THE ROLE OF PODOCYTE PROSTAGLANDIN E2 AND ANGIOTENSIN II RECEPTORS IN GLOMERULAR DISEASE

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Medicine

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ABSTRACT

The incidence of chronic kidney disease (CKD) is increasing. CKD is characterized by a gradual decrease in renal function leading to end stage renal disease (ESRD). Damage to the glomerular podocytes, is one of the first hallmarks of CKD. We hypothesized that podocyte prostaglandin E2 (PGE2) receptors contribute to the progression of glomerular injury in models of CKD. To test this hypothesis, transgenic mice were generated with either podocyte-specific overexpression or deletion of the PGE2 EP4 receptor ($EP4^{pod+}$and $EP4^{pod-/-}$ respectively). Mice were next tested in the 5/6 nephrectomy (5/6 Nx) or angiotensin II (Ang II) models of CKD. These studies revealed increased proteinuria and decreased survival for $EP4^{pod+}$ mice while $EP4^{pod-/-}$ mice were protected against the development of glomerular injury. Furthermore, our findings were supported by *in vitro* studies using cultured mouse podocytes where an adhesion defect was uncovered for cells overexpressing the EP4 receptor.

Additionally, our investigations have demonstrated a novel synergy between angiotensin II AT1 receptors and prostaglandin E2 EP4 receptors. This was revealed by *in vitro* studies using isolated mouse glomeruli. There we were able to show that Ang II stimulation leads to increased expression of cyclooxygenase 2 (COX-2), the enzyme responsible for synthesis of PGE2, in a p38 mitogen activated protein kinase (MAPK) dependent fashion. Moreover increased PGE2 synthesis was measured in response to Ang II stimulation. We confirmed the presence of this synergy in our cultured mouse podocytes and showed an adhesion defect in response to Ang II stimulation which was COX-2 and EP4 dependent. These findings suggest that Ang II AT1 receptors and PGE2

EP4 receptors act in concert to exacerbate glomerulopathies. Studies using mice with either podocyte-specific overexpression of a dominant negative p38 MAPK or mice with global deletion of the EP1 receptor did not provide conclusive results as to their respective signaling involvement in podocyte injury. Altogether our findings provide novel insight for podocyte PGE2 EP4 and Ang II AT1 receptor signaling in models of CKD. These studies provide novel avenues for pursuing therapeutic interventions for individuals with progressive kidney disease.

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LIST OF ABBREVIATIONS

5/6 Nx	5/6 Nephrectomy
AA	Arachidonic acid
AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
ACR	Albumin to creatinine ratio
ADR	Adriamycin
AMDCC	Animal Models of Diabetic Complications Consortium
α-GBM GN	Anti-glomerular basement membrane glomerular nephritis
AMPK	5' adenosine monophosphate-activated protein kinase
Ang II	Angiotensin II
ANOVA	Analysis of variance
Arg	Arginine
ARB	Angiotensin II receptor blocker
Asp	Aspartate
AT1	Angiotensin II type 1
AT1-Ad	Angiotensin II type 1 adenovirus
AT2	Angiotensin II type 2
bp	Base pair
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
CD2-AP	CD2-associated protein
cDNA	Complimentary deoxyribonucleic acid
cds	Coding sequence
Cr	Creatinine
CKD	Chronic kidney disease
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1

COX-2	Cyclooxygenase-2
COX-3	Cyclooxygenase-3
CV	Cardiovascular
CVD	Cardiovascular disease
DME-low	Dulbecco's Modified Eagles-low
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EGPF	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EP1	E-prostanoid type 1
EP2	E-prostanoid type 2
EP3	E-prostanoid type 3
EP4	E-prostanoid type 4
Epods	Immortalized podocyte cell line
ESRD	End stage renal disease
FBS	Fetal bovine serum
FITC-inulin	Fluorescein Isothiocyanate-inulin
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
GFP	Green fluorescent protein
GN	Glomerular nephritis
GPCR	G-protein coupled receptor
HA	Hemagglutinin
HCl	Hydrochloric acid
H-2Kb	Mouse major histocompatibility complex
His	Histidine
hrs	Hours

Hz	Hertz
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
Ile	Isoleucine
ITS	Insulin, human transferring, selenous acid
JNK	c-Jun N-terminal kinase
Kb	Kilobase
kDa	Kilodalton
Kg	Kilogram
M	Molar
MAPK	Mitogen activated protein kinase
MCD	Minimal change disease
MEK1	MAP kinase kinase 1
mg	Milligram
mL	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
ng	Nanogram
nM	Nanomolar
NaOH	Sodium hydroxide
Non-TG	Non-transgenic
NSAID	Non steroidal anti-inflammatory drugs
Nx	Nephrectomy
O.C.T.	Optimal cutting temperature
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAN	Puromycin aminonucleoside
PAS	Periodic acid Schiff
PBS	Phosphate- buffered saline

PFA	Paraformaldehyde
pfu	Plaque forming unit
Pgc	Glomerular capillary pressure
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGEM	Prostaglandin E2 metabolite
PGF2	Prostaglandin F2
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	Prostaglandin I2 or prostacyclin
Phe	Phenylalanine
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
Pro	Proline
RAS	Renin angiotensin system
RBF	Renal blood flow
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean
siRNA	Short interfering ribonucleic acid
Spods	Immortalized podocyte cell line
SV40	Simian virus 40
TAg	T antigen
TBS-T	Tris buffered saline-polysorbate 20
TG	Transgenic
TGF-β	Tumor growth factor beta
TRPC6	Transient receptor potential cation channel, subfamily C
TxA2	Thromboxane
Tyr	Tyrosine
Val	Valine
VEGF	Vascular endothelial growth factor

Wt	Wildtype
WT1	Wilms' tumor suppressor 1
ZO-1	Zona occludin 1
α	Alpha
β	Beta
β-actin	Beta actin
β-gal	Beta-galactosidase
γ	Gamma
γ-interferon	Gamma interferon
δ	Delta
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

ACKNOWLEDGMENTS

First and foremost I would like to thank my husband, Graham. Thanks for being there Graham on the days when the results were good as well as for those days when nothing went right. You have been my rock through it all providing unwavering support even when I was a bear. Thank you, I love you.

I would also like to thank my supervisor, Chris Kennedy, whose mentorship over the past six years has been a constant source of guidance. Through your supervision I have gained innumerable experiences that will continue to serve me into the next chapter of my life. Thank you for all your encouragement.

To my advisory committee – Richard Hébert, Rashmi Kothary and Mario Tiberi, thank you for your guidance throughout my graduate studies. Your insights and contributions have always been greatly appreciated.

I wish to sincerely thank all the members of my family. Mom and Dad, thank you for your support through it all. You have always encouraged me to be the best I can be. To my brother and sister, John and Julielee, I wish you every success in life. And to Brenda and Gord, thank you for everything you've done for Graham and I and for always expressing an interest in my research.

Next, the studies presented in this thesis would not have been possible without the help of our many collaborators; Matthew Breyer (Vanderbilt-Ingram Cancer Center, Nashville, TN) for kindly providing us with $EP4^{flox/flox}$ mice and Barbara Vanderhyden (Ottawa Hospital Research Institute, Ottawa, ON) for assistance in generating $EP4^{pod+}$ mice. Robin Parks (Ottawa Hospital Research Institute, Ottawa, ON) for generating adenoviral constructs. Both Stuart Shankland (University of Washington Medical Center, Seattle, WA) and Karlhans Endlich (University of Greifswald, Germany) for their generous gift of immortalized podocyte cell lines. And Jeffrey McClintock (Children's Hospital of Eastern Ontario, Ottawa, ON) for helping us process tissue for electron microscopy.

I would also like to thank all the members of the Kennedy lab (past and present), especially Anthony Carter, whose expertise in mouse experimental surgery was instrumental in carrying out a number of our studies. Warmest thanks to the Animal Care and Veterinary Services staff, especially Eileen and Kim. Finally, thanks to the entire group at the Kidney Research Centre, the journey would not have been the same without you!

CHAPTER 1 – INTRODUCTION

1.1 Chronic kidney disease

The incidence of chronic kidney disease (CKD) is increasing, owed largely to our aging population and the near epidemic levels of type 2 diabetes (Coresh et al., 2007). The progression of CKD has been associated with renal failure and cardiovascular disease (CVD) (Ayodele & Alebiosu). Importantly, CVD is the leading cause of mortality in CKD (Yerram, Karuparthi, Hesemann, Horst, & Whaley-Connell, 2007). Chronic kidney disease can be defined as the sustained and gradual decrease in kidney function over time. Renal function is estimated by evaluating a number of variables including glomerular filtration rate and urinalysis. Urinalysis may examine an array of components to determine whether there is a disruption in homeostasis including pH, osmolality, red blood cells, protein, bilirubin, glucose, as well as a host of electrolytes. Furthermore, biopsy and imaging techniques allow for determination of renal injury. There are many risk factors for CKD including hypertension, diabetes, obesity, and smoking (Fox et al., 2004; Haroun et al., 2003; Y. Wang, Chen, Song, Caballero, & Cheskin, 2007). Presence of one or more of these risk factors will ultimately lead to increased glomerular capillary pressure (Pgc) to cause podocyte damage (**Figure1.1**). Podocyte damage results in a condition called proteinuria where there is leakage of proteins into the urine. Importantly, proteinuria correlates with CV (cardiovascular) morbidity and mortality (Boersma et al., 2008). Therefore, early diagnosis of CKD in the years to come will prove essential in stemming the flow of patients progressing to CV events and renal failure.

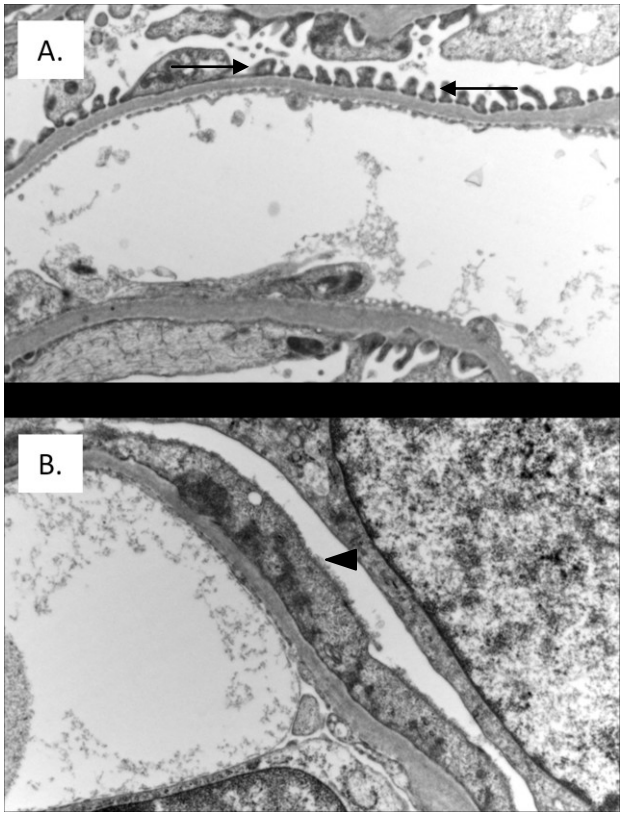


Figure 1.1 Electron microscopy images showing podocyte foot process effacement.

(A) Normal podocyte foot processes (arrows), (B) foot process effacement (arrow head) in mouse podocytes following induction of CKD using a subtotal nephrectomy model. Magnification, 15000X.

1.2 Glomerular structure and function

One of the major roles of the kidney is ultrafiltration of the blood. Resembling a mass of tiny tubules, the glomerulus is the filtering unit of the kidney (**Figure 1.2**). Each healthy kidney contains approximately one million glomeruli. Blood is filtered as it enters the glomerulus via the afferent arteriole, following its passage through the glomerular capillary loops; filtered blood exits the glomerulus through the efferent arteriole, through the medullarly plexus to the interlobular vein (Horacek, Earle, & Gilmore, 1986). At the level of the glomerulus, filtration occurs to restrict the passage of molecules based on their size, charge and shape. Water, electrolytes and small molecules are freely filtered out of Bowman's capsule as primary urine. There are three different components that make up the glomerular filtration barrier (GFB): a fenestrated endothelial layer, the glomerular basement membrane (GBM) and highly specialized epithelial cells called podocytes (**Figure 1.2**).

The porous endothelial layer of the GFB acts to prevent the movement of large substances such as red blood cells from moving into the urinary space (Palade, Simionescu, & Simionescu, 1979). The fenestrations are in fact transcytoplasmic holes approximately 60-70nm in diameter whose presence is dependent on VEGF (vascular endothelial growth factor) signaling initiated by podocyte VEGF production (Satchell et al., 2006). A proposed mechanism by which VEGF mediates endothelial fenestestrations is that VEGF, produced by the podocyte, activates its receptors in endothelial cells to induce cytoskeleton rearrangements through activation of the small GTPases, Rho and Rac (Wojciak-Stothard & Ridley, 2002; Yokomori et al., 2004). Activation of

endothelial VEGF receptors also promotes recruitment of plasmelemmal vesicle-associated protein-1 (PV-1). PV-1 is also thought to be involved in forming endothelial fenestrations (Ioannidou et al., 2006).

This first layer of fenestrated endothelial cells merely provides a coarse screen to hinder the passage of large plasma proteins. The second component contributing to the GFB is the glomerular basement membrane (GBM). The GBM is 240-370nm thick and composed of a number of proteins that come together to form a fibrous, mesh-like barrier. Some of the most abundant proteins present in the GBM include collagen IV, laminin and entactin, fibronectin (Laurie, Leblond, Inoue, Martin, & Chung, 1984). Another important component of the GBM is the negatively charged heparin sulfate proteoglycan groups, the most abundant of which are agrin and perelcan. It was initially thought that the presence of negative charges within the GBM was a major contribution to the filtration barrier, however more recent studies suggest a less central role for charge selectivity of the GBM (Batsford, Rohrbach, & Vogt, 1987; Bolton, Deen, & Daniels, 1998; Caulfield & Farquhar, 1974). Nevertheless, at the level of the GBM, large plasma proteins are physically barred from entry into the urinary space, smaller proteins such as the abundant 67kDa plasma protein, albumin, are still able to pass through.

The final and most selective component of the glomerular filtration barrier is formed by the highly differentiated epithelial cells called podocytes. It is as the level of the glomerular podocyte that movement of small plasma proteins across the glomerular filtration barrier is blocked. It is not surprising then that damage to this final specialized barrier accounts for many of the initial stages of glomerular injury that give rise to proteinuria.

1.3 Podocyte structure and function

As the final element of the GFB, podocytes are terminally differentiated epithelial cells that surround the glomerular capillaries. Podocytes do not completely encompass the capillary loops. Rather, primary processes extend from the podocyte cell body and further segregate into secondary foot processes (**Figure 1.3**). Podocyte foot processes adhere to the GBM and resemble pear shaped bodies when viewed using transmission electron microscopy (**Figure 1.1.A**). Podocytes are able to maintain this sophisticated architecture due to a highly structured actin cytoskeleton. Actin filaments project along the foot processes and in dynamic equilibrium allow for the movement and compensation of the foot processes in response to the pulsatile nature of glomerular capillary pressure. These actin filaments are bridged by the protein α-actinin-4. Work by several groups has demonstrated that mutations to the gene encoding α-actinin-4, *ACTN4,* lead to focal segmental glomerulosclerosis (FSGS) (Kos et al., 2003; Michaud et al., 2003). FSGS describes a set of glomerular lesions that are localized focally (not all glomeruli are affected) and segmentally in that the scarring pattern does not globally encompass those glomeruli that are damaged. Interdigitation of adjacent foot processes is responsible for the intricate filtration barrier provided by podocytes. The gap between neighbouring foot processes is termed the filtration slit and this 40nm space is bridged by a slit diaphragm. In 1998, nephrin was the first protein identified at the level of the filtration slit. Karl Tryggvason's group determined that nephrin, highly expressed in the podocyte, is a 180kDa transmembrane protein encoded by a gene (*NPHS1*) that is mutated in congenital nephrotic syndrome of the Finnish type (Lenkkeri et al., 1999). With this exciting

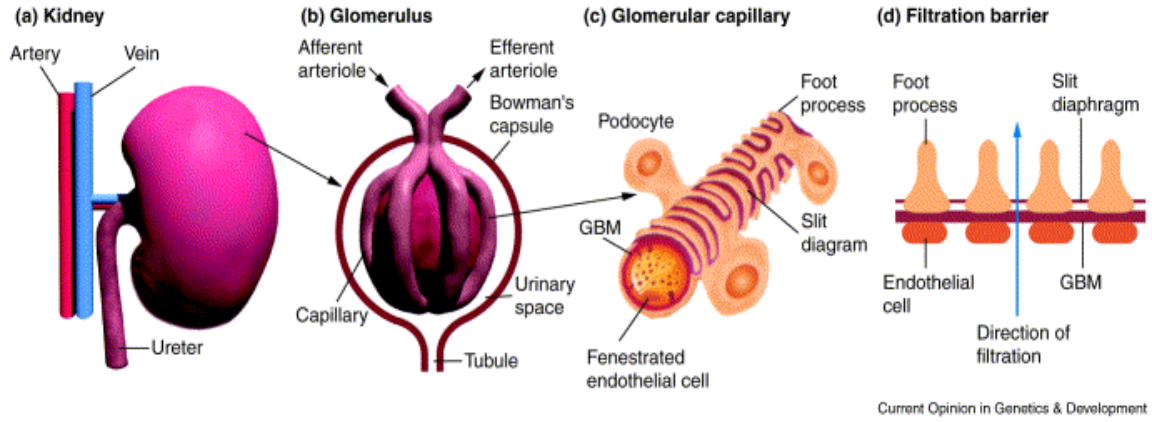


Figure 1.2 The kidney filtration system.

Within each kindey (A) there are are roughly one million glomeruli. Blood enters the glomerulus through the afferent arteriole, and filtered blood exits via the efferent arteriole (B). Chartieristic of glomerular capillaries is a fenestered endothelium, a glomerular basement membrane and the podocytes. Foot processes extend from the podocyte cell bodies (C). Together these components act to maintain the glomerular filtration barrier as illustrated in (D). *Figure adapted from* (Khoshnoodi & Tryggvason, 2001).

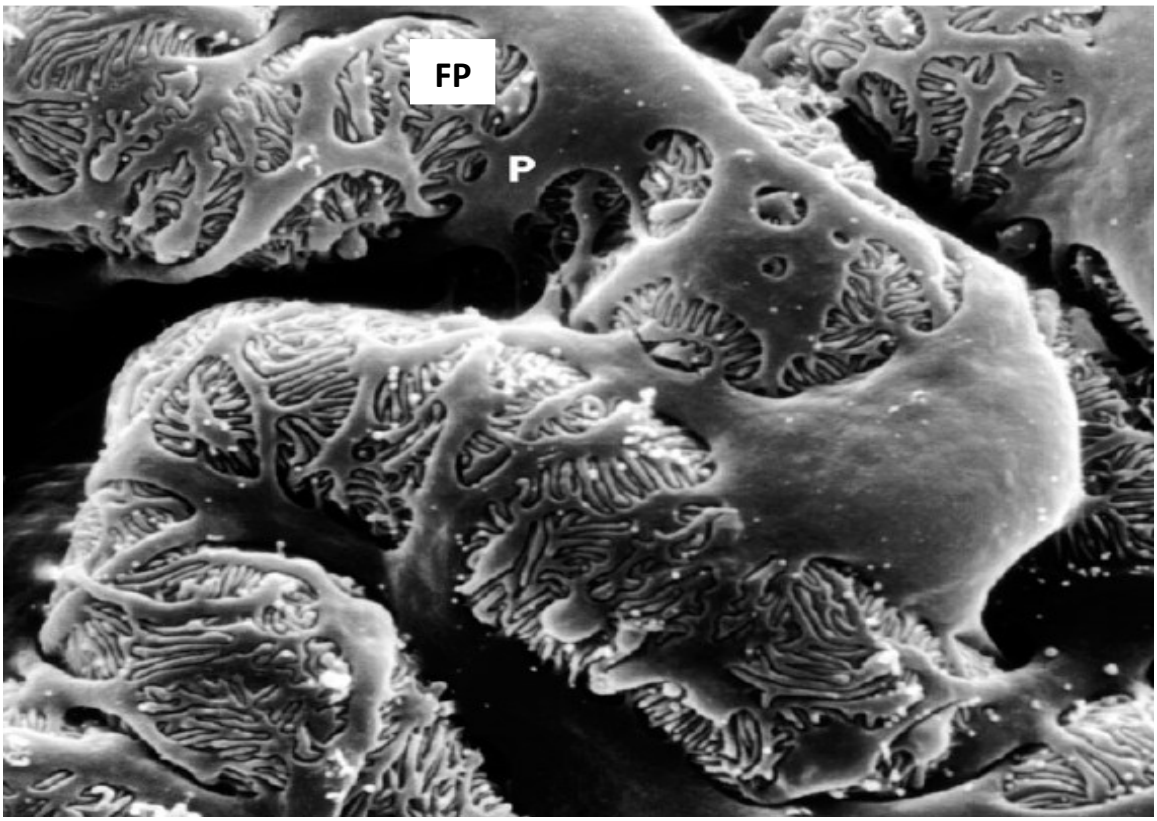


Figure 1.3 The podocyte.

Image obtained from scanning electron microscope, depicting podocytes surrounding the glomerular capillaries. Primary processes extend from the cell body (P) and further segregate into smaller secondary foot processes (FP) that interdigitate with neighbouring foot processes. Magnification ~6000X. *Adapted from (Pavenstadt, Kriz, & Kretzler, 2003).*

discovery, nephrin was deemed to be a critical transmembrane protein that bridges the filtration slit and works to maintain the barrier to protein (Ruotsalainen et al., 1999). Furthermore mice lacking nephin develop gross proteinuria, foot process effacement and die neonatally (Putaala, Soininen, Kilpelainen, Wartiovaara, & Tryggvason, 2001).

Together these findings point to a strong correlation between decreased neprhin expression, podocyte foot process effacement and increased proteinuria. Interestingly, nephrin interacts with a number of other proteins at the filtration slit such as podocin, CD2-associated protein (CD2-AP), the canonical transient receptor potential 6 (TRPC6) ion channel, P-cadherin, α- and β-catenin, NEPH1 and zona occludins-1 (ZO-1) (Barletta, Kovari, Verma, Kerjaschki, & Holzman, 2003; Reiser, Kriz, Kretzler, & Mundel, 2000; Reiser et al., 2005; Schnabel, Anderson, & Farquhar, 1990; Schwarz et al., 2001; Shih et al., 2001). Together these slit diaphragm proteins form what is described as a modified adherens junction (Reiser et al., 2000). Mutations affecting these proteins have also been linked to various kidney diseases including familial FSGS and congenital nephrotic syndrome (Fuchshuber et al., 1995; J. M. Kim et al., 2003; Shih et al., 1999).

While it is clear that nephrin provides a crucial physical barrier to protein, more recent findings highlight nephrin's involvement in podocyte signaling at the filtration slit. Major progress was made in terms of our understanding of nephrin signaling by the simultaneous discovery by two independent groups showing that the cytoplasmic tyrosine phosphorylation sites of nephrin serve as docking sites for the adapter protein Nck, which ultimately links nephrin to the actin cytoskeleton (Jones et al., 2006; Verma et al., 2006). Because podocyte foot processes are actin rich structures, these findings were pivotal in

providing the first link between nephrin signaling and foot process integrity (Tryggvason, Pikkarainen, & Patrakka, 2006).

What's more, a number of maladaptive endpoints have been identified for the podocyte in glomerular diseases including: foot process effacement; detachment from the GBM; apoptosis; hypertrophy; and dedifferentiation (E. Stitt-Cavanagh, MacLeod, & Kennedy, 2009; Ziyadeh & Wolf, 2008). In fact foot process effacement has been observed in many proteinuric glomerular diseases including: minimal change disease (MCD), glomerular nephritis (GN), FSGS and diabetic nephropathy (DN) (J. J. Li et al., 2007; Wolf & Ziyadeh, 2007). Additionally, studies have revealed that podocyte apoptosis results from increased TGF-β, Ang II, reactive oxygen species, and decreased p21 and p27 (cyclin dependent kinase (CKD) inhibitors) (Ding et al., 2002; Mundel & Shankland, 2002; Sanwal et al., 2001; Schiffer et al., 2001). Altogether, regardless of the injurious endpoint, damage to the podocytes ultimately results in proteinuria.

Proteinuria has been described as a marker of glomerular disease (Kriz, 2002; Morigi et al., 2005; Tryggvason & Pettersson, 2003). Hence, it is becoming increasingly evident that there is a tight link between the severity of foot process effacement, the amount of proteinuria and the progression of renal failure to end stage renal disease (ESRD). While the mechanistic link between proteinuria and glomerular disease remains elusive, one of the key players in this progression are the highly differentiated podocyte cells. With the incidence of kidney disease increasing and the availability of effective treatment limited, uncovering the role of podocytes in the development of glomerular injury is vital for developing novel therapeutic strategies.

1.4 Prostanoid signaling in the podocyte

In addition to their role as a physical component of the glomerular filtration barrier, podocyte cells possess the molecular machinery that allow for signaling events typical of epithelial cell lineages. One such signaling pathway characteristic of podocyte cells is the prostanoid cascade.

Overview of prostanoid signaling

The prostanoid singaling cascade begins with arachidonic acid (AA). Arachidonic acid is a polyunsaturated fatty acid found ubiquitously in phospholipids of cell membranes. Metabolism of AA is carried out by the enzyme, cyclooxygenase. There are at least two isoform members of the cyclooxygenase family, COX-1 and COX-2. Several studies have postulated the existence of a third isoform, COX-3, which is a splice variant of the gene encoding COX-1; however the physiological relevance of COX-3 is incompletely understood (Botting, 2000). The COX-1 isoform is thought to serve a "housekeeping" role, in that it maintains basic physiologic functions including protection of the gastrointestinal tract and promotion of platelet aggregation (Herschman, 1996; Smith, 1992; Smith & Langenbach, 2001). In contrast, COX-2 is thought to be the inducible isoform associated with angiogenesis, inflammation and tumorigenesis (Herschman, 1996; Hla, Bishop-Bailey, Liu, Schaefers, & Trifan, 1999; Smith, 1992; Smith & Langenbach, 2001).

For all isoforms, COX acts to convert AA to prostaglandin G2 (PGG2) and then to the unstable intermediate prostaglandin H2 (PGH2) in a two-step reaction. PGH2 is

quickly metabolized by specific synthases (PGE synthase, PGI synthase, PGF synthase, PGD synthase, and thromboxane synthase), to unique members of the prostanoid family (R. M. Breyer, Bagdassarian, Myers, & Breyer, 2001). Prostanoids are often segregated into two major categories, the prostaglandins and thromboxane (TxA2). There are four members of the prostagnalnind category: PGE2, PGI2 (also referred to as prostacyclin), PGD2, and PGF2α (**Figure 1.4**). Together these prostanoids are involved in a multitude of processes throughout the body both to maintain homeostasis as well as in the pathogenesis of disease. Due to the alacrity of metabolically driven prostanoid degradation, prostanoid physiological effects are extremely localized which accounts for their autocrine and paracrine signaling. Within the kidney, the arachidonic acid signaling cascade is vital for maintaining vascular tone, water and electrolyte balance and systemic blood pressure. Prostanoids are able to mediate such a wide range of effects due to their affinity for multiple G-protein coupled receptor subtypes. Furthermore, renal expression of COX-1 and COX-2 is not evenly distributed. COX-1 expression is highest in collecting duct with lower levels in the interstitial cells, mesangial cells, and within in the endothelium (Campean, Theilig, Paliege, Breyer, & Bachmann, 2003; Castrop, Schweda, Schumacher, Wolf, & Kurtz, 2001; R. C. Harris et al., 1994; Yang et al., 1998). By comparison, the highest levels of COX-2 are found in the medullary interstial cells, the cortical thick ascending limb, and the macula densa (Campean et al., 2003; Guan et al., 1997; Hao, Komhoff, Guan, Redha, & Breyer, 1999). As stated earlier, COX-2 expression is induced in response to stress. This was observed in studies performed by Wang et al., who showed induced COX-2 expression in the podocytes of rats following subtotal renal ablation (5/6 Nx) (J. L. Wang, Cheng, Zhang, McKanna, & Harris, 1998).

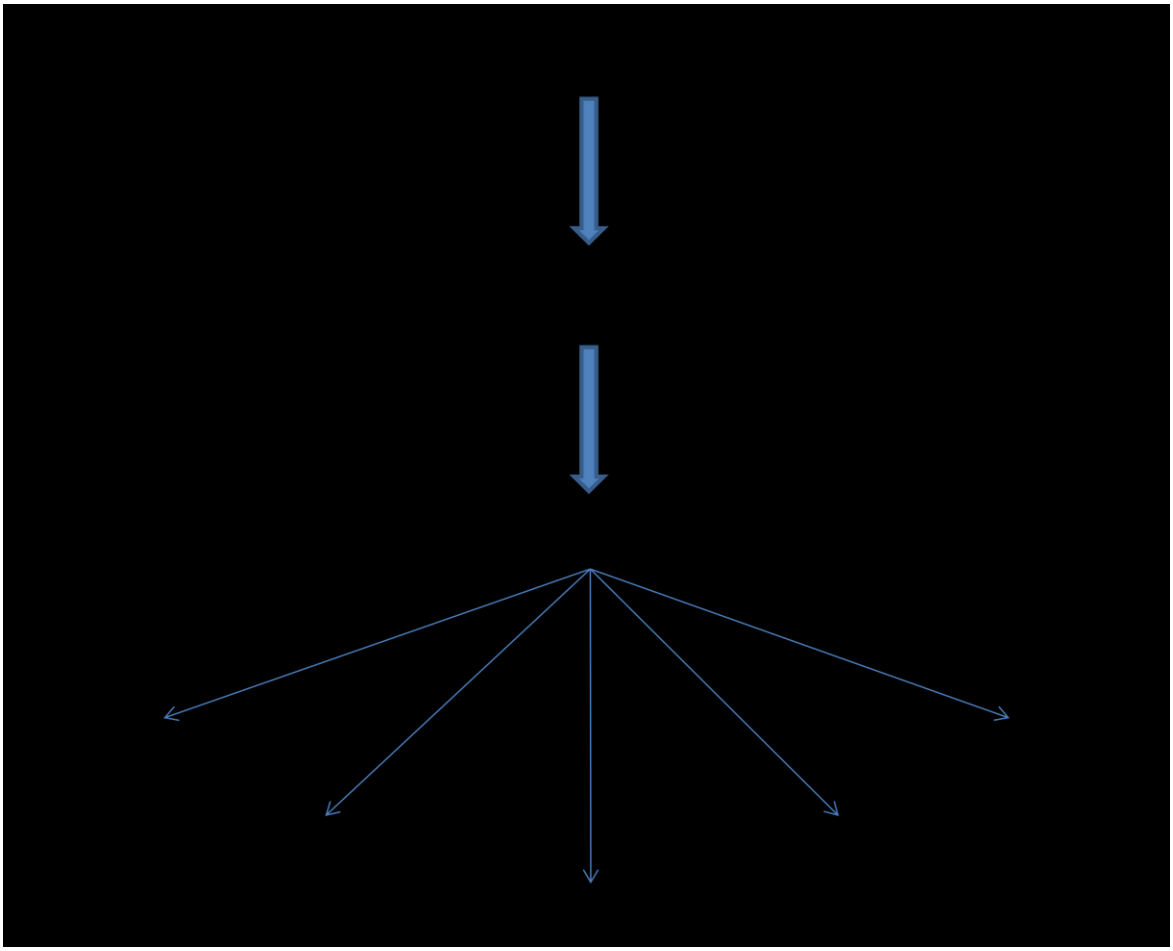


Figure 1.4 Prostanoid synthesis from arachidonic acid.

Isoforms of cyclooxygenase (COX), COX-1, COX-2, and COX-3 convert arachidonic acid to prostaglandin G2 (PGG2) and then to the unstable intermediate prostaglandin H2 (PGH2) in a two-step reaction. PGH2 is quickly metabolized by specific synthases (PGE synthase, PGI synthase, PGF synthase, PGD synthase, and thromboxane synthase), to PGE2, PGI2, PGD2, PGF2α and TxA2 respectively. Modified from (Taketo, 1998).

Furthermore, subtotally-nephrectomized rats treated with a selective COX-2 inhibitor displayed reduced glomerular PGE2 levels that correlated with improvements in proteinuria and glomerulosclerosis (J. L. Wang, Cheng, Shappell, & Harris, 2000).

Recall that inhibition of either COX-1 or COX-2 isoforms attenuates synthesis of at least five distinct prostanoids (PGE2, PGD2, PGI2, PGF2α, and TxA2) which interact with their respective G-protein coupled receptors (EP1-4, DP, IP, FP, and TP) to provoke a variety of physiological actions in the kidney and elsewhere. Within the kidney, COX inhibition reduces proteinuria. Unfortunately, use of COX inhibitors to treat proteinuric kidney disease is not feasible as they diminish vasodilatory prostanoid levels, resulting in reduced glomerular filtration rate (GFR) and renal blood flow (RBF) (M. D. Breyer & Breyer, 2001; Murray & Brater, 1993). Therefore, limiting prostanoid receptors that are pro-proteinuric, while retaining those necessary for normal GRF/RBF, may represent a potential therapy for preserving GFB function in renal disease.

Prostanoid receptors

Prostanoids are able to carry out a wide range of effects due to their ability to act through different G-protein coupled receptor (GPCR) subtypes. Specifically, PGE2, the most abundantly synthesized renal prostanoid, has four different E prostanoid (EP) receptors designated EP1-4. The EP1 receptor couples to a Gq protein resulting in an increase in intracellular Ca^{++} and protein kinase C (PKC) activation. In comparison, the EP3 receptor couples to a Gi protein to inhibit AC. Both the EP1 and EP3 receptors cause smooth muscle constriction. Lastly, the EP2 and EP4 receptors couple to a Gs protein resulting in activation of adenylate cyclase (AC). Adenylate cyclase increases

cAMP levels and protein kinase A (PKA) activity (Endlich & Endlich, 2002; Slipetz et al., 2001). Activation of EP2 and EP4 receptors results in smooth muscle relaxation.

