REMODELING OF THE COLLECTING DUCT WITH URINARY TRACT OBSTRUCTION

by

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ABSTRACT

Urinary tract obstruction is a major cause of morbidity and mortality worldwide. Obstruction occurs in both the fetus and adult, can lead to impaired renal development, substantial renal injury, dysplasia, or progressive fibrosis of the kidney. Though different in outcome, fetal and adult obstruction both impair normal kidney function and promote the onset of chronic kidney disease. While a substantial amount of research has identified key features in the pathogenesis of obstruction, our understanding of the early events in the progression of obstructive injury is incomplete.

In this thesis, I have used several experimental models to investigate the role of medullary and collecting duct injury following urinary tract obstruction. Research using these models has provided many key findings. First, I have established the previously unreported ontogeny of the collecting duct in the human fetal kidney. Second, I have defined the impact of urinary tract obstruction on the collecting duct and linked it to collecting duct injury. Third, I have characterized the response of the collecting duct and associated renal interstitium to obstruction, and identified cellular dedifferentiation and phenotypic transition as common outcomes of collecting duct injury. Last, I have characterized the expression, localization and protein-complex formation of TRPV4, and highlighted a mechanism for TRPV4 in the transduction of physically injury into a collecting duct epithelial response.
Through investigation of clinical cases of fetal urinary tract obstruction and experimentation using 2 models of fetal and adult obstruction, I have demonstrated that collecting duct epithelial injury and remodeling is a common and conserved feature of obstructive injury and I have described a role for TRPV4 in the mechanosensation and transduction of the physical stimuli caused by obstructive injury. I believe TRPV4-mediated signal transduction, in part, provides a unifying mechanism for the induction of a repair and fibrosis during both fetal and postnatal urinary tract obstruction.
PREFACE

Much of the work presented in this thesis has been previously published, with some of these publications under my former surname of Butt, and has been completed with the invaluable assistance of several contributors.

Publications and Contributions

A version of Chapter 3 has been published:


I designed and conducted all experiments, quantification and analyses, and performed all figure layout and manuscript writing under the guidance and supervision of my supervisor, Dr. Douglas Matsell. I thank my collaborators for their assistance in helping in the collecting and processing of the samples that made this research possible. Fetal and postnatal samples in this study were kindly provided by the Pathology and C.A.R.E programs of BC Children’s Hospital, and by Dr. Nuria Toran of the Vall D’hebron University Hospital. Our research technician, Dr. Larissa Ivanova provided technical assistance and in-lab support. We thank Dr. Alice F. Tarantal for her contributions to the project planning and manuscript revisions.
A version of Chapter 4 has been published under my previous surname, Butt:


I designed and conducted all experiments, quantification and analyses, downstream of the animal work and sample acquisition. I also performed all figure layout and manuscript writing under the supervision of Dr. Douglas Matsell. All animal work, including the recovery of renal tissues, and subsequent processing, sectioning and RNA extraction was performed by our collaborators Dr. Alice Tarantal and Daniel Jimenez at the Davis Campus of the University of California.

Chapter 5 and 6 are based on recent work that has not yet been published, but will be submitted as two separate publications. I have designed and conducted all experiments, quantification and analyses, and performed all figure layout and manuscript writing under the supervision of Dr. Douglas Matsell, Dr. Larissa Ivanova and Dr. Peter Trnka assisted me with all UUO surgeries. Dr. Ivanova also provided technical assistance with western blotting, immunoprecipitation and routine cell culture work.
Additional Publications and Work

In addition to the work presented in this thesis, I have contributed to a number of other relevant projects and publications.


This paper addresses the role of the IGF-I receptor in both glomerular maintenance and in podocyte growth and survival, and implicates IGF signaling in progressive glomerular disease. I performed the isolation, explanting, and analysis of glomeruli from Igf-1R^{H66FLAGstop} mice, and contributed Figure 10 to the publication.


Following publication of my Kidney International paper (2) describing mesenchymal transition of collecting duct epithelial cells in vivo, this paper confirmed that collecting duct-derived mIMCD3 cells undergo mesenchymal transition in vitro following treatment with the profibrotic factor, TGFβ-1. I planned and performed the initial TFGβ-1 stimulations and analyses identifying epithelial changes following treatment. Dr. Ivanova expanded these analyses and further investigated the role of signaling events that follow TGFβ-1 treatment. I assisted Dr. Ivanova, with technical expertise regarding immunohistochemistry, and microscopy.

This paper replicates in the human fetal kidney many of the findings of collecting duct injury and remodeling described previously in the fetal non-human primate (2). I participated in the planning of these experiments, and provided substantial technical assistance and expertise relating to immunohistochemistry, microscopy, renal histology, and data analysis.


Following my characterization of the fetal collecting duct ontogeny in human fetal kidneys (1), this paper similarly characterized the ontogeny of CD24 positive, putative mesenchymal progenitor cells in the developing human fetal kidney. I assisted Dr. Ivanova, with technical expertise regarding immunohistochemistry, microscopy, and renal histology.


This paper investigates biomarkers present in the urine of children with congenital urinary tract obstruction caused by posterior urethral valves and seeks to assess their diagnostic and prognostic value. I provided technical assistance in these
experiments, and consulted with Drs. Trnka and Ivanova regarding possible collecting-duct derived proteins that are altered by obstructive injury and that may have value as putative biomarkers.

**Ethics Approval**

The work described in this thesis was performed in accordance with the requirements of the University of British Columbia and with the permission of the Clinical Research Ethics Board (Chapter 3, Certificate #H06-70006) and the UBC Animal Care Committee (Chapter 4, Certificate #A03-0200; Chapter 5 and 6, A09-0200; and Chapter 6: A10-0228).
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LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>αSMA</td>
<td>α Smooth muscle actin</td>
</tr>
<tr>
<td>βCat</td>
<td>β Catenin</td>
</tr>
<tr>
<td>AQP2</td>
<td>Aquaporin 2</td>
</tr>
<tr>
<td>CA II</td>
<td>Carbonic anhydrase II</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>Coll IV</td>
<td>Collagen IV</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>E-Cad</td>
<td>E-Cadherin</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-derived neurotropic factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>K/DOQI</td>
<td>Kidney Disease Outcomes Quality Initiative</td>
</tr>
<tr>
<td>mIMCD3</td>
<td>Mouse inner medullary collecting duct cell line</td>
</tr>
<tr>
<td>MM</td>
<td>Metanephric mesenchyme</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-Angiotensin system</td>
</tr>
<tr>
<td>RhCG</td>
<td>Rhesus blood group C glycoprotein</td>
</tr>
<tr>
<td>RR</td>
<td>Ruthenium red</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid family</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteric obstruction</td>
</tr>
<tr>
<td>vATPase</td>
<td>vacuolar H+ ATPase</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
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ACKNOWLEDGMENTS

I wish to thank my supervisor, Dr. Doug Matsell, for his guidance, mentorship, and friendship throughout my time in the lab. I am deeply indebted to him for taking a chance on me so many years ago, and value his investment of time, wisdom, and patience in guiding my academic journey.

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Lastly, I wish to thank my family for their love and support. For my mother Sandie, who has always been my biggest supporter; for my grandmother Flora, who always believed in me; for uncle Lorne, who has been a father figure in my life; for Nick and Sonia, who welcomed me with open arms and hearts; and for Donna, who brings joy to my life and for whom I do all that I do; this thesis is for all of you....
For my loving wife,

and the family that has supported me.
CHAPTER 1 INTRODUCTION TO THE KIDNEY

1.1. **Evolution of the Kidney**¹

The true medium in which we live is neither air nor water, but the blood, the internal medium that bathes our muscles, glands and brain.

*Claude Bernard* (7), paraphrased by *H. W. Smith* (8).

The evolution of the kidney is one of the most important steps in the survival of early vertebrates. While their oceanic antecedents maintained an osmotic equilibrium with their saline surroundings, the adaptation of early chordates to fresh water environments necessitated a method to counteract the strong osmotic force driving water into their solute-rich tissues. The modern kidney was shaped from a preexisting structure that juxtaposed arteries and excretory tubules equipped for the elimination of nitrogenous wastes. The interaction between these vascular and tubular elements formed the first glomerular structure, a dense capillary bed surrounded by a collecting vessel contiguous with the lumen of the excretory tubule. Thus the basic functional unit of the kidney was born.

---

¹ This introductory section is based on Peter D. Vize’s reprint of Homer W. Smiths “*From Fish to Philosopher*” (1953).
CHAPTER 1

Adaptation to the terrestrial environment served as another influential stage in the evolution of the kidney. Survival in a non-aquatic environment imposed the immediate need not to exclude water from the body, as in the freshwater hypotonic environment, but instead to conserve it. This evolutionary step took two widely divergent paths. In reptilian and avian species, the conservation of body water was achieved by the excretion of nitrogenous wastes as uric acid, a nearly insoluble paste that eliminated the need to expel wastes in an aqueous solution. On the other hand, mammals achieved the ability of producing a hypertonic filtrate to eliminate highly concentrated wastes by reversing the direction of water transport inherited from their marine ancestors, and by evolving a specialized water-recovering segment of the tubular architecture.

The structure and arrangement of the functional unit of the kidney, the nephron, (discussed in section 1.3) recapitulates this evolution of the glomerular and tubular elements. In the developing mammalian fetus, the steps of the kidney’s evolution are replayed by the formation of three sequential renal structures: the pronephros, the mesonephros, and metanephros. In mammals, only the metanephros forms the mature kidney (discussed in section 1.4).

The underlying parallel between the structure, function, physiology, and embryogenesis of the mature, mammalian kidney closely recapitulates its evolutionary origins. In the words of Homer W. Smith...
...The human kidney manufactures the kind of urine that it does, and it maintains the blood in the composition which that fluid has, because this kidney has a certain functional architecture: and it owes that architecture not to design or foresight or any plan, but to the fact that the earth is an unstable sphere with a fragile crust, to the geologic revolutions that for 600 million years have raised and lowered continents and seas, to the predacious enemies, and heat and cold, and storms and droughts, the unending succession of vicissitudes that have driven the mutant vertebrates from the sea to freshwater, into desiccated swamps, out upon the dry land, from one habitation to another (8).

1.2. **Gross Anatomy of the Kidneys and Urinary Tract**

The urinary tract is comprised of the kidneys, the ureters, bladder and urethra (Figure 1.1). The ureters and urethra serve to transport the urine produced by the kidneys to and from the bladder, respectively, where it is stored until it can be appropriately eliminated from the body. The kidneys are located on either side of the vertebral column posterior to the abdominal cavity. In adults, on average, they are approximately 12 centimeters in length, 7 ½ centimeters in width, and 3 centimeters in thickness (9). The hilum on the medial side serves as the entry point for the single renal artery (Figure 1.2). This junction is also the entry point for the lymphatics and nerves, the egress of the renal vein, and is where the ureters exit, one per kidney, to deliver the urine generated by the kidney to bladder. The kidneys
Figure 1.1: The Urinary Tract. Urine formed by the kidneys is transported by the ureters to the urinary bladder prior to evacuation through the urethra. Reproduced from http://www.stomaatie.com/spiisvertering_urinewegen/normal-urinary-tract-2col.gif

Figure 1.2: The Kidney. Filtrate formed in the cortex passes through the medulla into the calyces which fuse to form the ureter. The ureter, renal vasculature, lymphatics, and renal nerves enter and exit the kidney through the hilum. Adapted from http://www.yalemedicalgroup.org/stw/images/125836.jpg
are surrounded by a fibrous capsule that is contiguous with the connective tissue surrounding the hilar structures (10).

The parenchymal tissue of the kidney is separated into two distinct zones: the cortex and medulla. The cortex forms the outermost region and contains the glomeruli, the individual filtration units, and the most functionally active tubular segments of the nephron. The medullary region contains the unique mammalian water reabsorption segments of the nephron, as well as the collecting duct system. The collecting duct system empties into the open lumen of the funnel-shaped calyces, which converge to form the enlarged renal pelvis that joins to the ureter. The epithelial walls of the calyces and pelvis are continuous with the transitional epithelial linings of the ureter. Furthermore, they share the ureter’s outer layer of smooth muscle that, through peristaltic action, forces the collected urine towards the bladder (9,10).

1.3. **Basic Function and Structure of Kidney**

As suggested previously, the overall function of the kidney is one of homeostasis. While the kidneys perform many diverse functions, including a crucial role as endocrine organs that regulate blood pressure and hematopoiesis, they have two principal roles. The first is to regulate the balance of wastes, nutrients and minerals in the blood, the second is to conserve and reabsorb water.
During evolution in the fresh water environment, the large osmotic gradient driving water into the body produced kidneys with a high rate of filtration to eliminate this excessive body water (8). However, during adaptation to the water-poor terrestrial environment, such a high filtration rate was contrary to the acquired need for water conservation. Evolution therefore resulted in the development of highly specialized water-reabsorbing segments of the nephron. As a result, the kidneys filter approximately 200 liters of filtrate and 3 kilograms of sodium per day, only to reabsorb approximately 98% of that volume and the majority of the excreted sodium. Paradoxically, therefore, but as a product of its evolutionary path, the mammalian kidney can be seen as grossly inefficient.

In order to accomplish these contradictory tasks of filtration and reabsorption, the kidney has several unique and distinct compartments. Approximately 80% of the kidney is comprised of epithelia that make up the numerous different tubular segments. Each tubular segment is functionally distinct and often contains several subtypes of epithelial cells. In turn, each epithelial subtype has a unique structure and function.

The kidney’s basic functional unit is the nephron (Figure 1.3). Each kidney contains approximately 500,000 nephrons (9), many of which span both the cortical and medullary regions. Each nephron consists of one filtering unit, the glomerulus, connected to a series of specialized tubular compartments. The glomeruli are
Figure 1.3: The Nephron. The nephron is the basic functional unit of the kidney. Filtrate is formed by the glomerulus, and is transported through several specialized tubular segments. These tubules selectively reabsorb nutrients, electrolytes and water while secreting unwanted wastes. The urine formed by multiple nephrons is transported through the collecting duct into the calyces and ureter for elimination from the body. Reproduced from http://www.beltina.org/pics/nephron.jpg
responsible for filtration, while the tubules perform the majority of the balancing and processing of the filtrate to produce the urine that is excreted.

1.3.1. Filtration

In the mammalian kidney, blood is delivered to the glomerulus by the afferent arteriole, which branches off the larger intralobular arteries (9). Upon entering the glomerulus at the vascular pole, the afferent arteriole splits into a dense bundle of capillary loops supported by the mesangium (Figure 1.4). Upon exiting the glomerulus, blood enters the efferent arteriole that supplies blood to the downstream tubular segments of the nephron.

