

ROLE OF TRANSFORMING GROWTH FACTOR BETA IN PROTEINURIA

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ABSTRACT

The incidence and prevalence of people suffering from end stage renal disease is increasing. Proteinuria, particularly the presence of albumin in urine is concerning because proteinuria is associated with the progression to end stage renal disease (ESRD). Understanding the mechanisms involved in damaging the glomerular filtration barrier is essential. Transforming growth factor beta (TGFB) is a key cytokine in mediating glomerulosclerosis and proteinuria. Not much is known about the downstream pathways that mediates the renal damage and proteinuria.

I hypothesize that TGFB induces proteinuria through podocyte de-differentiation and this occurs through SMAD dependent and independent pathways.

Methods: I used adenovirus mediated gene transfer of TGFB1 to rat renal artery to study the effects of TGFB1 on renal structure and functions. To study the importance of SMAD3 in mediating downstream effects of TGFB1 in proteinuria and podocyte effacement, I used an anti-glomerular basement membrane model in SMAD3^{+/+} and SMAD3^{-/-} mice to induce glomerulonephritis and proteinuria.

Results: Transient TGFB1 overexpression via AdTGFB1 induced significant proteinuria, podocyte foot process effacement, nephrin down-regulation, and nephrinuria. The expression of synaptopodin was also significantly down-regulated by TGFB1. TGFB1 increased the expression of the angiopoietin receptor, Tie2, in podocyte cell culture. In cultured podocytes, TGFB1 downregulated the gene and protein expression of both nephrin and synaptopodin. These findings suggest that locally produced TGFB1 can

cause podocyte de-differentiation marked by a loss of synaptopodin, nephrin, and foot process effacement; this process is partly regulated by angiopoietins. This process represents a novel pathway that may explain proteinuria in a variety of common renal diseases.

Both $\text{SMAD3}^{-/-}$ and $\text{SMAD3}^{+/+}$ mice had proteinuria after induction of anti-GBM glomerulonephritis, though to a lesser extent in $\text{SMAD3}^{-/-}$ mice. $\text{SMAD3}^{-/-}$ and $\text{SMAD3}^{+/+}$ mice developed significant glomerulonephritis with progressive interstitial fibrosis and chronic renal impairment. The $\text{SMAD3}^{+/+}$ mice were found to be more prone to fibrotic changes, interstitial damage and tubular and glomerulosclerosis than the $\text{SMAD3}^{-/-}$ mice. This suggests that TGF β 1 signals through pathways other than SMAD3 such as those triggered by hypoxia.

Conclusion: I have shown that TGF β 1 upregulation via AdTGF β 1 induces proteinuria through podocyte dedifferentiation and FP effacement. Angiopoietins are essential for TGF β 1 mediated podocyte injury. The effects of TGF β are partially mediated through SMAD3 as there is residual podocyte effacement and proteinuria in the $\text{SMAD3}^{-/-}$ mice. Hence there are SMAD3 dependent and independent pathways involved in proteinuria.

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LIST OF ABBREVIATIONS

MCEC	Mouse cardiac endothelial cells
PAI	Plasminogen activator inhibitor
Acta2	Alpha smooth muscle actin
SiRNA	Small-interfering RNA
LCM	Laser microdissection
IP	Intraperitoneal
FGAd	First generation adenovirus
CAR	Coxsackie adenovirus receptor
HGF	Hepatocyte growth factors
BMP-7	Bone morphogenic protein-7
ACE	Angiotensin-converting enzyme
ESRD	End stage renal disease
EAG	Experimental autoimmune glomerulonephritis
GN	Glomerulonephritis
ANGPT1	Angiopoietin-1
ANGPT2	Angiopoietin-2
HIF1A	Hypoxia inducible factor 1 alpha
EMT	Endothelial to mesenchymal transdifferentiation
PCR	Polymerase chain reaction
PSR	Picrosirius red stained
GFB	Glomerular filtration barrier
GFP	Green fluorescent protein
GBM	Glomerular basement membrane
NGS	Normal goat serum
TGFB1	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factors
GECs	Glomerular endothelial cells
PKC	Protein kinase C
CTGF	Connective tissue growth factor
LacZ	Betagalactosidase Z

CHAPTER 1 HYPOTHESIS AND AIMS

1.1 Hypothesis

TGFB1 induces proteinuria through podocyte de-differentiation and this occurs through SMAD dependent and independent pathways.

1.2 Aims

To determine:

- 1.** If upregulation of TGFB1 induces proteinuria.
- 2.** The effects of TGFB1 on podocytes.
- 3.** The role of vascular growth factors like angiopoietin-1 (ANGPT1) and angiopoietin-2 (ANGPT2) in TGFB1 induced proteinuria.
- 4.** If SMAD3 is required for TGFB1 induced podocyte de-differentiation and proteinuria.

CHAPTER 2 INTRODUCTION

2.1 Clinical Relevance and Rationale

Kidney diseases eventually lead to kidney failure and long term dependence on dialysis. According to the Kidney Foundation of Canada an estimated 1.5 million Ontarians have or are at increased risk for developing kidney disease and 9,800 Ontarians are currently on dialysis. Lack of effectiveness of currently available drugs mandate the use of other agents that would protect the kidneys and hence prevent end stage renal disease (ESRD). Due to the increasing burden of kidney disease there is a need to develop additional therapies that can be used solely or combined with current therapies to effectively prevent progressive renal disease.

The results of my experiments have revealed the importance of TGF β and angiopoietins in kidney injury. By blocking the angiopoietins and their receptor, we were able to reverse TGF β induce damage to podocytes. I propose that angiopoietin inhibiting drugs could be an alternative treatment strategy or could be combined with present day therapies to prevent renal injury in diseases like glomerulonephritis. One such agent is AMG-386 which is an angiopoietin antagonist that selectively inhibits the interaction of ANGPT1 and ANGPT2 with Tie2. It is currently undergoing a phase 3 clinical trials testing its use in patients with ovarian cancer (Amgen clinical trials United States 2013). I have recently done some preliminary work using AMG-386 in mice (SMAD3^{+/+} and SMAD3^{-/-}) with anti-GBM induced glomerulonephritis and the results have been promising. In this model AMG-386 caused a significant decrease in

proteinuria. Hence, based of my work I can propose that Blocking TGFB and ANGPT can be beneficial in reducing proteinuria.

2.2 Kidney Structure and Function

Kidneys are two bean shaped organs approximately 10 cm in length, 5-7cm in width and 2-3 cm thick. Internally, the kidney is divided into different compartments. The outer portion is the cortex and the inner portion is called the medulla. The structural and functional unit of the kidney is called a nephron. A nephron is composed of a glomerulus, which is a ball shaped cluster of capillaries, and a system of collecting ducts. Two renal arteries arise from the abdominal aorta and supply blood to each kidney. Outside of the glomerulus is a urinary space (Bowman's space) where urea and fluid filtered from the glomerular capillaries collect. This filtrate then travels in a series of convoluted tubules. The glomeruli are positioned in the cortex while the collecting system makes up the medulla.

Functions of the kidney:

1. Kidneys serve to rid the body of nitrogenous waste such as urea and ammonia.
2. They regulate levels of sodium, potassium and chloride in the blood.
3. They maintain a fluid balance. They are sensitive to blood salt and fluid levels and also act as sensors to monitor blood pressure. Kidneys regulate blood pressure via the renin-angiotensin-aldosterone system.
4. They secrete important hormones like erythropoietin (essential for red blood cell production).

5. Kidneys also produce prostaglandins, mainly PGI₂ PGE₂, thromboxane and PGF₂ (1;2).

Nitrogenous waste is excreted from the body as urine. The three stages of urine formation are glomerular filtration, reabsorption and finally secretion. Blood flowing into the glomerulus is filtered across glomerular filtration barrier (GFB). The filtration barrier of the kidney is composed of three layers: fenestrated endothelium, glomerular basement membrane (GBM), and podocytes. The kidney provides an effective barrier against the passage of proteins to the urine. The glomerulus serves as a size selective barrier, allowing the passage of water soluble and low molecular weight molecules.

Most of the solutes in the blood get filtered out except for plasma proteins because of their large size. Water and nitrogenous waste is filtered out into the Bowman's space (urinary space) (Figure 1.1) due to the hydrostatic pressure gradient. The filtered fluid then passes through a series of tubules where most of the filtered water, some electrolytes and other nutrients are reabsorbed. The tubules are divided into proximal and descending tubules and they differ in the types and amounts of electrolytes they reabsorb. For example, the proximal tubule reabsorbs sodium, chloride and bicarbonate whereas distal tubules mainly reabsorb sodium and also secrete ammonia and some drugs into the tubules. Whatever is left in the tubules after being reabsorbed or excreted passes down the collecting tubules and is carried to the urinary bladder.

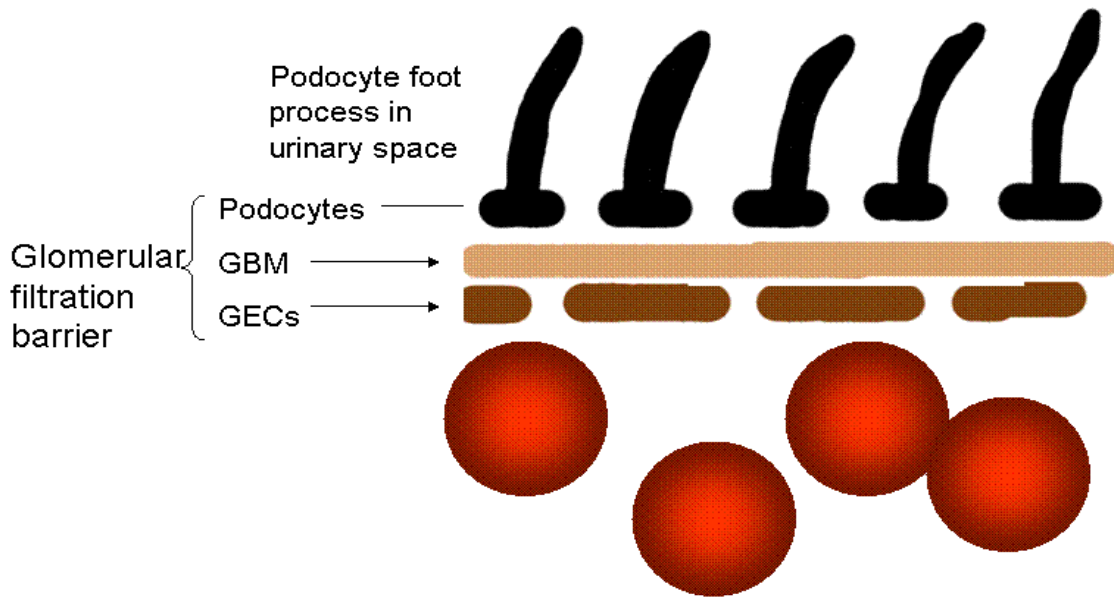


Figure 1.1: Showing components of glomerular filtration barrier. The GFB is composed of podocytes, GBM and glomerular endothelial cells (GECs).

2.3 Components of Glomerular Filtration Barrier

2.3.1 Glomerular Basement Membrane (GBM)

GBM serves as a size and charge selective barrier. GBM is composed of type IV collagen, laminin and proteoglycans and defects in the structural components such as type IV collagen leads to proteinuric diseases. Structurally, adult GBM consists of alpha 3 collagen chains forms a triple helix with the alpha 4 and alpha 5 chains. There are three distinct regions in each alpha chain. The N-terminal domain is rich in cysteine, the central portion is the 7S domain, which is the triple helix and the C-terminal non-collagenous NC1 domain, which is globular and help to assemble the alpha chain heterotrimer.

Thickening of GBM or loss of its negative charge can contribute to proteinuria. Multiple studies have demonstrated the importance of negative charge in maintaining filtration selectivity by GBM. Increase of GBM thickening is correlated to proteinuria and

is described well in diabetic states. Induction of GBM nephritis by anti-GBM antibodies result in interstitial fibrosis and proteinuria. Interaction between the GBM and the podocytes is carried out via the integrins and interruption of these integrins results in glomerulosclerosis (3).

2.3.2 Podocytes

The podocyte is a terminally differentiated epithelial cell with a highly specialized structure with foot processes that wrap around the glomerular capillaries. Podocytes possess numerous foot processes (FP) which envelope the blood vessels and these FP interdigitate with each other leaving between them filtration slits that are bridged by the slit diaphragm (SD) (4). Size and net charge of proteins determine the ease of filtration, size barrier however is more critical for the successful retention of albumin and other proteins (5).

In case of renal damage involving the glomeruli, podocytes undergo multiple adaptive changes which include effacement of FP and apoptosis (6). Protein leakage occurs through defective podocyte SD via podocyte structural alterations frequently described in proteinuric diseases (7). Podocyte effacement is simply the loss of interdigitating FP and formation of cells that look flat instead of the typical arborized pattern. Effacement is believed to represent podocyte response to injury. In podocyte effacement the length of FP decreases by 70% whereas the width goes up by 60% making them flat (8).

The essential components of podocyte SD include nephrin, CD2AP, podocin and P-cadherin. Zonula occludens-1 (ZO-1) has been suggested to be the in vitro equivalent of

podocyte FP (9). Selmabel et al. found ZO-1 to be abundantly present in the glomeruli on FPs where slit diaphragms insert (10). The presence of ZO-1 along the SD junctions of the glomerular epithelium makes it important for the integrity of selective-barrier function.

Nephrin, encoded by NPHS1 gene, was discovered by Dr. Karl Trygvason as a transmembrane adhesion protein of the Ig super family, is one of the major proteins of the SD (11). In kidneys, nephrin has been shown to be exclusively present in the SD (12). Mutations in NPHS1 lead to proteinuria. Mice which are made nephrin deficient by gene targeting show development of nephrotic syndrome (13). Immunogold double labelling has revealed that P-cadherin is located at the extracellular site of the SD whereas ZO-1 is located on the cytoplasmic side (14). Podocin, a constituent of SD is encoded by a gene NPHS2.

Synaptopodin is an actin associated protein present in dendritic cells in the brain and kidney podocytes and was recently discovered by Dr. Peter Mundel (15). It is present in the FP of the podocytes, is an actin binding protein and is essential to maintain the arborized shape of podocytes. Synaptopodin expression is down regulated in diseases like idiopathic nephritic syndrome of childhood, preeclampsia and idiopathic focal segmental glomerulosclerosis (16). Degradation of synaptopodin by activation of calcineurin is sufficient to cause proteinuria (17). Synaptopodin-mutant mice suffer from long term proteinuria when challenged with lipopolysaccharide (18).

Pathological conditions can induce a wide array of changes in podocyte morphology and down regulate all the above discussed markers. This loss of epithelial

markers was termed ‘de-differentiation’. Podocyte de-differentiation is an early event in glomerular injury while subsequent podocyte apoptosis and sloughing has been observed in advanced renal disease (19). Notably, apoptosis appears to be a later finding that occurs after the onset of proteinuria. Some of the changes occurring during podocyte damage include podocyte effacement and apoptosis, cyst formation and detachment from the underlying GBM. Depending on the nature of insult, the podocyte effacement and consequent proteinuria can be reversible or irreversible (20).

If podocytes are injured, the expression of the slit diaphragm protein nephrin in the glomerulus decreases (21) and nephrin appears in the urine (22). Later in the course of glomerular disease, podocytes detach from the basement membrane and also are found in the urine (23).

Podocytes also secrete Angiotensin-like-4 glycoprotein and this is upregulated in Minimal change disease and in rat model of puromycin nephropathy. In this animal model upregulation of Angiotensin-like-4 resulted in diminished charge of the GBM. MCD is sensitive to glucocorticoids and treatment of rats with glucocorticoids diminished Ang-like-4 levels and blunted the disease outcome.

2.3.3 Glomerular Endothelial Cells (GEC)

GECs are one of the components of the GBM. GECs are a specialized form of endothelial cells in that they possess fenestrations. The size of endothelial cells fenestrations does not explain its filtration selectivity. These fenestrae are much larger than most plasma proteins (60-100 nm). Despite this, albumin does not pass freely across the GECs fenestrations (24). The filtration selectivity of GECs is imparted by dense

negatively charged proteins coating the GECs. Rostgaard et al. have demonstrated that the GECs and the fenestrae are covered by thick layer of glycoproteins (25), which covers all the glomerular endothelial cell surfaces (26), called glycocalyx and contains negatively charged proteins which impart filtration selectivity. Any injury that perturbs the integrity of this glycocalyx renders GECs inefficient in preventing passage of protein through them hence leading to proteinuria.

GECs possess receptors for vascular endothelial growth factors (VEGF) and podocytes produce VEGF-A which is essential for the integrity of endothelial cells. VEGF secretion by podocytes is essential for mesangial and endothelial cell migration during development as VEGF specific knockout lacked the migration of mesangial cells and that of endothelial cells into the filtration barrier (27). Hence when bevacizumab (an antibody against VEGF) was used in cancer treatment this resulted in proteinuria and hypertension (28).

Khan et al. have shown that cross talk between endothelial cells and mesangial cells is also crucial for GECs integrity. GECs also produce the basement membrane underneath them and possess receptors for TGF β . Protein kinase C mediates increased TGF β and collagen IV synthesis by the GECs in response to glycated albumin in vitro (29). TGF β also diminished the replication of GECs (30).

One of the well understood mechanism by which TGF β induces endothelial cell dysfunction is endothelial to mesenchymal transition (EndMT). TGF β has a well established role in transition of cardiac endothelial cells into mesenchymal cells (31). These transitioned endothelial cells are demonstrated to be the source of fibrosis in three

well known mouse models (32). In all the three models studied, the fibroblasts co-expressed endothelial markers along with fibroblast markers.

2.4 TGFB1

Much more is known about TGFB than it was known in late 70s when Odaro and De Larco considered it a “factor” responsible for changing cells phenotype and called it "sarcoma growth factor" (SGF) (33). Most body cells secrete TGFB1 and express the receptor for it (34). TGFB1 comes from inflammatory cells such as eosinophils, macrophages and lymphocytes, along with structural cells such as fibroblast and epithelial cells and platelets. TGFB is a member of cytokine family collectively known as TGFB super family and it has three isoforms TGFB1, TGFB2, TGFB3 (35). TGFB1 is normally released as a latent complex associated protein and is bound non-covalently to latency associated protein and is released in its active form after undergoing proteolysis (36). In vitro factors which activate TGFB1 include heat, reactive oxygen species, MMP-2, MMP-9, binding to integrin alpha v beta 6, acid or alkali treatment and exposure to proteases like cathepsin (37).

TGFB1 plays a multiple role in renal disease including tubular atrophy (38), tubulointerstitial fibrosis (39), podocytes depletion (40;41), epithelial-to-mesenchymal transdifferentiation (42) and nephron loss. Strategies aimed at blocking TGFB1 have multiple limitations including adverse effects, cost and requirement for repeated dosage of blocking agent (43). Proteinuria caused by TGFB1 has been shown to be independent of SMAD signalling (44) and investigating the pathways and mediators involved in

causing protein leakage glomerulopathies can open avenues for treatment and will help identify potential therapeutic targets.

2.4.1 SMADS

SMAD proteins lack any known structural motifs but all the members of this family have highly conserved amino Mad homology domain 1 (MH1) and carboxyl terminal Mad homology domain 2 (MH2) domains. MH1 domain helps in nuclear import, DNA binding and interaction with transcription factors for influencing gene expression. MH2 is phosphorylated at the receptor, this helps in SMAD-SMAD4 interaction.

TGFB1 interacts with type II receptor which leads to phosphorylation of type I receptor kinase (45). The receptor activation signal is then transmitted to the nucleus via complex pathways which include SMADs, cross-talk with other transcriptional factors, and other SMAD independent pathways. SMADs are the most well characterized signalling pathway for TGFB1(46). SMAD2 and SMAD3 have similar functions to some extent but in the kidney, SMAD2 is less important due to its lack of sequence specific DNA binding activity (47).

Nine different human SMADs are known and they are divided into

- Receptor activated SMADs (R SMADs)
- Common-mediator SMADs (co-SMADs)
- Inhibitory SMADs (I SMADs)

R SMADs are directly phosphorylated by the type 1 receptor (48). SMAD4 and one of the receptor-activated SMAD partner up to form a complex which then translocates to the nucleus and activates or represses gene transcription (49).

SMAD2 knockout is embryonically lethal. Phanish et al. looked at different roles played by SMAD2 and SMAD3 in regulation of TGF β responses in human proximal tubular endothelial cells (139). They used an in vitro proximal tubular endothelial cell culture model and divided the response to TGF β 1 induced fibrosis in two phases. An early phase there is induction of connective tissue growth factor (CTGF) along with down regulation of E-Cadherin. These early phase responses are mediated by SMAD3. A later response manifested by induction of MMP-2 requires SMAD2. Alpha smooth muscle actin (Acta2) upregulation requires both SMAD2 and SMAD3 activity.

The unphosphorylated R SMADs are completely flexible at their C-terminal. Abdollah et al. has shown that phosphorylation of SMADs takes place in the C-terminal on two Serine residues within the flexible region (50), and it is directly mediated by TGF β 1 receptor. They also demonstrated that SMAD2 is phosphorylated by TGF β 1 on Serine 465 and 467 residues. Mutation of serines, S465 or S467 abolishes TGF β 1 receptor dependent activity of SMAD2 in a negative dominant fashion. The phosphorylation of these residues is an independent rather than sequential event. Following the ligand dependent activity of TGF β 1 receptor, SMAD2 and SMAD3 are recruited to the receptor. They are phosphorylated and this is a key event for the release of SMADs from the receptor and allows R-SMADs to form complexes with SMAD4 through their MH2 domains (50).

Phosphorylation of SMADs is critical for their proper functioning. Mutations in SMAD2 in colorectal cancer that block its phosphorylation yields inactive proteins. Phosphorylation of the carboxyl terminal residues is unusual by serine kinases and rarely

happens in other serine kinase driven pathways. Hence these residues may not be good targets for other serine kinase in the cells and this could provide one mechanism for the specificity of this pathway.

SMAD6 and SMAD7 are inhibitory SMADs, and they function as an antagonist to R SMADs and Co SMADs. SMAD7 lacks the C-terminal serine motif. It associates stably with the TGFB1 receptor and inhibits TGFB1 signalling by preventing access to SMAD2 and its phosphorylation by type 1 receptor.

Flandres et al. has shown that mice lacking in SMAD3 show significantly less fibrotic response when exposed to Gamma radiation. There was less ulceration and inflammation and significantly reduced number of myofibroblasts (51).

Wang et al. used streptozotocin induced diabetic mice. In this model SMAD3^{-/-} mice developed significantly less renal fibrosis than their wild type counterparts. SMAD3^{-/-} had reduced renal hypertrophy, fibronectin production, mesangial matrix expansion and glomerular basement thickening. The level of proteinuria, however, did not differ between the two groups (44). These finding are quite different from those of Fujimoto et al. They also used streptozotocin induced diabetic SMAD3 null mice and compared the urinary albumin excretion in null and wild type groups. Urinary albumin excretion increased dramatically after diabetes induction in wild type group but the null mice had no overt albuminuria (52).

Podocyte depletion has been the hallmark of different types of glomerulosclerosis and is considered the key component in the renal disease progression (38; 39) and podocyte apoptosis is associated with proteinuria (1; 40). Increased SMAD7 has been

shown to cause apoptosis on its own and this effect is potentiated in the presence of TGF β 1 (18). Apoptosis induced by TGF β 1 via SMAD pathway is well studied. TGF β up regulates and maintains prolonged mRNA and protein expression of p21 which is critical for epithelial cytotaxis (42). Other factors activated by TGF β 1 include SMAD7, p-38 mitogen activated protein kinase (MAPK), and caspase-3 which play a proapoptotic role in podocytes.

2.5 Glomerular Diseases

Proteinuria

2.5.1 Mechanism of Proteinuria

The kidney provides an effective barrier against the passage of proteins to the urine. The glomerulus serves as a size selective barrier, allowing water soluble and low molecular weight molecules to pass through. Higher molecular weight proteins such as albumin do not filter out in normal conditions. Another factor that determines the permeability of a molecule is its net charge. Neutral and positively charged molecules are more permeable.

Proteinuria greater than 150 mg in 24 h is abnormal and usually indicates renal pathology that requires further investigation. Proteinuria is not only a marker of renal injury but it further contributes to progressive renal failure (53). Greater amounts of proteinuria is associated with worse prognosis (54). Clinical trials have repeatedly emphasized that antiproteinuric treatment maximizes renoprotection (55). A better understanding of the intracellular pathways causing proteinuria would help control progression to chronic renal failure and uncover therapeutic targets.

Multiple pathways have been suggested to contribute to podocyte damage and progressive proteinuric nephropathies. Components of filtration barrier are arranged in a series whereby all the molecules that are filtered have to pass through different barriers each one allowing molecules of decreasing size to pass-through. Hence lack of proper functioning of any of these components can lead to proteinuria. The podocytes are critical to the maintenance of glomerular barrier function and the SD provides last and the major size barrier to protein loss (56).

2.5.2 TGFB and Glomerular Proteinuria

Direct inhibition of TGFB in models of renal disease reduces proteinuria (57-59). TGFB has been shown to cause proteinuria by altering the integrity of glomerular filtration barrier at the level of glomerular endothelial cells (GECs) (60) and podocytes (61).

Proteinuria was described in two animal models of TGFB overexpression (62). High levels of systemic TGFB, using a transgenic mouse model were shown to induce proteinuria, but the mechanism was not described (62). Krag and colleagues described a novel transgenic mouse with TGFB driven from the Ren-1^c promoter of the juxtaglomerular apparatus which developed Albuminuria (63). In an earlier publication, using the same mouse model of TGFB driven from the Ren-1^c promoter, Wogensen and colleagues included electron micrographs revealing extensive podocyte effacement, but this phenomenon was not commented on in the paper (64).

In a novel model of adenovirus mediated gene transfer to the rodent kidney, we have demonstrated that transient overexpression of TGF β primarily in the glomerular endothelium leads to podocyte effacement and proteinuria(61).

Proteinuria caused by TGF β has been shown to be somewhat independent of SMAD signalling and investigating the pathways and mediators involved in causing protein leakage glomerulopathies can open avenues for treatment and will help identify potential therapeutic targets.

2.5.3 Angiopoietins in Proteinuria

ANGPT2 has been associated with proteinuria and renal injury in several settings including lupus nephritis (65) and diabetic nephropathy (66). Davis and colleagues created a mouse model where ANGPT2 was overexpressed by a podocyte specific promoter (7). In this model, ANGPT2 overexpression led to proteinuria and decreased podocyte nephrin expression. Interestingly, they did not comment on podocytes foot process effacement in response to ANGPT2, but demonstrated endothelial cell dysfunction which they believed to underlie the proteinuric response.

In our work, we have identified that both ANGPT1 and ANGPT2 were upregulated in the glomeruli from animals exposed to AdTGF β 1 (61). Podocytes under resting conditions do not express Tie-2 receptors but we have shown that exposure of podocytes to TGF β 1 leads to Tie-2 being expressed on podocytes in vitro

Autoimmune Disease

2.5.6 Anti-GBM

One common animal model of renal disease is autoimmune anti-glomerular basement membrane antibody induced glomerulonephritis (GN). Anti-GBM GN is manifested by endothelial cell proliferation, mesangial matrix expansion, infiltration of inflammatory cells and fibrin deposition. Binding of antibodies activate the complement system and eventually leads to crescent formation, a hallmark of anti-GBM GN. Greater amounts of proteinuria are associated with worse prognosis. In this model a better understanding of pathways leading to proteinuria is still lacking.

2.5.7 History of anti-GBM Glomerulonephritis

The term Goodpasture's disease is coined after Ernest Goodpasture, an American pathologist who first described the syndrome in 1919. He described this syndrome in a patient presenting with fever, cough, hemoptysis and renal failure. This term is still used to describe a rare form of auto-immune disease affecting lungs and kidneys.

Antibodies are responsible for inducing glomerulonephritis was established by a key experiment carried out by Lerner et al. in 1967 (67). They transferred autoantibodies purified from anti-GBM patients to squirrel monkeys and showed that the antibodies bound to the basement membrane of these monkeys when injected in vivo and caused a disease pattern similar to that of anti-GBM glomerulonephritis. Not only is disease severity related to the antibody level in patients but depending on the target antigen of these antibodies the diseases can vary in severity (68).

The experimental autoimmune glomerulonephritis (EAG) was first described by Steblay in 1962 (69) and now this model is commonly used in rats and mice and it shares many features in common with the human disease.

Boyce et al. generated a pre-immunized and non pre-immunized model of anti-GBM GN in rats. The model generated without presensitization is macrophage independent model and the injury is mediated by neutrophils and complement. The macrophage dependent model (requires presensitization with immunoglobulins) is an accelerated form of the disease and TGF β is elevated only macrophage dependent model and the infiltrating macrophages are the source of TGF β (70).

2.5.8 Antigen and Autoantibodies

The basement membrane carries a potent immunogen present in a continuous distribution. The target auto antigen in GBM GN has been identified as type IV collagen. Autoantibodies play a pathogenic role in anti GBM GN is also supported by various renal transplant procedures. The diagnosis is based on the presence of anti GBM antibodies either in the blood or in kidneys. Patients suffering from GBM GN are not good candidate for renal transplant as they continue to produce autoantibodies and eventually exhibit same pathologic changes in the transplanted kidney which were observed in their own kidneys.

The pathognomonic findings are linear deposition of IgG along the glomerular basement membrane. Typically these antibodies belong to IgG (71) but IgM or IgA may also be present. In patients anti-GBM antibodies do not bind intact NCI hexamers. Under

normal conditions the epitope is sequestered such that antibodies cannot access it. But factors such as exposure to smoking, hydrocarbons, infections or reactive oxygen species disrupt the integrity of these hexamers and in doing so bring about some structural changes which induce antibody binding.