Within the kidney, EP1 receptor is expressed in the collecting duct, microvasulature and podocytes. Guan et al., showed that two different EP1 antagonists, AH6809 and SC19220 were able to prevent intracellular calcium release. The AH6809, EP1 antagonist, was also effective in blocking the inhibitory effect of PGE2 on Na^+ absorpotion in micropurfused rabbit cortical collecting ducts. (Gorin et al., 2005; Guan et al., 1998). These findings suggest a natriuretic role for the EP1 receptor within the collecting duct.

Furthermore, induction of glomerular nephritis in EP1 null mice resulted in increased glomerular and tubular damage, increased serum creatinine, urea, Na^+, and K^+ compared to Wt mice. Despite the differences observed in EP1-/- mice in a glomerular nephritis milieu, Rahal et al., suggest that that mice lacking the EP1 receptor have a urine concentrating defect which is centrally mediated (Kennedy et al., 2007; Rahal et al., 2006). Given the nature of the EP1 receptor, one would speculate that its deletion would cause a hypotensive phenotype. This was indeed reported by Stock *et al*., (Stock et al., 2001). Further evidence to substantiate these findings were provided when $EP1^{-/-}$ mice receiving chronic infusion of the vasoconstrictor, angiotensin (Ang) II, displayed milder hypertension than Wt mice (Guan et al., 2007). Whether EP1 receptors within the podocytes are contributing to these pheonotypes remains to be elucidated.

The EP3 receptor is also expressed in the kidney, specifically in the collecting duct and thick ascending limb. $EP3^{-/-}$ mice are unable to concentrate their urine in

response to the COX inhibitor, indomethacin, suggesting a concentrating defect for $EP3^{-/-}$ mice (Fleming et al., 1998). Next, while EP2 receptor expression is low in the kidney, EP2 receptor deficient mice exhibit salt-sensitive hypertension (Kennedy et al., 1999). In wiltype mice, stimulation of EP2 receptors in the collecting duct leads to cAMP production prompting inihibtion of antidiurectic hormone. In EP2 null mice, this break on antidiuretic hormone is remove and leads to hypertension (Hebert, 1994).

In contrast to the other PGE2 receptors, renal expression of the EP4 receptor is highest within the glomerulus. Investigaing the role of the EP4 receptor has been challenging in the past since since EP4 receptor deficiency causes patent ductus arteriosus. Nevertheless, this receptor is thought to contribute to regulation of renal hymodynamics (M. D. Breyer & Breyer, 2000). Interestingly, activity of the EP4 receptor in the afferent arteriole of the glomerulus is proposed to act as a counter to the pressor effects exhibited by increased Ang II levels by stimulating localized vasodilatation (Tang, Loutzenhiser, & Loutzenhiser, 2000).

Podocyte prostaglandin E2 signaling

Therefore, while many factors undoubtedly contribute to the initiation and progression of podocyte injury and dysfunction, non-steroidal anti-inflammatory drugs (NSAIDs) reduce proteinuria suggesting that prostanoids derived from cyclooxygenase (COX1 & 2) activity may comprise a portion of the etiological mosaic (Velosa & Torres, 1986; Vriesendorp, Donker et al., 1986). More recent studies using selective COX-2 inhibitors (COXIBS) have provided mixed results. In a 2007 study of patients with diabetic nephropathy, Celecoxib was not effective in lowering proteinuria after 6 weeks

of treatment (Sinsakul et al., 2007). In contrast, more promising results were observed more recently when Rofecoxib was administered to renal patients for a 6 week time course (Vogt, de Zeeuw, Woittiez, & Navis, 2009). Further investigations are required to fully elucidate whether COXIBs may be used successfully to reduce proteinuria and whether these therapies are beneficial over extended periods of time. A more promising approach may be pursuing localized targets of COX-2 activity.

It is known that glomerular PGE2 synthesis is enhanced following subtotal renal nephrectomy. This suggests that signaling via one or more of its EP receptor subtypes contributes to the deleterious effects of COX-2 activity on GFB function (J. L. Wang et al., 1998). Podocytes may be the targets of these actions as they express both EP1 and EP4 receptors (Bek et al., 1999). Rational for this theory is supported by Cheng et al., who showed that podocyte-specific overexpression of COX-2 in mice renders them more susceptible to glomerular injury in models of minimal change disease (H. Cheng et al., 2007; Jo, Cheng, Wang, Moeckel, & Harris, 2007). Additionally, our group uncovered a novel feedback loop in cultured mouse podocytes whereby an *in vitro* surrogate for glomerular capillary pressure (Pgc) (i.e., mechanical stretch) along with PGE2 stimulation of the EP4 receptor, induces COX-2 in a p38 MAPK-dependent manner (Faour, Gomi, & Kennedy, 2008; Martineau, McVeigh, Jasmin, & Kennedy, 2004).

1.6 p38 mitogen activated protein kinase

Supporting the idea that the prostaglandin EP4 receptor is involved in damage to the podocytes, studies showed that one of the actions of the EP4 receptor is to activate the stress sensitive p38 MAPK. p38 MAPK was first identified in 1994 as a 38 kDa protein (hence its name). There are four isoforms of p38 MAPK (α, β, γ, δ) all encoded by different genes and mediating tissue specific responses including inflammation, apoptosis, growth and differentiation. p38α is the most widely expressed isoform with broad tissue expression. In contrast, the remaining p38 members are expressed in a more tissue-specific pattern with highest expression levels of p38β, p38γ, and p38δ found in the brain, skeletal muscle and endocrine glands respectively. The homogeneity between isoforms is 60%. p38 MAPK is activated in a number of glomerular diseases, including MCD, GN, and FSGS as well as by a number of stimuli including Ang II, oxidative stress, and TGF-β, endorsing p38 MAPK as a viable target for therapy (Jiang, Jia, Dai, & Li, 2006; Landry & Huot, 1995; Naito et al., 2004; Stambe, Nikolic-Paterson, Hill, Dowling, & Atkins, 2004). Moreover, *in vivo* studies using a rat model of α-GBM GN demonstrated that pre-treatment with a specific p38 inhibitor results in the prevention of renal injury and significantly lower levels of proteinuria (Stambe, Atkins, Tesch et al., 2003). These findings suggest that p38 MAPK may be another key player in the progression of glomerular injury, with inhibition of p38 offering protection for podocytes in glomerular disease.

Podocytes continuously cope with pulsatile glomerular capillary pressure (Pgc). *In vitro* stretch experiments designed to mimic increases in Pgc a condition characteristic

of glomerular diseases, revealed that in response to mechanical stimulation, p38 MAPK becomes activated (Martineau et al., 2004). Activation of p38 MAPK results in its translocation to the nucleus where it signals the increase in COX-2 and PGE2 EP4 receptor expression in a cAMP/AMPK dependent, but PKA-independent signaling cascade (Faour et al., 2008). Amplified COX-2 expression enhances synthesis of PGE2 from AA. In a feedback manner, PGE2 then stimulates the activation of EP4 which we have shown to both increase cAMP as well as activate p38 MAPK (Lemieux, Rahal, & Kennedy, 2003).

Taking the sum of these findings into consideration, it is possible that this PGE2 (EP4 receptor dependent), p38MAPK, COX-2 pathway may be mediating effects that compromise the glomerular filtration barrier.

1.7 The Renin angiotensin system

The renin angiotensin system (RAS) is a major regulator of vascular tone and volume and electrolyte balance (Peach, 1977). The RAS pathway has been extensively researched since Tigerstedst's discovery of the proteolytic protein renin in 1889 (Marks & Maxwell, 1979). The classical RAS pathway begins with the production of angiotensinogen by the liver. Angiotensinogen is an inactive precursor that is cleaved to the biologically active decapeptide angiotensin I via the enzyme renin. Renin is produced by the juxtaglomerular cells of the kidney. Interestingly, in select strains of mice, renin is encoded by 2 autonomous genes, *Ren*-1 and *Ren*-2 (Piccini, Knopf, & Gross, 1982). Angiotensin I (Ang I) is further cleaved to the octapeptide, angiotensin II (Ang II) (Asp^{1}–

Arg^2–Val^3–Tyr^4–Ile^5–His^6–Pro^7–Phe^8), the primary effector of the RAS pathway. Cleavage of Ang I to Ang II is catalyzed by the lung derived dipeptidyl carboxypeptidase, angiotensin converting enzyme (ACE). More recently, angiotensin converting enzyme 2 (ACE2) was identified as a novel mono-carboxypeptidase member of the RAS family. As a homologue of ACE, it catalyzes the degredation of angiotensin II to Ang-(1-7), additionally ACE2 promotes the production of Ang-(1-9) from Ang I (**Figure 1.5**) Physiologically, it appears that ACE2 activity buffers the deleterious actions of angiotensin II by limiting its levels and additionally via the signaling of Ang-(1-7) through its cell surface MAS receptor (Dilauro & Burns, 2009). A number of immunohistochemical studies of human and rodent cortical sections have localized ACE2 and the MAS receptor in podocytes and mesangial cells (Ye et al., 2006; Zimpelmann & Burns, 2009).

Ang II mediates its effects through its two GPCR subtypes, Ang II type 1 (AT1) receptors and Ang II type 2 (AT2) receptors (**Figure 1.5**). Both Ang II AT1 and AT2 receptor subtypes couple to Gq/11, G12/13 Gi/o proteins (extensively reviewed by de Gaspara et al., (de Gasparo, Catt, Inagami, Wright, & Unger, 2000)). Similar to renin, mice and rats have two isoforms of the AT1 receptor ($AT1_A$ and $AT1_B$) while humans only have a single AT1 isoform (Yoshida et al., 1992). Taken together it is postulated that these genetic variations might explain, in part, the profound resistance rodents display in response to renal injury. Within the kidney, stimulation of Ang II receptors induces both arteriolar vasoconstriction and proximal tubule sodium and water retention (P. J. Harris, 1992; Navar, Saccomani, & Mitchell, 1991). Additionally Ang II stimulated aldosterone release promotes sodium reabsorption at the distal tubule.

While Ang II is able to activate both AT1 and AT2 receptors, it is known that activation of these receptors mediates diverse pathways. During pathological conditions, it is largely believed that activation of AT2 receptors, antagoniszes AT1-dependent maladaptive signaling (Nakajima et al., 1995). More recent investigations have determined that AT1 receptor is involved in regulation of blood pressure as well as growth-promoting and fibrotic effects (Mezzano, Ruiz-Ortega, & Egido, 2001; Ruiz-Ortega, Ruperez, Esteban, & Egido, 2003). The AT2 receptor on the other hand, participates in cell growth inhibition and sodium reabsorption (Touyz & Schiffrin, 2000). During renal injury, AT2 receptor expression increases to promote tissue remodeling or inflammation (Ruiz-Ortega, Esteban et al., 2003; Touyz & Schiffrin, 2000). In contrast, in response to inflammation, the AT1 receptor regulates proinflammatory genes (ie. cytokines, chemokines, and adhesion molecules) (Ruiz-Ortega, Ruperez et al., 2003).

Significantly, with respect to patients with chronic kidney disease, Ang II receptor blockers (ARBs) such as losartan and candesartan as well as ACE inhibitors are effective agents in reducing proteinuria and maintaining renal function (as reviewed in (Taal & Brenner, 2000)). However the therapeutic results of ARB and angiotensin converting enzyme inhibitor (ACEi) treatment exceed that which could be explained by hemodynamic actions alone. This raises the question as to whether localized RAS signaling events may account for the improved outcome in chronic kidney disease. In fact, in recent years, several studies have demonstrated the existence of locally acting renin angiotensin systems (reviewed in (Durvasula et al., 2004; Durvasula & Shankland, 2008; Paul, Poyan Mehr, & Kreutz, 2006; Sato et al., 2008). In the podocyte, it is known that mechanical stretch (a mimic of enhanced Pgc) results in increased Ang II expression

which in turn mediates a host of effects which contributes to a defective filtration barrier, including apoptosis, increased TGF-β, and reduced nephrin expression (S J Shankland, 2006). Additionally, transgenic rats with podocyte-specific overexpression of the AT1 receptor become proteinuric suggesting the renoprotection effects of ARB treatment.

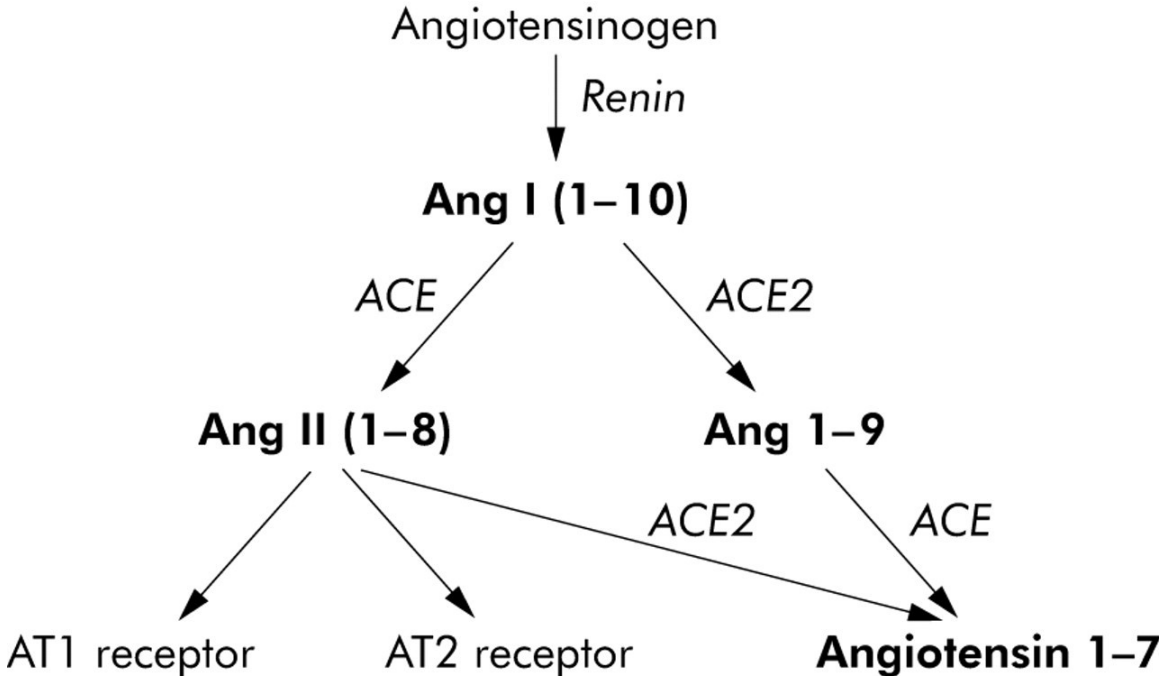


Figure 1.5 The renin angiotensin system.

Schematic representation of the renin angiotensin system (RAS). Angiotensinogen is cleaved to angiotensin I (Ang I (1-10))by renin which is further cleaved by angiotensin converting enzyme (ACE) to form the bioactive angiotensin II (Ang II (1-8)) which acts on its respective G-protein coupled receptors, AT1 and AT2. Ang I (1-10) is also cleaved by angiotensin converting enzyme 2 (ACE2) to produce Ang 1-9. ACE2 and ACE are both responsible for production of Angiotensin 1-7 from either Ang II or Ang 1-9. (Figure modified from (Paizis et al., 2005)).

1.8 Rationale

While it is known that damage to the podocytes is one of the first hallmarks of chronic kidney disease, the molecular mechanisms by which podocyte injury occurs is not fully elucidated. Evidence for involvement of a PGE2 signaling cascade is building following studies showing that mice with podocyte-specific overexpression of COX-2 are more susceptible to glomerular injury. However, while podocytes are known to express both EP1 and EP4 receptors; their involvement in the progression of chronic kidney disease is not completely understood.

Moreover, the recent discovery of a locally activing renin angiotesin system implies that signaling though Ang II receptors (AT1 and/or AT2), may contribute to podocyte injury in glomerular pathologies. This theory is further substantiatied by previous studies showing that overexpression of the AT1 receptor in rats leads to podocyte foot process effacement.

Importantly, it is known that mechanical stretch of cultured mouse podocytes leads to increased AT1 receptor expression alongside, rapid p38 MAPK activation. Our group previously showed that p38 MAPK activation in response to mechanical stretch caused amplified COX-2 expression and increased PGE2 synthesis to further stimulate EP4 receptors.

Accordingly, while the pathological relevance of podocyte PGE2 and Ang II signaling is unknown, studies aimed at investigating their involvement in the progression of glomerular injury are pivotal for improving the current pharmacological therapies.

1.9 Objectives

Objective 1 – Investigate the role of the podocyte PGE2 EP4 receptor in a mouse model of glomerular injury.

Objective 2 – Determine whether there is crosstalk between a local podocyte renin angiotensin system and podocyte PGE2 EP4 receptors.

Objective 3 – Develop mice with podocyte-specific overexpression of a dominant negative p38 MAPK.

Objective 4 – Study the involvement of the EP1 receptor in models of glomerular injury.

1.10 Hypothesis

Prostaglandin E2 and angiotensin II mediated signaling contributes to podocyte injury and leads to a compromised glomerular filtration barrier.

1.10 Relevance

Selective COX-2 inhibitors (COXIBs) such as Celebrex and Vioxx (discontinued) have proven effective in decreasing proteinuria in patients with chronic kidney disease. Unfortunately these drugs are contraindicated due to their association with adverse cardiovascular events as well as their long-term capacity to lower glomerular filtration rate and renal blood flow. The goals of the studies described within this thesis are directed toward providing novel targets under influence of COX-2 activity. The pursuit of unique targets able to provide therapeutic relief to current and future patients whose quality of life is compromised due to chronic kidney disease is the paramount objective. Advantages of this approach are that it encompasses a wide range of pathologies whose final end point is chronic kidney disease, including hypertension and diabetes.

1.11 Thesis content

The first chapter of this thesis (Chapter I) consists of a general introduction introducing glomerular/podocyte biology as well as prostaglandin E2 and angiotensin II. Chapter II describes the methodologies and materials used to carry out our studies. Chapter III contains an investigation of the role of prostaglandin E2 EP4 receptors in the progression of glomerular disease. Chapter IV looks at signaling crosstalk between prostaglandin EP4 receptors and a local podocyte renin angiotensin system. Chapter V investigates the involvement of the p38 mitogen activated protein kinase pathway in the development of podocytes injury. Chapter VI focuses on the prostaglandin E2 EP1 receptor and its role in the progression of glomerular injury. Finally, Chapter VII addresses major findings in a discussion format including relevance and future avenues for therapy.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Podocyte cell culture

Conditionally immortalized cell lines

Since podocytes are terminally differentiated, to study them in culture, conditionally immortalized podocyte cell lines must be generated. Importantly, it is known that the simian virus 40 (SV40) large tumor antigen (TAg) is able to immortalize many different cell types. Due to the increased risk of tumorigenesis associated with TAg expression, a thermolabile antigen, tsA58, is used instead to restrict TAg expression *in vivo*. Therefore, by generating transgenic mice expressing the SV40 tsA58 gene under control of the mouse major histocompatibility complex H-2Kb promoter (H-2Kb-tsA58 mice); many different immortalized cell types can be generated (Jat et al., 1991). In order to specifically generate immortalized podocyte cell lines, glomeruli are isolated from 10 week old H-2Kb-tsA58 transgenic mice, cultured and podocyte outgrowths are cloned (Mundel et al., 1997).

Two conditionally immortalized mouse podocyte cell lines were used for our *in vitro* studies. The first cell line was a kind gift from Dr. Karlhans Endlich, University of Greifswald, Greifswald, Germany (Epods), and the second cell line was generously provided by Dr. Stuart Shankland's laboratory, University of Washington, Seattle (Spods). Both cell lines were cultured as previously described (Endlich et al., 2001; Mundel et al., 1997). Briefly, cells were grown on collagen I coated culture plates (0.1 mg/ml; Sigma-Aldrich, Saint Louis, MI) in RPMI-1640 media supplemented with 10% (fetal bovine serum, Invitrogen Corporation, Carlsbad, CA), 100 μg/ml normocin (Cedarlane Labortories Ltd., Hornby, ON), and penicillin-streptomycin solution (1:100; Invitrogen). Podocytes were propagated at 33ºC in the presence of 10 U/ml recombinant

mouse γ-interferon (Invitrogen). To induce podocyte differentiation, cells were maintained at 38ºC for fourteen days in the absence of γ-interferon. Several key events occur during podocyte differentiation, the cells exit the cell cycle and cease to proliferate, the "cobblestone" appearance is exchanged for a more elaborate cytoskeleton with major processes extending from the cell body, and finally specific differentiation markers are expressed. One such differentiation marker used to assess podocyte differentiation is synaptopodin (Mundel et al., 1997).

Primary podocyte cultures

Primary podocyte cultures were generated as previously described (S. J. Shankland, Pippin, Reiser, & Mundel, 2007). Briefly, glomeruli were isolated from either $EP4^{flox/flox}$ or $EP4^{pod-/-}$ mice using magnetic DynalBeads (Invitrogen) as previously described (Takemoto et al., 2002). To this end, mice were anesthetized with isoflurane and perfused with 20 ml ice-cold phosphate buffered saline (PBS)/DynalBeads through the left ventricle. A small cut was made in the right atrium to allow the escape of return circulation. Kidneys were harvested from mice, de-capsulated, and the poles were removed and tissue was minced to facilitate digestion. Minced kidney was then digested with collagenase and filtered using a 100μm cell-strainer (BD Biosciences, Franklin Lakes, NJ). Isolated glomeruli were next seeded onto collagen I coated plates in 1:1 K-1 Media (Fisher Scientific, Ottawa, ON) : media harvested from cultured NIH-3T3 cells. K-1 Media contained Ham's F-12, nutrient mixture, DME (Dulbecco's Modified Eagle's)-low glucose (Fisher Scientific), 10% FBS, 0.1% ITS (insulin, human transferrin, selenous acid) Premix (Fisher Scientific), 1% sodium bicarbonate (Fisher Scientific) and

1% penicillin-streptomycin (Invitrogen). To confirm successful podocyte isolation, RNA was extracted from cultured podocytes and expression of the podocyte marker, Wilm's Tumor 1 (WT1) was assessed. Assaying for cAMP or for genomic DNA isolation was performed 7-10 days following isolation.

2.2 Adenoviral constructs and infection of cultured podocytes

EP4 receptor adenovirus

An adenoviral construct containing full length mouse double hemagglutinin (HA) tagged EP4 receptor sequence was generated by the University of Ottawa Viral Vector Core Facility. Briefly, a human cDNA coding sequence (cds) clone was obtained from Invitrogen via the NIH Mammalian Gene Collection (IMAGE Id 8069040; Accession BC101534). The EP4 cds (1621bp) was subcloned out of its host plasmid (pCMV-Sport6) into pcDNA3 using EcoRI / Xho I restriction enzyme sites. An N-terminal double HA tag was introduced by PCR-based cloning (**Table 2.1**).

For adenovirus production, the HA-tagged EP4 construct was subcloned into pShuttle-CMV (Stratagene, La Jolla, CA). This shuttle vector undergoes homologous recombination in bacteria with pAdEasy-1 to constitute the recombinant adenoviral plasmid. Linearized recombinant adenoviral plasmid was transfected into AD-293 cells to obtain a primary viral stock, which was amplified, purified and titred. To carry out the infection, podocytes were incubated with adenovirus using a minimal volume of serum free RPMI (Roswell Park Memorial Institute). Following 1 hour of incubation at 37°C,

infected cells were supplemented with RPMI containing 10% FBS. To optimize infection conditions differentiated mouse podocytes were infected with EP4 adenovirus at a 0-100 MOI (multiplicity of infection) for 72 hrs. Multiplicity of infection was caluculated based on the adenoviral titre (plaque forming units/ml). Cell lysates were then prepared by washing podocytes 2x with ice-cold PBS followed immediately by lysis. Lysis buffer (50mM Tris base, 150mM sodium chloride, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and TritonX 100). Protease inhibitors such as PMSF (phenylmethanesulfonylfluoride; Sigma-Aldrich), and protease inhibitor cocktail (Sigma-Aldrich) were added fresh. Following lysis, samples were then centrifuged at 16,000 x *g* for 15 minutes and supernatants collected. Equal quantities of protein (10 μg) lysate were loaded onto 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) resolving gel. Gels were run at 125 V using the Bio-Rad Power Pac 300 and then electro-transferred to Hybond-ECL nitrocellulose membrane (Fisher Scientific). After performing the electro-transfer, membranes were incubated in blocking buffer (5% milk in tris buffered saline Polysorbate 20 [TBS-T]) for 1 hour at room temperature. Following the blocking step, membranes were left to shake gently overnight at 4ºC with antibodies against either COX-2 (1:1000; Cayman Chemical, Ann Arbor, MI), HA-7 (1:1000; Sigma-Aldrich), or β-actin (1:1000; Sigma-Aldrich). Following overnight incubation with respective primary antibodies, membranes were washed in TBS-T and left to incubate under gentle shaking conditions for 1 hour at room temperature with anti-rabbit IgG horseradish peroxidase which served as the secondary antibody (1:1000; Cell Signaling Technologies, Danvers, MA). Finally, proteins were detected using SuperSignal West Fempto Maximum Sensitivity Substrate (Biolynx Inc., Brockville, ON)

and exposed to Kodak X-Omat Blue film for the appropriate exposure time. For all subsequent experiments, podocytes were infected at an MOI of 50.

AT1 receptor adenovirus

An adenoviral construct containing full length mouse double hemagglutinin (HA), Myc/Flag tagged AT1 receptor sequence was generated by Dr. Robin Parks (Ottawa Hospital Research Institute, Ottawa, ON). Briefly, a human cDNA coding sequence (cds) clone with N-terminal Myc and FLAG tags was obtained from Origene (Accession NM_000685.3). The AT1 cds was subcloned out of its host plasmid (pCMV6 Entry) into pcDNA3 using SgfI / MluI restriction sites. An N-terminal double HA tag was introduced by PCR-based cloning (**Table 2.1**).

For adenovirus production, the pcDNA3-2xHA-AT1-Myc-FLAG construct was subcloned. 2xHA-AT1-Myc-FLAG-Adenovirus (AT1-Ad) contains 2xHA-AT1-Myc-FLAG cDNA under regulation of the cytomegalovirus immediate-early promoter/enhancer and bovine growth hormone polyadenylation sequence. For the AT1-Ad, the AT1 expression cassette replaced the E1 region and transcription is directed rightward, relative to the conventional human adenovirus serotype 5 map. Additionally, our E1-deleted, first-generation adenovirus vector for the AT1 receptor was constructed by way of conventional cloning techniques and RecA-mediated recombination (Chartier et al., 1996; He et al., 1998). Following AT1 adenoviral construction, virus was grown and titered on HEK 293 cells, as previously described (Ng & Graham, 2002).

For cell culture experiments using the adenovirus for AT1 receptor (AT1-AD), differentiated mouse podocytes were infected with AT1 adenovirus at an MOI of 100 for

72 hrs. Cells were then stimulated with Ang II at a dosage of either 1μM or 100nM for 6 hours. Western blotting was carried out as described above. For experiments using inhibitors, p38 MAPK (SB202190), c-Jun N-terminal kinase (JNK; SP600125), MAP kinase kinase 1 (MEK1; PD98059), and apocynin were used at 1μM (Sigma-Aldrich) and the COX-2 inhibitor (NS-398; Cayman Chemical) was used at a concentration of 1μM.

2.3 cAMP assay

Cell lines

Cells were grown for 14 days on 12-well cluster dishes at 38˚C. Following 10 minutes of pre-incubation with 5μM indomethacin (to block endogenous prostanoid production; Sigma-Aldrich) and 0.5mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), podocytes were stimulated with 1μM PGE2 (Cayman Chemical) for 10 minutes. cAMP levels were determined by ELISA (enzyme-linked immunosorbant assay; Cayman Chemical). Briefly, samples were lysed in 0.1M HCl at room temperature for 30 minutes with gentle shaking. Undiluted supernatants were then directly assayed following the manufacturer protocol.

Glomerular isolation

All glomeruli were isolated as described above. Isolated glomeruli were then resuspended in RPMI containing 0.1% FBS. Following 10 minutes of pre-incubation

with 5μM indomethacin and 0.5mM IBMX, glomeruli were stimulated with 1μM PGE2 for 10 minutes. cAMP levels were determined by ELISA.

2.4 PGEM Assay

Due to the short half life of PGE2, PGEM, its stable metabolite was used as a surrogate marker. PGEM levels were determined by ELISA (Cayman Chemical). To this end, urine and glomular samples were derivitized directly following stimulation at 37°C for 24 hours to ensure that all PGE2 products were metabolized to PGEM. Following derivitization, samples were assayed directly following the manufacturer protocol.

2.5 Adhesion studies

Differentiated podocytes (14 days of differentiation) were seeded onto six-well collagen I-coated stretch plates (Flexcell Int., Hillsborough, NC) for 3 days. Plates were mounted onto vacuum based loading docks of the Flexcell FX-4000T apparatus (Flexcell Int.). Podocytes were incubated with 1μM PGE2 in RPMI containing 0.5% FBS and subjected to 12% elongation at a frequency of 0.5 Hz for 24 hrs in the presence of 5μM indomethacin. Non-stretched cells (control) were exposed to identical experimental conditions but without mechanical stretch. Following mechanical stretch, media was

collected and non-adhering cells were counted using a hemocytometer. Adherent cells were similarly quantified following trypsinization.

2.6 Generation and genotypic analysis of EP4^{pod+} transgenic mice

A construct containing the EP4 receptor (EP4^{del355}) but lacking the nucleotides that encode the 133 C-terminal amino acids was engineered and cloned into pcDNA 3 as described previously (Slipetz et al., 2001). An N-terminal double hemagglutinin (2xHA) tag was cloned in frame with the truncated receptor open reading frame using overlapping and complementary oligonucleotides (**Table 2.1**). An 8.3 Kb fragment of the mouse nephrin (*NPHS1*) promoter was cloned immediately upstream of the 2xHA-EP4^{del355} sequence and included the 5' untranslated and 5' flanking regions of the mouse *NPHS1* gene. Briefly, the plasmid was cut by restriction enzyme digestion with *Acc65I* and its 5' nucleotide overhang subsequently filled in and blunted with T4 DNA polymerase and the plasmid was then digested by *HindIII*. The 8.3 kb *NPHS1* promoter fragment was likewise excised in a stepwise procedure from its pcDNA3 host vector using XhoI and blunted as above with T4 DNA polymerase followed by *HindIII* restriction enzyme digestion. The *HindIII* / blunt end fragment was cloned into pcDNA3-2xHA-EP4^{del355} to generate p*NPHS1*-2xHA-EP4^{del355}. This *NPHS1*-2xHA-EP4^{del355} transgene was linearized with *Hin*d*III/RsrII* and isolated by agarose gel electrophoresis / QIAEX II gel extraction. Following preparation of the *NPHS1*-2xHA-EP4^{del355} transgene, Dr. Barbara Vanderhyden (Ottawa Hospital Research Institute, Ottawa, ON)

performed the microinjection into FVB/N mouse embryos. Embryos were then surgically transferred to the oviduct of pseudopregnant CD1 recipient female mice. Both the embryo donor mice and the recipient mice were purchased from the Jackson Laboratory, Inc. (Bar Harbor, ME). Genotyping of resulting pups was performed by PCR of genomic DNA isolated from tail snips of 3 week old mice (see **Table 2.1** for primers). A total of seven candidate founders were identified. Following determination of the highest expressing founders, two lines were initiated and maintained through intercrossing with non-TG FVB/N mice. Both EP4^{pod+} lines yield healthy, fertile, hemizygous transgenic offspring at the predicted Mendelian ratio (1:1).

2.7 Generation of CreEGFP^{pod+} mice

The CreEGFP plasmid was obtained from Dr. David Threadgill (University of North Carolina) and was constructed as follows: pBluescript containing the coding sequence for Cre-recombinase was enzymatically digested with *XhoI* and *BamHI* to isolate a 5' fragment of the Cre coding sequence (1068 bp). A corresponding 3' fragment of Cre, lacking the stop codon was isolated from pnCre-noStop following *BamHI* and *ApaI* restriction enzyme digestion. These two pieces were inserted into pEGFP-N2 (Clontech, Mountain View, CA) at the *XhoI* and *ApaI* sites to generate pCreEGFP. The CreEGFP sequence was subcloned into a pCDNA3.1 plasmid containing an 8.3 kb fragment of the 5' mouse nephrin promoter (pCDNA-*NPHS1*) as follows: the entire CreEGFP sequence was excised from the plasmid following *BglII* and *NotI* digestion.

This fragment was blunted with T4 DNA Polymerase and cloned into pCDNA-*NPHS1* to generate p*NPHS1*-CreEGFP. Orientation was verified by automated sequencing. The transgene was resolved as an 11.3 kb fragment following restriction digestion with *HindIII* and *FspI* and includes the BGH polyadenylation signal sequence, and purified using a QiaEX II kit according to the manufacturer's instructions (QIAGEN, Mississauga, ON). Following pronuclear injection of the *NPHS1*-CreEGFP transgene into oocytes, and subsequent implantation into pseudopregnant females, 3 founder mice were obtained as determined by PCR-based genotypic analysis of tail snip genomic DNA (isolated with a Qiagen tissue DNA purification kit) using Cre-specific primers (**Table 2.1**). Subsequent backcrossing to wild type C57Bl/6J mice yielded healthy, fertile, hemizygous transgenic offspring at the predicted Mendelian ratio (1:1). Mice were subsequently backcrossed for 10 generations to obtain a congenic FVB/N CreEGFP^{pod+} line.