In the glomerular capillary bed, filtration occurs through the interactions of the vascular endothelium with highly specialized visceral epithelial cells called podocytes (9). Both the endothelial and podocyte layers are highly fenestrated and reside on opposing sides of a mutually maintained basement membrane. This high degree of fenestration serves as the first level of filtration barrier. In the human glomerulus, the endothelial fenestrae form pores of approximately 70 - 100nm in diameter (11), however, these pores are still far too large for the restrictive requirements of ultrafiltration. Instead, this task lies with the podocytes that encircle the capillary. In the gap of roughly 25 - 60 nm that lies between their foot processes, adjacent podocytes form the highly specialized junctional structure of the slit diaphragm, which serves as the functional site of filtration (12). The slit diaphragm (Figure 1.5) residing 60 nm above the basement membrane, is formed of
Figure 1.4: The Glomerulus. Blood is delivered to the glomerulus by the afferent arteriole. The podocytes surrounding the glomerular capillaries form the filtration barrier, allowing the passage of small proteins, molecules and water into Bowman's space. This filtrate then passes into the proximal convoluted tubule for selective reabsorption of required nutrients. Adapted from http://coe.fgcu.edu/faculty/greenep/kidney/glomerulus.html
Figure 1.5: The Slit Diaphragm. The slit diaphragm composes the molecular sieve of the glomerular filtration barrier. The uniformly sized pores of the slit diaphragm are formed by specialized junctional complexes between the foot processes of the adjacent podocytes. Reprinted from Simons and Huber, Kidney Int, 2008, 7:671-673.
a central filament adjoined to the foot processes by regularly spaced cross-bridges. The resulting formation creates uniformly sized and spaced pores of approximately 4nm by 14nm (13). Though the exact molecular structure of the slit diaphragm remains unknown, several podocyte-specific proteins including nephrin and podocin have been identified that are integral to formation of the slit diaphragm (14,15). The structure and integrity of this filtration barrier is crucial for allowing the appropriate filtration of the urine while retaining the cells and large molecules within the blood.

The filtration barrier of the glomerulus is therefore equivalent to a molecular sieve that allows the passage of small molecules, proteins and water out of the blood stream while retaining cells and large proteins. Blood pressure drives ultra-filtration, with a glomerular filtration rate (GFR) of approximately 90-120 ml per minute (9). Once past the slit diaphragm, the filtrate enters the lumen of Bowman’s Space. However, because the major criterion of filtration is size, both wastes and essential proteins and molecules are filtered. Without downstream tubular reabsorption, the loss of vital minerals, nutrients, and water would preclude survival.

**1.3.2. Tubular Reabsorption and Excretion**

Once in Bowman’s Space, the filtrate begins its journey through the many tubular segments of the nephron (Figure 1.3) (9). Each of these tubular compartments has specialized functions that are crucial for maintaining blood and body homeostasis.
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The chief tubular functions include: secretion of unfiltered wastes from the bloodstream, the selective reabsorption of nutrients and minerals, blood acid-base balance, and the recovery of almost 98% of the excreted water. By the time the filtrate exits the kidney, it will have been concentrated by a factor of 100 and will contain, under normal circumstances, only wastes and unneeded nutrients and water (8).

To facilitate tubular reabsorption, each nephron segment is intimately associated with branches of the efferent arteriole (9). Upon exiting the glomerulus, the efferent arteriole takes one of two routes. In the outer cortex, the efferent arteriole forms an open network of capillaries that surround the cortical proximal convoluted tubules. In the inner cortex, the efferent arteriole forms a straight vessel that penetrates the adjacent medulla to form the peritubular capillary system of the medullary segments. These medullary capillaries, comprising the vasa recta, eventually return to the juxtamedullary region where they drain into the interlobular veins (10).

While a comprehensive discussion of tubular function is beyond the scope or need of this thesis, the structure and basic function of the nephron segments requires further examination.

The first tubular segment encountered by the filtrate is the proximal convoluted tubule, which adjoins the glomerulus at the urinary pole (9,10). The proximal convoluted tubule lies entirely within the cortex of the kidney and serves as an
active site of selective molecule transport and reabsorption. In order to perform
their task of active and passive transport, proximal convoluted tubule cells have a
highly developed brush border formed from extensive vilification of the their apical
plasma membrane. As seen in other types of absorptive cells (i.e. in the intestine)
these microvilli serve to increase the surface area of the apical membrane,
significantly increasing transport capability. The proximal convoluted tubule is
responsible for the uptake of the majority of molecules recovered by the kidney
including: phosphate, potassium, urea, and up to 100% of all organic solutes such as
glucose and amino acids. The proximal convoluted tubule is also the site of the
majority of sodium reabsorption, which facilitates the reabsorption of a similar
proportion of water to balance the osmotic gradient. The proximal tubule is also
responsible for most of the active secretion performed by the kidney.

The filtrate, now reduced in volume by approximately one third, enters into the
juxtamedullary portion of the nephron, or the loop of Henle, which represents the
primary evolutionary adaptation of the mammalian kidney to osmotic conservation
by allowing for the production of concentrated, hypertonic urine. Structurally, the
loop of Henle is composed of both thin and thick segments, characterized by the
vastly differing morphologies of the epithelial cells that form them (9,10). Thin limb
segments display a flattened, almost squamous morphology, while the segments of
the thick limbs exhibit a more characteristic, cuboidal or columnar shape.
Functionally, the tonicity of the urine in relation to the plasma allows water to pass
freely through the water permeable thin descending limb of the loop. This process is
facilitated by the close association of the loop of Henle with the vasa recta, which creates a counter-current flow that maximizes water retrieval. Upon turning to run back towards the cortex, the loop transitions into the water impermeable thick segment, trapping the concentrated urine in the luminal space. In the human kidney, the proportion of water reabsorbed by the loop of Henle is relatively small, however the action of the loop serves to generate a high osmotic gradient in the fluid of the medullary interstitium (10). This process is vital to the selective reabsorption of water by the collecting duct and will be discussed below. Upon its return to the cortex, the loop of Henle transitions into the final segment of the nephron proper, the distal convoluted tubule.

Histologically, the distal convoluted tubule resembles the proximal convoluted tubule but lacks the characteristic brush border. The distal convoluted tubule retains the water impermeability of the ascending limb of the loop of Henle and further contributes to the reabsorption of sodium. However, sodium transport in the distal convoluted tubule is selective and is controlled by the secretion of aldosterone from the adrenal glands. Calcium reabsorption in the distal convoluted tubule is similarly controlled by parathyroid hormone. The distal convoluted tubule also plays an important role in the secretion of potassium as needed to maintain the body’s overall potassium levels. Lastly, the distal convoluted tubule plays an important role in the regulation of blood pH through the secretion of acid (H+) or reabsorption of bicarbonate.
1.3.3. The Collecting Duct and Lower Urinary Tract

While the distal convoluted tubule is the last segment of the nephron proper, it is not the final segment encountered by the filtrate (9,10). The collecting duct system is a branched, tree-like network of ducts that serve to drain multiple nephrons. Filtrate from the distal convoluted tubule passes briefly though the connecting tubule, formed by an overlapping fusion of the distal convoluted tubule and collecting duct, before entering into the contiguous lumen of the collecting duct. The collecting ducts then transport the urine through the cortical and medullary tissues. Multiple terminal collecting ducts empty their urine into the calyces, which fuse to the join the ureters completing the transportation of urine from the kidney to the bladder.

In addition to the transport of urine, the collecting duct system is also responsible for fine-tuning of the final filtrate composition. The two cell types of the collecting duct serve many of the same transport functions as the distal convoluted tubule with intercalated cells regulating acid-base homeostasis, and principal cells selectively regulating sodium absorption and potassium secretion (9). In the case of water reabsorption, 95% has been reabsorbed from the filtrate prior to entry into the collecting duct system. However, depending on the hydration level of the body, and under the control of the water-regulating hormone vasopressin, principal cells are also responsible for up to 5% of the total water recovery of the kidney. This is achieved in conjunction with the high interstitial osmotic concentration produced by the loop of Henle. The expression of vasopressin promotes the apical localization
of the water channel aquaporin 2 (AQP2) allowing for the free passage of water into the surrounding interstitium, and serving to further concentrate the urine (16). Thus, the epithelium of the collecting duct is responsible for the final, adjustable regulation of the urinary composition before its departure from the kidney and subsequent elimination from the body.

1.4. Development of the Kidney

Chronic injury results in structural damage to mature adult kidneys that negatively impact their function. However, in the fetal kidney, chronic injury serves to derail the normal developmental process leading to alteration of structure and the loss of potential function. This ultimately leads to a decrease in filtration potential and predisposes the individual to complications later in life (to be discussed in Chapter 2). Furthermore, to understand how various components of the kidney will respond to injury at different points in gestation, the stage of development and differentiation of the various regions of the kidney must be considered. To understand the effect of fetal kidney injury upon development and therefore upon postnatal function, it is therefore important to explore the mechanisms of renal organogenesis.

As with most organs, the key steps in the evolution of the kidney are recapitulated in its embryonic development. During the organogenesis of the kidney this is especially evident in the sequential formation of 3 nephric structures; namely the
pronephros, mesonephros and metanephros (Figure 1.6) (17). In mammals, the first two of these constitute vestigial or temporary kidney-like structures that are lost or redeveloped during the maturation of the metanephros which derive the mature and functioning kidney.

1.4.1. Early Patterning of the Kidney

In the developing mammalian embryo, all renal structures derive from the intermediate mesoderm located between the axial/somatic mesoderm and the lateral plate mesoderm (18). As with all developmental processes in the fetus, the regional specification of the intermediate mesoderm and the renal tissues that derive from it, depend heavily on then expression of genes that define the axial patterning of the fetus. In particular, the definition of the anterior-posterior axis by the Hox family genes is crucial for the correct localization and orientation of renal structures. The formation of the intermediate mesoderm is defined by the expression of HOXB4. The subsequent expression of PAX2 and PAX8, two genes crucial for the specification of renal tissues, closely follows that of HOXB4 (17). PAX2 in particular plays a key developmental role in the morphogenesis of the final, permanent kidney (19-21).

The first step in the organogenesis of the kidney is the formation of the pronephric duct that arises from the intermediate mesoderm as a single-layered epithelial tube (17,18). Formation of the pronephric duct begins at embryonic day 22 in the human fetus (day 8 in the mouse) and extends caudally towards the most posterior portion of the cloaca or hindgut. Tubules extend medioventrally from the anterior portion of
Figure 1.6: Development of the Early Nephric Structures. Formation of the pro- and meso-nephric structures precedes that of the metanephros. The pronephric structures regress fully in mammals, while portions of the mesonephros contribute to the formation of the genital tract. The anterior portions of the nephric, or Wolffian, duct interact with the metanephric mesenchyme to form the metanephric, or mature, kidney. Adapted from http://commons.wikimedia.org/wiki/File:Development_of_embryonic_nephrons.png
the pronephric duct forming the rudimentary pronephros, which is not functional in mammals. The mesonephros, comprised of approximately 30 tubules in the human fetus, subsequently forms from the more caudal tubules and closely resembles the proximal tubule and glomeruli of the mature kidney (Figure 1.6). Some of these mesonephric tubules later contribute to the formation of sperm-carrying tubules in the males, while others regress during metanephric development.

1.4.2. Induction of the Metanephric Development

The organogenesis of the mature, permanent kidney occurs as a result of interactions between the Wolffian duct and the adjacent metanephric mesenchyme (MM) (17,18,22). The MM also derives from the intermediate mesenchyme and forms adjacent to the Wolffian duct. Localization of the MM, and therefore of the mature kidney, along the anterior-posterior axis is controlled by the expression of HOX11. Once formed, the MM is developmentally restricted to differentiation into renal tissues (18). At approximately 5 weeks gestation in the human fetus (day 10.5 in mouse), the MM induces an outgrowth from the adjacent Wolffian duct called the ureteric bud (22). This budding process constitutes the first step in the formation of the metanephros and initiates the cascade of reciprocal interactions between the ureteric bud and the condensed MM that guides the patterning of renal development.

Several signaling pathways are involved in the complex process of metanephric development and reciprocal inductive interactions are essential for the survival and
differentiation of both the mesenchymal and epithelial components of the developing kidney (17,18). Most of these signals are not freely diffusible, and require cell-cell or cell-matrix interaction. The central pathway controlling ureteric bud growth is one exception to this mechanism, and relies upon the production and secretion of glial-derived neurotrophic factor (GDNF) by the MM. GDNF acts upon the tyrosine receptor, RET, and its co-receptor GDNF family receptor alpha 1 (23) expressed along the Wolffian duct and at the tips of the ureteric bud. RET activation in turn promotes ureteric bud elongation and invasion of the MM by activating cell migration and proliferation at the ureteric bud tip.

The localization of the ureteric bud outgrowth along the anterior-posterior axis is closely controlled by several mechanisms that inhibit GDNF/RET signaling (17). These mechanisms serve to inhibit ectopic bud formation and ensure appropriate positioning of the ureteric bud. Non-MM mesenchymal cells surrounding the nephric duct express bone morphogenic protein 4, which inhibits GDNF/RET signaling and therefore ureteric bud budding. In the posterior region of the MM, the transcription factor FOXC1 expressed by the intermediate mesoderm and retained by the MM adjacent to the Wolffian duct suppresses the inappropriate expression of GDNF. Similarly, GDNF expression is inhibited at the anterior side of the MM via interaction of the ureteric bud-expressed transmembrane SLIT2 protein and its MM-expressed receptor roundabout homolog 2 (24). Lastly, RET expression along the Wolffian duct and ureteric bud is tightly controlled by sprouty 1, which limits RET expression to the ureteric bud tips (17,25,26). Disruption of these signaling
pathways, as demonstrated through the use of conditional gene targeting experiments in mice, results in either a loss of ureteric bud budding and therefore renal agenesis, or in the formation of ectopic buds causing development of duplicate collecting systems and other renal malformations. These mechanisms, and undoubtedly others yet to be uncovered, ensure that budding of the ureteric bud occurs appropriately.