Pedchenko et al. considered goodpastures disease an autoimmune “conformeropathy.” They postulate that the key event early in the disease is conformational transition in subunits forming the neoepitope that initiates antibody formation and subsequent binding (72). The autoantibodies recognize the non-collagenous 1 domain of alpha3 chain belonging to type IV collagen [alpha 3(IV) NCI]. Anti-GBM antibodies bind specifically to the c-terminal noncollagenous-1 domain of the a3 chain or in some case the a5 chain, of type IV collagen. This type of collagen is found in the basement membranes of kidney, lung, choroid plexus, retina and cochlea (73). Hence the disease manifests itself in these organs.

The administration of anti-GBM antibodies in presensitized mice leads to a well characterized acute crescentic glomerulonephritis with subsequent proteinuria and interstitial fibrosis. Sado et al. in 1998 reported that human alpha3 (IV) NCI was able to induce severe crescentic nephritis in Wistar Kyoto rats (74). Antigens used so far to induce EAG were GBM isolated from different animals using different isolation techniques and recombinant human alpha3 (IV) NCI. Ryan et al. were the first ones to isolate full length DNA sequence of alpha3 (IV) NCI and obtain mammalian recombinant protein which can induce severe EAG in WKY rats (75).

2.5.9 Role of TGFB in Anti-GBM Glomerulonephritis

TGFB plays an important downstream role in causing cellular injury in anti-GBM glomerulonephritis. Zhou et al. reported the effects of blocking TGFB1 in the early stages of disease in an animal model of anti-GBM glomerulonephritis. They did gene transfer of entire soluble extracellular domain of TGFB1 receptor via adenovirus and expressed it in rats' muscles. This resulted in blockage of TGFB action and there was a significant reduction in the degree of renal fibrosis and interstitial damage in the treated group, the degree of proteinuria however was similar in both groups (76).

Saegusa et al. used an herbally derived chemical compound TJN-331 and showed that it improved the degree of nephritis and proteinuria in rats by inhibiting the intraglomerular production of TGFB. They were able to show that TJN-331 was able to dose-dependently inhibited the increase in total and mature TGFB1 production from nephritic glomeruli (59).

Kanamaru et al. used transgenic mice that over expressed SMAD7 (antagonist of TGFB1/SMAD signaling) in T cells and by doing so they were able to block TGFB/SMAD signaling in T-cells (77). After induction of anti-GBM GN, SMAD7 over expressing mice had lower proteinuria and less severe glomerular damage.

Song et al. compared the level of renal damage and proteinuria in mice heterozygous for TGFB type II receptor and wild type animals. The level of proteinuria was significantly less in heterozygous mice at all the time points compared to WT mice, indicating a role of TGFB in proteinuria and renal pathology (78).

2.5.10 Role of T-cells

Autoreactive T-cells are sufficient and necessary for induction of anti-GBM GN in animals. Ryan et al. have shown that T-cells are present in the glomeruli of animals with EAG (79) and these T-cells have been used to transfer disease to naïve recipients. Several studies have demonstrated that anti T-cell immunotherapy can prevent or diminish the disease. Glomerular TGFβ1 is elevated in the macrophage dependent injury model and infiltrating macrophages were found to be the source of TGFβ1. TGFβ blockage in T-cells of mice with anti-GBM induced nephritis resulted in less glomerular damage, reduced proteinuria and improved renal function (77).

2.6 Renal Fibrosis

In US, 90,000 patients developed chronic kidney failure in the year 2000 and at present an estimated 300,000 are on dialysis. Another 80,000 patients in US have had renal transplants while the cost of treatment for these patients with end stage renal disease (ESRD) is around \$18 billion per year (80). As of 2012 according to kidney foundation of Canada, approximately 2.6 million Canadians have kidney disease or at risk of developing kidney disease and patients treated for kidney failure have tripled in the span of past 20 years (<http://www.kidney.ca/document.doc?id=1376>).

Multiple immune and non-immune pathologies contribute to chronic renal fibrosis. Diseases including hypertension, diabetes, infection, inflammation and autoimmune diseases, regardless of the underlying pathogenesis, follow a common pathophysiological pathway (43). These diseases first manifest with structural glomerular injury and proteinuria (81) followed by tubulointerstitial injury and fibrosis.

Renal fibrosis following glomerular injury takes a predictable course involving tubular epithelial cell activation, increase in interstitial myofibroblasts and inflammatory cells, decrease in peritubular capillaries, and interstitial fibrosis.

The situation is worsened by an attempt of remnant renal glomeruli to compensate for the lost renal mass and eventually succumbing to hemodynamic burden. This is what eventually constitutes the ESRD. Recent treatment strategies are able to slow down the progression of disease but have not proven successful in reversing the fibrotic changes. Hence decelerating this pathway or blocking it altogether will halt the process of fibrosis irrespective of the causative agent

Angiotensin II receptor blockers and angiotensin-converting enzyme inhibitors have been the mainstay of therapy in diabetes but lack of complete effectiveness of these agents clearly points out that there are other mechanisms leading to proteinuria and ESRD. Chronic renal failure patients have to either survive on lifelong dialysis or undergo renal transplant.

Large scale studies have proven the renoprotective effects of angiotensin II antagonism for hypertensive patients with type II diabetes (82) but a bigger challenge is yet to prove if these fibrotic changes are reversible to any degree. Evidence does exist to suggest that renal fibrosis may be a reversible process. Bledsoe G et al. showed that kallikrein through B2 receptor reverses salt-induced inflammation, renal fibrosis and glomerular hypertrophy (83). Major work is being done on three different hypothesized mechanisms, inhibition of inflammation-oxidative stress, blockade of vasoconstrictor

peptide action and inhibition of TGF β action. It is hoped that blockade of one or more of these pathways will eventually reverse the damage occurred in the fibrotic kidney

2.6.1 TGF β and its Role in Renal Fibrosis

TGF β is found in the glomerulus in many different models of renal fibrosis (84). Over-expression of TGF β 1 leads to diffuse deposition of fibrotic protein in renal parenchyma (85), and inhibition of TGF β in animal models decreases renal injury (86).

TGF β plays an important role in inducing the deposition of extracellular matrix (ECM). It directly stimulates synthesis of ECM (5) and block matrix degradation by downregulating ECM degrading proteases and up-regulating protease inhibitors (6). TGF β helps in fibrosis by enhancing collagen deposition (87), inhibits fibrinolysis through plasminogen activator inhibitor -1 (88) and inhibits collagenolysis through tissue inhibitor of metalloproteinase (89). It also plays a major role in mediating the fibrogenic action of angiotensin II. TGF β Receptor and TGF β have been reported to be up-regulated in human glomerulopathies. There have been previous reports of the effects of systemic TGF β 1 on the structure and function of the kidney. Systemic overexpression of TGF β appears to have an impact on the kidney either through a hemodynamic, vasoconstrictive mechanism or through a direct fibrogenic effect.

Decorin, a proteoglycan and an inhibitor of TGF β 1 was used by Isaka Y et al. in a rat model of glomerulonephritis (86). Fibrosis in the model was mediated by TGF β and cDNA of decorin was transferred into rat skeletal muscle. This lead to increased decorin protein present in skeletal muscles and in the kidney. Transfected glomerulonephritic rats

showed a significant reduction in levels of glomerular TGF β 1 mRNA and TGF β 1 protein, extracellular matrix accumulation and proteinuria.

In 2001 Kasuga H et al. showed that the use of soluble TGF β receptor antibody prevented the accumulation of extracellular matrix and renal fibrosis (90). However these treatment regimens have limitations such as decorin being non-specific in higher concentrations and the need for repeated administration of TGF β receptor antibody.

In the renal fibrosis model of unilateral ureteral obstruction (UUO), the use of monoclonal antibody against TGF β (1D11) was able to significantly decreased apoptosis, fibroblast expression and renal collagen deposition (91).

2.6.2 Hypoxia and Renal Fibrosis

Tubular hypoxia is proposed to occur through different mechanisms including: renal vasoconstriction through up regulation of angiotensin and endothelin (92), structural glomerular lesion affecting downstream perfusion, and direct vascular alteration with apoptosis of the peritubular endothelium (93).

The role of hypoxia as a link between glomerular disease and tubulointerstitial fibrosis has been clarified (92;94). These animal studies include both the remnant kidney model of chronic renal disease and models of acute glomerulonephritis. Supporting data includes a temporal association between injury, hypoxia, and subsequent tubulointerstitial fibrosis (92).

Pimonidazole is a small nontoxic hypoxia marker. In vivo, in normoxic conditions pimonidazole undergoes oxidative metabolism so that it is rapidly excreted from cells. In

hypoxic condition however, it cannot be easily excreted owing to the formation of its compound with thiol groups in proteins, peptides and amino acids.

Pimonidazole makes a good choice for studying hypoxia because it is stable in vivo and is rapidly distributed to all the tissues. Pimonidazole is then identified in tissue section with immunohistochemistry. Pimonidazole staining has been correlated with CA9 immunohistochemistry in a rat tumour model (95) and has been used in several experiments analyzing renal tubular hypoxia (92;96). We have used Pimonidazole as a hypoxia marker.

2.7 Vectors for Gene Transfer to the kidney

Choosing the vector for renal gene transfer is yet a big challenge. Several factors need to be considered. The functional heterogeneity of renal cells is one of the major hurdles in efficient transduction. The anatomical structure of kidney provides a limited access to renal sites. The different specialized compartments of the kidney are separated from each other by the basement membrane (BM), and this BM is a hurdle for the viral vectors to pass through (17). Since renal cells are terminally differentiated and non-dividing, not all the viral agents available can transduce such stable cell lines. Hence, there is ongoing research to identify a system that provides long term expression.

The most widely used vector for glomerular gene transfer is the HVJ-liposome technology (18). This Sendai virus based lipid – cDNA mixture has been shown to transduce 15% of glomerular cells (capillary and mesangial) with a limited time course of 7 days (19). The HVJ-liposome vector is delivered via the renal artery. The major

limitations with this vector are the low efficiency of gene transfer and the transient nature of gene transduction.

Other vectors such as Recombinant adeno-associated viral vectors (rAAV) provide transduction for up to 3 months but the transduction efficiency is lower than adenoviruses (17). Murine retroviruses are promising in providing long-term transduction but these require cell division after transduction to stably integrate into the host genome. In kidneys, even after folate-induced tubular regeneration, the extent of transduction was limited to a few proximal tubular epithelial cells around the site of injection (20). Non-viral methods of transfection include direct DNA or RNA delivery to the cells and include DNA injection, liposome, DNA-ligand conjugates, gene gun etc. However most of these methods are transient. We used first generation adenoviruses carrying green fluorescent protein (GFP) or Beta galactosidase Z as reporter proteins and TGFB1 as active transgene.

2.7.1 First Generation Adenovirus

Adenovirus is double-stranded, non-enveloped viruses with the genome of 36Kb. In humans more than 51 serotypes belonging to six groups A-F have been identified so far and are well known for causing upper respiratory tract infection in children and adults. They are capable of transducing a wide variety of host cells, including stable and dividing cell lines. The size of DNA insert that can be accommodated is 8-10 Kb and they can be purified to yield high titres.

As adenoviruses do not integrate into genomic DNA, their expression is transient and repeated injections have faced the problem of immunogenicity due to continued

synthesis of viral proteins (97). The adenovirus possesses some unique characteristics which justifies its use in some gene therapy trials. They are theoretically safer than viruses which integrate their DNA in host's genome hence virtually eliminating the potential threat of oncogenic transformation of host cells. In the class of non-enveloped viruses, adenovirus offers a larger packaging capacity. Serotype 2 and 5 are mostly widely used.

The fibres on the surface of adenovirus enable the virus to attach to the host cells. The knob at the end of the fibre interacts with the coxsackie adenovirus receptor (CAR). Heparan sulphate glucosaminoglycans mediate CAR independent attachment mostly for group C members. Group B uses CD46, a complement regulatory protein which is expressed by all cells possessing nuclei. Several group D viruses use sialic acids and are known to cause conjunctivitis. Adenovirus Group 5 also attaches to VCAM-1, on endothelial cells. Once the virus attaches to the CAR, the RGD (Arg-Gly-Asp) motifs located at the site of fibre attachment (penton base) interacts with $\alpha\beta3$ and $\alpha\beta5$ integrins and helps the virus get internalized (98).

In the first generation adenovirus, E1 region (early region 1) is replaced with the transgene. The newer second and third generation adenovirus have deletions of E1, E2 and E4 genes making them much less immunogenic than first generation viruses (99).

2.7.2 Lentiviruses

These are retroviruses belonging to the family Retroviridae. These vectors offer several advantages. They can infect and stably transduce terminally differentiated, non-dividing cells. They have a large packaging capacity (8-10 kb) for the delivery of

therapeutic genes, can be pseudotyped with heterologous envelopes (i.e. vesicular stomatitis virus glycoprotein, VSV-G) and have low immunogenicity (21). Several approaches for expressing multiple genes in a single vector are available. Lentiviruses also have low immunogenicity compared to the adenovirus.

There is however a potential risk of insertional mutagenesis. Lentiviruses tend not to integrate into oncogene promoters and hence have no reports of causing cancer in the situations studied so far. For safety reasons, lentiviral vectors are manufactured using a four plasmid approach. This virtually eliminates the possibility of creating replication competent virus (31). In Phase I clinical trials with five HIV positive patients, researchers found that an HIV-based lentiviral vector was a safe and potentially effective way to deliver an anti-HIV gene (22). For Phase I clinical trials, the researchers studied the effects of this vector in five HIV-positive men who had become resistant to standard antiviral treatments. They removed T cells from each patient, modified the cells with the lentiviral vector, and injected one dose of the modified T cells back into the patient. The subjects were then monitored for signs of toxicity and immunological responses. No evidence of replication-competent Lentiviruses was found in any of the patients. Patients receiving Lentivirus for HIV treatment were followed up for as long as 8 years and were found to have no adverse effects (100).

The envelope of Lentivirus was a limiting factor due to its narrow tropism for CD4 + cells. The number of envelope pseudotypes available for cell- and tissue-specific tropism continues to expand. Pseudotyping is constructing viruses with surface glycoproteins from other enveloped viruses. For Lentivirus, pseudotyping is most

commonly done by using vesicular stomatitis virus G glycoprotein. This has two advantages. It drastically increases the tropism of these viruses and makes them much more stable. Enhanced stability enables ultracentrifugation during the process of virus formation and helps to produce virus with higher titers (101).

2.7.3 Modes of Delivery

Genes may be delivered to the kidney by the intravenous, intra-arterial, ureteric, or intraparenchymal routes (25). Intraparenchymal therapy is invasive and leads to poor distribution of gene product which is limited to the needle tract. The intravenous approach is the least invasive but leads to systemic administration and toxicity (26) with decreased efficiency at targeting the kidney (27). The ureteric / renal pelvis approach requires a surgical approach in rodents but could be carried out using cystoscopy in humans. The targeting of renal tubular cells with this method could make it attractive for intervention in many kidney diseases.

We have used the intra-arterial technique for introduction of adenovirus in rodents as was previously done by Ye et al. (102). This requires a surgical approach in animals, but radiologic/angiographic techniques could be used in larger mammals or humans. In larger animals, Heikkilä and colleagues used an in vivo perfusion of a swine kidney for 60 and 120 minutes with a high dose (10^{11} pfu) of adenovirus (28). They found 75 % of glomeruli expressed transgene product and the expression lasted 14 to 21 days. The renal perfusion technique led to some non-specific renal damage including patchy areas of tubular necrosis.

2.8 Summary

In summary, the mechanisms leading to proteinuria or renal fibrosis after an acute injury are still not clearly understood. Hence our therapeutic impact is limited. The role of TGF β in this process is of great interest. An acute injury leads to tubular epithelial cell activation, an infiltrate of inflammatory cells and cytokines, including TGF β , and this eventually leads to interstitial fibrosis. TGF β could be the final common pathway or one of the key players and understanding how TGF β and the SMAD pathway play a role in this progression is invaluable.

I used a model of localized gene upregulation to study the effects of TGF β in the kidney and discovered a detrimental effect on the glomerular filtration barrier and the tubulointerstitium. I observed proteinuria and podocyte de-differentiation in this model. Since SMADs are the most well studied pathway used by TGF β for its downstream effects, I also used SMAD3^{-/-} mice to determine the importance of this particular pathway in a model where TGF β contributes to the renal injury. In the anti-GBM GN model, blocking SMAD3 did not completely obliterate the downstream effects of TGF β , such as proteinuria or tubulointerstitial fibrosis. This indicates that there are other pathways that could be activated by TGF β or are working alongside TGF β . I believed that these experiments would answer the question of the causative role of TGF β and SMAD3 in interstitial fibrosis and proteinuria.

CHAPTER 3 MATERIALS AND METHODS

3.1 Animal Studies

3.1.1 AdTGFB1 Rat Model

All animal studies were carried out according to Canadian Council on Animal Care Guidelines and approval was received from the university animal research ethics board. Female Sprague-Dawley rats (weight, 200 to 250 g; Harlan, Indianapolis, IN) were anesthetised with isoflurane (MTC Pharmaceuticals, Cambridge, ON, Canada). Ye and colleagues first described the intra-arterial technique in rodents using slow perfusion of First generation adenovirus vectors and we have used the same technique (103).

In short, Female Sprague Dawley rats weighing 200-250 g from Harlan were used. Animals were anesthetised with isoflurane. A midline incision was made and the tissue was dissected down to the aorta proximal to the origin of the left renal artery. The aorta was clamped above the left but below the right renal artery. A small puncture was made in the aorta using a 22G needle at the origin of renal artery, a PE10 catheter was advanced into the left renal artery and the catheter was secured in place by a clamp (Figure 3.1). Saline was flushed through the catheter and blanching of the left kidney was observed. The left kidney was perfused with 1.5 ml of cold saline containing 5×10^9 plaque forming units of AdLacZ, AdGFP or AdTGFB1 with 25 Units of heparin using an infusion pump (Razel Scientific, St. Albans VT). Five minutes after starting the infusion the renal vein was clamped. Total infusion time was 15 minutes. The kidney was packed with ice cold saline soaked sterile gauze during infusion. After removal of the catheter, the aorta was sutured with 8-0 or 9-0 proline under stereomicroscopic visualization. Clips were

removed and reperfusion of the kidney was observed. Right kidney served as control. Ischemic interval was kept below 30 minutes in each procedure to avoid ischemic damage to the kidney.

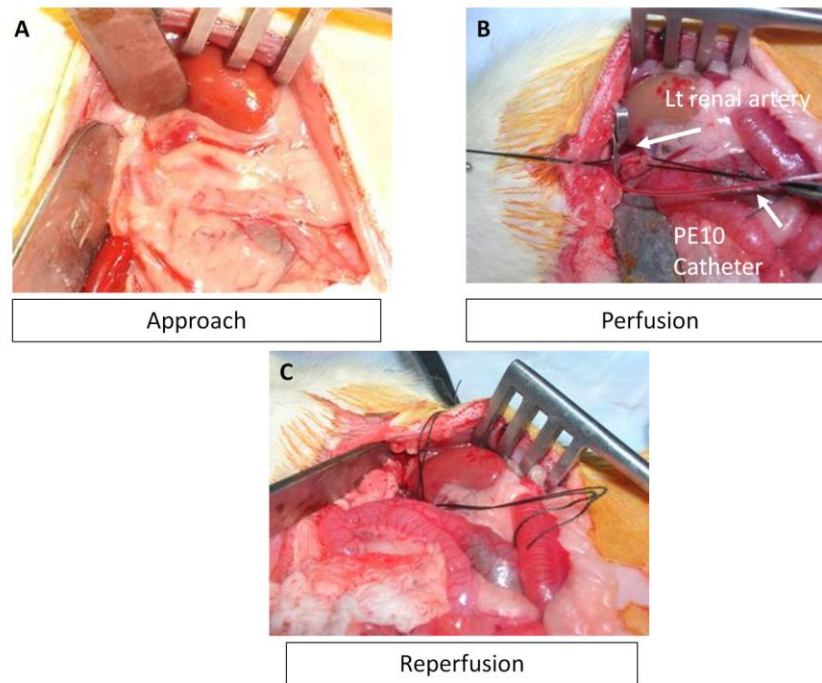


Figure 3.1: A) Surgical approach to the left renal artery in a rat. B) Insertion of PE10 catheter, blanching of the kidney with perfusion, and C) reperfusion after removal of PE10 catheter.

Twenty animals were divided into groups receiving AdLacZ, AdGFP or AdTGFB1 and were sacrificed at two time points at day 7 and day 28. After sacrifice tissues were taken for histology, protein and RNA analysis. Urine samples were obtained at days 4, 7, 14, 21 and 28.

3.1.2 Adenovirus

Adenoviral vectors expressing GFP, Beta-galactosidase, and active TGF β 1 were expanded, purified and plaque tittered on 293 cells, as previously described (104). This adenovirus construct is replication-deficient as the E1 genomic region is deleted. AdLac-Z was also used as a virus with reporter transgene.

Same batch of the virus was used for the entire experiment and repeated freeze thaw was avoided by making aliquots in advance. Recombinant adenoviruses have a wide host cell range and can transfect stable cell lines like that of kidney. Repeated administration of adenovirus is not possible due to its immunogenicity. AdDL is a null adenovirus and was used as a control for cell culture experiments.

3.1.3 Anti-GBM Animal Work

To generate a mouse model of anti-glomerular basement membrane glomerulonephritis, we used the same method as Wolf and colleagues (105). Eight week old C57Bl/6 (SMAD3^{+/+}) mice and SMAD3 knockout (SMAD3^{-/-}) transgenic animals were used. These mice lack exon 8 in the SMAD3 gene in the mixed background of 129SV3/EV X C57BL/6 (106). On day -4 we sensitized mouse's immune system by injecting normal sheep serum (115 μ l) by intraperitoneal (IP) in complete adjuvant, Titermax (Sigma Aldrich, Oakville ON). Four days later (day 0), animals received 5 mg/20 g of rabbit anti-mouse GBM via tail vein (100-150 μ l anti-GBM) injection. Animals were placed in metabolic cages on days 3, 7, 21, and 56 to collect urine for 8 hours to assess urine protein excretion as described below.

3.1.4 Anti-Glomerular Basement Membrane Antibody

Antibodies against glomerular basement membrane were generated using standard procedures. Isolated glomeruli were used to generate anti-GBM antibody. To isolate glomeruli rat kidneys were extracted and maintained at 4°C. The cortex was isolated from the medulla. Next the cortex is sieved through sequential sieves (250, 106 and 75 µm). The glomeruli are collected from the 75 µm sieve in ice cold PBS and spun down and resuspended in sterile PBS. Isolated glomeruli were then sent to Lampire, Pipersville, PA for custom antibody production. Non-specific antibody treated mice served as control. Normal sheep serum was also obtained from Lampire. The table below shows number of animals used in each group.

Table 1: Number of animals in each group for anti-GBM experiment

		SMAD3 ^{+/+}	SMAD3 ^{-/-}
Day 7	+ GBM	6	5
	No GBM	7	5
Day 21	+ GBM	7	6
	No GBM	6	6
Day 56	+ GBM	6	6
	No GBM	0	0

3.1.5 Urinalysis

For rats, urine was collected in a metabolic cage over 6 hours at 7, 14 and 28 days after treatment. For mice, urine was collected for 8 hours at day 3, 7, 21 and 56 after

initiation of treatment. Concentration of urea and creatinine in urine samples were determined using Hitachi 917 automated analyzer (Roche Diagnostics, Laval, QC). Urine total protein was also determined on the same instrument and these analyses were done in Core Histology laboratory here at St. Joseph’s Hospital, Hamilton.

3.1.6 Lentivirus in Rats

Lentivirus expressing LacZ reporter gene has been used by Gusella et al. (107). They performed the experiment in BALB/c mice and the Lentivirus used had the CMV promoter. They have shown transduction mostly of inner medullar region.

We used Female Sprague Dawley rats weighing 200-250 grams from Harlan and the gene transfer approach taken in my ADTGFB1 project was used. The animal groups are shown in table 1. Left Kidneys were treated with Lentivirus encoding for GFP. Right kidneys served as control. We were able to finish day 4 and 7 studies.

Table 2: Dose and number of rats used for Lentivirus experiment

Low Dose	High Dose	Urine collection	Organs to be collected
10 ⁷ pfu	10 ⁸ pfu	8 hours	
Day 7 (3 rats)	Day 7 (3rats)	Days 4 and 7	Kidneys, liver, spleen, lung and omentum. Serum for Liver function tests.

This approach was used in preliminary experiments to document localization of gene transfer and kinetics of gene expression after slow renal arterial perfusion. We used VSV glycoprotein pseudotyped Lentivirus and first generation adenovirus was used as the baseline to compare transfection efficiency and duration of Lentivirus. The virus was generously provided by Dr. Maria Medina (Assistant Professor Pathology and Molecular

Medicine, McMaster University). The titer of the virus used was 2×10^9 TU/ ml in 1.5 ml. We used two different doses, a high dose of 1×10^8 and used a low dose of 1×10^7 viral particles in 1 ml of cold saline.

3.2 Albuminuria Assays

Equal volumes of urine samples were electrophoresed on an SDS-PAGE gel and stained with Coomassie blue for twenty minutes. The gel was then washed with destaining solution overnight at 4 °C. The band at 60 kDa was quantified for albuminuria.

Nephrin was measured in the urine by probing electrophoresed urine with an anti-nephrin antibody. Band density was measured using Scion Image Software (Scion Corp, Frederick, MD) and corrected for urine creatinine concentration.

3.3 Western Immunoblotting

Tissue collected for protein extraction was snap frozen in liquid nitrogen immediately after sacrifice of animal, stored in -80 °C then ground with a mortar and pestle in protein lysis buffer (containing protease inhibitors) on dry ice to extract proteins. Protein concentration was measured by Bradford assay and samples were adjusted to 2 µg/µl protein concentration. To denature the proteins the samples were boiled at 100°C for 5 minutes with denaturing agent, sodium dodecyl sulfate (SDS).

For transgene quantification, kidney homogenate was run on an SDS-PAGE gel and probed with anti-GFP antibody. For podocyte maturity marker anti-synaptopodin and anti-nephrin antibodies were used. To quantify levels of ANGPT1 and ANGPT2, the

PVDF membranes were probed with respective antibodies. List of other antibodies used for staining and western is given below.

Table 3: Sources of antibodies and recombinant proteins used during all projects

Antibody	Source
Recombinant Human Angiopoietin-1	R&D Systems, Minneapolis, MN
Recombinant Human Angiopoietin-2	R&D Systems, Minneapolis, MN
Recombinant Human TGFB1	R&D Systems, Minneapolis, MN
Anti-human Angiopoietin-2 Antibody	R&D Systems, Minneapolis, MN
Anti-human Angiopoietin-1 Antibody	R&D Systems, Minneapolis, MN
Anti-mouse Tie-2 Antibody	R&D Systems, Minneapolis, MN
Acta2 Antibody	Dako Corp, Carpinteria, CA
Beta -actin Antibody	Sigma- Aldrich, Saint Louis, Missouri
HIF1A Antibody	Abcam, Cambridge, MA
Anti-nephrin antibody	Abcam, Cambridge, MA
Anti-GFP antibody	Santa Cruz Biotechnology, Santa Cruz, CA
Anti-synaptopodin Antibody	Progen Biotechnik, Heidelberg, Germany
Anti-Tie-2 Antibody	Millipore, Temecula, CA
CD34 Antibody	R&D Systems, Minneapolis, MN
All Fluorescent labeled Secondary Antibodies	Jackson Immunoresearch, West Grove, PA
Anti SMAD2	Cell signaling, Boston, MA
Anti SMAD3	Abcam, Cambridge, MA
Anti Zo-1	ZYMED Laboratories, Carlsbad, CA

3.4 ELISA

TGFB1 active and total was measured in urine samples, stored at -20°C, using standard ELISA (R&D Systems, Minneapolis, MN). ELISA was performed according to manufacturer's instructions at room temperature, in a 96-well plate. TGFB1 standard solution was prepared according to the manufacturers' instructions.

I used ELISA to measure total TGFB. The latent TGFB1 in the sample had to be activated because only the active TGFB1 could be measured using this technique. This was done by acidification of samples in 1N HCL for 10 minutes followed by neutralization with 1.2 N NaOH/0.5 M HEPES as per protocol. The concentration of active TGFB protein was analyzed on samples that were not acidified. The optical density of the samples was determined on a microplate reader (μ Quant, Bio-Tek Instrument) set at 450 nm and wavelength correction set to 540 nm.

3.5 Cell Culture

3.5.1 Podocyte Culture, Primary and Immortalized Podocytes

Podocyte primary culture cells should ideally differentiate into arborized cells and undergo growth arrest and express synaptopodin. Podocyte cell culture is a challenging task. Primary podocyte cultures can only be passaged a few times because after second passage they lose their proliferative capacity, morphology and maturity markers. We used immortalized cells which are highly proliferative when cultured under permissive conditions and even in vitro these retain a differentiation potential similar to podocytes in vivo.

Immortalized podocytes were a gift from Peter Mundel (Division of Nephrology, Massachusetts General Hospital, Boston, MA). Cells were plated on type I collagen-coated dishes and cultured cells were passaged. These cells can be maintained either in a proliferative state at 33 °C or be induced to differentiate at 37 °C. Immortalized podocytes are incubated with gamma-interferon at a temperature of 33 °C and these constitute permissive conditions in which the cells proliferate. At a temperature of 37 °C

and absence of gamma-interferon, the cells stop proliferating and after 14 days differentiate and express maturity markers such as synaptopodin.

Both proliferating and differentiating podocytes express the WT-1 protein (7). The differentiated cell showed an ordered array of actin filaments and microtubules extending into the forming processes during differentiation, reminiscent of podocyte processes in vivo. These cytoskeletal rearrangements and process formation were accompanied by the onset of synaptopodin synthesis.

For all experiments, podocytes were allowed to grow under non-permissive conditions for 14 days. 12 hours before treatment cells were washed twice with sterile PBS and serum starved. These cells were exposed to different concentrations (0.1, 2.5, 5 and 10ng/ml) of recombinant TGFB1 (rTGFB1; R&D Systems) for 8 hours. 5ng/ml was the most effective dose in downregulating podocyte markers, hence in further experiments we used this dose. Cells were also treated with rTGFB1 (5 ng/ml) and anti-human ANGPT1, ANGPT2 antibody, or a blocking Tie2 antibody. Cell lysates were then analyzed by using Western blot analysis and probed for Tie2, nephrin, podocin, or synaptopodin.