2.8 *In vivo* expression and catalytic activity of CreEGFP

Kidneys from 6 week-old non-transgenic (non-TG) wild type and CreEGFP^{pod+} mice were dissected, fixed with 4% paraformaldehyde (PFA), and embedded in paraffin wax. 7μm sections were processed for immunofluorescence using an FITC-conjugated mouse monoclonal anti-GFP antibody (Santa Cruz, Santa Cruz, CA; 1:100 dilution). Immunofluorescence was detected with a Zeiss Axioskop 2 fluorescence microscope (Zeiss Axioskop 2 MOT, Zeiss Germany) and images captured with a Zeiss AxioCam.

Next to determine whether the CreEGFP fusion protein could efficiently catalyze the excision of loxP-containing DNA sequences *in vivo*, CreEGFP^{pod+} founder mice were bred with homozygous ROSA26 mice (*R26R*), a reporter line that expresses LacZ upon Cre-mediated recombination (Soriano, 1999). More specifically, this mouse line contains 2 loxP sites inserted into the ROSA26 locus so that no functional β-galactosidase (β-gal) is expressed. Excision of the loxP-flanked DNA sequence by Cre restores β-gal expression (Soriano, 1999; Zambrowicz et al., 1997). Following appropriate intercrosses, the genotype of CreEGFP^{pod+}/ROSA26 compound mice were determined by PCR using the Cre-specific and ROSA26-specific primers (**Table 2.1**). Kidneys from double positive 6 week-old compound transgenic mice were dissected and processed for X-gal staining as follows: decapsulated kidneys were immersed in 0.1 M NaH_2PO_4 / 4% PFA solution (pH 7.3) for 1 hr at 4ºC and were subsequently washed in phosphate buffer containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40. Samples were then placed in X-gal staining solution (1mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$) for 6 hrs at 37ºC, followed by three brief washes in PBS. Kidneys were post-fixed for 24 hrs in 0.1 M NaH_2PO_4 / 4% PFA solution (pH 7.3) at 4ºC. Whole kidneys from ROSA26 and CreEGFP^{pod+} mice were grossly sectioned by razor blade and visualized under low power light microscopy.

2.9 EP4$^{flox/flox}$ mice

EP4$^{flox/flox}$ mice were obtained from Dr. Matthew Breyer (Vanderbilt University, Nashville, TN). These EP4$^{flox/flox}$ mice are characterized by flanking loxP sites on exon 2 of the EP4 gene. Using this mouse allows for generation of a conditional EP4 knockout (under influence of Cre recombinase activity). Importantly, loxP sites do not affect wildtype EP4 receptor in the absence of Cre recombinase (Schneider et al., 2004).

2.10 EP4$^{pod-/-}$ mice

EP4$^{pod-/-}$ mice were generated by intercrossing CreEGFP^{pod+} mice with EP4$^{flox/flox}$ mice. Genomic DNA was isolated from cultured podocytes using the QIAamp DNA Mini Kit (QIAGEN). In order to determine whether Cre recombination had occurred in podocytes, primers were designed to flank exon 2 of the EP4 receptor (**Table 2.1**). Generation of a 200bp PCR product was indicative of successful recombination.

p38αDN-TG mice

To generate mice with podocyte-specific overexpression of a dominant negative (DN) p38α isoform (p38αDN mice) Dr. Philipp Scherer (University of Texas, Dallas, TX) kindly provided the mouse p38α construct. Briefly, site-specific mutations were introduced within the p38α coding sequence (cds) at critical amino acids of the phosphorylation rich kinase domain of the protein (T180A; Y182F) (p38αDN). A C-terminal FLAG tag (-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was introduced for facilitated detection of the mutated p38α (p38αDN-FLAG). The resulting fragment was cloned into

the pOPI3 vector at *NotI*. Next, a double hemagglutinin (2xHA) tag (3` end) was introduced using overlapping and complementary oligonucleotides (**Table 2.1**). Podocyte-specific expression was achieved by cloning the 2XHA-p38αDN-FLAG construct downstream of an 8.3kb fragment of the mouse *NPHS1* promoter. To this end, the plasmid was cut by restriction enzyme digestion with *Acc65I* and its 5' nucleotide overhang subsequently filled in and blunted with T4 DNA polymerase and the plasmid was then digested by *HindIII*. The 8.3 kb *NPHS1* promoter fragment was likewise excised in a stepwise procedure from its pcDNA3 host vector using *XhoI* and blunted as above with T4 DNA polymerase followed by *HindIII* restriction enzyme digestion. The *HindIII* / blunt end fragment was cloned into pcDNA3-2XHA-p38αDN-FLAG to generate p*NPHS1*-2XHA-p38αDN-FLAG. This *NPHS1*-2XHA-p38αDN-FLAG transgene was linearized with *HindIII/RsrII*, isolated by agarose gel electrophoresis / QIAEX II gel extraction, and then microinjected into FVB/N mouse embryos. Embryos were then surgically transferred to the oviduct of pseudopregnant CD1 recipient female mice. Both the embryo donor mice and the recipient mice were purchased from the Jackson Laboratory, Inc. (Bar Harbor, ME). Genotyping of resulting pups was performed by PCR of genomic DNA isolated from tail snips of 3 week old mice (**Table 2.1**). Fifteen founders were identified using PCR of tail-snip DNA. Following determination of the highest expressing founders, two lines were initiated and maintained through intercrossing with non-TG FVB/N mice. Both p38αDN-TG lines yield healthy, fertile, hemizygous transgenic offspring at the predicted Mendelian ratio (1:1).

2.11 Animal models of glomerular injury

All procedures were approved by the University of Ottawa Animal Care Committee. Surgical procedures were carried out under isoflurane-induced anesthesia, in age matched male and female mice (8-10 weeks).

5/6 Nephrectomy

Mice underwent renal ablation via removal of 5/6th of total renal mass. The surgical procedure was carried out by ressecting the left kidney, and cauterizing the upper and lower poles of the right kidney. Briefly, surgeries were carried out under standard sterile conditions with the mouse lying ventrally with shaved area of back exposed. A one inch incision was made along the skin of the back followed by dissection down the right lateral side to expose the muscle. A small incision was made through the muscle and the right kidney was exposed and tied off at its blood supply at three different locations before its removal. The site was then stitched closed before proceeding with the left kidney. The left kidney was located and exposed and the poles were excised to achieve 75% reduction in total renal mass. The ends of the kidney were cauterized and the organ was placed back into the mouse. Once the left side was stitched, the dorsal incision was closed using stainless steel wound clips before the mouse was placed into recovery. Control mice underwent sham operations without the removal of any renal mass.

Angiotensin II Chronic Infusion

Mice were subcutaneously implanted with Alzet mini-osmotic pumps (Durect Corporation, Cupertino, CA) for chronic Ang II infusion. Ang II was prepared in saline solution at a concentration that accounted for mouse weight, pump holding volume and pump flow rate such that Ang II was delivered at a constant rate of 1000ng/kg/min. Pumps were inserted posteriorly in anesthetized mice.

α-GBM Glomerular Nephritis

To induce glomerular nephritis (GN) in our mice, sheep α-rat GBM nephrotoxic serum was injected via the tail vein. A non-accelerated model of GN, was employed, in this respect 5µl of α-GBM (kindly provided by Dr. Tomoko Takano, McGill University, Montreal, QC) was diluted in (145µl) PBS and injected into the tail vein of the mouse. The non-accelerated model of α-GBM GN differs from the accelerated model in that there is no pre-immunization with Freund's adjuvant, resulting in milder and more transient glomerular injury.

2.12 Urinalysis

Morning spot urine samples were collected from mice at specified time points following induction of glomerular injury and analyzed for albumin and creatinine content using the Albuwell M Test Kit and Creatinine Companion Kit (Exocell, Inc., Philadelphia, PA) respectively, consistent with the AMDCC (Animal Models of Diabetic

Complications Consortium) guidelines. In order to obtain results that fit within the standard curve range, urine was diluted 1:80 for albumin and 1:15 for creatinine. Results were reported as μg albumin: mg creatinine.

2.13 Blood pressure measurements

Systolic blood pressure of mice was measured by tail-cuff plethysmography (BP-2000; Visitech Systems, Apex, NC). Briefly, mice were placed on a heated platform (30ºC) in an isolated chamber and systolic blood pressure measurements were obtained. Prior to nephrectomy or Ang II pump implantation, mice were trained for 5 days followed by an additional 3 days of measurements. Beginning at two weeks post 5/6 Nx, and 1 week of chronic Ang II infusion, measurements were collected bi-weekly. Each session included 5 preliminary measurements followed by 10 measurements of data acquisition.

2.14 Determination of glomerular filtration rate

Glomerular filtration rate was estimated in conscious mice based on Fluorescein Isothiocyanate-inulin (FITC-inulin) following a singe bolous tail-vain injection according to Animal Models of Diabetic Complications Consortium. Briefly, 5% FITC-inulin was dissolved into 0.9% NaCl and dialyzed over night. Following dialysis, FITC-inulin was

intravenously injected into anesthetized mice. Following the injected, blood was collected from the saphenous vein in heparinized capillary tubes at 3, 7, 10, 15, 35, and 55 minutes. Samples were then assayed according to AMDCC guidelines. GFR values were calculated using Graph Pad PRISM 5 software, based on a two-compartment clearance model.

2.15 Renal pathology

Mice were anesthetized with isoflurane and perfused with 20ml ice-cold PBS through the left ventricle. A small cut was made in the right atrium to allow the escape of return circulation. Kidneys were harvested from mice, de-capsulated, and the poles were removed. Kidneys were either placed in 4% paraformaldehyde (PFA)/PBS for paraffin embedding, or embedded in O.C.T. (Optimal Cutting Temperature) Compound (Sakura Finetek USA Inc., Torrance, CA) for frozen sectioning. Kidneys to be used for light microscopic analysis were sectioned at 4μm and stained with periodic acid Schiff (PAS) and visualized with a Zeiss AxioCam HRc (Zeiss Axio Imager.A1, Zeiss Germany).

2.16 Immunohistochemistry and immunofluorescence

Embedded kidney sections were deparaffinized in mixed xylenes (Fisher Scientific), re-hydrated and antigen unmasking was carried out by immersing the slides in

citrate buffer, microwaving for 2 min and exposing to steam for 25 min. Sections were blocked in 5% goat serum, incubated with antibody against WT1 (undiluted; Spring Bioscience, Fremont, CA) followed by anti-rabbit IgG, horseradish peroxidase conjugated secondary antibody (dilution 1:100; Cell Signaling Technologies, Danvers, MA). Sections were then washed in PBS before applying the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA). Slides were stained using 3,3'-diaminobenzidine (DAB) substrate solution according to manufacturers' instructions (DAB Substrate Kit for Peroxidase; Vector Laboratories). Images were then captured using a Zeiss AxioCam HRc.

Frozen tissues embedded in OCT were cut in serial 6 μm sections. Sections were allowed to thaw, washed three times with PBS and then blocked with 5% goat serum (Vector Laboratories**)** in PBS. Following blocking, sections were incubated with primary antibodies (rabbit anti-HA, 1:1000, Zymed, San Francisco, CA and mouse anti-synaptopodin 1:100, Fitzgerald Industries International, Concord, MA). Sections were then incubated with either Alexa 488 or 584 conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR). Images were then captured using a Zeiss AxioCam.

2.17 Electron microscopy

Kidney tissue for electron microscopy was collected concurrently with tissue dissected for paraffin and O.C.T. embedded sectioning. To prepare tissue for electron microscopy, four 1mm^3 cortex samples were collected from each mouse. Cortex (as

opposed to medullary region) was used to maximize the number of glomeruli. Cortex samples were then quickly preserved in 2.4% glutaraldehyde (70% glutaraldehyde diluted in PBS). Preserved samples were then prepared by Jeff McClintock (Children's Hospital of Eastern Ontario, Ottawa, ON) for visualization of podocyte structure.

2.18 RNA extraction and quantitative RT-PCR

Snap frozen kidneys were retrieved from -80 ºC storage and RNA was extracted using an RNeasy Kit (QIAGEN). Following RNA isolation mRNA levels of EP4 receptor, EP1 receptor, COX-2, and nephrin were determined by quantitative RT-PCR using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems Research, Foster City, CA) and an ABI Prism 7000 Sequence Detection System. See **Table 2.1** for all primer and TaqMan probe sequences. All values were normalized to rodent GAPDH (TaqMan Rodent GAPDH Control Kit, Applied Biosystems).

2.19 Statistical analysis

All experimental data were analyzed using GraphPad PRISM 5 software. Data is represented as the means ± SEM. Where appropriate either *t* test or ANOVA followed by Newman-Keuls multiple comparison test was used.

Name	Description	Sequence (5'-3' direction)
2xHA Tag	Forward	gatccatgtatccatatgacgtcccagactctgcctatccatatgacgtcccagactctgccg
	Reverse	aattcggcagagtctgggacgtcatatggataggcagagtctgggacgtcatatggatacatg
$EP4^{pod+}$	Forward	gagaaagaactgttaacggg
	Reverse	accaccccgaagat
$CreEGFP^{pod+}$	Forward	aggtgtagagaaggcacttag
	Reverse	ctaatcggcatcttccagcagg
ROSA26	Forward	gcgaagagtttgtcctcaacc
	Reverse-1	ggagcgggagaaatggatatg
	Reverse-2	aaagtcgctctgagttgttat
$EP4^{pod-/-}$	Forward	gaagggtcttgctcatctcg
	Reverse	ctaacccgcactctctctc
p38αDN-TG	Forward	gagaaagaactgttaacggg
	Reverse	ttgtatcaaaagcagcacac
EP4	Forward	atggtcatcttactcatcgccac
	Reverse	ccttcaccacgtttggctgat
	Probe	6FAM-catctgctccattccgctcgtggt-TAMRA
EP1	Forward	agtgccaagggtggtccaa
	Reverse	ccgggaactacgcagtgaac
	Probe	6FAM- tgggcctaaccaagagtgcctgta-TAMRA
COX-2	Forward	ggggtgcccttcacttctttca
	Reverse	tgggaggcacttgcattga
	Probe	6FAM-tgtgcaagatccacagcctaccaaaaca-TAMRA
Nephrin	Forward	aagctggacgtgcattatgct
	Reverse	cggtgcagactatatccacagaac
	Probe	6FAM-tgccctgaaccctactgaggtgaa-TAMRA
WT1	Forward	tcttccgaggcattcaggat
	Reverse	tgctgaccggacaagagttg
	Probe	6FAM-tgcggcgtgtatcgtgagtggc-TAMRA

Table 2.1 Primer and probe sequences.

A summarized table of all primers and probes used for cloning, genotyping and real time PCR. All sequences are displayed in the 5' to 3' direction.

CHAPTER 3 – RESULTS

PODOCYTE PGE2 EP4 RECEPTOR SIGNALING

3.1 5/6 Nx induces renal EP4 receptor and COX-2 expression

Enhanced renal COX-2 induction is associated with PGE2 synthesis following subtotal renal ablation in rats (J. L. Wang et al., 1998). COX-2 inhibition can blunt albuminuria in such models suggesting that prostanoid(s) contribute to GFB damage (J. L. Wang et al., 2000). In the present study, we investigated whether similar changes are observed in a mouse version of this CKD model. For this purpose, FVB/N mice were subjected to 5/6 Nx as described in the methods section. Filtration barrier injury in FVB/N mice at 12 weeks post 5/6 Nx was evident as renal mRNA levels for the slit diaphragm component nephrin were significantly reduced as compared with sham operated controls (**Figure 3.1.A**). Similar to changes found in rats, renal COX-2 mRNA expression was increased in nephrectomized mice (**Figure 3.1.B**). Additionally, renal EP4, but not EP1, mRNA levels were also upregulated (**Figure 3.1.C & 3.1.D**). As expected, 5/6 Nx significantly increased albumin:creatinine ratios (ACR). Administration of NS-398 (1mg/kg/day) to attenuate COX-2 activity over a 12 week period in the drinking water, markedly desceased 5/6 Nx-induced proteinuria (**Figure 3.1.E**). However, COX-2 inhibition did not restore nephrin mRNA expression at 12 weeks post Nx, indicating that nephrin protein expression may persist despite the reduced mRNA levels (**Figure 3.1.F**). Dissociation between nephrin protein and mRNA levels have been previously reported (Davis et al., 2007; S. X. Wang et al., 2002). These data suggest that the intrarenal COX-2/PGE2/EP4 signaling axis is induced and may participate in this model of progressive renal disease.

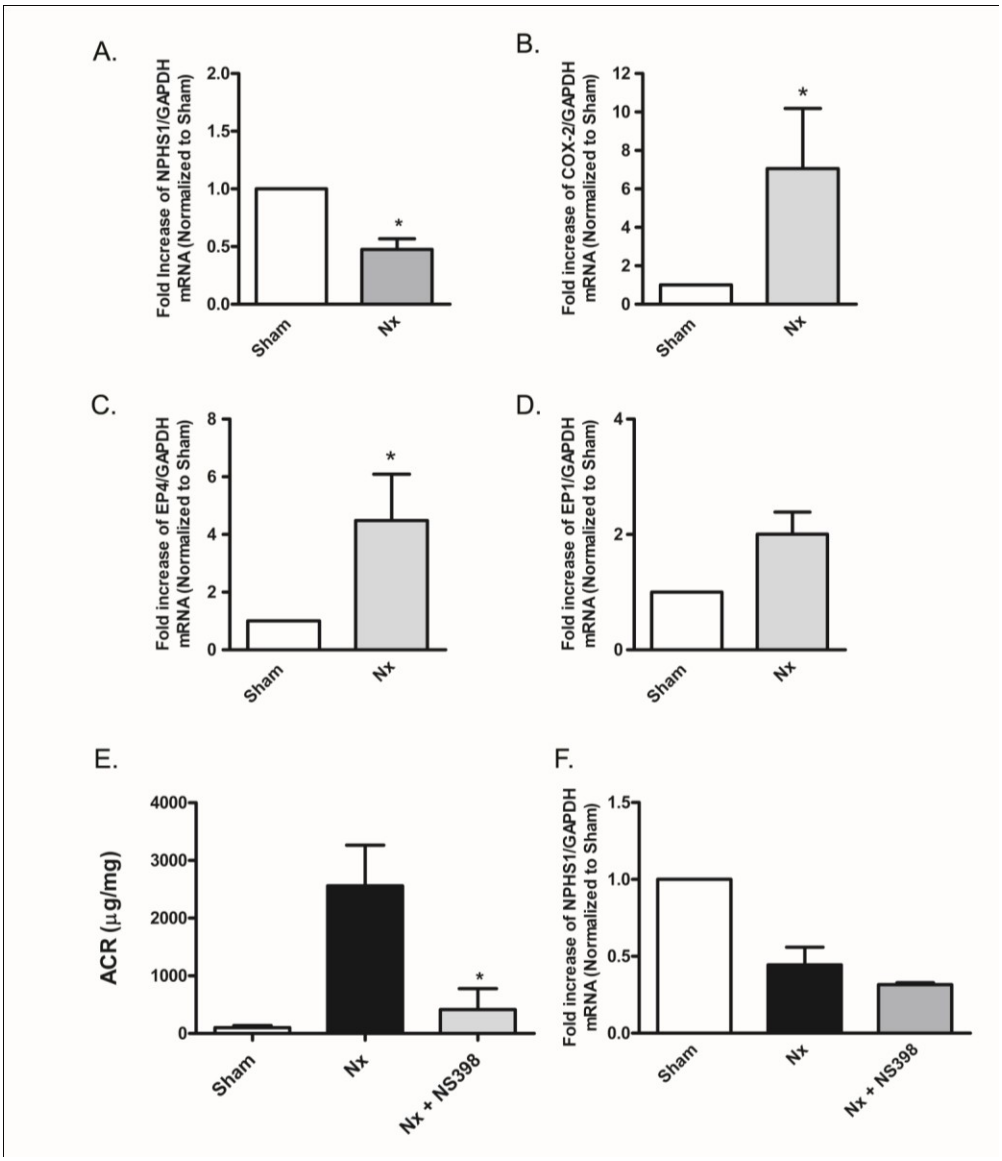


Figure 3.1 COX-2 is upregulated and contributes to 5/6 Nx–induced albuminuria.

Eight week-old FVB/N mice are subjected to 5/6 Nx or sham operation. At 12 weeks after 5/6 Nx, remnant renal mass is removed and total mRNA is isolated. (A) GFB damage is evident as glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalized nephrin mRNA levels are significantly decreased in 5/6 Nx mice when compared with sham-operated animals as determined by quantitative RT-PCR. **P* < 0.05 *versus* sham group. (B through D) Renal COX-2 (B) and EP4 receptor (C) but not EP1 (D) mRNA levels are upregulated in 5/6 Nx mice. **P* < 0.05 *versus* sham group. (E) A subset of mice are administered NS-398 (1 mg/kg per d), and ACR is determined. **P* < 0.05 *versus* 5/6 Nx group. (F) At 12 weeks after 5/6 Nx, NS-398 is not able to recover nephrin expression.

3.2 Generation of transgenic mice with podocyte-specific EP4 expression (EP4^{pod+} mice)

To test the role of podocyte EP4 receptors in filtration barrier injury in a CKD model, mice were generated with podocyte-specific EP4 receptor overexpression (EP4^{pod+} mice). In order to diminish the impact of heterologous desensitization of the overexpressed receptor, an EP4 construct lacking a significant portion of its C-terminal intracellular tail was engineered. This region (from amino acid 355 onwards) was shown previously to contain several phosphorylation sites that contribute to its rapid desensitization and sequestration (Slipetz et al., 2001). A double hemagglutinin (2xHA) tag was added to the 5'end of the truncated EP4 receptor open reading frame. Neither the epitope tag nor the loss of the C-terminus had any appreciable impact upon acute receptor function (**Figure 3.2A**). An 8.3 Kb fragment of the mouse nephrin (*NPHS1*) promoter region was incorporated upstream of the 2xHA-EP4del355 sequence for expression in mice (**Figure 3.2.B**) (Michaud et al., 2003). Transgenic mice on a FVB/N background were produced and genotyping of the resulting 36 pups (candidate founders) was performed by PCR using tail-snip derived DNA (**Figure 3.2.C**). Seven EP4^{pod+} founder lines were initiated and otherwise healthy and fertile offspring were obtained in a Mendelian ratio. For all subsequent studies, two lines were chosen exhibiting the highest transgene expression as determined semi-quantitatively by immunofluorescence of renal sections with an HA-antibody. **Figure 3.2.D/E/F** depicts co-localization of the HA-tagged EP4 transgene in one such founder line with the podocyte marker synaptopodin, thereby confirming its podocyte-restricted expression. To verify that transgene expression resulted in a functional GPCR, glomeruli

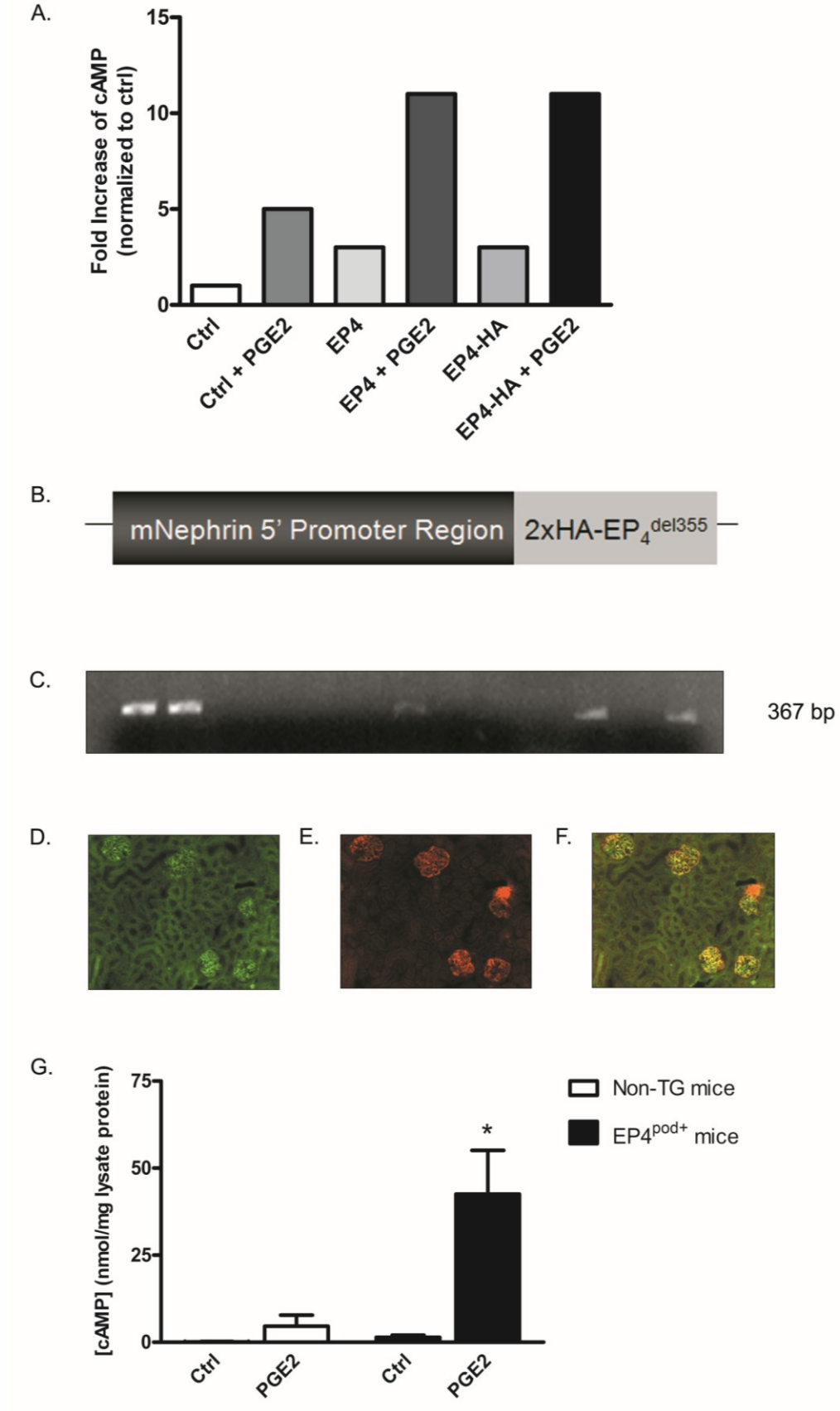

A.
Fold Increase of cAMP (normalized to ctrl)
15
10
5
0
Ctrl
Ctrl + PGE2
EP4
EP4 + PGE2
EP4-HA
EP4-HA + PGE2
B.
mNephrin 5' Promoter Region
2xHA-EP4del355
C.
367 bp
D.
E.
F.
G.
[cAMP] (nmol/mg lysate protein)
75
50
25
0
*
Ctrl
PGE2
Ctrl
PGE2
Non-TG mice
EP4pod+ mice

Figure 3.2 Podocyte-restricted functional expression of an EP4 receptor transgene in mice.

(A) COS-7 cells are transfected with full-length or truncated (del355) EP4 constructs. cAMP production is not affected by incorporation of a 2XHA tag. (B) Illustration of the construct used to generate $EP4^{pod+}$ mice. An 8.3-kb fragment of the mouse *NPHS1* immediate promoter region is incorporated upstream of a human EP4 open reading frame that lacks the coding sequence for amino acid 355 onward. A 5` 2XHA epitope tag is inserted immediately distal to the *NPHS1* promoter sequence. (C) TG mice are identified by PCR of tail-snip DNA. The representative image shows agarose gel electrophoresis of the 367-bp PCR product amplified from the transgene (five of 13 mice shown are positive for the transgene). Seven founders with germline transmission of the transgene are obtained from a total of 36 pups. (D) Immunofluorescence of EP4 receptor transgene in glomeruli of $EP4^{pod+}$ mice using a rabbit HA antibody. (E) Mouse synaptopodin antibody (podocyte marker) immunofluorescence identifies podocytes. (F) Merged images (C/D) reveal colocalization of HA-EP4 and synaptopodin in $EP4^{pod+}$ mouse kidney sections. (G) Glomeruli isolated from $EP4^{pod+}$ mice respond to 10 minutes of stimulation with 1 μM PGE2 with cAMP production that significantly exceeds that of non-TG mice. $*P < 0.05$ *versus* non-TG group with PGE2. Magnification, X200.

were isolated from age-matched (8 week old) non-TG and EP4^{pod+} littermates and assayed for PGE2-induced cAMP synthesis. As shown in **Figure 3.2.G**, in response to 1μM PGE2, glomerular cAMP levels in EP4^{pod+} mice were more than 8 fold higher than those of their non-TG littermates (43nmol/mg lysate protein vs. 5nmol/mg lysate protein respectively, $P<0.05$).

3.3 Exacerbated renal phenotype of EP4^{pod+} mice following 5/6 Nx

To evaluate the impact of podocyte-specific overexpression of the EP4 receptor on the filtration barrier, groups of EP4^{pod+} mice underwent 5/6 Nx. Subtotal renal ablation in mice results in hypertension, albuminuria, and FSGS (L. J. Ma & Fogo, 2003). In the present studies, 5/6 Nx non-TG mice developed elevated systolic blood pressure compared to sham operated mice by 4 weeks post-Nx (~Δ25mmHg) (**Figure 3.3.A**). Similar increases were observed in 5/6 Nx EP4^{pod+} mice, suggesting that expression of the transgene in podocytes is without effect on systemic blood pressure. Spot urine ACR analyses revealed that 5/6 Nx non-TG mice became significantly albuminuric as compared to sham operated mice. However, 5/6 Nx EP4^{pod+} mice were significantly more albuminuric (3438μg/mg, n=7) than 5/6 Nx non-TG mice (773μg/mg, $P<0.0001$, n=12) as early as 2 weeks post-Nx, becoming severely affected by 4 weeks (**Figure 3.3.B**). Furthermore, as compared with their 5/6 Nx non-TG littermates, 5/6 Nx EP4^{pod+} mice exhibited increased mortality by 8 weeks post-Nx (**Figure 3.3.C;** 67% vs. 16%). Of

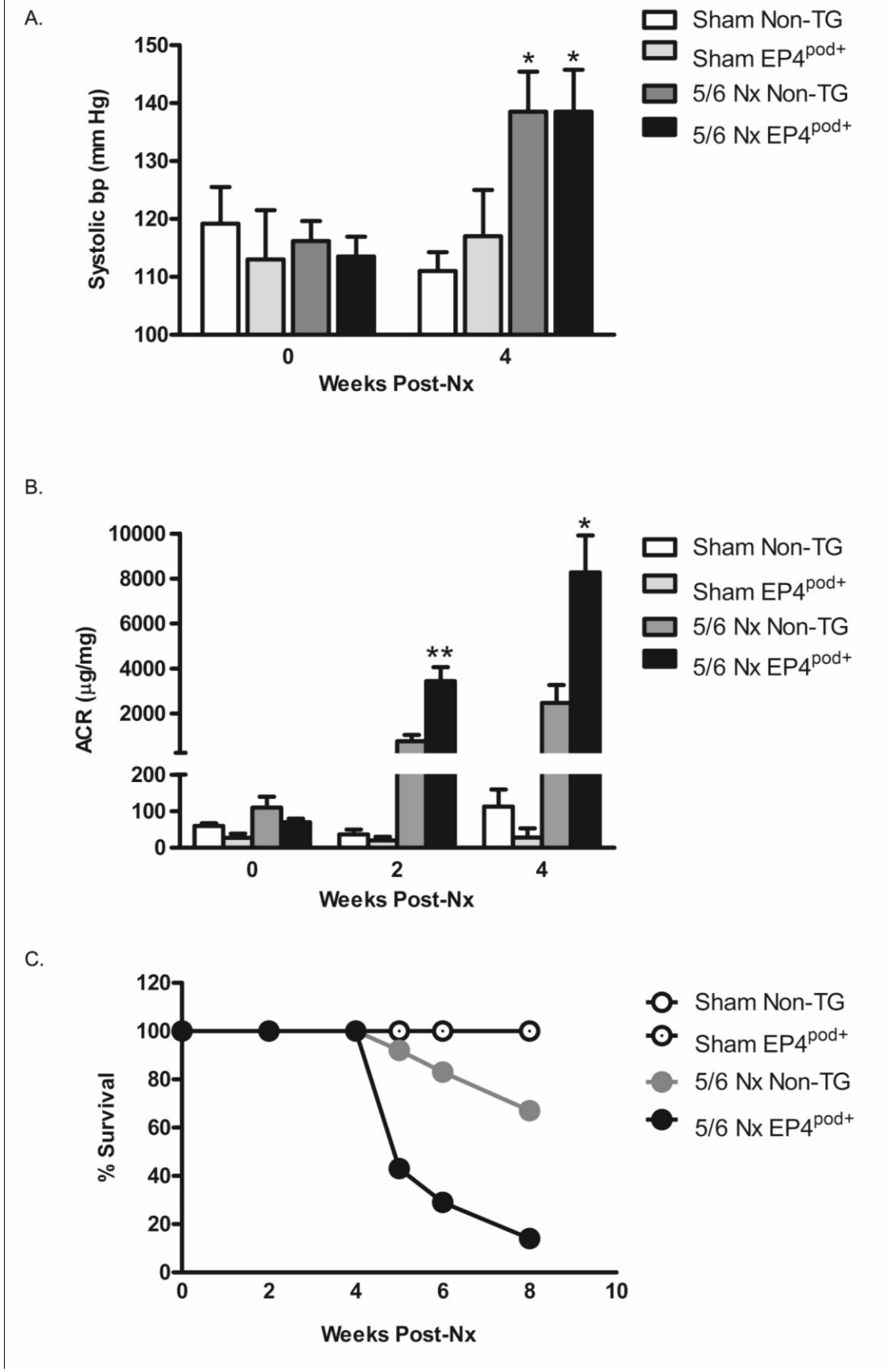

A.
Systolic bp (mm Hg)
150
140
130
120
110
100
0
4
Weeks Post-Nx
*
*
Sham Non-TG
Sham EP4pod+
5/6 Nx Non-TG
5/6 Nx EP4pod+
B.
ACR (μg/mg)
10000
8000
6000
4000
2000
200
100
0
0
2
4
Weeks Post-Nx
**
*
Sham Non-TG
Sham EP4pod+
5/6 Nx Non-TG
5/6 Nx EP4pod+
C.
% Survival
120
100
80
60
40
20
0
0
2
4
6
8
10
Weeks Post-Nx
Sham Non-TG
Sham EP4pod+
5/6 Nx Non-TG
5/6 Nx EP4pod+

Figure 3.3 $EP4^{pod+}$ mice are significantly more proteinuric following 5/6Nx.