1.4.3. Branching Morphogenesis

Following its migration into the neighboring MM, the ureteric bud develops into a branched tree-like structure that will ultimately form the ducts of the collecting system. During the formation of this tree-like structure, the reciprocal interaction of the ureteric bud and MM described previously continues and results in the dramatic remodeling of both tissues (Figure 1.7). The remodeling of the latter tissue includes nephrogenesis and is discussed in the next section. The remodeling of the ureteric bud includes branching morphogenesis and is characterized by many successive reiterations of branching and elongation, followed by maturation and differentiation of the epithelia (17,18,22,27-29). Branching and nephrogenesis occur within a nephrogenic zone that progresses centrifugally, leaving the oldest nephrons and ureteric bud segments in the innermost portions of the kidney. Throughout this process, the ureteric bud tips retain the inductive potential characteristic of the early ureteric bud, while the ureteric bud stalks left behind in the wave of branching and nephrogenesis begin to differentiate into mature epithelia.
**Figure 1.7: Nephrogenesis.** Reciprocal interactions between the ureteric bud and metanephric mesenchyme (MM) govern both branching morphogenesis and nephrogenesis. Condensation of the MM around the ureteric bud tips forms renal vesicles, which mature into nephrons via the intermediate S-shaped body. Signals from invading stromal cells are important for the growth, survival and differentiation of both MM and ureteric bud derived structures. Adapted from Dressler GR., *Annu. Rev. Cell Dev. Biol.* 2006, 22:509-29
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Through its capacity to induce nephron formation and constitute the final collecting duct system, appropriate branching is fundamental in establishing the architecture and determining the final nephron number of the kidney.

While the ureteric bud is capable of branching in a number of unique ways, the mechanism of branching is largely conserved. Following elongation, the individual tips of the ureteric bud swell to form hollow and rounded ampullae (17,18). These structures are then remodeled into the necessary branched structures. Amazingly, this remodeling does not occur by delamination or migration of cells from the ampulla’s epithelial sheet (28). Instead, remodeling occurs simply by the differential growth, death, and alterations in cellular morphology. The initial formation of the nascent branches appears to be driven by localized proliferation at opposing ends of the ampulla and facilitated by the acquisition of a wedge-shaped morphology to promote folding of the epithelial layer. Another proposed mechanism relies on the “purse-string” model of epithelial folding (30) whereby the localized contraction of apical actin filaments in several cells promotes the evagination of the epithelial layer. Apoptosis is also thought to contribute to the formation of the dividing cleft that separates the nascent branches (28). Despite the lack of a complete understanding of the exact mechanisms of branch formation, it is clear that cellular dynamics, and not migration, play a key role.

Though generally described as dichotomous, the ureteric bud is capable of many branching patterns including terminal bifid, terminal trifid, and lateral branching.
(28,29). Terminal bifid branching is the most common form, and sees the ampulla remodeled into two separate branches. Terminal trifid branching similarly sees the ampulla form three branches, though many of these formations are later remodeled into two bifid branches. Lateral branching, most easily demonstrated during the original formation of the ureteric bud from the Wolffian duct, is rare and occurs mostly in the early stages of branching morphogenesis. From this repertoire of branching patterns the ureteric bud is progressively remodeled and, as will be discussed in the next section, the sequence in which these patterns of branching are employed is crucial to the final architecture of the kidney. Indeed, several developmental stages of branching morphogenesis have been described beginning with the initial outgrowth ureteric bud, progressing through phases of early and late ureteric bud branching, and ending with the cessation of branching and cellular differentiation (28,29).

In the developing human kidney, the ureteric bud undergoes approximately 15 rapid branching events (approximately 8 events in mouse) (29). The first stage of branching begins with the induction of the ureteric bud as a lateral branch of the Wolffian duct at approximately 5 weeks gestation in human (embryonic day 10.5 in mouse), as previously discussed (28). Soon after its invasion of the adjacent MM (embryonic day 11.5 in mouse), the ureteric bud undergoes its first bifurcation forming a unique T-junction. This first branching sets the stage for the early branching events, which occur largely by trifid branching. The first 5 or so of these branches produce the renal pelvis and calyceal structures (29). In humans, the next
5 branching events occur predominantly by terminal bifurcation and comprise the collecting ducts of the papilla and medulla. How the papilla forms its final inverted, pyramidal structure in the calyceal space is unknown. However, it appears likely that its formation must result from the gradual broadening of calyces followed by the collapse or inversion of the early, post-calyceal branches of the ureteric bud. The human kidney houses an average of 8 papillae, with approximately 44 collecting ducts exiting the tip of each one (29). This would equate to a total of 352 ducts or between 8 or 9 branching events \( (2^8 = 256 \text{ branches}, 2^9 = 512 \text{ branches}) \).

In the human fetal kidney, the subsequent 15 branching events also occur by terminal bifurcation, and produce the remaining segments of the medulla and inner cortex (29). At this stage, branching coincides with nephron induction, discussed below. During the 13-15th branching events, the ureteric bud tips induce multiple nephrons simultaneously that connect to the mature collecting duct via shared connecting tubule arcades in the deep cortex. Following the 15th branching event, the terminal branch continues to extend through the cortex towards the capsule. During this growth, several successive ampullae, differing from those seen previously, are formed near the elongating ureteric bud tip. These ampullae are less rounded and more triangular than the ampulla of earlier branchings, and serve to induce the budding of lateral branches connected to individual nephrons. This process results in approximately 10 laterally branched nephrons per cortical collecting duct. At 36 weeks gestation in humans the induction of new nephrons
ceases and the final maturation of the newly formed structures concludes. Approximately two-thirds of nephrons are formed during the last trimester (31-33).

In the fetal mouse kidney, 8 branching events occur between embryonic day 11.5 and 15.5, forming the branches that will comprise the collecting ducts of the inner and outer medulla (28). From embryonic day 15.5 until birth, only 2 to 3 cortical branching events occur, with the remainder of cortical branching and nephrogenesis occurring in the first two weeks postnatal.

The characteristic structure of the final collecting duct system is not determined by branching alone. During branching morphogenesis, periods of increased proliferation and restricted branching are responsible for the elongation of the ureteric bud tip and the spacing of branching events (28,29). Earlier branching events have shorter branch segments, while later branches have longer elongation periods producing longer segments. Following behind the wave of branching and nephron induction, differential growth of the ureteric bud stalks again remodels the ureteric bud by altering the relationship of the various branched segments and contributing to the overall growth of the kidney. The elongation of previously formed segments of the ureteric bud occurs by a process of convergent extension, whereby the combination of cellular proliferation and morphological change result in the thinning and lengthening of the structure. In the 4th month of human gestation, segments of the inner medulla show little growth while the 10th - 15th branching events elongate dramatically by convergent extension producing the long,
straight segments of the outer medullary collecting duct. In the mouse, the 6th-8th branching events undergo similar elongation following embryonic day 15.5 (28). Postnatally, juxtamedullary and cortical segments exhibit similar high rates of elongation, accounting for much of the postnatal growth of the kidney.

Many of the factors that control the initial budding of the ureteric bud continue to serve a similar role in controlling branching morphogenesis. In particular, the GDNF/RET pathway acts as a permissive signal controlling reiterative cycles of elongation and branching of the ureteric bud. This signal is permissive, rather than instructive, in that it “green-lights” the predetermined developmental response but does not guide the outcome (18). Cells at the ureteric bud tip under the control of RET activity provide most of the proliferation that drives ureteric bud elongation and serve as the driving force behind branching morphogenesis (34,35).

Many additional factors have been implicated in various aspects of branching morphogenesis, however the function of many of these signaling events remains to be elucidated. The expression of one such factor, WNT11, is activated by GDNF signaling in RET expressing cells of the ureteric bud tip (36), and in turn maintains Gdnf gene expression in the adjacent MM. Wnt11 and GDNF therefore appear to form a positive feedback loop that potentiates continued GDNF signaling. Several other growth factors have been implicated in the maintenance of branching. Knockout studies in mice have shown that deficiencies in fibroblast growth factor (FGF) signaling via FGF-receptor 2 (37), FGF7 (38) and FGF10 (39) reduce epithelial
branching. The hepatocyte growth factor and epidermal growth factor pathways have been shown to interact synergistically to influence branching morphogenesis (40).

While GDNF/RET signaling serves as the predominant permissive signal of branching, appropriate branching requires close spatial and temporal control over the phases of elongation and branching. Several factors have been identified which limit or restrict the effect of GDNF signaling. Sprouty 1 again plays a key role in inhibiting inappropriate branching by limiting RET expression and opposing inappropriate GDNF signaling (25). Intriguingly, many members of the Transforming Growth Factor superfamily serve to oppose GDNF signaling. Bone morphogenic protein 4 expression in the MM adjacent to the ureteric bud stalks inhibits inappropriate branching in these regions while serving to promote differentiation of the adjacent ureteric bud epithelium. Gremlin 1 expressed by the surrounding MM the ureteric bud tips prevents the bone morphogenic protein 4-mediated inhibition of GDNF signaling (41). Transforming growth factor β (TGFβ) controls the spacing of tubular branching in other branching systems, and may serve as the arbiter determining the frequency of branching points.

The process of branching morphogenesis is crucial not only to the formation of the collecting duct, but also to the overall patterning of the kidney. Several studies have demonstrated that factors that impair appropriate branching negatively impact the induction of nephron formation leading to a reduction of functional filtration units
(38,42-45). As will be discussed in Chapter 2, this reduction in nephron number is a fundamental factor underlying the predisposition to and progression of kidney disease.

1.4.4. Nephrogenesis

In parallel with branching morphogenesis, cells of the MM undergo differentiation into non-ureteric bud derived portions of the nephron. This process of nephrogenesis occurs in response to induction from the developing ureteric bud tip and is subject to its own host of patterning and signaling pathways.

Following induction by the ureteric bud, cells from the surrounding MM condense to form pretubular aggregates adjacent and inferior to the ureteric bud (17,18,22). These aggregates subsequently undergo mesenchymal-epithelial transdifferentiation to form epithelialized structures called renal vesicles. To transform, the mesenchymal cells must alter their intracellular adhesion via expression of adherens and tight junctional proteins. Novel cell-cell adherence allows the establishment of columnar morphology and cell polarization necessary for epithelialization and allows for the deposition of a provisional basement membrane and formation of a lumen. Once formed, the immature epithelium of the renal vesicle will eventually derive all of the epithelial components of the nephron including the podocytes and parietal epithelial of the glomerulus and all of the tubular epithelia up to and including the distal tubule.
The differentiation of the simple renal vesicle to the complex structure of the final nephron proceeds through two recognizable intermediates. The first intermediate, the comma-shaped body, is created by the formation of a cleft by invagination of the epithelium nearest the ureteric bud (17,18). This forms the comma-shaped body, with the distal tail of the vesicle fusing with the ureteric bud to form a contiguous lumen. The formation of the second intermediate, the S-shaped body, occurs following the formation of a second, proximal cleft. The invasion of FLK1-positive angioblasts into this cleft provides the precursors for the vascular bundles comprising the future glomerular capillary network. Meanwhile, invading mesangial precursors provide structural support for the growing capillary loops, and serve an important role in the patterning and final development of the glomerular tuft.

Within the S-shaped structure, regions of epithelial precursors can be identified for each epithelial compartment of the developing nephron (17,18). The most proximal end of the S-shaped body, together with its vascularized cleft, differentiates into the functional filtering unit of the nephron, the glomerulus. The epithelium in this region specializes into two regions of columnar and flattened epithelium, serving as precursors to the podocyte and parietal epithelium respectively. Through interaction of the developing vascular endothelium, the adjacent columnar epithelial precursors form the highly specialized podocyte layer. Through a reversal of the mesenchymal-epithelial transdifferentiation process that potentiated the transition from aggregate to renal vesicle, the podocyte precursors revert to a mesenchymal phenotype allowing for their invasion into the glomerular tufts and envelopment of
the capillaries. Ultimately, podocytes revert to a unique epithelial phenotype that facilitates the significant fenestration. Adjacent podocytes produce modified junctions that form the slit diaphragm (discussed in section 1.3.1), a crucial feature of the glomerular filtration barrier. The remaining epithelium of the proximal arm of the S-shaped body subsequently forms the parietal epithelial layer of the Bowman’s capsule and encloses the glomerular urinary space, or Bowman’s Space.

The tubular portions of the nephron also develop from the S-shaped body in a proximal-distal sequence (17,18). The adjacent tubular region to the nascent glomerulus forms the proximal convoluted tubule, followed by the loop of Henle. The distal convoluted tubule forms from the most distal portion of the S-shaped body immediately adjacent to the ureteric bud tip. Throughout the epithelialization of the developing nephron precursor, signaling via the Notch pathway is central to the establishment of local proximal-distal patterning. Specifically, NOTCH2 expressed in the MM has been shown to control the differentiation of proximal epithelial types, but not of the distal convoluted tubule. The role of distal patterning may reside with other notch ligands yet to be identified. Following their formation, these tubular precursors undergo extensive proliferation to establish their mature structure. In particular, the long segments of the loop of Henle proliferate in conjunction with the convergent extension of the later branches of the ureteric bud, allowing the loop to maintain its position in the medullary region.
1.4.5. Stromal Differentiation

During nephrogenesis, some cells of the MM do not undergo condensation to form the tubular epithelium. These cells, recognizable by their expression of the transcription factor FOXD1 (formerly BF-2) (46) differentiate into the renal stroma that populates the interstitial spaces between the developing tubules (Figure 1.7). The production of several growth factors by the stroma has been shown to support and promote the process of ureteric bud growth and branching, including FGF2, FGF7 and FGF10 (38,47). The stromal expression of retinoic acid receptors α and β2 has been similarly shown to be crucial for facilitating branching by maintaining the expression of RET in the ureteric bud tip through production of unknown paracrine signals (18). Conversely, bone morphogenic protein 4 produced by stroma inhibits the ectopic budding of the Wolffian duct and ureteric bud as described previously (48). In addition to regulating ureteric bud growth and branching, stromal signals have proven important in maintaining the mesenchyme. The combined activity of FGF2 and bone morphogenic protein 7 is important in the maintaining the competence of the uninduced mesenchyme (49), and in return is an important regulator of the balance of growth, survival, and differentiation of tubular progenitors (18). The lack of stromal activity, as in mice deficient of FOXD1, produces kidneys with significant inhibition of normal ureteric bud branching, abnormal condensation of the MM, and a lack of mesenchymal-epithelial transdifferentiation required to promote tubulogenesis (46). It therefore appears that reciprocal signaling between stroma and epithelial cells is crucial for the survival and proliferation of both cell types (18). Though still poorly understood,
these studies and others suggest that stromal cells serve a critical dual role in maintaining both appropriate branching and appropriate MM condensation.

