculated. Heart rate of mice was also recorded for stress level monitoring.
Figure 2.4. Systolic blood pressure measurements of mice were taken by using tail cuff plethysmography. Conscious mice were first trained to be handled and restrained in the mouse restrainers prior to experimental data collection. A cuff was used on the tail of the mouse to detect the pulse and measure the blood pressure. At least 3 or 4 systolic blood pressure measurements were taken at any one setting per mouse and mean blood pressure were calculated. Heart rate of mice was also recorded for stress level monitoring.
2.7 Drug administration

Treatment drugs were given orally whenever possible either in their drinking water or food diet. ACEi captopril was dissolved in distilled water and given at 200mg/L (or 32mg/Kg/day). While ETaRA sitaxsentan was initially given in drinking water at 350mg/L (40mg/Kg/day). However, due to formation of precipitate when captopril and sitaxsentan were mixed together in the drinking water, the administration method for sitaxsentan was changed. Instead of giving sitaxsentan in drinking water, the drug was added to the food. Control food was made using the same method but omitting the drug as detailed below.

ACEi captopril and control drinking water
Captopril (Sigma Life Sciences, Ref. No: C8856-5G) powder was weighed and added to distilled water at 200mg/L concentration and placed on a shaker rotator at 4ºC to fully dissolve (the solution is colourless with a distinct drug smell). The solution is stable for up to 14 days at room temperature (Schlatter J et al 1997). Water bottles for animals were filled with an estimated weekly consumption (plus 20% extra solution) for individual cages according to the number of mice housed per cage. They were wrapped in tin foil to protect from light and were changed weekly. Control water consisted of normal drinking water given routinely at the animal house.

ETaRA sitaxsentan and control food
Sitaxsentan (Compound number: UK-372882 (AKA PF-1228305-02), Pfizer Inc) was added to the food diet of mice. Normal mice diet consisted of solid food pellets, therefore it was not possible to mix or coat the drug evenly to allow appropriate monitoring of drug intake. Using dry ground powder chow to add in the drug was also an issue as the method involved high dispersal and wastage of food/drug. Since sitaxsentan provided was limited and expensive, special food diet aliquots were made weekly adapting the protocol previously used by Nicholas Kirkby (PhD thesis 2009, Edinburgh University).
To prepare 1kg of sitaxsentan food at 110mg/kg (or 40mg/Kg/day assuming that mice weigh 31g/mouse and eat 11.5g/mouse/day), the following recipe was used. 38g of gelatine (Dr Oetken beef gelatine) was dissolved in 302ml boiling water and cooled down to approximately 40° C prior to use. Sitaxsentan (110mg) was dissolved in 300ml distilled water and added to 360g powdered chow and mixed homogenously. This was mixed in well with the dissolved gelatine to bind the food together to obtain 1kg of food. Food aliquots ranging from 20-50g were made in batches and stored in 60ml universal containers (VWR International, Ref No: 2162612-X) in -20°C freezer to be changed and given daily to mice for the allocated time. Control food was made using the same protocol by simply omitting the drug.
CHAPTER 3: Characterisation of the transgenic Podo-DTR mouse model

3.1 Background
   • Breeding of transgenic Podo-DTR mice

3.2 Untreated transgenic animals
   • PCR genotyping
   • Antigen expression of human HB-EGF receptor (hDTR)
     ▪ Immunohistochemistry of hDTR staining showed positive staining in the glomeruli
     ▪ Real-time quantitative PCR of hDTR gene expression tested positive for transgenic but not wild-type mice

3.3 Transgenic lines are susceptible to diphtheria toxin
   • Mode of action of diphtheria toxin
   • Line and toxin susceptibility

3.4 Detailed dose ranging and response
   • Podo-DTR mice develop podocyte injury after diphtheria toxin injection
   • Albuminuria and serum urea peak at week two
   • Morphological glomerular damage is progressive
   • Podocyte number falls and remain low
   • Podo-DTR line 47 is much more sensitive to diphtheria toxin than line 57

3.5 No signs of liver damage in serum liver function test and liver histology in mice with toxin-induced renal failure

3.6 Discussion
**Characterisation of the transgenic Podo-DTR mouse model**

**3.1 Background**

The transgenic Podo-DTR mice also referred to as pIN mice were created by a previous research fellow in our laboratory (Ihmoda Ihmoda PhD thesis, 2006, University of Edinburgh). They had been genotyped but not tested for toxin sensitivity. However, the aim was to generate transgenic mice expressing the diphtheria toxin receptor (DTR) specifically on podocytes allowing targeted cell injury when injected with the diphtheria toxin.

Murine cells are naturally 1000 fold less susceptible to diphtheria toxin that human cells (Mitamura et al., 1995). Transgenic mice with podocytes fully susceptible to diphtheria toxin were made by expressing the human diphtheria toxin receptor (hDTR), also known as human heparin binding epidermal growth factor receptor (hHB-EGF), under the control of a fragment of the nephrin promoter (Fig. 3.1) that was previously shown to be expressed solely in podocytes when coupled to β-galactosidase (Moeller et al., 2002). This technique was first applied to hepatocytes (Saito et al., 2001) but has subsequently been successfully applied to a number of other cell types (Duffield et al., 2005, Wharram et al., 2005).

Unlike previous models of podocyte injury that involved use of toxins of uncertain specificity, such as adriamycin in mice (Guo et al., 2008, Zheng et al., 2006), puromycin in rats (Inokuchi et al., 1996), and possibly pamidronate toxicity and HIV infection in humans (Dijkman et al., 2006), our Podo-DTR model permits a graded specific podocyte injury that can be delivered by a single injection of diphtheria toxin. Therefore, the model will allow investigation of mechanism of podocyte role and action, as well as intervention studies with potential therapeutic agents after induction of targeted podocyte injury.

**Breeding of transgenic Podo-DTR mice**

Transgenic Podo-DTR (pIN) mice expressing hDTR receptor were generated as described in the Materials and Methods section. Briefly, the DNA fragment was
injected into F2 embryos, derived from B6CBAF1 mice. These mice originated from Harlan UK, and were offspring of a cross between the C57BL/6JOlaHsd inbred female and the CBA/CaOlaHsd inbred male. These mice were intercrossed to produce F2 embryos, which were then used for microinjection. Subsequent breeding used wild type littersmates or in house C57BL/6 strains mice between 2006 and 2011.

3.2 Untreated transgenic animals
Out of 68 animals that received pronuclear microinjection of murine fertilized ova with the pIN plasmid, four transgenic founders (pIN 21, 47, 57, 65) were generated showing positive polymerase chain reaction (PCR) results for the transgene (Fig. 3.2).

While line 65 was terminated early due to poor reproduction of offspring, all mice from transgenic (Tg) lines 21, 47 and 57 were healthy and had no problems in reproducing. These transgenic founders were then backcrossed to normal C57 black/6 (C57BL/6) mice or wild-type (WT) littersmates in the subsequent breeding. The ratio of transgenic mice was 1:1, with half desired heterozygous genotype, no negative effects of the transgene were observed.

**PCR genotyping**
Transgenic offspring were identified by PCR analysis of ear notch DNA using the following primers: 5’-GGA AGA GAG AAG GGC GAG TT-3’ and 5’-GGG TCC ATG GTG ATA CAA GG-3’ for a 243bp nephrin gene/intron genotyping product; 5’-GGT GGT GCT GAA GCT CTT TC-3’ and 5’-GCT TGT GGC TTG GAG GAT AA-3’ for a 228bp human HB-EGF gene genotyping product.
Figure 3.1. Schematic representation of the transgene.  
Human diphtheria toxin receptor (hDTR), or human heparin binding-epidermal growth factor receptor (hHB-EGFR), is expressed as a transgene under the control of the murine nephrin promoter.

Figure 3.2. Agarose gel showing positive bands for the PCR products of a 243bp fragment from podocyte promoter and rabbit β-globin intron of transgenic Podo-DTR mice.
Antigen expression of human HB-EGF receptor (hDTR)

a. Immunohistochemistry of hDTR staining showed positive staining in the glomeruli

Although the PCR results of the transgene of line 21, 47 and 57 were positive, it was important to make sure that the antigen expression of the human diphtheria toxin receptor (hDTR) was specific to the podocytes in the glomeruli. To provide the necessary evidence, immunohistochemical staining for hDTR or hHB-EGF receptor were carried out on formalin fixed kidney tissues (see Materials and Methods for details). The results showed positive segmental staining in the glomeruli of the transgenic mice but no staining in the respective negative controls (Fig. 3.3). No obvious difference between the three lines was recorded (the only exception was in line 65 that stained more strongly, but due to poor reproduction of offspring, the line was terminated early as mentioned previously).

b. Real-time quantitative PCR of hDTR gene expression tested positive for transgenic but not wild-type mice

In order to check the specificity and the level of expression of hDTR in various organs, real-time or quantitative (q)PCR for hDTR was carried out on 8 different organs (kidney, liver, brain, lung, heart, spleen, stomach, pancreas) from line 47 transgenic, wild-type littermates and unrelated C57BL/6 mice as negative control (n=1).

On the first attempt, a SYBR® Green detection method was applied by using in-house designed primers (hDTR-For: 5’-TGGGGCTTCTCATGTTAGG-3’, hDTR-Rev: 5’-CATGCCCAACTTCACCTTCTC-3’). However, the assay was not successful, as results from both transgenic, wild-type littermate, as well as negative control (C57BL/6) mice appeared to show positive expression of the hDTR receptor in all of the organs used.

Following inspection and comparison of the mouse hDTR (Hbegf) and human (HB-EGF) gene using the BLAST (basic local alignment search tool) program (http://blast.ncbi.nlm.nih/Blast.cgi), a homology of 79% was observed. The positive
expression in transgenic and wild-type mice was therefore due to homology of the gene site where the primers were designed.

In the following experiment, a commercially available TaqMan gene expression assay for human HB-EGF (cat. no. Hs00181813_m1 from Applied Biosystems) was used to detect the expression (see Materials and Methods). cDNA from human placenta (obtained from the Edinburgh Reproductive Tissue BioBank Ref. No. ERTBB-038) as positive control and kidney cDNA from unrelated C57BL/6 mouse as negative control were used.

The results showed that the kidney, liver and brain but not pancreas from the transgenic mice had positive expression, while tissues from wild-type littermate and C57BL/6 mice all tested negative with the exception of pancreas from C57BL/6 where one of the replicate samples was positive (Fig. 3.4a). Due to one high threshold cycle (Ct) value for hDTR (39.5 versus 32.2 of the transgenic kidney) in combination with one negative result, it is not unreasonable to assume that this is likely due to contamination of one of the C57BL/6 pancreas wells. Nonetheless the assay needs to be repeated in triplicate to confirm the result.

The level of expression of the human transgene was calculated using the comparative Ct method. First the delta relative Ct (dCt) was normalized by subtracting the signals of endogenous gene (18S) from the gene of interest (GOI) signals (hDTR) using the formula: \( Ct_{GOI} - Ct_{18S} = dCt_r \). Then each dCt value was normalized to the chosen calibrator (cb) (dCtcb) value of the assay (which consists of a mixture of cDNA from different organs from the transgenic mouse): \( dCt_r - dCt_{cb} = ddCt_r \). Therefore \( ddCt_{cb} \) is equal to zero. The relative target number (N) is \( 2^{ddCt_r} \). In this case, the target number is normalized to the transgenic kidney and is expressed in percentage with the following results: transgenic kidney (100%), liver (16.4%), brain (4.7%) versus calibrator (39.1%) (Table 3.4).

Although human placenta was used as a positive control for the transgene and positive expression was detected, this was not suitable for direct comparison with
mouse cDNA that was reverse-transcribed separately. In addition, the eukaryotic 18S housekeeping gene was amplified more effectively in human (placenta cDNA) than in mouse (transgenic kidney cDNA) with an average Ct value of 6.4 versus 23.6 respectively, despite the Ct values of the gene of interest (hHB-EGF) being relatively similar between the two samples (placenta: 31.0 versus transgenic kidney: 32.1) (Fig. 3.4b and Table 3.4).
Figure 3.3. Immunohistochemistry staining of human DTR antigen. (a) Positive control showing brown staining of human placenta trophoblast cells. (b) Negative human placenta control. (c) Transgenic Podo-DTR mouse kidney with positive and (d) negative staining in the glomeruli of wild-type C57BL/6 kidney. x200 magnification.
Figure 3.4. RT-PCR of hHB-EGF (hDTR). (a) Raw data showing positive expression of hHB-EGF gene of human placenta (top 2 lines), transgenic (Tg) mouse kidney (bottom 2 lines) and their respective –RT (minus reverse transcriptase) negative controls. (b) Relative % of hHB-EGF expression to Tg kidney in various organs and controls. Results showing positive expression in the kidney, liver and brain but not pancreas from the transgenic mice, and negative expression from wild-type littermate and C57BL/6 mice tissues with the exception of pancreas from C57BL/6 where one of the duplicate samples resulted positive due to contamination. The level of expression of the transgene in the transgenic mouse kidney is normalised to 100%, and the expression in the liver (16.4%) and brain (4.7%) is substantially lower. Although the human placenta was used as positive control for the transgene and positive expression was detected, this was not suitable for direct comparison with mouse cDNA that was reverse-transcribed separately.
3.3 Transgenic lines are susceptible to diphtheria toxin

Mode of action of diphtheria toxin
Diphtheria toxin (DT) is produced by Corynebacterium diphtheriae as a single polypeptide which is cleaved to form two subunits: A and B. Briefly, the larger B subunit binds to its membrane receptor (hDTR) and gets internalised. The smaller A subunit dissociates in the acidic environment of endosome and passes to the cytoplasm where it inhibits protein synthesis by inactivating elongation factor 2 (EF-2) leading to cell death (Palmiter, 2001).

Line and toxin susceptibility
In order to determine the susceptibility of various transgenic lines to diphtheria toxin and subsequently determine relevant doses of toxin to use, preliminary experiments of diphtheria toxin dose response were carried out. Podo-DTR mice and wild-type littermates as controls were injected with one single dose of diphtheria toxin ranging from 1-166ng/g body weight (bw). At various time points, urine samples were collected for proteinuria measurements, and animals were sacrificed and their kidneys analysed histologically.

Seven animals from each line were tested for proteinuria and haematuria with dipsticks (scale from zero to ++++) prior to any diphtheria toxin treatment using freshly collected urine (see Table 3.1.1 for proteinuria reference). All animals tested negative for blood, while proteinuria measured either trace or one plus, (only one animal from line 57 measured ++ for proteinuria). Of note, normal healthy rodents, in particular males tend to have some degree of protein in their urine.

Animals from line 21 were unaffected by diphtheria toxin at any dose used at day seven. However, one mouse that was injected with the highest dose (166ng/g bw) of diphtheria toxin died at day 20 post-toxin injection. All animals from line 47 and 57 had heavy proteinuria at day 7 with +++ or++++. At higher doses, mice were culled due to poor health between 7-11 days (Table 3.2).
Histologically, all treated animals from line 21 had normal glomerular morphology at day 20 and 28. Kidney samples of only one animal from line 47 was obtained at day nine after 50ng/g bw diphtheria toxin, which showed presence of increased number of dilated tubules, with a few cysts, juicy, swollen nuclei around glomerular tuft. At day 28 both animals from line 57 with lower toxin doses showed signs of early segmental scar and some chronic tubular injury.

Due to high mortality at higher end toxin doses, additional dose ranging experiments were carried out with lower toxin dose (0.1, 1 and 25ng/g diphtheria toxin) with more susceptible lines 47 and 57. Line 57 survived up to day 28 at both 1ng/g and 25ng/g bw toxin doses, while at 0.1ng/g bw toxin, glomerular histology was comparable to transgenic or wild-type untreated controls. Due to low availability of line 47 mice at the time, only a limited number were used for day seven timepoint at 25ng/g bw (Fig. 3.5b) and 1ng/g diphtheria toxin.

In summary, three out of four transgenic lines that were established were subsequently characterized (Podo-DTR 47, 57 and 21), two of which (47 and 57) were susceptible to diphtheria toxin. All three lines were entirely healthy and had normal glomerular morphology. Non-transgenic animals and also animals from line 21 had normal morphology regardless of the dose of toxin received. Mice from lines 47 and 57 developed renal injury in response to low doses of toxin but no major extra renal pathology was detected.
Table 3.1. Multistix© 10 SG reference for protein and blood measurement in urine.

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<th>Blood concentration (haemolysed)</th>
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<tr>
<td>+</td>
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<tr>
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Table 3.2. Effects of diphtheria toxin in transgenic Podo-DTR line 21, 47, 57.

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Note: see Table 1a for proteinuria reference
Figure 3.5. Glomerular histology of diphtheria toxin treated and untreated mice. (a) Normal glomerulus in untreated transgenic mouse. (b) Damaged glomerulus with abnormal morphology and vacuolated cytoplasm (*) from treated Podo-DTR line 47 (25ng/g bw DT) at D7. (c-d) Sclerosed glomeruli from treated Podo-DTR line 57 (1ng/g bw DT) at D28; (c) Partially damaged glomerulus with normal morphology at 4 o’clock, with tuft/capsular adhesion in the area of segmental scar at 9 o’clock. (d) Almost completely sclerosed glomerulus. Periodic acid Schiff (PAS) staining, at x400 magnification.
3.4 Detailed dose ranging and response

_Podo-DTR mice develop podocyte injury after diphtheria toxin injection_

In dose-ranging studies, animals were given 0.1-166ng/g body weight and culled at timepoints from one to twenty-six weeks. Higher doses caused early acute renal failure with elevated urine albumin:creatinine ratio (ACR) and serum creatinine and urea levels at two to three weeks in Podo-DTR line 47 (Fig. 3.8), but at lower doses, there seemed to be dose-dependent glomerulosclerosis at four to eight weeks, which progressed in severity to six months (twenty-six weeks) (Fig. 3.6a). Urinary ACR results showed that Podo-DTR line 47 was more sensitive to toxin than line 57 (Fig. 3.9).

Proteinuria occurred within days after diphtheria toxin administration (Fig 3.6a). Toxin treated transgenic (Podo-DTR) mice showed a progressive reduction in normal glomeruli with time (Fig 3.6b). At one month, early focal segmental glomerulosclerosis (FSGS) and chronic renal damage can be seen (Fig 3.5 c-d). Non-transgenic animals were unaffected by diphtheria toxin.