At 4 weeks after 5/6 Nx, both $EP4^{pod+}$ and non-TG mice display similar BP elevation compared with sham-operated animals (grouped as non-TG and $EP4^{pod+}$ mice). (B) At 2 weeks after 5/6 Nx, ACR of $EP4^{pod+}$ mice (3438µg/mg; n=7) is significantly higher than that of non-TG mice (773 µg/mg; $P < 0.0001$; n=12).This effect persists at 4 weeks after 5/6 Nx, when $EP4^{pod+}$ mice (8280 µg/mg; n =7) are significantly more albuminuric than non-TG mice (2471 µg/mg; n=12). *$P < 0.0001$. (C) Severe albuminuria observed in 5/6 Nx $EP4^{pod+}$ mice is associated with a significant drop in mouse survival over the course of the experiment. Percentage survival of nephrectomized mice over 8 weeks for $EP4^{pod+}$ mice (16%) is much lower than that for non-TG Nx mice (67%). No sham-operated animals are lost at 8 weeks after 5/6 Nx.

the mice that did not survive to the end of this 12 week study, renal pathology showed tubulointerstitial fibrosis along with significant tubular abnormalities including dilatations and filling with protein casts (**Figure 3.4.A**) along with severe glomerular scarring (**Figure 3.4.B**) — all suggesting that renal deterioration subsequent to exacerbated proteinuria was more likely in $EP4^{pod+}$ mice than in non-TG mice following 5/6 Nx. Sham operated controls were devoid of pathological features (**Figure 3.4.C**). On the other hand, for mice surviving to 12 weeks, renal pathology was much less severe with milder glomerulosclerosis for both 5/6 Nx non-TG and $EP4^{pod+}$ mice (**Figure 3.4.D/E**).

3.4 Generation of $EP4^{pod-/-}$ mice

In order to further test the role of podocyte EP4 receptors in the regulation of the GFB, a line of mice with conditional deletion of this PGE2 receptor subtype was generated. Briefly, transgenic mice with podocyte-specific expression of a Cre-recombinase/enhanced GFP fusion protein were engineered using an 8.3 kb fragment of the mouse *NPHS1* promoter ($CreEGFP^{pod+}$ mice). Three founders of mixed C57Bl/6J x C3H/HeJ background, carrying the *NPHS1*-CreEGFP transgene, were identified by PCR-based genotypic analysis and confirmed by Southern analysis (data not shown). Confirmation of podocyte-specific CreEGFP expression and activity was carried out by immunofluorescent detection of EGFP and by intercrossing with the ROSA26 reporter

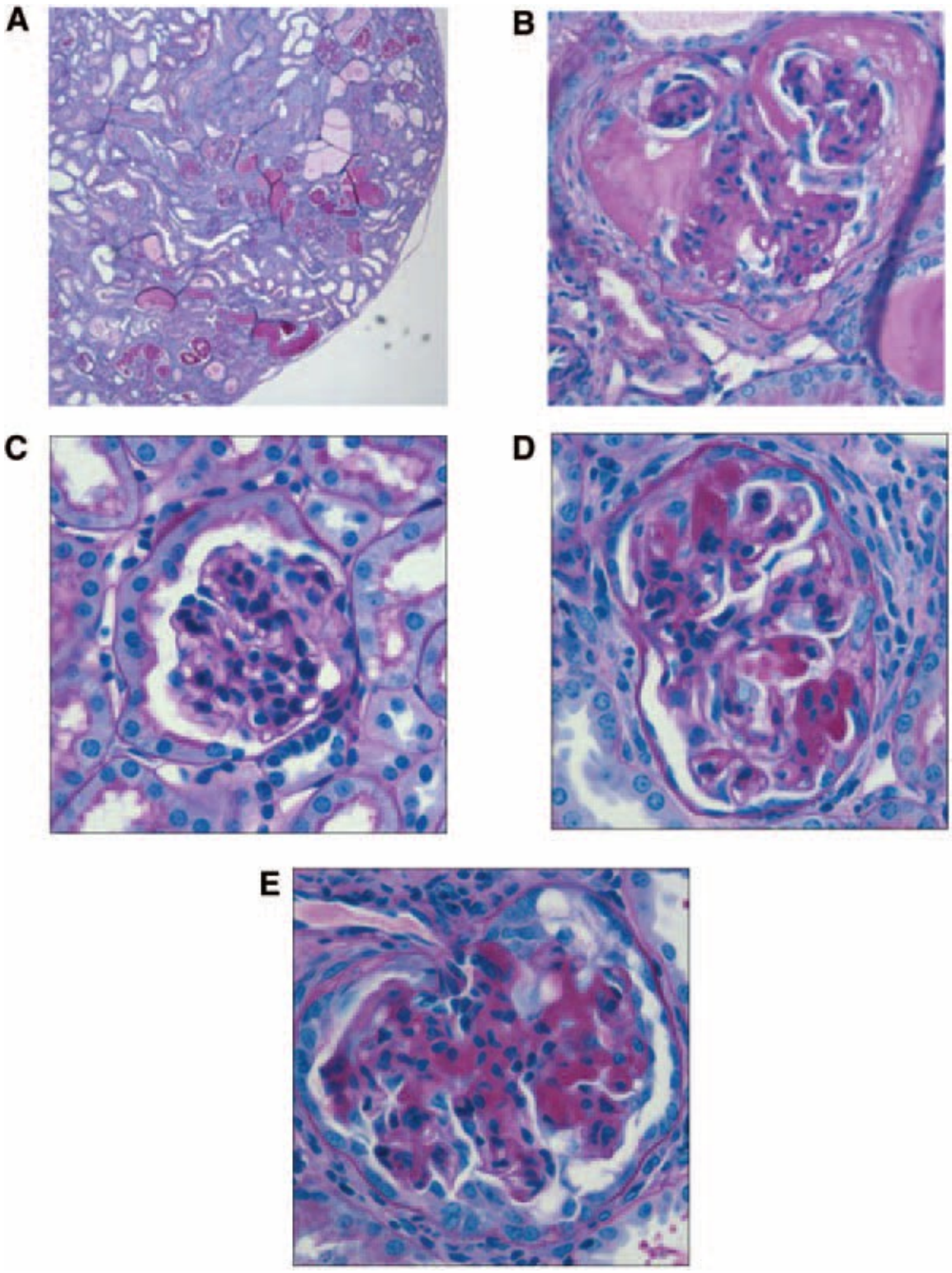

A
B
C
D
E

Figure 3.4 Mice exhibit severe renal pathology following 5/6Nx.

A subpopulation of 5/6 Nx EP4^{pod+} mice (n=6) die suddenly between 5 to 8 weeks after Nx. Kidneys are recovered and disease pathology is visualized by periodic acid–Schiff staining of sections. (A and B) Of the mice that do not survive for 12 weeks, renal pathology shows severe glomerular scarring, tubulointerstitial fibrosis, and widespread accumulation of tubular protein casts. For mice that survive to 12 weeks, renal pathology is milder with less glomerulosclerosis and no grossly apparent differences between 5/6 Nx non-TG and EP4 $^{pod+}$ mice. (C) Sham (12 weeks after 5/6 Nx). (D) 5/6 Nx non-TG (12 weeks after 5/6 Nx). (E) 5/6 Nx EP4^{pod+} (12 weeks after 5/6 Nx). Magnifications: X40 in A; X400 in B through E.

strain (experiment performed by Wissam H. Faour; **Figure 3.5**). Expression of the fusion protein was restricted to the podocytes (**Figure 3.5.A**). Expression was absent from any other tissues examined, including liver, lung, heart and muscle (data not shown). Organs from 6 week old ROSA26/CreEGFP^{pod+} mice were dissected and stained with X-gal. As shown in **Figure 3.5.B**, extensive and widespread glomerular staining was achieved in ROSA26/CreEGFP^{pod+} mice. Staining intensities were similar between littermates, as well as across the 3 founder lines (data not shown). Weaker X-gal staining was observed in brain, but no signal could be found in any of the other tissues examined, a finding generally consistent with the expression pattern observed by others employing this promoter fragment (Moeller, Kovari, & Holzman, 2000). CreEGFP^{pod+} mice were subsequently backcrossed 10 generations onto the FVB/N strain and the retention of Cre-recombinase activity was confirmed using the ROSA26 mice (data not shown).

CreEGFP^{pod+} mice were then bred to floxed EP4 receptor mice (EP4$^{flox/flox}$) obtained from Dr. Matthew Breyer (Vanderbilt University) to generate podocyte-specific EP4 deleted mice (EP4$^{pod-/-}$). EP4$^{flox/flox}$ mice have loxP sites flanking exon 2 of the EP4 gene making it a conditional knockout (Schneider et al., 2004). EP4$^{flox/flox}$ mice are healthy and fertile, exhibiting no obvious renal or other phenotypes and were backcrossed for 10 generations to obtain congenic mice on the FVB/N background. For EP4$^{pod-/-}$ studies, EP4$^{flox/flox}$ mice were used as controls. EP4$^{pod-/-}$ mice appeared healthy, normoalbuminuric and were obtained in a Mendelian ratio. Initial attempts to verify the efficacy of the conditional deletion of the EP4 gene using immunohistochemical approaches employing EP4 receptor antibodies were unsuccessful. Immunoblotting was negative for EP4 protein in glomeruli isolated from either EP4$^{flox/flox}$, EP4$^{pod-/-}$ and even

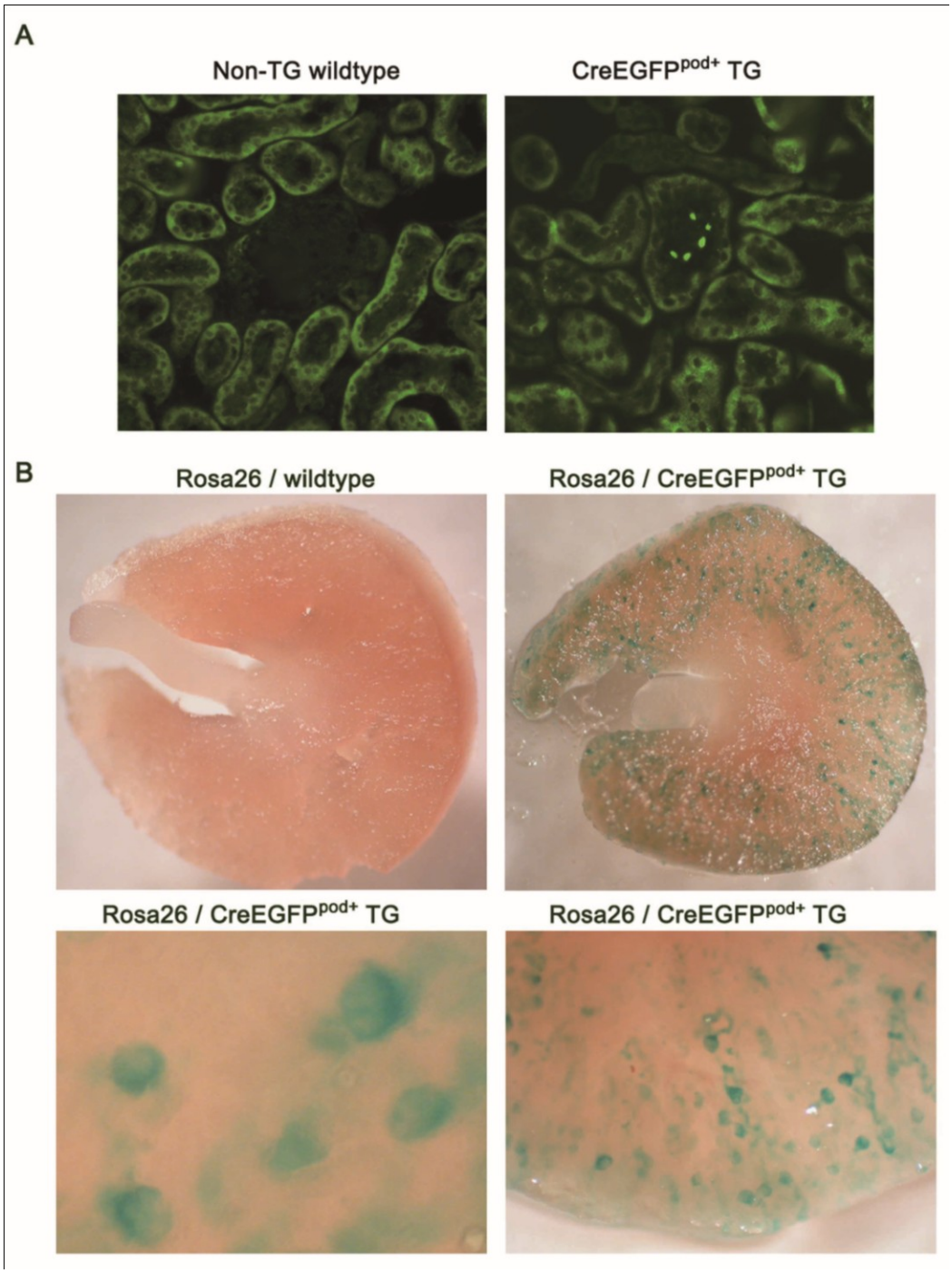

A
Non-TG wildtype
CreEGFP^{pod+} TG
B
Rosa26 / wildtype
Rosa26 / CreEGFP^{pod+} TG
Rosa26 / CreEGFP^{pod+} TG
Rosa26 / CreEGFP^{pod+} TG

Figure 3.5 *In vivo* expression and catalytic activity of CreEGFP is confirmed in TG mice.

(A) Expression of the CreEGFP fusion protein in TG mice. Kidneys from 6-week-old non-TG wild-type and CreEGFP$^{pod-/-}$ mice are dissected and processed for immunofluorescence as described in the Concise Methods section using an FITC-conjugated mouse monoclonal anti-GFP antibody. Shown are representative images of kidney sections from non-TG wild-type and CreEGFP $^{pod-/-}$ mice. Only glomerular cells of the CreEGFP $^{pod-/-}$ mice display immunofluorescence. (B) *In vivo* catalytic activity of the CreEGFP fusion protein in TG mice. CreEGFP $^{pod-/-}$ mice are intercrossed with ROSA26 mice, and kidneys from double-positive 6-week-old compound TG mice are dissected and processed for X-gal staining as described in the Concise Methods section. Whole kidneys from ROSA26 and CreEGFP $^{pod-/-}$ mice are grossly sectioned by razor blade and visualized under low-power light microscopy. X-gal staining is restricted to glomerular cells of the CreEGFP $^{pod-/-}$ mice. Magnification, X200 in A, X20 and X50 in upper and lower B panels respectively.

$EP4^{pod+}$ transgenic mice. Therefore, we isolated and cultured primary podocytes from $EP4^{flox/flox}$ and $EP4^{pod-/-}$ mice. As shown in **Figure 3.6.A**, quantitative RT-PCR of mRNA obtained from primary cultured podocytes of $EP4^{pod-/-}$ mice showed a statistically significant 50% reduction in EP4 receptor expression as compared to $EP4^{flox/flox}$ mice. In contrast, WT1 expression remained similar between groups (**Figure 3.6.B**). Furthermore, in contrast to $EP4^{flox/flox}$ podocytes, PGE2-stimulated cAMP production was absent in $EP4^{pod-/-}$ podocytes, suggesting functional EP4 receptor deletion in podocytes of $EP4^{pod-/-}$ mice (**Figure 3.6.C**). Cre-mediated recombination of the floxed EP4 alleles was verified by PCR of genomic DNA obtained from $EP4^{pod-/-}$ primary podocyte cultures (**Figure 3.6.D**).

3.5 Milder renal phenotype of $EP4^{pod-/-}$ mice following 5/6 Nx

We next investigated the impact of EP4 receptor deletion from the podocytes upon the filtration barrier in progressive kidney disease. For this purpose, groups of $EP4^{pod-/-}$ mice underwent 5/6 Nx as described for the $EP4^{pod+}$ mice. Similar to our findings with $EP4^{pod+}$ mice (**Figure 3.3.A**), systolic blood pressures increased equivalently for $EP4^{flox/flox}$ and $EP4^{pod-/-}$ mice by 4 weeks post 5/6 Nx (Δ+25 mmHg) compared to sham operated controls (**Figure 3.7.A**). However, partial attenuation of filtration barrier damage was evident as early as 4 weeks post-Nx and became statistically significant by 6 weeks post-Nx as ACR of $EP4^{pod-/-}$ mice (753 μg/mg) was significantly

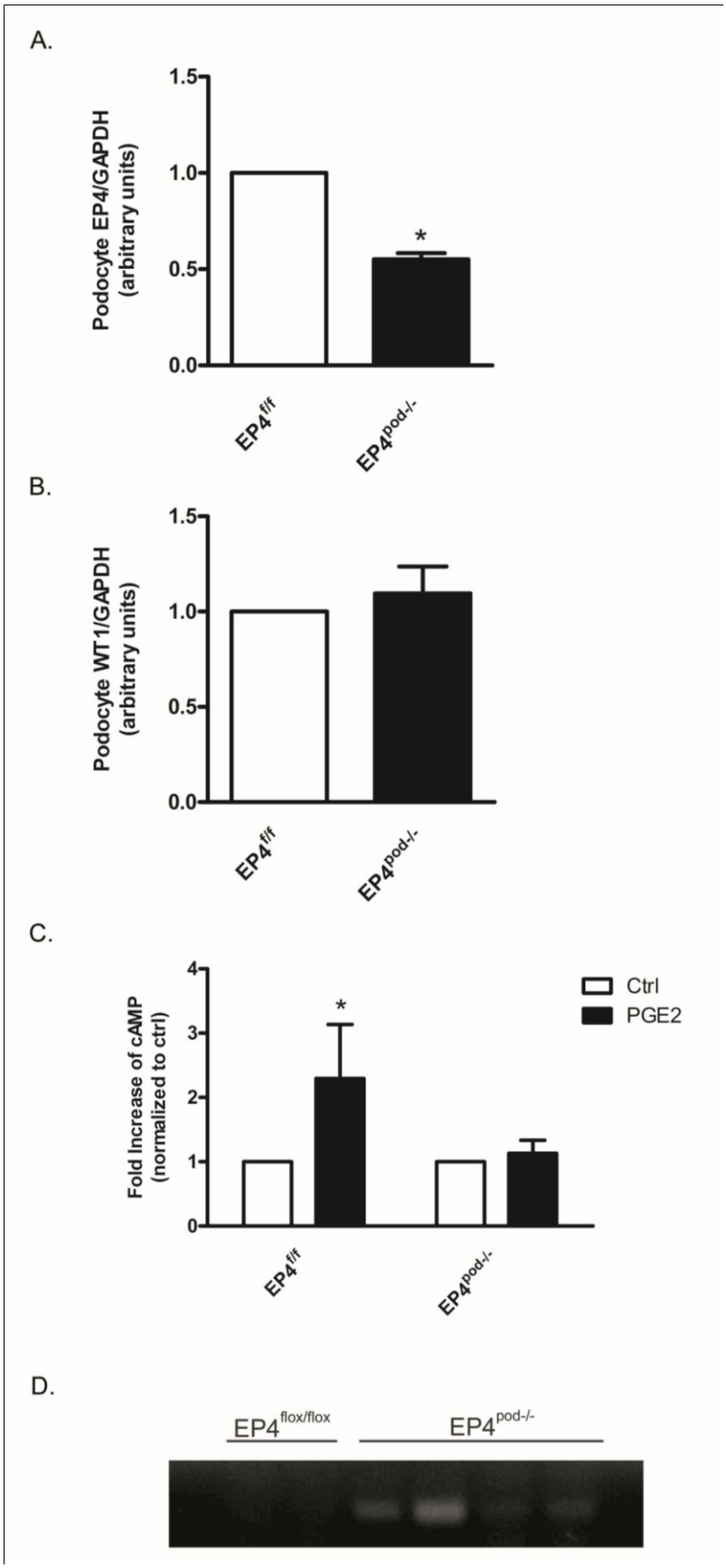

A.
Podocyte EP4/GAPDH (arbitrary units)
1.5
1.0
0.5
0.0
*
EP4f/f
EP4pod-/-
B.
Podocyte WT1/GAPDH (arbitrary units)
1.5
1.0
0.5
0.0
EP4f/f
EP4pod-/-
C.
Fold Increase of cAMP (normalized to ctrl)
4
3
2
1
0
*
Ctrl
PGE2
EP4f/f
EP4pod-/-
D.
EP4flox/flox
EP4pod-/-

Figure 3.6 EP4 mRNA expression is reduced in primary $EP4^{pod-/-}$ podocytes.

The efficacy of Cre recombinase–mediated EP4 receptor deletion is assessed indirectly. (A) Podocytes from $EP4^{pod-/-}$ mice (n = 5) are isolated and cultured as described in the Concise Methods section. Subsequently, total mRNA is isolated from these cultures, and EP4 mRNA levels are determined by quantitative RT-PCR. $EP4^{pod-/-}$ podocytes express significantly lower EP4 receptor mRNA compared with EP4flox/flox mice (normalized to GAPDH mRNA). *P < 0.05. (B) No differences are observed between groups with respect to Wilms' tumor expression. (C) PGE2- dependent cAMP production is blunted in podocytes isolated from $EP4^{pod-/-}$ (*versus* $EP4^{flox/flox}$ podocyte cultures). (D) PCR confirmation from genomic DNA isolated from either $EP4^{pod-/-}$ or $EP4^{flox/flox}$ podocytes showing Cre recombination in $EP4^{pod-/-}$ podocytes only (representative image shows agarose gel electrophoresis of the 200-bp PCR product amplified where recombination has occurred; each lane represents podocytes isolated from either $EP4^{flox/flox}$ [n= 2] or $EP4^{pod-/-}$ [n = 4 mice]).

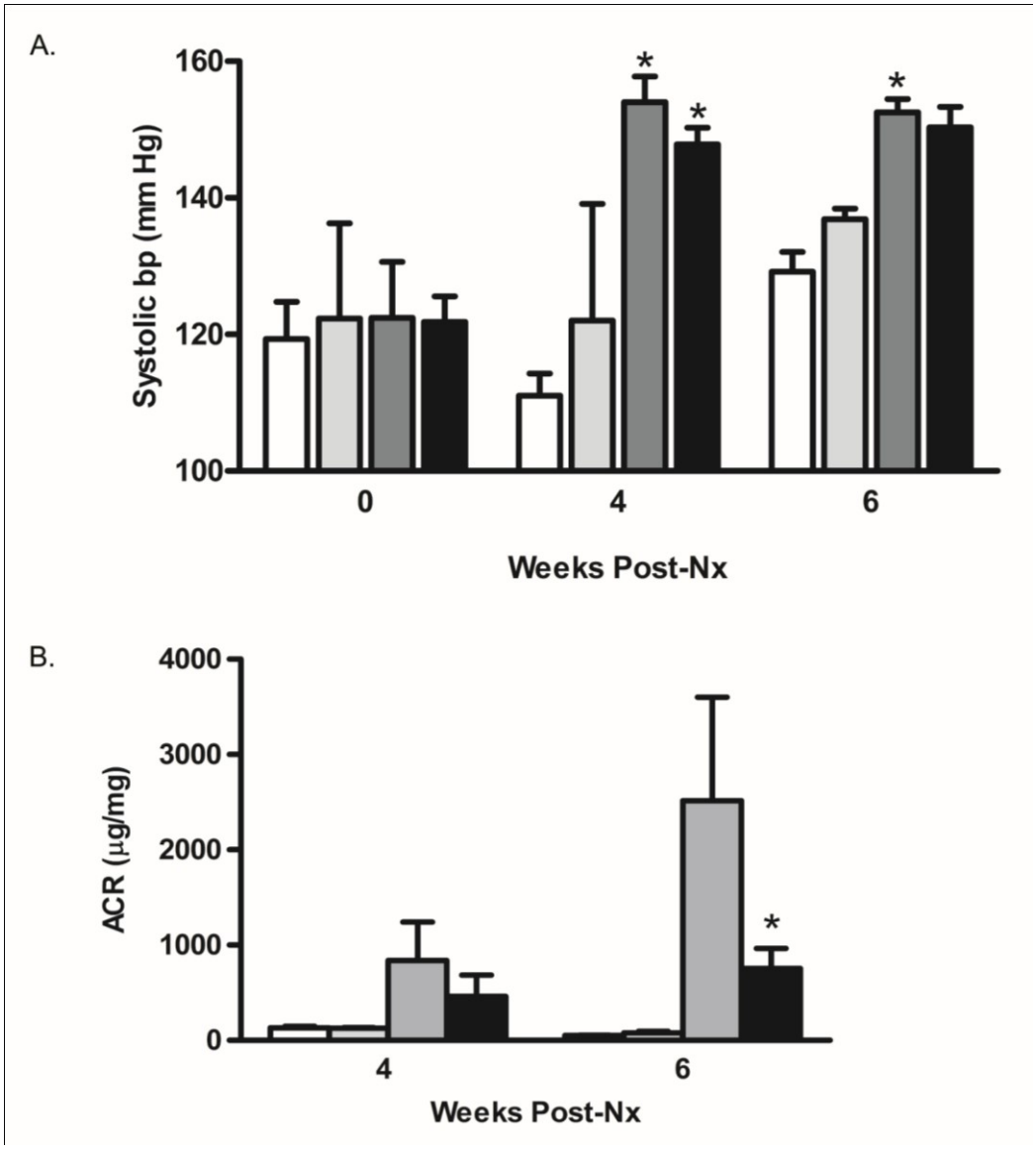


Figure 3.7 EP4$^{pod-/-}$ mice are less proteinuric following 5/6Nx.

(A) SBP increases in 5/6 Nx mice. SBP is assessed *via* tail-cuff plethysmography. Beginning at 2 weeks after Nx, both EP4$^{pod-/-}$ (black bars) and EP4$^{flox/flox}$ (dark gray bars) mice displayed similar elevated SBP as compared with sham-operated animals (white and light gray bars respectively). *$P < 0.05$ *versus* sham. (B) Proteinuria increases after 5/6 Nx. At 6 weeks after Nx, ACR is significantly lower for EP4$^{pod-/-}$ mice (753 µg/mg; $n = 12$) than for EP4$^{flox/flox}$ mice (2516 µg/mg; $n = 6$). *$P < 0.05$

lower than EP4$^{flox/flox}$ mice (2516 μg/mg, P<0.05, **Figure 3.7.B**). Consistent with the partial preservation of GFB function in mice lacking podocyte EP4 receptors, glomerular pathology of 5/6 Nx EP4$^{pod-/-}$ mice exhibited qualitatively milder glomerular lesions, with less matrix deposition than their 5/6 Nx EP4$^{flox/flox}$ littermate controls (**Figure 3.8.**).

3.6 EP4 receptor overexpression exacerbates stretch-induced detachment from the extracellular matrix

Podocyte loss through detachment from the glomerular basement membrane is associated with proteinuria and subsequent sclerosis in glomerular disease (Kriz, 2005). Excessive mechanical forces due to enhanced Pgc could facilitate podocyte detachment *in vivo*. We previously demonstrated that *in vitro* mechanical stretch of conditionally-immortalized mouse podocytes induces both EP4 receptor and COX-2 expression (Martineau et al., 2004). Moreover, we recently showed that PGE2 acting via the EP4 receptor can likewise induce COX-2 in cultured podocytes (Faour et al., 2008). In the present study, EP4 overexpression by adenoviral infection and subsequent stimulation with PGE2 resulted in a significantly greater level of cAMP production (**Figure 3.9.A**) and COX-2 expression (**Figure 3.9.B/C**) compared to non-infected controls. Thus, both mechanical stretch and PGE2 trigger an autocrine feedback loop in podocytes. However, whether PGE2 acting via EP4 receptors, along with mechanical stretch could promote podocyte detachment is not known. We therefore exposed

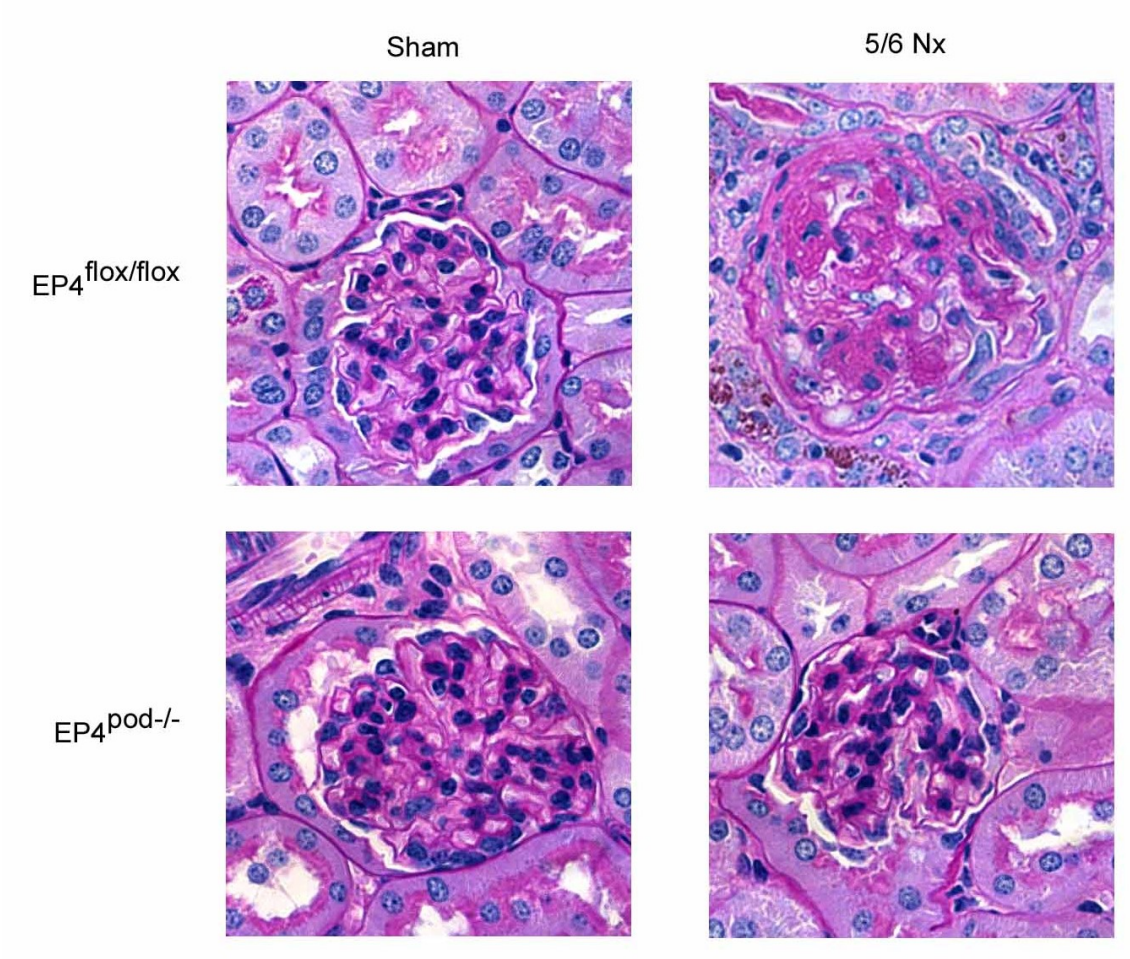


Figure 3.8 Milder renal pathology is observed in 5/6Nx EP4$^{pod-/-}$ mice.

Kidneys are removed and disease pathology is visualized by periodic acid–Schiff staining of paraffin embedded sections at 8 weeks after 5/6 Nx. Milder glomerular pathology is observed in 5/6 Nx EP4$^{pod-/-}$ mice with less matrix deposition than 5/6 Nx EP4$^{flox/flox}$ mice. Magnification, X400.