1.5. **Conclusion**

The kidney displays immense complexity in its function, structure, and development. Functionally, the kidney serves the essential role of maintaining the homeostasis of our internal environment by removing wastes and toxins from the blood stream while retaining or reabsorbing the essential nutrients, minerals, electrolytes, and water needed for the fundamental biological processes necessary for survival. Structurally, the kidney is defined by its functional unit, the nephron, which is itself comprised of up to a dozen unique functional compartments. The kidney is a highly epithelialized organ, with most of its functional compartments formed of tubular epithelia, each comprised of numerous distinct epithelial cell types. The specific morphology and function of each individual cell type underlies the kidney's overall function and plays its own essential role in homeostasis and survival. Developmentally, the kidney is similarly diverse, forming from the interaction and cooperation of two tissues, the MM and the ureteric bud, of distinct embryological origins. Through exceedingly complex reciprocal interactions, the ureteric bud and MM progressively drive the growth, branching and maturation of the collecting system, while repeating the process of nephrogenic induction, patterning and growth hundreds of thousands of times.
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All of this complexity is delicately balanced to produce and maintain the function of the kidney as a whole. Currently, our knowledge of the interactions between the various compartments of the kidney, including the glomerulus, tubular epithelium, and interstitium, is incomplete. Significant questions remain regarding the proliferative, regenerative and pluripotent capacities of most of the cells in the kidney, and we do not fully understand the extent to which one compartment contributes to injury or compensates for repair of the other. As will be described in the next chapter, injury or disease can have a significant impact on the development, and therefore on the structure and postnatal function, of the kidney. Furthermore, the developmental stage of the kidney at the time of injury can also significantly alter the outcome and severity of renal disease. Therefore, when studying injury and disease affecting the fetal kidney it is crucial to know the developmental stage, renal compartment, and cellular context in which the event is occurring.
CHAPTER 2 INTRODUCTION TO CHRONIC KIDNEY DISEASE

2.1. Clinical Burden of Chronic Kidney Disease

Chronic kidney disease (CKD) is a major cause of morbidity and mortality worldwide and leads to a variety of complications including hypertension, anemia, acidosis, renal malnutrition and altered mineral metabolism (50). Although due to a number of different etiologies, CKD has the common end point of declining renal function. This decline can result from injury to any of the kidney’s distinct structures including the glomerulus, renal vasculature, or the tubulointerstitial compartments and can have a diverse range of functional consequences. The variety of mechanisms of renal injury coupled with the complexity of the kidney significantly complicates the treatment and management of CKD. The most severe clinical manifestation of CKD is termed end-stage renal disease (ESRD) (50,51). ESRD is “end-stage” in that the patient’s kidney function is no longer capable of sustaining life, and renal replacement therapy through dialysis or transplantation is required. While these therapies can prove effective, they bear a substantial economic cost and have long-term impacts on the recipient’s quality of life.

2.1.1. Incidence, Mortality and Morbidity of ESRD

Historically, the best tracked patients were those suffering from ESRD. During the 1990s, ESRD increased dramatically in the United States with the incidence doubling during this period (50). A review of data from the U.S. Renal Data System, found that more than 85,000 patients were diagnosed with ESRD in 1998 with 351,406 total
ESRD patients at the calendar year’s end (reviewed in (50)). Of these patients, 72% were treated by dialysis while the other 28% had received organ transplants. Despite these renal replacement treatments, it is estimated that more than 63,000 ESRD-patient deaths occurred, with a 20% mortality rate observed within the dialysis population. Life expectancy within the remaining dialysis population is dramatically shorter than their age-matched peers, with the expectancy of 7.1 to 11.5 years of life for patients aged 40-44 years, and 2.7 to 3.9 years for those aged 60-64 years. While serving as effective replacements of kidney function, the replacement therapies are associated with significant morbidity and lower self-reported quality of life. In 1998, patients on dialysis exhibited an average of 4 co-morbidities, and spent a mean number of 15 days in hospital per year. In total, the U.S. annual costs for such replacement therapies totaled $16.7 billion dollars.

There is substantial evidence that the burden of kidney disease continues to increase. The most current US Renal Data System data indicate that the prevalence of ESRD patients has increased to 547,982 in 2008 (52). In Canada, 2009 figures indicate 37,744 registered ESRD patients (53). In both countries, the adult population over the age of 20 years old constitutes more than 98% of the ESRD populations with a total of 540,373 and 37,196 patients, respectively. In both countries, the predominant cause of ESRD is diabetes, accounting for 35% of ESRD patients in the U.S. and 26% in Canada. In the US, the next two most common causes of ESRD are hypertension (23%) and inflammatory diseases of the glomerulus, or glomerulonephritis (14%). Canadian figures list glomerulonephritis and renal
vascular disease as the second and third most common causes of ESRD (22% and 13% respectively). With the prevalence of obesity, hypertension and cardiovascular diseases increasing in our society, it is clear that kidney disease is a rapidly growing health problem.

2.1.2. Classification of Chronic Kidney Disease

In general terms, progressive CKD is defined as the slow loss of kidney function over time. In the clinic, CKD is defined as either structural or functional damage of the kidneys, or the significant decline in GFR, lasting more than 3 months (50). While the earlier forms of CKD are associated with little or no symptoms, CKD often proves to be progressive leading to more significant damage and injury. Thus, the ESRD group is the most visible group of patients with CKD and requires the most care, but is also the smallest population in number. Prior to 2002 little was reported about the incidence, prevalence, or morbidity associated with milder forms of CKD. This lack of epidemiological information was largely due to poorly defined or inconsistent definitions of what constitutes mild or moderate CKD. Furthermore, the inability to accurately identify patients with earlier forms of CKD made it difficult to effectively treat patients to minimize disease progression (50).

When CKD is identified early, therapeutic interventions can be effective at staving off the progression to ESRD. To take advantage of this, the U.S. National Kidney Foundation formed the Kidney Disease Outcomes Quality Initiative (K/DOQI) in 1999 to create a representative classification system of CKD that would allow for standardization of care and outcome tracking of all stages of CKD (50). This system
needed to utilize clinically measurable criteria to determine both the structure and function of the kidney, and to apply these values to the severity of CKD. Kidney structure, or more specifically the extent of kidney injury, can be determined through urinalysis, bioimaging, and biopsy. In the clinic, kidney function is determined by measuring the clearance of a relevant substance or marker from the serum to determine the GFR (54). Calculated estimations of GFR require complicated normalizations for age, height, weight, gender and ethnicity of a patient in order for the result to be informative. An in-depth discussion of these formulae is beyond the scope of this review, however, intuitively GFR can be thought of as the rate of filtration and generalized as a percentage of total normal function. Notably, GFR declines as kidney injury worsens making it a suitable classification criterion in the context of CKD.

In the K/DOQI system, each stage has been defined by a specific range of function based upon GFR in the presence or absence of renal damage (50). Stage 1 denotes the presence of kidney damage in the absence of any decline in function or GFR. Stages 2, 3, and 4 represent worsening kidney injury associated with a stepwise decline of function (loss of 11-40%, 41-70%, and 71-85% function respectively). Stage 5 represents the population previously defined as ESRD, signifying a GFR of less than 15ml/min or kidney function approximately 15% of normal. This classification system allows for better recognition of patients in the early stages of CKD, and for the application of standardized treatment regimens targeted to slow or halt the progression of CKD.
2.1.3. **ESRD as a Threshold Disease**

The K/DOQI classification system highlights a startling feature regarding kidneys and their function; namely the onset of severe clinical disease requires the loss of a majority of kidney function, approximately 85% (50). This implies that under normal circumstances, the kidneys possess a significant degree of redundancy in their function. This redundancy is similarly evident in the survival of living donors, who voluntarily reduce their kidney function by 50% to provide an organ for transplant. In this regard ESRD can be thought of as a threshold disease, for its onset is only realized when the decline in one’s renal function reaches a threshold value that is no longer capable of sustaining life.

How then, with the extensive redundancy present in the function of our kidneys, does such a dramatic loss of function occur? As described in the previous chapter, the global function of the kidney results from its many individual filtration units, or nephrons. At birth, we are endowed with our full complement of approximately one million nephrons (500,000 per kidney) (55). During our lifetime, no new nephrons will be formed. Unfortunately, as we age, injury gradually decreases the number of healthy, functioning nephrons. Under normal circumstances, it has been estimated the after the age of 18 we lose approximately 4,500 glomeruli per year (56). Clinical examination suggests that this may amount to a reduction of glomerular number by 30-50% by age 70 (57). At this rate, our endowment of nephrons at birth will more than serve us over the reasonable course of our life expectancy. However, renal injury and disease often serve to accelerate the decline in our renal function by
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stressing or damaging our nephrons. Renal injury can be linked to diseases such as cardiovascular disease. In the case of hypertension, high blood pressure causes the eventual breakdown and scarring of susceptible glomeruli, leaving fewer filtration units filtering the same volume of blood (58). This results in even higher intraglomerular blood pressure and further glomerular disruption. Not all kidney diseases promote this self-sustaining cycle of glomerular injury, however each insult is likely to induce the damage and dropout of some proportion of nephrons. While nephron number does not relate directly to GFR or the percentage of function, eventually the loss of a sufficient number of nephrons will overcome the compensatory capacity of the kidneys and hasten a decline in renal function.

To further complicate this concept of declining function and disease thresholds, nephron endowment exhibits a standard distribution within the population ranging from 600 000 to 1.4 million nephrons per individual (59,60). This wide distribution led to the nephron endowment theory, championed by Dr. Barry Brenner, which suggests that low nephron number predisposes one to cardiovascular and renal diseases in later life. Evidence suggests that in kidneys with fewer glomeruli there is a compensatory increase in glomerular size and volume (61,62). The maintenance of normal rates of renal filtration in these low nephron kidneys appears to be maintained by hyperfiltration and hypertrophy, contributing to increased glomerular stress and damage and an increased the rate of functional decline over time. Therefore, an individual’s nephron endowment may ultimately influence the likelihood of their decline in function reaching the ESRD threshold.
How then is an individual's endowment of nephrons determined? It appears that both genetic and developmental factors play a role (reviewed in (55)). Clinical data show that hypertension and renal disease is more prevalent in certain ethnic groups including Australian Aboriginals, Native Americans and those of African descent (63-65). Similarly, genetic predisposition to hypertension and kidney disease can be transmitted within families. Clearly, genetic factors are a major determinant of nephron endowment. To date, few genes have been shown to directly influence nephron endowment, though studies have demonstrated a key role for PAX2 (43,45). In many cases, fetal programming may play a role in influencing the developmental pathways of the kidney. Low birth weight is an indicator of sub-optimal intrauterine growth, and is well described as a risk factor for the progression of renal (59,66) and cardiovascular disease (67,68). Low birth weight has been similarly linked to a reduction of nephron number (69) with estimates suggesting a correlation of 257,426 glomeruli per kilogram birth weight ((70)). Decreased nephron number has also been observed in pre-term babies and correlates with gestational age (69). This reduction is likely due to the arrest of later gestational development in favor of renal differentiation and maturation needed for survival outside the womb. Many additional studies similarly support the nephron endowment theory and suggest a relationship between birth weight and nephron number.
Factors determining our endowment of nephrons are therefore crucial in establishing our functional capacity at birth and will, in case of low endowment, predispose us to potential complications as we age. While the specific mechanisms that influence nephron endowment are not yet fully understood, they inevitably act by influencing the normal processes of renal branching morphogenesis and nephrogenesis. Due to the interrelatedness of these two processes during development, it has been shown that an impairment of branching as small as 2% can result in a 50% reduction in total nephron number over the course of development (71). An appropriate fetal environment during kidney development is therefore key to the realization of maximal genetically programmed nephron endowment. In addition to genetic factors and fetal programming described here, fetal and pediatric diseases can also have a significant impact on both endowment and early functional declines.

2.1.4. CKD in Pediatric Populations

Children represent a small portion of the CKD population. Pediatric ESRD has a prevalence and incidence of less than 2% of the total ESRD population in the U.S and Canada (prevalence of 7,607 and 548 patients respectively (52,53)). However, for the reasons described in the previous discussions on nephron endowment and functional decline, CKD in those under 18 can have profound implications on long-term health and life expectancy. While in humans nephrogenesis is complete by birth, nephron maturation occurs in the first 2 to 3 years of life and kidney growth continues until age 12 (72). Therefore, pediatric kidney injury can have a substantial impact on postnatal maturation and growth, increasing the likelihood of progressing
though the stages of CKD. Furthermore, diseases that affect children during critical
times can negatively impact kidney organogenesis. In particular, congenital
disorders can disrupt kidney development, and account for greater than 40% of
paediatric CKD (73).

Not surprisingly, the etiologies leading to pediatric CKD are vastly different from
those causing adult disease. In the US and Canada, two of the primary causes of
pediatric ESRD necessitating transplantation are hypoplasia/dysplasia of the kidney
and obstructive nephropathy (53,74). Hypoplasia/dysplasia is a general description
covering a host of disorders resulting in the abnormal or under-development of the
kidney. Obstructive nephropathy describes conditions involving the restriction of
urinary flow causing subsequent renal injury. These two disorders are both
congenital anomalies and together account for 31% of U.S. pediatric transplants
(15.8 and 15.3% respectively) (74). In Canada, hypoplasia/dysplasia and
obstructive nephropathy comprise 24% of the transplant population (16.5% and
7.5% respectively) (53). Congenital disorders such as these occur during the fetal
development of the kidney, and serve to impair the normal processes of branching
morphogenesis and nephrogenesis. As a result nephron endowment, and therefore
future functional potential, is negatively impacted.
2.2. **Urinary Tract Obstruction**

Obstructive nephropathy occurs as a result of urinary tract obstruction when the passage of urine through the urinary tract is restricted or blocked. As a consequence, urinary volume and pressure increase proximal to the obstruction causing hydrenephrosis, or swelling, of the kidney (75). Clinically, the blockage of urinary flow is usually not complete, and the degree of urinary restriction determines the severity of hydrenephrosis and urinary pressure. Acute obstruction can result in minimal damage if quickly reversed, however prolonged or chronic obstruction can lead to widespread damage to the kidney, to scarring, and ultimately to the impairment of long-term kidney function (75,76).

Urinary tract obstruction can affect adults but is most common in children, especially young boys. In the U.S., urinary tract obstruction accounts for up to 24% of pediatric transplants, but only 0.3% of transplants in adults (77). This discrepancy reflects the very different etiologies of urinary tract obstruction in the adult and pediatric populations, and highlights the impact of childhood disease on kidney development, maturation, and long-term function. In adults, urinary tract obstruction occurs most frequently in men over the age of 60 as a result of prostate diseases or cancers that impair urine flow (78). However, because adult urinary tract obstruction affects the mature and developed kidney and can often be treated before significant renal damage occurs, its overall impact is usually limited to acute declines in kidney function. In children, urinary tract obstruction results
predominantly from congenital anomalies, and therefore has more widespread implications on kidney development and long-term function.