_Albuminuria and serum urea peak at week two_

Diphtheria toxin injection caused proteinuria that reached very high levels (2048mg/mmol) at two weeks, falling to much lower levels by five weeks (57.6mg/mmol) and remaining low but abnormal at twenty-six weeks (12.4mg/mmol), (n=3-6) (Fig. 3.6a). No significant albuminuria was seen in transgenic animals not given toxin or wild-type littermates (3.0-5.4mg/mmol) (data not shown). Serum urea showed a similar acute profile to ACR. Although the number of experimental animals were small (n=3-7), it appeared that there was an acute rise in blood urea at week two (10.9 mmol/L), followed by temporary recovery at week five (5.13mmol/L) and then by slow deterioration (7.9, 8.1, 9.2mmol/L at week six, eight and twenty-six respectively) (data not shown).
Morphological glomerular damage is progressive
While serum urea normalized around six weeks post toxin injection, glomerular histology deteriorated progressively. Importantly additional glomeruli became morphologically abnormal long after six weeks. Almost all glomeruli in wild-type treated controls (Fig. 3.6b) and transgenic untreated animals (data not shown) were normal (96%). However, toxin treated animals showed a progressive reduction in the proportion of normal glomeruli with time (74% and 53% at six and twenty-six weeks respectively)(Fig. 3.6b).

Podocyte number falls and remains low
The number of podocytes per glomerular cross section (GCS) also fell at two and possibly further at twenty-six weeks after toxin injection compared to the controls (6.2 and 5.3 versus 10.0 podocyte per GCS respectively, p< 0.02) (Fig. 3.7a). The fall at early time points appeared to be dose-dependent: in line 57, the 14 day count after 20ng/g bw toxin fell from 8.5 to 6.5 podocyte/GCS, p =0.004 (Fig 1.7b). This is almost double the change seen with 5ng/g bw toxin (8.2 to 7.1 podocyte/GCS from day zero to day 56 respectively) (see additional details in chapter 4).
Figure 3.6. Urinary albumin:creatinine ratio (ACR) and glomerulosclerosis of Podo-DTR line 47 mice. (a) Urinary ACR at various timepoints before and after injection of diphtheria toxin at 1ng/g body weight. (b) Glomerulosclerosis score at 6 and 26 weeks (6 months) post diphtheria toxin injection at 1ng/g bw. 100 glomeruli were scored per animal. ctrl, control; wk, week; WT, wild-type.
Fig 3.7. (a) Podocyte quantification of Podo-DTR line 47 mice at 2 and 26 weeks after diphtheria toxin (DT) injection at 1ng/g bw. Podocyte numbers were significantly reduced at 2 and 26 weeks after toxin injection compared to the controls (6.2 and 5.3 versus 10.0 podocyte/GCS respectively, p<0.02).

Figure 3.7. (b) Podocyte quantification of short-term ACEi intervention study using Podo-DTR line 57 mice after diphtheria toxin injection at 20ng/g bw. There was no significant difference between ACEi- and water-treated mice at respective timepoints in both transgenic (Tg) and wild-type (WT) mice. Podocyte number were significantly reduced in transgenic mice at day 7 and 14 after toxin injection versus controls (p<0.05).
**Podo-DTR line 47 is much more sensitive to diphtheria toxin than line 57**

Following a detailed time course experiment using Podo-DTR line 47 at 1ng/g bw diphtheria toxin, only 26% and 47% glomerulosclerosis was achieved at six weeks and six months respectively. In order to increase the degree of injury at a shorter time point e.g. eight weeks, the diphtheria toxin dose was subsequently increased to 2.5-20ng/g bw.

Unexpectedly, Podo-DTR line 47 developed renal failure at week two to three following diphtheria toxin injections at 2.5 and 5ng/g bw dose (Fig 3.8 & 3.9). At week three, after 5ng/g bw toxin injection, some of the mice lost excessive weight (>20% of their body weight), developed heavy albuminuria (Fig 3.8a), increased serum creatinine and urea (Fig 3.8b and 3.8c). Physically mice had scruffy and dull fur, they decreased food and water intake and became lethargic. Even when the experiment was repeated with a lower dose at 2.5ng/g bw, similar outcome occurred (Fig. 3.9a). As result, mice were culled at week two and three, before the established time point (eight weeks).

In the contrary, Podo-DTR line 57 mice all survived up to week eight after 5ng/g bw (see details of ACEi intervention study, chapter 4 results) and 20ng/g bw diphtheria toxin injections (Fig. 3.10). At 5ng/g bw toxin dose, mice developed relatively low but significantly higher level of glomerulosclerosis than wild -type controls at week eight (17% versus 7% of wild -type controls, p ≤0.001) (Fig. 4.5). At 20ng/g bw diphtheria toxin dose, although urinary ACR were much higher (772.0mg/mmol) at week two compared to the 5ng/g bw dose (272mg/mmol), the difference in histological damage in the kidney between the two doses were not as pronounced at week eight (20ng/g bw toxin: 21% versus 5ng/g bw toxin: 17% glomerulosclerosis, p=0.90) (Fig. 3.10).
Figure 3.8. (a) 24h urinary albumin:creatinine ratio (ACR) of Podo-DTR line 47 mice after diphtheria toxin injection at 5ng/g bw from experiment 4.
Transgenic mice that were injected with DT at 5ng/g bw were significantly affected at wk 2 and 3 with an increase of mean ACR from 1.9mg/mmol at D0 to 13250mg/mmol and 18233mg/mmol at week 2 and 3 respectively (n=4-6). At week 1, wild-type controls (38mg/mmol) injected with DT at 5ng/g bw but not Tg mice (1.9mg/mmol) injected with saline had transient albuminuria (n=3). However, at later time points, both transgenic and wild-type controls stayed at baseline level at week 2 and 3 (range: 0.0 to 4.0mg/mmol).
Figure 3.8. (b) Serum creatinine and (c) serum urea of Podo-DTR line 47 mice after diphtheria toxin injection at 5ng/g body weight from experiment 4 at week 3. DT, diphtheria toxin; Tg, transgenic; WT, wild-type.
No signs of liver damage in serum liver function test and liver histology in mice with toxin-induced renal failure

To check that the diphtheria toxin administration had no direct negative effects on the liver, histology and serum analysis for liver function tests were run in previously collected samples from transgenic Podo-DTR line 47 mice that received toxin at 5ng/g bw. These mice developed renal failure at week three after the toxin injection as demonstrated by highly elevated levels of urinary albuminuria, serum creatinine and urea (Fig. 3.8 a-c).

Particular attention was paid to the liver as low but positive expression of hDTR transgene was detected in this organ by qPCR. Apart from examining liver histology in transgenic animals with toxin-induced renal injury, liver function test was also carried out to measure liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin levels in the serum.

Of note, both ALT and AST are enzymes found in the cytoplasm, therefore raised levels would suggest hepatocellular damage. While ALT is more specific to liver, AST can also be found in cardiac, skeletal muscle and red blood cells. On the other hand, bilirubin is a by-product of haem breakdown in the red blood cells, which is normally taken up and conjugated in the liver and excreted via urine, therefore increased levels of bilirubin can indicate liver dysfunction or bile duct blockage.

The results showed that the levels of serum bilirubin and AST from affected mice were comparable to the controls, as no significant difference was detected between transgenic- (group one) and wild-type-toxin treated (group two) or group two and three (transgenic saline treated) combined results (Table 3.3). Comparison for alanine aminotransferase (ALT) was not applicable, as the levels were <15u/l for all the samples. This decreased level of enzyme assay sensitivity could be due to dilution (one in two) and/or prolonged sample storage, which may have led to enzyme degradation. Two out of nine animals had increased alkaline phosphatase (ALP) levels (Table 3.3), but they are less than double the upper limit of normal range (ALP control range: 120-150 u/l).
Figure 3.9. 24h urine albumin:creatinine ratio (ACR) of (a) Podo-DTR line 47 injected with diphtheria toxin at 2.5ng/g bw and (b) Podo-DTR line 57 injected with 20ng/g bw diphtheria from experiment 5a.
Figure 3.10. Glomerulosclerosis score of transgenic (Tg) Podo-DTR line 57 mice at 8 weeks post diphtheria toxin (DT) injection at 5ng/g and 20ng/g body weight. The difference in histological damage with glomerulosclerosis in the kidney between the two doses were not significant at week (WK) 8 (5ng/g DT: 17% versus 20ng/g DT: 21%, p=0.90). Group mean and standard error of the mean (SEM) are presented.
However, on examination of liver sections, all animals had normal histological appearance under light microscopy with no obvious pathological signs or inflammatory infiltrates even in mice that showed slightly higher AST than normal (Fig. 3.11). Although liver was not directly affected by diphtheria toxin administration, future work should include histological analysis of extra-renal tissues expressing the transgene. Closer attention needs to be paid to look for potential negative effects of the toxin in other organs.
Figure 3.1. Representative liver histology of wild-type control (left column) and transgenic (right column) diphtheria toxin treated mice at week 3. Normal liver histology showing (a-c) hepatocytes arranged into hexagonal shaped lobules and the gall bladder structure; (d-f) portal tract between adjacent liver lobules containing hepatic portal vein V, hepatic artery A, and bile duct D. Periodic acid Schiff (PAS) staining, at x25, x100 and x200 magnifications.
### Table 3.3. Serum analysis of liver function tests of Podo-DTR line 47 mice after diphtheria toxin (DT) injection at 5ng/g bw at week 3 (DT experiment 4).

No significant difference was detected between transgenic- (group 1) and wild-type-toxin treated (group 2) or group 2 and 3 (transgenic saline treated) combined results for bilirubin, AST, ALP (normal range for: bilirubin, 8-15μmol/l; AST, 38-50u/l, ALP, 120-150u/l). Comparison for ALT was not applicable, as the levels were <15u/l for all the samples. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Approx, approximately; Grp, group; Tg, Transgenic; WT, wild-type.
3.6 Discussion

*Line susceptibility to diphtheria toxin*

The results from the characterization part of the project have shown that line 47 was more sensitive to diphtheria toxin than to line 57. While the lowest diphtheria toxin dose at 0.1ng/g bw induced no or very little change in terms of proteinuria or histological damage in the kidney, higher diphtheria toxin doses used in the initial dose-range experiments at 25, 50 and 166ng/g bw caused severe acute injury in line 47. These animals were either culled early or died prior to established timepoints between week one and four.

However, at 1ng/g bw diphtheria toxin, mice developed proteinuria within days after toxin administration, and progressive glomerular damage and scarring was seen from week four to twenty-six (six months) with no major illnesses (Fig. 3.6). In the attempt to increase more severe glomerulosclerosis at a shorter timepoint of eight weeks, slightly higher doses were tested. Regrettably, at increased toxin doses of 2.5 and 5ng/g bw, the animals from line 47 developed early acute renal failure indicated by severe albuminuria at week two to three, elevated serum creatinine, serum urea and lowered serum albumin at week three (Fig. 3.8 and 3.9). These animals were culled at week three instead of the established timepoint at week eight due to poor health.

As a result, a single diphtheria toxin dose administered intraperitoneally at 1ng/g bw was optimised and selected to induce approximately 50% glomerular damage and sclerosis in line 47 at week eight, and subsequent intervention study with ACEi captopril and ETaRA sitaxsentan was carried out based on this optimized dose. However, the strengths and limitations of the intervention studies will be discussed separately later on.

Unlike line 47, mice from line 57 were less sensitive to the toxin, as animals survived up to week four following toxin injection at a wider concentration range (1, 25, 50ng/g bw). Proteinuria tested positive on dipstix at all doses used (0.1, 1, 25, 50,
166ng/g bw) ranging from ++ to ++++ at day seven or eleven. At 1ng/g and 25ng/g bw diphtheria toxin dose, histologically, damaged glomeruli were observed at week one, but sclerosis formation with matrix accumulation, tuft and Bowman’s adhesion and FSGS were more prominent at later timepoint (week four) with 46% and 57% damaged glomeruli respectively.

In order to ensure longer survival at later timepoints (week eight) to permit chronic damage development and subsequent analysis such as glomerulosclerosis, additional reduced toxin doses were used at 5ng/g and 20ng/g bw in line 57. These two doses were later applied in the intervention studies with the ACEi captopril, which will be discussed later on. Similarly to line 47 results, very high dose of toxin at 166ng/g bw induced early acute injury leading to premature cull (day 11), while at the lowest dose (0.1ng/g bw), no or very little damage was observed.

In summary, with dose range experiments, I have shown that line 47 is extremely susceptible to diphtheria toxin. While this is advantageous in using low doses of toxin thus avoiding large volume injections and therefore more cost effective to induce substantial injury in a big cohort of animal experiments, one of the disadvantages is that a relatively narrow range of the toxin dose can be applied to induce increased kidney damage without excessive adverse effects and mortality in line 47.

On the other hand, the advantage of having a different line with lower toxin sensitivity means that it can permit more subtle and milder induction of injury to study cell function, interaction and morphology of podocytes in conjunction with other cell types in the glomeruli.

However, thorough characterization of a different line is laborious, costly and requires additional time, and the results from the two lines are not necessarily comparable at this stage. Therefore additional dose and time response with higher number might be required to characterise the two lines in more detail. Line 47 and 57 could be used in separate settings in future work, e.g. use line 47 for short-term acute
injury experiments, while line 57 can be applied in the elucidation of cellular and molecular mechanism of podocyte in a chronic disease setting.
Table 3.4. Data of hHB-EGF gene expression in transgenic (Tg), wild-type (WT) and C57BL/6 mice with respective positive and negative controls.

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Note: Data are presented as Ct values for each sample with corresponding Ct values for 18S rRNA. The table includes the average Ct values for hDTR and 18S rRNA, the ddCt (dCt sample-calibrator) values, and 2^(-ddCt) values relative to Tg kidney. % relative to Tg kidney is calculated by dividing the 2^(-ddCt) value by the 2^(-ddCt) value for the calibrator sample and multiplying by 100.

**Table 3.4.** Data of hHB-EGF gene expression in transgenic (Tg), wild-type (WT) and C57BL/6 mice with respective positive and negative controls.
**Podocyte injury and number alteration**

Diphtheria toxin administration to transgenic but not wild-type mice clearly induced renal injury indicated by presence of proteinuria and histological damage, suggesting transgene-specific effect. In order to investigate whether the damage caused by the toxin is indeed podocyte-specific, WT-1 positively stained podocytes were quantified. The results from the more sensitive line 47 showed that after induction of injury with diphtheria toxin at 1ng/g bw, a progressive decrease in podocyte number was observed for up to six months with no or limited recovery in number.

In summary, these results indicate that the injury induced is toxin-receptor mediated and that podocytes are specifically targeted. The lack of podocyte number recovery after the injury also suggests that no or very limited regeneration occurs in these highly specialised cells in our Podo-DTR model.

Although the podocyte numbers have been quantified using a podocyte specific marker, this technique does not allow us to follow the fate of injured or lost cells. Whether podocytes die from apoptosis, necrosis or simply detach from the glomerular tuft and are washed away in the urinary space has not been elucidated in this project. Therefore an ideal model would be to have our hDTR expressing podocytes fluorescently labelled to allow investigation of the fate of damaged podocytes and possible replacement of lost cells from intrinsic renal progenitor cells.

**hDTR antigen and RNA expression**

To check the specificity of expression of hDTR in the kidney, immunohistochemistry and RT-PCR were used. One anti-hDTR antibody was used and stained glomeruli in the kidney specifically. However, unexpectedly the staining was segmental, where only part of some glomeruli are positively stained. This is somehow puzzling, and we don’t have an explanation as why the murine nephrin promoter used for the hDTR transgene would only be expressed in some part of some glomeruli instead of a homogenous expression.

In order to provide additional support of the expression of hDTR protein, RT-PCR was used to measure hDTR mRNA levels in the kidney. The results showed that the
kidney of the transgenic mouse expressed the hDTR transgene, while the wild-type and unrelated C57BL/6 kidneys tested negative as expected.

Although the fragment of nephrin promoter used in our Podo-DTR mouse has been previously confirmed by Moeller et al 2002 to be exclusively expressed by podocytes when coupled to β-galatosidase (Moeller et al., 2002), RNA expression from seven other organs including liver, pancreas, brain, spleen, stomach, lung and heart from transgenic and wild-type Podo-DTR mice were tested using the highly sensitive RT-PCR technique to further confirm the findings.

Preliminary results showed that the commercially available TaqMan gene expression assay for human HB-EGF (cat. no. Hs00181813_m1 from Applied Biosytems.) was suitable for hDTR detection. Unlike previously designed primers that picked up both human and murine DTR (due to high homology of the two genes), this assay was specific to human and not mouse DTR gene. This was demonstrated by the positive expression in the exogenous positive human placenta control but absence in the respective negative wild-type mouse kidney control.

Therefore, using the RT-PCR technique, I have successfully shown that the transgenic kidney does indeed express the hDTR transgene, while organs from wild-type mice and unrelated C57BL/6 all tested negative for the transgene as expected. However, unlike the negative chemiluminescence results presented by Moeller and colleagues (2002) in extra-renal organs, low level of expression was detected in the liver and the brain but not pancreas in our Podo-DTR model.

The difference in the results presented by our group and that of Moeller et al could be explained by the level of sensitivity of the two methods used. While high levels of expression can be easily detected in the kidney using the chemiluminescence method applied by Moeller’s group, it is possible that low levels of expression in other organs such as liver and brain might have been missed. Since RT-PCR is an extremely sensitive method of gene expression detection, it is not surprising that even extremely low levels of hDTR were detected.
However, the expression levels of the transgene in various organs need to be further analysed in different lines (47 and 57) to allow comparison, and additional n number from each line need to be included to ensure homogeneity of the transgene expression within the same line.

In summary, transgenic mice express both hDTR protein and mRNA in the kidney supporting the specific toxin-receptor mediated renal damage induced by diphtheria toxin injection, hence demonstrated the functionality of the transgenic model. Although low levels of extra-renal hDTR expression have also been detected, no obvious negative effects have been observed.