Podocytes were also exposed to rTGFB1 (5 ng/ml) with cyclosporine (1 µg/ml) (Sigma- Aldrich, Saint Louis, Missouri) for 8 hours. Cell lysates were taken and assayed for synaptopodin or Zo-1. Three samples were assayed for each condition, and the experiments were repeated in duplicate.

3.5.2 Immortalized Mouse Cardiac Endothelial Cells Culture

Immortalized mouse cardiac endothelial cells (MCEC) (Cedarlane, Burlington, ON) were grown on 0.2% gelatin-coated plates. When 80% confluent, cells were infected with AdDL (control adenovirus) or AdTGFB1 at 10 plaque-forming units per cell. Twenty-four hours later, cells were washed with PBS and fresh media were placed for 12 hours. The media was then collected (without the cells) and was inactivated by ultraviolet-light and then added to cultured podocyte cells for 8 hours. Podocyte were grown and serum starved as explained previously. Cell lysates were then taken and assayed for nephrin and synaptopodin by Western blot analysis.

3.5.3 Transfection of Podocytes

Small-interfering RNA (siRNA) against Tie2 (Stealth; Invitrogen) or nontargeting siRNA was transfected into immortalized podocytes. Twenty-four hours after transfection, cells were treated with rTGFB1 (5 ng/ml) for 8 hours. Cell lysates were assayed for synaptopodin or nephrin by using Western blot analysis

In a separate experiment, immortalized podocytes were also transfected with scrambled siRNA, SMAD2, SMAD3 siRNA and a combination SMAD2 and SMAD3 siRNA. *Smartpool* siRNA was purchased from Dharmacon, Thermo Scientific, and was performed in 6 well plates according to the manufactures' instructions. Podocytes were grown at non permissive conditions (37 °C and absence of gamma–interferon) for 14 days to ensure podocytes express all the maturity markers. One day before transfection podocytes were trypsinized and spit into 6 well plates. The control group scrambled SMAD2, SMAD3^{-/-} and co-SMAD2-SMAD3 siRNA groups were treated with or without

recombinant TGFB1, 8 hours prior to harvesting. Non treated cells served as negative control. The cells were harvested after 48 hours for gene expression analyses and 72 hours for protein expression analyses.

3.6 Tissue Analysis

3.6.1 Immunohistochemical Analyses

Immunostaining was performed using antibodies against ACTA2, CD34 and synaptopodin. The slides were deparaffinized in xylene, then hydrated by through graded alcohols. After antigen retrieval in 0.01 M Citrate Buffer (pH 6.0), slides were blocked with 1% NGS and incubated with primary antibodies for 1 hour at RT. Secondary antibody used was biotinylated. Slides were developed with streptavidin peroxidase conjugate (Dako) followed by 0.05 M Acetate buffer then freshly prepared and filtered chromogen/substrate solution for 20 min. Slides were counterstained in Mayer's hematoxylin for 1 min. Staining was controlled using concurrently run isotype primary antibody (Dako). Formalin-fixed sections were also stained for Masson's trichrome.

Frozen sections, 0.5 μ m, were cut and stained for β -galactosidase after fixing for 2 hours with 2% formaldehyde and 0.2% glutaraldehyde. The staining solution contained 5 mM potassium ferricyanide, crystalline 5 mM potassium ferrocyanide, trihydrate 2 mM magnesium chloride 0.01% SDS, 0.01% Triton-X and 1 mg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) in DMSO. AdTGFB1 sections served as control.

3.6.2 Picrosirius Red Staining

Fibrosis was assessed using quantification of Picrosirius red staining. Picrosirius red stained sections were imaged under polarized light. Polarizer (U-POT) and Analyzer (U-ANT) attachments were purchased from Olympus Corporation and sections were quantified using Image J. Total collagen was quantified by percentage of the green birefringent area while new collagen was quantified by subtracting green from the red area.

3.6.3 Dual Immunofluorescence

Renal samples were initially fixed in 10% formalin for 48 hours, transferred to 70% ethanol and paraffin embedded. The tissue were deparaffinized in xylene then hydrated in graded ethanol. The sections were then blocked with 1% Normal goat serum and the incubated in the cocktail mixture of two primary antibodies, anti GFP from rabbit and Acta2 from mouse. For anti-GFP, the secondary antibodies used was FITC labelled donkey anti-rabbit and for Acta2, Texas red goat anti-mouse was used.

Similar protocol was followed for dual staining of GFP and synaptopodin and for GFP and CD34. For both synaptopodin and CD34, secondary antibodies were Texas red. Primary and secondary antibodies were left for 2 hours each at room temperature. Sections were mounted with a DAPI nuclear stain (Vectashield, Vector Laboratories, Burlingame, CA). 0.05 M PBS (pH 7.6) was used to wash the sections between incubations.

3.6.4 Assessment of Hypoxia

One hour before sacrifice, animals received 60 mg/kg IP injection of Pimonidazole (50 µl) (Chemicon). Hypoxic tissues are unable to get rid of pimonidazole and this can be detected on staining. Renal tissue used for immunohistochemistry was formalin fixed and sections were cut at a thickness of 6 µm. These were probed with a monoclonal antibody for pimonidazole, Hypoxyprobe Mab-1.

3.7 Gene Expression

3.7.1 Laser-Capture Microdissection of Glomerular for RNA

For rat tissues frozen sections of the left kidney (8 µm thick) were used for laser capture microdissection (LCM). The glomeruli were identified based on morphological characteristics and were captured using a PixCell II System (Arcturus, Mountain View, CA). RNA from laser-captured tissue was extracted using a Pico Pure RNA Isolation Kit (Arcturus), followed by amplification using a Message Amp aRNA Kit (Ambion, Austin, TX).

The RNA from LCM tissue was reverse transcribed using a standard protocol (Invitrogen, Burlington, ON, Canada). Quantitative real-time PCR was performed using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA). Pooled mRNA from control animals was used to generate a standard curve for comparative quantification. The correlation coefficient for the standard curves for all samples was >0.90. Samples were run in duplicate. Negative control samples (no template or no reverse transcriptase) were run concurrently.

3.7.2 Whole Tissues Gene Analysis

mRNA was extracted from frozen kidney samples using Trizol reagent (Invitrogen) under RNase free conditions. The concentration of RNA obtained was measured by Nanodrop Spectrophotometer (NanoDrop Technologies, USA). RNA (1 µg) obtained was reverse transcribed by standard methods (Invitrogen, Mississauga, ON) and then gene expression was determined by 7500 Real Time PCR (Applied BioSystems, Foster City, CA) using forward and reverse primers and probes. 18S was used as an internal standard. The list of probes and primers used is given in the table below (Table 4 and Table 5).

3.7.2 Cell Culture Gene Analysis

Cells were washed three times with ice cold PBS and mRNA was extracted from cells using Bio-RAD Aurum Total RNA mini kit. mRNA was reverse transcribed and gene expression was determined as discussed above.

Table 4: Mouse probes and primers used. Table shows the sequence of forward and reverse primers used and the sequence of probes used

Gene	Forward Primer	Reverse Primer	Probe
Nephrin	AAGCTGGACGTG CATTATGCT	CGGTGCAGACTAT ATCCACAGAAC	TGC CCT GAA GGA CCC TAC TGA GGT GAA
Snail-2	CGGGAGCATACA GCCCTATTACT	GGCCACTGGGTAA AGGAGAGT	TGGACATCGTCGGCA GCTCCAC
18-S	CGAACGTCTGCCC TATCA	GCTGCCTTCCTTGG ATGT	TAG CCG TTT CTC AGG CTC CCT CTC C
E-cadherin	CCCAAACGTAAC GAGGGTATC	GTGCTGCTCAGGTA TTCGTATC	AACCCACGAAGTCCC TGGACTATGAAG
SNAIL-1	CCACTGCAACCGT GCTTTT	CACATCCGAGTGG GTTTGG	CTGACCGCTCCAACC TGC GTGC
HIF1A	AGGAGCCTGATG CTCTCACTCT	TCATCGCTGCCAAA ATCCA	CCAGCTGCCGGCGAC ACCAT
CTGF	TCCCGAGAAGGG TCAAGCT	TCCTTGGGCTCGTC ACACA	CCTGGGAAATGCTGC AAGGAGTGG
VEGF	CATCTTCAAGCCG TCCTGTGT	CAGGGCTTCATCGT TACAGCA	CCGCTGATGCGCTGT GCAGG
ANGPT1	GCAACCAGCGCC GAAAT	GGCACATTGCCCAT GTTGA	CAGAAAACGGAGGG AGAAGATATAACCG GA
ANGPT2	GACTTCCAGAGG ACGTGGAAAG	CTCATTGCCAGCC AGTACTC	ATACAAAGAGGGCT TCGGGAGCCCTCT
Collagen	CTTACCTACAGC ACCCTTGTG	GATGACTGTCTTGC CCCAAGTT	ACGGCTGCACGAGTC ACACCG
Desmin	TCCAAGCCGGAC CTCACA	TTAGCCGCGATGGT CTCATAC	TGCCCTCAGGGACAT CCGGG

Table 5: Rat probes and primers used. Table shows the sequence of forward and reverse primers used and the sequence of probes used

Gene	Forward Primer	Reverse Primer	Probe
Nephrin	ACGAGAAGCTC CACGGTTAG	CCTTCAGCACCTTG GTGATAC	TGGACCCAAATT ACTACTCCATGAG GGA
PAI-1	TTCCTCCACAGC CATTCTAGTCT	GAAAGGATCGGTC TAAAACCATCTC	AGCCCGCATGGC CCCCAC
E-cadherin	CACACTGATGG TGAGGGTACA	CTGGGCTTCAGGA ACACATA	TGTTTCCTAGCTG GAATCCTGTCCAT G
SNAIL-1	GCCGGAAGCCC AACTATAGC	AGGGCTCGTGGAA GGTGAA	AGCTGCAGGACG CGTGTGTGGA
ANGPT1	AGATACAACAG AATGCGGTTCA AA	TGAGACAAGAGGC TGGTTCCTAT	CCACACGGCCAC CATGCTGG
ANGPT2	CTGCAGGATTC ACCTTACAGGA CTCA	CTTCCTGGTTGGCT GATGCT	AGGCACGGCGGG CAAAATCA
Beta-2 microglobulin	ACTCTGAAGGA GCCCAAACC	TCCAGATGATTCAG AGCTCCATAG	CACCTGGGACCG AGACATGTAATC AAGC
Acta2	CACGGCATCAT CACCAACCTG	CCACGCGAAGCTC GTTAT AGA	CGACATGGAAAA GATCTGGCACCA CTC
Collagen	CCCAGCCAAGA ATGCATA CA	TCAAACCTGGCTGCC AT	TGCCAGGCCAA CAAGCATGTC

3.9 Electron Microscopy

Kidney samples for electron microscopy were taken from formalin-fixed, paraffin-embedded blocks, then dehydrated and embedded in Spurr's epoxy resin. Sections were stained with uranyl acetate, followed by lead citrate, and viewed with a Jeol 1200 electron microscope (Jeol, Kanata, ON) at Electron microscopy facility, McMaster University.

Twenty glomerular electron micrographs were taken at random for each sample, and podocyte foot process density was measured using Northern Eclipse image processing software (Empix Imaging). The podocyte FP effacement was quantified by counting the number of FP along a measured strip of GBM.

3.10 Statistics

Data are presented as mean \pm SEM. Groups were compared using an analysis of variance with Tukey's post hoc test. Data were analyzed using SPSS version 17.0 software (SPSS, Chicago, IL). $P < 0.05$ was considered significant.

CHAPTER 4 RESULTS AdTGFB1 and AdLacZ

4.1 Animal Model and Transgene Expression

AdTGFB1 was slowly perfused into the left kidneys of Sprague-Dawley rats via an intra-arterial approach with AdGFP used as control. Seven days after transfection, GFP expression was observed in the glomeruli and vasculature of the perfused left kidney (Figure 4.1). We did not observe any GFP expression in the right kidney, spleen, or lung, but it was expressed in the liver. Twenty eight days after infection, we found decreased but persisting expression of GFP in the left kidney. We used dual Immunofluorescence to assess cellular localization of adenovirus delivered GFP (Figure 4.1). Seven days after gene transfer, there was significant expression localized to CD34 positive endothelial cells of the glomeruli (Figure 4.1A-C). There was no co-localization with synaptopodin positive podocytes (Figure 4.1D-F). In the interstitium, we observed expression in the vascular structures that co-localized with Acta2 positive perivascular (Figure 4.1G-I) and CD34 positive endothelial cells (Figure 4.1J-L).

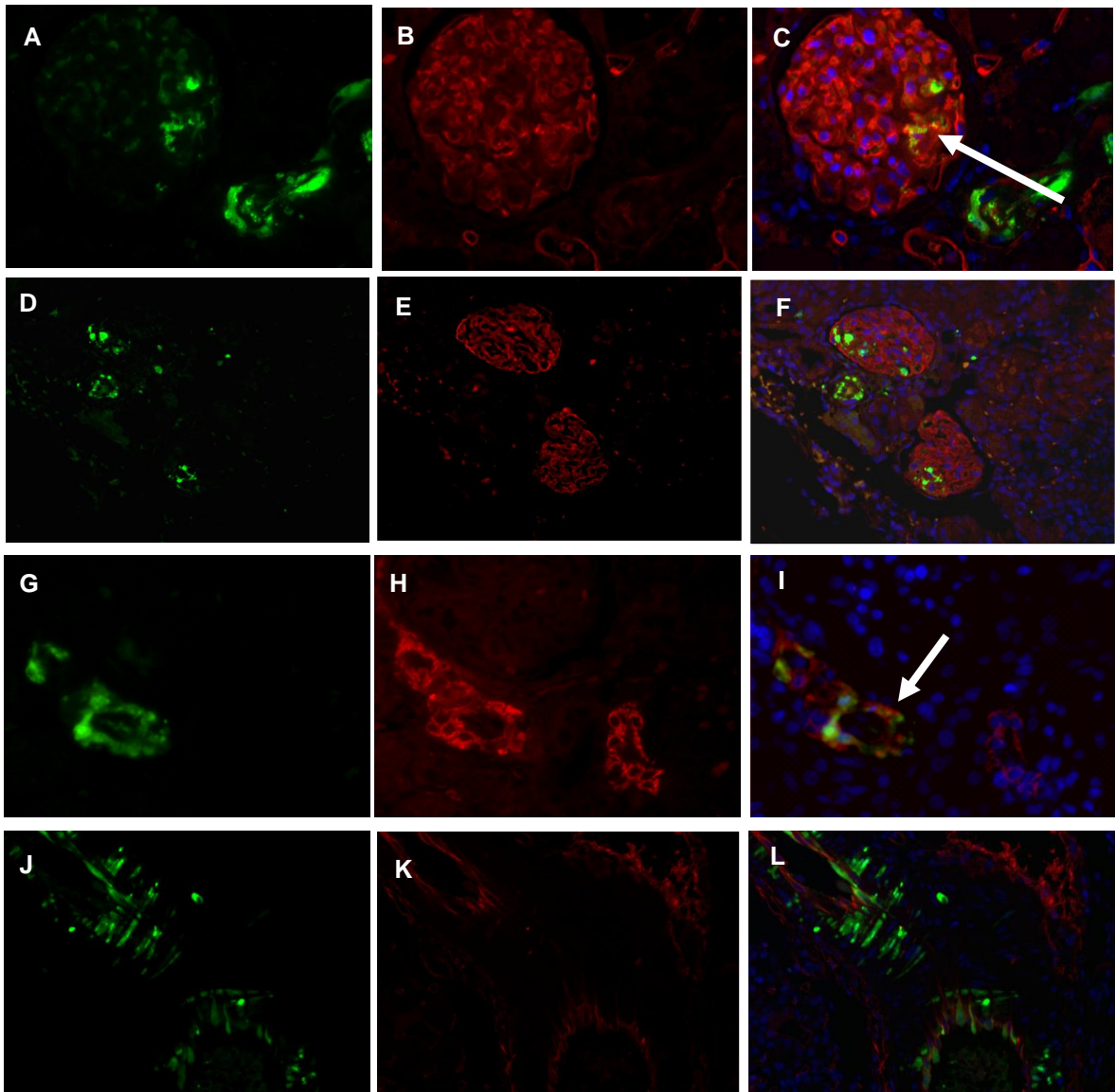


Figure 4-1: Localization of adenovirus-mediated transgene expression 7 days after GFP gene transfer. A–C: In the glomerulus, dual immunofluorescence for GFP (green) and CD34 (red) demonstrates endothelial colocalization (arrows). D–F: Dual immunofluorescent staining for GFP (green) and synaptopodin (red) shows increased glomerular GFP staining with no overlap with synaptopodin staining. G–I: In the interstitium, dual immunofluorescence for GFP (green) and alpha SMA (red) demonstrates colocalization (arrow in I). J–L: GFP and CD34 dual staining demonstrates some endothelial uptake in the interstitial arterioles (arrow in L). Original magnification: X400 (A–C and G–L); X100 (D–F).

We confirmed that expression was limited to the left kidney using whole tissue homogenate and examining for GFP expression (Figure 4.2).

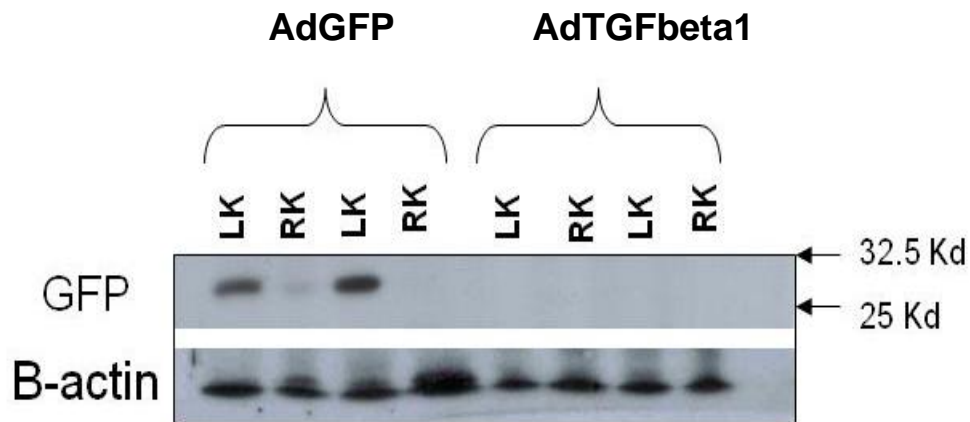


Figure 4.2: In AdGFP treated rats the transgene expression was determined by western Immunoblotting. (A) GFP expression measured by Western blot of whole kidney homogenate in animals treated with AdGFP or AdTGFB1. GFP expression was evident in the left kidney (LK) of AdGFP treated animals with no expression seen in the right kidney (RK) or in animals treated with AdTGFB1. Day 7 samples were used. This correlates well with the staining done for GFP which shows only AdGFP treated left kidney expresses the control transgene

We measured the urinary concentration of TGFB1 using ELISA (Figure 4.2B, C). Active TGFB1 concentration was transiently increased in the urine of AdTGFB1 treated animals. Total urinary TGFB1 was increased significantly 4 days after infection, returning back to baseline at 14 days after infection (Figure 4.2C).

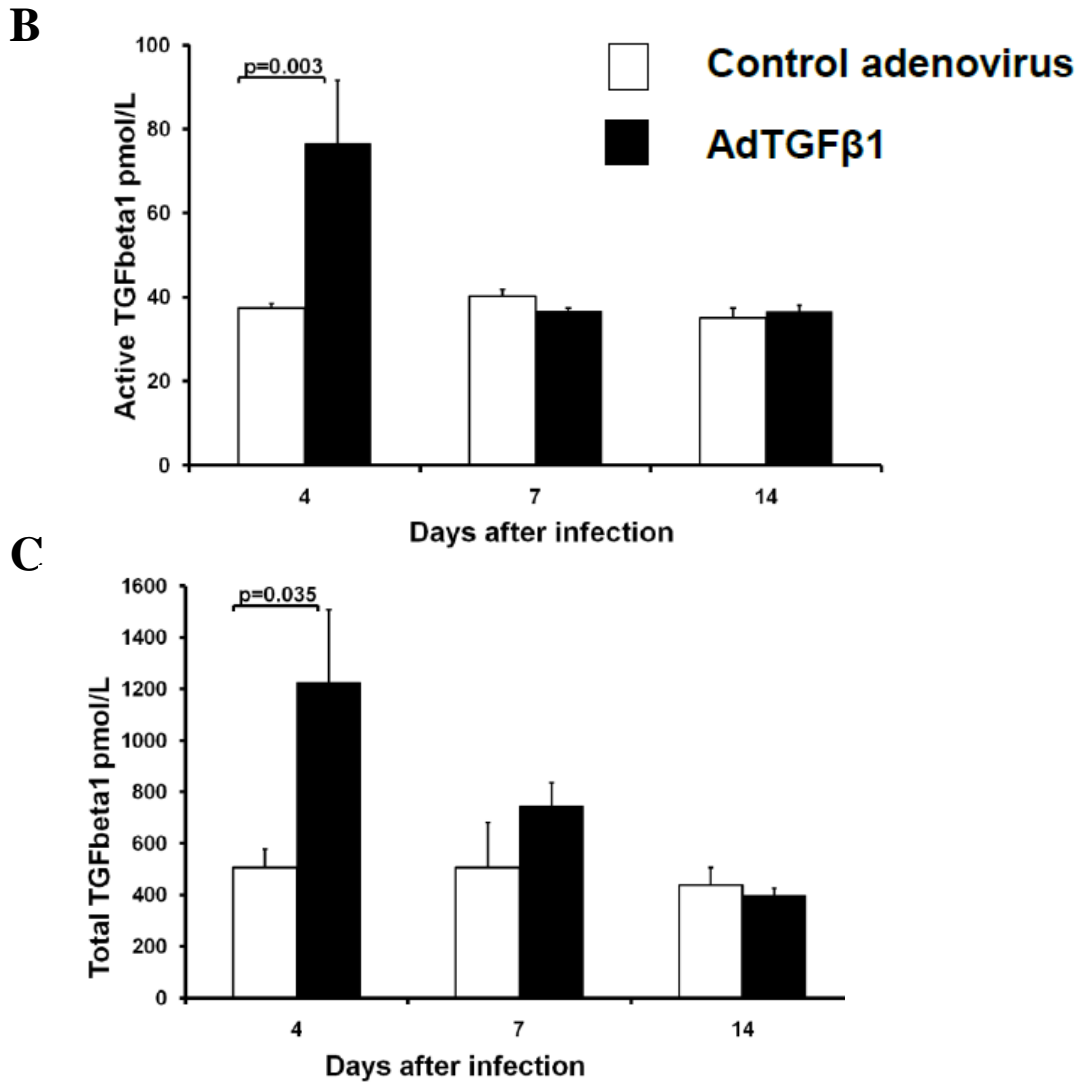


Figure 4-2: urine from AdTGFβ1 treated animals was collected to determine Active (B) and total (C) TGFβ1 by ELISA. There was a significant, transient TGFβ1 excretion in the urine of AdTGFβ1 treated rats.

There was also an abundant uptake of AdLacZ by the liver as shown in (Fig 4.3A and B). We confirmed the extent of expression in the adenovirus infected kidneys in a small group of animals treated with AdLacZ. β-galactosidase expression was found in approximately 75% of glomeruli (Figure 4.3D) of the left kidney with no expression seen

in the right kidney (Figure 4.3E). (Figure 4.3F) is a higher power magnification of β -galactosidase staining in the glomeruli. The perivascular cells are shown in (Figure 4.3G).

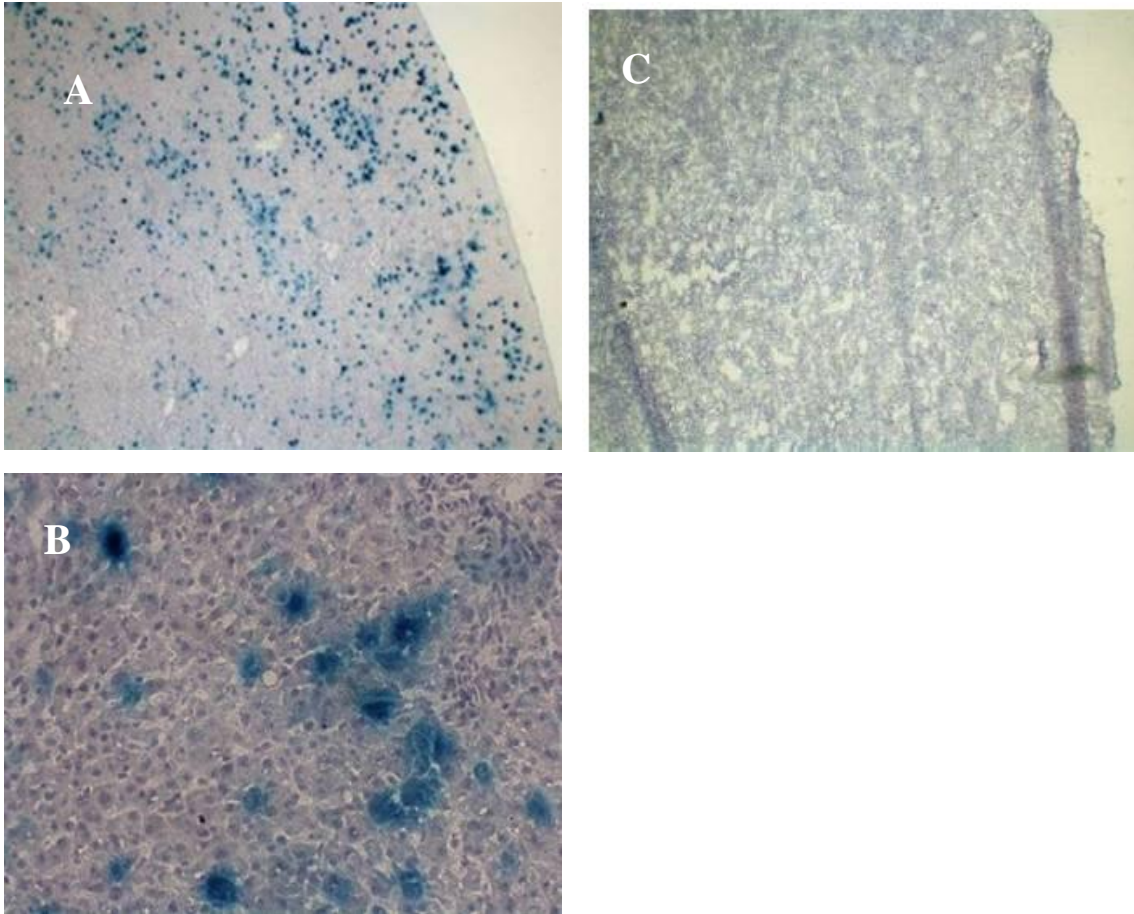


Figure 4-3: Liver sections from AdTGFB1 treated animals were stained for GFP. **A.** Liver, day 7, AdLacZ treated animals, shows abundant uptake of AdLacZ by the liver. Fig B shows staining at a higher magnification. C is the liver of AdTGFB1 treated rat serving as a negative control showing no staining for Lac-Z.