2.2.1. Congenital Urinary Tract Obstruction

Even in utero the human fetal kidney produces urine, beginning as early as the 8th week of gestation when the first waves of newly formed nephrons mature. The filtrate formed by the fetal kidney is not waste, per se, as the placenta performs the majority of excretory and homeostatic functions. Therefore, congenital obstruction is not associated with the toxic affects of impaired waste excretion present during adult obstruction. Instead, the hypotonic filtrate produced serves to maintain the amniotic fluid volume after gestational week 16 and is crucial for the appropriate development of the lungs in later gestation (72).

Congenital urinary tract obstruction has several etiologies, long-term functional implications, and limited treatment options (reviewed in (75)). Congenital malformations of the urinary tract are the most frequently diagnosed anomalies during routine sonography with urinary tract obstruction occurring at a rate of about 1/2000 pregnancies. In affected fetuses, obstruction is an important cause of morbidity and mortality (79-83). Most congenital obstructions are diagnosed in the second trimester (17-19 weeks) during routine dating sonography, by observation of dilatation of the renal pelvis, distension of the bladder, or thickening of the bladder walls (72). More severe cases with advanced dilatation of the collecting system can often be diagnosed as early 12-14 weeks. Unfortunately, by the time gross changes in urinary tract structure are observable via sonographic
visualization, the obstructive injury has already begun to negatively impact the
development and function of the kidney. Earlier detection of obstruction implies
earlier onset of functional urinary restriction and is therefore associated with
increased renal damage and poorer prognosis.

Congenital obstruction is caused by a host of possible malformations or
abnormalities and can affect one or both kidneys. Unilateral malformations are
more common than bilateral occurrences (84), and often bear a more favorable
prognosis as the opposing or contralateral kidney remains largely unaffected.
Bilateral obstruction is more variable in its effects. In mild cases the impact of
obstruction on the kidneys can be partially mitigated by the compliance of the
ureter, bladder or urethra. However, when a significant restriction of urinary flow is
present, bilateral obstruction can result in widespread dysplasia, abnormal
glomerular and tubular function, and fibrosis in both kidneys. Severe restriction of
urinary output also causes a deficiency of amniotic fluid, called oligohydramnios
(75). Oligohydramnios is associated with the worst prognosis as other
developmental complications, including pulmonary hypoplasia, arise in the absence
of adequate amniotic volume.

Restriction of urinary flow can occur in either the upper or lower urinary
tract (Figure 1.1). When affecting the upper urinary tract, namely the renal pelvis
and ureters, the obstruction is more commonly unilateral (75,76). The primary site
of upper urinary tract obstruction is the ureteropelvic junction where the renal
pelvis transitions into the ureter. Malformation of the ureteropelvic junction occurs
due to ureteric strictures, duplications, or other abnormalities and accounts for 39
to 64% of reported instances of hydrenephrosis. In contrast, obstruction of the
lower urinary tract results from malformations of the bladder outlet or urethra and
therefore restricts the flow in both kidneys. Lower urinary tract obstruction most
commonly results from the formation of posterior urethral valves at the site of the
bladder outlet, or from urethral atresia. These two malformations account for 64%
and 29% of lower urinary tract obstructions respectively (approximately 2.2 per
10,000 births) (79).

In cases of upper urinary tract obstruction, the severity of the outcome depends
upon whether the injury is bilateral, and upon the extent of hydrenephrosis.
Antenatal hydrenephrosis often stabilizes (85) with the postnatal pathology
proportional to the severity of the hydrenephrosis (86). Outcomes for bilateral
obstruction, including those of the lower urinary tract, are more variable and
potentially more severe. Mortality resulting from untreated lower urinary tract
obstruction is approximately 45%, with 25-30% of surviving children developing
ESRD and requiring renal replacement therapy (87). In the presence of
oligohydramnios, mortality jumps to as high as 80% (88).

Treatment of congenital urinary tract obstruction is significantly limited by the
inaccessibility of the fetus. Milder cases can be conservatively monitored through
gestation, and can expect relatively good postnatal prognosis with appropriate
management. In severe cases, treatment is limited to the termination of the pregnancy or fetal surgery. In current practice, fetal interventions are primarily limited to the cytosscopic ablation of posterior urethral valves or the insertion of a vesico-amniotic shunt (89, 90). In the latter process, a catheter is inserted through the fetus’ abdomen into the fetal bladder diverting the flow of urine into the amniotic space. Despite years of application, the efficacy of amniotic shunting still remains unclear. Future work will seek novel approaches to better identify occurrences and severity of congenital urinary tract obstruction, such as informative biomarkers present in the fetal urine or amniotic fluid. The efficacy and safety of vesico-amniotic shunting is being comprehensively examined by the Percutaneous Shunting in Lower Urinary Tract Obstruction study (91) while numerous antifibrotic agents and therapeutics are currently being tested to mitigate parenchymal damage and maximize renal function. However, despite a significant amount of research, we still do not have a complete understanding of the underlying pathophysiology of urinary tract obstruction nor of the mechanics of progression of obstructive injury.

2.2.2. Experimental Models of Urinary Tract Obstruction

Over the past several decades a number of different animal models have been developed to study fetal urinary tract obstruction (92). These models have employed both large and small animal species, and included fetal, postnatal and adult urinary tract obstruction. Though limitations exist, each model has provided significant contributions to our understanding of the mechanisms and interactions involved in the pathogenesis of urinary tract obstruction.
Several fetal models of urinary tract obstruction have been used to study congenital obstructive injury, in particular, *in utero* urinary tract obstruction in the fetal lamb (93-97) and the fetal monkey (98-100). The development of the fetal lamb resembles that of the human fetus, with a long fetal gestation of 145-150 days, and proportionate duration of nephrogenesis beginning at 27 days and proceeding to near-term. Due to the large relative size of the lamb fetus, fetal manipulation is possible and several modes of surgical obstruction have been employed to induce obstruction of either the ureters or urethra (96,97,101). This ovine model reflects some of the key features of human congenital urinary tract obstruction. Studies with complete blockage of the bladder outlet recapitulate the effects of bilateral obstruction at birth with hydronephrosis, oligohydramnios, pulmonary hypoplasia, and neonatal death (95).

Experiments using unilateral ureteric obstruction (UOO) via surgical ligation performed in early gestation (days 45-70 days) resulted in significant renal dysplasia, consistent with that seen in human congenital urinary tract obstruction. However, kidneys obstructed in the last half of gestation (more than 70 days) produced marked hydronephrosis without dysplasia. These results clearly demonstrate that the timing of fetal obstruction is crucial to the outcome (93,96).

The second large animal model of fetal urinary tract obstruction is the rhesus monkey (*Macaca mulatta*) model (98,99). This non-human primate model has, in
addition to its genetics, gestational and nephrogenesis durations most similar to humans. The primate fetal gestation lasts 165 ±5 days with nephrogenesis ending near term as in humans. In this model, partial unilateral obstruction is induced by injection of alginate beads into the renal hilum using an ultrasound-guided needle at various stages throughout the 2nd and early 3rd trimester (70 – 120 days gestation). Upon injection, the beads expand to fill the hilar space restricting the flow of urine through the renal pelvis. Histological analysis reveals the presence of renal dysplasia, which, as in the ovine model, is worse in earlier obstruction, and includes glomerular and tubular malformation, cystogenesis, reduced nephron endowment, mesenchymal expansion, and myofibroblast activation. The histopathology of these obstructed kidneys confirms observations made using the ovine model and more closely replicates that of obstructed human fetal kidneys.

While the large animal models of fetal obstruction accurately parallel the changes observed during congenital disease in humans, they suffer from several limitations. In addition to high costs required for housing and maintenance of the necessary colonies, their long gestational times and slow reproductive cycles make large-scale experimentation difficult. To overcome these limitations a number of small animal models have been developed. These models are much cheaper and have faster birth rates and shorter gestational ages allowing for larger study sizes. While fetal manipulation of these models is technically very difficult, researchers have taken advantage of the unique features of several species. The opossum (102-105) and rodent models have taken advantage of the fact that nephrogenesis continues after
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birth to allow for the approximation of fetal obstruction without the need for in utero manipulation.

The North American opossum gives birth to pups 12-13 days post-conception that are developmentally comparable to an 8 week old human fetus (106). For the remainder of their gestation the pups reside within the mother’s pouch, and are readily accessible for surgical manipulation. When unilaterally obstructed by ureteric cauterization and examined postnatally, this model replicates many of the histopathological features of the other fetal models (102). Notably, dysplasia of the cortical and medullary compartments occurs, with interstitial and periglomerular inflammation, medullary tubular disorganization and peritubular collar formation. The replication of events representative of early gestation obstruction makes this model unique among the postnatal models.

The rodent models of urinary tract obstruction have been best characterized in the adult animal (107-111). Obstruction via UUO results in reliable and reproducible injury in both adult rat and mouse, with chronic UUO resulting in significant renal fibrosis (reviewed in (112-119). Studies of fibrotic progression in this model have provided a wealth of information about the underlying mechanisms of obstructive injury. In order to specifically replicate congenital urinary tract obstruction, some researchers have taken advantage of a window of postnatal kidney development that occurs in the rodent species. Unlike the larger mammals, rodent nephrogenesis and renal maturation is incomplete at birth. In rodent neonates, continuing
nephrogenesis in the early postnatal period (14 days in mouse, 10 days in rat) approximates the third trimester of human kidney development (112,114). Obstruction during this time results in decreased nephron number, glomerular and tubular pathology, cell death and interstitial fibrosis. However, as in the sheep model, obstruction in later development does not reproduce the dysplastic components common to many forms of human congenital obstruction. Despite this limitation, the postnatal and adult rodent models have provided significant insight into the pathophysiology of both obstruction and fibrosis.

2.2.3. Pathogenesis of Urinary Tract Obstruction: Dysplasia Versus Fibrosis

The outcome of urinary tract obstruction depends heavily upon the timing, severity and duration of the obstruction. As seen in the human (120,121), sheep (93,96,101), and primate models (98,99), early obstruction leads to the development of renal dysplasia. In contrast, obstruction during later stages of kidney development or in the adult kidney results predominantly in fibrosis (97,122). However, both dysplastic and fibrotic kidneys share a key endpoint: the reduction of kidney function.

The etiologies of renal dysplasia are diverse and the pathogenesis of the dysplastic kidney is poorly understood. The hallmarks of dysplasia, namely the presence of undifferentiated and metaplastic tissues, suggest that this outcome is caused by dysregulation of the normal processes of kidney development (123-125). However, kidney development is complex, and at a cellular level is regulated by a large number of transcription factors, growth factors, cell adhesion molecules, and
survival factors (Reviewed in (124)). The central mechanism of renal dysplasia is
the disruption of the critical balance between kidney cell proliferation and
apoptosis. This balance is required for appropriate development, as seen in the
budding and growth of the ureteric bud ampulla during branching morphogenesis.
Similarly, survival factors from the ureteric bud and stroma are necessary to
maintain the survival and proliferation of the condensing MM. This delicate balance
of survival is critical, as inhibited cell death results in impaired branching and a
reduction of nephron number (126), while excessive cell death leads to renal
hypoplasia.

In the large animal models, urinary tract obstruction early in fetal gestation results
in the formation of dysplastic kidneys with reduced ureteric bud branching and
nephron number (98,116,123,124,127,128). In these kidneys abundant apoptosis
occurs in the mesenchymal and stromal cells surrounding the cystic tubules. The
epithelia of the ureteric bud and tubules exhibit increased expression of several pro-
proliferation and pro-survival factors including PAX2, BCL-2, and galectin-3 (124).
These epithelial cells have correspondingly high rates of proliferation. Intriguingly,
while the expression of these markers is normally lost during postnatal maturation,
the fetal expression pattern of these markers is maintained after birth, suggesting
that UUO skews normal renal development.

Late gestation and postnatal obstruction decreases nephron number by a different
mechanism of injury than that of earlier gestation obstruction. Obstructive injury in
later kidney development, after several rounds of successful ureteric bud branching and nephrogenesis, reduces nephron number, in part, by inhibiting the development and maturation of nephrons later formed. However, these models also result in significant fibrosis with progressive and sustained injury leading to reductions in the number of functional nephrons, glomerulosclerosis, and ultimately in glomerular dropout.

2.2.4. Mechanisms of Fibrosis

Most of our knowledge of the mechanisms of progression of fibrosis following urinary tract obstruction is derived from the postnatal and adult rodent models. These models include complete and partial obstruction with both unilateral and bilateral obstructions. These studies have identified a number of events in the sequence of obstructive injury (reviewed in (112,114-116)), beginning with hemodynamic changes in renal blood flow, hydronephrosis, interstitial inflammatory cell recruitment, tubular atrophy, and ultimately ending with fibrosis.

**Early Events.** Immediately after obstruction, the accumulation of urine proximal to the site of obstruction results in increased intrarenal volume and pressure (129,130). The accumulation of urine transmits pressure into the renal calyces, collecting system and eventually the tubular segments of the nephron (131). Upon reaching the glomerulus, increased luminal pressure counteracts the hydraulic pressure gradient across the filtration membrane, decreasing GFR. Alterations in renal blood flow occur soon after urinary obstruction (78). Initially, a brief period of afferent arteriolar vasodilatation occurs resulting in increased renal
blood flood and GFR (132). Subsequently, GFR decreases (132) as the
vasoconstrictive effects of factors such as angiotensin II reduce renal blood flow.
These effects are also believed to contribute to tubular injury by exacerbating cellular hypoxia. Examination of renal blood flow and intratubular pressure following bilateral ureteric obstruction in the rat suggests that proximal tubular pressure increases in advance of, and in fact may induce, changes to renal blood flow (133).

**Inflammation.** The infiltration of inflammatory cells into the kidney represents an early response following the onset of urinary tract obstruction. Macrophages and T-cells have been identified as the main classes of immune cells recruited and are increased following obstruction in rats (134,135). During the early stages of obstruction, infiltrating macrophages selectively localize to distinct rings around tubules, particularly around the distal tubule (134). Several studies have clearly demonstrated that impairing the recruitment of macrophages results in attenuation of tubular injury, apoptosis and fibrosis (136,137). Accordingly, several cytokines, chemoattractants and selectins have been implicated in promoting infiltration, and thus fibrosis, following obstruction. Deficiency of either colony stimulating factor-1 or macrophage chemoattractant protein -1 results in reduced macrophage and T-cell infiltration and associated apoptosis (137,138). Blockade of CCR2, the receptor for macrophage chemoattractant protein-1, also ameliorates fibrosis (139). Depletion of T-cell lineages is similarly associated with reduced injury and fibrosis with decreased interstitial expansion and collagen deposition (140). Loss of T-cells
has no effect on macrophage recruitment and reconstitution of CD4+ T_h.cells, but not CD8+ T_c-cells, restores fibrotic injury. This suggests a role for T_h.cells in modulating the activation and activity of macrophages following obstruction.