In conclusion, the results from the first part of my project have demonstrated that both line 47 and 57 were sensitive to diphtheria toxin, and proteinuria and kidney damage was induced in a dose dependent manner following toxin administration. Podocyte numbers were reduced progressively with time in conjunction with an increase of damaged and sclerosed glomeruli post toxin injection, suggesting specific toxin-receptor mediated injury. In agreement with many other studies, these data further support the hypothesis that podocyte damage and continuing loss is the primary cause of proteinuria and progressive glomerulosclerosis (Ichikawa et al., 2005, Kim et al., 2001, Kriz et al., 1998, Matsusaka et al., 2005, Wharram et al., 2005).

Therefore, if this was a good model of glomerular injury, we would expect ACEi to protect against progressive kidney disease as seen in human setting. So in the next part of the project, we set out to test the viability and functionality of the Podo-DTR model with the well-established therapeutic agent captopril in our first intervention study.
CHAPTER 4: ACEi intervention study-testing model with known therapeutic agent

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ACEi interventional study: testing model with known therapeutic agent

4.1 Background

Proteinuria reduction can slow down/arrest kidney deterioration
There is strong evidence that angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) can arrest deterioration in renal function in proteinuric kidney diseases of any aetiology, both in animal models (Adamczak et al., 2003, Gross et al., 2003, Ma et al., 2005, Remuzzi et al., 2002) and in man (Lewis et al., 2001, Praga et al., 2003). Response to this therapy appears to be closely correlated with proteinuria reduction. This protection was first attributed to haemodynamic effects. Recent evidence suggests additional mechanisms.

Hypothesis: The beneficial effect of ACEi is achieved by supporting podocyte
Our Podo-DTR model described in detail in the previous chapter (chapter 3) permits a graded specific podocyte injury that can be delivered by a single injection of diphtheria toxin. Here I describe the use of this model to investigate the nephroprotective potential of ACE inhibitor captopril in podocyte injury.

The Renin Angiotensin Aldosterone System
The renin-angiotensin-aldosterone system (RAAS) (Fig. 4.1) regulates the blood pressure, fluid and electrolyte balance through coordinated effects of a combination of hormones, blood vessels and various organs such as kidney, lungs, liver and heart.

In man, drop in blood pressure e.g. due to loss of blood via haemorrhage causes the kidney to produce the enzyme renin. This enzyme is required to cleave the circulating inactive precursor angiotensinogen (produced in the liver) to the active form angiotensin I, which in turn increases the blood pressure (BP) partially. For an increased effect, angiotensin I (AngI) needs to be converted into the more potent Angiotensin II (AngII) in the lung by angiotensin converting enzyme (ACE). In addition, ACE also functions to inactivate the vasodilator peptide bradykinin. AngII
exerts effect on heart and kidney by binding mainly to the G-protein coupled angiotensin receptor type 1 (AT1) to cause vasoconstriction of the blood vessels, which in turn increase the blood pressure even further.

In the kidney, AngII acts to increase the vascular tone, in particular that of efferent glomerular arterioles and regulates intraglomerular capillary pressure and glomerular filtration rate (GFR). Persistent and disregulated intracapillary hypertension and increased ultrafiltration of plasma proteins is likely to contribute to the onset and progression of chronic kidney damage (CKD) (Remuzzi & Bertani, 1998).

However, deleterious non-haemodynamic effects of AngII may also play roles in the progression of CKD. These include upregulation of cytokines, profibrotic growth factors and cell adhesion molecules (Hobo et al., 2009, Remuzzi et al., 2005, Ruiz-Ortega et al., 2001); increased production of reactive oxygen species (ROS) (Kramer et al., 2006); induction of transforming growth factor-β expression (TGF-β) (De Albuquerque et al., 2004), increased extracellular matrix proteins synthesis (Ma et al., 1998); stimulation of plasminogen activator inhibitor-1 production by endothelial and vascular smooth muscle cells (Ma et al., 2005); and activation and infiltration of inflammatory cells (Ruiz-Ortega et al., 2001).

More importantly, increased levels of AngII can exhibit many deleterious effects on podocyte directly. AngII has been shown to cause podocyte apoptosis (Ding et al., 2002, Durvasula et al., 2004), increased VEGF and TGF-β synthesis (Chen et al., 2005), actin rearrangement, reduction of ZO-1 and nephrin levels that leads to proteinuria (Blanco et al., 2005). In addition, Hoffmann et al demonstrated that overexpression of human AngII type 1 (AT1) receptor on podocytes in a transgenic rat model caused marked proteinuria and glomerulosclerosis, providing direct evidence of the increased deleterious effect of AT1 signalling (Hoffmann et al., 2004).

AngII also stimulates release of another hormone, aldosterone from the adrenal gland. Aldosterone influences water and salt balance by regulating re-absorption and
retention of salt in the kidney, and are likely to mediate direct effects on podocytes
due to the expression of its receptor on these cells as previously mentioned in chapter 1 (section 1.3 aldosterone antagonists). Therefore, the principle behind using ACEi
captopril is to reduce the levels of AngII and thereby abolish or decrease its
downstream deleterious effects.
Figure 4.1. Simplified diagram of renin-angiotensin-aldosterone system (RAAS).
The RAAS system regulates the blood pressure, fluid and electrolyte balance in the body. In man, a drop in blood pressure causes the kidney to produce the enzyme rennin. This enzyme is required to cleave the circulating inactive precursor angiotensinogen (produced in the liver) to the active form angiotensin I, which in turn increases the blood pressure partially. For an increased effect, angiotensin I (AngI) is converted into more potent Angiotensin II (AngII) in the lung by the angiotensin converting enzyme (ACE). ACE also function to inactivate the vasodilator peptide bradykinin. AngII exert effect on heart and kidney by binding mainly to the G-protein coupled angiotensin receptor type 1 (AT1) to cause vasoconstriction of the blood vessels, which in turn increase the blood pressure even further.

In the kidney, AngII acts to increase the vascular tone of both afferent and efferent glomerular arterioles and regulates intraglomerular capillary pressure and glomerular filtration rate (GFR). In a domino effect, the increase of AngII levels stimulates release of the hormone aldosterone from the adrenal gland. Aldosterone is a potent vasoconstrictor, it influences water and salt balance by regulating re-absorption and retention of water and salt in the body, and it affects the baseline filtering activity of kidney.
The mode of action of ACEi and ARB

ACEi together with ARB are one of the main classes of blood pressure lowering drugs. Although the mode of action of ARB will be covered briefly, the focus of this chapter will be on ACEi. Early study by Lewis et al in a randomized controlled trial of patients with diabetic nephropathy showed that reduction in glomerular hypertension and improved renal outcome was achieved by ACEi captopril compared to non-ACEi regimen that achieved similar or equivalent systemic blood pressure control (Lewis et al., 1993). The data demonstrated that captopril treatment was more effective in protecting against renal function deterioration in insulin-dependent diabetic nephropathy compared to blood pressure control alone. This beneficial blood pressure independent effect of ACE inhibition was further highlighted by Smeets et al in a model of murine collapsing FSGS (Smeets et al., 2006).

ACEi lowers glomerular capillary pressure by affecting and increasing vasodilation of the efferent arteriole. This is likely achieved by inhibiting ACE activity that converts Ang I to the active vasoconstrictor AngII, but could also be mediated by inhibiting degradation of the vasodilator bradykinin. On the other hand, ARB blocks the binding of AngI and AngII to angiotensin type 1 (AT1) receptors in the blood vessels, leaving angiotensin type 2 (AT2) receptors active. While AT1 receptors mediate vasoconstriction on the blood vessels, AT2 receptors partially counteract the AT1 effects and mediate mild vasodilation as well as inhibition of growth and apoptosis, therefore limiting the subsequent vasoconstriction effect (Ma et al., 1998, Siragy, 2000, Steckelings et al., 2005, Stoll et al., 1995, Yamada et al., 1996).

Unlike ACEi, ARBs do not increase bradykinin levels (Okada et al., 2004) or reduce renal injury to the extent of that seen with ACEi in some of the experimental studies (Tang et al., 2008). However, both ACEis and ARBs demonstrated to be more effective in slowing progressive CKD in both experimental models and in human compared to other (non-ACE-dependent) antihypertensive therapies (Kasiske et al., 1993, Lewis et al., 1993, MacKinnon et al., 2006, Maschio et al., 1996, Smeets et al., 2006).
Why haemodynamic effects of angiotensin inhibition cannot be enough

Both ACEi and ARB in combination or alone have the nephroprotective effect of reducing proteinuria and the rate of progression in proteinuric renal diseases (Berl, 2009, Campbell et al., 2003, Jacobsen et al., 2003, Luno et al., 2002, Mogensen et al., 2000, Nakao et al., 2003, Yusuf et al., 2008). Several pieces of evidence support the notion that the beneficial effects of ACEi and ARB extend beyond their haemodynamic effects.

Firstly, most other antihypertensive drugs (e.g. calcium channel blockers, aldosterone antagonist) are not as effective in reducing protein excretion when compared to ACEi or ARBs (35-45% reduction), even though equivalent blood pressures were achieved. In a 2008 meta-analysis of randomized trials, the proteinuria reduction at 5 to 12 months has been shown to be comparable between the ACEi and ARB (Kunz et al., 2008). The evidence for the preferential benefit with ACE inhibition comes from the same study, where different classes of calcium channel blockers were compared to achieve equivalent blood pressure. The results showed that non-dihydropyridine calcium channel blocker (e.g. diltiazem and verapamil) were more effective at reducing proteinuria compared to dihydorpiridine such as amloidipine and nifedipine in both diabetic and non-diabetic patients despite achieving same blood pressure control (Bakris et al., 2004), further supporting the notion that simply lowering blood pressure is not enough in achieving nephroprotection.

Secondly, Heeg JE et al. showed that acute administration of AngII did not reverse the antiproteinuric effect of long-term ACE inhibition despite inducing renal and systemic vasoconstriction, suggesting that the beneficial effect may be due to improved permselectivity of the filtration barrier that is independent of changes in glomerular haemodynamics (Heeg et al., 1991). Evidence further supporting this hypothesis has been provided by Gansevoort RT et al., where the authors showed that while blood pressure was lowered shortly after the start of ACEi treatment, the antiproteinuric effects of ACE inhibition was not as rapid, instead it had a gradual
progressive decline over a period of weeks to months (Gansevoort et al., 1993).

Thirdly, as mentioned earlier angiotensin inhibition may have direct influence on podocytes, as angiotensin receptors have been demonstrated on podocytes in both \textit{in vitro} and in vivo settings. Differentiated podocytes predominantly express AT1 receptors (approximately 75%) compared to the lower proportion (25%) of AT2 receptors (Liebau et al., 2006, Wang et al., 2003). Previous \textit{in vitro} studies with mouse podocytes provided evidence of activation of local renin angiotensin system in podocytes induced by mechanical strain and increased glucose levels (Durvasula et al., 2004, Durvasula & Shankland, 2008). While in an \textit{in vivo} model of murine autoimmune nephritis, Crowley S et al 2009 showed that glomerular AT1 receptors could augment kidney injury and inflammation. Gene expression of the renin-angiotensin system in human kidney and human podocytes has also been demonstrated (Lai et al., 1998, Liebau et al., 2006).

Since AngII exerts biological effects through the interaction with the AT receptors on cells such as the podocyte, we hypothesized that inhibition of AngII production with ACEi could have direct beneficial influences on podocytes, providing nephroprotection. To test this and establish the validity of the model, an ACEi intervention study with captopril was carried out using our Podo-DTR (line 57) mouse model.

\textbf{4.2 Experimental outline of ACEi intervention study}

Groups of 16 transgenic (Tg) mice and 8 wild-type (WT) littermates were given captopril (200mg/L) in their drinking water or placebo (water alone) 24h after a single intraperitoneal (i.p.) injection of diphtheria toxin at 5ng/g bw (Product no150, Lot 15023A1; List Biological Laboratories Inc., California, USA). Animals were allocated to three groups: Tg DT+H\textsubscript{2}O received water alone; Tg DT+ACEi were treated with captopril; WT DT+ACEi were wild-type littermate controls treated with captopril (Fig. 4.2).
Of note, captopril dose (200mg/L or 32mg/kg/day, assuming a daily fluid intake of 5ml and average mice weight of approximately 30g) was chosen based on previous study that demonstrated blood pressure lowering effect of the drug on a murine lupus nephritis models (De Albuquerque et al., 2004), but similar or higher doses have also been used by others (Smeets et al., 2006, Tang et al., 2008). Captopril is stable over 23 days in sterile water at 4ºC and 14 days at room temperature (22ºC) protected from light (>90% parent). When dissolved in water, the solution is clear, colourless with a distinct drug smell and is changed weekly in our experiments.

Urine samples were collected over 24h in metabolic cages at days zero, 14, 42, 49 and 56 and analysed for albumin:creatinine ratio (ACR). Systolic blood pressures were measured by tail cuff plethysmography on trained conscious animals during week seven after toxin injection, and the mean of three to four measurements was recorded for each animal. Untreated Podo-DTR mice (n=5) (transgenic not given diphtheria toxin or captopril) were also included to obtain the baseline blood pressure. Terminal blood samples were collected at week eight from anaesthetised animals for serum analysis.

Kidneys from each animal were bisected sagitally and fixed as required by overnight incubation at 4ºC in fixative (10% neutral formalin, Methyl-Carnoy fixative, or Karnovsky’s glutaraldehyde (700mOsm) or snap frozen in liquid nitrogen. For light microscopy, formalin-fixed samples were embedded in paraffin wax and 2µm sections cut and stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS).
Figure 4.2. Schematic representation of the experimental outline of ACEi intervention study. Groups of 16 transgenic (Tg) mice and 8 wild-type (WT) littermates were given captopril (200mg/L) in their drinking water or placebo (water alone) 24h after a single intraperitoneal (i.p.) injection of diphtheria toxin (DT) at 5ng/g bw. Animals were allocated to 3 groups: Tg DT+H₂O received water alone as placebo; Tg DT+ACEi were treated with captopril; WT DT+ACEi were wild-type littermate controls treated with captopril.
**Statistical analysis**

Results are expressed as mean ± standard error of the mean (SEM) or median where specifically stated. Statistical differences between groups were established by the Student t-test, one-way Anova analysis of variance with Bonferroni's multiple comparison test or Wilcoxon Rank Sum as appropriate. A p-value of <0.05 was considered to be significant.
4.3 Results

**ACEi lowered blood pressure and proteinuria**

Systolic blood pressure was lower in ACEi treated animals regardless of them being transgenic or wild-type compared to DT+H₂O treated or naïve untreated controls (mean: 84±1.7 and 73±1.9 mmHg respectively versus 114 ±3.8 mmHg, p<0.001) (Fig. 4.3).

Urine ACR was lowered in ACEi treated animals at all time points, but in toxin-treated animals did not reach baseline levels. At day zero, prior to diphtheria toxin injection, mice from all three groups had baseline level of urine ACR (range: 0.0-6.8mg/mmol). The DT+ H₂O treated group peaked at week two (272±128 mg/mmol), while the DT+ACEi treated group had the urinary ACR level blunted substantially (39.1±9 mg/mmol). At longer timepoints, albuminuria was lowered in both DT+ACEi treated and DT+H₂O groups (Fig. 4.4). The WT DT+ACEi controls had baseline level of urine ACR throughout the experiment (mean range value: 3.0-4.6mg/mmol).

**ACEi reduced histological damage**

Glomerular scarring and matrix accumulation was reduced almost to baseline level by ACEi captopril (Tg DT+H₂O: 17%, Tg DT+ACEi: 10%, p<0.04; wild-type control: 7%) (Fig. 4.5). At week eight, mice in the Tg DT+ H₂O group had the highest percentage of abnormal glomeruli (17%), significantly higher than the proportion in ACEi treated animals (10%, p=0.017) or wild-type controls. The degree of scarring and matrix accumulation in the Tg DT+ACEi approached that seen in the wild-type control group (7% abnormal glomeruli, p= 0.31) (Fig. 4.5a-b).
Figure 4.3. Tail cuff blood pressure (BP) of Podo-DTR line 57 mice.
At 7 weeks post DT injection (5ng/g bw), the BP of ACEi treated mice, whether Tg or WT (84 and 73mmHg respectively) were significantly lower (p<0.001) than the untreated groups (114mmHg).
DT, diphtheria toxin; ACEi, angiotensin converting enzyme inhibitor; Tg, transgenic; WT, wild type; bw, body weight; vs, versus; *, p< 0.0001 vs Tg DT+H2O; #, p≤ 0.0001 vs Tg DT+ACEi; +, p= 0.002 vs WT DT+ACEi.
Figure 4.4. Urine albumin:creatinine ratio (ACR) of Podo-DTR line 57 mice.
At day 0, prior to DT injection, mice from all 3 groups had baseline level of urine ACR (range: 0.0-6.8 mg/mmol). The DT+H₂O treated group peaked at week 2 (271.5±128 mg/mmol), the DT+ACEi treated group had the urinary ACR level blunted substantially (39.1 ±9 mg/mmol). The long-term albuminuria was lowered in both DT+ACEi treated and DT+H₂O groups. The WT controls had baseline level of urine ACR throughout the experiment (mean range value: 3.0-4.6 mg/mmol).
DT, diphtheria toxin; ACEi, angiotensin converting enzyme inhibitor; Tg, transgenic; WT, wild type; bw, body weight; vs, versus.
Figure 4.5. Glomerulosclerosis score and kidney histology of ACEi intervention study. (a) Glomerulosclerosis score of Podo-DTR line 57 mice at 8 weeks post DT injection at 5ng/g bw treated with or without captopril (200mg/L). Glomerular scarring was reduced almost to baseline level by ACEi captopril (Tg DT+H₂O (n=8) : 17%, Tg DT+ACEi (n=16):10%, p<0.04; wild-type control (n=16): 7%). (b) Kidney histology of captopril treated and untreated Podo-DTR line 57 mice. (A) Cluster of 4 glomeruli with varying degree of scar from a Tg DT+ H₂O treated mouse injected with 5ng/g bw DT; (B) Improved glomerular histology of a Tg DT+ACEi treated mouse injected with 5ng/g bw DT, PAS staining, x200 magnification.