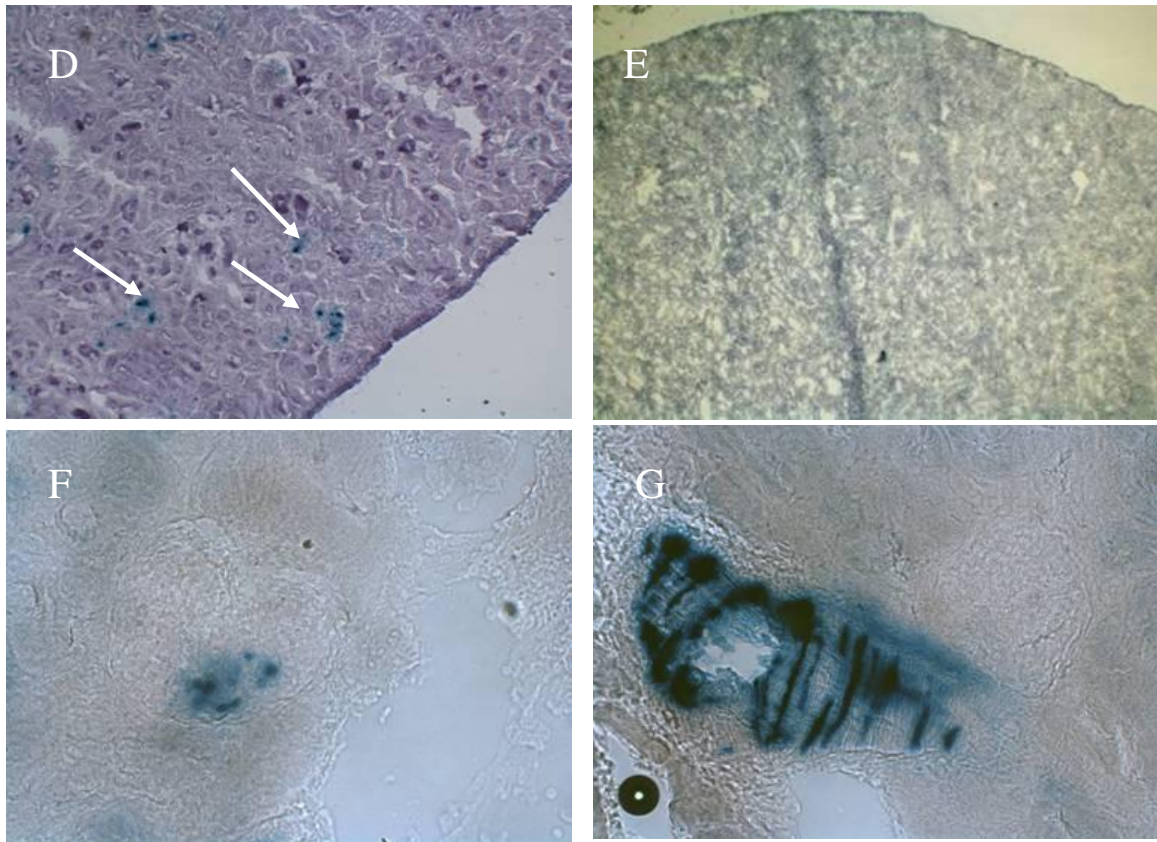


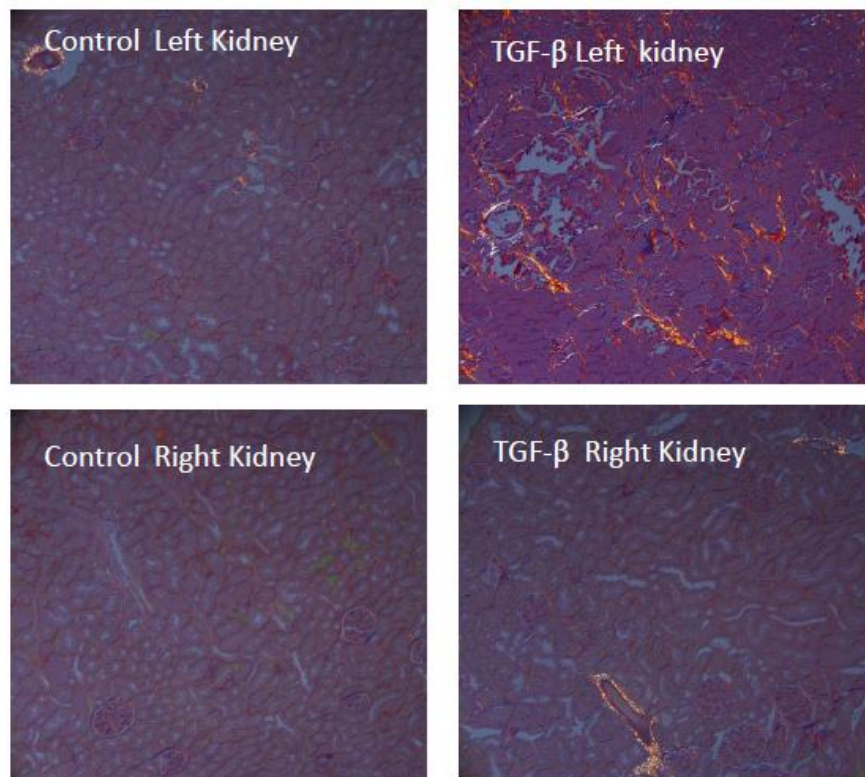
Figure 4.3: Animals were treated with an adenovirus expressing β -galactosidase (AdLacZ). Lac-Z staining (blue) shows successful uptake of Lac-Z gene into multiple glomeruli (D) and higher magnification of glomeruli shows uptake of Lac-Z staining in (F). The perivascular cells are shown in (G). Figure 4.3E is right kidney section from AdLac-Z treated animals and there is no glomerular or perivascular staining in these sections.

4.2 Picrosirius Red

There was an increase in renal fibrosis in the left kidneys of TGFB1-treated animals as demonstrated by Picrosirius red staining (Figure 4.4A and B). AdTGFB1 treated left kidney was compared to the right kidney of the same mice and to the left and right kidneys control treated mice. There was no fibrosis seen in the right kidney of

AdTGFB1 treated animals, or in either kidney of control adenovirus treated animals. Only AdTGFB1 treated left kidneys exhibited extensive staining with PSR and was also significant when quantified (Figure 4.4B).

A



B

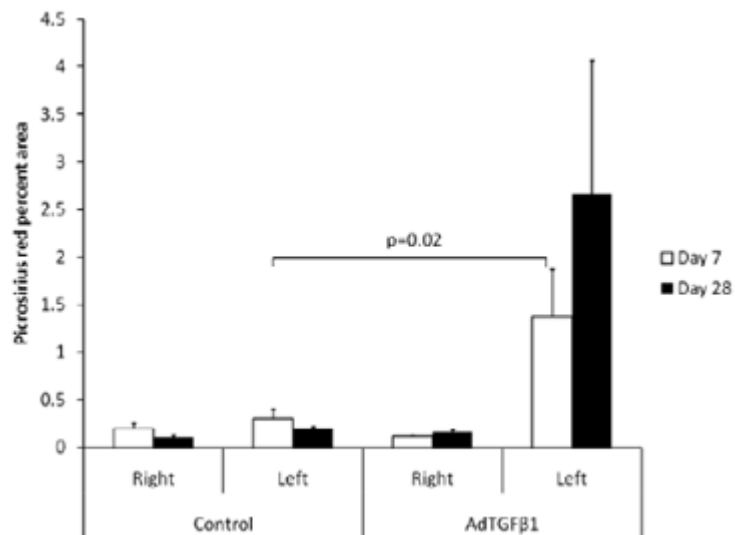


Figure 4.4: Picrosirius red staining of renal tissue demonstrates a significant increase in interstitial fibrosis in the left kidney of animals treated with AdTGFB1. Birefringence was quantified and results shown in B.

4.3 Renal Histology

The control adenovirus treated kidneys displayed no histological changes at day 7 or 28 after treatment (Figure 4.5A). Acta2 expression was confined to the vascular smooth muscle cells of the renal interstitium (Figure 4.5D). AdTGFB1 induced a mild interstitial fibrosis manifest by collagen accumulation and increased Acta2 expression (Figure 4.5B, C and E). On light microscopy, there were no changes identified in the glomeruli in either control adenovirus or AdTGFB1 treated kidneys (Figure 4.5F, G). Furthermore, the right untreated kidneys of both AdGFP and AdTGFB1 treated animals had normal histology (not shown).

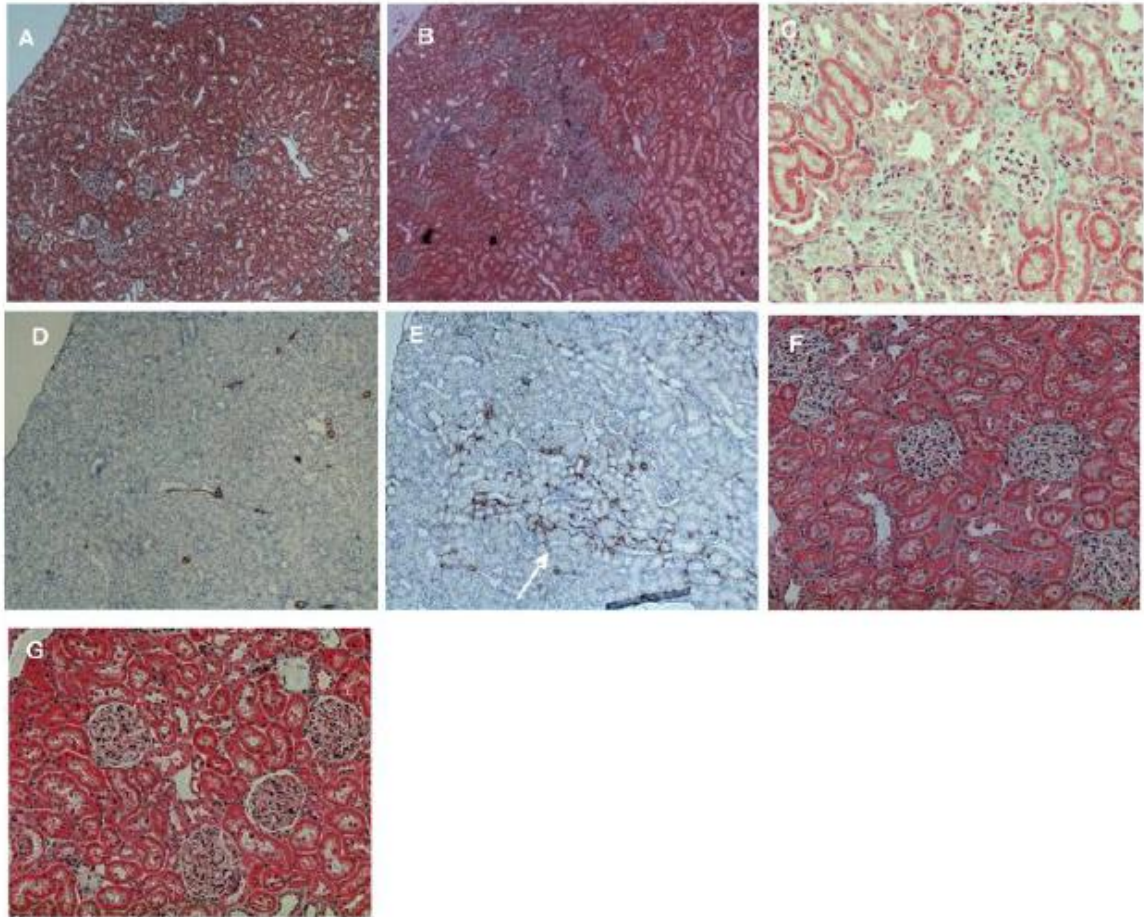


Figure 4.5: Renal histology after adenoviral infection. A, B) Masson's trichrome staining of left kidney 7 days after treatment with (A) AdGFP treatment or B) AdTGFB1. After AdGFP, the kidney reveals normal histology. B) Patchy interstitial fibrosis is observed 7 days after AdTGFB1 treatment in the left kidney. C) Higher power view of interstitial fibrosis. D, E) Acta2 staining of AdGFP treated left kidney on day 7 (D) shows perivascular Acta2 staining only. Seven days after AdTGFB1 treatment (E), the left kidney demonstrated patchy interstitial Acta2 expression (arrow). F, G) Masson's trichrome of glomeruli from AdGFP (F) and AdTGFB1 (G) treated animals' reveals normal histology. A, B, D, E: 50x magnification, C: 400x, E, F: 200x magnification. The right kidneys (untreated) of both AdGFP and AdTGFB1 treated animals demonstrated normal histology (not shown).

4.4 Proteinuria

Animals treated with AdTGFB1 demonstrated an increase in protein excretion (Figure 4.6) as assessed by a timed urine protein to creatinine ratio. Progressive rise in urine protein excretion in TGFB1 treated animals was obvious starting from as early as

day 4 and up to day 28, as measured by total urine protein. There was a significant ($P < 0.04$) rise in urine protein excretion in animals treated with TGFB1 compared to controls. This indicates the progression of renal dysfunction with time. Urine electrophoresis showed primarily 65 kDa protein in AdTGFB1 treated animals, suggesting predominant albuminuria.

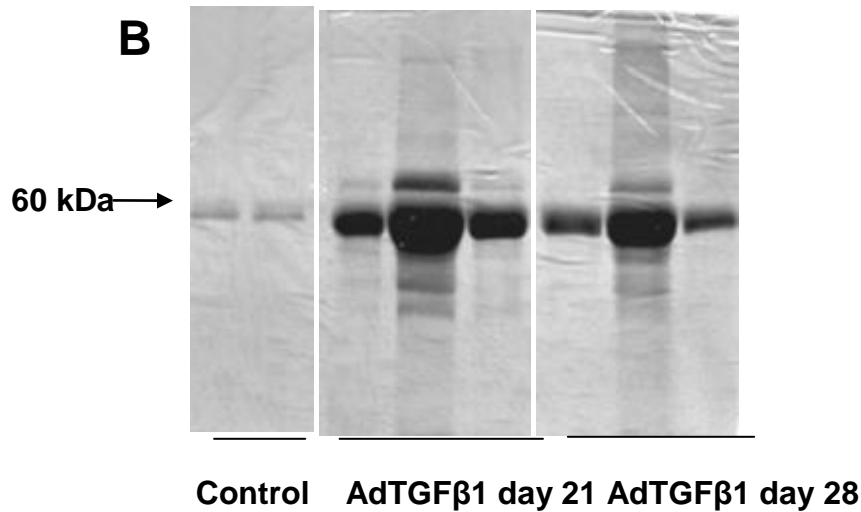
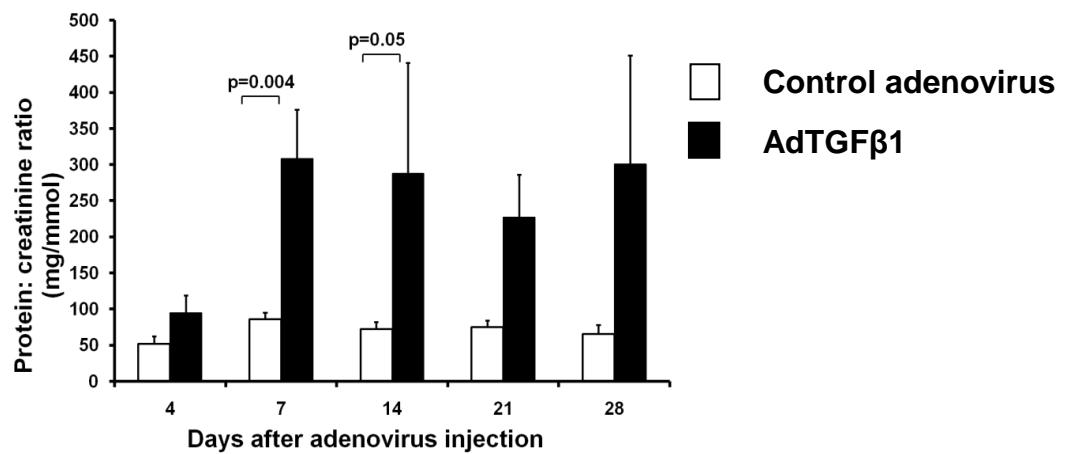
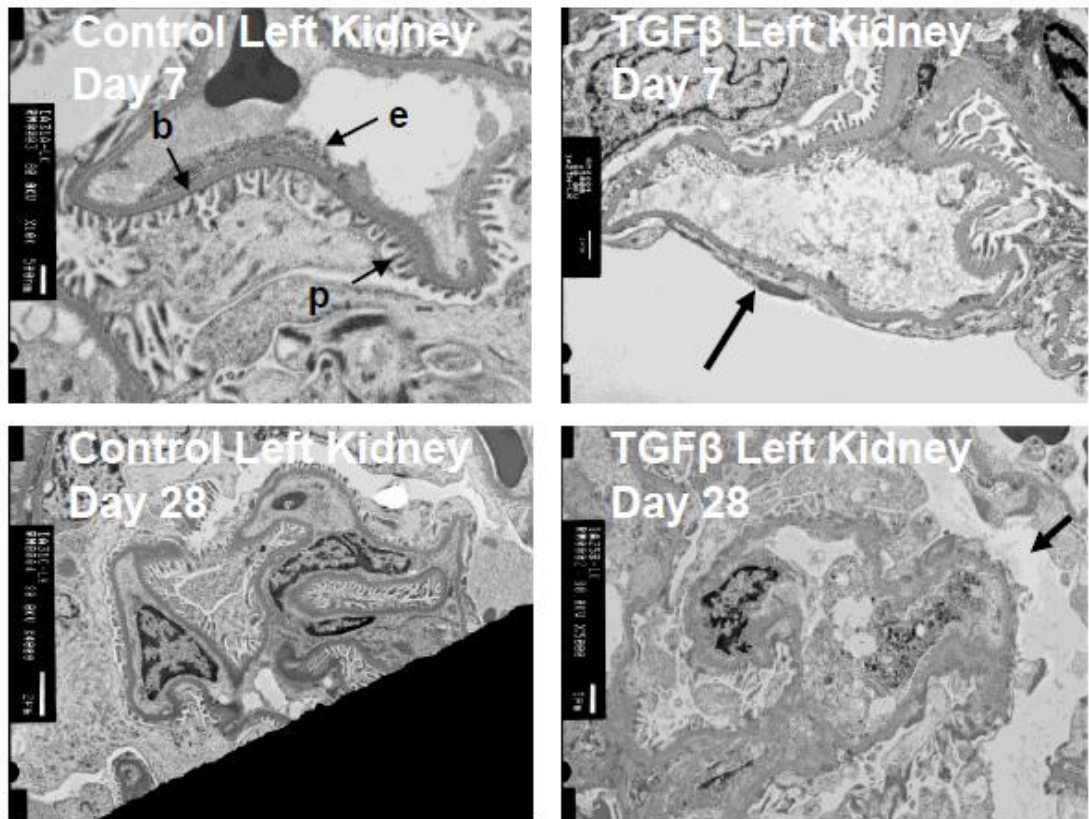


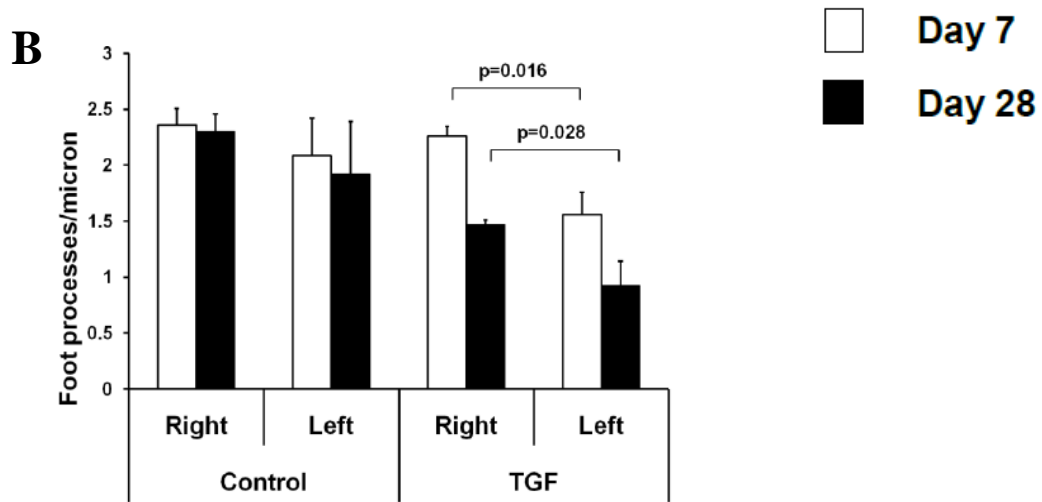
Figure 4.6: Timed urine collected demonstrates significantly increased proteinuria in rats exposed to AdTGFB1. B: Coomassie stained SDS-PAGE gel of equal volumes of urine demonstrates a predominance of albumin.

4.5 Podocyte Effacement

This increased protein excretion was associated with changes in the podocyte phenotype. Normal glomerular structure is shown in the control kidney (Figure 4.7) with the filtration barrier composed of the endothelium (e), basement membrane (b), and podocytes with foot processes visible (p). On electron microscopy, there was significant podocyte effacement (Figure 4.7A). This was quantified (Figure 4.7B) and there was significant decrease in the number of podocyte foot process in the left versus the right kidneys of AdTGF β 1 treated animals. We did not see any evidence of apoptosis of podocytes or endothelial cells on low power EM sections (Figure 4.7C).

A





C

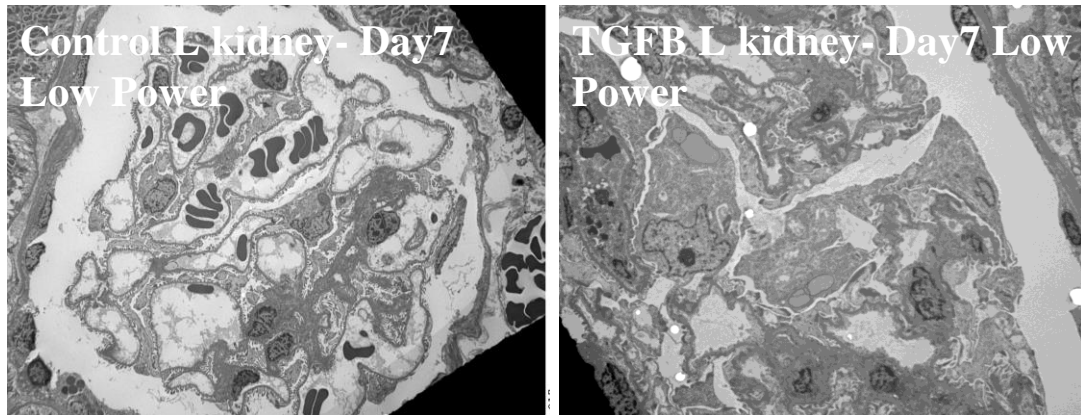


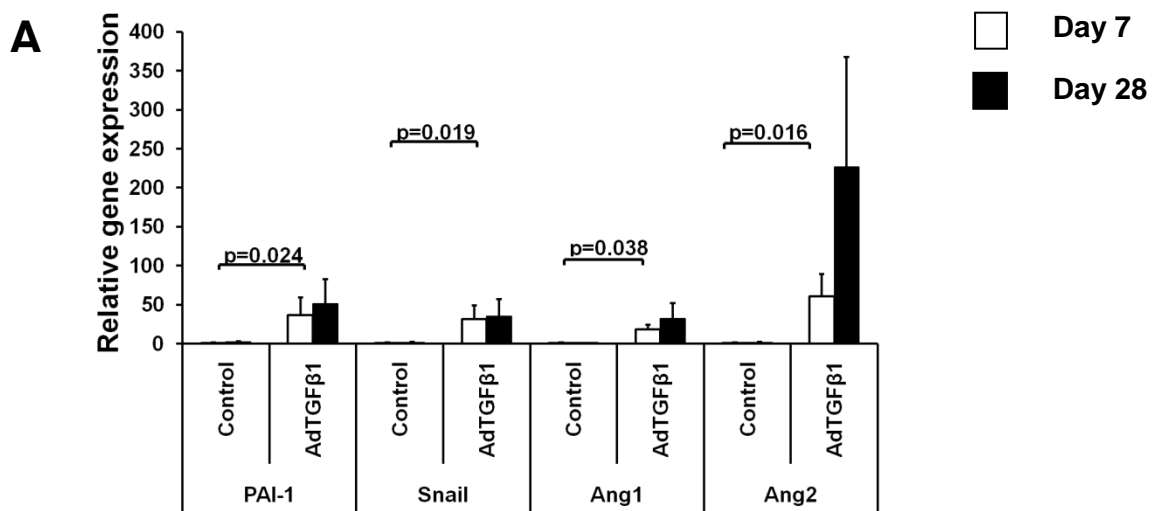
Figure 4.7: AdTGFB1 infection leads to foot process effacement (A) 7 and 28 days after treatment (arrows). Original magnification: x200 (low in C); x10,000 (high in A).

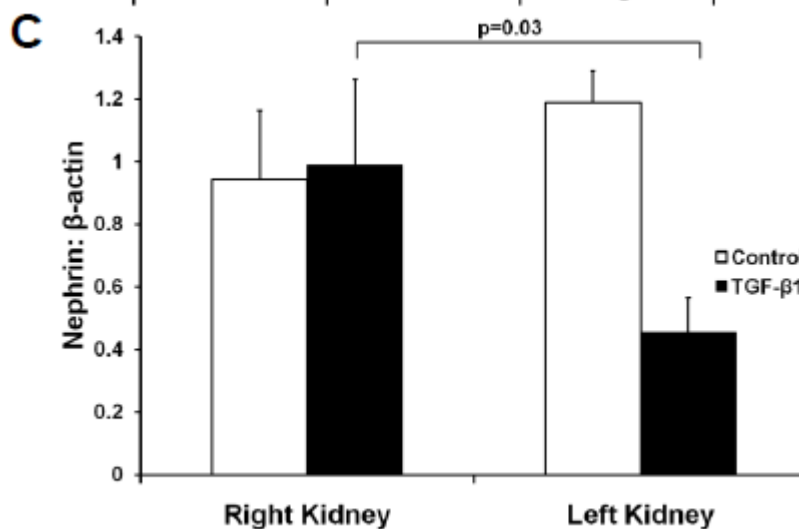
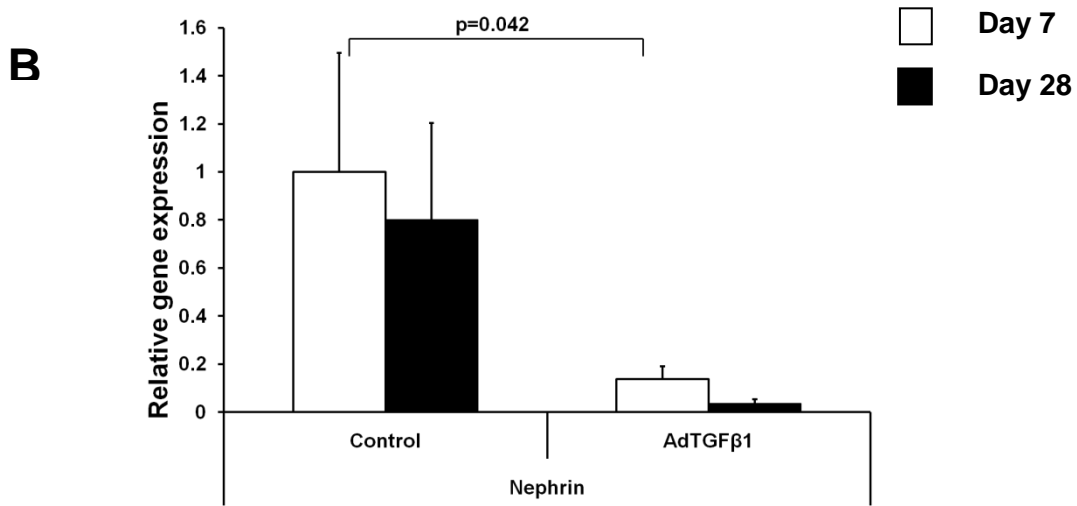
Number of podocyte foot processes was quantified and showed significant decrease in the AdTGFB1 treated kidneys compared with the untreated, contralateral kidney and kidneys from control-treated animals (B)

4.6 Glomerular Gene and Protein Expression

Glomeruli were isolated from renal tissue using laser capture microdissection. There was a significant increase in gene expression of the TGFB1 responsive cytokine plasminogen activator inhibitor (PAI)-1 seven days after AdTGFB1 compared to control adenovirus kidneys (Figure 4.8A). There was also a significant induction of the EMT associated regulatory protein Snail. Angiogenic cytokines ANGPT1 and ANGPT2 were also upregulated. There was a concurrent decrease in gene expression of the epithelial slit diaphragm protein nephrin (Figure 4.8B) in kidneys exposed to AdTGFB1.

From whole tissue homogenates, we found that nephrin protein expression was significantly decreased in the left kidney of AdTGFB1 treated animals after 28 days (Figure 4.8C, D). Of interest, a significant increase in nephrin protein was observed in the urine of animals treated with AdTGFB1 (Figure 4.8E, F).





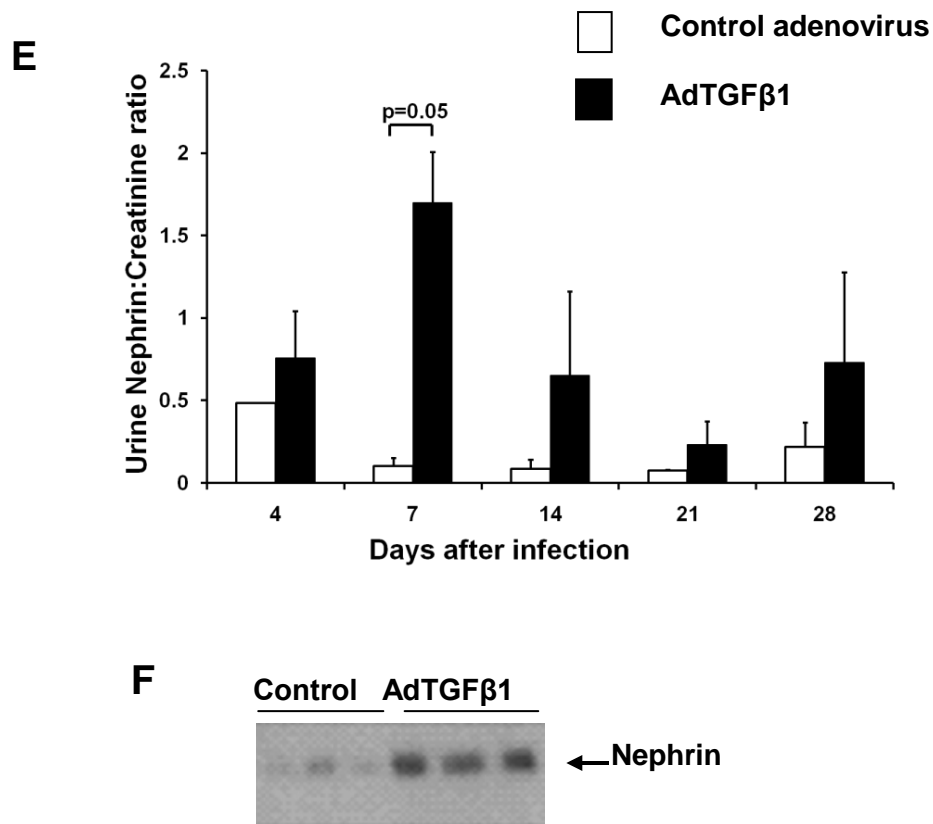


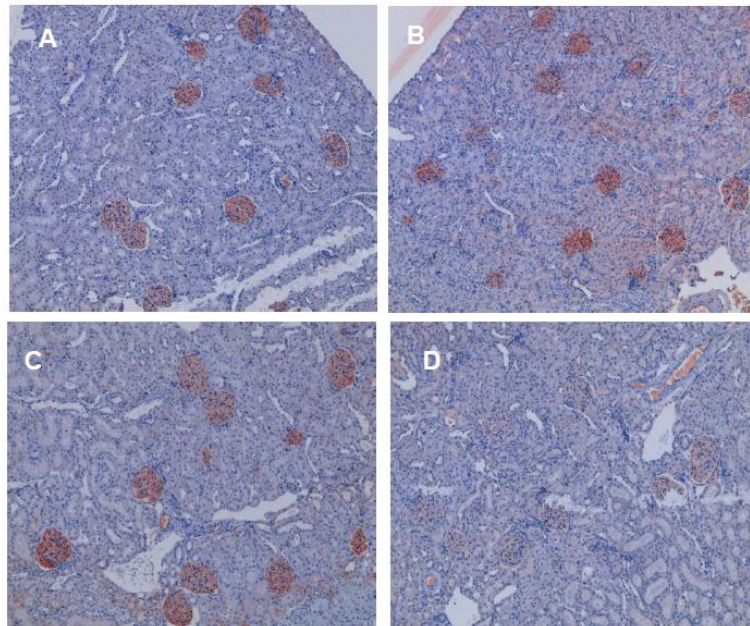
Figure 4.8: A and B: Laser-capture microdissection of glomerular tissue from left kidneys of animals treated with AdTGFβ1 or control adenovirus. A: There was a significant up-regulation of plasminogen activator inhibitor (PAI)-1, Snail, ANGPT1, and ANGPT2 at 7 days after adenovirus infection. B: There was a significant down-regulation of the gene expression of the podocyte adhesion molecule nephrin.