**Tubular Atrophy and Apoptosis.** Subsequent to the influx of inflammatory cells, significant tubular atrophy occurs. The role of apoptosis in parenchymal loss and tubular injury has been widely reported (141-146). Using the rat UUO model, Truong et. al performed a comprehensive examination of apoptosis and proliferation following obstructive injury (141). Following UUO, tubular apoptosis was first noted at day 1 in the inner medulla, before involving the entire medulla by day 15, and the cortex by day 25. The frequency of apoptosis increased dramatically from day 6 to day 25 post-obstruction before declining rapidly until day 43. Tubular proliferation exhibited a rapid peak of almost 60 times normal levels, beginning at day 1 and returning to baseline by day 10. Tubular proliferation was most common in the ducts and tubules of the outer medulla. Interstitial apoptosis remained low during the peak in tubular apoptosis, but increased steadily as obstruction progressed. Interstitial proliferation exhibited a moderate peak between days 1 and 25, with a second, more substantial peak from day 60 to 90.

A close correlation between tubular dilatation and apoptosis has been described following mouse UUO (144). Following obstruction, tubular dilatation was most pronounced in the distal convoluted tubule and collecting duct at 12 days post-obstruction while proximal tubules exhibited limited dilatation. Furthermore,
dilatation progressively increased in a retrograde fashion with medullary collecting duct dilatation exceeding that of the cortical collecting duct, and cortical distal convoluted tubule exceeding medullary distal convoluted tubule/ascending loop of Henle dilatation. Apoptosis similarly peaked at 12 days, with the majority of events in the collecting duct epithelium, and to a lesser extent, in the distal convoluted tubule. A correlation between distal convoluted tubule and collecting duct cell apoptosis in response to stretch was confirmed by application of in vitro axial strain, with higher incidence of apoptosis in collecting duct cells.

Several key factors have been shown to drive tubular apoptosis (reviewed in (112)). Decreased endogenous antioxidant production following obstruction has been linked to increased apoptosis due to reactive oxygen species. UUO also induces tumor necrosis factor α and FAS ligand expression, while increasing caspase activation. Conversely, reduction of endogenous epidermal growth factor, a pro-survival factor in human and rat tubular epithelium, may make kidney cells more susceptible to pro-apoptotic stimuli. Expression of the anti-apoptotic protein BCL-2 is also decreased in dilated tubules. Ultimately, the rate of tubular atrophy will depend upon the balance of pro- and anti-apoptotic stimuli experienced by the tubular epithelium at a given stage of development.

**Fibrosis.** Fibrosis is a common end-point of CKD and results from the accumulation of excessive extracellular matrix at the expense of normal parenchymal tissue (147-150). Under normal conditions, a balance of matrix synthesis and degradation
serves to maintain the extracellular matrix. However in the presence of persistent injury, the acute wound healing response gives way to fibrosis, as the expression of profibrotic signals perturbs this balance to heavily favor matrix accumulation. These signals control the differentiation, activation and regulation of the principal matrix-producing cell, the myofibroblast. Studies have shown that myofibroblasts have several potential origins, and can arise from the conversion of resident fibroblasts, the bone marrow derived-cells, or by the transformation of endothelial and epithelial cells. Once activated, these cells actively produce and deposit matrix proteins, such as collagens.

The best-described profibrotic agent is TGFβ-1, which is increased in both fetal and animal models of obstruction (110,135,151,152). While the cellular origin of TGFβ-1 following obstruction is unclear, it is reported to be produced by both the renal epithelium and the infiltrating macrophage population (134,151-153). The initial role of TGFβ-1 activity is the recruitment of fibroblasts and the differentiation of myofibroblasts. Subsequently, TGFβ-1 maintains matrix deposition by promoting the expression of both matrix-encoding genes and inhibitors of matrix degrading enzymes. Matrix deposition is further influenced by TGFβ-1-induced increases in protease inhibitors expression, including plasminogen activator inhibitor-1, concurrent with TGFβ-mediated degradation of matrix degrading enzymes such as the metalloproteinases. Disruption of TGFβ-1 activity by either disruption of TGFβ signaling, or by treatment with a TGFβ-1 neutralizing antibody significantly ameliorates tubular apoptosis and associated interstitial fibrosis (151,154).
A number of other profibrotic factors influence the progression of matrix accumulation, and often act in concert with TGFβ-1. Connective tissue growth factor is an important mediator of TGFβ activity, which potentiates fibrosis by contributing to fibroblast proliferation and matrix synthesis (148). Angiotensin II has been shown to be an important stimulator of TGFβ-1 production, and may account for up to 50% of observed fibrosis following obstruction (155). The profibrotic activity of angiotensin II appears to be closely linked with the regulation of other profibrotic cytokines and transcription factors, including TGFβ-1, tumor necrosis factor α, and the NFκB pathway (156). Tumor necrosis factor α is a potent pro-inflammatory mediator that may participate in fibrotic progression mainly by facilitating the recruitment of inflammatory cells via NFκB’s stimulation of pro-inflammatory cytokines. NFκB activity has been shown to increase 5-7 days after obstruction and has been correlated with increased interstitial proliferation and fibrosis (157).

### 2.2.5. A Paradigm of Injury

Inflammation, tubular atrophy and fibrosis are key events in the progression of obstructive injury and have been well characterized in the literature. How the physical manifestations of injury induce this cascade of events is not understood. However, the paradigm of injury from the work of Klahr, Morrissey and Chevalier highlights a central role of the renin-angiotensin system (RAS) in bridging this gap (78,115,158-161). Within the kidney, all of the components of the RAS exist to facilitate angiotensin production. Following obstruction, the hydrodynamic stresses
created by increasing urinary volume and pressure, and the mechanical strain on
the proximal tubular epithelium increases the production of RAS components (162).
Increased RAS activity results in angiotensin II production, thereby drives
production of autocrine and paracrine acting chemokines and influences subsequent
pathological processes through promotion of TGFβ expression, inflammatory influx,
and profibrotic factors (163). In UUO, activation of the RAS has also been described
through angiotensin II-induced activation of NFκB, promoting proinflammatory
gene expression (164). Inhibition of NFκB, reduces fibrosis and apoptosis (157).
Several studies support a significant role for RAS in obstruction. Knockout or
alterations in copy number of the angiotensinogen gene has shown that up to 60%
of fibrosis is dependent on angiotensinogen expression (155). Disruption of RAS by
inhibitors of renin, angiotensin-converting enzyme, or angiotensin receptors results
in similar attenuation of profibrotic events including apoptosis, macrophage
infiltration, TGFβ expression, interstitial expansion, and collagen deposition (165-
167). While this paradigm clearly demonstrates a link between the RAS system and
the progression of fibrosis, it centers on cortical events following obstruction and
primarily describes the role of the proximal convoluted tubule. A greater
understanding the pathogenesis of urinary tract obstruction will require a broader
investigation of other events throughout the kidney during the progression of
obstructive injury.
**Underappreciated factors.** Despite a significant amount of research utilizing several experimental models of both fetal and postnatal obstruction, a comprehensive understanding of the events that connect urinary tract obstruction to renal pathology is lacking. Owing to the popularity of the experimental obstructive models in studying fibrosis, we now have a detailed understanding of the later stages of renal injury that drive the progression of fibrosis. However, there remain several unappreciated factors that have not yet been extensively investigated. In particular, an examination of the early events during obstruction is required, defining the contribution of other compartments and nephron segments in the progression of this early injury. In addition, an understanding of the physical manifestations of obstruction and how they may injure the kidney is needed.
2.3. **Research Objective and Hypotheses**

In light of the underappreciated factors described previously, and based upon the significant histopathology observed in the medullary collecting duct following obstruction in the primate model, the overarching aims of this thesis are to characterize the early cellular events following urinary tract obstruction and to explore putative mechanisms of injury.

My work set out to test the following hypotheses:

(1) collecting duct injury with its concomitant epithelial cell response is an early and significant event in the progression of obstructive injury, and

(2) mechanosensation modulates both the collecting duct epithelial and tubulointerstitial response to injury following urinary tract obstruction.
CHAPTER 3 OBSTRUCTION AND THE HUMAN FETAL KIDNEY

3.1. Introduction

As discussed in Chapter 1, the development of the kidney occurs via the reciprocal interactions of the MM and ureteric bud. The MM will eventually form the glomerular and tubular compartments of the functional nephron while the ureteric bud exclusively determines the collecting duct system through a process called branching morphogenesis (17,18,22,168). Following branching, the ureteric bud matures into the differentiated epithelia of the collecting duct, occurring first in the initial medullary ureteric bud branches and proceeding outwardly as branching and nephrogenesis occurs in the cortex.

In the early gestation fetus, urinary tract obstruction causes a disruption of these developmental processes producing dysplastic kidneys. Alterations of the medullary architecture is a major feature of obstruction, and is associated with a reduced number of collecting ducts (93,96,98,99,101,120,121). However, in the human fetal kidney little is known about the developmental process of maturation and differentiation of the ureteric bud into the collecting duct epithelium, or how obstruction alters this process. Therefore, we sought to first describe and detail normal collecting duct ontogeny and physiology in the developing human kidney

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2 This chapter is based on published work. Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
and characterize the effects of congenital urinary tract obstruction on normal
collecting duct development and maturation.

3.1.1. Development and Physiology of the Collecting Duct

In addition to the transport of filtrate from the many nephron segments, the
collecting duct also serves a crucial role in the fine-tuning of the urinary filtrate
through selective reabsorption and secretion. To perform these tasks, the collecting
duct epithelium consists of two unique cell types. Principal cells are responsible for
vasopressin-regulated water reabsorption via AQP2 and balancing of sodium and
potassium levels in the blood and filtrate, while intercalated cells regulate acid-base
homeostasis (169). Intercalated cells can further be subdivided into type A, type B,
and non-A non-B cells that are identifiable by the expression of transporter proteins
(Figure 3.1). Vacuolar H+-ATPase (vATPase) is expressed in all intercalated cells,
and localizes to apical membrane in type A and non-A non-B intercalated cells, and
the basolateral membrane in type B intercalated cells. The bicarbonate transporter
pendrin is expressed in type B and non-A non-B cells while anion exchanger 1 is
only detectable in the basolateral membrane of type A intercalated cells. Lastly, the
ammonium transporter Rhesus blood group C glycoprotein (RhCG) is present in
type A and non-A non-B intercalated cells (170-174). In addition to pH regulation,
intercalated cells exhibit phenotypic plasticity in vitro allowing them to differentiate
into other intercalated cell subtypes and into principal cells (175,176). This putative
pluripotency raises the possibility that these subtypes may be responsible for
collecting duct epithelial renewal and/or the adaptive response to injury.
**Figure 3.1: Classification of Intercalated Cells.** Intercalated cells and their functionally specific subtypes are identifiable by their expression of transport proteins including the hydrogen ion transporter vATPase, ammonium transporter RhCG, and the chloride/bicarbonate exchangers pendrin and anion exchanger 1 (AE1). Reprinted from Hiatt et al., *Am J Pathol.* 2010 Feb; 176(2), 630-7.
While the adult distribution of intercalated cells is known across many species including human (171,177-180), most of our knowledge of fetal kidney intercalated cell development derives from studies in rodents (181-183). Unlike human nephrogenesis, which is completed in utero, renal development in the rodent continues into the early postnatal period. In the mouse and rat, immature intercalated cells first appear in the medullary collecting duct and connecting tubule early in kidney development (embryonic day 10.5 - 11.0) with type A cells being the first differentiated subtype to appear, and type B and non-A non-B cells emerging near the end of fetal gestation (embryonic day 21). Of note, intercalated cells are not observed in the rodent cortical collecting duct at any fetal age, and it has been suggested that the intercalated cells of the ureteric bud-derived medullary collecting duct and metanephric mesenchyme-derived connecting tubule may arise independently (183). In the final stages of rodent nephrogenesis, which occurs postnatally, intercalated cells are observed in the cortical collecting duct and subsequently are lost from the papillary inner medullary collecting duct.

Little is known about the normal development and ontogeny of intercalated cells in the human fetal kidney. The purpose of the work described in this chapter is to define the normal ontogeny of human intercalated cell development, and to characterize the changes in intercalated cell abundance and distribution following fetal urinary tract obstruction.
3.2. Results

3.2.1. Characterization of Intercalated Cells

In the adult kidney, intercalated cell subtypes can be readily identified by their expression of various transporter proteins including vATPase, RhCG, anion exchanger 1 and pendrin. However, the human fetal kidneys studied expressed these proteins at differing stages of development. vATPase was present in all intercalated cells and was observed as early as 8 weeks gestation (not shown). Diffuse RhCG expression was first observed in isolated collecting duct cells of the 18 week gestation kidney, but displayed high background immunoreactivity in kidneys of 18 weeks gestation or less. Similarly, neither pendrin nor anion exchanger 1 expression was observed prior to 26 weeks gestation. The disparate expression of these functional intercalated cell proteins at early gestational ages impedes the ability to identify and classify intercalated cells into their classical subtypes (namely type A, type B and non-A non-B) and suggests that many of the intercalated cells observed in the early gestation kidney have an undifferentiated or immature phenotype. For the purpose of this study, intercalated cells are classified into specific subtypes based strictly upon the expression of the obligate transporters described above (Figure 3.1). Anion exchanger 1 was found to localize with RhCG expression in type A intercalated cells and not in non-A non-B cells, and was therefore not included in subsequent characterization of these cells.
Figure 3.2: Intercalated Cells in the Early Human Fetal Kidney. Intercalated cells (arrowheads in B) in the 10 week gestation fetal kidney are restricted to the medullary ureteric bud (outline in A, magnified in B) and are not seen in the cortical ureteric ducts or in the ureteric tips of the nephrogenic zone (arrows). In the 18 week gestation fetal kidney, intercalated cells are present in the medullary collecting duct (C) and are seen rarely in the cortical collecting duct (D). Stains: red – vATPase, green – RhCG, blue – DAPI. Scale Bars: A – 200µm, B through D – 25µm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
3.2.2. The Early Gestation Fetal Kidney

Based on their expression of vATPase, intercalated cells were first observed in the early medullary branches of the ureteric duct of human fetal kidneys studied at 8 weeks gestation (not shown), and constituted 1.6% of collecting duct cells in this segment by 10 weeks gestation (Figures 3.2 and 3.3). In 18 week gestation kidneys, intercalated cells were present in the inner medullary collecting duct and outer medullary collecting duct at approximately equal abundance (3.5% and 3.9% of collecting duct cells respectively). Based upon the lack of pendrin expression, the majority of intercalated cells are classified as immature intercalated cells with a small fraction of type A intercalated cells present (15.4% and 3.7% for the inner and outer medullae respectively) (Table 3.1). In the medullary rays of the inner cortex, only immature intercalated cells (1.9% of collecting duct cells) were observed while no intercalated cells were observed in the outer cortex or nephrogenic zone.