ACEi: angiotensin converting enzyme inhibitor, bw: body weight; DT, diphtheria toxin; PAS, Periodic Acid Schiff; Tg, transgenic; *, p< 0.02; **, p≤ 0.001; NS, not significant (p=0.33); score 0= normal, 1= <50% sclerosis, 2= >50% sclerosis.
**ACEi did not protect from podocyte loss**

At week 8, toxin-treated susceptible mice had lower podocyte counts than wild-type controls irrespective of additional treatment with water or ACEi (median podocytes per GCS was 7.1 for Tg DT+H₂O; 7.3 for Tg DT+ACEi; and 8.2 for wild-type mice) and both comparisons were significant, p<0.05 (Wilcoxon Rank Sum). The variability in podocyte number per glomerulus made it impossible to exclude a minor degree of protection against podocyte loss, but there was no significant difference in podocyte count per GCS between the DT+ H₂O and DT+ACEi groups (Fig. 4.6a). This remained the case if count per GCS was corrected for glomerular area (Fig. 4.6b).

**ACEi or DT did not alter overall glomerular size**

Glomerular area of 50 consecutive GCS was measured using ImageJ 1.4r software. The results showed no significant difference in mean glomerular area between the WT DT+ACEi, Tg DT+H₂O and Tg DT+ACEi groups (group mean 9676, 10708, 9693µm² respectively), p>0.08 (data not shown).

**Serum results change were minimal**

In this experiment, differences of serum results between the groups were very small or absent as shown for serum creatinine (Fig. 4.7), but also for urea and albumin (data not shown).

**No significant difference between young and old mice in sclerosis and serum analysis**

Mixed genders and a wide range of mice age were used in this experiment (3-12 months, with the median age set to 7.5 months old), therefore comparison between age and sex were carried out retrospectively.

Although the trend seemed to show higher glomerulosclerosis score in transgenic old (>7.5 months) mice compared to the respective young (<7.5 months) mice in both water- and ACEi-treated groups, no significant difference was observed in this ACEi study (Fig. 4.8). Similarly, no significant difference was detected in serum results of
creatinine, urea and albumin between the respective young and old mice groups (data not shown).
Figure 4.6. Box plots of the (a) podocyte count or (b) podocyte count per unit glomerular cross section area for Podo-DTR mice treated with toxin with or without ACEi compared with wild-type (toxin + ACEi treated) mice at week 8. Significance was assessed using the Wilcoxon Rank Sum test.

Tg, transgenic; WT, wild-type; ACEi, angiotensin converting enzyme inhibitor; GCS, glomerular cross section; DT, diphtheria toxin; NS, non significant; *, p = 0.03; **, p < 0.003.
**No significant difference between female and male mice in sclerosis and serum analysis**

To see whether sex had an effect on the experimental outcome in regards to glomerulosclerosis and serum results, female (F) and male (M) mice were sub-grouped in Tg DT+ H₂O and Tg DT+ACEi treatment groups and analysed subsequently.

The glomerulosclerosis results showed that statistically female mice sclerosis was not significantly different from the male mice in the respective groups (Fig 4.9). However, sclerosis in male DT+ACEi (0.09) was significantly reduced compared to male DT+ H₂O (0.21) group, p<0.01. While female DT+ACEi (0.12) retained similar levels to female DT+ H₂O (0.13) mice. This suggests that the beneficial effect of ACEi treatment may be more effective in lowering glomerulosclerosis in male mice than the respective female mice.

Serum results of creatinine, urea and albumin also showed no significant difference between female and male mice in the respective groups of either water- or ACEi-treated mice (Fig 4.10a-b) (data not shown for serum albumin). Interestingly, WT DT+ACEi controls (24.8±4.4µmol/L) had higher serum creatinine than water treated mice (F: 20.8±1.9, M: 21.1±4.5µmol/L), but no significant difference was observed. Another interesting observation recorded was that ACEi treated mice (whether transgenic or wild-type) tended to have slightly higher serum creatinine compared to water treated mice, but no significant difference was detected (Fig 4.10a).

Although male mice had higher serum urea than female mice in the respective DT+ H₂O and DT+ACEi groups, the difference observed was not significant (Fig 9b). However, both female and male mice of DT+ H₂O group (7.2 and 8.4mmol/L) were significantly lower than male DT+ACEi group (p<0.001 and <0.01 respectively). Both female ACEi treated groups (whether transgenic (9.2mmol/L) or wild-type (9.1mmol/L) had higher serum urea than the female water treated group (7.2mmol/L).
The serum albumin of the water treated group generally had slightly lower levels (F: 29.40, M: 29.30g/L) compared to female WT controls (30.88g/L) and ACEi treated mice (F:30.67, M:30.20g/L), however, the difference observed was not significant (data not shown).
Figure 4.7. Serum creatinine of ACEi intervention study at week 8. Serum creatinine of Tg DT+H2O is lower than both Tg DT+ACEi treated and WT controls mice (21.0±3.7 versus 24.4±6.3 and 24.8±4.4 μmol/L respectively), but the results were not significantly different.)
Figure 4.8. Young (<7.5 months) versus old (>7.5 months) glomerulosclerosis index of Podo-DTR line 57 mice from ACEi experiment 3 at week 8. There is no significant difference between old and young mice in the respective groups. However, the trend seems to show that the old mice have higher sclerosis score compared to the respective young mice in both water- and ACEi-treated groups, but not in the wild-type controls (WT CTRL). The only significance seen between the groups is that old DT+H2O is significantly higher than old WT CTRL and young DT+ACEi (* p<0.05).

Figure 4.9. Female (F) versus male (M) glomerulosclerosis index of ACEi experiment 3 at week 8. Statistically female mice sclerosis was not significantly different from the male mice in the respective groups.
Figure 4.10. Female versus male (a) serum creatinine and (b) serum urea of ACEi experiment 3 at week 8. No significant difference was detected between female and male mice from the respective groups in the serum results.
4.4 Discussion

In order to test the hypothesis that podocyte protective drugs can protect against self-perpetuating mechanism of podocyte damage, we set out to use our Podo-DTR model to assess the proven nephroprotective ACEi drug, captopril. Other groups have developed toxin receptor-mediated conditional podocyte knockout models (Matsusaka et al., 2005, Wharram et al., 2005) similar to ours, but no intervention studies had been published.

ACEi and ARB were originally thought to provide protection via haemodynamic effects, the key to their protective effect assumed to be reduction of glomerular filtration pressure. Although blood pressure reduction by any means had been shown to protect renal function in proteinuric diseases (Klahr et al., 1994), this only explains part of the effect of ACEi and ARB agents. Some have suggested that ACEi/ARB may also modulate the toxicity of filtered protein by effects on non-glomerular cells (Rosenberg et al., 1994): it is thought that filtered protein may be toxic to renal tubular cells and thereby promote interstitial fibrosis (Baines & Brunskill, 2008).

An alternative hypothesis is that continuing podocyte damage or loss is central to the progression of proteinuric renal diseases and development of chronic renal failure (Gassler et al., 2001, Ichikawa et al., 2005, Wharram et al., 2005). Under this hypothesis, drugs that protect podocytes may also be generally nephroprotective. Many drugs employed in renal disease have been shown to have direct effects on podocytes raising the possibility that podocyte protection is an important mode of action in addition to (or instead of) that formerly supposed.

As well as ACEi and ARB (Gross et al., 2003, Nemeth et al., 2009), direct effects on podocytes have been reported for: immunosuppressive drugs including corticosteroids (Ransom et al., 2005, Shibata et al., 2006), calcineurin inhibitors such as cyclosporine (Faul et al., 2008) and tacrolimus (Bhimma et al., 2006), and mizoribine (Nakajo et al., 2007); and non-immune drugs including the endothelin A
receptor antagonist (ET\(_A\)-RA) (Dhaun et al., 2007), and peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) agonists (Kanjanabuch et al., 2007, Miyazaki et al., 2007). As not all patients respond to existing treatments of their renal disease, there is a need for additional and more effective agents.

The results showed that our Podo-DTR mouse model has advantages for studying podocyte rescue as it is possibly through selection of dose of toxin to produce a consistent non-lethal degree of podocyte-specific injury. Mild degrees of injury were shown to superficially heal over eight weeks with normalisation of glomerular morphology under light microscopy and reduction of proteinuria, but with continuing reduction in podocyte numbers and low level proteinuria.

On this background we were able to test the benefit of captopril upon histopathology and on podocyte number. Captopril significantly reduced blood pressure and proteinuria and protected against progressive histopathological change, but there was no discernible preservation or restoration of podocyte number. Previous studies have shown a protective effect of ACEi upon podocyte number in certain settings (Gross et al., 2003, Nemeth et al., 2009), but our results showed no significant change. However, we cannot rule out a small change.

Direct comparison between the studies is complicated by the variation in techniques used to assess podocyte number. Our study employed quantification of podocyte per glomerular tuft in a 2D image similar to the approach of many other groups (Logar et al., 2007, Pippin et al., 2009a, Vaughan et al., 2005). Another approach is to estimate the total number of podocytes per whole glomerulus by extrapolating a volume from a series of 2D images (Sanden et al., 2003), which may have some advantages but is elaborate and assumes all glomeruli have spherical shape and the same size. The best technique with which to quantify podocytes is still a matter of debate, but whilst this difficulty complicates comparison between studies, it does not prevent conclusions from the comparisons made within the various studies where measurements have been made using a consistent technique.
Our results suggest that protection against podocyte loss cannot be the only mechanism by which ACEi achieve long-term nephroprotection, and that change of podocyte phenotype or function may also be important. Therefore future work with EM studies of podocyte ultrastructures may provide additional insights.

The exact mechanism of action of captopril has not been elucidated in this study. Therefore, the potential beneficial haemodynamic effect cannot be excluded. In order to show the supplementary effect of blood pressure independent benefit of ACEi, additional control group treated with non-ACE dependent antihypertensive drugs such as hydralazine would need to be included in the study.

Quantification of positively stained WT-1 cells showed a decrease in podocyte number after toxin administration, however the number was not rescued by captopril treatment, despite significant histological improvement in the glomeruli. In order to further support the results, additional podocyte markers such as nephrin, podocalyxin or CD2AP should be included in future studies.

In this study, a wide range of mice age and mixed genders were used. Undoubtedly these factors would add more variability to the experiment, however we believe that the experimental design perhaps reflected more closely to the actual human patients setting. The n numbers of the groups used were relatively big (Tg n=16, WT n=8) to compensate the increased variability. Although no significant difference was detected between young and old, female and male mice in the respective treatment groups, ACEi treatment seemed to be more effective in lowering glomerulosclerosis in more severely affected male than female mice.

In addition, serum creatinine and urea were found to be higher in ACEi treated transgenic or wild-type mice compared to the Tg DT+H2O group. Whether these increased levels have relevant impact and cause any physiological effects might need to be further elucidated with an additional control group (i.e. WT DT+H2O). Therefore future studies with single sex and more narrow age range might give tighter results with smaller intra-variation within the treatment groups, hence have
increased chance to detect more subtle change from any interventional drug treatment.

The level of histopathological damage seen in this study was relatively low (17%), therefore higher diphtheria toxin dose could be implemented in future studies to induce higher degree of injury. This would allow easier and possibly more pronounced detection in any improvement after rescue therapies with potential nephroprotective drugs.

In summary, the results from this study have shown that ACEi captopril achieved protection against progressive glomerulosclerosis after specific induction of podocyte injury in our Podo-DRT model. This partly proves the hypothesis that the beneficial effect of ACEi is via podocyte support. However, the lack of evidence of podocyte number rescue despite improvement in proteinuria and histology indicates that it might be through to change of podocyte function and/or phenotype. Therefore, I have successfully proven that the model is functional, viable and is ideal for testing other potential renal therapeutic drugs. However, the mechanism of action of captopril still needs to be further elucidated and the blood pressure independent effect needs to be proven in this model. Our model could be used to identify or screen new compounds to reduce podocyte damage and preserve renal function.
CHAPTER 5: Combined intervention study with ETaRA sitaxsentan and ACEi captopril

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Combined intervention study with ETaRA sitaxsentan and ACEi captopril

5.1 Background
Previously we have shown that the ACE inhibitor captopril had a nephroprotective effect in our Podo-DTR mouse model of specific podocyte injury. I wanted to test whether other proteinuria-reducing drugs can protect kidney function in the same way.

Endothelin type A receptor antagonists (ETaRA) have been shown to reduce proteinuria in animals (Opocensky et al., 2006, Ortmann et al., 2004, Vernerova et al., 2008, Watson et al., 2010) and in man (Dhaun et al., 2007, Mann et al., 2010, Wenzel et al., 2009). This study tested whether the ETaRA sitaxsentan (SITA) can protect against kidney damage on its own or in combination with captopril to achieve a synergistic or additive beneficial effect in progressive proteinuric kidney disease.

The role of endothelin-1
Endothelins are a family of peptides, of which endothelin 1 (ET-1) is the predominant isoform found in the vasculature and will be addressed mainly in this thesis. ET-1 is a potent vasoconstrictor and mediates growth-promoting properties when bound to the ETa receptor subtype. While ETa receptors mediate deleterious effects and are believed to play a central role in the pathogenesis of proteinuria and glomerulosclerosis, ETB receptors mediate vasodilatory and anti-inflammatory effects via release of nitric oxide and prostacyclin (Haynes & Webb, 1994). In addition ETB receptor also play a role in the clearance of ET-1 in the circulation (Fukuroda et al., 1994).

Role of endothelin in the kidney in health and disease
In the kidney, under physiological conditions endogenous endothelin regulates water and sodium excretion and acid-base balance, and maintains normal renal cell proliferation and tonic vasoconstriction (Kohan, 2006). However, in animal models of salt-induced hypertension and renal mass reduction of hypertensive glomerular
disease, augmented endothelin production can also promote the development of glomerulosclerosis (Barton et al., 2000, Benigni et al., 1993, Hocher et al., 1997).

A role for endothelin in chronic proteinuric kidney disease was shown by Benigni and colleagues in a rat renal mass reduction model of hypertensive glomerular disease where the authors demonstrated striking improvement in proteinuria and glomerulosclerosis after selectively inhibiting ETa receptors (Benigni et al., 1993). Hocher et al later demonstrated that systemic overexpression of the human ET-1 gene in a transgenic mouse model caused glomerulosclerosis in a blood pressure independent manner (Hocher et al., 1997). These data were supported by the follow up preclinical and clinical studies, where evidence of the nephroprotective effects of endothelin blockade were thought to be via inhibition of the proliferative properties of this peptide (Barton et al., 2000, Boffa et al., 2001, Honing et al., 2000, Opocensky et al., 2006, Vernerova et al., 2008).

Endothelin exerts effects on various components of the glomerular filtration barrier. It promotes mesangial cell proliferation, hypertrophy of the glomerular basement membrane, which is likely to be a podocyte effect, and can mediate direct effects on podocytes due to expression of a fully functional endothelin system including endothelin receptors, preproendothelin-1 and endothelin converting enzyme 1 (Rebibou et al., 1992). Both in vivo and in vitro studies showed that endothelin, similar to angiotensin II, promotes disruption of the podocyte actin cytoskeleton, increases glomerular albumin permeability and stimulates podocyte apoptosis (Barton et al., 1997, Morigi et al., 2006, Ortmann et al., 2004).

Preclinical prevention studies and transgenic animal models support the evidence that endothelin pathway plays a role in the pathogenesis of proteinuria and glomerulosclerosis, which makes it an attractive new therapeutic target. Hence endothelin receptor antagonists could be promising candidates for the clinical treatment of proteinuric renal diseases.
Properties of ETaRA sitaxsentan

Sitaxsentan (SITA) is a sulfonamide-based ETa receptor antagonist licensed for treatment of pulmonary arterial hypertension (O'Callaghan & Gaine, 2006). The drug is administered orally and it is rapidly absorbed and highly bound to plasma proteins (>99.5%), in particular albumin. Sitaxsentan is extensively metabolized but the metabolites are shown not to contribute significantly to its therapeutic efficacy (Inveresk Research 2004). In healthy human subjects, sitaxsentan displays linear steady-state pharmacokinetics at the 100mg therapeutic dose (with nonlinear kinetics at higher doses) (Inveresk Research 2004).

Sitaxsentan is relatively stable over 24h in deionised, distilled water (>90% parent). When dissolved in water, the solution is clear, pale yellow and can be stored frozen (-20°C) for up to 23 days. The recommended dose for oral administration in food or drinking water in rat model studies is 40-45mg/kg/day (personal communication with Pfizer representative Simon Teal).
5.2 Pilot studies with ETaRA sitaxsentan

**Sitaxsentan pilot study (i): measuring baseline BP, water and food intake**

In order to determine the appropriate dilution of sitaxsentan for our study to achieve 40mg/kg/day drug intake, a pilot study part (i) was carried out to measure the baseline water, food intake and blood pressure.

Six cages of mice containing one to six mice per cage were selected with mean mice weight of 35g (range 24.3-50.4g). Standard drinking water and dry food pellet diet were given to mice as under normal condition and their water and food intake were recorded in two separate occasions (see Table 5.1). The results showed that at baseline, assuming mice weigh an average of 35g and consume 4ml of water per mouse per day, and sitaxsentan was given in drinking water at 40mg/kg/day, the drug should be diluted to 350mg/L.

To obtain a baseline blood pressure (BP) of Podo-DTR line 47 mice, 13 mice of 12-15 months old (male n=7, female n=6) were selected (Table 5.2). Tail cuff blood pressure was taken on trained conscious mice using tail cuff plethysmography as described previously (see materials and methods). The results showed that the average male mice blood pressure (104±15 mm/Hg) was lower compared to the respective female mice (115± 5mm/Hg). However, they were not significantly different (p= 0.10) due to high variation in the male group. The average blood pressure of all mice was 109±12mm/Hg.

**Sitaxsentan pilot study (ii): effects of sitaxsentan on BP, food and water intake**

For the sitaxsentan pilot study part (ii), mice (n=9) aged 12-14 months housed evenly in 3 different cages were given water with (n=6 SITA) or without (n=3 WATER) sitaxsentan for three weeks. Of note, mice from group one and two were given sitaxsentan at 350mg/L for eight days, followed by an increase in dosage to 700mg/L for 13 days. The blood pressure of mice was recorded weekly (Table 3), while water and food consumptions were recorded on a regular basis.
The average water consumption was not significantly different between the SITA treated (5.3±1.9 ml/mouse/day) and water treated (5.2±1.3 ml/mouse/day) groups during the 3 weeks (Fig. 5.1a). In addition the weekly group mean water consumption of SITA male, SITA female and WATER female at week one, two and three were not significantly different (Fig 5.1b). No significant difference was detected in the level of water consumption between male and female mice whether receiving the drug or water as placebo (Fig. 5.1b).