C and D: Western blot analysis of whole kidney homogenate 7 days after infection with control adenovirus or AdTGFβ1 demonstrates decreased nephrin expression only in the treated (left) kidneys. D: Representative blot (L, left kidney; R, right kidney)

E and F: Nephtrin is significantly increased in the urine of rats treated with AdTGFβ1. Nephtrin was measured in the urine by using Western blot analysis (F), and the relative band density from the Western blot analysis was normalized to urine creatinine.

4.7 Synaptopodin Immunohistochemistry

We assessed the expression of the podocyte associated protein synaptopodin using immunohistochemistry (Figure 4.9). There was a significant decrease in synaptopodin expression the left kidney of animals treated with AdTGFB1. This change was not seen in the right kidney of these animals or in the glomeruli of control animals. This downregulation was also found in whole kidney tissue homogenates run on a western blot and probed for synaptopodin. (Figure 4.9E, F).



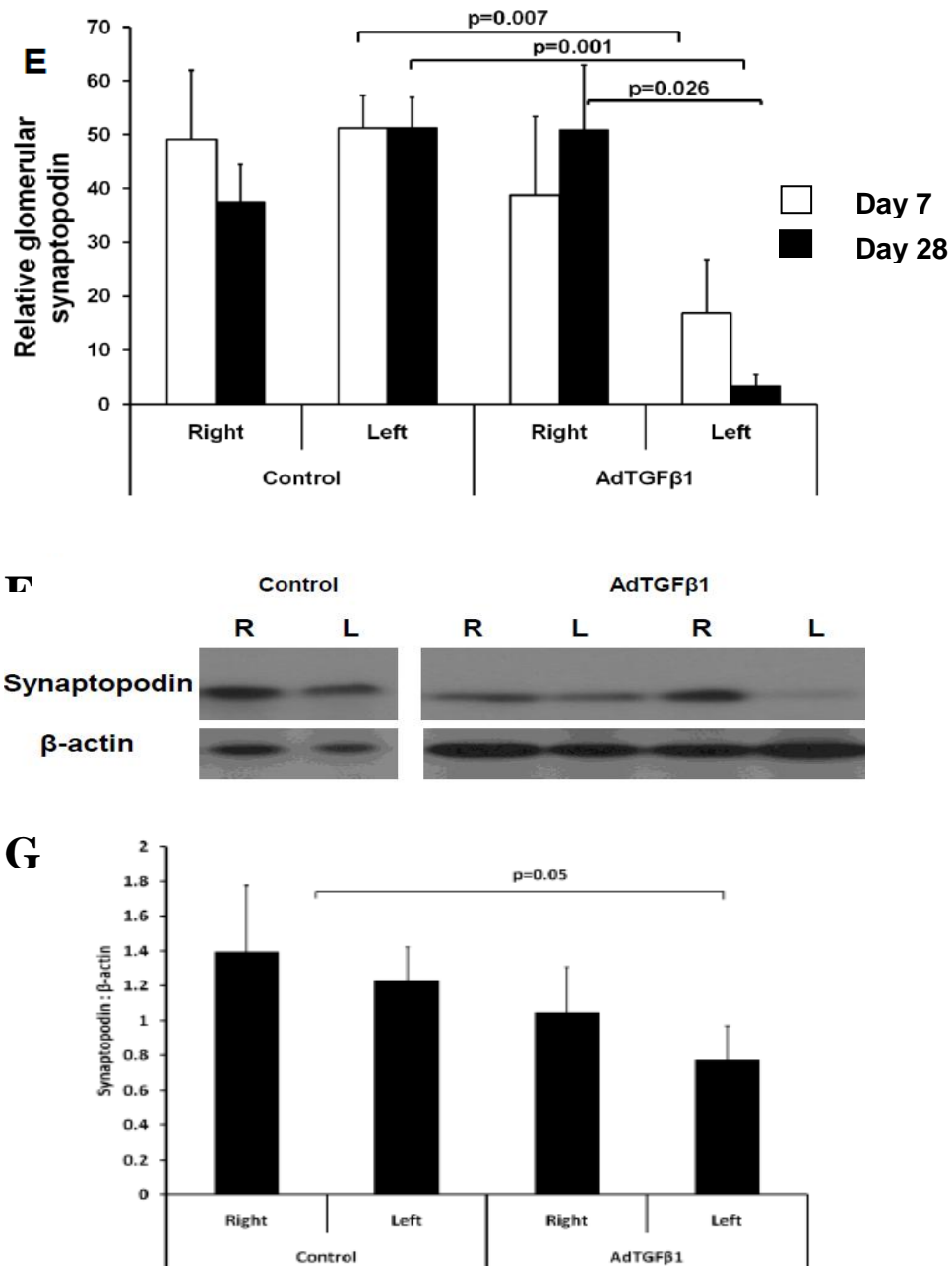


Figure 4.9: Immunohistochemistry for kidney sections for synaptopodin. Right (A) and left (B) kidneys from control adenovirus-treated animals at day 7. Right (C) and left (D) kidneys from AdTGFβ1 treated animals. Glomerular staining density was quantified (E) and demonstrated significantly decreased staining in glomeruli of the left kidney of AdTGFβ1 treated animals. Original magnification is x 50. F: Synaptopodin protein was assessed from whole kidney homogenates using Western blot analysis and quantified in G. AdTGFβ1 treated kidneys demonstrated a significant down-regulation of synaptopodin protein. L, left kidney; R, right kidney.

4.8 Effects of TGFB1 on Podocin

We assessed the effects of TGFB1 on cultured podocytes. Podocytes treated with TGFB1 were probed with podocin (Figure 4.10). Podocin, a slit diaphragm protein, was significantly down-regulated by TGFB1. Untreated mouse cardiac endothelial cells (MCEC) were used as a positive control for Tie2 expression. Hence we confirmed the increase expression of Tie2 by TGFB1.

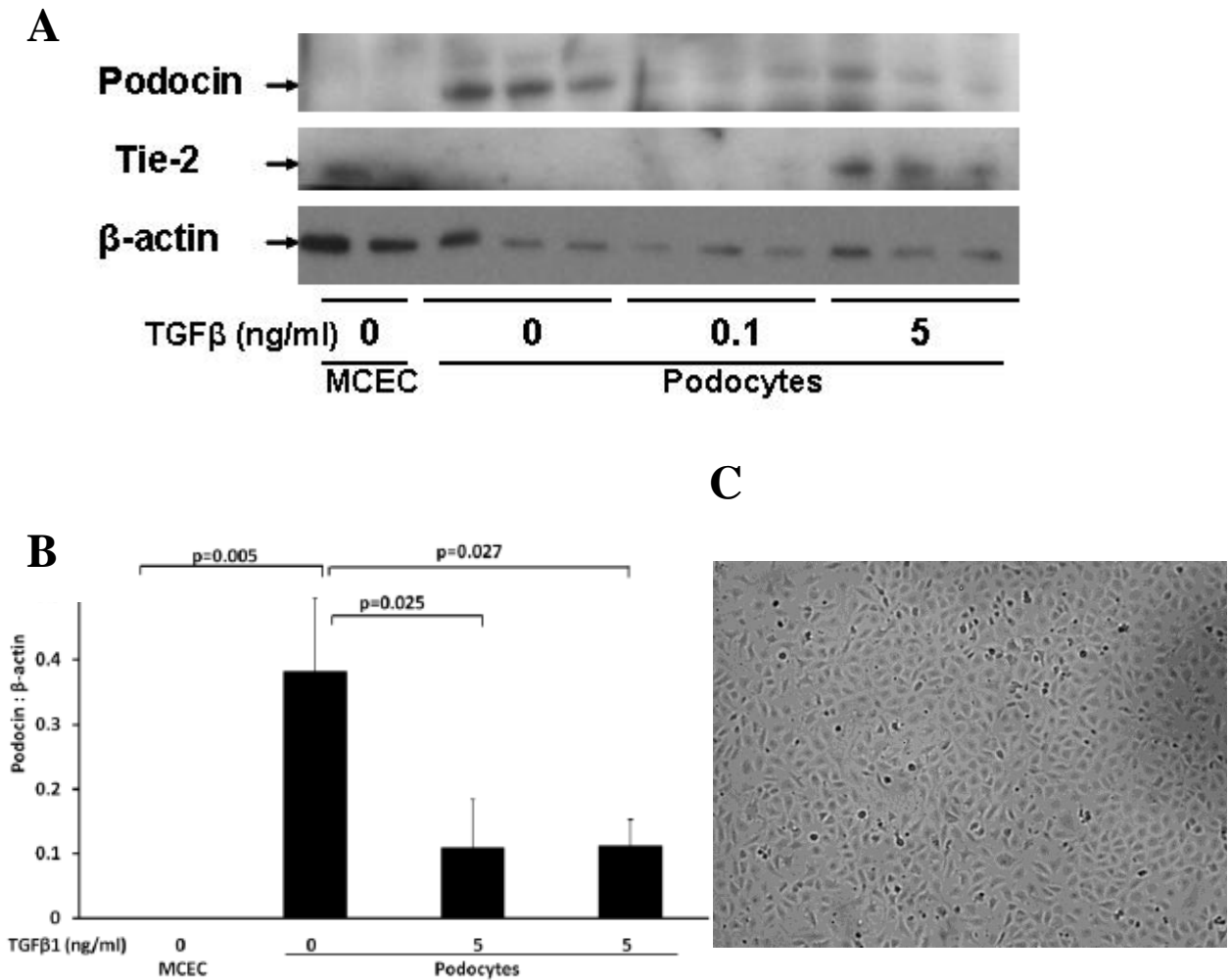
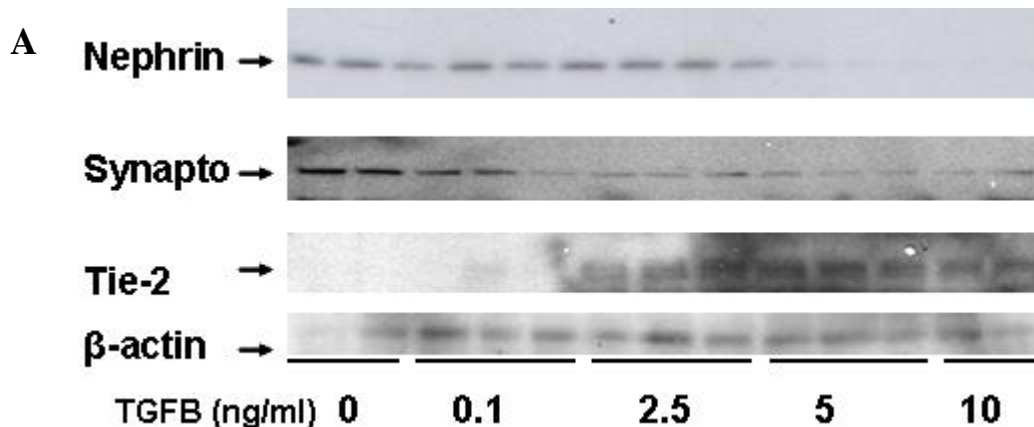
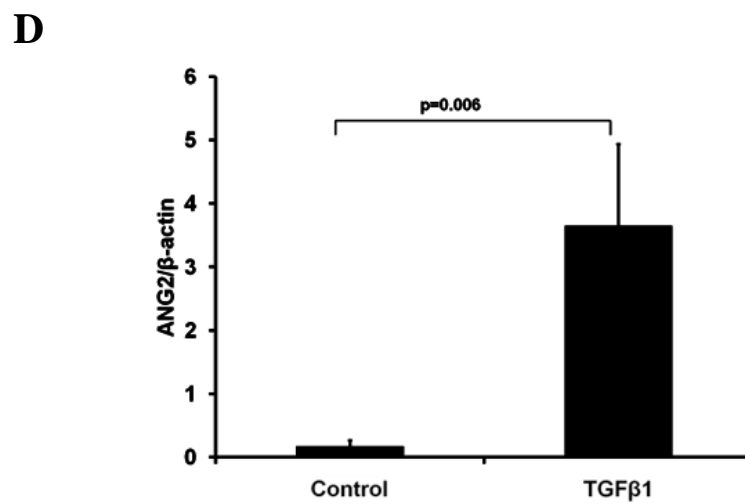
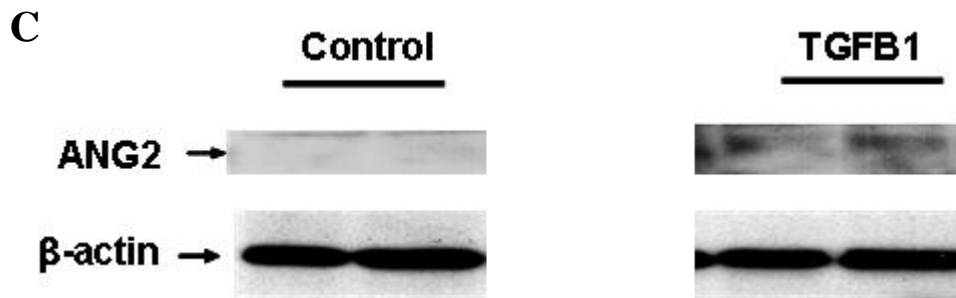
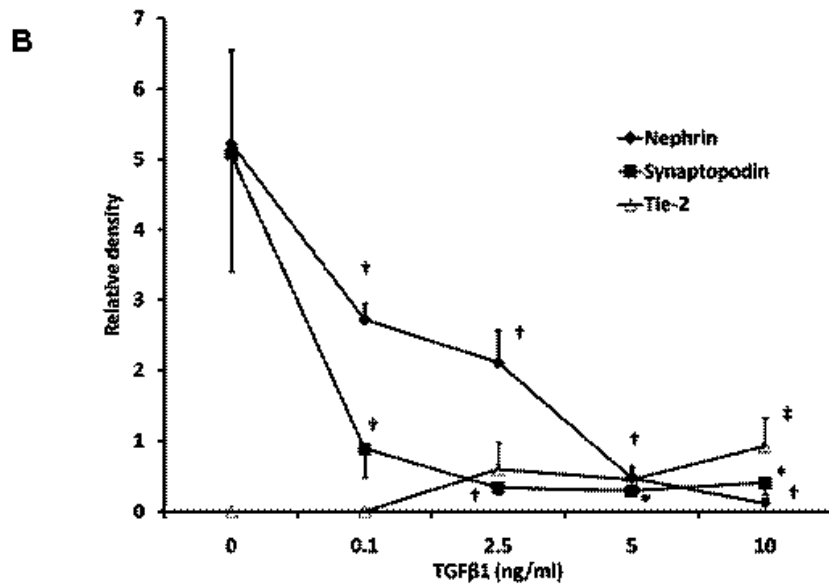


Figure 4.10: (A) Podocin protein was assessed from cultured podocytes using Western blot analysis and quantified in B. Untreated mouse cardiac endothelial cells (MCEC) shown in culture in C

4.9 Interaction between TGF β 1 and Angiopoietins

Because of our observations of angiogenic gene regulation after AdTGF β 1 gene transfer to the kidney, we hypothesized that angiopoietins may be involved in podocyte changes induced by TGF β 1. We found that recombinant TGF β 1 suppressed the expression of nephrin and synaptopodin in a dose dependent manner (Figure 4.11A, B). TGF β 1 also significantly increased the expression of the angiopoietin receptor Tie2 (Figure 4.11A, B). We demonstrated that the virus mainly infected CD34 positive endothelial cells (Figure 1A-C). We therefore attempted to mimic this in vitro by infecting MCEC with AdTGF β 1 and exposing podocytes to this conditioned media. We found that after infection with AdTGF β 1, MCEC conditioned media significantly downregulated nephrin expression in podocytes (Figure 4.11E, F). The effect on synaptopodin expression was not as obvious (Figure 4.11G).





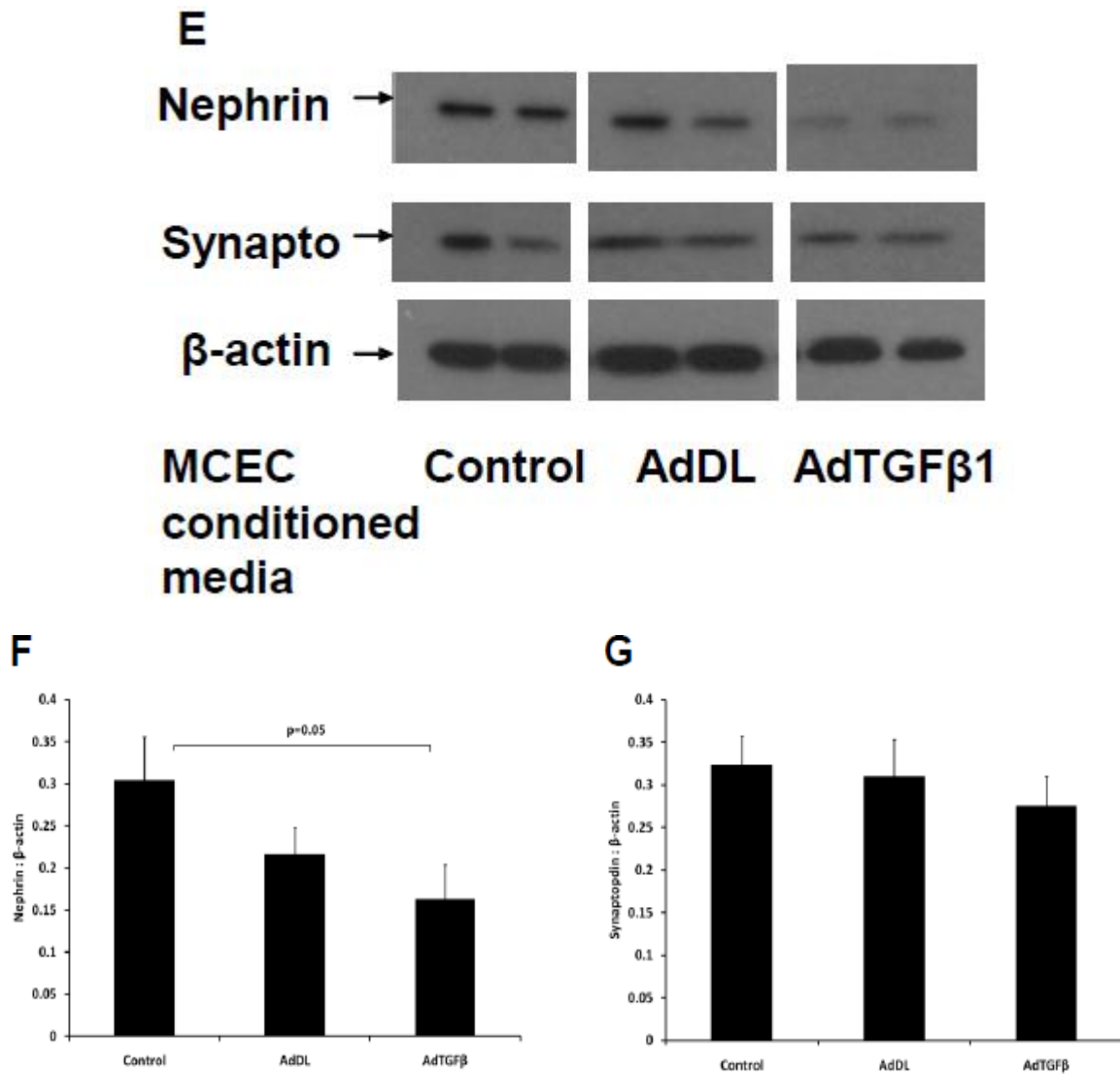
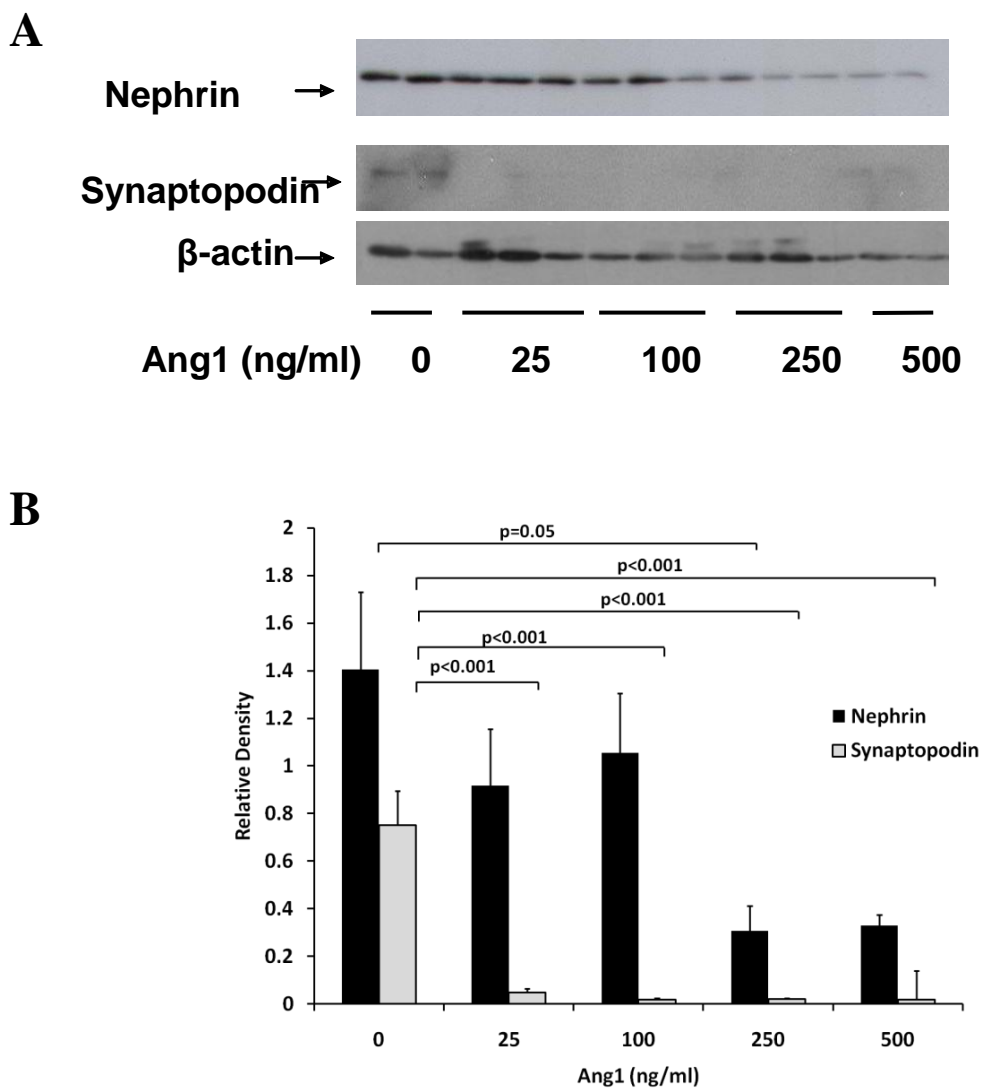


Figure 4.11: A: Immortalized podocytes were treated with rTGFβ1. B: TGFβ1 significantly inhibited nephrin and synaptopodin expression in a dose-response manner, while increasing the expression of the angiotensin receptor Tie2. *P < 0.001, †P < 0.01, and ‡P < 0.05 versus untreated cells. C and D: Treating podocytes with rTGFβ1 significantly increased ANGPT2 expression by podocytes. E: MCECs were grown to confluence and exposed to AdDL, AdTGFβ1, or no adenovirus (Control). Conditioned media were then added to podocyte culture. F: Nephrin expression was significantly down-regulated by MCEC media condition with AdTGFβ1. G: Synaptopodin expression was not significantly affected.

4.10 Effects of ANGPT1 and ANGPT2 on Glomerular Proteins in Cell Culture

In immortalized podocyte cell culture there is a dose dependent down regulation of both synaptopodin and nephrin protein expression by ANGPT1 and ANGPT2 (Figure 4.12). However, ANGPT2 was less potent in downregulating synaptopodin



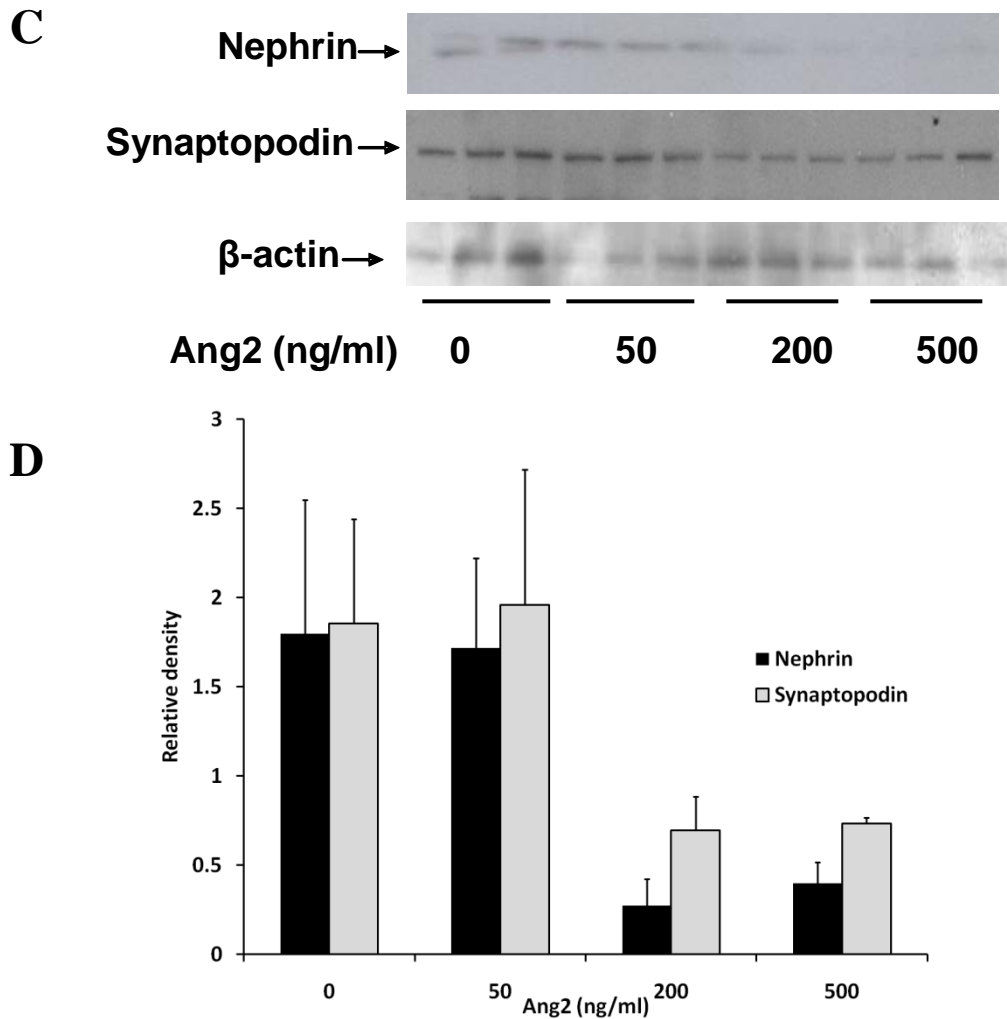


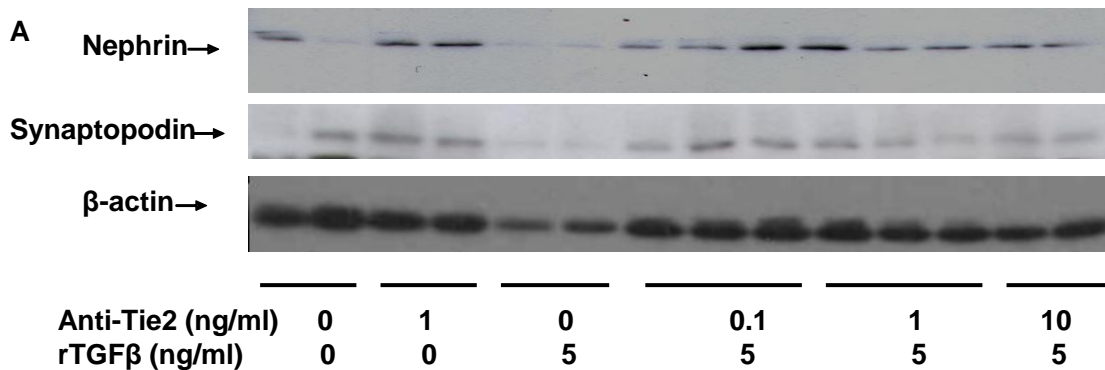
Figure 4.12: Effects of increasing dose of ANGPT1 on nephrin and synaptopodin protein expression (A). Western blot was quantified in (B). Synaptopodin is significantly down regulated starting at 25ng/ml. For Nephrin significant downregulation was seen at the dose of 250ng/ml. (C) Effects of ANGPT2 on nephrin and synaptopodin protein expression. (D) Western blot was quantified.