3.2.3. The Mid and Late Gestation Fetal Kidney

As in the early gestation kidney, the intercalated cells of the mid gestation 26 week human kidney were distributed from the inner medulla to inner cortex (Figures 3.3, and 3.4). However, in the inner medullary collecting duct, the relative number of intercalated cells increased compared to earlier gestation kidneys (8.8% of collecting duct cells) with type A cells predominating (96.2% of intercalated cells) (Table 3.1). In the outer medullary collecting duct, intercalated cells constituted 3.3% of the collecting duct epithelium with type A intercalated cells representing
Figure 3.3: Intercalated Cell Abundance in the Normal Fetal and Postnatal Kidney. For each segment of the collecting duct and for each gestational age, the abundance of intercalated cells is expressed relative to the total number of collecting duct cells. *The intercalated cell abundance of the inner medullary collecting duct of the 1 year postnatal kidney was evaluated qualitatively. Error bars = standard error of the mean. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
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<th>% of CD cells</th>
<th>% of ICs (% of CD Cells ± Standard Deviation)</th>
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<td>IMCD</td>
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<td>96.2 (8.5 ± 1.5)</td>
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<td>54.4 (1.8 ± 1.6)</td>
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<td>CCD</td>
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<td>IMCD</td>
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<td>97.1 (7.5 ± 1.1)</td>
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<tr>
<td>OMCD</td>
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<tr>
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<td>41.0 (1.4 ± 0.8)</td>
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Table 3.1: Abundance of Intercalated Cells Subtypes. Intercalated cell fractions expressed as percentage of collecting duct cells ± standard deviation. Bolded numbers express intercalated cell subtypes as a percentage of total intercalated cells. Immature cells are calculated as percent of total cells minus Type A, B and non-A non-B subtypes and therefore have no standard deviations. *Pendrin-positive cells not observed in 36 week gestation kidney. CD – collecting duct, IC – intercalated cell, CCD – cortical CD, IMCD – inner medullary CD, OMCD – outer medullary CD. Adapted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
54.4% of intercalated cells. In the cortical collecting duct, intercalated cells are less abundant (1.4% of collecting duct cells) with type A intercalated cells continuing to predominate (43.9% of intercalated cells), and non-A non-B, type B, and immature intercalated cells comprising 34.2, 10.6 and 11.3% of intercalated cells respectively. Intercalated cells were absent from the nephrogenic zone of the cortex.

In the 36 week gestation human fetal kidney, the abundance of intercalated cells and their subtypes in the inner and outer medullae was found to be similar to that of the mid gestation kidney (Figure 3.3 and 3.4). While the proportion of Type A intercalated cells the inner medullary collecting duct remained similar to the mid gestation kidney (97.1% of intercalated cells), the abundance of type A intercalated cells in the outer medullary collecting duct increased to 78.4% of intercalated cells. In the cortical collecting duct, which is more developed than previous gestations but has not yet fully differentiated, the fraction of type A intercalated cells remained unchanged however the total number of intercalated cells doubled to 3.3% of collecting duct cells. Unfortunately, type B or non-A non-B intercalated cells could not be observed in the normal 36 week collecting duct. This was due to a lack of pendrin staining, and likely results from issues with tissue processing of the limited samples available, rather than an absence of the intercalated cells or a lack of sensitivity of the pendrin antibody.
Figure 3.4: Intercalated Cells in the Mid to Late Gestation Human Fetal Kidney. In the 26 week kidney (A, B), type A intercalated cells (arrowheads) predominate. Type B intercalated cells (arrows) are first observed in the cortical collecting duct at this age (B). In the 36 week gestation fetal kidney (C, D), a mix of type A (arrowheads), type B and immature intercalated cells is observed in both the outer medullary (C) and cortical (D) collecting duct. Stains: red – vATPase, green – RhCG (A, C, and D) or pendrin (B), blue – DAPI. Scale Bars: A through D – 25µm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
3.2.4. The Postnatal Kidney

In the postnatal human kidney, the distribution of intercalated cells changed dramatically compared to the fetal kidney, and has differentiated to resemble that of the adult collecting duct. As in the adult kidneys of most species, intercalated cells were rare in the inner portions of the inner medulla (Figure 3.3 and 3.5). Throughout the outer medulla, and compared to the 36 week gestation kidneys, the intercalated cell population nearly tripled to 11.0% of collecting duct cells with type A intercalated cells comprising 70% of intercalated cells (Table 3.1). Surprisingly, type B or non-A non-B intercalated cells are not observed. As expected, the most significant change in intercalated cell abundance in the postnatal kidney occurred in the cortical collecting duct. In this segment, the total number of intercalated cells increased almost 10-fold to 32.8% of collecting duct cells. It is at this time point that the pendrin-positive type B and non-A non-Bs intercalated cells become the most abundant differentiated intercalated cells (29.4 and 8.9% of intercalated cells respectively) while the proportion of differentiated type A intercalated cells decreased (6.9% of intercalated cells). Surprisingly, a large proportion of intercalated cells retained an immature phenotype (54.8% of intercalated cells), indicating that the postnatal remodeling of the collecting duct epithelium was still in progress one year after birth.

3.2.5. The Connecting Tubule

The connecting tubule is a unique segment of the nephron intermediary to the metanephric mesenchyme-derived distal convoluted tubule and the ureteric bud-
Figure 3.5: Intercalated Cells in the Postnatal Human Kidney. At 1 year postnatally, intercalated cells were lost in the inner medullary collecting duct (A) but persisted in the outer medullary collecting duct with type A predominating (arrowheads in B). In the postnatal cortex (C, D), the abundance of intercalated cells increased dramatically with type A (arrowheads), type B (arrows), and non-A non-B intercalated cells (asterisk) present. Stains: red – vATPase (A-C) or pendrin (D), green – RhCG, blue - DAPI. Scale Bars: A – 200μm, B through D – 25μm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
Figure 3.6: The Connecting Tubule. At 10 weeks gestation, rare nephron segments resembling the distal convoluted tubule are observed that expresses uniform apical vATPase expression and contain isolated cells with stronger vATPase expression characteristic of intercalated cells (arrowheads in A). These ducts may represent the earliest formations of the distal nephron, specifically, of the connecting tubule. By 18 weeks, intercalated cells (arrowheads in B) became more distinct and by 29 weeks (C) the connecting tubule (asterisk) can be clearly identified as a transition segment between the distal convoluted tubule (arrows) and the collecting duct (outline). The connecting tubule appears to represent a progressive overlapping of the mesenchymally-derived distal tubule and ureteric bud-derived collecting duct with intercalated cells derived from the latter, as depicted in (E). Stains: red – vATPase, green – RhCG, blue – DAPI. Scale Bars: A and B – 25μm, C and D – 50μm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
derived cortical collecting duct. In the 10 week gestation human kidney, the tubules of the distal nephron express uniform apical vATPase with rare juxtamedullary segments displaying interspersed cells containing more abundant and cytoplasmic vATPase expression (Figure 3.6). These strongly vATPase positive cells resemble the immature intercalated cells of the collecting duct, described in the early gestation kidney, and suggest that these tubular segments represent the nascent connecting tubule. In kidneys of 18 weeks gestation and older, the connecting tubule could be distinguished from distal convoluted tubule (expressing apical vATPase and strong membranous RhCG) by the appearance of defined and abundant intercalated cells. As the connecting tubule nears its point of intersection with the cortical collecting duct, intercalated cells persist, while the expression of vATPase and RhCG in non-intercalated cells fades, similar to that observed in the collecting duct.

3.2.6. The Obstructed Human Fetal Kidneys

Observed collecting ducts from obstructed 18 week gestation kidneys exhibited an increase in intercalated cell fraction per duct to 6.0% of collecting duct cells from 3.9% in age-matched control (Table 3.2). In early kidneys and in medullary collecting ducts with mild to moderate dilatation, these ducts also exhibited an increased proportion of differentiated intercalated cells (Figure 3.7) expressing RhCG and pendrin immunoreactivity, in contrast to age-matched controls. In addition to an increase in type A cells (33.1% of intercalated cells versus 8.4% in...
Figure 3.7: Intercalated Cells in the Early Obstructed Human Fetal Kidney. At 18 weeks gestation, an increase in intercalated cell fraction can be observed (A, B). Furthermore, unlike the normal 18 week gestation kidneys where immature intercalated cells predominate, differentiated type A (arrowheads), and type B (arrows) intercalated cells can be observed in medullary collecting ducts of the obstructed kidney. Intercalated cells are also observed in the cortical collecting duct (asterisk) near the nephrogenic zone highlighting premature differentiation of intercalated cells in the region (C). Stains: red – vATPase (A C) or pendrin (B), green – RhCG, blue - DAPI. Scale bar: A – 100μm, B – 25μm, C – 50 μm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
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<tr>
<td>Control</td>
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<td>Obstructed</td>
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<td><strong>36 weeks</strong></td>
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<td>Control</td>
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<td>92.9 (5.3 ± 2.8)</td>
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<td>Obstructed</td>
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<td>68.8 (7.4 ± 6.0)</td>
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<td><strong>1 year postnatal</strong></td>
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<tr>
<td>Control</td>
<td>10.7 ± 6.2</td>
<td>70.2 (7.5 ± 4.8)</td>
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<td>Obstructed</td>
<td>3.8 ± 4.8</td>
<td>70.8 (2.7 ± 4.2)</td>
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Table 3.2: Abundance of Intercalated Cells Subtypes During Obstruction. Intercalated cell fractions expressed as percentage of collecting duct cells ± standard deviation. Bolded numbers express intercalated cell subtypes as a percentage of total intercalated cells. Immature cells are calculated as percent of total cells minus Type A, B and non-A non-B subtypes and therefore have no standard deviations. *Pendrin-positive cells not observed in 36 week gestation kidney. CD – collecting duct, IC – intercalated cell. Adapted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
age-matched control), these results demonstrate the novel presence of medullary type B (23% of intercalated cells) and non-A non-B intercalated cells (7.0%) that were not observed in the normal fetal medullary collecting ducts at any fetal age. These results suggest an accelerated differentiation of the medullary collecting duct in response to urinary tract obstruction.

Overall, obstructed kidneys studied at 36 weeks gestation also showed an increased fraction of total intercalated cells when compared to controls, similar to that seen in the early gestation kidneys (10.8% of collecting duct cells versus 5.7% in age-matched control) (Table 3.2, Figure 3.8). While the majority of medullary intercalated cells remained type A intercalated cells (68.8% of intercalated cells), this increase in total intercalated cells was also accompanied by an unexpected presence of differentiated type B (5.1% of intercalated cells) and non-A non-B cells (7.1% of intercalated cells) in the medullary collecting duct. However, late gestation kidneys displayed heterogeneous histopathology ranging from mild to severe injury; severe injury included increased tubular dilatation and interstitial expansion, with altered cellular morphology and peritubular collar formation, as previously described (2). In this study, intercalated cell fraction varied inversely with the degree of duct dilatation, a quantifiable surrogate measure of injury (Figure 3.8).

In the congenitally obstructed kidneys studied postnatally, intercalated cells were less abundant in the outer medullary collecting duct than in age-matched controls.
Figure 3.8: Intercalated Cells in the Late Gestation Obstructed Fetal Kidney. As with the early gestation obstructed kidney, mildly affected collecting ducts of the 36 week gestation obstructed fetal kidney (arrows in A, magnified in B) exhibit an increase in intercalated cell fraction. However, more severely dilated collecting ducts displayed decreased intercalated cell abundance (asterisks in A, magnified in C). In the late gestation fetal kidney, injury and progressive dilation of the obstructed collecting duct (measured as relative luminal surface area in pixels), correlated with a progressive decline in the relative number of intercalated cells in the collecting duct (D) (R2=0.255). Stains: red – vATPase, green – RhCG, blue – DAPI. Scale Bars: A – 200μm, B through C – 100μm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176(2), 630-7.
(3.8% of collecting duct cells versus 10.7% in age-matched control) (Table 3.2). As in obstructed kidneys studied during fetal gestation, some mildly affected collecting ducts exhibited increased intercalated cell abundance while dilated collecting ducts exhibited a depletion of intercalated cells (Figure 3.9). As with the normal postnatal kidney, differentiated type A intercalated cells were predominant in the medullary collecting duct (70.8% of intercalated cells versus 70.2% in age-matched control), while rare type B intercalated cells (1.0% of intercalated cells) were observed in the medullary collecting duct despite being absent from the normal postnatal kidney. Furthermore, the expression of AQP2 was also disrupted in more severely injured, dilated collecting ducts indicating that in addition to intercalated cell depletion, these ducts also demonstrate a loss of principal cell function.