Food record showed that over a period of three weeks, on average SITA treated male mice consumed more food than the respective SITA treated and water treated female mice (4.1±1.0 versus 3.1±0.8 (p<0.01) and 3.4±0.9 g/mouse/day respectively) (Fig. 5.2a). However, no significant difference was observed between female SITA treated and WATER treated mice (Fig. 5.2a). This meant that the higher level of food consumption seen in male mice was likely due to bigger body mass (mean body weight: 48.5g) compared to the female mice weight (32.1g) of similar age (range 12-14 months) (Fig. 5.3a).
Figure 5.1. Water consumption of SITa pilot study (ii). Mice (n=9) housed evenly in three different cages (aged 12-14 months) were given normal dry food chow pellets, water with (n=6) or without (n=3) sitaxsentan (SITa) for three weeks at 350mg/L for the first 8 days followed by an increase in dosage to 700mg/L for the following 13 days. (a) Record of water consumption of three weeks time of individual groups. (b) Weekly record of water consumption of individual groups. Each point represent daily water intake per mouse per cage. Mean ± SD are shown. 
F, female; M, male; NS, not significant; SITa, sitaxsentan.
Figure 5.2. Food consumption of SITA pilot study (ii). Mice (n=9) housed evenly in three different cages (aged 12-14 months) were given normal dry food chow pellets, water with (n=6) or without (n=3) sitaxsentan (SITA) for three weeks at 350mg/L for the first 8 days followed by an increase in dosage to 700mg/L for the following 13 days. (a) Record of food consumption of 3 weeks timeline of individual groups. (b) Weekly record of food consumption of individual groups. Mean ± SD are shown. F, female; M, male; NS, not significant; SITA, sitaxsentan.
**Sitaxsentan pilot study (iii): comparing serum levels of sitaxsentan when changing the drug daily or every two days**

In a parallel pilot study (iii), we wanted to test the stability of the drug diluted in sterile water and compare the level of the sitaxsentan reached in the serum to see if there was any difference between the methods of changing the drug daily (every 24h) or every other day (48h). If the latter method were viable, we would benefit from reducing the labour time and drug waste. For this experiment, 12 male mice aged 13-16 months were used (Fig. 5.3b).

The initial plan was to give fresh sitaxsentan at 350mg/L every day for five days to all mice, then split the animals into two groups (n=6), group one received fresh SITA water every 24h for an additional two days, while group two received the same SITA water bottle for 48h (5 days + 48h). However, unexpectedly, in one of the cages, two of the three mice that were housed together were being attacked and scratched by the dominant mouse (ID 915), as result, the two wounded mice (ID 924 and 983) lost substantial weight in the first five days probably due to lack of food and water intake. Similarly, ID 910 was also bullied by a more dominant mouse that was housed in the same cage and lost excessive weight. The animal was culled prematurely at day 5 before reaching the end-point at day 14.

To avoid losing the two affected mice from dehydration and aborting the whole experiment, the animals were housed individually in separate cages for the remaining of the experiment and were given water soaked food pellets until the following day to recover. We also ceased giving sitaxsentan for one day to all mice to keep the consistency of the experiment. As a result of this incidence, the experiment was prolonged to two weeks timeline (group one: changed drug everyday for 14 day, group two: changed drug every day, then at day 12 left the drug for 48h). The blood was collected via the inferior vena cava after terminally anaesthetizing the mice, and serum samples were store at -80ºC freezer until the day of analysis.
Figure 5.3. (a) Mice weight record of sitaxsentan pilot study part (ii). No significant change in body weight was observed in SITA treated or water treated mice over the 3 weeks period.

Figure 5.3. (b) Mice weight record of sitaxsentan pilot study part (iii). The weights of 4 out of 12 mice were affected substantially. ID 910 was culled prematurely at day 5, 2 out of 4 mice (963 and 924) stabilized after day 11 and 12 respectively. ID 983 progressively lost weight until it was culled at day 14.
Summary of sitaxsentan pilot studies

From the sitaxsentan pilot study part (i) and (ii), we concluded that the food and water intake was not affected by the addition of sitaxsentan to the drinking water in the three weeks time period. The weights of the mice in the three groups from pilot study (ii) as well as their blood pressure were not significantly affected by oral sitaxsentan at 350mg/L after one week or in the following two weeks at 700mg/L dose as shown in Fig. 5.3a and Table 5.3 respectively (blood pressure at week zero: 103±9.7, week one: 105±9.9, week two: 103±6.9, week three: 98±5.3mm/Hg). It was not possible to make statistical comparison in blood pressure between the SITA treated and the WATER treated groups at week two and three, as one out of three mice in the WATER treated group died prematurely. All mice survived to the end of the experiment, and no obvious illness symptoms were observed, with the exception of ID 991 from water treated group that died at day 14 while blood pressure measurements were being taken. Overall the animals looked healthy with shiny fur coat and were physically active. Histologically, the kidneys had normal morphology with occasional protein casts. No obvious kidney damage or major organs defects were observed.

The levels of serum sitaxsentan from the pilot study (iii) have not yet been analyzed by our collaborator. So to be on the cautious side, in our actual SITA study the drug was changed daily. From this last pilot study we also learned that some male mice could be very territorial and aggressive, especially when there is limited water availability. Of note, for the first 12 days 50ml drug aliquot (instead of normal 250ml weekly drinking water) was changed daily for every cage housing two to four mice per cage to avoid excess drug wastage. This adequate but decreased drinking volume may have caused the fighting between the mice in some of the cages leading to weight loss in four out of twelve mice (Fig. 5.3b). Therefore, future experiments should take these factors into account and house male mice individually (especially if they are not littermates) and provide considerable excess drinking volume.
Table 5.1. SITA pilot study (i) a: baseline food and water consumption of Podo-DTR line 47 mice.

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<th>Grp No</th>
<th>Genotype</th>
<th>age</th>
<th>cage</th>
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<th>BP (mmHg)</th>
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Mean weight: 35.1, 3.8, 4.0, 3.7, 3.6
Range: 24.3-50.4, 3.6-4.4, 3.5-4.1, 3.3-4.4, 2.7-4.1

Table 5.2. SITA pilot study (i) b: baseline blood pressure of Podo-DTR line 47 mice.

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<tr>
<td>991</td>
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Table 5.3. Blood pressure measurement of sitaxsentan pilot study (ii) at various time points. No significant difference was detected between sitaxsentan and water treated mice at different time points.

BP, blood pressure; gr, group; sex 1= male; 0= female; SITA, sitaxsentan; treat, treatment; wk, week.
5.3 Experimental outline of sitaxsentan experiment

The experimental protocol was phased to keep numbers manageable, so the study was split into two parts: SITA 8a and SITA 8b. Briefly transgenic mice and wild-type (WT) controls were given placebo or oral sitaxsentan (110mg/kg) and/or captopril (200mg/L) in their food and water respectively 24h after intraperitoneal injection of diphtheria toxin at 1ng/g body weight. Five groups received different treatments. Group one mice were given sitaxsentan in food (SITA); two: ACEi captopril in drinking water (ACEi); three: sitaxsentan plus captopril (SITA+ACEi); four: water as placebo (WATER); five: wild-type controls given both sitaxsentan and captopril (WT SITA+ACEi) (Fig. 5.4).

In the first half of the experiment, a total of 32 mice (Tg n= 27, WT n= 5) were used. While in the second half 35 mice (Tg n= 29, WT n= 6) with similar n number distribution were allocated. Although the initial intention was to combine the results from the two parts of the experiments to obtain higher statistical power to detect any difference between the experimental groups, due to a number of unexpected changes and factors in the first part of the experiment, the results of part a and b were analyzed independently. In this chapter SITA 8b results are presented principally, as the experimental study was improved and better designed than part a, hence more representative results were generated. However, results from SITA 8a will also be covered and discussed. Primary endpoints from this study were glomerulosclerosis and kidney function (serum creatinine). Other readouts included blood pressure, albuminuria, serum albumin, podocyte quantification and collagen staining of kidney.

It is important to note that five mice (two from SITA, one from ACEi and two from WATER group) were excluded from the result analysis (SITA 8b) due to normal urinary ACR throughout the study and sclerosis index at week eight, indicating that these mice were possibly not affected by the toxin injection. Since the toxin was injected intraperitoneally, the needle could be misplaced elsewhere e.g. in the intestine, thereby not eliciting the same level of toxin effect.
Figure 5.4. Schematic representation of combined drug intervention study with ETaRA sitaxsentan and ACEi captopril. Transgenic (Tg) mice and wild-type (WT) controls were given placebo or oral sitaxsentan (110mg/kg) and/or captopril (200mg/L) in their food and water respectively 24h after intraperitoneal injection of diphtheria toxin at 1ng/g body weight. Five groups received different treatments. Group 1 mice were given sitaxsentan in food (SITA); 2: ACEi captopril in drinking water (ACEi); 3: sitaxsentan plus captopril (SITA+ACEi); 4: water as placebo (WATER); 5: wild-type (WT) controls given both sitaxsentan and captopril (WT SITA +ACEi). Urine samples were collected in metabolic cages at different timepoints for urinary albumin:creatinine measurements; blood pressure was taken at week 0, 2, 7 and blood samples were collected prior to culling the mice at week 8.

ACEi, angiotensin converting enzyme inhibitor; BP, blood pressure; bw, body weight; DT, diphtheria toxin; WK, week; SITA, sitaxsentan; WT, wild type;
**Statistical analysis**

Results in this chapter are expressed as mean ± standard deviation (SD) unless otherwise specified. Statistical differences between groups were established by the Student t-test, one-way ANOVA analysis of variance with Bonferroni’s multiple comparison test as appropriate. A p-value of <0.05 was considered to be significant.
5.3.1 Results

**ACEi but not SITA lowered blood pressure in toxin injected sick mice**

To see the effect of the toxin on transgenic and wild-type mice, unpaired Student t-test was used to compare the blood pressure of transgenic mice in group one, two, three or four versus wild-type control group five mice at week seven. The results showed that ACEi (group two) and SITA+ACEi (group three) mice had their blood pressure significantly reduced compared to the WT SITA+ACEi controls (90±12, 97±12 versus 111±9 mm/Hg), p≤0.04; while WATER treated mice (group five) had the blood pressure significantly raised (136±17mm/Hg) compared to the wild-type controls, p<0.05 (Fig. 5.5).

To see the difference between drug treated and water (placebo) treated mice, unpaired Student t-test was applied again. The results showed that SITA and ACEi alone or in combination lowered blood pressure significantly compared to the water treated group (SITA: 109±17, ACEi: 90±12, SITA+ACEi: 97±12 versus WATER: 136±17mm/Hg), p ≤0.03. However, the combined treatment lowered blood pressure further than SITA as a single agent (97±12 versus 109±17mm/Hg respectively). In order to see whether there was any difference between the single and combined therapies, one-way Anova was used and no significance difference was detected (Fig. 5.5).

Furthermore, blood pressure of week zero (prior to diphtheria toxin or drug treatment) and week seven timepoint of respective five groups were compared using paired, two tail t-test. The results showed that only ACEi alone but not SITA lowered blood pressure significantly from week zero to week seven (ACEi: 108±9.8 versus 90±12mm/Hg, p=0.006). While in water treated group, although there was a trend of blood pressure increasing from week zero to week seven (113±5.0 versus 136±17mm/Hg respectively), the difference was not quite significant (p=0.08) (data not shown).
Figure 5.5. Tail cuff systolic blood pressure (BP) of Podo-DTR line 47 mice at week 7 post DT injection at 1ng/g bw. Blood pressure of ACEi treated and SITA+ACEi treated mice were significantly lowered compared to the water treated group (90±12 and 97±12 vs 136±17mm/Hg respectively, p<0.04), but SITA had little or no effect compared to the WT SITA+ACEi control (109±17mm/Hg vs 111±9mm/Hg respectively, p=0.72). Significance was assessed using the Student t-test when comparing Tg vs WT or drug vs placebo (water) groups. For multiple comparisons, ANOVA was used and post hoc analyses were done with Bonferroni’s multiple comparison test, mean ± SD are shown in the graph.

DT, diphtheria toxin; ACEi, angiotensin converting enzyme inhibitor; WT, wild type; bw, body weight; vs, versus; SD, standard deviation; *, p≤ 0.03 vs WATER; +, p≤ 0.04 vs WT SITA+ACEi, NS, not significant.
SITA and ACEi alone or in combination reduced histological damage

Glomerulosclerosis score also showed beneficial effect of the drug treatments in kidney histology (Fig. 5.6a-d). The water treated group had the highest glomerulosclerosis index score (0.5±0.16). While the WT SITA+ACEi control had the lowest sclerosis index (0.04±0.03), with 98.2% normal/not sclerosed glomeruli (Fig. 5.6c). Drug treated mice, either alone or in combination had significantly reduced histological damage compared to the placebo (water) treated mice (SITA: 0.17±0.03, ACEi: 0.16±0.03, SITA+ACEi: 0.08±0.02 versus WATER: 0.5±0.16, p ≤0.02) (Fig. 5.6d). However, only combination drug treatment with SITA+ACEi reduced glomerular damage comparable to wild-type control (0.08±0.02 versus 0.004±0.003, p=0.086) (Fig. 5.6d).

Serum analysis: Combined but not ACEi alone lowered serum creatinine

ACEi and WATER treated groups had significantly higher serum creatinine levels than wild-type controls (11±1.6, 13±3.6 versus 9.0±1.3μmol/L respectively), p<0.03. While SITA (9.0±3.0μmol/L) and SITA+ACEi (8.2±2.0μmmol/L) were not significantly different compared to the WT SITA+ACEi mice. Only combined treatment reached a significant level of reduction in serum creatinine when compared to water treated group, p<0.02. When comparing the groups that received either single or combined drug treatments, ACEi animals seemed to have a higher trend than both SITA alone and SITA+ACEi, but no significant difference was detected (Fig. 5.7a).

Also serum urea and albumin levels were measured, but little change was seen in serum urea results (Fig. 5.7b). Although the water treated group had the highest serum urea (12.7±4.5mmol/L), no significant difference was detected when compared to wild-type controls (10.0±2.1mmol/L) or drug treated groups (8.7±1.8, 12.2±1.9, 11.6±3.8mmol/L for SITA, ACEi and SITA+ACEi respectively) (Fig. 5.7b).
Figure 5.6. (a) Glomerular histology at various degree of sclerosis (A) score 0 = 0\% sclerosis, (B) score 1= 1-50\% sclerosis, (C) score 2= >50-95\% sclerosis, (D) score 3= 100\% sclerosis. Periodic acid Schiff staining (PAS), (A-D) at x400 magnification.
Figure 5.6. (b) Kidney histology of transgenic and wild-type (WT) drug treated (SITA, ACEi, SITA+ACEi) and untreated (WATER) Podo-DTR line 47 mice injected with diphtheria toxin at 1ng/g body weight at week 8. Periodic acid Schiff staining (PAS), (A-F) at x200 magnification, (H-L) at x400 magnification. SITA, sitaxsentan; ACEi, angiotensin converting enzyme inhibitor; WT, wild-type.
Figure 5.6. (c) Glomerulosclerosis score in percentage (d) glomerulosclerosis index of Podo-DTR line 47 mice at 8 weeks post DT injection at 1ng/g bw with or without drug treatment (ACEi captopril: 200mg/L and/or ETAi sitaxsentan: 110mg/kg). Significance was assessed using the Student t-test when comparing Tg vs WT or drug vs placebo (water) groups. For multiple comparisons, ANOVA was used and post hoc analyses were done with Bonferroni’s multiple comparison test, mean + SEM are shown in the graph.
DT, diphtheria toxin; ACEi, angiotensin converting enzyme inhibitor; ETAi, endothelin type a receptor antagonist; *, p ≤ 0.02 vs WATER; ++, p ≤ 0.007 vs WT SITA+ACEi.
Figure 5.7. Serum analysis of Podo-DTR line 47 mice at week 8. (a) Serum creatinine and (b) serum urea. Serum creatinine was significantly lowered in combined (*, p<0.02) but not in SITA or ACEi alone treatment groups when compared to water treated mice. ACEi and water treated mice had their serum creatinine significantly raised compared to WT controls, p<0.03.

ACEi, angiotensin converting enzyme inhibitor; SITA, sitaxsentan, WT, wild-type; +, p<0.03 vs WT control.
No significant difference in urinary ACR at week 8

In this experiment differences in urinary albumin creatinine ratio (ACR) were not significant at eight weeks (Fig. 5.8). Prior to diphtheria toxin injection, mice from all five groups had baseline urinary ACR with a mean value of 2.3mg/mmol (range: 0.9-6.1mg/mmol). This remained more or less constant throughout the experiment. All transgenic toxin injected mice peaked at week one, but the drug treated groups were not significantly different from the water treated group (SITA: 4759±2261, ACEi: 4770±1459, SITA+ACEi: 5577±2300 versus WATER: 3427±3732mg/mmol) (Fig. 5.8).

The albuminuria levels were reduced at later time points at week two, six, and eight regardless of mice receiving the drug treatment or placebo (water). At week two, the trend seemed to show that ACEi was most effective at reducing albuminuria, followed by combined treatment and SITA alone, but the results were not significantly different (284±97, 474±352, 918±436 versus WATER: 1622±1892mg/mmol respectively). However, at later time points, although the albuminuria progressively dropped, there was no significant difference between the drug treated groups at week six (64±16, 64±73, 71±44mg/mmol) or week eight (31.5±10.5, 32.5±25.3, 37.4±23.5mg/mmol) for SITA, ACEi and SITA+ACEi respectively.