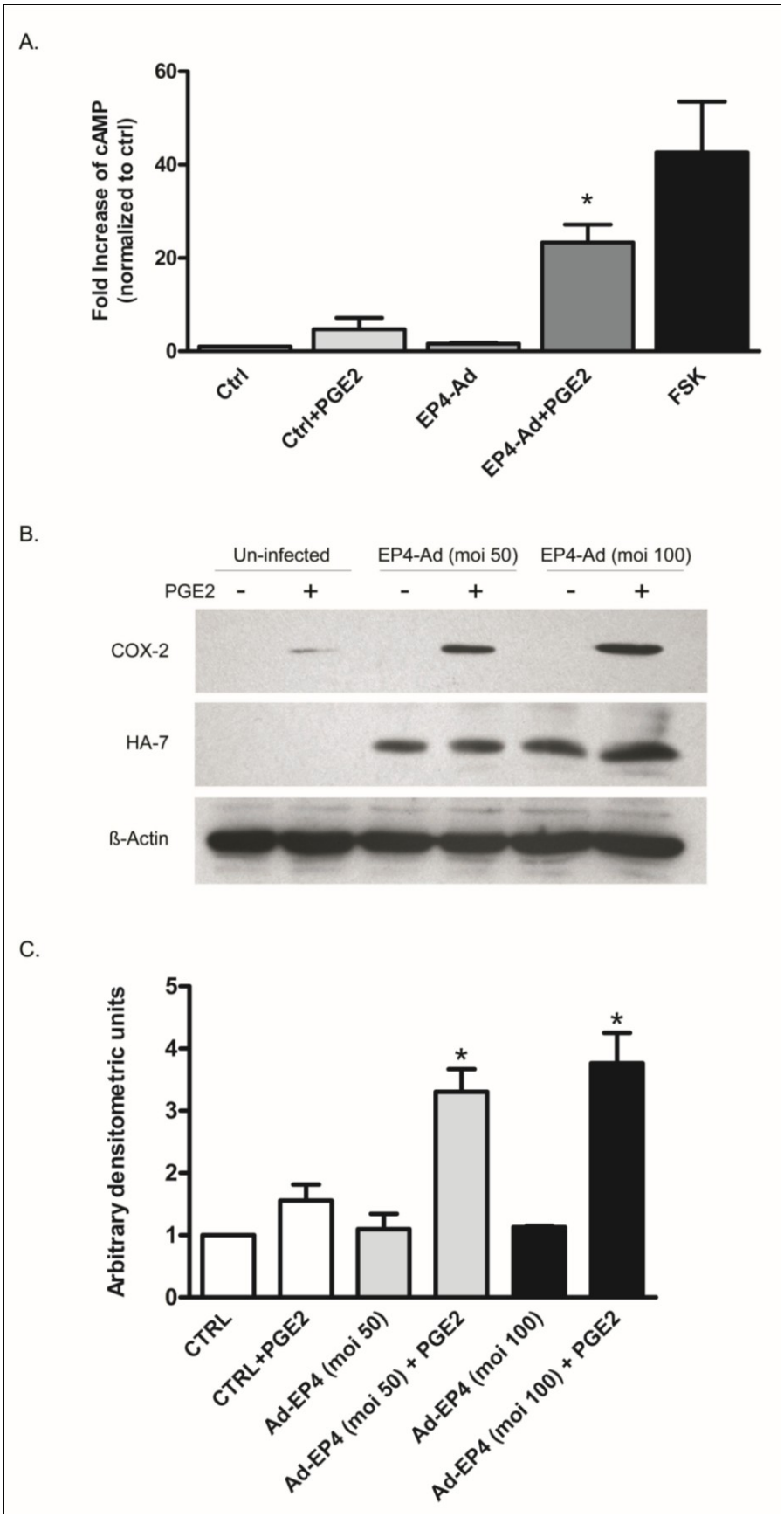

A.
Fold Increase of cAMP (normalized to ctrl)
60
40
20
0
*
Ctrl
Ctrl+PGE2
EP4-Ad
EP4-Ad+PGE2
FSK
B.
Un-infected
EP4-Ad (moi 50)
EP4-Ad (moi 100)
PGE2 - + - + - +
COX-2
HA-7
ß-Actin
C.
Arbitrary densitometric units
5
4
3
2
1
0
*
*
CTRL
CTRL+PGE2
Ad-EP4 (moi 50)
Ad-EP4 (moi 50) + PGE2
Ad-EP4 (moi 100)
Ad-EP4 (moi 100) + PGE2

Figure 3.9 EP4 overexpression and stimulation with PGE2 results in significantly greater cAMP production and COX-2 expression.

(A) After 14 days of differentiation, conditionally immortalized mouse podocytes are transduced with adenovirus for the human full-length HA-tagged EP4 receptor (MOI=50). Three days after infection, cells are stimulated with 1 μM PGE2 for 10 minutes in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 5 μM indomethacin. Forskolin (FSK; 10 μM) is used as a positive control. Cells infected with EP4 adenovirus and stimulated with PGE2 display a significant increase in cAMP production compared with uninfected controls (n = 3). *P < 0.05). (B) Podocytes infected with HA-tagged EP4 adenovirus are stimulated with 1 _M PGE2 for 6 hours, inducing a significant increase in COX-2 expression over noninfected controls. Western blots are probed with COX-2, HA, and β-actin antibodies (n = 3). MOI = 50 or 100 as shown. (C) Quantification of COX-2 expression is achieved using densitometric values normalized to β-actin levels. Cells infected with EP4 adenovirus had significantly higher COX-2 expression than uninfected controls after PGE2 stimulation. *P < 0.05.

conditionally immortalized mouse podocytes to mechanical stretch as previously described (Martineau et al., 2004). In cell detachment assays, PGE2 alone was without effect upon the number of podocytes adhering to the surface of the culture dish (**Figure 3.10**). Furthermore, the application of mechanical stretch either alone, or in the presence of PGE2 were likewise without effect upon podocyte adhesion. In contrast, when EP4 expression was enhanced via adenoviral-mediated transduction, a significant increase in cell detachment following mechanical stretch was observed. Curiously, the addition of PGE2 to EP4-transduced cells did not contribute further to stretch-induced podocyte detachment from the substratum, suggesting possible agonist-independent receptor activation in response to mechanical stretch. GFP overexpression by adenoviral transduction was without effect upon podocyte adhesion.

Acknowledgements

Dr. Wissam H.Faour initiated experiments with Cre-EGFP^{pod} and EP4$^{pod-/-}$ mice.

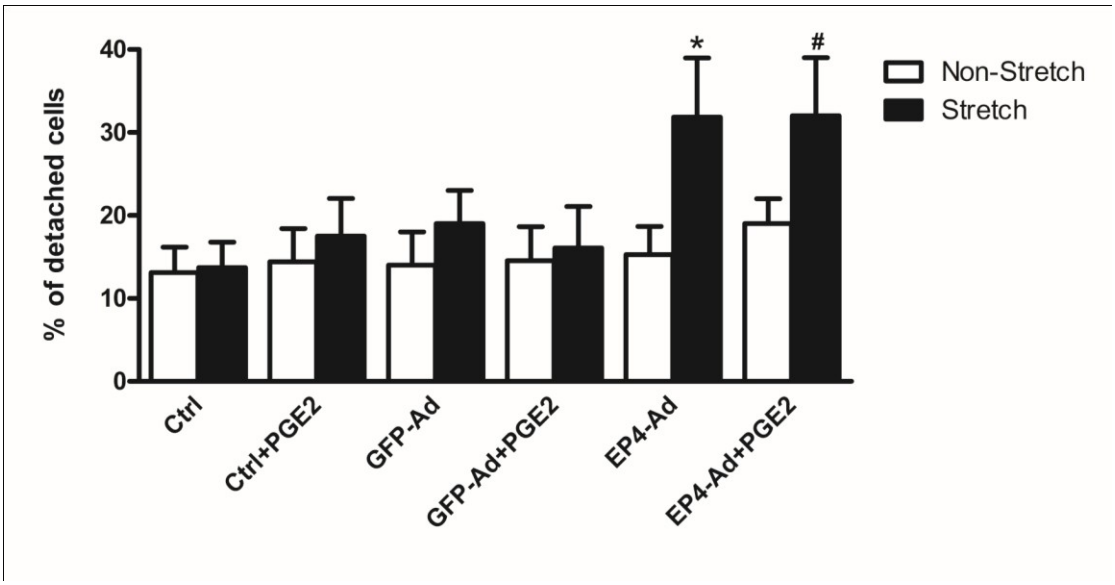


Figure 3.10 EP4 signaling and mechanical stretch promotes podocyte detachment.

After 14 days of differentiation, conditionally immortalized mouse podocytes are plated onto collagen I–coated silicone stretch plates and transduced with adenovirus for either the EP4 receptor or GFP control (MOI = 50). Three days after infection, cells are stimulated with 1 μM PGE2 and exposed to 12% equibiaxial mechanical stretch for 24 hours in the presence of 5 μM indomethacin. After mechanical stretch, medium is collected and nonadherent cells are counted using a hemocytometer. For determination of the number of adherent cells, trypsin is added to wells, the dislodged cells are centrifuged, the pellets are resuspended, and cells are counted (n = 5). *$P < 0.05$, $^{\#}P < 0.01$.

CHAPTER 4 – RESULTS

PODOCYTE EP4 AND AT1 RECEPTORS

4.1 *In vivo* Ang II pump studies reveal increased susceptibility in EP4^{pod+} mice

Following 5/6 Nx studies, EP4^{pod+} mice were next tested in a milder model of glomerular injury, progressive kidney disease secondary to chronic angiotensin II infusion. The goal of the Ang II pump studies was to determine whether the increased susceptibility exhibited by EP4^{pod+} mice would be sustained in an alternative model of renal injury. Accordingly, mice were subcutaneously-implanted with osmotic mini-pumps to deliver chronic Ang II infusion (1000ng/kg per day) in 8 week old male/female non-TG and EP4^{pod+} mice on an FVB/n background. EP4^{pod+} mice were normotensive and similar to non-TG littermates in their systolic blood pressure response to chronic Ang II infusion over time (**Figure 4.1.A**). However, at 2 weeks, ACR of Ang II infused EP4^{pod+} mice (760 μg/mg) was significantly greater than non-TG mice (303 μg/mg, $P<0.05$) (**Figure 4.1.B**). Despite these albuminuria differences, nephrin mRNA levels were significantly and to a similar extent reduced in both EP4^{pod+} and non-TG Ang II-infused mice compared vehicle-infused control mice ($P<0.05$) (**Figure 4.2**).

4.2 Crosstalk between PGE2 EP4 and Ang II AT1 receptors

A synergy between Ang II and PGE2 synthetic pathways was suggested when glomeruli were isolated and stimulated with Ang II *ex vivo.* **Figure 4.3** shows that after 2 h, Ang II-stimulated EP4^{pod+} glomeruli produced significantly higher PGE2 compared to glomeruli from non-TG mice (205 pg/mg vs. 56 pg/mg, respectively,

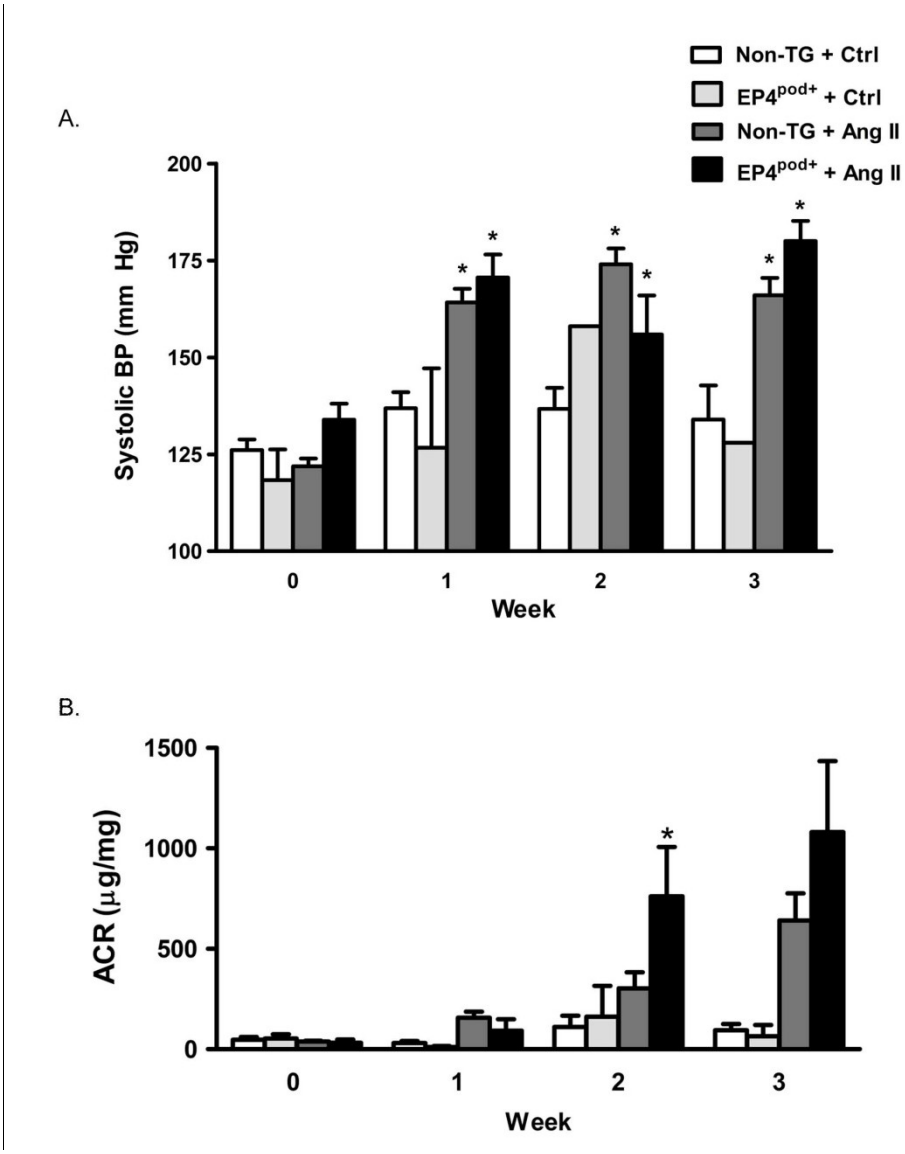


Figure 4.1 ***In vivo*** **Ang II pump studies reveal increased susceptibility in EP4^{pod+} mice.**

(A) Systolic blood pressure was assessed via tail-cuff plethysmography. Both EP4^{pod+} and non-TG mice receiving Ang II (n=6 and n=26 respectively) displayed elevated blood pressure compared to vehicle control animals (n=4 and n=11 for EP4^{pod+} and non-TG mice respectively). (B) At 2 weeks, ACR of Ang II infused EP4^{pod+} mice (760µg/mg, n=6) was significantly greater than non-TG mice (303µg/mg, n=26, *P < 0.05).

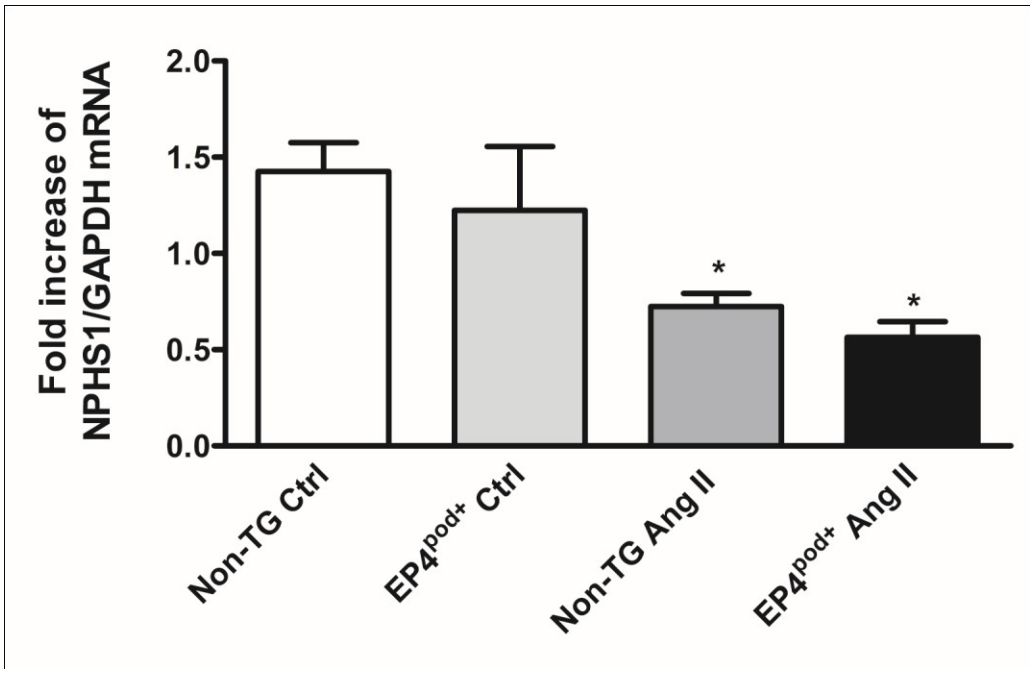


Figure 4.2 Nephrin expression decreases following Ang II administration.

mRNA levels were significantly and equally reduced in both $EP4^{pod+}$ and non-TG Ang II-infused mice compared vehicle-infused control mice ($P<0.05$), n=3.

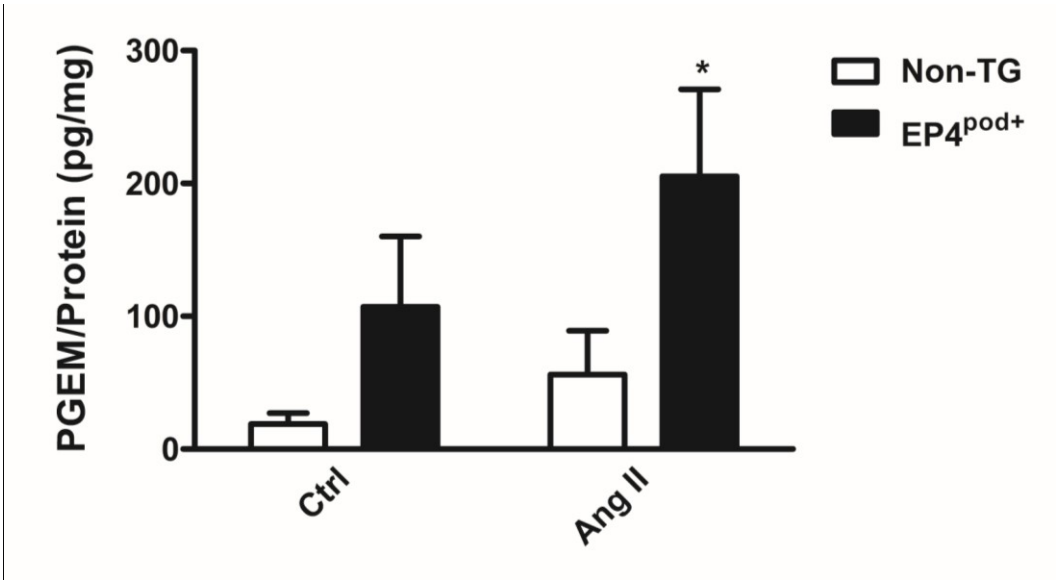


Figure 4.3 Glomerular PGEM production increases in EP4^{pod+} mice with Ang II stimulation.

Glomeruli isolated from EP4^{pod+} mice showed increased PGEM production following Ang II administration (1μM) vs. non-TG mice (205pg/mg vs. 56pg/mg, respectively, $P<0.05$, n=3-4).

P < 0.05). Furthermore, studies using isolated glomeruli showed that 2 hr Ang II (1μM) stimulation increased COX-2 protein in a p38MAPK dependent fashion, since the p38 MAPK inhibitor, SB202190 was able to prevent COX-2 induction (P<0.05) (**Figure 4.4**). Using immortalized mouse podocytes adenovirally transduced to overexpress an AT1 receptor construct, COX-2 induction was detected following 6 hrs of Ang II stimulation (1μM; P<0.05; **Figure 4.5**). As with isolated glomeruli, induction of COX-2 in cultured mouse podocytes is p38 MAPK dependent since the p38 MAPK inhibitor, SB202190 was able to block Ang II dependent increases in COX-2 expression. Neither of the additional kinase inhibitors, SP600125 (JNK) and PD98059 (MEK1), nor the NADPH oxidase inhibitor, apocynin were able to prevent COX-2 induction (**Figure 4.6)**.

4.3 AT1 receptor overexpression exacerbates stretch-induced detachment from the extracellular matrix

Podocyte detachment was also investigated in cells overexpressing the Ang II AT1 receptor following mechanical stimulation. Recall that increased mechanical strain is a mimic of enhanced glomerular capillary pressure characteristic of a number of glomerular diseases. To this end a significant adhesion defect was observed for podocytes overexpressing the AT1 receptor following simultaneous exposure to Ang II and equibiaxial mechanical stretch compared to uninfected cells (52% detachment vs. 29% detachment respectively; P<0.05; 12% elongation, 0.5 Hz, 24 h). Importantly, this decrease in podocyte extracellular matrix attachment was reversed by COX-2 inhibition (31% detachment; **Figure 4.7**).

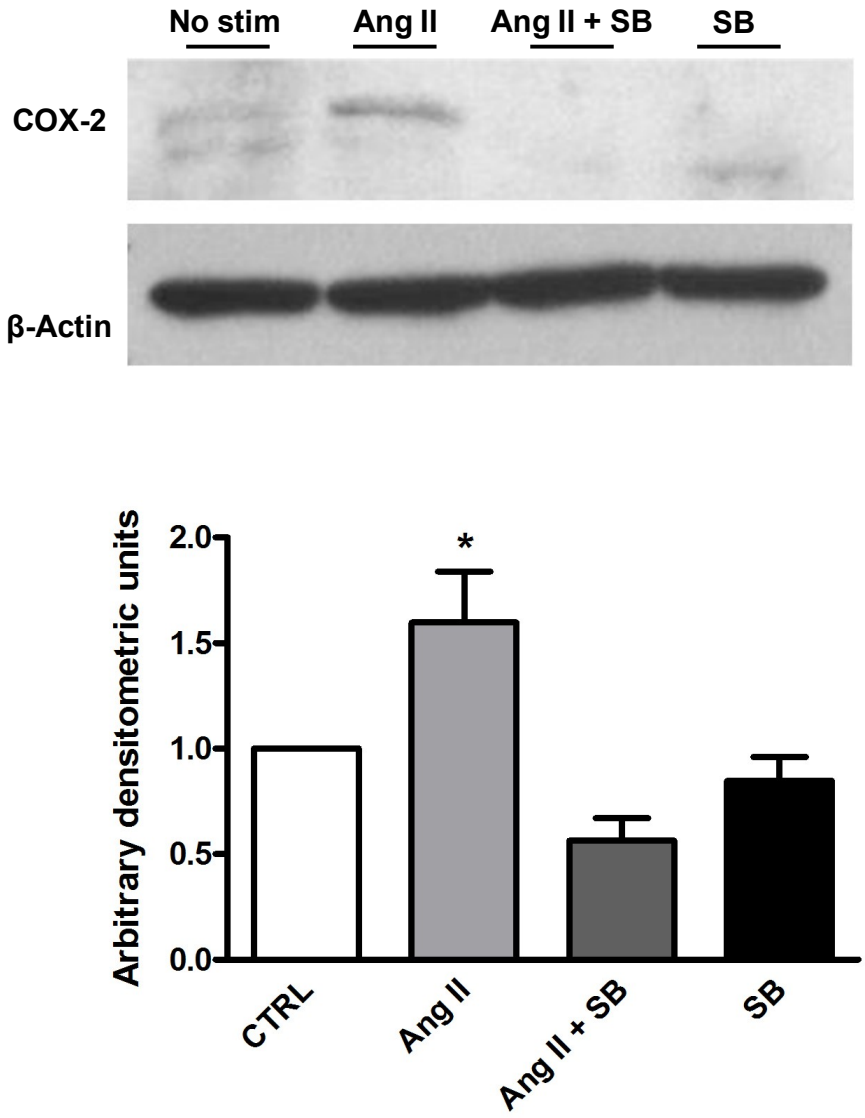


Figure 4.4 Glomerular COX-2 expression increases following Ang II stimulation and is p38 dependent.

COX-2 expression increases in glomeruli isolated from non-TG mice and stimulated with Ang II (1μM) for 2 hrs. This increase was blocked following co-treatment with the p38 MAPK inhibitor, SB (5μM), n=3.

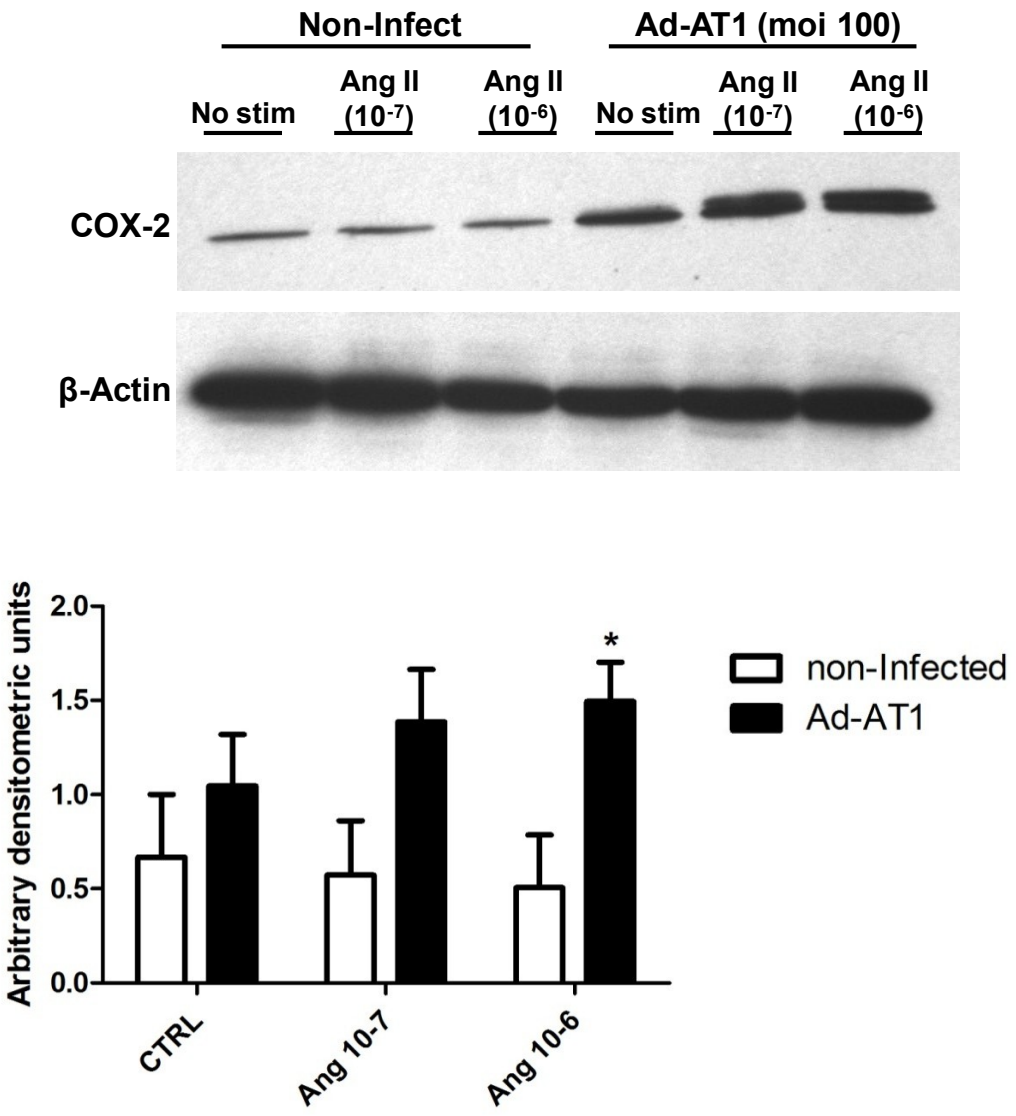


Figure 4.5 Stimulation of podocyte AT1 receptors increases COX-2 expression. Conditionally-immortalized mouse podocytes, adenovirally transduced to overexpress an AT1 receptor construct, showed increased immunodetectable COX-2 expression as compared to both uninfected and unstimulated controls (*P < 0.05) following 6 h exposure to Ang II (0.1μM – 1μM), n=3.

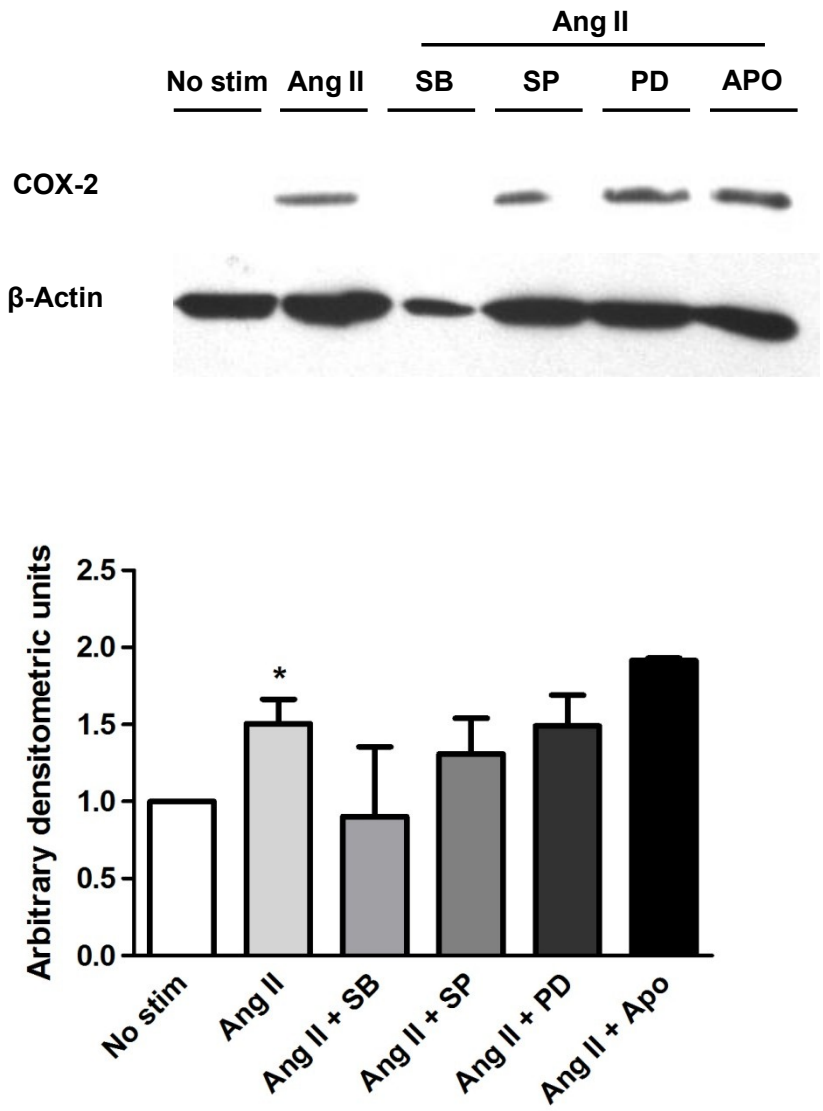


Figure 4.6 Induction of podocyte COX-2 is p38 dependent.

Conditionally-immortalized mouse podocytes, adenovirally transduced to overexpress an AT1 receptor construct, showed increased COX-2 expression following 6 h exposure to Ang II (1μM). The p38 inhibitor, SB202190 (5μM) blocked COX-2 induction while JNK and MEK1 inhibitors, and apocynin (SP, PD and APO respectively, 5μM) had no effect, (*$P < 0.05$; n=3).

4.4 $EP4^{pod-/-}$ mice are protected from Ang II mediated podocyte injury

Following podocyte detachment experiments, *in vivo* studies were conducted to determine whether the EP4 receptor was involved in Ang II receptor mediated signaling. In this regard, mice lacking podocyte EP4 receptor expression ($EP4^{pod-/-}$ mice) were subcutaneously implanted with mini-osmotic pumps for chronic Ang II infusion. As expected, systolic blood pressure increased rapidly (by 1 week) for all groups receiving chronic Ang II infusion compared to control groups (Δ+25 mmHg; **Figure 4.8.A**). Significantly, mice lacking the EP4 receptor in podocytes were protected against the development of Ang II mediated albuminuria. ACR levels for $EP4^{pod-/-}$ mice (125μg/mg) was significantly lower than $EP4^{pod+}$ mice (3545μg/mg, $P<0.05$; **Figure 4.8.B**)

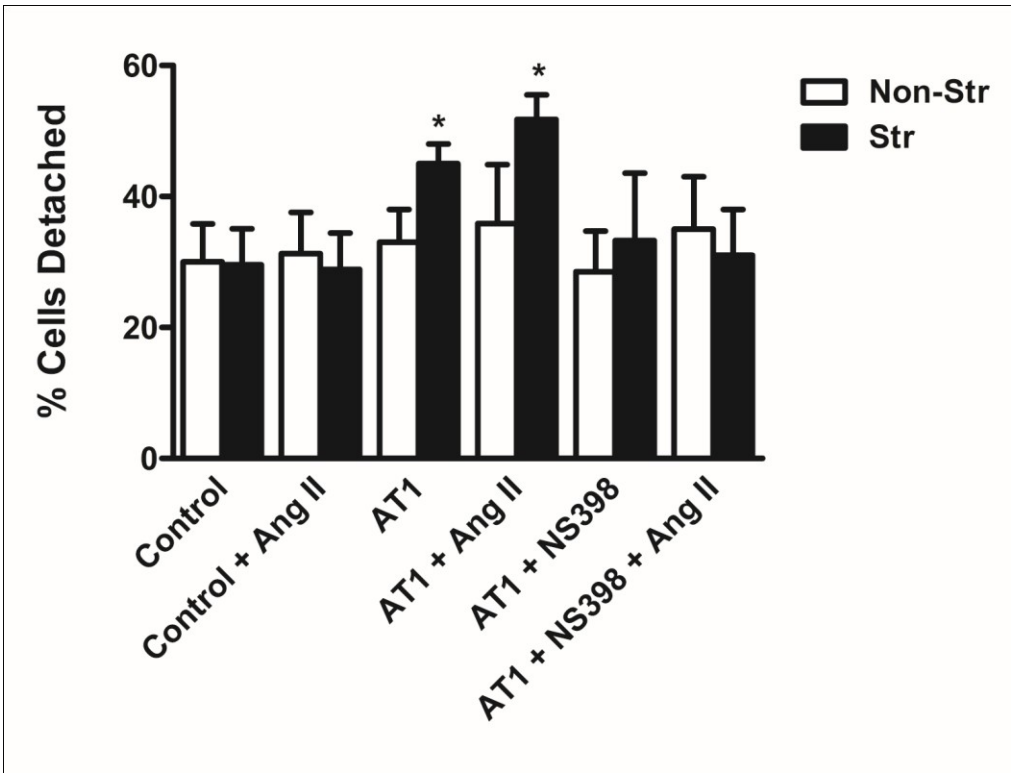


Figure 4.7 AT1 signaling and mechanical stretch promotes podocyte detachment in a COX-2 dependent manner.