4.11 Effects of Blocking Tie-2 on TGFB1 Treated Podocytes

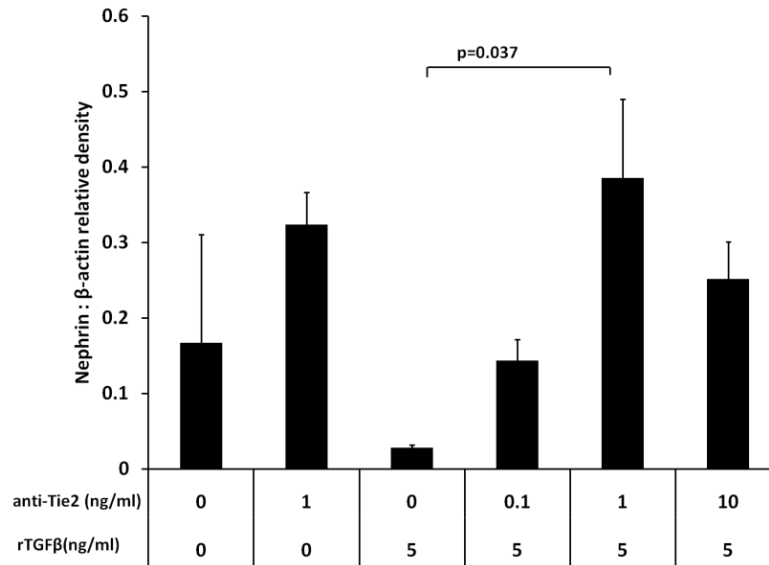
The TGFB1-induced downregulation of nephrin and synaptopodin could be reversed by an antibody against anti-Tie2 (Figure 4.13A-C). Podocytes incubated with a

standard dose of 5ng/ml were treated with Anti-Tie-2 antibody in increasing doses from 0-10ng/ml. Tie-2 blockage will block both ANGPT1 and ANGPT2 mediated effects on podocytes. At a dose of 1ng/ml, both synaptopodin and nephrin were significantly up regulated compared to the podocytes treated by TGFB1 only.

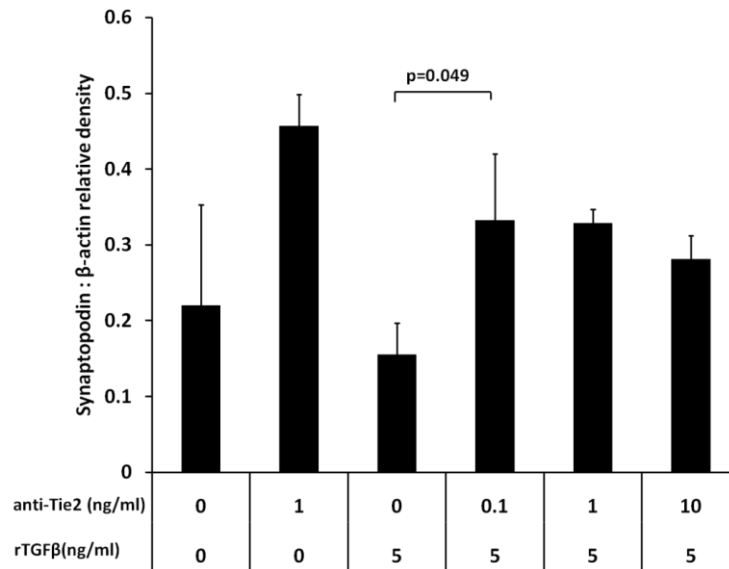
There was a dose response between 0 and 1 ng/ml of anti-Tie2 antibody, but no additional effect at 10ng/ml. Furthermore, we could block the effect of TGFB1 on podocytes using siRNA against Tie2 (Figure 4.13D). In this experiment, podocytes were transfected with siRNA against Tie2 or a control siRNA. TGFB1 significantly reduced nephrin and synaptopodin expression, and this effect was reversed with siRNA against Tie2 (Figure 4.13E). The non-specific siRNA had no effects.



B



C



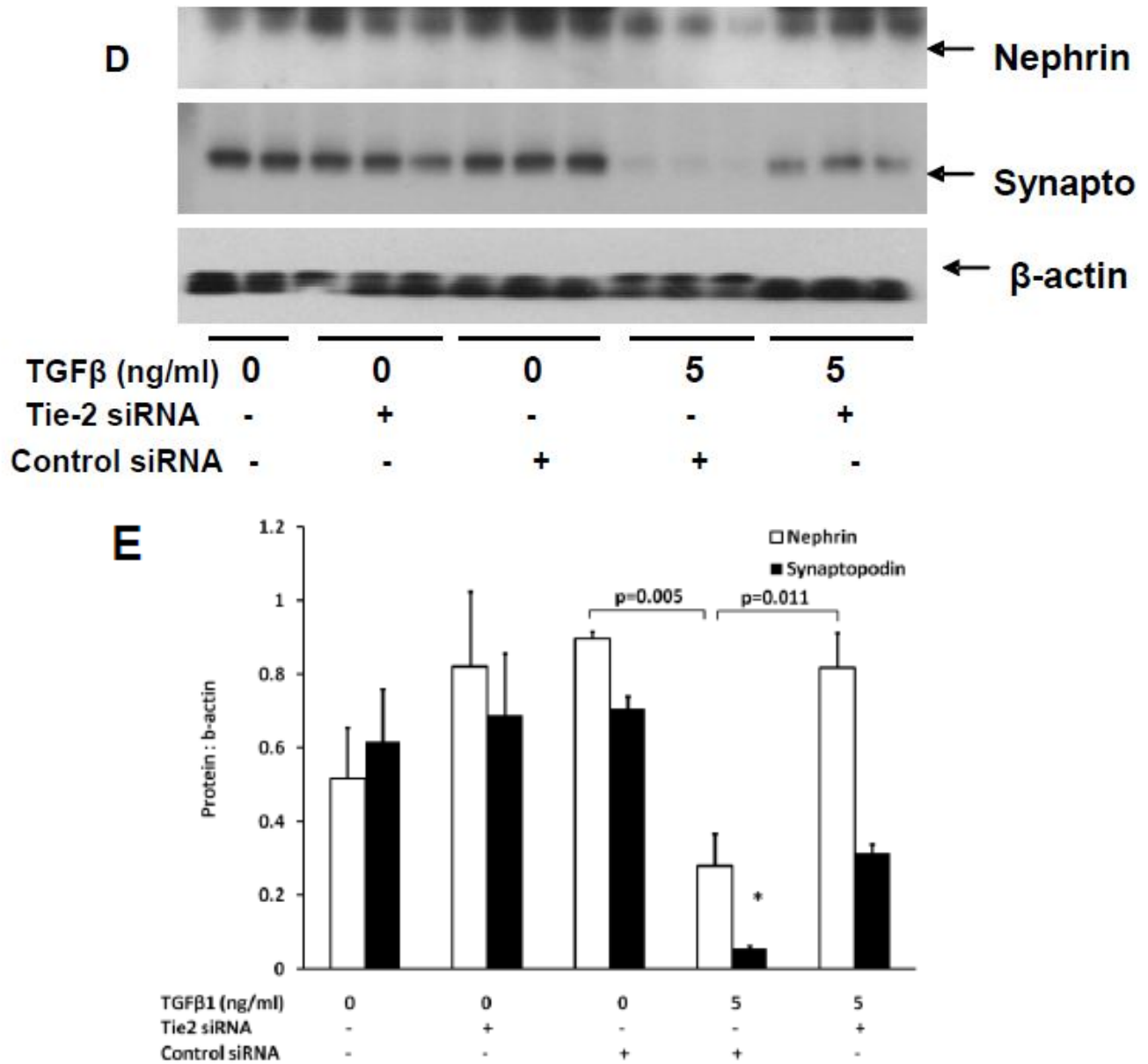
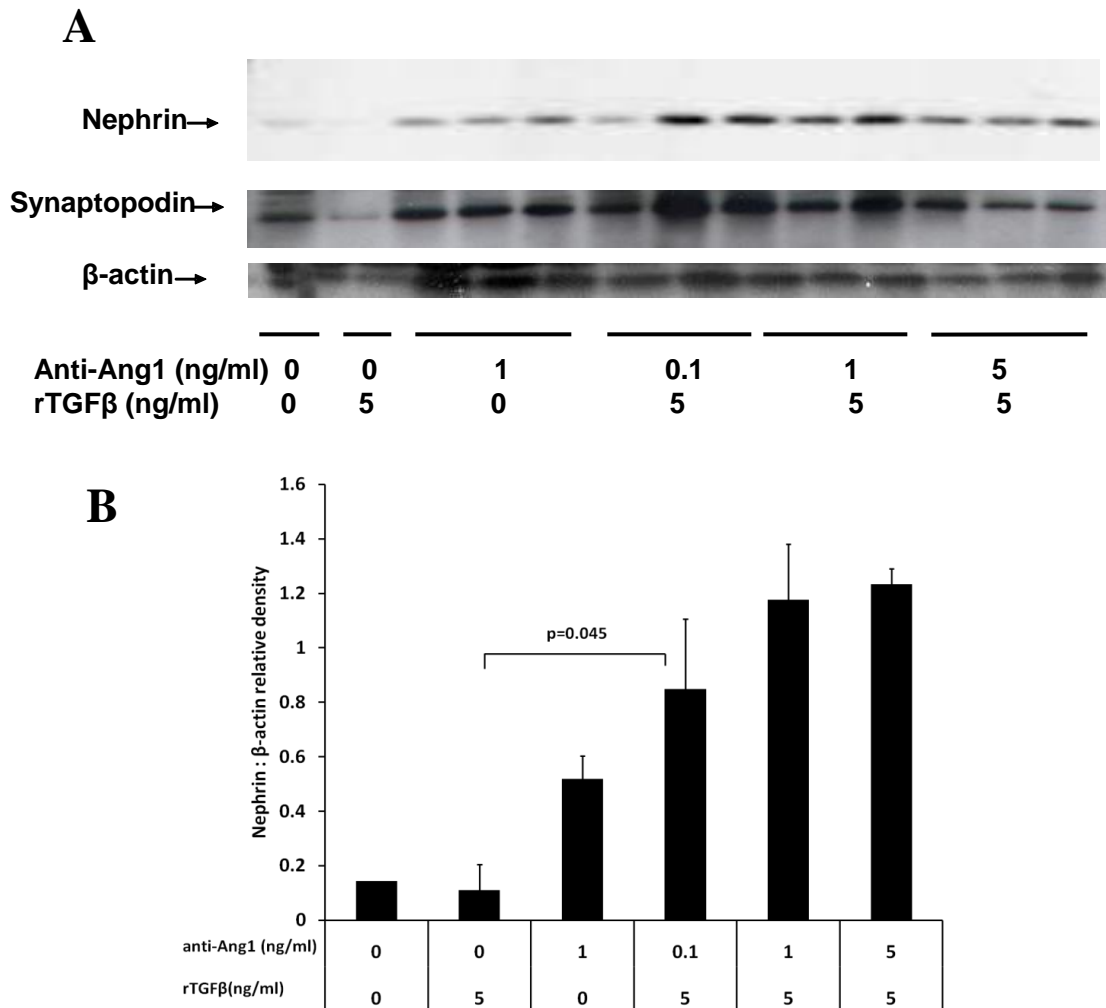


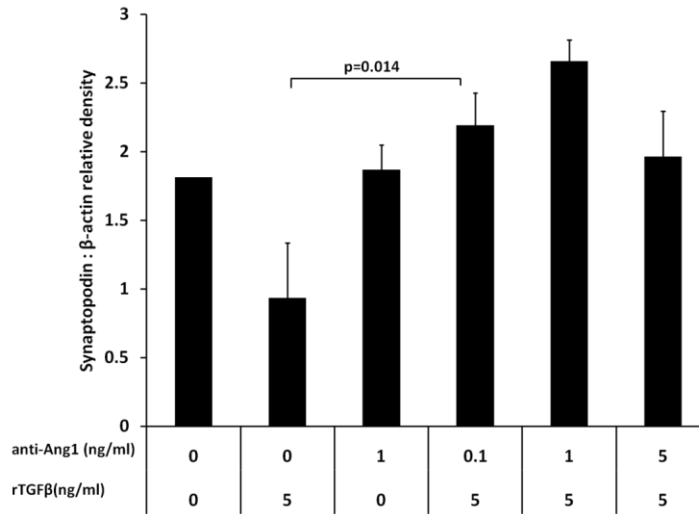
Figure 4.13: A: Podocytes were treated with rTGF β 1 concurrently with increasing doses of an antibody against Tie2. Tie2 antibody was able to reverse the TGF β 1 induced inhibition of nephrin (B) and synaptopodin (C). D: Podocytes were treated with rTGF β 1 (5 ng/ml) and siRNA for Tie2 (controlled with nontargeting siRNA). E: SiRNA to Tie2 also reversed the TGF β 1 induced inhibition of nephrin and synaptopodin. * $P < 0.01$ versus all.

4.12 Effects of anti-ANGPT1 and anti-ANGPT2 on TGFβ1 Treated Podocytes

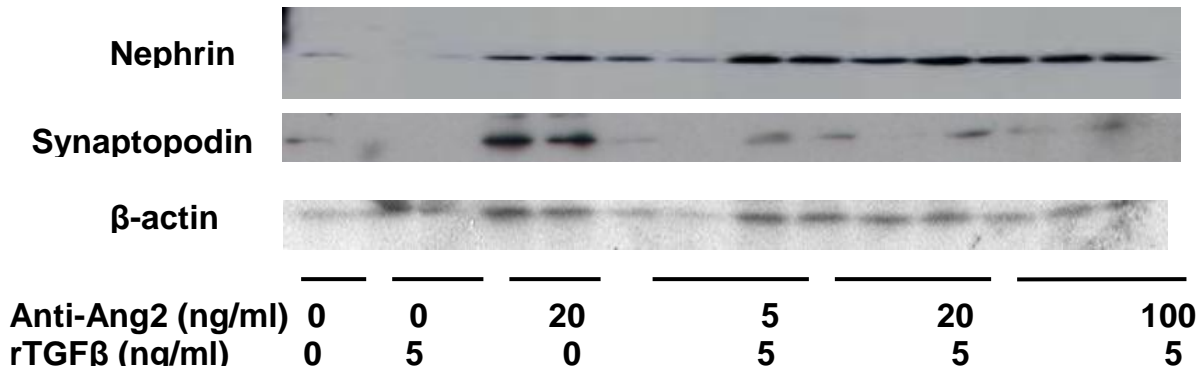
An antibody against ANGPT1 significantly blocked the TGFβ1 down regulation of nephrin and synaptopodin (Figure 4.14A-C). An antibody against ANGPT2 significantly reversed the TGFβ1 downregulation of nephrin (Figure 4.14D, E), but did not block the effect on synaptopodin (Figure 4.14F).



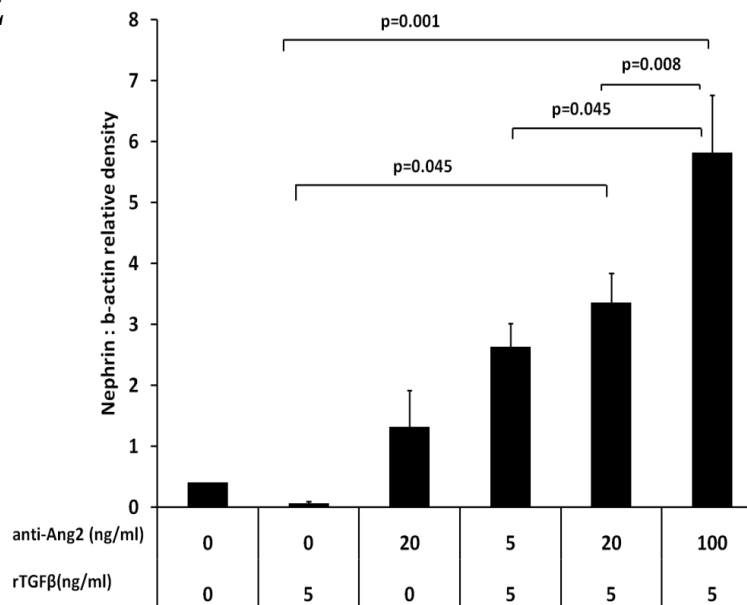
C



D



E



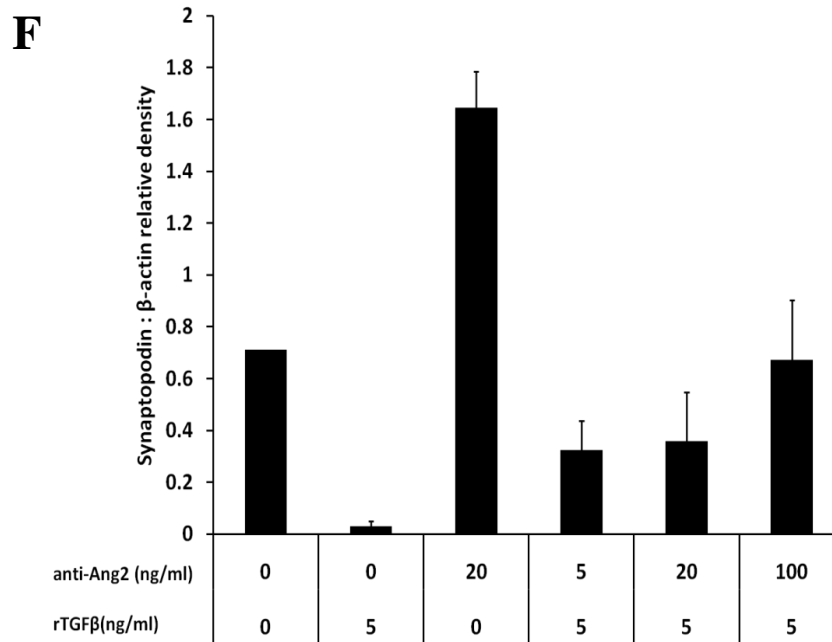


Figure 4.14: A: Podocytes were treated with rTGFβ1 (5 ng/ml) concurrently with increasing doses of an antibody against ANGPT1. B and C: ANGPT1 antibody was able to reverse the TGFβ1-induced inhibition of nephrin (B) and synaptopodin(C). D: Similar experiment was repeated but with increasing doses of an antibody against ANGPT2. E and F: ANGPT2 antibody was able to reverse the TGFβ1 induced inhibition of nephrin (E) but not synaptopodin (F).

4.13 Cyclosporins Effect on Synaptopodin

It was previously demonstrated that cyclosporine can prevent cathepsin-L-mediated degradation of synaptopodin (17). We, therefore, exposed podocytes to both TGFβ1 and cyclosporine (Figure 4.15B) and found that cyclosporine was able to block the TGFβ1 mediated down-regulation of synaptopodin (Figure 4.15B), but had no effect on Zo-1, a tight junction-associated epithelial protein (Figure 4.15C).

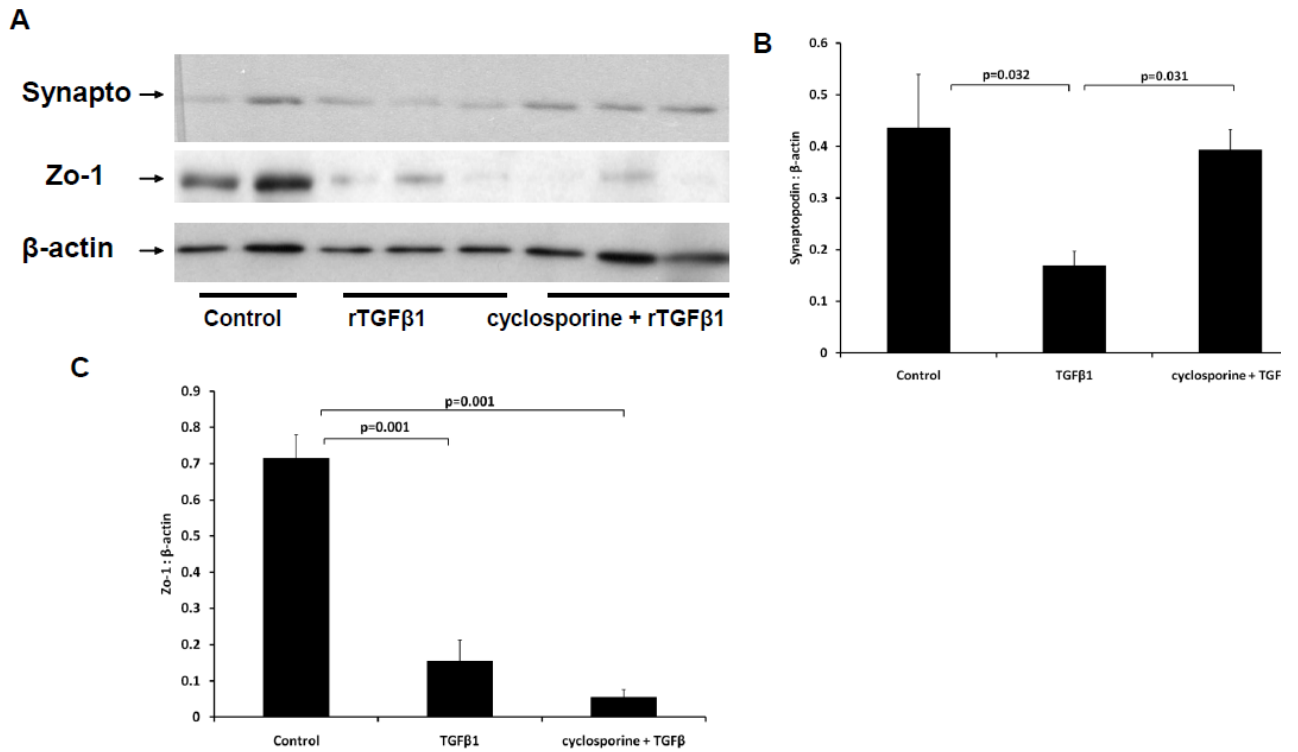


Figure 4.15: A: Cultured podocytes were treated with TGFβ1 (5 ng/ml) and cyclosporine (1 μg/ml). B: As previously demonstrated, TGFβ1 down-regulated synaptopodin and this effect was blocked by cyclosporine. C: The epithelial protein Zo-1 was also down-regulated by TGFβ1 and this was not reversed by cyclosporine.

CHAPTER 5 RESULTS ANTI-GBM GLOMERULONEPHRITIS

5.1 Animal Model

We used a standard model of antibody induced glomerulonephritis to induce significant glomerular damage in mice. In order to understand the SMAD3 pathways involved in proteinuria and interstitial fibrosis, we induced anti-glomerular basement membrane (GBM) glomerulonephritis in SMAD3^{+/+} and SMAD3^{-/-} mice. Animals received normal sheep serum in adjuvant for pre-immunization via an intraperitoneal injection and 4 days later received sheep anti-GBM antibody by tail vein injection (Figure 5.1). This created an acute inflammatory glomerulonephritis with proteinuria, glomerulosclerosis and tubulointerstitial fibrosis. Therefore, this mimics human proliferative glomerulonephritis. The mice were sacrificed on day 7, 21 and 56.

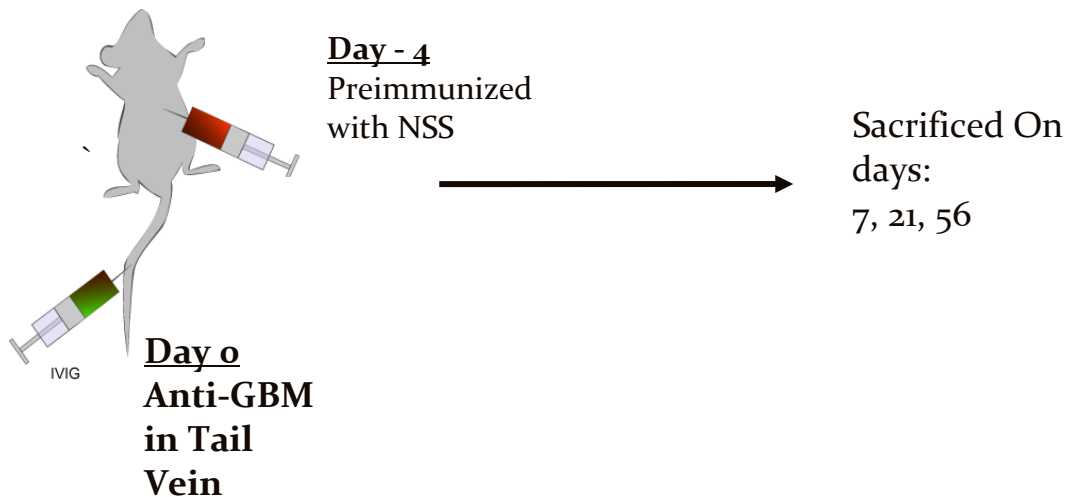


Figure 5.1: 8 week old SMAD3^{+/+} mice and SMAD3^{-/-} transgenic animals were used to induce glomerulonephritis. Animals were preimmunized with normal sheep serum and four days later received sheep anti-mouse GBM via tail vein.

5.2 Kidney Histology

SMAD3^{+/+} and SMAD3^{-/-} mice treated with anti-GBM antibody after sensitization show glomerulonephritis with evidence of focal proliferation and necrosis (Figure 5.2).

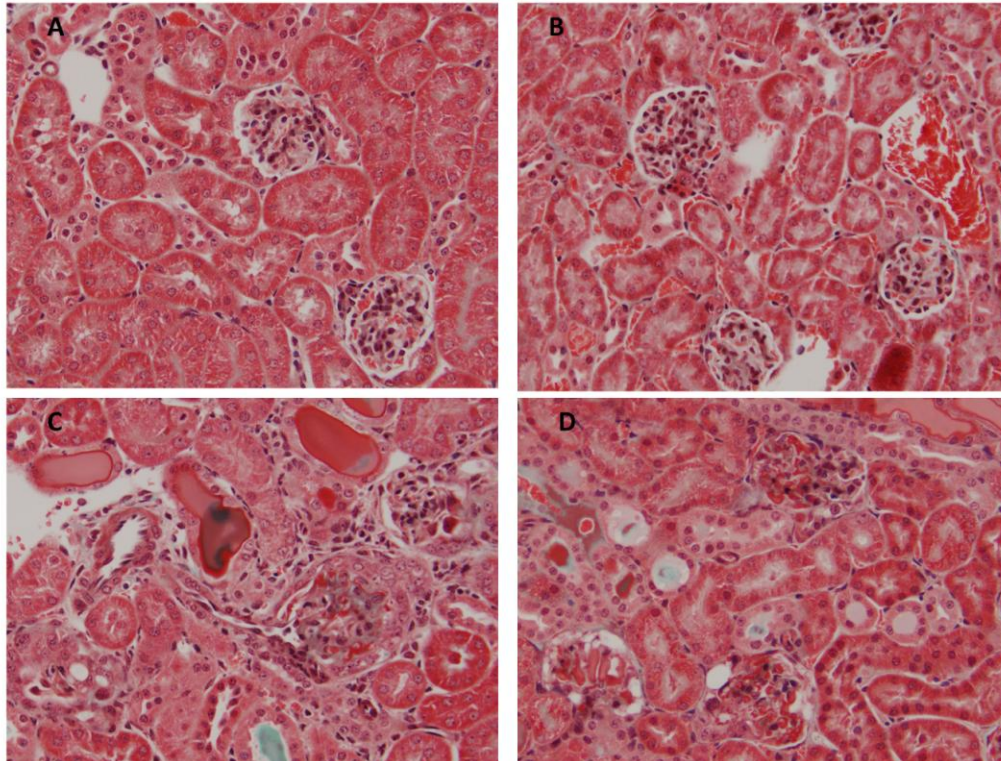


Figure 5-2: Renal histology 7 days after antibody injection in SMAD3^{+/+} and SMAD3^{-/-} mice A) Control antibody treated SMAD3^{+/+} and B) SMAD3^{-/-} mice show normal glomerular histology. After induction of glomerulonephritis, both C) SMAD3^{+/+} and D) SMAD3^{-/-} mice demonstrate a proliferative glomerulonephritis. Masson's trichrome sections were photographed at 200x magnification.

Twenty one days after induction of glomerulonephritis, mice developed tubular dilatation, and interstitial fibrosis (Figure 5.3).

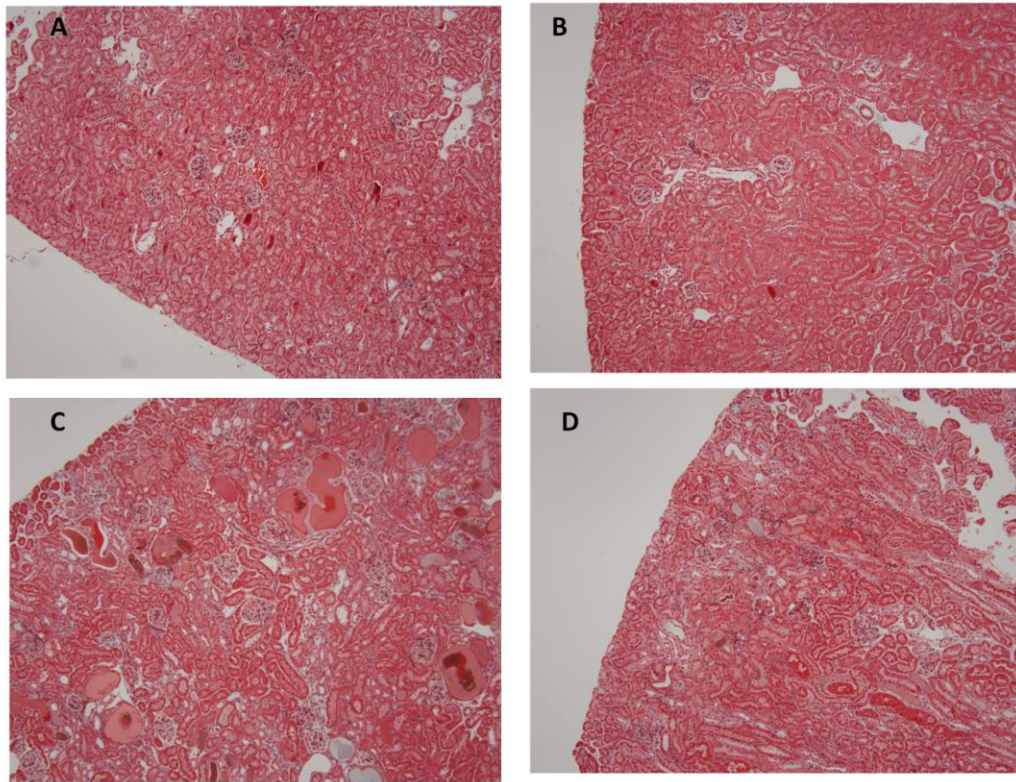


Figure 5.3: Renal histology 21 days after antibody injection. A) Control antibody treated SMAD3^{+/+} and B) SMAD3^{-/-} mice show normal renal histology. After induction of glomerulonephritis, both C) SMAD3^{+/+} and D) SMAD3^{-/-} mice show tubular dilatation, and interstitial fibrosis. Masson's trichrome sections were photographed at 100x magnification.