Surprisingly, the water treated group, despite having the worst histological score in the kidneys (Fig 5.6), the albuminuria was lower than the drug treated groups at both week six and eight (WATER at week six: 33.9±16.3, week eight: 10.9±5.1mg/mmol respectively) (Fig. 5.8), which could be due to decreased GFR. However, the significant difference was only observed with SITA alone (SITA at week six: 63.7±16.4, p=0.02, week eight: 31.5±10.5mg/mmol, p=0.007) but not ACEi or SITA+ACEi groups (Fig. 5.8). All ACR values were expressed as means ± SD. Significance was assessed with unpaired Student t-test when comparing transgenic versus wild-type mice and drug treated versus placebo treated mice for each timepoint at week zero, one, two, six and eight. For multiple comparisons, ANOVA
was used and post hoc analyses were done with Bonferroni’s multiple comparison test.
Figure 5.8. Urine albumin:creatinine ratio (ACR) of Podo-DRT line 47 mice (n=4-7). Significance was assessed with Student t-test. Mean and SD are shown in the graph. +, p< 0.05 vs WT SIT+ACEi; *, p<0.05 vs WATER.
*Podocyte number was not significantly protected by drug treatments*

Podocyte number and the respective glomerular area of 30 glomeruli per animal were counted and measured using ImageJ (Tg n= 4-7, WT n= 6) as previously described (see materials and methods). At week eight, transgenic toxin treated susceptible mice had lower podocyte counts than wild-type controls regardless of additional treatment with drugs or water (median podocytes per glomerular cross section (GCS) for SITA: 6.0, ACEi: 5.5, SITA+ACEi: 5.4, WATER: 4.2 versus wild-type control: 7.9). Only the SITA group was not significantly different from wild-type controls, p=0.14 (Fig. 5.9a). Although it looks like there could be some protection from drug treatments, when compared to the water treated animals, no significant difference was detected. This remained the case if count per GCS was corrected for glomerular area (Fig 5.9b).
Figure 5.9. Box plots of the (a) podocyte count or (b) podocyte count per unit glomerular cross section area of Podo-DTR mice injected with DT at 1ng/g bw treated with or without drugs (ACEi captopril: 200mg/L and/or ETaRA sitaxsentan: 110mg/kg) at week 8. Significance was assessed using the Student t-test.

DT, diphtheria toxin; bw, body weight; SITA, sitaxsentan; ACEi, angiotensin converting enzyme inhibitor; ETaRA, endothelin type a receptor antagonist; WT, wild type.
**PSR but not collagen IV or I were reduced by drug treatments**

Picosirius red (PSR) staining of water-treated mice had the highest percentage level of total area (5.4±3.2) (Fig. 5.10 and Fig. 5.11a), indicating the increased level of total collagen formation in the kidney cortex, which supported the increased scarring seen in the glomeruli (Fig. 5.6d). Unlike the glomerulosclerosis index results, where the combination treatment reached the lowest level amongst the drug treated groups, PSR data showed that ACEi was most effective in reducing total collagen (ACEi: 2.2±1.7). While all three drug treated groups had the PSR staining reduced compared to the placebo (water) treated mice, reaching comparable levels to wild-type controls (SITA: 2.9±0.6, ACEi: 2.2±1.7, SITA+ACEi: 3.1±1.2 versus wild-type controls: 3.3±0.4), SITA and combined treatment were not significantly different from water-treated mice (WATER: 5.381±3.166) (Fig. 5.11a).

Collagen IV results showed that all transgenic mice injected with the toxin had their staining elevated compared to the wild-type controls, regardless of them being treated with the drugs or placebo (water) (SITA: 11.7±1.8, ACEi: 10.8±2.3, SITA+ACEi: 9.9±1.1, WATER: 9.6±5.3 versus wild-type controls: 6.9±3.5). However, only SITA and ACEi treated mice were significantly higher than the wild-type controls, p ≤ 0.03 (Fig. 5.11b).

Results from collagen I staining were totally unexpected, as the worst affected water treated group (indicated by glomerulosclerosis index and serum analysis) had similar and very low levels of staining to wild-type controls (WATER: 0.2±0.1 versus wild-type controls: 0.1±0.08) when compared to the increased levels of drug treated groups (SITA: 4.5±1.2, ACEi: 1.9±0.8, SITA+ACEi: 1.1±0.5), p<0.05 (Fig. 5.11c). SITA was significantly higher than ACEi and combination treatment (p<0.001), while no significant difference was detected between ACEi and SITA+ACEi (Fig. 5.11c).
Figure 5.10. Kidney histology pictures of PSR (column one), collagen IV (column two), collagen I (column three) staining of Podo-DTR line 47 mice treated with or without the drugs at week 8. (A-C) sitaxsentan, SITA, (D-F) ACEi, (G-I) SITA+ACEi, (J-L) WATER, (M-O) WT SITA+ACEi controls. X100 magnification.
ACEi, angiotensin converting enzyme inhibitor; PSR, picrosirius red; WT, wild-type.
Figure 5.1. Histogram graphs of percentage of total area of kidney cortex staining of (a) picrosirius red (PSR), (b) collagen IV and (c) collagen I of Podo-DTR line 47 mice at week 8.
5.4 Blood pressure control study with SITA and ACEi in DT untreated mice

From the combined therapy study results, the renal damage caused by diphtheria toxin caused hypertension in transgenic mice that were treated with placebo (water), and blood pressure was not significantly lowered in sitaxsentan treated mice compared to wild-type controls (SITA: 109±17mm/Hg versus wild-type controls: 111±9 mm/Hg). To investigate further the effect of sitaxsentan and/or captopril on healthy mice, a follow up blood pressure experiment was carried out using transgenic Podo-DTR line 47 mice without diphtheria toxin injections (Fig. 5.12).

Briefly, groups of eight transgenic mice with age ranging from 6-12 months (median age: ten months) old were give oral sitaxsentan and/or captopril or placebo (no added drug) in their food and water respectively for four weeks at the same concentrations as described for the combined experiment above. The drugs were taken off in the following two weeks where mice returned to their normal food and water diet until the endpoint at week six. The blood pressure measurements were taken using tail cuff plethysmography (see materials and methods) prior to drug treatment at week zero, week two and four post treatment and week six after the animals have been off the drug for two weeks. Food and water consumption were monitored weekly on regular basis.
Figure 5.12. Schematic representation of blood pressure control study with SIT and ACEi in diphtheria toxin untreated mice.

Groups of eight transgenic mice with age ranging from 6-12 months (median age: ten months) old were given oral sitaxsentan and/or captopril or placebo (no added drug) in their food and water respectively for four weeks at the same concentrations as described for the combined experiment. The drugs were taken off in the following two weeks where mice returned to their normal food and water diet until the endpoint at week six. The blood pressure measurements were taken using tail cuff plethysmography prior to drug treatment at week zero, week two and four post treatment and week six after the animals have been off the drug for two weeks. Food and water consumptions were monitored weekly on regular basis.
5.4.1 Results

*SITA alone or in combination reduced blood pressure in toxin untreated mice*

Healthy transgenic mice not injected with diphtheria toxin treated with SITA, ACEi alone or in combination have their blood pressure significantly reduced at week two post treatment when compared to the respective groups at week zero timepoint (week zero to two, SITA: 117±7.7 to 100±5.1; ACEi: 108±5.2 to 92±6.2; SITA+ACEi: 109±4.5 to 99±7.1mm/Hg, p<0.01). As expected blood pressure of placebo (water) treated mice was not significantly lowered from week zero to two (WATER: 115±4.9 to 109±7.1mm/Hg respectively) (Fig. 5.13c).

However, when comparing blood pressure of drug treated mice versus water treated mice at week 2, only ACEi and SITA+ACEi were significantly lowered (92±6.2 and 99±7.1 versus 109±7.1mm/Hg, p<0.05), but not SITA alone (110±5.1mm/Hg). This is likely due to the group variation, as the starting blood pressure of the SITA mice was highest (117±7.7mm/Hg) as shown in Fig. 5.13a and 5.13c. This was demonstrated by calculating the percentage change in blood pressure where both SITA and ACEi reached similar levels (86.3 versus 85.5% from 100% of the original measurements respectively) (Fig. 5.13b). Surprisingly, the combined treatment did not lower blood pressure further when compared to the single therapies (91.2%). This led to further analyses of the food and water consumption of mice as detailed below.

At week 6, after mice were taken off the drugs for 2 weeks (between week 4-6), blood pressure returned back to baseline levels (SITA: 111±5.1, ACEi: 111±5.3, SITA+ACEi: 111±2.9). These measurements were significantly higher than the respective week two blood pressure, but were comparable to the week six water treated mice (WATER: 107±7.7mm/Hg). No significant difference was detected between the groups at week six.
Of note, due to change in personnel handling and taking the blood pressure at week four, the measurements were not suitable for comparison due to substantial variation, therefore only blood pressure results of week zero, two and six were presented.
Figure 5.13. (a) Systolic blood pressure (b) percentage blood pressure change, (c) scatter plot of individual systolic blood pressure of mice treated with sitaxsentan (SITA), ACEi captopril (ACEi), combination (SITA+ACEi) or placebo (WATER) at different timepoints without diphtheria toxin (DT) injection.

*, p < 0.05; NS, not significant. For multiple comparisons amongst the same treatment group, ANOVA was used and post hoc analyses were done with Bonferroni's multiple comparison test.
**No significant difference in food intake between drug and placebo treated groups**

Unlike in the pilot study where sitaxsentan was given in the drinking water at 350mg/L, in the later experiments, sitaxsentan was given in the food at 110mg/kg to obtain a dosage of 40mg/kg/day. Although mice in the sitaxsentan group on average consumed less than ACEi and SITA+ACEi mice, the difference was not significant amongst the groups (SITA: 11.8±3.7, ACEi: 14.5±4.5, SITA+ACEi: 12.5±3.2, WATER: 11.1±1.8g/mouse/day) (Fig. 5.14a).

**SITA treated mice drink less water than ACEi- and placebo-treated animals**

The results from the water consumption data showed that mice receiving sitaxsentan in their food from week 1-4 (whether alone or in combination) tend to drink less water compared to ACEi- and placebo-treated mice (SITA: 2.0±0.6, SITA+ACEi: 3.4±1.8 versus ACEi: 4.3±1.7, WATER: 4.1±0.9ml/mouse/day) (Fig. 5.14b). However, the level of significance was only seen in the SITA treated mice when given as a single agent (p<0.05) (Fig. 5.14b). Untreated wild-type mice (given standard water and solid/dry food diet throughout the experiment) were also included as an extra control, and the level of water intake was similar to the transgenic placebo (water) treated mice (4.5±2.1 versus 4.1±0.9ml/mouse/day respectively) (5.14b).

At week 5-6, the level of water intake was normalised in all drug treated groups after the animals were taken off from the treatment (SITA: 4.7±0.4, ACEi: 5.6±1.0, SITA+ACEi: 5.0±0.5 ml/mouse/day). These levels were comparable to the wild-type untreated controls (5.0±0.9ml/mouse/day) (Fig. 5.14c). On the other hand, the placebo (water) treated mice had their water intake significantly lowered compared to the rest of the groups at week 5-6 (WATER: 3.4±0.5ml/mouse/day, p<0.05). Unlike in the previous four weeks, where the mice were given dry chow pellets (WATER: 4.1±0.9ml/mouse/day) (Fig. 5.14a-b), in the following two weeks (week 5-6), the animals were given gelatine/chow mixed wet food to obtain baseline food consumption for comparison with drug treated mice (Fig. 5.14c).
Figure 5.14. (a) Average food (b) water (week 1-4), (c) water (week 5-6) consumption of blood pressure control study with SITA and ACEi in diphtheria toxin untreated mice. Significance was assessed using ANOVA, for multiple comparisons, post hoc analysis were done with Bonferroni's multiple comparison test. Mean ± SD are shown in the graph. *, p<0.05
5.5 Discussion

Summary of sitaxsentan and combined intervention study
In the combined drug intervention study, sitaxsentan treatment on its own and in combination with captopril improved matrix accumulation and scarring seen following specific podocyte injury in our Podo-DTR model (Fig. 5.6b-d). From the glomerulosclerosis index (Fig. 5.6d) and serum creatinine (Fig. 5.7a) results, the combined therapy (ETaRA plus ACEi) had a synergistic effect on progressive kidney disease. Similar to the previous ACEi intervention study results, the protection does not seem to be attributed entirely to podocyte number rescue, as no significant increase in podocytes have been observed at week eight after the toxin injection followed by drug treatments. This raises the possibility that alternative mechanisms such as amelioration of podocyte phenotype or function may be involved in achieving long-term protection.

PSR and collagen staining
Although the level of significance was not reached in the PSR results for SITA and combination treatment, the trend showed a decrease in total collagen in the kidney cortex of all drug treated mice compared to the water treated mice (Fig. 5.11a). These reduced levels were comparable to wild-type controls (Fig. 5.10 and Fig. 5.11a).

From the PSR and sclerosis results, we expected a decrease in collagen IV and I levels in the kidney cortex in drug treated mice, unexpectedly, the levels were either similar (in the case of collagen IV) or higher (in collagen I) when compared to the water treated group. These data were different from the glomerulosclerosis results, where marked improvements were observed in glomerular scarring in mice that received the drugs (Fig. 5.6d). This difference could be accounted to the area of kidney cortex being covered and assessed, as both glomeruli and tubules were included in collagen measurement instead of glomeruli alone in the glomerulosclerosis index, suggesting that the additional collagen formation was possibly from tubular and/or interstitial fibrosis (Fig. 10-11).
While the difference could account for collagen IV results, the data from collagen I analysis was puzzling. Not only the drug treated mice had much higher level of collagen I than the wild-type controls, but the water treated group had similar and extremely low levels that was comparable to the wild-type controls. Taking into account that water treated mice had worst glomerulosclerosis, and highest PSR levels, the photo taken for collagen I analysis were re-examined and re-assessed. It turned out that the intensity of the staining was different in various groups, where SITA mice were prominently darker than the water treated mice (Fig 5.10 C and L), raising the possibility of batch difference during the DAB (3,3’-diaminobenzidine) or counterstain steps despite the fact that a single immunohistochemistry run was carried out in the same day.

The potential variations between e.g. rack one and two in any of the two steps described is likely to affect the analysis of the staining and subsequent result outcomes. Therefore additional care should be taken in the future to minimize the incubation or staining time between the batches, as well as mixing the slides from different groups if batching was unavoidable. Also when taking photos for analysis, it is important that the settings of the microscope and camera need to be same for all the slides.

**Blood pressure effects of SITA and ACEi in toxin treated and untreated mice**

In the combined intervention study, blood pressure comparison of the respective experimental groups at week zero and week seven showed that only ACEi captopril reduced blood pressure significantly after seven weeks of treatment, demonstrating that captopril was a more effective drug agent in lowering blood pressure in toxin treated i.e. diseased animals. While sitaxsentan alone and in combination maintained the blood pressure in toxin-affected mice to baseline level similar to wild-type controls.

Unlike the results of the pilot study (ii), where no significant change was seen in blood pressure after short-term treatment of oral sitaxsentan in healthy mice (Table 5.3), a significant drop was observed in the later blood pressure control study with
SITA and/or ACEi in diphtheria toxin untreated mice (Fig. 5.13). Two main factors could attribute and explain the different outcomes. Firstly, although sitaxsentan was administered orally in both settings to achieve a dose of 40mg/kg/day, in the pilot study the drug was given in the drinking water, while in the later study sitaxsentan was given in the food. The latter method of administering the drug with the food possibly resulted in a more consistent drug intake, which could partly explain the significant change in blood pressure. Therefore depending on the rate and how the drug is metabolized, the level of active compound may have substantial effect in blood pressure outcomes. Secondly, unlike the pilot study where small group number were used (n=3-6), the later study involved bigger experimental settings (n=8) (Fig. 5.12), hence giving a higher power to detect any difference in statistical change.

In addition, in human studies, the use of endothelin receptor antagonists have been shown to cause fluid retention (Mann et al., 2010), although oedema formation was not observed or obvious in our experimental animals, the drug may have caused a decreased water intake as demonstrated later (section 5.4) in the blood pressure control study with SITA and ACEi in diphtheria toxin untreated mice (Fig. 5.14b).

Although all three factors could potentially contribute to the result outcome either on its own or in combination, the degree of variability of the tail cuff method for blood pressure measurement certainly require large experimental number to detect any small blood pressure change.

*Urinary ACR was not significantly lowered by sitaxsentan*

Urinary ACR results showed that SITA and ACEi alone or in combination might improve proteinuria at earlier time points (i.e. week 2-4) when the mice were worst affected by the toxin as supported by substantial weight loss (Fig. 5.15). However, they did not contribute in lowering albuminuria further than the placebo treated mice at later time points as shown in Fig. 5.8. Some transient albuminuria was seen in two out of six WT SITA+ACEi control mice at week eight only (ID 1237 and 1240 with 27.5 and 16.4mg/mmol ACR respectively), but the kidney histology was normal with 99 and 98% normal glomeruli respectively. This could be due to a transient infection
(e.g. presence of pinworms infection in the animal unit at the time the study was carried out but learned about the fact later). In contrast to previously published results and our expectation, in this study ETaRA sitaxsentan did not improve albuminuria in our toxin induced diseased Podo-DTR model. This could be due to small n numbers and low levels in our experimental groups, in particular the water treated mice where high variation within the animals’ albuminuria results were measured.

Mechanism of action of sitaxsentan needs to be further elucidated
Although it was not possible to determine the mechanism of action of sitaxsentan in this experiment, the ETaRA seemed to have a trend in providing protection in lowering serum urea and creatinine levels at week eight. However, bigger n number in experimental groups and possibly an additional wild-type untreated control group without drug administration (WT DT+WATER) might be useful in elucidating and pinpointing the true difference between toxin treated transgenic versus wild-type mice and drug treated versus placebo (water) treated groups.
Figure 5.15. Group mean weight record of Podo-DTR line 47 mice of SITA experiment 8b. Weight loss was most prominent between week 1 and 3 in transgenic diphtheria toxin treated mice, while wild-type control mice were not affected by the toxin, instead there was an overall gain of weight.
Susceptibility and mortality rate in SITA 8a and 8b study

There were a number of weaknesses in the first part of the experiment that we came across and later addressed the issues and incorporated the improvements in the second half of the study (phase b). In SITA 8a study, due to late arrival of the ETaRA sitaxsentan from our collaborator, the originally allocated mice for the experiment were much older (14-17 months of age) than we previously planned. Also in order to make up for numbers to replace the old mice that we had to cull, very young mice (>5-6 weeks old) were used as replacement; this resulted in an unbalanced and very wide age range.