A significant adhesion defect for podocytes overexpressing the AT1 receptor was observed following simultaneous exposure to Ang II and mechanical stretch (12% elongation, 0.5Hz, 24h, *P<0.05). Which was reversed by the COX-2 inhibitor NS-398, n=4.

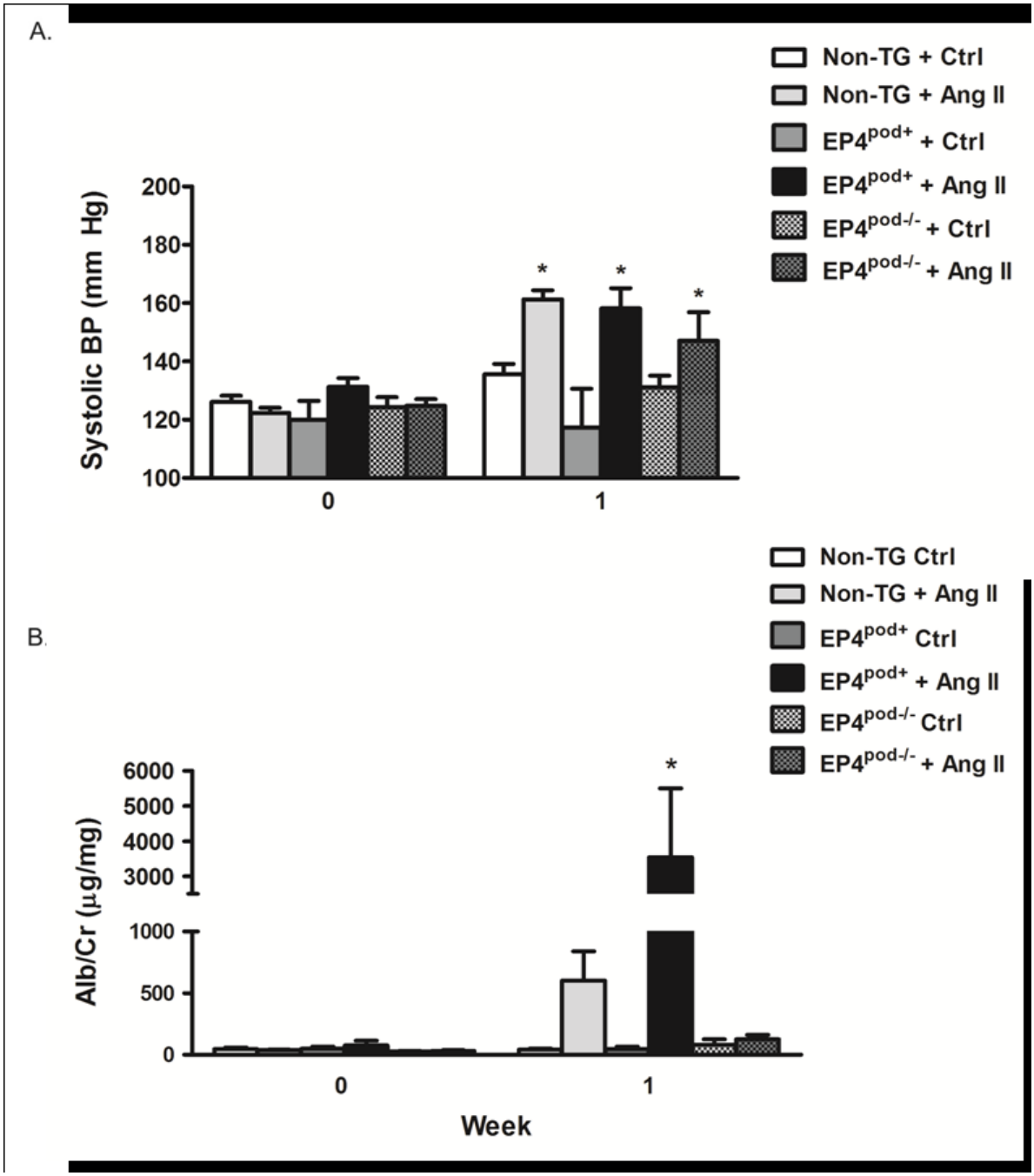


Figure 4.8 ***In vivo*** **Ang II pump studies reveal protection in EP4$^{pod-/-}$ mice.**

(A) Systolic blood pressure was assessed via tail-cuff plethysmography. All groups receiving Ang II displayed elevated blood pressure compared to vehicle control animals. (B) After 1 week of chronic Ang II infusion, ACR of EP4$^{pod-/-}$ mice (125µg/mg, n=5) was significantly lower than EP4^{pod+} mice (3545µg/mg, n=12, *P<0.01).

CHAPTER 5 – RESULTS

GENERATION OF P38DN-TG MICE

5.1 Generation of transgenic mice with podocyte-specific p38αDN-expression

p38 MAPK activity is induced in a number of models of glomerular injury (F. Y. Ma, Liu, & Nikolic-Paterson, 2009). In order to ascertain the role of p38 MAPK signaling in the podocyte, mice were generated with podocyte-specific expression of a dominant negative (DN) p38α isoform (p38αDN mice). The pro-apoptotic isoform of p38 (p38α) is activated in a number of glomerular diseases, including MCD, GN as well as FSGS making p38 MAPK a viable target for therapy (Stambe et al., 2004). To effectively reduce p38 activity, site-specific mutations were introduced at critical amino acids of the phosphorylation rich kinase domain of the protein (T180A; Y182F) (Engelman, Lisanti, & Scherer, 1998; Ludwig et al., 1998; Rincon et al., 1998; Y. Wang et al., 1998). The construct included a double hemagglutinin (2xHA) tag added at the 5'end and a C-terminal FLAG tag, which like the HA tag, facilitates transgene detection. Podocyte-specific expression was achieved by cloning the 2XHA-p38αDN-FLAG construct downstream of an 8.3kb fragment of the mouse nephrin (*NPHS1*) promoter (see **Figure 5.1.A** for a schematic of the construct). This promoter has been successfully used in a number of different transgenic lines including $EP4^{pod+}$ transgenic mice. Forty-six pups were obtained following injection of the above mentioned transgene into FVB/N oocytes, and 15 founders were identified using PCR of tail-snip DNA, presence of the 432bp PCR product confirmed the presence of the transgene **(Figure 5.1.B)**. As with $EP4^{pod+}$ mice, p38αDN-TG mice appear healthy, and are non-proteinuric. Quantitative RT-PCR of mRNA from whole kidney was used to identify 2 founder lines with significant podocyte-specific expression of the p38αDN transgene (**Figure 5.1.C**).

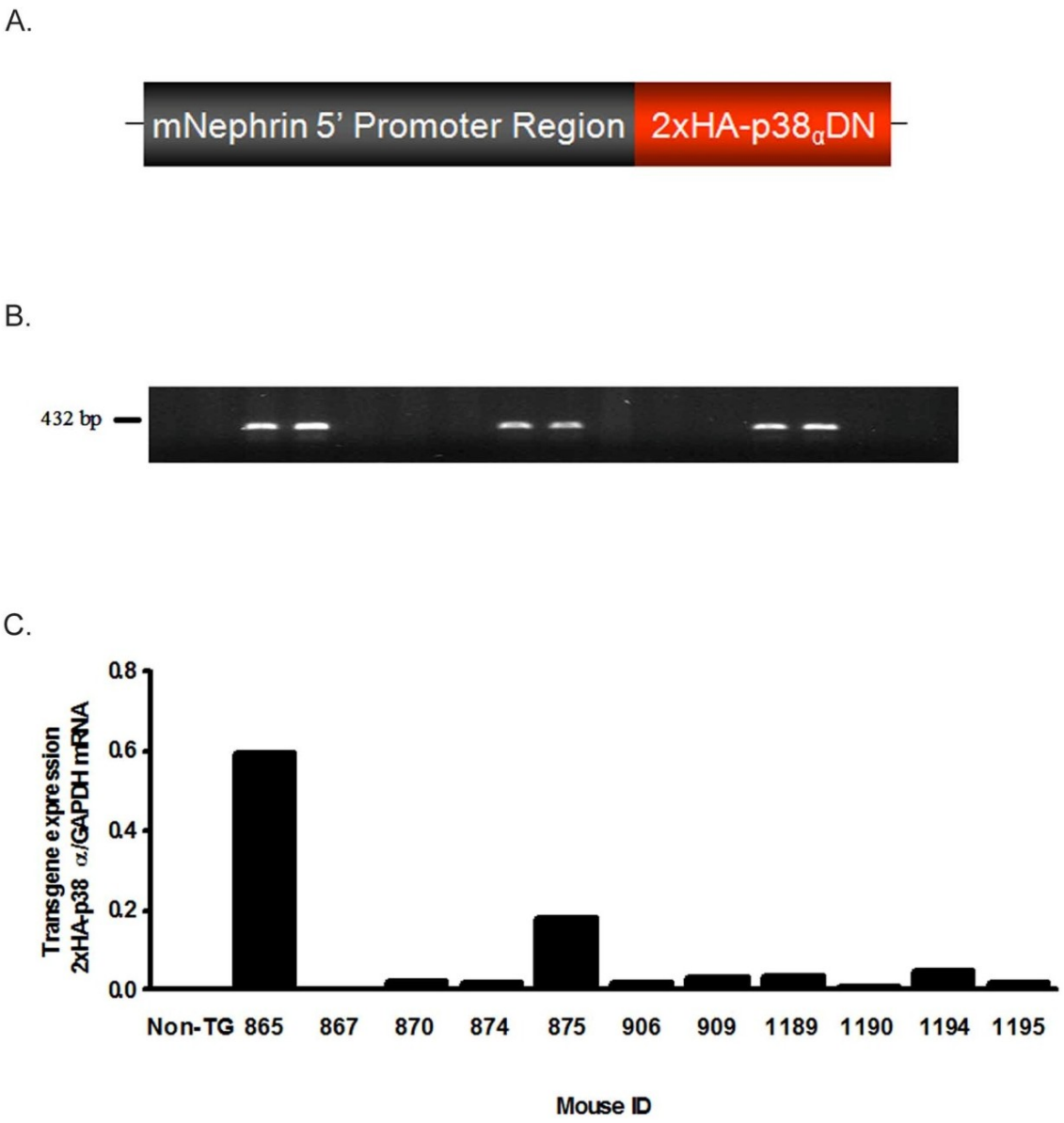


Figure 5.1 Characterization of p38αDN transgenic founders.

(A) Illustration of the construct used to generate p38αDN mice. An 8.3-kb fragment of the mouse *NPHS1* immediate promoter region is incorporated upstream of a human p38α open reading frame that has site-specific mutations were introduced at critical amino acids of the phosphorylation rich kinase domain of the protein (T180A; Y182F). A 5` 2XHA epitope tag is inserted immediately distal to the *NPHS1* promoter sequence. (B) Transgenic mice were identified by PCR of tail snip DNA. The representative image illustrates the 432 bp product amplified from the transgene. 15 founders were obtained from a total of 46 pups. (C) Quantitative RT-PCR was performed using specific HA-p38αDN primers to identify founders with high podocyte-specific levels of the transgene (normalized to GAPDH).

5.2 Anti-GBM GN in p38αDN-TG mice

Upon characterization, p38αDN-TG mice were next tested in various models of glomerular injury known to involve PGE2, COX-2 and p38 MAPK. The first model employed was a non-accelerated α-GBM model of glomerulonephritis. The α-GBM GN variant of GN is distinguished by its production of antibodies against the α_3 chain of type IV collagen which invokes a quick immune response involving initial neutrophil infiltration and subsequent complement activation and leukotriene synthesis (Bergs, 2005). Studies by others have demonstrated that podocyte p38 activity increases in rodent models of GN (Stambe, Atkins, Hill, & Nikolic-Paterson, 2003).

Testing our p38αDN-TG mice in the α-GBM model of GN involved injecting an α-GBM antibody via tail vein to mice and collecting urine over a given time course. Albumin from spot urine samples were normalized to urinary creatinine (Alb/Cr). Following tail-vein injection of the α-GBM antibody, we observed rapid and transient development of proteinuria. One day following induction of GN, p38α-DN-TG mice (n=12) were slightly less proteinuric (2631 μg/mg) than non-TG mice (3507 μg/mg; n=12), however this trend did not reach statistical significance (**Figure 5.2**).

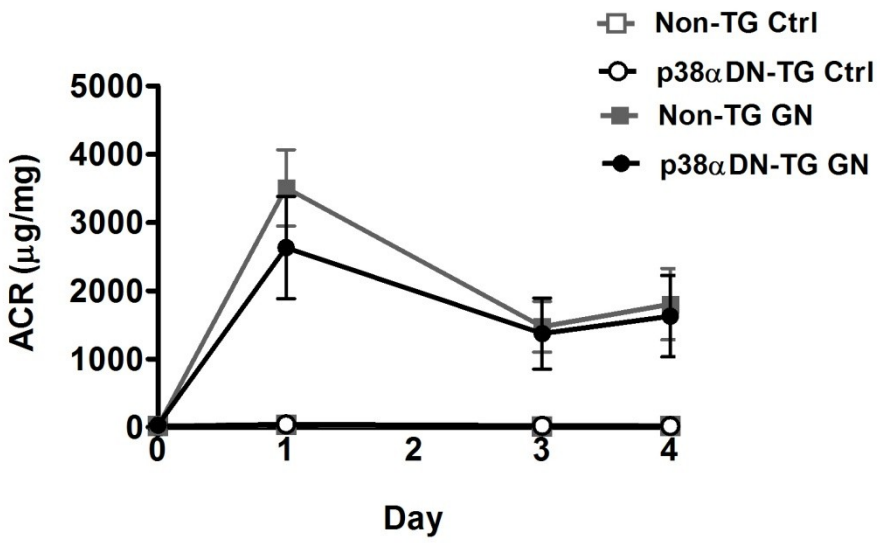


Figure 5.2 Anti-GBM GN in p38αDN-TG mice.

Following tail-vein administration of the α-GBM antibody, p38αDN-TG mice (n=12) were less proteinuric (day 1) as compared to non-TG mice (n=12). ACR levels were not elevated for either non-TG-Ctrl (n=5) nor p38αDN-TG-Ctrl (n=6) mice.

5.3 Ang II-dependent hypertension

Subsequently, p38αDN-TG mice were tested using an Ang II dependent hypertension model of hypertension-associated renal injury. Both non-TG and p38αDN-TG mice exhibited similar increases (~+Δ25mmHg) in systolic blood pressure as early as 1 week following insertion of the Ang II pump (**Figure 5.3.A**). Increased systolic BP was accompanied by slightly lower ACRs for p38αDN-TG mice receiving Ang II at the 3 week time point (433μg/mg vs. 697μg/mg respectively) however this trend did not achieve statistical significance (**Figure 5.3.B**).

In addition, nephrin expression was significantly lower from whole kidney RNA samples following chronic Ang II infusion. Both non-TG and p38αDN-TG mice displayed a 50% reduction in nephrin mRNA levels compared to mice receiving vehicle control ($P<0.05$; **Figure 5.4.A**). A slight but significant decrease in WT1 expression was also detected in non-TG mice, implying a reduction in podocyte number ($P<0.05$; **Figure 5.4.B**). p38αDN-TG mice with chronic Ang II infusion exhibited a slightly higher WT1 expression level (vs. non-TG mice receiving Ang II) however this trend was not statistically significant.

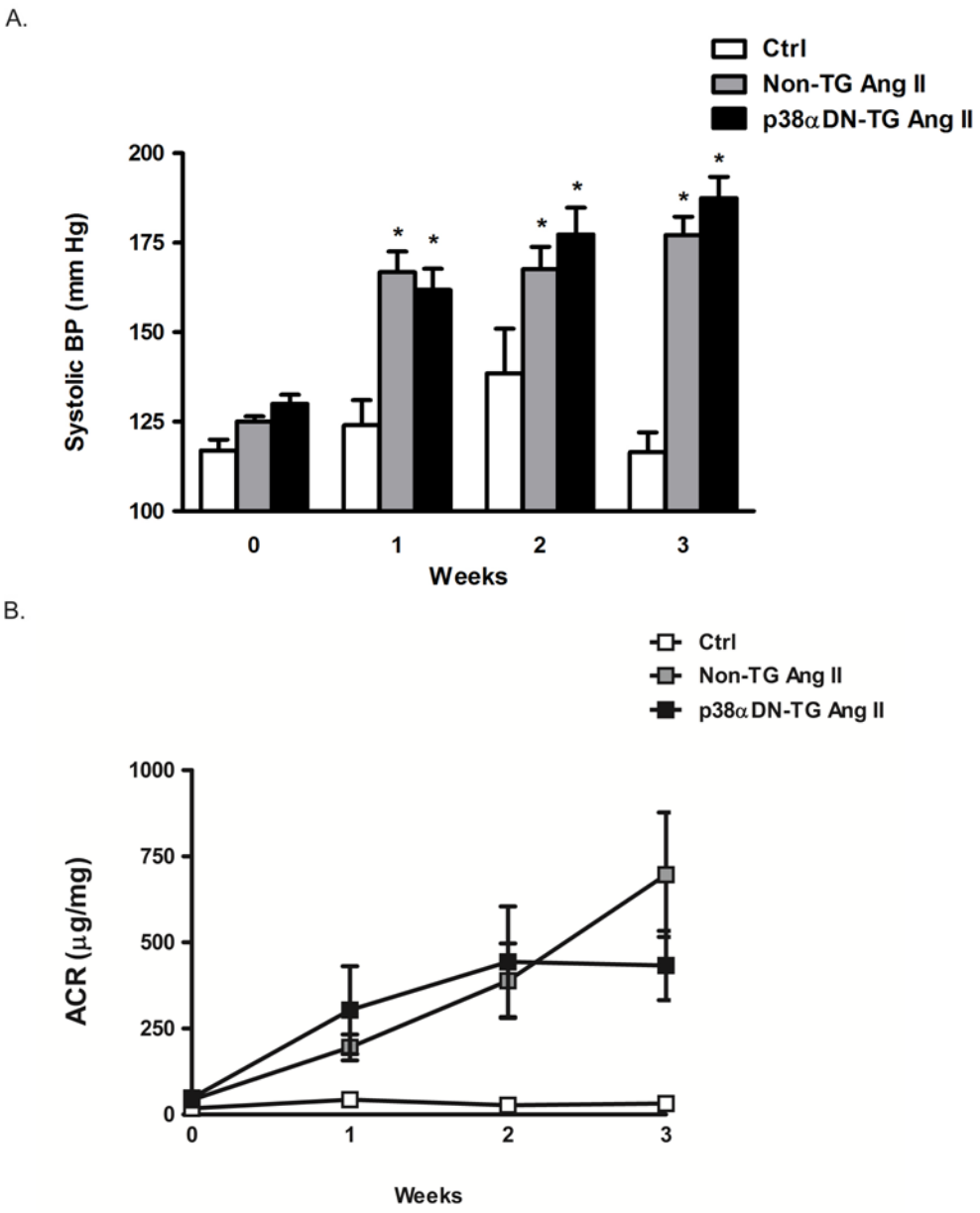
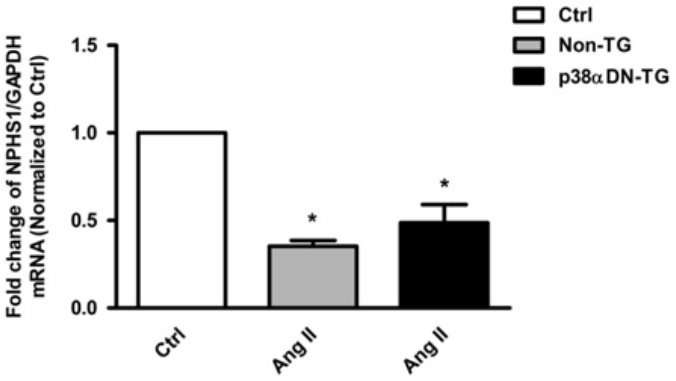


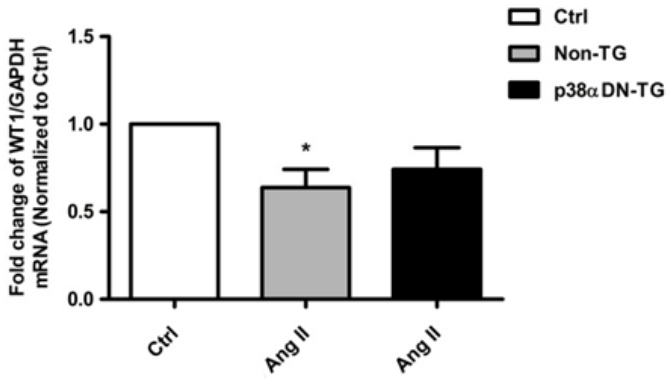
Figure 5.3 ***In vivo*** **Ang II pump studies in p38αDN-TG mice.**

(A) Systolic blood pressure was assessed via tail-cuff plethysmography. Both p38αDN-TG (n=27) and non-TG (n=18) mice receiving Ang II exhibited similar increases in systolic blood pressure as early as 1 week following insertion of the Ang II pump compared to vehicle control animals (Ctrl; n=4). (B) Chronic infusion of Ang II, revealed slightly lower ACR for p38αDN-TG compared to non-TG mice at the 3 week time point (433 µg/mg vs. 697 µg/mg, n=27 and n=18 respectively).

A.


Ctrl
Non-TG
p38α DN-TG
Fold change of NPHS1/GAPDH mRNA (Normalized to Ctrl)
1.5
1.0
0.5
0.0
*
*
Ctrl
Ang II
Ang II

B.


Ctrl
Non-TG
p38α DN-TG
Fold change of WT1/GAPDH mRNA (Normalized to Ctrl)
1.5
1.0
0.5
0.0
*
Ctrl
Ang II
Ang II

C.

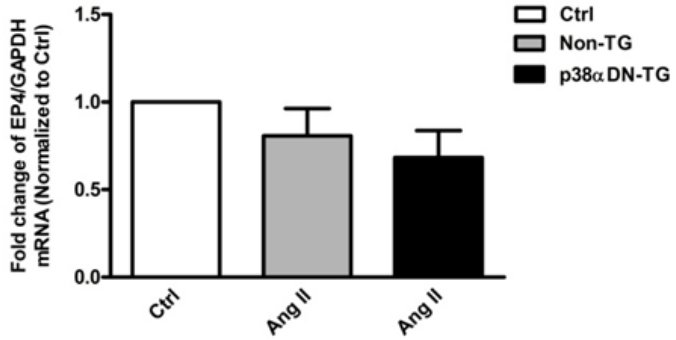

Ctrl
Non-TG
p38α DN-TG
Fold change of EP4/GAPDH mRNA (Normalized to Ctrl)
1.5
1.0
0.5
0.0
Ctrl
Ang II
Ang II

Figure 5.4 Decreased renal *NPHS1* following chronic Ang II infusion.

Quantitative RT-PCR confirmed that renal nephrin mRNA was significantly decreased in mice receiving chronic Ang II infusion compared to vehicle control (n=2) at the end of this 4 week study (mRNA normaluized to GAPDH and then to control). There was also a significant decrease in WT1 expression for non-TG mice (n=5) but not for TG mice (n=4) receiving Ang II (*P < 0.05 vs. mice receiving vehicle control)

CHAPTER 6 – RESULTS

INVESTIGATING THE ROLE OF THE EP1 RECEPTOR

6.1. EP1$^{-/-}$ mice become proteinuric following 5/6Nx

Since podocytes express both EP4 and EP1 receptor subtypes, our next objective was to determine the involvement of EP1 signaling in the podocyte. Previously it was demonstrated that in response to mechanical stretch, EP1 receptor levels did not change (as opposed to the increases observed with respect to the EP4 receptor subtype). In order to investigate the role of EP1 receptors in the progression of glomerular injury EP1$^{-/-}$ mice were tested in the 5/6 nephrectomy model of progressive kidney disease. In addition to the urinary ACR measurements, blood pressure was also monitored for each mouse by tail-cuff plethysmography.

Given that the EP1 receptor exhibits vasoconstrictor effects it came as a surprise when 5/6 Nx studies revealed similar increases in systolic blood pressure for Wt and EP1$^{-/-}$ mice following nephrectomy (**Figure 6.1.A**). Furthermore, albuminuria was not significantly different among nephrectomized groups (**Figure 6.2.B**).

6.2. Chronic Ang II infusion in EP1$^{-/-}$ mice

We next tested whether chronic infusion with Ang II, which is thought to confer both hemodynamic and non-hemodynamic actions on the podocyte, would exacerbate proteinuria in mice lacking the EP1 receptor. Studies showed that EP1$^{-/-}$ mice on a C57Bl/6 background are slightly hypotensive (Guan et al., 2007 ; Stock et al., 2001). This phenomenon was not observed in the current study, where $EP_1^{-/-}$ mice on an FVB/N background are normotensive and in response to chronic Ang II infusion display similar increases in systolic blood pressure compared to Wt (**Figure 6.2.A**). Interestingly at 1

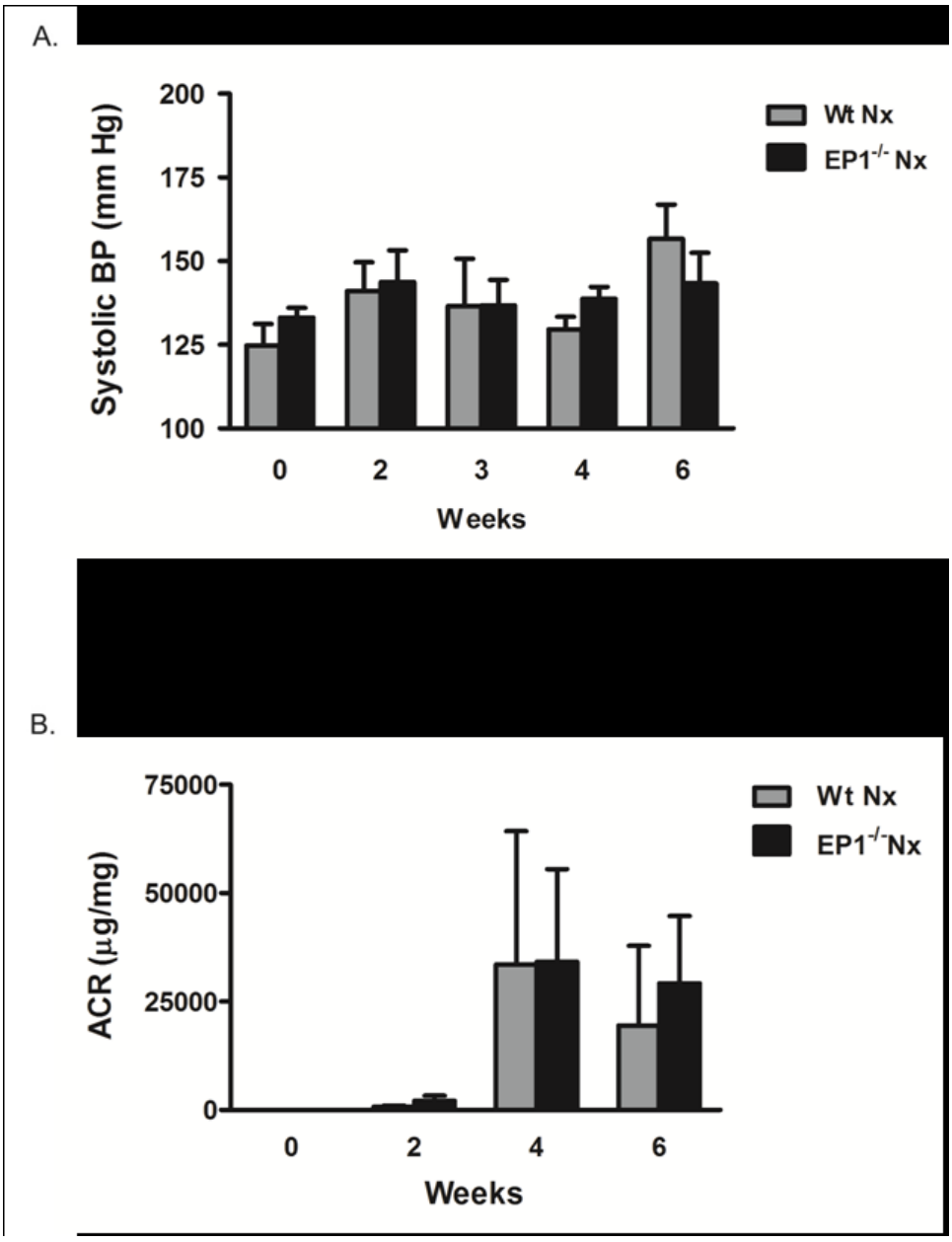


Figure 6.1 EP1$^{-/-}$ mice become proteinuric following 5/6Nx.

(A) Following 5/6 Nx, both EP1$^{-/-}$ (n=6-7) and Wt (n=4-5) mice displayed similar elevations in systolic BP. (B) No significant difference was observed in ACRs between EP1$^{-/-}$ and Wt mice with 5/6 Nx at the end of six weeks.

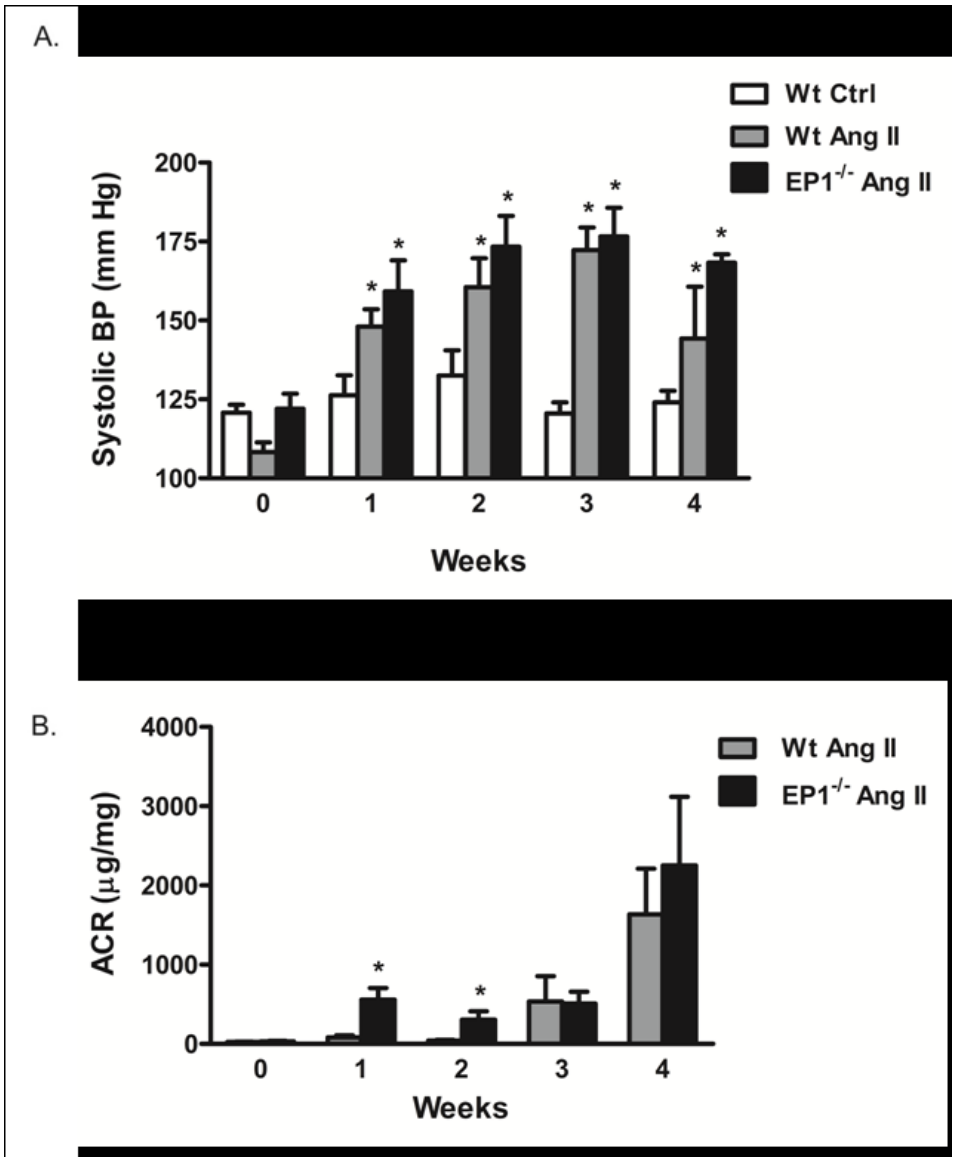


Figure 6.2 ***In vivo*** **Ang II pump studies in EP1$^{-/-}$ mice.**

(A) Systolic blood pressure was assessed via tail-cuff plethysmography. Both EP1$^{-/-}$ (n=21) and Wt (n=26) mice receiving Ang II exhibited similar increases in systolic blood pressure as early as 1 week following insertion of the Ang II pump compared to vehicle control (n=11) animals. (B) Chronic infusion of Ang II, revealed significantly higher ACR for EP1$^{-/-}$ mice compared to Wt mice as early as 1 week following insertion of the Ang II mini-pump (558 µg/mg vs. 81 µg/mg, $P < 0.05$ respectively).

week following the insertion of the Ang II osmotic mini-pump $EP1^{-/-}$ mice have significantly higher proteinuria than Wt (558 μg/mg vs. 81 μg/mg, $p<0.05$), an effect which holds at the 2 week time point (306 μg/mg vs. 43 μg/mg, $p<0.05$) but is lost during week 3 and 4 of the study (**Figure 6.2.B**). This implies that initially, non-hemodynamic effects (ie. activation of various signaling pathways), may be altered in $EP1^{-/-}$ mice, ultimately to disrupt the filtration barrier. Following 3 weeks of sustained high Ang II levels, hemodynamic effects dominate.