Interstitial fibrosis and glomerulosclerosis was scored in a blinded fashion. Anti-GBM treated SMAD3^{+/+} mice had more interstitial fibrosis on day 7 (p=0.007), day 21(p=0.002) and day 56 (p=0.001). Glomerulosclerosis was also significantly greater in anti-GBM treated SMAD3^{+/+} mice (Figure 5.4).

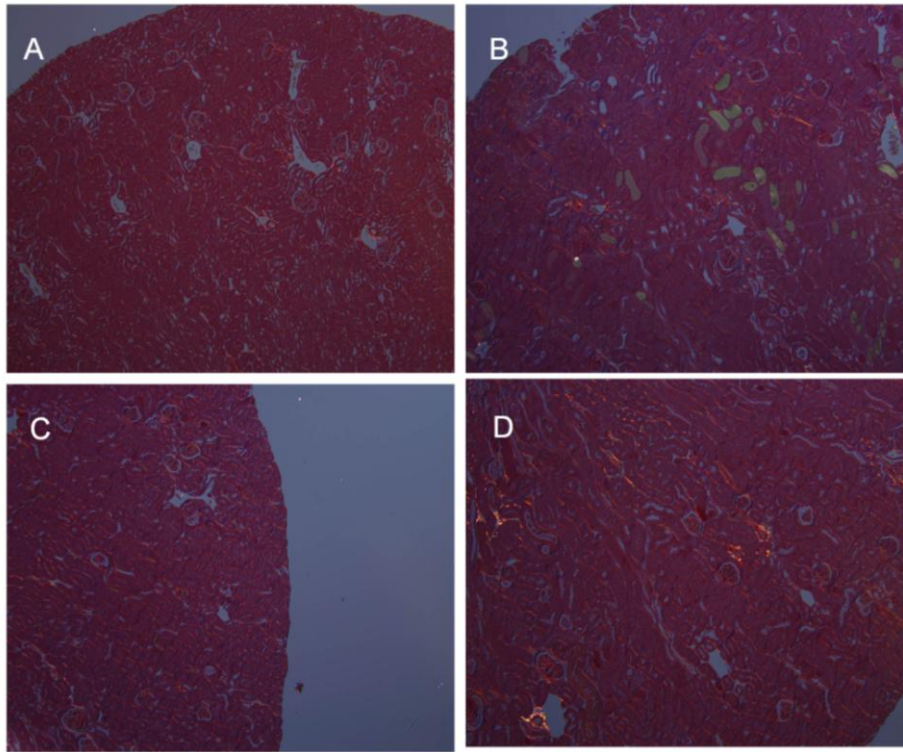


Figure 5.4: SMAD3^{+/+} and SMAD3^{-/-} mice sections were stained with picrosirius red and sections were quantified. A) and B) are SMAD3^{-/-} control and SMAD3^{-/-} treated with anti-GBM. C) and D) show SMAD3^{+/+} control and SMAD3^{+/+} treated with anti-GBM.

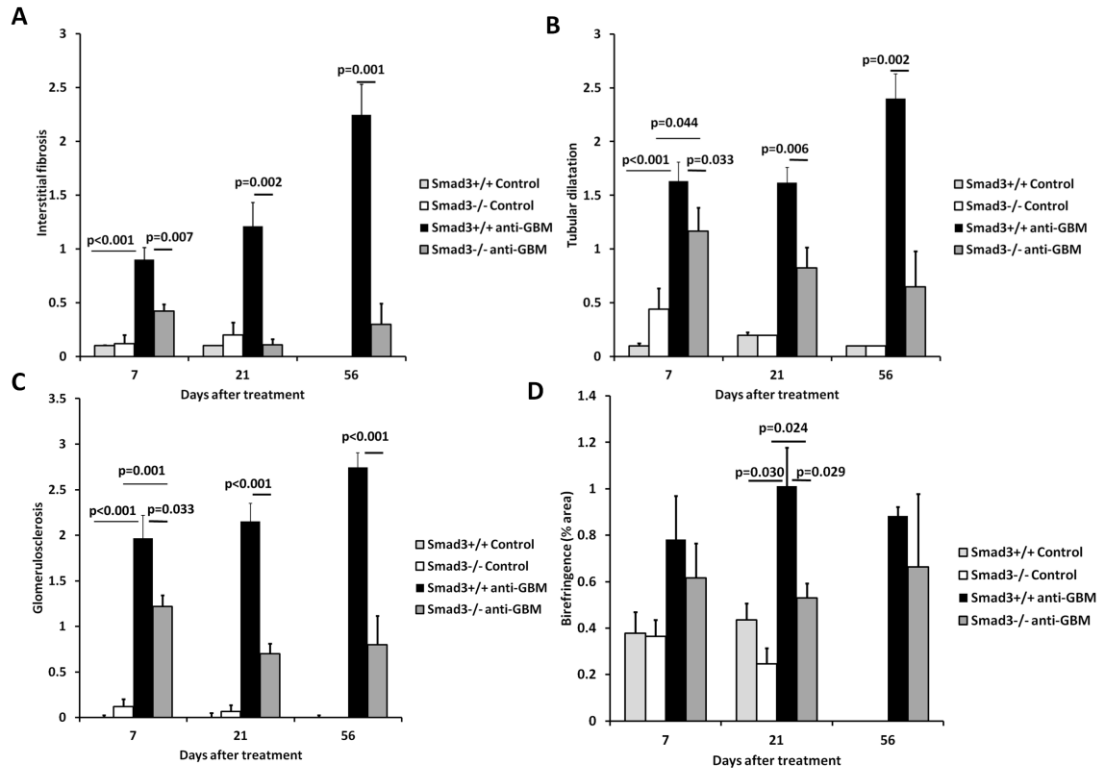


Figure 5.5: Quantification of renal histology changes. A) Blinded, semiquantitative scoring of Masson’s trichrome sections demonstrated significant, progressive interstitial fibrosis in SMAD3^{+/+} mice with no response in SMAD3^{-/-} mice treated with anti-GBM antibody. B) SMAD3^{+/+} mice treated with anti-GBM antibody demonstrated significant tubular dilatation which was also observed to a lesser extent in SMAD3^{-/-} mice treated with anti-GBM antibody. C) There was evidence of significant glomerulosclerosis in SMAD3^{+/+} mice treated with anti-GBM antibody, with some residual glomerulosclerosis seen in the SMAD3^{-/-} mice. D) Picosirius red sections were quantified based on intensity of birefringence. Again, SMAD3^{+/+} mice demonstrated significant fibrosis evident 21 days after treatment with anti-GBM antibody, with a less pronounced, but still significant response in SMAD3^{-/-} mice treated with anti-GBM antibody.

Picrosirius red stained sections (Figure 5.4) were quantified in (Figure 5.5D). In general, picrosirius red staining demonstrated that SMAD3^{-/-} mice had some tubulointerstitial fibrosis but significantly less than anti-GBM treated SMAD3^{+/+} mice which had higher percentage of birefringent area representing new collagen deposition. The birefringence was quantified using imageJ and on day 21 birefringence is significantly higher in anti-GBM treated SMAD3^{+/+} compared to anti-GBM treated SMAD3^{-/-} mice (P=0.029).

In order to assess the role of hypoxia in tubulointerstitial fibrosis, and the effect of deletion of SMAD3 on hypoxia, we used pimonidazole as a marker of tissue hypoxia. Animals were administered pimonidazole at 60 mg/kg body weight 1 hour before sacrifice. Pimonidazole is taken up by all tissues but is reduced in low oxygen environment. The reduced pimonidazole is unable to exit from cells, and can be stained to identify hypoxic tissue. Tissues from both SMAD3^{-/-} and SMAD3^{+/+} mice, treated with anti-GBM were stained for pimonidazole (Figure 5.6). Minimal background staining was observed in control antibody treated animals. There was clear tubular hypoxia in a patchy distribution in animals treated with anti-GBM antibody. Both SMAD3^{+/+} and SMAD3^{-/-} mice demonstrated tubular hypoxia, indicating that this response was not driven by TGF β mediated SMAD3 signalling.

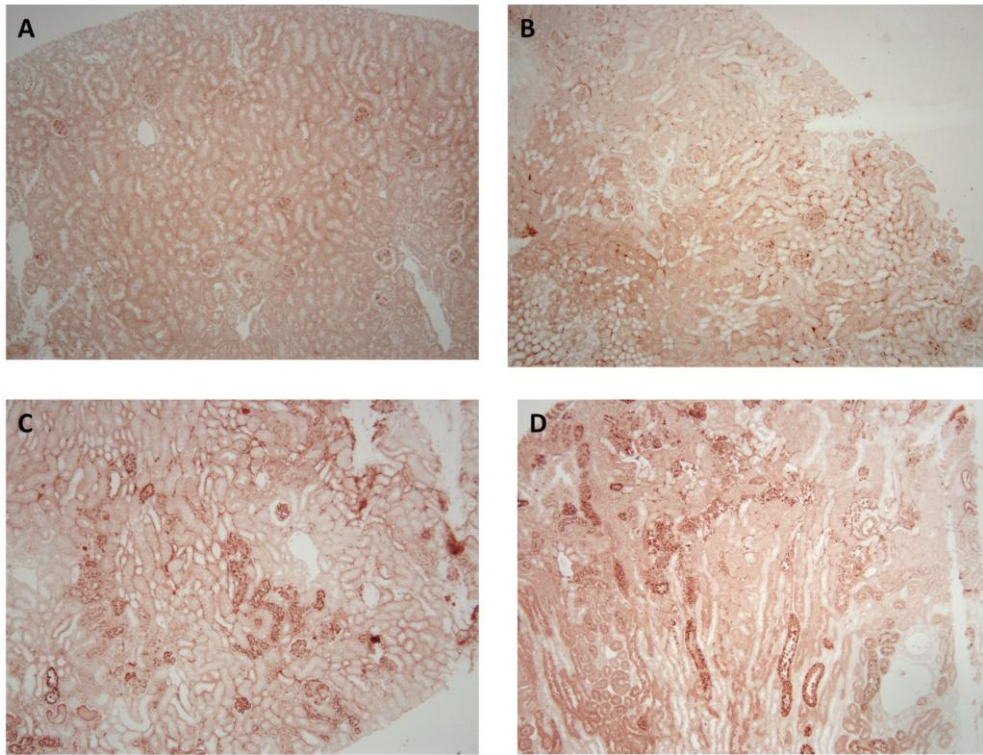


Figure 5.6: Pimonidazole staining as a marker of tubular hypoxia. A) Control antibody treated SMAD3^{+/+} and B) SMAD3^{-/-} mice show minimal background pimonidazole staining. Seven days after induction of glomerulonephritis, both C) SMAD3^{+/+} and D) SMAD3^{-/-} mice show increased patchy pimonidazole staining reflecting tubular hypoxia. Photomicrographs were taken at 100x magnification.

Western blot analysis was done for the whole kidney homogenate 7 days after treating with anti-GBM antibody. Tissue from SMAD3^{+/+} and SMAD3^{-/-} mice was run on SDS-gel to determine the level of HIF1A. Representative blot in Figure 5.7 shows HIF1A is highest in SMAD3^{-/-} mice and is down regulated by treatment with anti-GBM in both SMAD3^{+/+} and SMAD3^{-/-} mice.

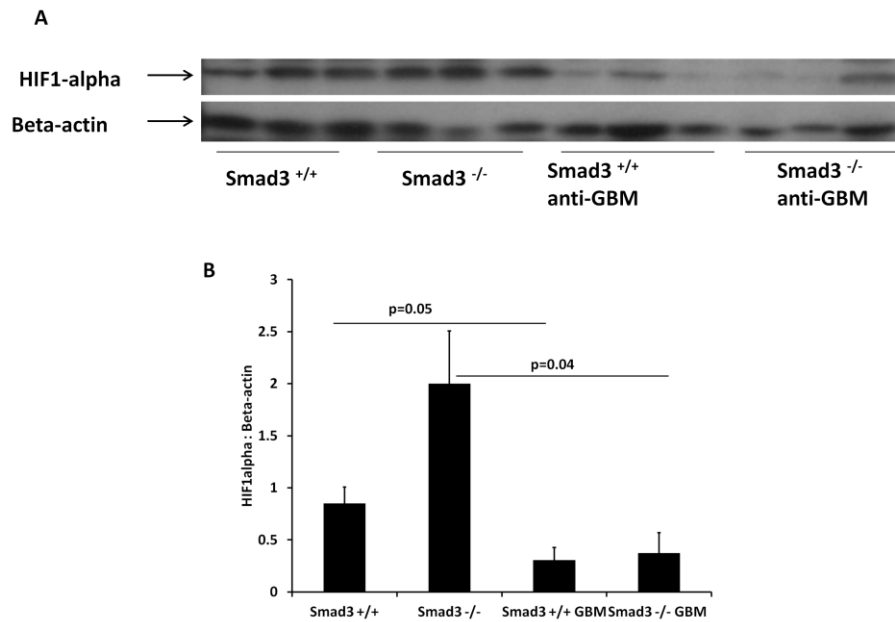


Figure 5.7: Seven days after induction of anti-GBM glomerulonephritis, there was a significant decrease in HIF1A protein measured by whole tissue western blot (A). This decreased expression was observed in both SMAD3^{+/+} and SMAD3^{-/-} mice treated with anti-GBM antibody (B).

5.3 Proteinuria

Proteinuria was quantified after induction of anti-GBM nephritis (Figure 5.8). Urine protein excretion was evaluated using a timed urine collection in a metabolic cage for 8 hours and protein to creatinine ratio was measured. Anti-GBM antibody induced glomerulonephritis leads to proteinuria in both SMAD3^{+/+} and SMAD3^{-/-} mice. Non-specific antibody treated mice are shown as control. SMAD3^{-/-} mice had 50% less protein excretion than SMAD3^{+/+} mice, and this proteinuria was transient and resolved by 7 days after induction of glomerulonephritis. In the SMAD3^{+/+} mice, proteinuria is significantly elevated early in the course of this model, and persists at a lower level to 56 days after

injection. The highest level of proteinuria was recorded on day 3 with protein to creatinine ratio was higher than 4g/mmol.

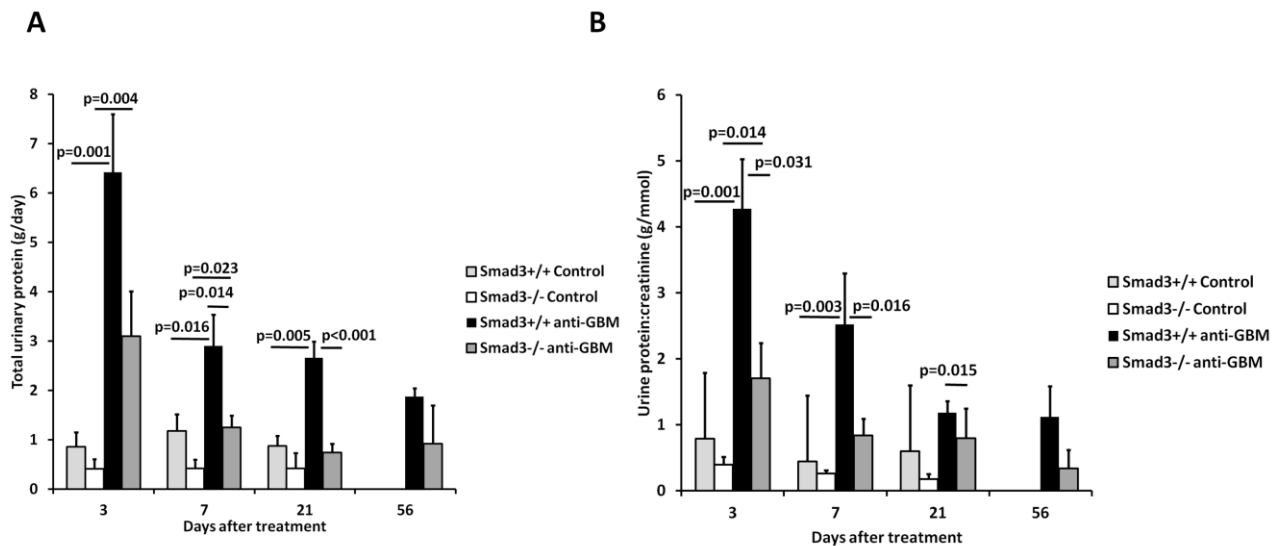


Figure 5.8: For both SMAD3^{+/+} and SMAD3^{-/-} mice proteinuria was measured by 24 hour urine protein concentration (A), or protein: creatinine ratio (B). Proteinuria was significantly elevated in SMAD3^{+/+} mice treated with anti-GBM antibody. This response was transient and declined toward baseline by day 56 after induction of glomerulonephritis. SMAD3^{-/-} mice treated with anti-GBM antibody also demonstrated a very transient proteinuric response, seen only at day 3 after induction of anti-GBM glomerulonephritis.

5.4 Electron Microscopy

Injured podocytes respond through a de-differentiation process that morphologically appears as effacement of the podocyte foot processes. In order to quantify this, we used electron microscopy. The number of foot processes/mm GBM length was quantified. Both SMAD3^{-/-} and SMAD3^{+/+} mice had significant foot process

effacement at day 7 after induction of glomerulonephritis suggesting that podocyte de-differentiation occurs by non-SMAD mechanisms (Figure 5.9). Foot process effacement persists to day 21, with some resolution in the SMAD3^{-/-} mice. This ongoing morphological change in podocytes persists despite resolution of proteinuria at this time point in the SMAD3^{-/-} mice.

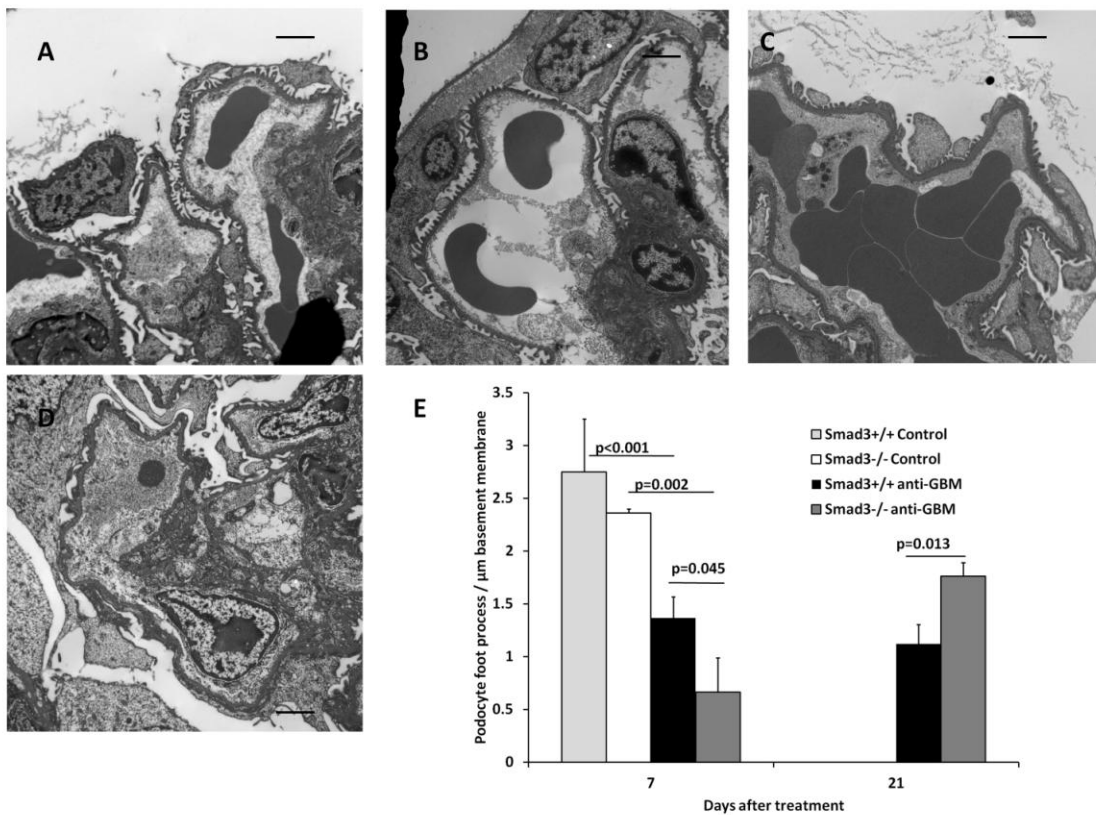


Figure 5.9: Electron micrographs of glomeruli in anti-GBM antibody induced glomerulonephritis. A) Control antibody treated SMAD3^{+/+} and B) SMAD3^{-/-} mice show no foot process effacement. Seven days after induction of glomerulonephritis, both C) SMAD3^{+/+} and D) SMAD3^{-/-} mice show significant foot process effacement quantified by the number of foot processes per μm of GBM (E). Electron micrographs at 1000x magnification, black bars represent 2 μm .

5.5 Renal Protein Expression

Western blot analysis was done for the whole kidney homogenate 7 days after treating with anti-GBM antibody (Figure 5.10). Tissue from SMAD3^{+/+} and SMAD3^{-/-} mice was run on SDS-gel to determine synaptopodin and nephrin protein concentration.

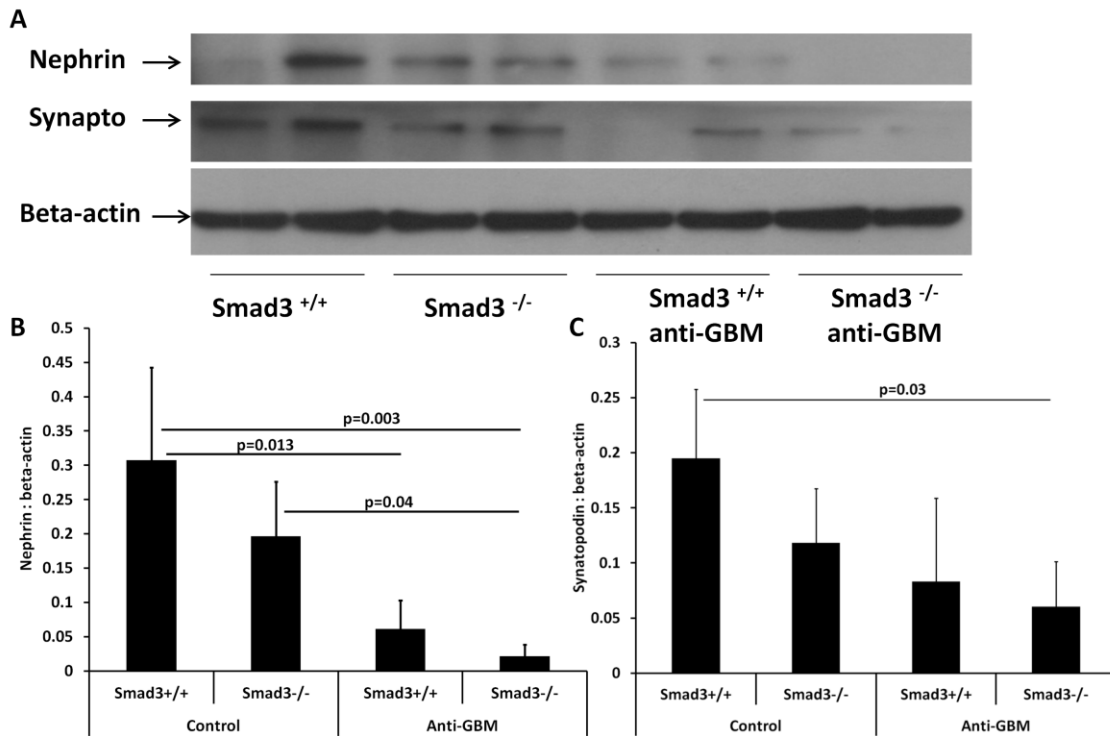


Figure 5.10: Nephrin and synaptopodin protein was measured by whole tissue western blot in whole kidney homogenate (A). Both nephrin (B) and synaptopodin (C) protein was reduced 7 days after induction of anti-GBM glomerulonephritis. Nephrin was reduced significantly in both SMAD3^{+/+} and SMAD3^{-/-} mice treated with anti-GBM antibody, but synaptopodin reduction was only significant in only in SMAD3^{-/-} mice.

Nephrin protein concentration is not significantly different between untreated SMAD3^{+/+} and SMAD3^{-/-} mice. Nephrin protein in whole tissue decreased significantly in both SMAD3^{+/+} and SMAD3^{-/-} mice treated with anti-GBM antibody. A similar, but not as robust response was observed for synaptopodin (Figure 5.10C).

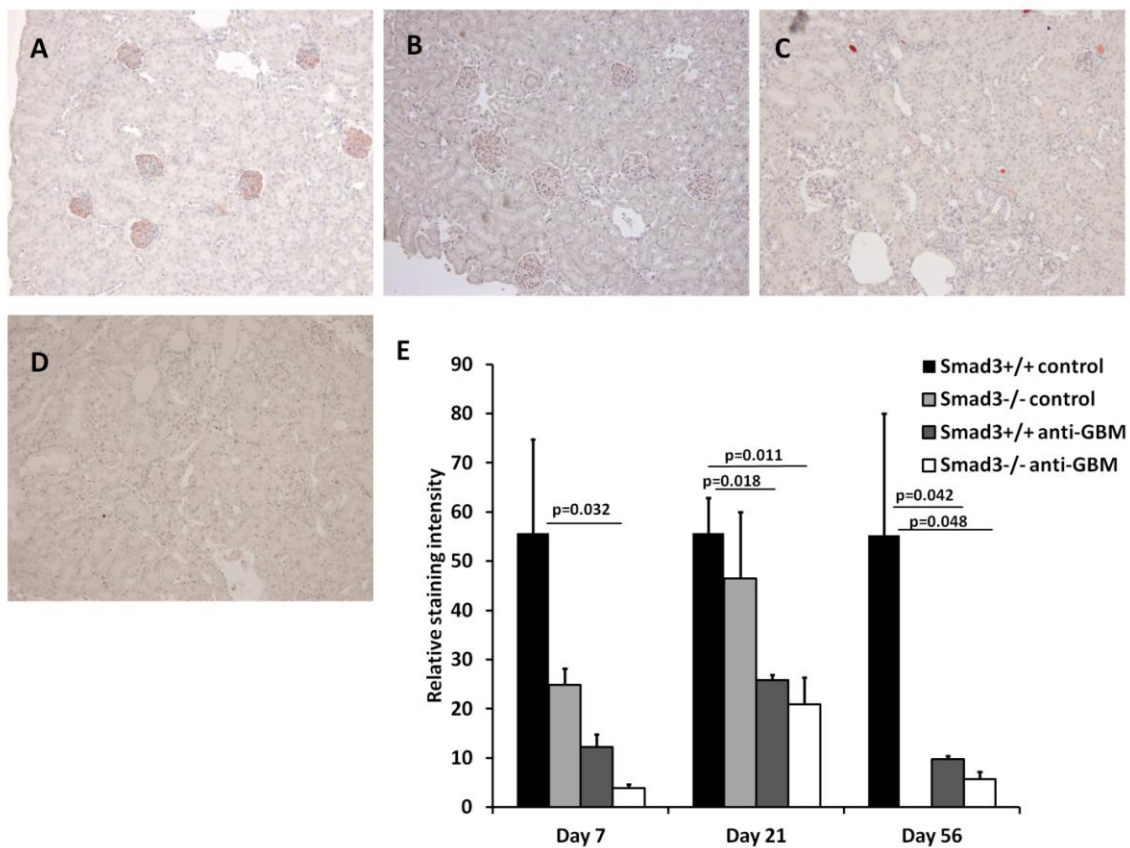


Figure 5.11: Kidney sections were stained for synaptopodin and demonstrated glomerular staining. Synaptopodin expression in anti-GBM antibody induced glomerulonephritis. A) Control antibody treated SMAD3^{+/+} and B) SMAD3^{-/-} mice demonstrate similar synaptopodin glomerular expression. In anti-GBM antibody treated C) SMAD3^{+/+} and D) SMAD3^{-/-} mice, there was a significant decrease in glomerular synaptopodin staining compared with control antibody treated SMAD3^{+/+} mice. Glomerular staining intensity was normalized to background staining; quantification is shown in E).

5.6 Synaptopodin Staining

Kidney sections were stained for synaptopodin (Figure 5.11) and demonstrated glomerular staining. Relative staining intensity was quantified using imagej and demonstrated significantly decreased synaptopodin staining in glomeruli of anti-GBM treated animals. Both SMAD3^{+/+} and SMAD3^{-/-} mice demonstrated significantly decreased glomerular synaptopodin protein expression that persisted to day 56. Again, it is noted that decreased synaptopodin expression in SMAD3^{-/-} mice persisted well past the resolution of proteinuria which occurred by day 7.

In both Figure 5.10 and Figure 5.11, we note a non-significant decrease in both nephrin and synaptopodin expression in untreated SMAD3^{-/-} mice compared to untreated wild type mice.

5.7 SMAD2 and SMAD3 SiRNA in Immortalized Podocytes

Our in vivo work evaluated the role of SMAD3 in proteinuria and interstitial fibrosis. In order to further evaluate the role of SMAD2, we used a podocyte cell line in vitro. Immortalized podocytes were a kind gift from Dr. P Mundel (Department of Medicine, University of Miami Miller School of Medicine) and were cultured as previously described (108). Podocytes were grown in permissive conditions then allowed to differentiate at 37°C. Cells were treated with siRNA to inhibit SMAD2 or SMAD3.