At week 2-3, transgenic mice injected with diphtheria toxin were most susceptible and worst affected with substantial weight loss (29% of mice (nine out of 31) lost >20% body weight at week two. In this case seven out of 32 mice were culled or died prematurely between day 10 and day 30. All seven mice (two each from SITA and ACEi groups, one from SITA+ACEi group and two from wild-type controls) were aged between 14 and 16 months, suggesting that old mice were more susceptible either to toxin and/or treatment drugs. Of the mice that died or culled early, the majority had renal failure indicated by elevated levels of serum creatinine, urea and decreased serum albumin (Table 5.4), while the remaining mice had moderate increase in serum creatinine in SITA, ACEi and WATER treated group when compared to combination treatment or wild-type controls (Fig. 5.16). Surprisingly, no mortality or early cull occurred in water treated group four, even though very old mice from 15-17 months old were included and histologically kidney damage and urinary albuminuria were most prominent.

In the second part of the SITA experiment (8b), mice age range were reduced (4-6 months) to avoid mortality, only one out of 35 mice was culled prematurely at day 36 (SITA+ACEi group) due to excessive weight loss (30% body weight loss set as maximum limit). The reduced mortality in the second part of the experiment demonstrated that the age of the mice did play an important role in the injury and recovery of the Podo-DTR mice. Therefore future experiments could address more in details the influence of the age in the therapeutic intervention studies.
Figure 5.16. Serum creatinine of combined intervention study phase 8a with Podo-DTR line 47 mice at week 8.
### Table 5.4. Serum results of mice that died or culled early from combined intervention study with sitaxsentan and ACEi phase 8a.

Markedly raised serum creatinine, urea levels in combination with decreased level of serum albumin of sick mice indicated that the animals had renal failure when compared to the baseline levels of one year untreated controls mice. The normal range alanine aminotransferase (ALT) levels indicated that the liver function was normal.

<table>
<thead>
<tr>
<th>ID no</th>
<th>DT (ng/g)</th>
<th>Approx. Age</th>
<th>SEX</th>
<th>Genotype</th>
<th>Drug Treatm</th>
<th>Timepoint</th>
<th>Creat mmol/L</th>
<th>Urea mmol/L</th>
<th>Albumin g/L</th>
<th>ALT u/L</th>
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<tr>
<td>1044</td>
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<tr>
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<td>F</td>
<td>Tg</td>
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<td>F</td>
<td>Tg</td>
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<td>D22</td>
<td>34</td>
<td>17</td>
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<tr>
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<tr>
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<td>9</td>
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<tr>
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<td>F</td>
<td>Tg</td>
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<tr>
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<td>1yr old</td>
<td>13</td>
<td>6.9</td>
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ACEi, angiotensin converting enzyme inhibitor; creat, creatinine; D, day; DT, diphtheria toxin; F, female; n/a not applicable; M, male; mo, month; SITA, sitaxsentan; Tg, transgenic; WT, wild-type; yr, year; untreat, untreated.
5.6 Future improvements

To improve future study, additional n numbers will be required to obtain more representative results and hence higher power to detect statistical significance between the treatment groups. More 24h urine samples should be obtained from each group at additional time points to monitor the change in ACR levels more closely, although this can be very labour intensive and is subject to equipment availability. Furthermore, additional WT DT+WATER control, and transgenic SITA treated but not toxin-injected group might be useful to further elucidate the effects of sitaxsentan on normal mice.

In the follow up blood pressure control study with SITA and ACEi in diphtheria toxin untreated mice, a decreased level of fluid intake was observed in the SITA and SITA+ACEi treatment groups. Although the significance level of water consumption in the combination treatment group was not reached, the decreased intake of captopril containing water (SITA+ACEi: 3.4±1.8 versus WATER: 4.1±0.9ml/mouse/day) during the four weeks treatment might have affected the blood pressure outcome as observed in this study (Fig. 5.14b). This could explain why the combination treatment group did not have their blood pressure lowered even further than the respective single therapies (Fig. 5.13b).

On the other hand, the placebo (water) treated mice had their water intake significantly lowered compared to the rest of the groups at week five to six (WATER: 3.4±0.5ml/mouse/day). This was because these mice were given gelatine/chow mixed wet food that contained approximately 50% water content, instead of dry food pellet given to the rest of the groups, hence affecting and lowering their drinking water consumption. Of note, this method of mixing the ground powdered chow together with the dissolved gelatine and dissolved drug or water alone (detailed in materials and methods) is to prevent unnecessary dispersion of the drug, allow homogenous mixture and reduce wastage of the treatment drug.
These results suggest that by giving gelatine mixed wet food (regardless of presence of the drug or not) could affect the level of water intake. Therefore future studies need to take this factor into account by either adjusting the concentration of the drug in the water or apply an alternative method of delivering the drug (e.g. by gavages) to ensure more accurate dosage to reach desired concentration.

From the sitaxsentan intervention study, we observed that toxin treated transgenic mice were worst affected between week two to three and albuminuria results were not improved at later time points by drug treatment. Therefore an interesting experiment would be to see whether targeted treatment at more specific time points would provide similar if not better degree of protection avoiding potential SITA side effects seen in human (e.g. oedema development).

In a clinical study of diabetic nephropathy, Mann J et al showed that the endothelin antagonist avosentan significantly reduced ACR in the short term, however, the trial was terminated prematurely after a median follow up of four months (maximum 16 months) due to adverse effects of the drug that caused significant fluid overload and congestive heart failure. Because of the early termination of the trial, the authors were unable to conclude whether or not the drug had any significant beneficial effects against kidney progression (Mann et al., 2010). In a different study, Dhaun et al carried out a short-term study where they showed haemodynamic and renal effects of endothelin receptor antagonist TAK-044 in a small cohort of patients with chronic kidney disease (Dhaun et al., 2007). Therefore long-term effects of endothelin antagonist still need to be further elucidated in both experimental and human studies.

The results from our study showed that although glomerulosclerosis was significantly lowered in all drug-treated groups, podocyte number was not significantly protected by drug treatments, though there is a trend toward protection (Fig. 5.9a). Although our intention of intervention study with potential therapeutic agents after induction of injury with diphtheria toxin was to affect the chronic phase, the early treatment started 24h after toxin injection may have also improved the acute phase injury. Nevertheless, whether the beneficial effect of sitaxsentan and/or
captopril affected the acute or chronic or both phases, the actual result outcome is still valid and representative. Even if some of the effect in our model was amelioration of acute damage, this may well be clinically relevant, as it is likely that in human disease new podocytes become injured over a long period of time. In a different model of podocyte injury, Smeets et al showed that protection was achieved even when captopril treatment was delayed to day three post-injury induction (Smeets et al., 2006).

The possible fate of podocyte number after diphtheria toxin injection is illustrated in Fig. 5.17a. Depending on the severity of injury induced, hypothetically one option is that the cell number will eventually gradually increase if replacement from progenitor cells was to occur as demonstrated by recent studies (Appel et al., 2009, Ronconi et al., 2009). Another possible outcome is that podocyte number will continue to fall as the disease progresses, either due to lack of replacement combined with continuing loss or rate of loss is greater than rate of cell replacement. We favour the latter hypothesis, as results from our study showed a progressive loss with time in the diphtheria toxin injected but not drug treated mice (Fig. 3.7a). However, the experimental numbers used were small (n=3-4), therefore more animals need to be used at more timepoints in future studies to confirm the results and reach higher significance.

In the drug treatment setting after induction of podocyte injury, we think that nephroprotective agents could ameliorate renal outcome by either reducing acute injury to reach stability or achieve improved function by reducing late injury. On the other hand, without the treatment, podocyte number will continue to fall. However, if replacement from progenitor cells were to occur, then we may see a third scenario e.g. a gradual recovery in podocyte number after the treatment stabilized the disease progression (Fig. 5.17b).

Although podocyte numbers in drug treated groups were slightly higher than the placebo (water) treated mice in the combined intervention study, the level of significance was not reached. Therefore we cannot be certain from our current data
whether the drugs provide any protection in podocyte number from toxin-induced injury.

Further analysis and long-term study of effects on podocyte numbers and morphology may provide additional insight on the mechanism of action of the drugs and future therapeutic potential.
Figure 5.17. (a) Hypothetical effects of diphtheria toxin on podocyte number in the untreated model and (b) drug treated Podo-DTR mice at various timepoints. (a) Depending on the severity of injury induced, hypothetically one option is that the cell number will eventually gradually increase if replacement from progenitor cells was to occur (dotted line); another possible outcome is that podocyte number will continue to fall as the disease progresses (dashed line), either due to lack of replacement combined with continuing loss or rate of loss is greater than rate of cell replacement. (b) In the drug treatment setting after induction of podocyte injury, nephroprotective agents could ameliorate renal outcome by either reducing acute injury to reach stability or achieve improved function by reducing late injury (dotted line). In the animal without the treatment (unmodified with solid line), podocyte number will continue to fall. However, if replacement from progenitor cells were to occur, a gradual recovery in podocyte number may be possible after disease stabilization (dashed line).
**CHAPTER 6: Discussion and Conclusion**

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Discussion and Conclusion

6.1 Summary of the project outcomes

This project is based on the key hypothesis that podocyte damage and continuing loss is the primary problem that causes proteinuria and progressive glomerulosclerosis (Elger & Kriz, 1998, Gassler et al., 2001, Ichikawa et al., 2005, Kim et al., 2001, Shankland, 2006, Wharram et al., 2005) instead of the alternative hypothesis of proteinuria being the principal cause of renal damage (Baines & Brunskill, 2008). We believe that disruption of the dynamic interactions between the podocytes triggers the vicious cycle of “podocyte-damage-damaging-podocyte” (Ichikawa et al., 2005). It is my hypothesis that interventions that reduce the disruption by rescuing susceptible podocytes next to injured ones are potential therapies to restore podocyte phenotype and filtration behaviour, thereby protecting the kidney from progressive deterioration.

To address the above questions, a transgenic Podo-DTR mouse model expressing human diphtheria toxin receptor (hDTR) specifically on podocytes was used in this project. The aim was first to characterize the model, secondly test the model with a known therapeutic agent to prove its viability, and thirdly carry out intervention studies with potential podocyte protective agents to assess whether combination treatment has additive effects compared to single therapies.

The results from our studies provided evidence that partly excluded the alternative hypothesis. In our combination study (chapter 5), proteinuria was not different between the drug- and water-treated mice. If the alternative hypothesis were true in that proteinuria was the principal cause of renal damage, then we would expect the histological outcome of the kidney to be similar amongst the groups. However, this was not the case as the serum and glomerulosclerosis index results showed that all drug treated mice had lowered serum creatinine and improved renal histology with additional benefit seen in the combination treatment group, suggesting that the
beneficial effect of the drug therapy must be via other means other than or on top of proteinuria reduction.

In addition, the urinary ACR results showed that reduction of proteinuria alone is not sufficient to stop the progression of the kidney deterioration as demonstrated by the worst kidney histology outcome of water treated mice despite reaching very low levels of urinary albuminuria at later timepoints. In support of our data, the evidence that prolonged proteinuria present in nephrotic minimal change disease does not lead to rapid and marked renal disease progression also suggest that proteinuria is likely a consequence of e.g. podocyte damage/loss rather than the main cause of renal damage and fibrosis.

Below I will briefly describe the major outcomes of each of the three project sections highlighting the pros and cons of the approach used and discuss the level of success of the project with suggestions for possible improvements and future work.
6.2 Podo-DTR model and intervention studies

The characterization results of our transgenic Podo-DTR mouse model showed that we have in our hands two viable and functional lines that express hDTR, both of which are sensitive to diphtheria toxin. Administration of diphtheria toxin to transgenic but not wild-type littermates induced specific toxin-receptor mediated injury of podocytes, causing proteinuria, progressive glomerulosclerosis and podocyte loss. These data support the notion that healthy and normal podocytes are essential for functional and effective glomerular filtration. Damage to this cell type leads to progressive glomerular scarring which seems identical to that seen in other types of glomerular injury.

I have demonstrated that line 47 is more susceptible to diphtheria toxin than line 57. At eight weeks timepoint, approximately 50% of glomeruli are damaged to various degrees with increased collagen, cystic parietal epithelial cell, tuft/capsular adhesion, focal and segmental glomerulosclerosis in line 47 with 1ng/g bw diphtheria toxin dose compared to 17% of line 57 despite a five fold increase in the toxin dose used (5ng/g bw). However, the extent and basis of variability between the two lines and the level of mRNA expression need to be further characterized in the future.

Since the insertion of the transgene in the genome is random, the level of expression could be affected depending on the point of insertion, as by chance this could inactivate a gene that is important for e.g. toxin internalisation, or the ability of withstanding infections. This could potentially explain the lack of susceptibility of line 21 to diphtheria toxin, despite positive detection of the transgene via PCR analysis. Although all the animals were healthy and reproduce normally, it is important to point out that the mice were housed in a very clean facility, and we did not challenge, test or investigate the immune response of mice to other pathological stimuli such as stress or infection.

To confirm that Podo-DTR is indeed a good model of human proteinuric glomerular disease, we set out to test the transgenic mouse model with a known and widely
applied therapeutic agent, the ACEi captopril in our first intervention study. ACEi have been used widely in both animal and human settings to treat proteinuric diabetic (Jacobsen et al., 2003, Jennings et al., 2007, Lewis et al., 1993, Lewis et al., 2001) and non-diabetic renal diseases (Blanco et al., 2005, Campbell et al., 2003, Coppo et al., 2007, De Albuquerque et al., 2004, Jacobsen et al., 2003, Jennings et al., 2007, MacKinnon et al., 2006, Maschio et al., 1996, Nakao et al., 2003, Reiser & Mundel, 2007, Remuzzi et al., 2002, Shinosaki et al., 1997, Smeets et al., 2006, Tang et al., 2008), therefore if we had produced a useful model, we would expect captopril to protect against the progressive glomerulosclerosis induced by diphtheria toxin administration.

The results from the ACEi intervention study showed that captopril successfully reduced blood pressure, proteinuria and most importantly improved kidney histology by almost abolishing glomerulosclerosis to levels comparable to the wild-type controls. Although the readouts of the study were simple, these results clearly demonstrated the viability and functionality of the model, providing the proof of concept that the model could be applied to test other potential nephroprotective agents.

This led us to the next stage of the project where we wanted to test the novel ETaRA sitaxsentan either on its own or on top of ACEi to investigate any potential additive or synergistic effect. Various ETRA are licensed for clinical use in pulmonary hypertension (Agapitov & Haynes, 2002, Boniface & Reynaud-Gaubert, 2009, Gross et al., 2003, Kermeen et al., 2010, Valerio et al., 2010). Previous experimental studies (Opocensky et al., 2006, Ortmann et al., 2004, Watson et al., 2010) and some clinical studies have shown that inhibition of ETa receptors have anti-proteinuric effects (Dhaun et al., 2007, Gross et al., 2003, Mann et al., 2010). However, whether these agents can achieve long-term protection in terms of histopathological improvement in the kidney has not been fully elucidated.

The results from the combination therapy experiments (chapter 5) showed that mice treated with both sitaxsentan and captopril had the highest reduction in
glomerulosclerosis at eight weeks when compared to the respective single therapies and controls, suggesting a synergistic effect when using the two agents together. Although there seemed to be an improvement in the urinary ACR at the early timepoint at week 2 in the drug treated animals, unexpectedly the levels were not significantly different amongst the groups at week eight when compared to the respective placebo treated group.

The reason why proteinuria was not reduced significantly at later timepoints, despite apparent renal histological amelioration in drug treated mice is uncertain. A possible explanation could be that in order for the animal to survive and partly preserve the kidney function after the severe induction of podocyte injury by the toxin is to form scarring in the glomeruli to prevent the prolonged potential deleterious effect of severe protein leakage. On the other hand, the intervention of the drug treatment by directly affecting the podocytes and possibly lowering the glomerular pressure may have permitted the remaining podocyte to signal and interact more effectively to induce reparative mechanism, hence lowered sclerosis and matrix accumulation. In both settings (drug- or placebo-treated) mice have their proteinuria substantially reduced from early (week one and two) to late (week eight) timepoint, but not quite reaching the baseline levels, suggesting that the glomerular filtration barrier was not completely restored.

However, it is worth pointing out that the n number of urine samples for each group was relatively small (n=4-7). This was partly due to exclusion of some animals from the analysis where a proportion of mice did not have proteinuria or glomerulosclerosis induced (12.7%: four out of 32 and five out of 39 mice from phase a and b respectively). Bearing this factor in mind, future studies may need to include bigger experimental group size in order to obtain more robust results and statistical analysis.

In addition, blood pressure results from the study with ACEi and SITA suggested that podocyte damage and loss might lead to systemic hypertension in this model. This was demonstrated by the increased blood pressure of toxin injected placebo-
(water) treated mice (136±17.0mmHg versus 106±7.9 mmHg of baseline level, p<0.0001). The level of hypertension is likely to be dependent on the degree of renal injury, as no prominent blood pressure change was seen in the ACEi captopril experiment with line 57 where milder injury was induced (toxin treated: 114±8.4mmHg versus untreated control 114±6.7mmHg).

However, the blood pressure increase could be due to a number of factors. For example, a reduction in glomerular filtration rate due to renal mass reduction (as seen in experimental models of 5/6 nephrectomy) (Adameczak et al., 2003, Shinosaki et al., 1997), or loss of functional nephrons due to toxin administration and subsequent scarring (Asano et al., 2005, Kim et al., 2001, Wharram et al., 2005) can lead to decreased salt excretion, which in turn causes the blood pressure to rise. The resultant high blood pressure might then in turn cause podocytes stretch and stress leading to the vicious cycle of podocyte damage damaging podocytes as well as other neighbouring cells in the glomeruli.
6.3 Pros and cons of Podo-DTR model compared to the existing models

Our Podo-DTR mouse model has a number of advantages over the existing animal models. Unlike the rat puromycin aminonucleoside (PAN) (Diamond & Karnovsky, 1986, Inokuchi et al., 1996, Shiiki et al., 1998) and mouse adriamycin models (Chen et al., 1995, Guo et al., 2008, Zheng et al., 2006) of human minimal change disease/FSGS where the toxin specificity is uncertain due to potential deleterious effects to other cell types apart from podocytes, our model permits specific toxin-receptor mediated injury of podocytes after administration of diphtheria toxin via expression of the hDTR transgene on the cell of interest.

This method of cell targeting injury has previously successfully been applied to other cell types by other groups (Duffield et al., 2005, Saito et al., 2001), including a DTR rat model generated by Wiggins’ group (Wharram et al., 2005) and a human (h)CD25 mouse model using LMB2 immunotoxin by Ichikawa’s group in Japan (Matsusaka et al., 2005). A comparison highlighting the similarities and differences with these models will be presented in the following sections below.