Following 4 weeks of subcutaneous Ang II infusion spot urine samples were collected and analyzed for PGE2 levels. Since PGE2 has a very short half-life, PGEM, its stable metabolite was used as a surrogate. Chronic infusion of Ang II, revealed higher urinary PGE2 concentrations for Wt mice (3404 pg/mg) compared to Wt mice receiving vehicle control (1097 pg/mg), this increase in PGE2 production was prevented in $EP1^{-/-}$ mice (1337pg/mg, **Figure 6.3.**)

Finally, decreased renal function was detected in mice following chronic Ang II infusion. FITC-inulin was used to establish glomerular filtration rate. An approximate 50% reduction in GFR was observed for wt and $EP1^{-/-}$ mice (4.7 μl/min/gBW and 3.7 μl/min/gBW respectively) compared to those mice receiving vehicle control infusion (10 μl/min/gBW, **Figure 6.4.**).

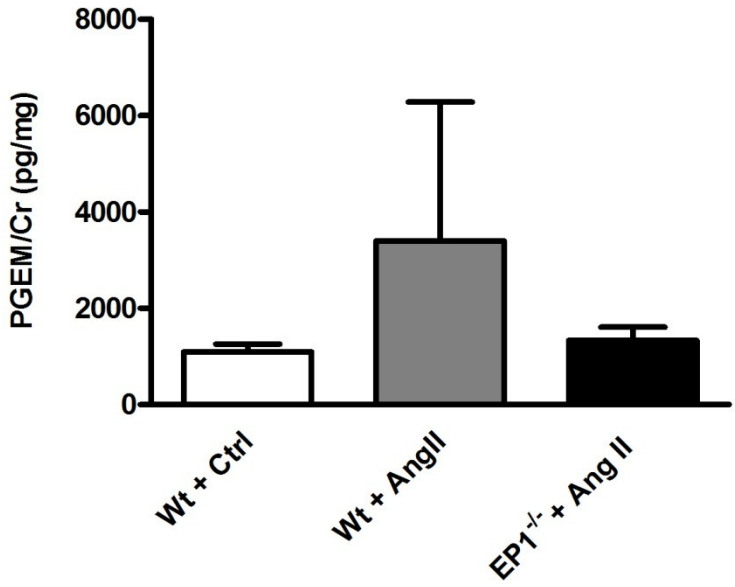


Figure 6.3 Chronic Ang II infusion increases urinary PGEM levels.

Chronic infusion of Ang II, revealed higher urinary PGEM (normalized to creatinine; Cr) concentrations for Wt mice (Wt+Ang II; 3404 pg/mg) compared to Wt mice receiving vehicle control (Wt+Ctrl; 1097 pg/mg) following four weeks of chronic Ang II infusion (1000ng/kg/d), no increase was detected in $EP1^{-/-}$ mice ($EP1^{-/-}$+Ang II; 1337pg/mg). PGEM was assayed by enzyme immunoassay (EIA).

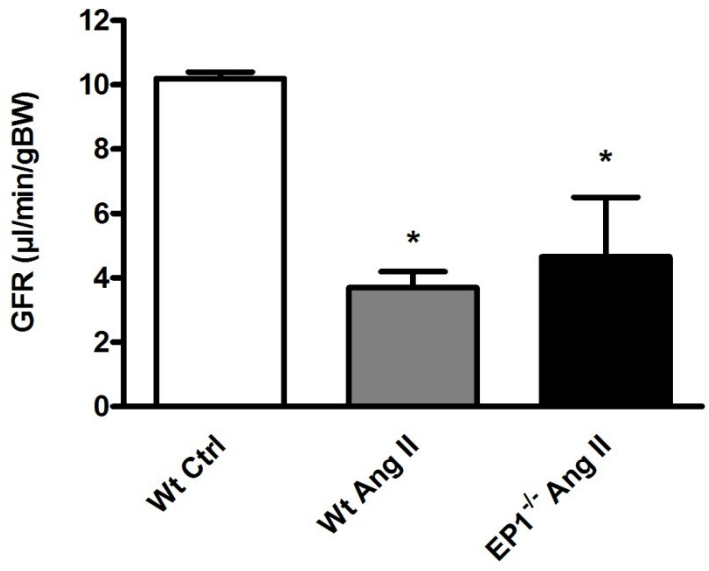


Figure 6.4 Chronic Ang II infusion decreases GFR.

Chronic infusion of Ang II, resulted in lower GFR for both $EP1^{-/-}$ and Wt mice (4.7 µl/min/gBW, n=4 and 3.7 µl/min/gBW, n=4 respectively, where $*P < 0.05$) compared to Wt mice receiving vehicle control (10 µl/min/gBW, n=3) following four weeks of chronic Ang II infusion.

CHAPTER 7 – DISCUSSION

7.1 A maladaptive role for EP4 receptor signaling in podocytes

COX inhibition reduces albuminuria in clinical and experimental renal disease. Work by Harris' group showed that COX-2 inhibition with SC58236 blunted PGE2 synthesis and proteinuria while improving glomerulosclerosis in nephrectomized rats (J. L. Wang et al., 2000). The present data are consistent with these findings, showing that in a mouse version of the 5/6 Nx CKD model, renal COX-2 was upregulated while its inhibition with NS-398 significantly attenuated albuminuria. A maladaptive role for COX-2 in podocytes is supported by studies using transgenic mice with podocyte-specific COX-2 overexpression. The elevated COX-2 levels render the mice susceptible to filtration barrier injury following either adriamycin (ADR) or puromycin aminonucleoside (PAN)-induced nephrosis, while non-TG mice remain resistant (H. Cheng et al., 2007; Jo et al., 2007).

The factors that initiate COX-2 upregulation in podocytes have only recently been investigated. Spurney and co-workers showed that Gq-dependent signaling induces COX-2 expression in cultured podocytes which was associated with increased PGE2 synthesis (L. Wang et al., 2005). Previous work showed that both mechanical stretch and PGE2 induce COX-2 expression in cultured mouse podocytes (Faour et al., 2008; Martineau et al., 2004). Specifically, it is the Gs-coupled EP4 receptor, and not the Gq-coupled EP1 subtype, that mediates PGE2-stimulated COX-2 induction in conditionally-immortalized mouse podocytes. The work presented in this thesis asked whether the EP4 receptor expressed in podocytes mediates the injurious effects of COX-2 activity and therefore contributes to GFB damage encountered in a model of CKD. Two approaches were chosen to test this hypothesis. First, overexpression of desensitization-resistant EP4

receptors in podocytes of mice allowed for maximal PGE2 signaling. This strategy rendered the mice more susceptible to injury during disease progression. In contrast, sham-operated EP4^{pod+} mice were normoalbuminuric indicating that overexpression of this GPCR was without effect on filtration barrier function. These findings suggest that in addition to overexpression of the EP4 receptor, glomerular COX-2 induction coupled with enhanced PGE2 synthesis is likely required to activate the EP4 signaling pathway. Although other prostanoids could potentially interact with the EP4 receptor expressed at such high levels, this possibility seems remote as the EP4 receptor is selective for PGE2 over most other prostanoids (Ki for PGE2, 1.1 nM; PGE1, 0.66 nM; PGD2, 1240 nM; PGF2α, 570 nM; iloprost – an IP selective agonist, 277 nM; and U46619 – a TP receptor ligand, 2330 nM) (Boie et al., 1997). Although overexpression of the wild type EP4 receptor may have yielded a similar phenotype, due to the potential for heterologous desensitization it was not thought that there would be observable enhanced expression/signaling. The second approach taken was to genetically ablate podocyte EP4 expression. Accordingly, podocyte-specific conditional EP4 deletion from mice conferred significant GFB protection following 5/6 Nx. This is in contrast with overexpression of the receptor which significantly enhanced the glomerular/tubulointerstitial damage following 5/6 Nx. In fact, renal damage was so severe that nearly 80% of EP4^{pod+} mice died earlier than 8 weeks post-5/6 Nx. Although loss of the EP4 receptor offered protection, it did not prevent the 20% mortality observed in 5/6 Nx wild type mice. Others showed that renal pathology and GFR following subtotal renal ablation in rats are ameliorated with an EP4 agonist (CP-044,519-02) (Vukicevic et al., 2006), which suggests that vascular EP4 receptor activation may be

beneficial in preserving renal blood flow in chronic kidney disease. However, in contrast to the present work renal ablation in those studies failed to induce EP4 expression. Furthermore, the effects upon the podocyte were not explored. The EP4 receptor may therefore exert pleiotropic effects on the kidney during the onset of injury. The current findings also contrast with those of Cheng et al., who showed that thromboxane receptor deletion, but not podocyte-specific EP4 receptor knockout in COX-2 overexpressing mice, protected against ADR-induced albuminuria and renal pathology (H. Cheng et al., 2009). This led to speculation that in addition to the obvious differences in the severity and etiology of the models employed (ADR vs. 5/6 Nx; the latter being significantly more injurious than the former), another factor contributing to the observed GFB phenotypes could be the background strain of mice (C57Bl/6J x B6/D2 in the Cheng study vs. FVB/N used in the present study). While C57Bl/6J mice are relatively resistant to a variety of kidney disease models, (L. J. Ma & Fogo, 2003) the FVB/N strain of mice employed in the current study are susceptible to various forms of renal injury (Qi et al., 2005; Teiken et al., 2008; Z. Wang et al., 2005).

In the present study, attenuation of albuminuria in 5/6 Nx $EP4^{pod-/-}$ mice was incomplete, which may reflect the inefficient nature of the Cre-mediated EP4 gene deletion. Despite the fact that $CreEGFP^{pod+}$/ROSA26 mice exhibited a qualitatively robust glomerular Cre-recombinase activity, EP4 mRNA expression was not reduced beyond 50% in podocytes isolated from $EP4^{pod-/-}$ mice. While this could be due to incomplete excision of the floxed allele, basal podocyte EP4 expression may be relatively weak (and therefore difficult to detect reliably) in healthy glomeruli, being induced to significant levels only under pathophysiological conditions. Consistent with this

possibility, glomeruli isolated from healthy non-TG mice responded to PGE2 with a modest cAMP synthesis, whereas those obtained from $EP4^{pod+}$ transgenics produced cAMP levels in excess of 40-fold above non-stimulated controls. And while the possibility of off-target activity of Cre-recombinase in podocytes of $EP4^{pod-/-}$ mice cannot be discounted, functional deletion of EP4 receptor expression was sufficient to abrogate cAMP synthesis induced by PGE2 in $EP4^{pod-/-}$ primary podocyte cultures. On the other hand, the contribution to podocyte damage by additional factors not directly affected by the loss of EP4 expression cannot be discounted in this model (e.g., AngII, TGF-β, etc.). However, the interplay between these and the COX-2/PGE2/EP4 expression and activity is not yet known. The mechanism by which the EP4 receptor might alter podocyte structure/function will require additional studies. An impact upon cell survival is unclear, however Takano et al., demonstrated that PGE2/EP4 receptor coupling protects rat glomerular epithelial cells from apoptosis (Aoudjit, Potapov, & Takano, 2006). The present study revealed no detectable changes in apoptosis for conditionally immortalized mouse podocytes in response to PGE2. Similarly, Harris and co-workers showed that PAN-induced apoptosis was attenuated in response to antagonism of the Gq-coupled TP receptor, but not antagonism of the Gs-coupled EP4 receptor (H. Cheng et al., 2009). The work of Spurney and colleagues is consistent with these findings as they demonstrated that overexpression of a constitutively-active Gq α subunit stimulated calcineurin activity and was associated with podocyte apoptosis in vitro (L. Wang, Flannery, Rosenberg, Fields, & Spurney, 2008). The notion that Gq but not Gs-coupled signaling promotes podocyte apoptosis is supported by a number of studies examining effects of the Ang II AT1 receptor. Mechanical stretch of conditionally immortalized

podocytes contributed to AT1-dependent apoptosis (Durvasula et al., 2004). Transgenic overexpression of the AT1 receptor in rats produced glomerulosclerosis and podocyte loss (Hoffmann, Podlich, Hahnel, Kriz, & Gretz, 2004). In light of these findings, how then might activation of the Gs-coupled EP4 receptor be detrimental to podocyte health? Our findings indicate that exposure of immortalized podocytes, transduced to overexpress the EP4 receptor, to both mechanical stretch and PGE2, results in detachment from the extracellular matrix. Furthermore, as demonstrated previously, *in vitro* mechanical stretch of podocytes followed by acute PGE2 stimulation dramatically reduced actin stress fiber content (Martineau et al., 2004). Therefore, excessive EP4 activation might promote cell survival pathway activation, yet nevertheless contribute to injury through its detrimental effects upon podocyte attachment and cytoskeletal architecture. The loss of stress fibers along with the detachment defect observed in the present study may be mechanistically related. Stress fiber formation is dependent upon the small GTPase RhoA. PKA can phosphorylate RhoA on Ser188 to inhibit its activity by sequestering it away from active sites associated with the membrane (Ellerbroek, Wennerberg, & Burridge, 2003). For example, cAMP-dependent PKA induction in cultured human trabecular meshwork cells directly phosphorylates and inactivates RhoA resulting in stress fiber dissociation (Shen, Koga, Park, SundarRaj, & Yue, 2008). Whether PGE2 modulates its effects on the podocyte cytoskeleton and adhesion through regulation of Rho GTPase activity or other pathways such as those involving integrin receptor expression or focal adhesion kinase activity remain to be determined.

Taken together, these findings point to a maladaptive role for PGE2 acting through its Gs-coupled EP4 receptor subtype in podocytes. Activation of this receptor

contributes to the early deterioration of the GFB in a mouse model of chronic progressive renal injury and highlights the pleiotropic nature of the intrarenal actions of the prostanoid family.

7.2 Crosstalk of podocyte PGE2 EP4 and Ang II AT1 receptors

Recent studies by a number of groups have uncovered locally acting renin angiotensin systems in a number of tissue/cell types (Dzau, 1988). The classic renin angiotensin system (RAS) is a vital mediator of vascular tone and homeostasis. It has come to light that the glomerular podocytes are another cellular location for localized RAS activity (Durvasula & Shankland, 2008; Yoo et al., 2007). In the previous chapter we described a maladaptive role for the prostaglandin E2 EP4 receptor in podocytes (E. M. Stitt-Cavanagh et al.). Interestingly, studies using Sprague-Dawley rats and cultured mesangial cells revealed that Ang II induces glomerular COX-2 (Jaimes, Tian, Pearse, & Raij, 2005). Furthermore, it has been known for nearly 30 years that prostaglandins can stimulate renin release in canine kidneys (Gerber, Data, & Nies, 1978). Therefore we hypothesized that signaling through the EP4 receptor and activation of a local RAS act in concert to promote podocyte injury. The current study investigated whether there is crosstalk between the PGE2 EP4 receptors and the angiotensin II AT1 receptors in podocyte injury. To this end, both non-TG and the previously described $EP4^{pod+}$ mice were tested in an Ang II dependent model of hypertension associated with progressive kidney disease. Chronic Ang II infusion causes a rapid increase in systolic blood

pressure with increased proteinuria observed following 2 weeks of exposure in both non-TG and EP4^{pod+} groups. Interestingly, EP4^{pod+} mice were significantly more proteinuric than their non-TG littermates at this time point, suggesting that signaling through the EP4 receptor propagates podocyte injury in response to Ang II signaling. This phenomenon seems to be an early/transient event, as the increased susceptibility in EP4^{pod+} mice is lost by the 3 week time point. Furthermore, evidence for the EP4 receptor acting as an initiating factor of podocyte injury was further supported by tissue collected from sacrificed mice at the 4 week time point. At this later time point nephrin expression for both non-TG and EP4^{pod+} mice receiving Ang II infusion is similarly decreased compared to control groups, supporting the notion that increased susceptibility evidenced in EP4^{pod+} mice is a transient, initial event.

Next it was demonstrated that isolated glomeruli respond to Ang II stimulation with marked increases in PGEM production. Augmentation of PGEM production in response to Ang II stimulation is further enhanced in glomeruli from EP4^{pod+} mice, supporting a possible synergy between the two independent signaling pathways. Additionally when isolated glomeruli were stimulated with Ang II there was a significant increase in COX-2 protein levels. Recall that increased COX-2 expression is associated with podocyte injury in experimental glomerular disease models. Increased glomerular COX-2 following Ang II stimulation was abolished by a p38 MAPK inhibitor, underlining the importance of p38 MAPK activity in Ang II dependent COX-2 induction.

To explore a possible link between PGE2 mediated EP4 signaling and Ang II mediated AT1 signaling in a more direct and controlled environment, a conditionally immortalized podocyte cell line was employed. While it is true that podocytes express

AT1 (and AT2) receptors *in vivo*, expression *in vitro* is very low. It appears that conditionally immortalized podocytes lose AT receptor (both AT1 and AT2) expression following isolation and repeated passaging over time. Because of this, several groups have employed different strategies to boost AT1 receptor expression in cultured podocytes (Hsu et al., 2008). Similarly, we developed an adenovirus for the AT1 receptor in order to allow for Ang II mediated signaling in cultured podocytes, as occurs *in vivo*. To this end we showed, in cultured mouse podocytes adenovirally transduced to express AT1 receptor, increased COX-2 production in response to Ang II stimulation. As with isolated glomeruli, this COX-2 induction mediated through a p38 MAPK dependent mechanism. Inhibitors of alternative kinase pathways, JNK and MEK1/2 as well as the NADPH inhibitor apocynin, had no effect on COX-2 expression. Furthermore *in vitro* stretch experiments showed that for cultured mouse podocytes overexpressing AT1 receptors, there was a significant attachment defect in response to mechanical stimulation. Promisingly, this adhesion defect was blunted by selective COX-2 inhibition. Once again, these findings support the underlying hypothesis that COX-2 (thus potentially EP4 receptors) and AT1 receptors cooperate to promote podocyte injury.

Finally, to tease out whether the EP4 receptor itself is directly involved in increased podocyte injury in response to Ang II signaling; the *in vivo* Ang II infusion model of progressive kidney disease was once again used and included an additional group of $EP4^{pod-/-}$ mice. This approach is different from our studies using either isolated glomeruli or cultured podocytes where we considered the localized effects of Ang II signaling alone, chronic infusion of Ang II will also exhibit vascular / hemodynamic events.

As with previous studies using the chronic Ang II infusion model of chronic kidney disease, in our hands, mice quickly became both hypertensive and proteinuric. Podocyte-specific deletion of the EP4 receptor offered protection against the development of proteinuria, providing evidence for crosstalk between Ang II and PGE2 signaling pathways. Whether the protection offered by $EP4^{pod-/-}$ mice was due to interference of a local RAS or an external, downstream hymodynamic protective mechanism could not be addressed from the current data. Future studies using specific agents to lower blood pressure (ie. hydralazine) while co-administering Ang II should elucidate the nature of the protection offered by podocyte EP4 receptors. Nonetheless, taken together, this study implicates for the first time an interactive relationship between Ang II AT1 receptors and PGE2 EP4 receptors. Further investigations are required to explore the therapeutic benefits of this association.

7.3 Generation of transgenic mice with podocyte-specific p38αDN-expression

To reiterate, it is becoming increasingly evident that there is a close association between podocyte damage, proteinuria and the progression of glomerular disease. To date, experiments designed to expose this relationship show that in rat models of progressive kidney disease both COX-2 expression and proteinuria increase. In support of this link, recall that proteinuria can be reduced using a specific COX-2 inhibitor (J. L. Wang et al., 2000; J. L. Wang et al., 1998). COX-2 inhibitors block PGE2 production

which results in decreased signaling through the EP4 receptor. Importantly, GPCRs such as the EP4 receptor are known activators of p38 MAPK. There are four isoforms of p38 MAPK (α, β, γ, δ) mediating tissue specific responses including inflammation, apoptosis, growth and differentiation (Cuadrado & Nebreda). A maladaptive role for p38 MAPK is supported by studies using TGF-β transgenic mice where, TGF- β induces podocyte p38 MAPK leading to caspase-3 activation. Signaling though this pro-apoptotic pathway in TGF- β mice, lead to progressive glomerulosclerosis (Schiffer et al., 2001). Additonally, Kang *et al*., demonstrated that Ang II stimulates VEGF synthesis and extracellular matrix accumulation through a p38 MAPK pathway in cultured mouse podocytes (S. W. Kang et al., 2003; Y. S. Kang et al., 2006). Finally p38 activation in cultured podocytes following puromycin aminonucleoside exposure causes actin reorganization (Koshikawa et al., 2005). Importantly, the pro-apoptotic isoform of p38 (p38α) is activated in a number of glomerular diseases, including MCD, GN as well as FSGS making p38 MAPK a viable target for therapy (Stambe et al., 2004). Moreover, *in vivo* studies using a rat model of α-GBM GN showed that pre-treatment with a highly-selective p38α inhibitor reduced renal injury and significantly blunted proteinuria (Stambe, Atkins, Tesch et al., 2003). This suggests that p38 MAPK may be another key player in the progression of glomerular injury and that inhibition of p38α may play a protective role in podocytes.

Podocytes continuously cope with pulsatile glomerular capillary pressure (P_{gc}). *In vitro* stretch experiments, designed to mimic increased Pgc encountered in many glomerular diseases revealed that in response to mechanical stimulation, p38 MAPK is rapidly activated to induce both COX-2 and prostaglandin EP4 receptor expression kennedy. No similar induction of EP1 receptor was observed. Amplified COX-2

expression enhances synthesis of PGE2 from AA. It is known that in an autocrine feedback manner, PGE2 stimulates EP4 receptors to promote p38 activation through a PKA independent pathway (Faour et al., 2008). Therefore stimulation of this signaling cascade may result in podocyte damage following the activation of various downstream mechanisms and thus compromise the integrity of the filtration barrier. For this reason it was hypothesized that p38 MAPK activation contributes to podocyte injury and leads to increased proteinuria. To test this hypothesis a transgenic mouse with podocyte-specific overexpression of a dominant negative p38α was developed. Following characterization of p38αDN-TG mice, mice were tested in an α-GBM GN model of Goodpasture's syndrome. Goodpasture's syndrome is characterized by an immune response targeting the Goodpasture antigen present in both the lungs and kidneys (Weber, Kohler, Manns, Baum, & Meyer zum Buschenfelde, 1987).

Our studies revealed that while there was a slight trend for p38αDN-TG mice to have lower ACRs, these values did not reach statistical significance. This was surprising since Stambe *et al*., previously reported that p38 activity increased following induction of GN (Stambe, Atkins, Hill et al., 2003). It is possible that podocyte expression of the dominant negative p38α was not sufficient to invoke a positive renal outcome. It is also possible that other p38 isoforms (ie. the β isoform is also expressed in podocytes) are able to compensate in response to inactivity of the α isoform. In the event that p38α is not an active player following induction of α-GBM GN, p38αDN-TG mice were next tested in the Ang II hypertension dependent model of chronic kidney disease. As with previous studies, chronic Ang II infusion causes a rapid increase in systolic blood pressure. No discernable blood pressure differences were detected for p38αDN-TG mice

(vs. non-TG) following Ang II infusion, suggesting that expression of this mutant transgene has no detectable effect on glomerular hemodynamics. Unfortunately, as with the α-GBM GN model, p38αDN-TG mice were not protected against the development of podocyte injury. This finding was in opposition of the central hypothesis that p38 activity is linked to the progression of podocyte damage. Once again, expression level and compensation of other p38 isoforms could account for the absence of filtration barrier protection. Because p38 activation is both rapid and transient, confirmation of increased p38 activity in response to glomerular injury is very difficult to detect and quantify. Thus, while we attempted a number of different experimental approaches, we were unsuccessful is showing p38 MAPK activation. Other than a non-statistically significant increase in WT1 expression (which would indicate increased podocyte number), quantitative PCR did not provide any indication that the p38αDN-TG mice were protected under circumstances of glomerular injury. Nephrin expression was similarly reduced for both non-TG and p38αDN-TG mice following chronic Ang II infusion, decreased nephrin serving as an indicator of podocyte injury.

While the current study using a transgenic mouse approach did not reveal a significant role for p38 MAPK in the progression of podocyte injury, this does not rule out a role for p38 MAPK as a maladaptive effector. Cell culture studies have repeatedly indicated that p38 MAPK is involved in the stress response to Ang II stimulation, increased mechanical stress, oxidative stress and elevated glucose levels (Durvasula et al., 2004; Durvasula & Shankland, 2008).

It would be beneficial to attempt generating a mouse with podocytes-specific deletion of p38 MAPK, not only the α isoform but also the other isoforms as well. This

would address the question as to which isoform(s) are involved in a podocytes stress environment.

7.4 Induction of glomerular injury in EP1$^{-/-}$ mice

Highest levels of the EP1 receptor are found in the kidney with expression to a lesser extent also in the gastric muscularis mucosa and adrenal tissue. Stimulation of the EP1 receptor results in phosphatidyl inositol turnover and intracellular Ca^{++} elevation through a Gq protein to mediate constrictor effects in the vasculature (Zonta et al., 2003). In the collecting duct of the kidney, the EP1 receptor is abundantly expressed. In fact due to its high expression in the collecting duct it was postulated that the EP1 receptor might be involved in Na reabsorption ultimately leading to natriuresis (Guan et al., 1998). Studies by Kennedy et al., showed that mice lacking the EP1 receptor do indeed have a urine concentrating defect however this was postulated to be under control of EP1 receptors within the hypothalamus to promote arginine vasopressin (antidiuretic hormone) release (Kennedy et al., 2007). Previous studies have also indicated that the renal EP1 receptor is involved in blood pressure control with several groups reporting mild hypotension in EP1$^{-/-}$ mice, especially in males (Audoly et al., 1999; Stock et al., 2001).

As mentioned previously, podocytes express both EP4 and EP1 PGE2 responsive receptors. To date the role of the PGE2 EP1 receptor in the podocytes is incompletely understood. While EP1 receptor expression was not induced in cultured podocytes by

mechanical stress, this does not preclude its involvement in the progression of podocyte injury since other factors are known to induce EP1 receptor expression (ie. high glucose) (Nasrallah, Landry, Singh, Sklepowicz, & Hebert, 2003). To investigate the role of podocyte EP1 receptors further, mice deficient for the EP1 receptor (global EP1 knockout mice; $EP1^{-/-}$ mice) were tested in the 5/6 Nx model of chronic kidney disease. Based on previous studies by our group demonstrating increased renal impairment in $EP1^{-/-}$ mice following induction of GN, we hypothesized that deletion of the EP1 receptor would confer a maladaptive podocyte phenotype (Rahal et al., 2006). This was in contrast to several studies demonstrating a beneficial impact of antagonizing EP1 receptors in the prevention of diabetic nephropathy (Makino et al., 2002). From our investigations we showed that $EP1^{-/-}$ mice on an FVB/N background displayed similar levels of proteinuria, with no observable differences in systolic blood pressure compared to Wt mice. The disparity in our experimental results from previous studies may be explained by the genetic background of our mice. Previous studies using $EP1^{-/-}$ mice that showed a hypotensive phenotype were on a mixed 129xC57BL/6, and DBA/2 background, while all of our experiments were carried out using mice on an FVB/N background. Furthermore, our $EP1^{-/-}$ mice are normotensive, supporting our *in vitro* findings where mechanical stimulation failed to induce EP1 expression. One cannot discount that given the global nature of this EP1 deletion; other cell types may invoke different cellular signaling events that cancel podocyte-specific EP1 receptor driven effects on the cell. In fact, within the glomerulus, mesangial cells are also known to express the EP1 receptor. Previous studies using $EP1^{-/-}$ mice showed increased mesangial proliferation compared to Wt mice following induction of GN (Rahal et al., 2006). Thus, further studies are

required using cell specific approaches to ensure a more comprehensive understanding of EP1 signaling.

Significantly, chronic infusion of Ang II resulted in rapid increases in systolic blood pressure along-side transient and significantly increased proteinuria in $EP1^{-/-}$ mice. These findings were in contrast to studies showing milder hypertension in $EP1^{-/-}$ mice compared to Wt mice following chronic Ang II infusion (Guan et al., 2007). Once again we speculate that genetic background may account for differences observed in our studies. Given the rapid overshadowing of this increased susceptibility to podocyte damage, it was postulated that any beneficial signaling of podocyte EP1 receptor is quickly masked by compounding and outweighing renal factors. Subsequent results showed that Ang II stimulation increased urinary PGE2 in Wt mice but was abrogated in $EP1^{-/-}$ mice. These findings suggest that the EP1 receptor subtype is required to promote Ang II dependent PGE2 synthesis and is consistent with the belief that there is crosstalk between Ang II and PGE2 signalning pathways. The mechanism underlying such a relationship remains to be elucidated. Finally, as expected, glomerular filtration rate (GFR) decreased following Ang II infusion, supporting a progressive loss in renal function for mice in a hypertensive enhanced RAS signaling milieu. Given that there were no observable differences in ACR for $EP1^{-/-}$ mice, it was not surprising to see similar decreases in renal function for this group (vs. Wt). Altogether, further studies using tissue specific approaches to either overexpress or knockdown expression of the PGE2 EP1 receptor are required to fully elucidate the role of this receptor in podocytes.

7.5 Summary and future studies

While there have been many recent exciting advances in our understanding of podocyte structure and function for both health and disease there are still many unanswered questions. The findings presented in this thesis provide novel insight to podocyte signaling using both *in vivo* and *in vitro* models of glomerular injury. We uncovered a maladaptive role for the PGE2 EP4 receptor through a complimentary approach employing not only *in vitro* podocyte cell culture experiments but also by successfully generating mice with either podocyte-specific overexpression or deletion of the EP4 receptor. We also propose for the first time crosstalk of PGE2 EP4 receptors and Ang II AT1 receptors.

Taken together, our findings showed for the first time that increased signaling through both PGE2 EP4 receptors and Ang II AT1 receptors leads to p38 MAPK dependent activation and leads to amplified COX-2 expression. Together, activation of these signaling pathways contributes to podocyte injury and leads to compromised filtration barrier function (**Figure 7.1**).

Thus, we have provided original, viable targets for improved pharmacological treatment of patients afflicted with chronic kidney disease. With the ever increasing number of patients diagnosed with CKD, providing treatment that is more target selective than traditional therapies, adverse drug effects may be significantly reduced. In turn we would be successful in reducing the financial health care burden while improving overall quality of life.

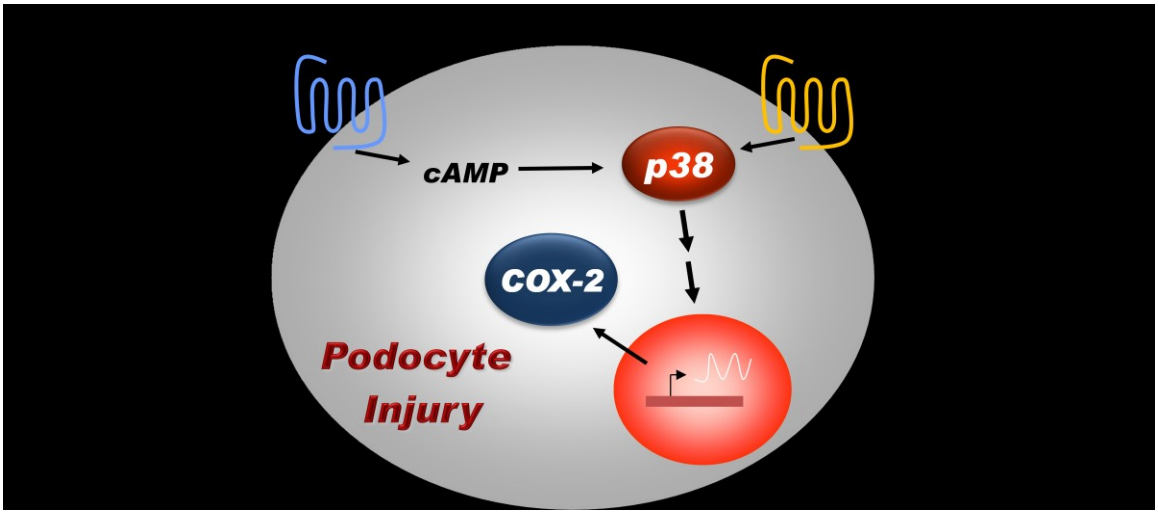


Figure 7.1 Podocyte prostaglandin E2 EP4 and angiotensin II AT1 receptors in glomerular disease.

Enhanced podocyte PGE2 EP4 and Ang II AT1 receptor signaling leads to p38 MAPK activation and enhanced COX-2 expression. These signaling events contribute to podocyte injury and leads to a compromised glomerular filtration barrier.

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APPENDIX

The Podocyte in Diabetic Kidney Disease

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Key words: Podocyte, Diabetic Kidney Disease, Albuminuria

Domains: 1°, Nephrology; 2°, Cell Signaling; Metabolism & Metabolic Disease; Endocrinology

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Abstract – Approaching epidemic levels, diabetic kidney disease (DKD) is now the leading cause of end stage renal disease (ESRD). Microalbuminuria is an early clinical marker of DKD that results from damage to the glomerular filtration barrier at the level of the highly differentiated glomerular podocyte cells. Injury these epithelial cells – podocytopathies, includes cellular hypertrophy, foot process effacement, detachment from the glomerular basement membrane and apoptosis. Here we review the role of a number of recently identified factors that contribute to podocytopathies in DKD. These factors include members of the renin-angiotensin system (RAS), including angiotensin converting enzyme (ACE) type 1 and 2, prorenin and its receptor, reactive oxygen species (ROS), prostanoids, peroxisome proliferator-activated receptors (PPAR), advanced glycation end products (AGEs) and their receptors (RAGE), adiponectin and micro RNAs. As the number of therapeutic options that slow but do not halt the progression of DKD to ESRD remains limited, a more comprehensive understanding of the signaling events that contribute this increasingly prevalent disease may identify novel avenues for treatment and prevention.