To confirm effective knockdown, Western blot for siRNA treated cells was run (Figure 5.12).

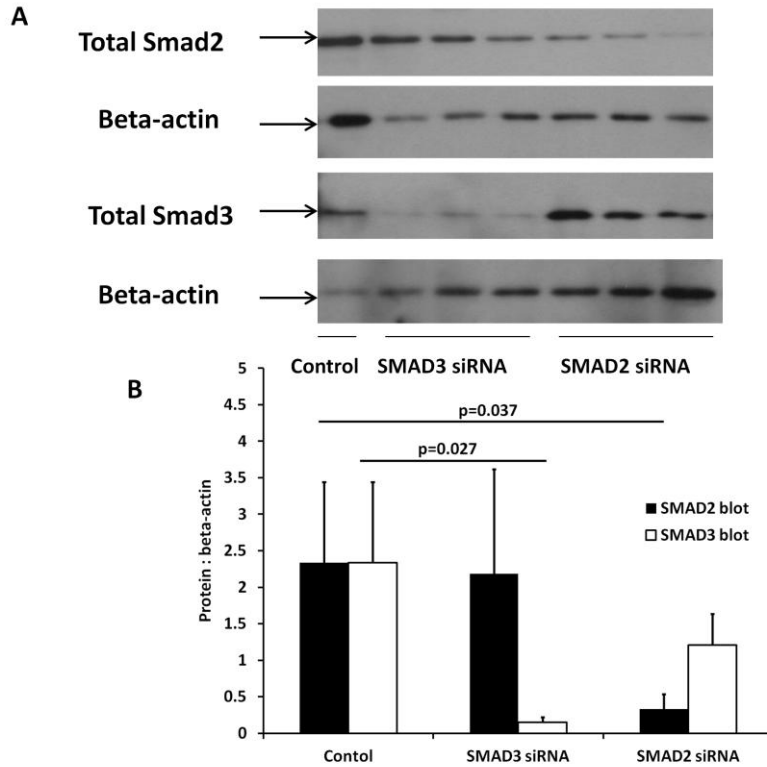


Figure 5.12: Cultured podocytes were treated with SMAD2 or SMAD3 siRNA. There is significant, specific, downregulation of corresponding proteins by siRNA.

We then evaluated the effect of knocking down SMAD2 or SMAD3 on TGF β -mediated podocyte de-differentiation. As shown in Figure 5.13, nephrin and synaptopodin protein concentrations were both significantly reduced by TGF β , but this effect was not reversed by SMAD2 or SMAD3 siRNA.

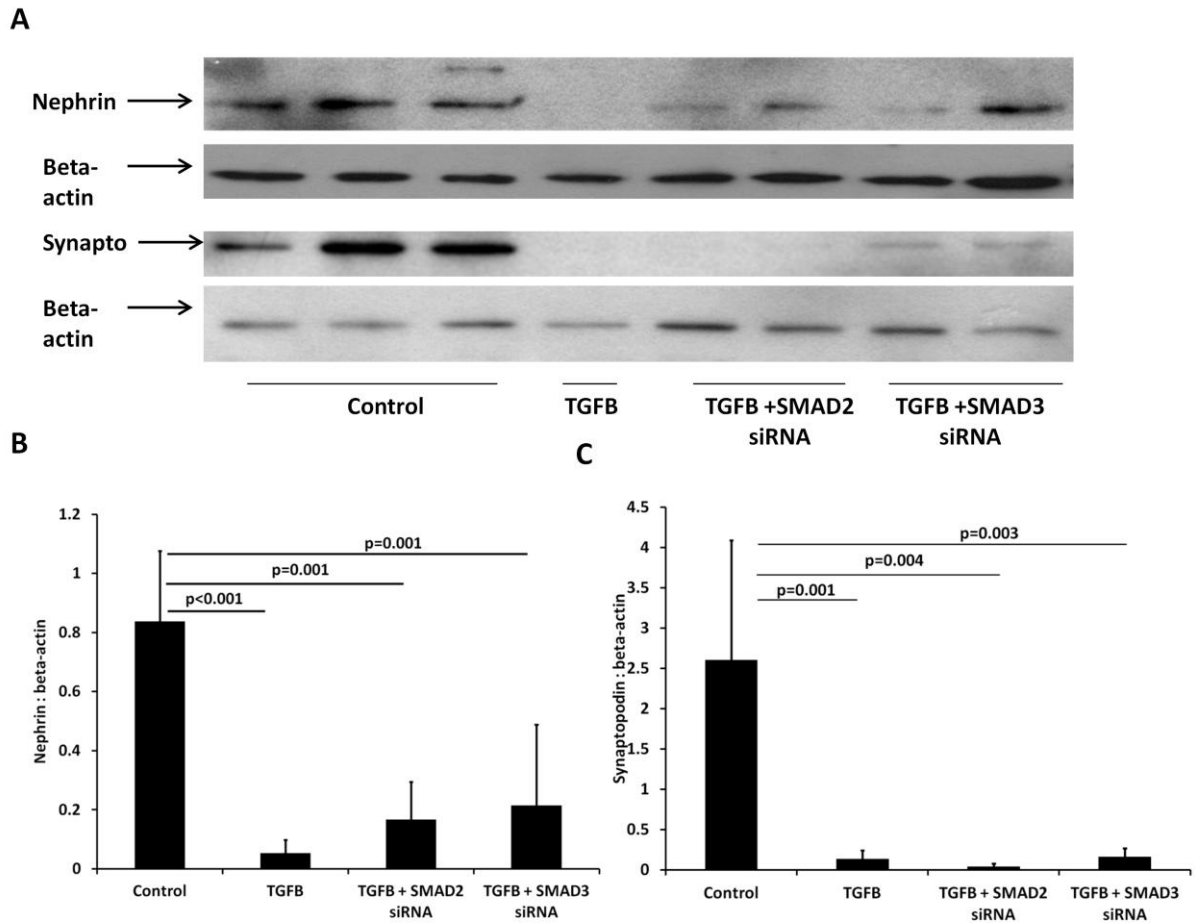


Figure 5.13: Cultured podocytes were treated with SMAD2 or SMAD3 siRNA in the presence of TGFB1. Recombinant TGFB significantly suppressed nephrin and synaptopodin protein (A) concentration in podocyte cell culture. SiRNA to SMAD2 or SMAD3 was unable to rescue TGFB1 mediated suppression of nephrin (B), or synaptopodin (C).

CHAPTER 6 RESULTS LENTIVIRUS

6.1 Western Blotting

Sprague-Dawley rats underwent slow perfusion of the left kidney with Lentivirus expressing GFP. There was no GFP expression in left or right kidneys of the rats injected with GFP Lentivirus, at day 7 or day 28. For a positive control we used the left kidney from AdGFP treated rat (Chapter 4). None of the other tissues were positive for GFP either.

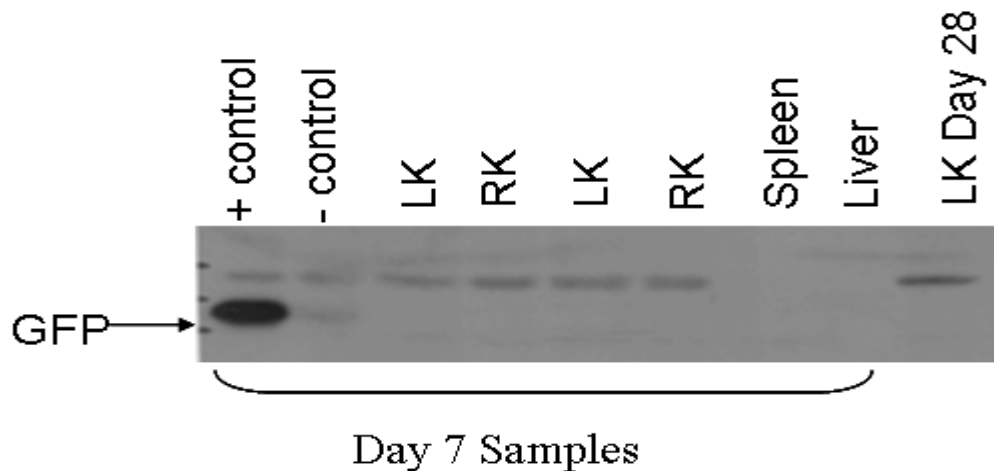


Figure 6.1: Immunoblot of Lentivirus treated left and right kidneys of rats. No signal detected for GFP. Positive control of Adenovirus GFP treated rat shown as positive control (+ control) and untreated rat kidney was used as negative control (- control). LV-RK represent Lentivirus treated Left kidneys and RK represent contralateral kidneys of Lentivirus treated rats.

6.2 Immunostaining

Formalin fixed and frozen sections of Lentivirus treated kidneys were stained for anti-GFP antibody along with positive and negative controls. Contralateral kidneys were also stained along with liver and spleen sections. Left rat kidneys Figure 6.2A, transfected with AdGFP served as positive control. Liver and spleen sections were stained too but there was no GFP signal detected. Shown in the Figure are frozen sections. Formalin fixed sections also showed no GFP signal.

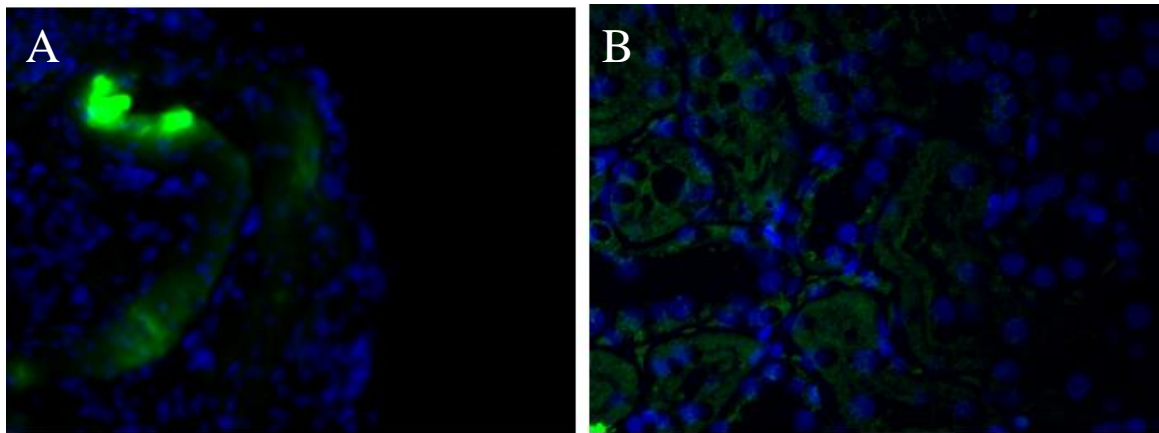


Figure 6.2: Formalin fixed and frozen sections of Lentivirus treated kidneys were stained for anti-GFP antibody Positive control in (A) and Lentivirus treated left mice kidney (B). No GFP signal detected in Lentivirus treated kidneys.

CHAPTER 7 DISCUSSION AND CONCLUSION

7.1 TGFB Induced Proteinuria Requires Angiopoietin

The molecular basis of proteinuria in diseases such as diabetes or chronic glomerulonephritis has yet to be fully understood. Glomerular hypertension, hyperglycemia, and an increase in local angiotensin II expression are common findings in proteinuric renal disease (66). These factors have all led to increased expression of TGFB (109) suggesting that TGFB may play a significant role in alterations of the glomerular filtration barrier.

We have adopted a novel model of TGFB1 overexpression in the kidney. By using a slow perfusion method to infect the kidney with adenoviruses expressing TGFB1, GFP, or beta-galactosidase, we have demonstrated renal-specific expression. The technique we adopted was first described by Ye et al. (110) and has also been used successfully in larger animals (111). Similar to our findings, these studies demonstrated that up to 75% of glomeruli expressed the transgene and the expression lasted up to 21 days (111).

We observed a diffuse uptake of adenovirus in glomeruli and peritubular capillaries. We were able to detect transgene expression in the urine up to 7 days after infection. One benefit of this approach is that we selectively infect the left kidney, leaving the right kidney as an internal control. There is liver uptake and expression of the adenovirus using this technique. Aside from this, we could find no evidence of systemic effects of TGFB1 expression in the animals treated with AdTGFB1; the control right kidneys did not show any of the effects seen in the infected left kidney.

There have been previous reports of the effects of systemic TGFB1 on the structure and function of the kidney. Systemic overexpression of TGFB1 appears to have an impact on the kidney through either a hemodynamic, vasoconstrictive mechanism (112) or a direct fibrogenic effect (113). In a previous report (62), high levels of systemic TGFB1 using a transgenic mouse model had a proteinuric effect, but the mechanism of this renal injury was not described. Wogensen et al. (114) described a novel transgenic mouse with TGFB1 driven from the Ren-1c promoter of the juxtaglomerular apparatus, which developed albuminuria. Other studies have used proximal tubule promoter (115) or gene transfer of TGFB1 (116), but the effect on protein excretion was not addressed.

We have demonstrated that transient overexpression of TGFB1 primarily in the glomerular endothelium leads to podocyte effacement and proteinuria. The resulting phenotypic change of podocytes was associated with a glomerular specific increase in the regulatory protein Snail1 as measured in laser capture microdissected samples. We also observed a decrease in podocyte specific markers nephrin, synaptopodin, and podocin along with suppression of the epithelial marker Zo-1. These findings are indicative of a de-differentiation or EMT-like process. Recently, TGFB1 has been shown to induce de-differentiation and features consistent with EMT in podocytes (9). Li and colleagues argue that this de-differentiation may explain proteinuria in many clinical conditions. We have extended their findings with our in vivo evidence of this process. Podocyte de-differentiation appears to be an early event in glomerular injury and subsequent podocyte apoptosis and sloughing has been observed in advanced renal disease (19). However,

podocyte apoptosis appears to be a later finding that occurs after the onset of proteinuria (117).

The role of Snail in nephrin gene transcription repression has been demonstrated in a model of puromycin induced nephrosis (118). Podocyte EMT has also been suggested in a recent study in patients where the mesenchymal marker FSP-1 was identified in podocytes in the urine of patients with diabetes (119). The downregulation of synaptopodin by TGFB1 in our model is a novel finding. Synaptopodin is involved with the structure of the actin cytoskeleton and of critical importance for the maintenance of the podocyte slit diaphragm. Decreased synaptopodin expression has been associated with proteinuria (17;120) and related to steroid unresponsiveness in primary focal segmental glomerulosclerosis (121). Abbate and colleagues found that injury to podocytes in a remnant kidney model led to increased TGFB expression and loss of synaptopodin (122). In a study by Lai and colleagues (123), human mesothelial cells were exposed to pathogenic IgA and podocytes were then grown in the presence of this conditioned media. They observed a downregulation of podocin and synaptopodin. The downregulation of nephrin by this conditioned medium could be blocked by antibodies to TGFB1 but the effects on synaptopodin were not assessed. Furthermore, high glucose has been shown to downregulate synaptopodin and this was demonstrated to be inhibited by BMP7 which is a TGFB1 antagonist (124).

Recent work (125) has identified the central role of cathepsin L-mediated proteolytic cleavage of synaptopodin in proteinuria. Sever et al. (126) identified a

cytoplasmic form of cathepsin L, which induced dynamin degradation. Proteinuria was inhibited when cathepsin L was blocked.

Further work by Faul et al. (17) showed a novel non-immunologic role for cyclosporine in preventing cathepsin–L mediated degradation of synaptopodin. Most recently, Yaddanapudi et al. (127) identified a role for TGFB1 induction of cathepsin L in CD2AP knockout mice. Our experiments provide direct evidence of TGFB1 induced downregulation of synaptopodin. We also found that TGFB1 mediated down-regulation of synaptopodin could be prevented with co-treatment with cyclosporine. Cyclosporine did not prevent the TGFB1–mediated down-regulation of Zo-1, suggesting a specific action of cyclosporine on Synaptopodin.

The loss of this structural associated protein may lead to foot process retraction (25) and may be the predominant cause of proteinuria induced by transient overexpression of TGFB1. The loss of nephrin expression in the glomeruli, along with the increase of urine nephrin, is further evidence for a direct damage or de-differentiation of podocytes in our model. The potential of increased urinary nephrin as a marker of podocyte injury has been shown previously (128). Indeed, urine nephrin excretion has been observed very early in the course of experimental nephritis, even before overt proteinuria (129).

Angiopoietins are known to modify the angiogenic effects of vascular endothelial growth factor (VEGF). In quiescent vasculature, ANGPT1 is produced by perivascular cells and has a paracrine effect on endothelial cells. ANGPT1 binds to the Tie2 receptor and signals through PI3K /Akt to maintain vascular integrity (130). In pathologic settings,

VEGF and ANGPT2 are upregulated and lead to vascular destabilization, angiogenic sprouting, and new vessel formation (131). ANGPT2 similarly binds to the Tie2 receptor and is thought to be an antagonist for ANGPT1. In the glomerulus, the role of the angiopoietins are not clearly defined (132). In the quiescent state, ANGPT1 and VEGF are produced by podocytes and have protective and anti-proteinuric effects on endothelial cells (133;134).

ANGPT2 has been associated with proteinuria and renal injury in several settings including lupus nephritis (65) and diabetic nephropathy (66). In previous work, Davis and colleagues created a mouse model where ANGPT2 was overexpressed on a podocyte specific promoter (7). In this model, ANGPT2 overexpression led to proteinuria and decreased nephrin expression, similar to what we found in our study. There was also downregulation of nephrin in podocytes as seen in our model too. Interestingly, they did not describe phenotypic changes in podocytes in response to ANGPT2, but found that it induced endothelial cell dysfunction which was believed to underlie the proteinuric response.

We identified that both ANGPT1 and ANGPT2 were upregulated in the glomeruli from animals exposed to AdTGFB1, similar to observations by Campean and colleagues in the Thy 1.1 model of glomerular injury (135). In unstimulated podocytes, we found no expression of Tie2, but TGFB1 increased Tie2 expression in a dose responsive manner. Tie2 expression is normally confined to endothelial cells, but two previous papers have described the expression of Tie2 on podocytes (7;136). The current understanding is that ANGPT1 is a Tie2 agonist, while ANGPT2 acts as an antagonist (65). In our

experiments, we were able to block the inhibitory effect of TGF β 1 on nephrin with an antibody and siRNA directed against Tie2, an antibody directed against ANGPT1, and also with an anti-ANGPT2 antibody. This suggests that ANGPT2 may have similar effects to ANGPT1 on podocytes and Tie2 signalling is different in epithelial as opposed to endothelial cells.

In our model unstimulated podocytes did not express the receptor for angiopoietins. We were able to see an increased Tie-2 expression in TGF β 1 stimulated podocytes (figure 7.1) and this suggests that TGF β 1 could be signalling through angiopoietins (137). An ANGPT2 agonist activity has been suggested in endothelial cells in some settings (138). Furthermore, in the original description of angiopoietin/Tie2 signalling, it was noted that ANGPT2 may act as a Tie2 agonist in non-endothelial cells (65). Finally, angiopoietins may signal through other non-Tie2 mechanisms such as integrins (139). The lack of podocyte effacement after ANGPT2 overexpression as seen in the study by Davis and colleagues (7) suggests that ANGPT2 alone is not sufficient to induce phenotypic changes in podocytes. We hypothesize that TGF β is required for podocyte de-differentiation either as a direct co-factor or through the induction of Tie2 receptors on podocytes (figure 7.1)

The role of angiopoietins in TGF β 1 mediated podocyte de-differentiation will need to be integrated with the observed effects of TGF β 1 on cathepsin L-induced synaptopodin degradation and proteinuria. These may be steps within the same pathway, or they may represent separate simultaneous processes that augment the proteinuric effects of TGF β 1.

In summary, we have developed a novel model of proteinuria and podocyte de-differentiation that involves the isolated upregulation of a single key pro-fibrotic cytokine – TGFB1 – in glomerular endothelial cells. The mechanism appears to involve an EMT – like phenomenon with increased expression of Snail and downregulation of podocyte epithelial markers such as nephrin and synaptopodin. The effects of TGFB1 are at least partly mediated by angiopoietins.

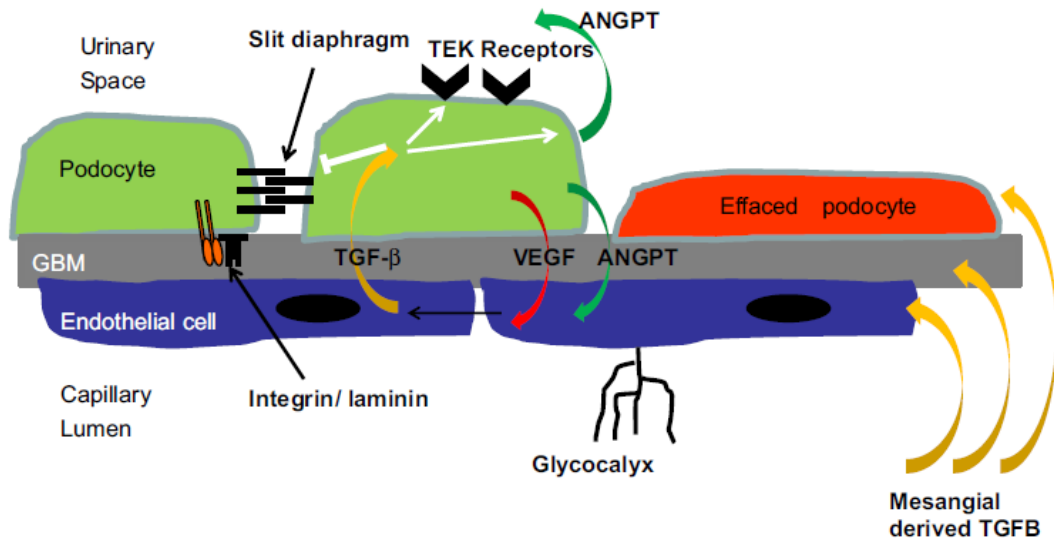


Figure 7.1: Effect of TGFB on the Glomerular Filtration Barrier. The glomerular filtration barrier consists of podocytes with their intracellular slit diaphragm, the glomerular basement membrane (GBM), and the endothelial cells with associated glycocalyx. Podocytes express vascular growth factors such as VEGF and angiopoietins. Podocyte derived VEGF and ANGPT1 is required to maintain endothelial health and an intact filtration barrier. TGFB is produced by endothelial cells in response to increased VEGF, or from mesangial cells exposed to injury such as high concentration of glucose. We have demonstrated a role for TGFB in the induction of ANGPT1 and ANGPT2 in podocytes, with an increase in the ANGPT receptor TEK (TEK is the gene segment encoding the Tie-2) that is responsible for some of the TGFB-induced podocyte changes.

7.2 The Role of SMAD in Anti-GBM Antibody Induced Glomerulonephritis

Anti-GBM model is a well established model of renal glomerulonephritis and proteinuria. It has been demonstrated by multiple groups that the injury is mediated by TGF β 1 upregulation and blocking the action of TGF β 1 can alter the disease severity (59;140). Kanamaru et al. specifically blocked TGF β signalling in T-cells using a transgenic mouse expressing SMAD7 in peripheral mature T-cells (77). They then induced anti-GBM nephritis in both wild type and transgenic mice. They compared the two groups in terms of their renal histology and urinary protein excretion. The transgenic mice were healthier and survived longer and had very little glomerular pathology than their wild type counterparts. Hence they were able to determine that blocking TGF β pathway in T-cells can result in a much milder form of nephritis in this model. We also observed milder damage to the kidney in SMAD3^{-/-} versus SMAD3^{+/+} mice.

We used both SMAD3^{+/+} and SMAD3^{-/-} mice to study the importance of this pathway in proteinuria and podocyte de-differentiation. In our AdTGF β 1 rat model we were able to show the importance of TGF β 1 in mediating podocyte effacement and proteinuria (61). The importance of SMAD pathway in podocyte effacement and proteinuria has not been extensively investigated. Two groups have looked at the effects on proteinuria in SMAD3^{-/-} mice. Both groups used streptozotocin-induced diabetic SMAD3^{-/-} and SMAD3^{+/+} mice. Wang et al. reported overt albuminuria in both the groups; glomerulosclerosis was however less well established in SMAD3^{-/-} mice (44). Fujimoto et al. reported lack of overt albuminuria in SMAD3^{-/-} mice (141).

In both our SMAD3^{+/+} and SMAD3^{-/-} treated groups, there was significant foot process effacement, suggesting that there are non-SMAD mediated pathways which lead to podocyte FP effacement and de-differentiation. SMAD3^{-/-} mice also had proteinuria suggesting that absence of SMAD3 did not protect the filtration barrier from damage and hence the consequence of proteinuria. We also note that the proteinuria seen in the SMAD3^{-/-} mice was transient and only seen 3 days after the induction of glomerulonephritis, but evidence of podocyte de-differentiation persisted. We saw podocyte foot process effacement and downregulation of synaptopodin at 28 days after induction. This suggests that podocyte phenotypic changes are necessary for proteinuria, but do not appear to be sufficient. Different aspects of the glomerular filtration barrier – for example the endothelium – may play an important role in proteinuria. We did not investigate changes in the endothelium, but our findings suggest that TGFB induced endothelial injury may be SMAD3 dependent.

The relative significance of non-SMAD TGFB-mediated pathways in proteinuria has been less well characterized. We have demonstrated that despite inhibition of SMAD2 or SMAD3, we continue to see TGFB induced podocyte de-differentiation.

In previous work from our lab, we found that TGFB induced peritoneal mesothelial cell EMT occurs in SMAD3^{-/-} mice (142). We demonstrated that TGFB1 induced EMT could be blocked by the mTOR inhibitor rapamycin in SMAD3^{-/-} mice. We hypothesize that mTOR / beta-catenin may be an important auxiliary pathway in TGFB induced podocyte de-differentiation. Future work will be required to investigate this further.

Other important pathways to consider are the Notch and phosphatidylinositol-3-kinase/Akt (PI3K/Akt) pathway and the consequences of Notch and PI3K/Akt blockage in vivo and in vitro. Important information about the timeline of TGF- β 1 action came from the study of J. Zavadil et al. (143). They have shown that TGF- β 1 stimulates two different waves of activation that up regulates Hey1, an essential component of EMT. Immediate early activation of Hey1 requires SMAD3 and SMAD4 (independent of Notch signalling) and the second cycle of activation requires classical Notch signalling which is activated by TGF β 1 via SMAD3 and MAP kinase

We demonstrate that SMAD3^{-/-} mice are generally protected against interstitial fibrosis, but do show evidence of damage such as tubular dilatation. Other pathways that might be functional in SMAD3^{-/-} mice could be hypoxia driven. Renal hypoxia has been repeatedly shown to be associated with progressive renal disease in chronic renal failure, in diabetic rat kidney and polycystic kidney disease (144-146). Sekiguchi et al. in our lab has previously shown that hypoxic response in peritoneum is TGF β 1 driven and leads to peritoneal fibrosis and angiogenesis (142) and this occurs in a two step manner. Initially after injury, there is a TGF β 1 driven fibrotic response followed by hypoxia driven fibrosis and angiogenesis.

Song et al. demonstrated that renal hypoxia plays a role in diabetic nephropathy, mediated by over activity of renin angiotensin aldosterone system and both hypoxia and fibrosis were attenuated by ace inhibitor (147). HIF1A was reno protective and they used DMOG that activates HIF1A. DMOG infusion prevented further increase of proteinuria and attenuated renal injury (148).

In our model, animals treated with anti-GBM antibody showed positive staining for pimonidazole (Figure 5.6). Specifically, we saw isolated renal tubules demonstrating strong pimonidazole staining indicating hypoxia. Pimonidazole staining was positive in both SMAD3^{+/+} and SMAD3^{-/-} anti-GBM treated group suggesting the hypoxic response was not dependent on SMAD3. We also observed an aberrant angiogenic response. Despite the increase in hypoxia seen in the tubules, we observed a suppression of HIF1A seven days after induction of anti-GBM GN (Figure 5.7). We have previously found that this aberrant response is related to an induction of pigment epithelium derived factor that suppresses the normal angiogenic response to hypoxia.

Picrosirius red staining for fibrosis revealed more extensive fibrosis in SMAD3^{+/+} versus milder fibrosis in SMAD3^{-/-} mice. Hence SMAD3^{+/+} mice are more prone to fibrotic damage by anti-GBM antibody. Trichrome staining also reveals that there is less interstitial damage, less tubular and glomerulosclerosis in SMAD3^{-/-} pool.

In summary we hypothesized that there are both SMAD dependent and independent pathways that mediate proteinuria and podocyte effacement and by using SMAD knockout animals we have clearly shown that SMAD3^{-/-} mice are not protected from renal damage and proteinuria. Other pathways that could be activated by TGFB1 are detrimental in kidney diseases and further work is required to identify these mediators.

7.3 Gene therapy for renal disease

Slow perfusion of adenovirus in our experiment led to transient gene expression in renal glomeruli and perivascular cells. We used the intra-arterial technique for introduction of adenovirus. Though the transfection efficiency was lower than that found in the lung or peritoneum, there was clear reporter gene expression in our system.

Lentivirus expressing LacZ reporter gene has been used by Gusella et al. (107) They performed the experiment in BALB/c mice and the Lentivirus used had CMV promoter. Intrarenal artery, intrarenal vein, ureteral infusion and four different sites for intraparenchymal injections were used to infuse the virus and the transduction efficiency was compared. Intrarenal artery and vein infusions were inefficient and resulted in the transduction of very few cells compared to intraparenchymal or ureteral infusion.

We think there are two different possibilities for the lack of GFP gene expression in our model. The dosage of Lentivirus used may be low. We had a limited quantity of virus available for this project and we calculated the dose based on previous work done by Gusella et al. (107). We attempted to repeat this intra-arterial approach in mice. Due to the invasive nature of the procedure it was not successful. The mice performed poorly and most did not survive the procedure. We carried out this experiment in rats and there is also a possibility that Lentivirus does not infect rat renal glomeruli. In vitro studies need to be done on renal podocytes or glomeruli culture to confirm Lentivirus transfection of these cells.

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