6.3.1 Similarities and differences of various animal models

While diphtheria toxin (DT) was used to deplete podocyte in both our and Wiggin’s group using similar technology, a mouse instead of a rat model was chosen by our group. Although the two rodent models have many similarities, there are many aspects that also differentiate them considerably. The advantages of using a mouse model, is that genetic manipulations are more feasible and crossing with other models to study different diseases or mechanisms are more easily achievable than the rat model. The breeding turnover is quicker with higher litter number, the maintenance cost is lower and more manageable, the reagents for mouse are more readily available and lower volume of toxin or drugs are required for treatment. On the other hand, rats are bigger therefore easier to handle when taking the blood pressure, or performing any surgical procedures, multiple blood sample withdrawal is easier, there is more tissue available for analysis and there is possibly closer similarity in kidney histology to human.
The transgenic Podo-DTR mice had normal phenotype and life expectancy (over 17 months old) unless injected with diphtheria toxin. Wild-type littermates or C57BL/6 mice had no negative long-term effects and showed no functional or morphologic abnormalities after diphtheria toxin administration up to 25ng/g bw dose.

At the highest dose used at166ng/g bw, early mortality was induced in both transgenic line 47 and 57 mice at early timepoints (day 7 and 11 respectively). However, after diphtheria toxin treatment at lower dose in the more sensitive line 47 (1ng/g bw), Podo-DTR mice survived up to six months (chapter 3), unlike the hCD25 model, where only relatively short-term timepoints (up to four weeks) have been presented even after low dose of immunotoxin treatment at 0.625ng/g bw.

The pattern of urinary results from our model were similar to hCD25 mice, with ACR values peaking at week 1 to 2, decreasing substantially at week five post toxin injection in Podo-DTR mice, while almost reaching normal levels in hCD25 mice at week four. These data suggest that glomeruli subjected to mild injury by toxin have the ability to recover partially in our model or almost completely in the hCD25 model after four to five weeks.

Unlike the intravenous injection method applied in hCD25 model, diphtheria toxin administration was delivered via intraperitoneal injection in our model. While the latter method has the advantage of being straightforward, easy and quick, the level of toxin absorption may have higher margin of error than the direct intravenous injection methodology. However, the downside of intravenous injection with larger volume may affect the lungs and spleen more prominently possibly due to higher immune cell infiltration. Therefore future work could investigate on alternative route of toxin administration to increase higher percentage and more homogenous disease induction.

Mouse nephrin expression has been previously demonstrated in tissues other than the kidney, including brain and pancreas (Moeller et al., 2000, Moeller et al., 2002, Putaala et al., 2001). In order to overcome this problem, a fragment of the mouse
nephrin gene (1.25kb) was used in our model in comparison to the larger 5.5kb gene used in Matsusaka’s model to localize the expression exclusively in podocyte in the glomeruli previously demonstrated by Moeller (Moeller et al., 2002). Unexpectedly, similar to the hCD25 mouse model by (Matsusaka et al., 2005), we also detected positive but very low level of expression in extra-renal tissues such as liver and brain using the highly sensitive RT-PCR technology. However, diphtheria toxin injection caused no injury in the liver, and no obvious pathology was observed in other organs. This could be due to low level of expression of the transgene in these tissues and possibly additional protection provided by the blood-brain barrier.

Although untreated transgenic mice displayed no abnormal physical or health features, it is important to bear in mind that the actual transgene expression of hDTR by podocytes themselves could potentially influence the result outcome. Previous studies in rat models demonstrated increased level of HB-EGF protein expression in podocytes after puromycin aminonucleoside injury (Khong et al., 2000, Mishra et al., 2002, Takemura et al., 1999), but it is important to note that these animals were not transgenic, and had the natural promoter. In addition, in vitro studies have shown that mesangial cells also express EGF receptors and are capable of producing HB-EGF (Mishra et al., 2002, Takemura et al., 1999). Although the glomerular cells and structure were normal and comparable to wild-type mice, the possibility of the hDTR transgene expression having some impact on the model cannot be ruled out completely.

Contrary to other groups’ data where they showed podocyte number protection after ACEi treatment (Gross et al., 2003, Nemeth et al., 2009), our results did not show substantial podocyte number rescue despite improvement in glomerulosclerosis, indicating that the protection at the level of damage induced may be via change in podocyte phenotype or function. This is in agreement with two other animal models, where despite histological improvement and proteinuria reduction after drug treatment with enalapril in the subtotally nephrectomised rat model (Adamczak et al., 2003) or darbepoetin alpha in the antibody induced nephritic mouse model (Logar et al., 2007), podocyte number did not go back up. A number of factors could
contribute to the different result outcome of various studies. These include different rodent (rat versus mouse) models used; degree of injury induced by different methods or mediators; timescale of the disease induction and progression as well as variation in treatment type and dose of agents used. Although the method of podocyte quantification used in our study has been applied by other groups (Logar et al., 2007, Pippin et al., 2009a, Vaughan et al., 2005) and allowed adequate comparisons between the treatment groups within the study, it is important to acknowledge the limitation of the technique that only allows quantification on a 2D image and not the absolute value.
6.4 Possible improvement

From the studies carried out in this project, the degree of damage between male and female was not significantly different. This is in agreement with Wharram’s study using the rat DTR model (Wharram et al., 2005). However, the data indicates that old or male mice may have an increased tendency of developing higher glomerulosclerosis as shown in chapter 4 (Fig. 4.8 and 4.9). Therefore parallel studies with gender differentiation and larger group number may be useful in addressing the issue more specifically, thereby reducing the variability between the groups to get more accurate and specific drug effect. Age is likely to play a role in the disease severity, as mice over 12 months of age are more likely to fall sick, lose substantial weight and have higher mortality rate as observed in phase a of the combination study with ACEi and SITA (chapter 5). Another viable explanation for the difference could be due to weight-related issue, as male mice of the same age tend to be bigger and fatter than the respective female mice. Therefore, narrower age range should be used in future experiments where young versus old mice need to be addressed separately to obtain more homogenous results.

Although unlikely, variability of results due to different stages of backcrossing at the time of earlier experiments cannot be ruled out completely. Of note, from the time of transgenic mouse generation in 2006 up to date (2011), 7 backcrosses to C57BL/6 mice have been carried out.
6.5 Translational potential from Tg mouse model to human treatment

We used our Podo-DTR to test various potential nephroprotective agents. So far, we demonstrated that both ACEi and sitaxsentan either on its own or in combination could achieve protection against diphtheria toxin induced glomerulosclerosis. However, whether the beneficial effect of the drug treatment is via podocyte rescue alone or in combination with the haemodynamic effect needs to be further investigated in this model. Blood pressure reduction in the renal disease setting has always been considered beneficial, but past studies from experimental models as well as clinical data have shown that at least in the case of rennin angiotensin system inhibition with ACEi and or ARB, the benefit is more than just blood pressure lowering effect (Lewis et al., 2001, Nemeth et al., 2009, Smeets et al., 2006).

Unlike many proteinuric disease models, where induction of the disease is not necessarily directed to one cell type in the glomeruli, our Podo-DTR model adopts a very clean system of targeting only hDTR expressing podocytes. This toxin-receptor conditional knockout system therefore is ideal for testing other potential podocyte-protective drugs and would be a good model to screen and/or assess the efficacy and safety of new or re-discovered drugs in an in vivo setting in the future.

In vivo experiments using the transgenic model certainly provide a more complete system to test various drugs. However, we do need to bear in mind that these studies require longer time scale, are more expensive to run due to breeding and animal maintenance, and are more laborious to carry out. Therefore in vitro work with tissue culture or cell lines systems could be applied in conjunction to screen for drug toxicity and dose response to obtain quicker preliminary data to reduce animal usage as well as additional application to elucidate molecular mechanism.

Therefore, the “bench to bed” translation from animal model to human diseases to find and discover ideal therapeutic agents is an extremely complex, costly and slow process. However, development of good and suitable models that mimic closely human diseases is certainly an ideal start to reducing the timescale of delivering the right treatment to the right patients.
6.6 Future work

Having established a functional transgenic mouse model in which specific podocyte injury can be induced by a single injection on diptheria toxin to cause dose dependent glomerular damage, the immediate future work would be to test other potential nephroprotective agents. ACEi and ETaRA are two drugs that we have chosen to target two of many mediators that promote podocyte derangements (with their respective receptors being demonstrated on podocytes): angiotensin and endothelin respectively, but there are many other agents such PPAR-gamma agonist, calcineurin inhibitor such as tacrolimus, statin, vitamin D that may promote direct and beneficial effect on podocyte as illustrated in Table 6.1 Therefore exploration of some of the existing candidates as a single agent or combined treatment as well as testing new upcoming drugs could provide additional and more effective treatment to slow down or stop the progression of the costly and impairing renal disease in human settings, especially in patients who are particularly challenging to treat due to lack or low responsiveness to conventional therapies.

In order to elucidate structural changes of podocyte phenotype and the effects of the toxin and the drugs used, scanning and transmission electron microscopy should be applied to investigate more subtle structural alterations of these highly specialised cells. The results may provide insight and evidence to whether amelioration of drug treatment is mainly due to change in podocyte phenotype and/or function or whether podocyte number preservation and or replacement also has a role to play. In addition, quantification of gene expression of angiotensin II, endothelin-1 and their respective receptors in the glomeruli or podocyte may provide more direct evidence of the drug effects.
<table>
<thead>
<tr>
<th>Promote mobility in podocyte:</th>
<th>Promote differentiation in podocyte:</th>
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<tbody>
<tr>
<td>Angiotensin</td>
<td>ACEi/ARB</td>
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<tr>
<td>Endothelin</td>
<td>Endothelin antagonist</td>
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<tr>
<td>Anti-VEGF</td>
<td>VEGF</td>
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<tr>
<td>TRPC6 signalling</td>
<td>PPARγ signalling</td>
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<td>LPS via TLR4 (and B7-1)</td>
<td>Aldosterone antagonist</td>
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<td>TGF-β</td>
<td>All-trans retinoic acid</td>
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<td>Stretch /tension</td>
<td>CDK2 inhibition</td>
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<td>Circulating factors in</td>
<td>Corticosteroids</td>
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<tr>
<td>-Minimal change disease</td>
<td>Calcineurin inhibitor</td>
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<tr>
<td>-Idiopathic FSGS</td>
<td>-ciclosporin</td>
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<td>-tacrolimus</td>
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Table 6.1. A list of mediators that promote podocyte mobility (left) and a list of agents that promote differentiation in podocyte (right) (Adapted from Winn M et. al. 2006).

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CDK2, cyclin-dependent kinase 2; FSGS, focal segmental glomerulosclerosis; LPS, lipopolisaccharide; PPARγ, peroxisome proliferator-activated receptor gamma; TGF-β, transforming growth factor beta; TLR4, toll-like receptor 4; TRPC6, transient receptor potential cation channel 6; VEGF, vascular endothelial growth factor.
In conjunction with the above methods, podocyte damage should be assessed with additional podocyte specific staining such as synaptopodin, nephrin, podocalyxin and/or GLEPP1 to further support the findings shown by WT-1 staining in our model. As decreased WT-1 marker in injured but still alive podocyte may affect the quantification and end-result outcome of cell number.

It would be interesting to investigate the fate of damaged podocyte. What is the mechanism of cell death and where do they go following detachment or death. Do phagocytes clear them or do they simply detach from the glomerular tuft to be washed away in the urine?

If podocyte depletion were indeed a major mechanism underlying glomerulosclerosis, then the potential clinical utility of monitoring glomerular injury by measuring podocyte products in urine would be ideal as a non-invasive method of assessing the degree of glomerular damage and level of progression.

To date various groups have provided evidence of podocyte and its components in the urine using a number of methods such as antibody detection (Hara et al., 2001, Hara et al., 2005, Nakamura et al., 2000), western blotting (Patari et al., 2003) and RT-PCR (Szeto et al., 2005, Wang et al., 2008) in various human diseases (Garovic et al., 2007, Lemley et al., 2002, Vogelmann et al., 2003, Yu et al., 2005) as well as experimental models (Kim et al., 2001, Petermann et al., 2004, Sato et al., 2009). However, the feasibility, sensitivity and reliability of the assay will need to be further evaluated prior to the clinical application in patients to assess various proteinuric glomerular diseases.

A useful assay would be to have the ability to assess quickly the efficacy of a given treatment regimen, so that ineffective treatment could be stopped before reaching the irreversible state with some of the deleterious side effects. This could be tested using our Podo-DTR model to see any correlation between histology improvement and downregulation of podocyte markers (i.e. podocalyxin, nephrin, podocin mRNA) in the urine after the drug treatment. Ultimately, if feasible, these non-invasive assays
could provide additional information to improve management of proteinuric glomerular diseases in humans in the future.

Another important issue to address in these intervention studies is to determine the ideal timepoint to intervene to achieve the best outcome. In most experimental studies, the drug treatment is either initiated before the insult (Smeets et al., 2006) or shortly after as shown in ours (24h post toxin injection) and other studies (Smeets et al., 2006, Tang et al., 2008). In a murine model of podocyte injury, Smeets et al showed protective effect of ACE inhibition three days after injury induction. While this may be an ideal situation, in reality this is often not the case in human patients, as diagnosis of kidney diseases often occurs much later than the initial insult or cause of injury. Therefore, it is also important to determine the effectiveness of the drugs at later timepoints, i.e. when the injury is at its acute or even chronic phase, e.g. at week two and four post toxin injection.

Another interesting angle of investigation would be to see the possibility of podocyte regeneration and replacement after injury and loss. Previously evidence for the incorporation of bone marrow-derived cells into podocytes has been reported in a mouse model of Alport syndrome as well as in kidney transplant (Becker et al., 2007, Prodromidi et al., 2006, Sugimoto et al., 2006). However, these studies are controversial, as the possibility of cell fusion between the donor and recipient cells cannot be excluded (Terada et al., 2002, Ying et al., 2002). In addition, several report to date have also shown the existence of the intrinsic niche of renal progenitor cells (Appel et al., 2009, Humphreys et al., 2008, Kreidberg, 2003, Ronconi et al., 2009), further supporting the possibility of podocyte replacement and possibly potential induction of disease remission and/or regression of renal lesions.

Using two different approaches, both studies by Appel D et al. and Ronconi E et al. provided evidence that the parietal epithelial cells (PEC) from urinary pole of the inner lining of the Bowman’s capsule posses the progenitor cell properties and are able to migrate onto the vascular tuft to differentiate into podocytes (Appel et al., 2009, Ronconi et al., 2009) (Fig. 6.1). Unlike bone marrow-derived cells where they
require to cross the anatomical barrier of GBM, the location of PEC makes them an ideal and more feasible candidate for resident renal progenitor cells, as they are located within the same compartment as podocytes and are in direct continuity at the glomerular vascular stalk. In support to this evidence, PEC also express several stem cell marker as shown by Romagnani’s group in human kidney and have the ability to transdifferentiate in vitro into other cell types such as tubular cells, adipocytes or neuronal cells, suggesting that these cells have pluripotent properties (Lazzeri et al., 2007, Ronconi et al., 2009, Sagrinati et al., 2006).

In rodents, specific PEC and stem cell markers are still lacking, therefore tracing of these cells in mice and rat models may still be an obstacle. Although great advancement has been made in recent years in the field of podocyte replacement research, many issues such as extent of podocyte replacement in adulthood in health and disease, the mechanism and rate of replacement still remain to be elucidated. However, the possibility of remodelling the glomerular architecture and reconstitution of glomerular tuft through podocyte replacement certainly present an exciting prospect for researchers and a hopeful view for renal patients with progressive glomerular diseases.
Figure 6.1. Schematic representation of the renal glomerulus with various components. The glomerular epithelium consists of parietal epithelial cells (PECs) and podocytes (Podo) shown in red and blue respectively in the diagram. Both epithelial cells adjoin directly at the vascular pole (VP; arrow), while at the tubular pole (TP), the parietal epithelium joins the proximal tubule epithelium. The glomerular basement membrane shown in black line forms a continuous barrier between the glomerular epithelium and the endocapillary compartment that contains mesangial cells (shaded grey) and endothelial cells of the glomerular capillaries (*). Primary urine is filtered across the glomerular filtration barrier (composed of endothelial cells, glomerular basement membrane, and podocyte) into Bowman’s space (BS). Adapted from Appel D et al 2009.
6.7 Conclusion

In summary, our model system possesses many advantageous characteristics. Firstly, proteinuric and progressive glomerular damage and sclerosis can be induced by a single intraperitoneal injection of diphtheria toxin mimicking podocyte injury in human diseases. Secondly, the level of injury is dose and time dependent and transgenic mice are healthy with normal phenotype unless receiving the toxin, allowing easy breeding and maintenance. Thirdly, combination of this model with other transgenic diseased mice such as diabetes, hypertension or Alport syndrome can potentially be used to elucidate and study the function and role of podocytes in other disease settings. Also if coupled with mice expressing fluorescently labelled stem cell markers, it could become a powerful tool to investigate and trace podocyte fate and replacement after injury, thereby study remodelling of damaged glomerular architecture.

Therefore, our Podo-DTR model is ideal for studying strategies to protect the kidney from progressive injury following podocyte depletion. Further elucidations on the mechanism of action of the drugs and replacement of injured/lost podocytes by intrinsic renal stem cell or progenitor cells are crucial for development of superior future therapeutic treatments of kidney diseases.
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Appendix

Papers

Full manuscript in preparation:
Following specific podocyte injury captopril protects against long term renal damage with minimal effect on podocyte number.

Endothelin A receptor antagonist (ETaRA) sitaxsentan and ACEi captopril provided a synergistic effect in reducing long-term glomerulosclerosis after podocyte damage.

Abstracts

2011 British Renal Society/Renal Association Joint Annual conference: Birmingham, UK
“Endothelin receptor antagonist (ET-RA) sitaxsentan reduces long-term glomerulosclerosis after podocyte damage”

2009 Renal Association and British Transplantation Society Annual Meeting: Liverpool, UK
“ACE inhibitors protect against progressive renal damage following podocyte injury”

2007 Renal Association Annual Conference: Brighton, UK
“Targeted podocyte injury causes acute proteinuria and glomerular injury followed by delayed FSGS”
Y.S Zhou, I.A. Ihmoda, C.O.S. Bellamy, A.N. Turner
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