Synopsis – Diabetes mellitus is the leading cause of end stage kidney disease. One of the earliest clinical manifestations of diabetic kidney disease is microalbuminuria arising from injury to the glomerular filtration barrier. A key player in the renal filtration barrier is the glomerular epithelial podocyte cell, which undergoes a series of maladaptive changes resulting in severe morphological abnormalities, detachment from the glomerular

basement membrane, and programmed cell death. In this manuscript, we review the factors which drive the development of these podocytopathies in the progression of diabetic kidney disease.

INTRODUCTION - DIABETIC KIDNEY DISEASE

Diabetes mellitus (DM) now accounts for more cases of end stage renal disease (ESRD) than any other cause of chronic kidney disease (CKD). In North America, 4 of every 10 new cases of ESRD arise due to diabetic kidney disease (DKD), a proportion that has risen steadily over the past few decades and shows no signs of slowing. Roughly 30% of individuals with type 1, and 10% of those with type 2 diabetes will develop DKD ("Incidence of end-stage renal disease among persons with diabetes--United States, 1990-2002.," 2005). Glycemic control along with currently available pharmacotherapies slow, but do not stop the progression of DKD towards ESRD (Lewis & Lewis, 2003; Remuzzi et al., 2004; Ruggenenti et al., 2004; Wolf, Chen, & Ziyadeh, 2005). For these patients the only available solution is renal replacement therapy (i.e., transplantation or dialysis). Given the epidemic levels of obesity and type 2 DM in the populations of many Western nations, a comprehensive understanding of the etiology of DKD is urgently needed so that novel therapies can be developed. Along these lines, many efforts are being aimed at identifying the earliest signs of DKD so that treatments can be instituted before irreversible renal injury occurs. Indeed, it is now widely recognized that proteinuria,

specifically microalbuminuria, is one of the earliest clinically identifiable markers of diabetes-induced renal damage (Dronavalli, Duka, & Bakris, 2008; Kanwar et al., 2008; Wolf & Ziyadeh, 1999, 2007).

PROTEINURIA IN DIABETIC KIDNEY DISEASE

The appearance of protein in the urine (predominantly the 67 kilodalton albumin) indicates a compromised glomerular filtration barrier. A strong correlation has been identified between the likelihood of progression to ESRD and the level of albuminuria. Albuminuria is now considered a continuous variable (Ruggenenti & Remuzzi, 2006), in that even quantities of albumin that are in the upper end of what is considered the 'normal' range (<30 mg/day) are associated with an increased risk for the development of later stage CKD (Anavekar et al., 2004; Karalliedde & Viberti, 2004, 2005). Not only does proteinuria predict the pace of renal decline, it is an indicator of cardiovascular disease progression (Basi & Lewis, 2006). Indeed, individuals with chronic kidney diseases such as DKD presenting with a high urinary protein excretion rate advance more quickly to ESRD than do those with low proteinuria (A. Remuzzi et al., 2006). Proteinuria-lowering interventions such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), along with strict glycemic control, are associated with a slower deterioration of renal function and a delayed onset of ESRD in those with established DKD (Remuzzi, Benigni, & Remuzzi, 2006). However, recent

clinical trial data may have tempered enthusiasm for renin angiotensin system (RAS) blockade as a means for preventing DKD (Mauer et al., 2009). Mauer and colleagues showed that early RAS blockade with either losartan or enalapril were unable to prevent albuminuria or slow the progression of nephropathy in normalbuminuric patients with type 1 diabetes despite a significant reduction in the incidence of retinopathy. It is therefore possible that both the choice of target and the timing of the intervention are critical for effective prevention of DKD.

It is thought that proteinuria is not simply an indicator of glomerular damage, nor just a predictor of cardiovascular disease (CVD)/CKD progression, but has causative effects on these disease processes (G. Remuzzi et al., 2006). Excessive albumin levels likely overwhelm the metabolic capacity of the renal tubules thereby promoting inflammation, leading to tubulointerstitial fibrosis and an ensuing reduction of renal filtration rate (GFR). Intervention prior to these events is critical since tubulointerstitial and glomerular scarring accounting for this decline in renal function are currently, irreversible. The ensuing activation of the renin-angiotensin system (RAS) has well known effects on the vasculature and therefore contributes to the increased CVD risk. Additionally, the appearance of albuminuria may reflect widespread vascular endothelial glycocalyx dysfunction that may manifest similarly in the glomerulus (Satchell & Tooke, 2008). However the available ultrastructural evidence showing glomerular endothelial cell injury in the progression of DKD is currently lacking. Taken together, this emerging picture of DKD has made identifying the cellular loci that yield proteinuria an important goal. Numerous studies over the past decade have increasingly pointed to the podocyte

as a key target of injury at the outset of DKD progression (as reviewed in (Jefferson, Shankland, & Pichler, 2008; Reddy, Kotlyarevska, Ransom, & Menon, 2008; Wolf et al., 2005)).

PODOCYTOPATHIES IN DIABETIC KIDNEY DISEASE

Despite some recent studies questioning the role of the glomerular capillary as the primary means for maintaining serum albumin levels (Russo et al., 2009), most data continue to point towards this remarkable architectural structure as the essential renal filtration sieve. According to this classical view, passage of albumin into Bowman's space is overwhelmingly impeded by the size and charge selective glomerular filtration barrier consisting of 3 interdependent layers; the endothelial cells, the glomerular basement membrane (GBM) and the podocytes. The form and function of the glomerular filtration barrier has been reviewed extensively elsewhere (Deen, 2004; Deen, Lazzara, & Myers, 2001; Haraldsson, Nystrom, & Deen, 2008). The emerging picture is that the morphologically intricate podocyte and its slit diaphragm structure are of primary importance to the maintenance of the filtration barrier, and that injury to this cell is a key event in the initiation of DKD.

Over the past few decades, mesangial cells and the GBM have tended to be the focus of many DKD studies. While glomerular hypertrophy, mesangial matrix expansion and GBM thickening are classical hallmarks of diabetic glomerular lesions, studies of diabetic patients and animal models reveal that the onset of albuminuria is most closely associated with podocytopathies such as foot process effacement, podocyte hypertrophy, detachment, apoptosis and perhaps epithelial to mesenchymal transition (EMT) (Jefferson et al., 2008). The well documented characteristics of diabetes-induced podocyte injury have been comprehensively reviewed elsewhere (Jefferson et al., 2008; Reddy et al., 2008; Wolf et al., 2005). Therefore, we will only briefly highlight some key aspects of podocytopathies while focusing on the most recent discoveries.

In human and experimental DKD both foot process effacement and a decreased number of podocytes per glomerulus upon biopsy are well documented (Steffes, Schmidt, McCrery, & Basgen, 2001). Experimental evidence supporting these clinical observations is abundant. During the early stages of the Akita model of type 1 DKD, as well as the leptin receptor-deficient db/db mouse model of type 2 DKD, podocytes lose nephrin expression, become effaced, and detach from the GBM or undergo apoptosis – events that correlate with the emergence of albuminuria (Susztak, Raff, Schiffer, & Bottinger, 2006). Similarly, more recent studies of OVE26 transgenic mice, a model of type 1 DKD, show reduced podocyte number and density, although such changes are only evident following the onset of microalbuminuria, and may have emerged subsequent to more subtle podocyte injury (e.g., foot process fusion) (Teiken et al., 2008).

Irrespective of these minor differences, podocyte effacement and loss appear to be a key event in the early progression of DKD.

Another possible route of podocyte loss in DKD is EMT. During this process, epithelial cells undergo morphological changes resulting in loss of cell-cell contacts, alteration in cell polarity and reorganization of the actin cytoskeleton. Cells often revert to an immature undifferentiated phenotype reminiscent of an earlier developmental stage. Transforming growth factor beta (TGF-β), a potent fibrogenic cytokine and EMT inducer is upregulated in the glomeruli and nephron segments in a number of kidney diseases including DKD (as reviewed by (Liu, 2006)). Studies by Li et al., suggest that in podocytes, the actions of TGF-β suppress expression of key slit diaphragm proteins, induce extracellular matrix proteins expression (e.g., fibronectin and collagen I), and lead to secretion of matrix metalloproteinase-9 (MMP-9). Together, these changes are consistent with the hypothesis that podocyte EMT contributes to a defective glomerular filtration barrier and albuminuria under pathological conditions (Y. Li et al., 2008). More recently, Yamaguchi et al., assessed a role for EMT in DKD progression (Yamaguchi et al., 2009). They examined levels of TGF-β -induced fibroblast-specific protein 1 (FSP1), a member of the calmodulin S100 troponin C superfamily, and a fibroblast marker in human DKD (Iwano et al., 2002; Strutz et al., 1995; Yamaguchi et al., 2009). It was previously shown that tubular epithelial cells overexpressing FSP1 displayed reduced cell adhesion, along with upregulation of cytokeratin and vimentin (Okada, Danoff, Kalluri, & Neilson, 1997; Strutz et al., 1995). Using renal biopsy samples and urine sediment from patients with type 2 DM they showed that FSP1 levels in podocytes were associated with

macroalbuminuria, greater podocyte detachment and more severe glomerular pathology (Yamaguchi et al., 2009). Thus an EMT-like phenotype may arise in podocytes during DKD progression, resulting in detachment from the GBM and promoting podocytopenia.

MEDIATORS OF PODOCYTE INJURY IN DKD

A picture is now emerging of DKD-associated podocytopathies and how they account for alterations to the glomerular filtration barrier. Along with this more comprehensive understanding of the predominant role played by podocyte injury in DKD progression, additional studies aimed at identifying the mechanisms that give rise to these debilitating endpoints have likewise contributed invaluable insights. The importance of this field to the nephrology research community is evident from the number of comprehensive reviews provided over the past few years, including most recently, those discussing the role of vascular endothelial growth factor (VEGF) (Chen & Ziyadeh, 2008), mechanical stress (Lewko & Stepinski, 2009), the NOTCH pathway (Mertens, Raffetseder, & Rauen, 2008) and TGF-β (Ziyadeh, 2008). For the remainder of this review, we will therefore focus our attention on recent advances of our understanding of several other important mediators of podocyte injury in DKD (summarized in Figure 1).

The Renin-Angiotensin System. The renin angiotensin system (RAS) has for many years been implicated in the progression of diabetic kidney disease. Angiotensin converting enzyme inhibitors (ACEi) and angiotensin II type 1 (AT1) receptors blockers (ARBs) slow DKD progression (Langham et al., 2002; Lewis & Lewis, 2003; Mifsud et al., 2001; Philips, Weekers, & Scheen, 2001). For many years ACEi and ARBs were thought to improve the clinical outcome in diabetic patients via their blood pressure lowering effects, acting in part, to mitigate hyperfiltration-enhanced glomerular capillary pressure (Pgc). Indeed, the podocyte is susceptible to the mechanical forces brought about by elevated Pgc. Several studies showed that *in vitro* mechanical stretch, a mimic of elevated Pgc encountered *in vivo*, rendered podocytes more susceptible to a number of injurious endpoints such as apoptosis and actin cytoskeleton reorganization (Durvasula et al., 2004; Endlich et al., 2001; Martineau et al., 2004; Petermann et al., 2005). But these studies also led to subsequent discoveries of a podocyte-based, locally acting RAS that appears to induce apoptosis and TGF-β mRNA (Durvasula et al., 2004). Under conditions that reproduce a diabetic milieu (e.g., high glucose, mechanical stretch), podocytes are driven to express several RAS components, including angiotensinogen, as well as both prorenin and AT1 receptors (Durvasula et al., 2004; Durvasula & Shankland, 2008; Yoo et al., 2007). Furthermore, angiotensin I and II levels appeared to be enhanced by high glucose. Moreover, recent work by Xu et al., showing that inhibition of the 12-lipoxygenase pathway reduces AT1 expression, lowers proteinuria and decreases glomerular hypertrophy in a rat streptozotocin (STZ) model of type 1 diabetes mellitus (DM) is consistent with the notion that a local glomerular RAS is directly implicated with filtration barrier injury (Z. G. Xu et al., 2009). Finally, results from

recent clinical trials, revealed that combined administration of losartan and the renin selective inhibitor, aliskiren, reduce proteinuria in patients with type 2 DM, independent of the blood pressure lowering effects of the drugs (Parving, Persson, Lewis, Lewis, & Hollenberg, 2008).

Angiotensin converting enzyme 2 (ACE2) is a novel mono-carboxypeptidase member of the RAS family. As a homologue of ACE, it catalyzes the conversion of angiotensin II to Ang-(1-7). It appears that ACE2 activity buffers the deleterious actions of angiotensin II by limiting its levels and additionally via the signaling of Ang-(1-7) through its cell surface MAS receptor (Dilauro & Burns, 2009). A number of immunohistochemical studies of human and rodent cortical sections have localized ACE2 and the MAS receptor in podocytes and mesangial cells. Interestingly, glomerular ACE2 levels appear to be reduced in human (Mizuiri et al., 2008; Reich, Oudit, Penninger, Scholey, & Herzenberg, 2008) and rodent models of DKD (Leehey, Singh, Bast, Sethupathi, & Singh, 2008; Tikellis et al., 2003; Ye et al., 2006), which may allow for enhanced AT1 signaling in podocytes. The impact of Ang-(1-7) signaling and ACE2 activity on the known podocytopathies remains incompletely understood. However, administration of the ACE2 inhibitor MLN-4760 to either db/db mice (Ye et al., 2006), or STZ-induced diabetic mice (Soler et al., 2007), as well as targeted deletion of the ACE2 gene (Tikellis et al., 2008; Wong et al., 2007) exacerbates albuminuria. Taken together this suggests that the glomerular cells, including mesangial cells and podocytes may be an important renoprotective ACE2 activity locale.

Another potential therapeutic DKD target is the (pro)renin receptor. First identified in 2002, the (pro)renin receptor is highly expressed in the podocyte and can bind both renin and prorenin (Nguyen et al., 2002). An interesting feature of the (pro)renin receptor is that binding of prorenin results in activation of two independent pathways: an angiotensin II-dependent pathway as well as an angiotensin II-independent, (pro)renin-receptor-dependent intracellular mitogen-activated protein kinase (MAPK) pathway (Ichihara, Sakoda, Mito-Kurauchi, & Itoh, 2008). Activation of the MAPK pathway results in increased phosphorylation of p42/44 MAPK (Nguyen, 2006). To determine the predominant pathway in DKD, Ichihara and co-workers studied both STZ-induced and db/db models of type 1 and type 2 DKD, respectively. The handle region of the (pro)renin receptor which binds the receptor and blocks prorenin activity was administered to diabetic animals. Overexpression of the (pro)renin receptor handle region peptide significantly inhibited the DKD progression and was more effective than ACE inhibitors in diabetic angiotensin II type 1a receptor deficient mice (Ichihara et al., 2008). Similar findings were reported when STZ rats were infused with the handle region peptide (Ichihara et al., 2008). Altogether these results suggest an important role for prorenin and the (pro)renin receptor in slowing the progression of DKD and could prove a useful therapeutic target.

Reactive Oxygen Species. Within the kidney, as in other organs and tissues (i.e., vasculature), hyperglycemia and RAS activation promote oxidative stress, defined as

damage to macromolecules caused by reactive oxygen species (ROS; i.e., $\cdot O_2^-$, H_2O_2, NO and $ONOO^-$) (Gill & Wilcox, 2006; J. M. Li & Shah, 2003). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or Nox is a rich source of ROS production in the kidney, and indeed within the podocyte (Gorin et al., 2005; Greiber et al., 1998; Y. S. Kang et al., 2006; Satoh et al., 2005). Nox catalyzes production of $\cdot O_2^-$ by the one electron reduction of O_2 using NADH or NADPH as the electron donor: $2O_2 + NAD(P)H \rightarrow 2 \cdot O_2^- + NAD(P)^+ + H^+$. Seven Noxes have been identified (Nox1-5, Duox1 and Duox2). Although ROS generation undoubtedly has pleiotropic effects along the nephron and renal vasculature, a role for ROS in damaging the glomerular filtration barrier and more specifically, the podocyte is beginning to emerge from the experimental data. Inhibition of NADPH activity by apocynin in mice with STZ-induced diabetes reduces albuminuria, strongly suggesting a role for ROS in glomerular injury associated with type 1 DKD (Susztak et al., 2006). The identity of specific Nox isoform(s) accounting for glomerular ROS generation awaits confirmation. However, of the Nox isoforms, Nox4 is most abundantly expressed in the kidney and hence was originally termed renal oxidase (Renox) (Geiszt, Kopp, Varnai, & Leto, 2000). Nox4, which localizes within the renal vasculature, mesangial cells, tubular cells and podocytes, appears to be the major renal ROS source, and may therefore play a critical role in oxidative stress associated with kidney disease (Aoudjit et al., 2006). Recent *in vivo* studies support a role for Nox4 in DKD. In STZ-treated mice, mRNA expression of Nox4 was increased and renal production of ROS was enhanced (Etoh et al., 2003). A causative relationship between Nox4-induced ROS production and DKD was demonstrated in STZ-induced diabetic rats that were treated with antisense

oligonucleotides to reduce Nox4 expression (Gorin et al., 2005). In these Nox4 antisense-infused animals, renal ROS production and albuminuria were reduced (Gorin et al., 2005). While these studies provide compelling initial evidence for a role for Nox4 in type 1 DN, a comprehensive examination of Nox4 expression and downstream effects within podocytes has not been performed.

The notion that podocyte-derived ROS are implicated in DKD was obtained in studies using db/db and Akita mice where inhibition of Nox activation blunted podocyte apoptosis and detachment, lowered proteinuria and reduced mesangial matrix expansion (Susztak et al., 2006). Similar findings were more recently reported in the OVE26 model of type 1 DKD (Eid et al., 2009). In these studies, exposure of cultured mouse podocytes to high glucose promoted Nox-induced ROS generation, along with Nox1 and 4 upregulation. Similarly, Nox1 and 4 mRNA and protein expression were elevated in glomeruli of OVE26 mice. Whether one or both of these Nox isoforms contributes to the deleterious production of ROS in podocytes awaits investigation. Additionally, ROS generation in the podocyte may have other deleterious roles in addition to its pro-apoptotic actions. ROS could induce actin filament polymerization leading to cytoskeletal dysfunction and resulting in structural changes to foot processes and slit diaphragms. Furthermore, podocytes exposed to high glucose generate ROS via Nox activity in a cytochrome P450-dependent manner. Treatment of OVE26 diabetic mice with N-hydroxy-N'-(4-butyl-2-methylphenol) formamidine (HET0016), an inhibitor of CYP4A, blocked Nox activity and expression while reducing albuminuria (Eid et al., 2009).

In addition to NADPH dependent generation of ROS, the nitric oxide (NO) pathway has been implicated in the kidney as an important regulator of vascular tone and glomerular ultrafiltration coefficient. NO is generated from L-arginine via the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Significantly, eNOS deficient db/db mice display severe glomerular lesions and albuminuria (Zhao et al., 2006). NO may abrogate VEGF-mediated macrophage migration in DKD via inhibition of Flt-1 VEGF receptor expression, and its loss via eNOS deletion may contribute to glomerular injury (Sato et al., 2008). However, further studies are required to determine whether podocytes are involved in the progression of this glomerular injury subsequent to local NO production.

Cyclooxygenases and Prostanoids. The prostanoids are comprised of prostaglandins E2, F2α, I2, and D2, along with thromboxane A2. These lipid mediators are derived from arachidonic acid via the sequential catalytic activities of cyclooxygenases (COX), of which there are two isoforms (COX-1 and COX-2), followed by that of specific synthases (PGE, PGF, PGI, PGD & thromboxane synthase). We have known for decades that COX inhibitors (non steroidal anti-inflammatory drugs - NSAIDs) which block prostanoid synthesis, reduce proteinuria in a number of glomerulopathies (Golbetz, Black, Shemesh, & Myers, 1989; Velosa & Torres, 1986; Velosa et al., 1985; Vriesendorp, Brentjens, & Donker, 1985; Vriesendorp, de Zeeuw et al., 1986), including DKD (Dunn, 1990). Although COX inhibition delivers anti-proteinuric effects, NSAIDs including the

gastrointestinal-sparing COX-2 selective inhibitors can be nephrotoxic for CKD patients (Noroian & Clive, 2002; Perazella, 2002). By blocking the synthesis of vasodilatory prostaglandins these drugs can elicit a precipitous decline in renal blood flow and glomerular filtration rate (GFR) (Blume et al., 1999; M. D. Breyer & Breyer, 2001; Epstein, 2002; Murray & Brater, 1997; Whelton, 1999). Clearly, if the beneficial proteinuria-lowering effects of COX inhibition are to be realized, we will need to identify the cellular targets that modulate the anti-proteinuric effects of COX inhibitors. Furthermore, strategies must move downstream of COX blockade and differentiate between those prostanoids and their respective receptors, that deliver protective effects from those that impair renal function.

COX-2 inhibitors decrease proteinuria and reduce the severity of glomerular lesions in STZ-induced DN (H. F. Cheng & Harris, 2002). In several experimental models, as well as in human biopsy specimens, COX-2 expression becomes detectable in podocytes during early disease (Hirose et al., 1998; Komers, Lindsley, Oyama, & Anderson, 2007; Komers et al., 2001; J. L. Wang et al., 1998). These data suggest that in addition to their effects on the renal vasculature, prostanoids may have direct interactions with the cells of the glomerular filtration barrier. Indeed, prostanoids act locally from their sites of synthesis to elicit a variety of actions via a set of classical G-protein coupled receptors (GPCRs). PGE2 and TXA2 are major renal prostanoids whose actions are mediated via specific GPCRs known as the E and T-Prostanoid receptors (EP1-EP4, TP), respectively. The mesangium (Ishibashi et al., 1999; Mene & Dunn, 1986) and podocytes (Bek et al., 1999) express a number of prostanoid receptors including EP1,

EP4, and TP receptors, among others. EP1 receptors may modulate maladaptive aspects of DKD such as glomerular hypertrophy, matrix expansion, TGF-β production and proteinuria (Makino et al., 2002). Makino and colleagues showed that an EP1 antagonist diminished glomerular damage and proteinuria in rats with STZ-induced type 1 diabetes (Makino et al., 2002). Interestingly, glomerular expression of both EP1 and EP4 receptor mRNA both increased in these STZ rats. We have likewise observed enhanced glomerular EP4 and TP receptor mRNA levels in db/db mice (unpublished results). In addition, we recently uncovered a novel feedback loop in podocytes whereby an *in vitro* surrogate for glomerular capillary pressure (i.e., equibiaxial mechanical stretch) along with PGE2 stimulation of the prostaglandin EP4 receptor, induces COX-2 in a p38 MAPK-dependent manner resulting in actin cytoskeletal rearrangements (Faour et al., 2008; Martineau et al., 2004). We speculate that PGE2 dependent stimulation of the EP4 receptor in podocytes exacerbates glomerulopathies, such as DKD, that are associated with enhanced Pgc.

A role for the TP receptor in modulating the glomerular filtration barrier is suggested by several studies. Thromboxane levels rise in experimental DKD (Craven, Melhem, & DeRubertis, 1992) and administration of a TP antagonist markedly reduces albuminuria in rodent models of DKD (Matsuo et al., 1995; Sebekova, Eifert, Klassen, Heidland, & Amann, 2007; S. Xu et al., 2006). Inasmuch as these studies are consistent with a role for EP and TP receptors in filtration barrier dysregulation, whether podocytes are cellular targets for these deleterious actions of PGE2/TXA2 remains incompletely

defined. Furthermore, the precise mechanisms that might account for these effects on the filtration barrier await identification.

Peroxisome Proliferator-Activated Receptors. A role for peroxisome proliferator-activated receptor (PPAR) signaling in DKD progression has emerged from a number of studies. Belonging to the nuclear receptor super family, PPARs were first discovered by Issemann et al., in 1990 (Issemann & Green, 1990). Acting as transcription factors, PPARs modulate gene transcription by heterodimerizing with the retinoic acid receptor α (RXRα). Three PPAR subtypes (α, β, γ) have been cloned and characterized, all of which are encoded by specific genes. Potent synthetic ligands of PPARs are effective therapeutics for dyslipidemia as well as diabetes (Reviewed in (Berger & Moller, 2002)). Specifically, PPARγ agonism with rosiglitazone improves insulin resistance, hyperinsulinemia and hyperglycemia (Campbell, 2005; Kumar et al., 1996).

Rosiglitazone has a direct and protective effect on glucose uptake in cultured human podocytes via upregulation of the GLUT1 glucose transporter. This phenomenon was dependent on nephrin expression, as podocytes lacking nephrin were incapable of modulating glucose uptake (Lennon et al., 2009). Interestingly, studies by Benigni et al., showed that nephrin expression is regulated by the PPARγ agonist pioglitazone which in a model of Heyman Nephritis results in decreased proteinuria (Benigni et al., 2006).

Advanced Glycation End Products. Advanced glycation end products (AGEs) are generated in diabetes through the interaction of glucose with protein or lipid molecules. These molecules have a number of toxic effects throughout the body including the vasculature and within renal glomeruli where they accumulate in the mesangium, endothelial cells, GBM and podocytes of individuals with DKD (Fukami, Yamagishi, Ueda, & Okuda, 2008). The podocyte appears to be a sensitive target of AGE actions as it expresses the receptor for AGE (RAGE) (Wendt et al., 2003). Accordingly, Doublier et al., demonstrated that glycated albumin inhibited nephrin expression in cultured podocytes through engagement of RAGE which is consistent with reduced levels of this key slit diaphragm component observed in human and experimental DKD (Doublier et al., 2003). Blockade of the AGE pathway with anti-AGE agents, ALT-711, KIOM-79, and LR-90 slow the progression of DKD in Zucker diabetic fatty rats and db/db mice (Crowley et al., 2009; Figarola et al., 2003; Peppa et al., 2006). The mechanism of action for anti-AGE agents appears to involve, at least in part, a reduction in glomerular cytokine levels, as KIOM-79 administration to Zucker diabetic fatty rats reduced TGF-β levels and decreased podocyte apoptosis (Y. S. Kim et al., 2009). Other actions of AGEs in the podocyte were identified by Ruster et al., who showed that podocyte exposure to AGE-bovine serum albumin (AGE-BSA) induced activation of the cell cycle regulatory protein, p27(Kip1) leading to podocyte apoptosis (Ruster et al., 2009). Taken together, the AGE/RAGE system appears to contribute significantly to podocyte injury in DKD, and could emerge as a definitive therapeutic target.

Adiponectin Produced in adipose tissue, adiponectin is an important peptide hormone involved with glucose regulation and fatty acid catabolism. Decreased adiponectin levels result in oxidative stress, fusion of podocyte foot processes, and microalbuminuria (Sharma et al., 2008; Tao et al., 2007; Tsioufis et al., 2005). Consistent with these findings, adiponectin knockout mice have increased susceptibility to podocyte injury in a subtotal renal ablation model of progressive chronic kidney disease (Ohashi et al., 2007). Based upon such studies, some have suggested that adiponectin may be a biomarker for kidney disease and, due to its involvement in protecting the filtration barrier, a useful therapeutic target in slowing DKD progression (Ahima, 2008). Evidence to support a role for adiponectin in podocyte function is accumulating. A recent study by Cammisotto et al., revealed that stimulation of the adiponectin receptor in podocytes yielded activation of AMP-activated protein kinase, which controls oxidative stress and apoptosis (Cammisotto & Bendayan, 2008).

MicroRNA. MicroRNAs (miRNAs) are single-stranded non-coding RNA molecules which regulate gene expression by interfering with protein translation of mRNA targets – resulting in the degradation of mRNA species. The role of miRNAs in the kidney was recently reviewed (Kato, Arce, & Natarajan, 2009) and thus we will only provide a brief overview of this emerging field. Processing of miRNAs is facilitated by the enzyme Dicer, which when deleted from podocytes leads to filtration barrier dysfunction in mice (Harvey et al., 2008; Ho et al., 2008; Shi et al., 2008). Furthermore, five kidney specific miRNAs have been identified (miRNA-192, -194, -204, -215, and -216) and further segregated into two classes: those found predominantly in the cortex and those present in

the medulla (Sun et al., 2004; Tian, Greene, Pietrusz, Matus, & Liang, 2008). When investigating the role of miRNA from isolated diabetic glomeruli, Kato et al., showed that miRNA-192 levels were significantly higher in STZ-induced diabetic and db/db mice compared to non-diabetic controls (Kato et al., 2007). Elevated miRNA-192 positively correlated with increased TGF-β and collagen 1α2 levels which may have an impact upon GBM thickness and podocyte health (Kato et al., 2007). Clearly, our understanding of the role played by various miRNA species in not only the podocytes, but in the kidney as a whole is at a nascent stage. Undoubtedly, future studies will continue to unravel the complex miRNA based gene regulation in the context of the podocyte during DKD progression, and it is foreseeable that novel targets will be identified for therapeutic intervention with specific miRNA inhibitors.

CONCLUSION

The large number of factors that contribute to the etiology of podocyte dysfunction in DKD is indicative of the complexity of this condition. Nevertheless, as the number of therapeutic options that slow but do not halt the progression of DKD to ESRD remains limited, a more comprehensive understanding of the underlying signaling events that contribute to this increasingly prevalent disease is needed in order to identify novel avenues for treatment and prevention.

Acknowledgements. Erin Stitt-Cavanagh is an Ontario Graduate Scholarship in Sciences and Technology Scholarship awardee.

APPENDIX 1

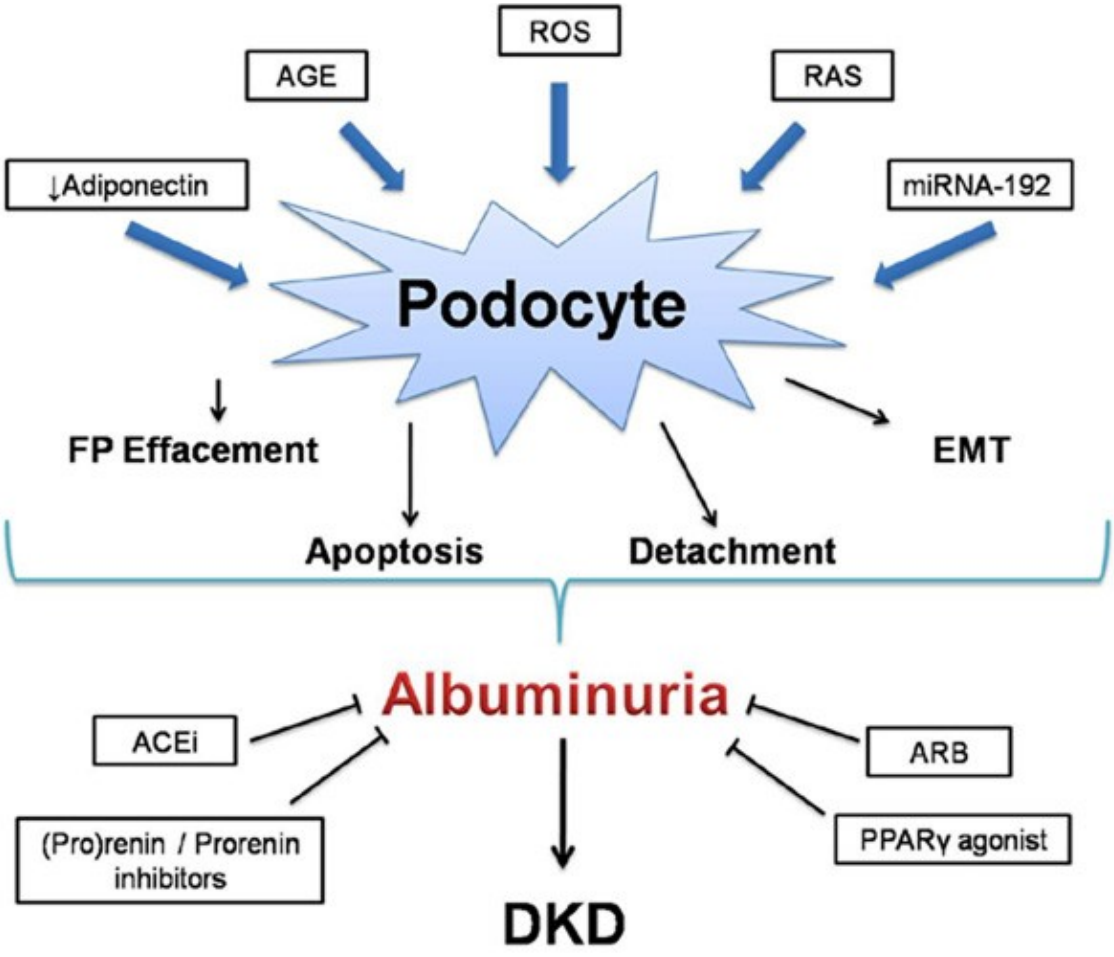
ROS
AGE
RAS
↓Adiponectin
miRNA-192
Podocyte
FP Effacement
EMT
Apoptosis
Detachment
Albuminuria
ACEi
ARB
(Pro)renin / Prorenin inhibitors
PPARγ agonist
DKD

Figure 1 – Mediators of podocyte injury in DKD

Several molecular pathways are known to contribute to podocyte injury in diabetic kidney disease (DKD). As depicted, these include, but are not limited to the following: decreased adiponectin, production of advanced glycation end products (AGEs), increased reactive oxygen species (ROS), activation of the renin angiotensin system (RAS) and miRNA-192 all contribute to podocyte injury. Podocyte injury exhibits itself as foot process (FP) effacement, apoptosis, detachment and epithelial to mesenchymal transition (EMT). Damage to the podocyte leads to increased albuminuria and exacerbates DKD. Agents known to inhibit this process include, angiotensin II receptor blockers (ARBs), angiotensin converting enzyme inhibitors (ACEi), peroxisome proliferator-activated receptor (PPAR) agonists and (pro)renin / prorenin inhibitors.