3.3. Discussion

While fetal urinary tract obstruction has been studied in a number of experimental models (93,98,99,102,107,184-187), little is known about its effects on the development and differentiation of the collecting duct and its differentiated cell types in the human fetus. In fact, little is known about the ontogeny of these functional cell types, particularly intercalated cells, in the human fetal kidney. The current study defines the origins, distribution and maturation of intercalated cells in the collecting ducts of the normal human fetal kidney and identifies how congenital obstruction alters this development.
Figure 3.9: Intercalated Cells in the Postnatal Obstructed Fetal Kidney. As with the 36 week kidney, some mildly affected collecting ducts in the obstructed postnatal kidney displayed abundant intercalated cells (arrows in A, magnified in B) while more severely affected, dilated collecting ducts exhibited a depletion of intercalated cells (asterisks in A, magnified in C). Correspondingly, dilated collecting ducts exhibiting intercalated cell depletion (arrowheads) also demonstrated a significant decrease in AQP2 expression compared with mildly affected collecting ducts (arrow). Stains: red – vATPase, green – RhCG (for Figures A-C), green- AQP2 (for Figure D), blue – DAPI. Scale Bars: A – 200μm, B through D – 100μm. Reprinted from Hiatt et al., Am J Pathol. 2010 Feb; 176 (2), 630-7.
During normal human nephrogenesis, spatial and temporal changes can be observed both in the distribution of intercalated cells, and in their differentiation into mature subtypes. Intercalated cells first appear in the inner medulla at 8 weeks gestation and progressively differentiate in the outer medulla and inner cortex in later gestation. However, unlike the postnatal and adult kidney, intercalated cells in the fetal kidney remain most abundant in the inner medulla, with low abundance in the outer medulla and most differentiated regions of the inner cortex. Intercalated cells are not observed in the nephrogenic zone, or in the differentiating region of the outer cortex. Initially, intercalated cells are identified solely by their vATPase expression but lack the expression of other important functional transporters. These intercalated cells likely represent an immature or undifferentiated intercalated cell. Type A intercalated cells are the first differentiated subtype observed, as early as 14 weeks gestation, and remain the predominant intercalated cell of the inner medulla throughout fetal development. Type B and non-A non-B intercalated cells are not observed until 26 or 36 weeks, respectively, and are restricted to the cortex. Postnatally, the distribution of intercalated cells changes dramatically. Similar to other species, the intercalated cells of inner medulla are lost while the abundance of intercalated cells in the cortex increases dramatically (171,177-180). Surprisingly, a large proportion of these newly acquired cortical intercalated cells appear to exhibit an undifferentiated or immature phenotype, suggesting that even at one year after birth the renal collecting duct system continues to undergo significant remodeling.
Our description of human fetal kidney intercalated cell development parallels that described previously in the mouse and rat, with a few notable exceptions. First, type B and non-A non-B intercalated cells were not observed in the normal human medullary collecting duct at any gestational age, or in the early postnatal period. Second, while intercalated cells do not appear in the rodent cortical collecting duct until postnatal development, rare intercalated cells can be observed in the human cortical collecting duct at 18 weeks gestation, highlighting the ability of the less differentiated cortical collecting duct to develop intercalated cells. Third, in the human fetal kidney the appearance of a distinct connecting tubule segment in the juxtamedullary region of the inner cortex with intercalated cells occurs at approximately the same time as, and adjacent to, the appearance of intercalated cells in the cortical collecting duct. The lack of a clear delineation between each segment of the distal nephron supports previous descriptions of a gradual transition and mixing of tubular segments in the distal nephron of rat, mouse and human (Figure 3.6E) (188-191). The connecting tubule therefore represents a hybrid segment composed of elements from the adjacent metanephric mesenchyme-derived distal tubule and ureteric bud-derived collecting duct, with the latter nephron segment the mostly likely source of intercalated cells.

Extensive phenotypic changes occur in the collecting duct epithelium of the obstructed fetal kidney. In addition to previously described changes, the fraction of intercalated cells increased in early gestation collecting ducts with mild obstruction. However, as previous studies in the non-human primate have demonstrated a
CHAPTER 3

decrease in collecting duct mass following obstruction (98) this increased
intercalated cell density is unlikely due to an overall increase in intercalated cell
number throughout the whole kidney. Furthermore, following mild obstruction
intercalated cells extend further into the developing cortex, and collecting ducts
exhibit more differentiated subtypes including medullary type B and non-A non-B
intercalated cells not observed in age-matched normal kidneys. The increased
fraction of intercalated cells and intercalated cell subtypes may signify a
compensatory mechanism to maintain acid-base homeostasis in spite of a decrease
in collecting duct mass and therefore overall intercalated cell number. Alternately,
the increased intercalated cell fraction may be the result of a selective loss of
adjacent principal cells. In the late gestation and more severely affected kidneys,
overall intercalated cells are more abundant, but are depleted in the most severely
dilated collecting ducts. Together, these results suggest that the collecting duct
epithelium undergoes premature remodeling in response to congenital obstruction.

The impact of this early collecting duct remodeling on further fetal kidney
development, and in particular on ureteric bud branching, is unknown. However,
premature epithelial maturation and remodeling may occur at the expense of
branching morphogenesis and subsequent nephrogenesis. With ductal dilatation
and when epithelial integrity is threatened, differentiated functions of the tubule,
including intercalated cell function, may be lost in favor of the formation of
peritubular muscular collars and phenotypic changes of the epithelium as described
previously in non-human primates (2).
3.4. **Conclusions**

We have found that the development and maturation of the collecting duct in the fetal human resembles that of other previously described species. Notably, the maturation of the collecting duct occurs first in the initial branches of the ureteric bud and progresses outwards. During the fetal period, intercalated cells are most abundant within the inner medulla and rare in the outer portions of the renal cortex. Postnatally, this distribution of intercalated cells changes dramatically with intercalated cells becoming highly abundant in the cortex, yet largely absent from the inner medulla. This transition represents the natural remodeling process of the collecting duct.

Following fetal urinary tract obstruction, collecting duct maturation is altered from that of the normal kidney. Surprisingly, intercalated cell distribution and maturation is more advanced in the early obstructed kidney. However, with continued obstruction these intercalated cells are eventually depleted from the collecting duct epithelium. The associated reduction of AQP2 in the principal cells constituting the remaining collecting duct population highlights a general pattern of epithelial dedifferentiation, which impairs collecting duct function and may, as examined in the next chapter, represent an adaptive response of the epithelium to mitigate cellular injury.
CHAPTER 4 OBSTRUCTION AND COLLECTING DUCT

REMODELING

4.1. Introduction

Developmental anomalies and associated fetal obstructive nephropathies are the most important causes of childhood kidney failure. Congenital urinary tract obstruction results in an alteration in normal patterns of gene and protein expression responsible for nephrogenesis and nephron endowment, an alteration in the expression of vasoactive and renotrophic factors responsible for the establishment and maintenance of normal glomerular filtration, and the induction of dysplasia, including concomitant mesenchymal reaction and renal fibrosis (112,114-117). Postnatal renal outcome is directly linked both to nephron endowment and to the severity of mesenchymal fibrosis. While the role of obstruction and fibrosis in the postnatal kidney has been well studied, the role of obstruction and fibrosis in these congenital disorders has not been explored.

Prior publications from our group have reported on the fetal non-human primate model of obstructive renal dysplasia, demonstrating that ultrasound-guided UUO during the early second trimester results in renal pathology characteristic of human fetal obstructive renal disease (92,98,99). These studies have highlighted the

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3 This chapter is based on work published under my previous surname. Butt et al., Kidney Int, 2007; 71(2), 936-944.
disruption of normal nephrogenesis, the defects in branching morphogenesis and
the decrease in glomerular endowment.

In the previous chapter, we defined the normal ontogeny of collecting duct
maturation in the human fetal kidney, and demonstrated the alteration of this
process following urinary tract obstruction. To further investigate the effect of
obstructive injury on the collecting duct, and to examine the role of the collecting
duct epithelial response to injury, we employed the fetal primate model of
obstruction described above. The intent of the present work was to test the
hypothesis that the collecting duct epithelium’s response to congenital urinary tract
obstruction includes mesenchymal transformation, which then contributes to the
development of progressive fibrosis.

4.2. Results

4.2.1. Induction of Fetal Obstructive Nephropathy

We examined both obstructed and contralateral kidneys of non-human primates at
approximately 150 days gestation and of similarly aged control kidneys. As in
previous reports (99), unilateral obstruction with alginate beads at 70 days
gestation successfully induced renal dysplasia, with distortion of the renal
architecture, including altered glomerular development with a decrease in
glomerular number and the development of glomerular cysts. In particular, we
observed dilatation and cystic transformation of medullary collecting ducts, the
formation of peritubular smooth muscle collars, and the expansion of the medullary
interstitium (Figure 4.1). We observed corresponding increases in medullary vimentin (VIM) and α smooth muscle actin (αSMA) immunoreactivity in both the renal interstitium and in the peritubular cellular collars with E-Cadherin (E-Cad) expression decreased in the medullary collecting duct epithelium.

4.2.2. Quantitative Changes in the Obstructed Fetal Kidney

To quantify the histopathological changes, we extracted RNA from dissected medullary and cortical tissue from obstructed, contralateral, and control fetal kidneys and performed quantitative PCR analysis. As expected, mRNA changes corresponded to the changes in immunohistochemistry with a significant increase in Vim, decrease in E-Cad and no change in TGFβ. Of note, these differences were more pronounced in medullary than in cortical samples (Figure 4.2).

4.2.3. Characterization of the Normal Collecting Duct

In the control kidney, medullary collecting ducts were identified by the expression of principal cell-specific water channel AQP2 in a majority of cells (Figure 4.3) (16,192). This late gestation collecting duct epithelium expresses the expected basolateral adherens junction protein E-Cad and the apical structural protein cytokeratin, characteristic of a differentiated epithelial cell layer. Notably, and as expected, the epithelia of normal unobstructed collecting ducts did not show any evidence of VIM immunoreactivity. However, and at variance with previous reports in adult human kidney biopsy specimens (193) and postnatal mouse kidneys, we
Figure 4.1: Immunohistochemical Features of Fetal Urinary Tract Obstruction.
(A) αSMA in the expanded interstitium, periglomerular region and peritubular collars following obstruction. Strong αSMA expression in the medial layer of arterioles with weaker expression in the renal capsule. (B) Disruption of E-Cad in the obstructed kidney and loss of interepithelial localization (arrowheads) and diffuse cytoplasmic expression (arrow), (C) VIM in the expanded interstitium. (g = glomerulus, d = collecting duct, * = interstitium) Scale bars = (A) 100 µm and (B, C) 50µm. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
Figure 4.2: mRNA Expression in Obstructed Fetal Kidneys. Quantitative real-time RT-PCR demonstrates the down-regulation of E-Cad and up-regulation of VIM gene expression in obstructed versus control and contralateral kidneys. Of note, these differences were more pronounced in the medulla than in the cortex. *p < 0.05, obstructed versus control. Error bars = standard error of the mean. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
identified cells in the collecting ducts of control kidneys that exhibited diffuse αSMA immunoreactivity that, to our knowledge, have not been observed in normal, postnatal kidneys. This was unexpected and surprising since αSMA expression implies a mesenchymal or less differentiated phenotype. This αSMA expression colocalized with the expression of the intercalated cell marker, carbonic anhydrase II (CA II) (194,195), and was mutually exclusive with AQP2. Furthermore, and unlike the columnar epithelium that constitutes the majority of the collecting duct, these cells display a pyramidal morphology with a widened basolateral and narrowed apical surface.

4.2.4. Collecting Duct Features in Fetal Urinary Tract Obstruction

Fetal kidneys obstructed at 70 days gestation and studied near term contained both normal and segmentally dysplastic medullary elements. In particular, collecting ducts in affected segments of the inner medulla exhibited significant dilatation. In addition, dramatic expansion of the adjacent interstitial compartment was observed in regions of the more severely dilated ducts. Obstructed collecting ducts also exhibit an increase in peritubular αSMA-positive cells ranging from localized expression adjacent to one or two collecting duct epithelial cells through to the formation of dense peritubular collars fully surrounding the duct (Figure 4.4). Typically, though not exclusively, more severely dilated ducts exhibited more fully developed peritubular collars. Since this was the most predominant and consistent feature of fetal urinary tract obstruction, we used the degree of peritubular collars formation to characterize the severity of collecting duct injury.
Figure 4.3: Normal Fetal Medullary Collecting Duct. (A) Normal medullary collecting duct epithelium displays AQP2, E-Cad, and cytokeratin immunoreactivity and absent VIM immunoreactivity (arrows demarcate epithelial layer). (B) Co-localization of αSMA and CA II immunoreactivity in AQP2 negative epithelia (from part A) of the normal collecting duct (arrowheads highlight examples of αSMA+/CA II+/AQP2-subpopulation). Scale bars = 25 μm. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
**Figure 4.4: Peritubular Collar Formation in Obstructed Fetal Collecting Duct.**
Obstruction of the fetal kidney results in collecting duct injury that can be classified by the extent of αSMA immunoreactivity in the peritubular cellular collars, ranging from ‘mild’ with limited peritubular αSMA immunoreactivity (arrowhead), to ‘severe’ with full, circumferential collars (arrows). Scale bar = 25 μm. Reprinted from *Butt et al., Kidney Int, 2007; 71(2), 936-944.*
4.2.5. Loss of Epithelial and Gain of Mesenchymal Cell Markers

In conjunction with the prominent dilation of collecting ducts and adjacent peritubular collar formation observed following obstruction, these kidneys also displayed a temporal and spatial sequence of phenotypic changes that suggests the occurrence of epithelial mesenchymal transition (EMT) (Figure 4.5). In particular, the epithelia of obstructed collecting ducts exhibited disruption of E-Cad whereby its localization at the inter-epithelial junctions became less distinct with a corresponding increase in diffuse cytoplasmic immunoreactivity. Furthermore, localized regions of the epithelium exhibited a complete loss of E-Cad, presumably highlighting sites of active EMT. Expression of the principal cell marker AQP2 also appears to be down regulated and exhibits altered cellular localization when compared to both adjacent undilated collecting ducts and the collecting ducts of normal kidneys. In contrast, VIM expression, which is absent from control epithelium, was acquired in the basolateral aspect of collecting duct cells adjacent to the formation of peritubular αSMA collars. This VIM expression is seen throughout the obstructed collecting duct in both AQP2-positive and αSMA+/CA II+ intercalated cell populations but likely represents divergent responses between migrating and non-migrating cells. Like VIM, expression of fibroblast specific protein was minimal in the control collecting duct but markedly increased in the obstructed duct epithelium. As expected, interstitial fibroblasts expressed fibroblast specific protein. These results suggest a loss of epithelial and a gain of mesenchymal characteristics in the obstructed collecting duct epithelium.
Figure 4.5: Loss of Epithelial and Gain of Mesenchymal Characteristics.
(A) In the obstructed fetal kidney, the medullary collecting duct epithelium demonstrates disruption of interepithelial E-Cad immunoreactivity (arrowheads) with concomitant de novo expression of basolateral VIM (arrows). (B) In addition, in the obstructed kidney there is a decrease in AQP2 immunoreactivity in the epithelium of dilated collecting ducts (*) versus adjacent normal and control collecting ducts. (C) Similar to Vim, the obstructed collecting duct epithelium exhibits increased basolateral expression of fibroblast specific protein (FSP, arrows) associated with αSMA collar formation. Scale bar = (A) 25 μm, (B) 100 μm (C) 25μm. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
4.2.6. Cell Proliferation During Urinary Tract Obstruction

Among the phenotypic changes observed within the dilated collecting duct epithelia, an increase in the absolute number of epithelial cells was noted. This observation suggests that proliferation may be occurring in these collecting ducts to maintain an intact epithelial layer when dilation occurs. Immunohistochemical staining for Ki-67, a cell cycle protein indicating cell proliferation (196), revealed increased nuclear expression in selected obstructed collecting ducts versus control specimens. The expression of Ki-67 in the collecting duct epithelium was restricted to the AQP2-positive principal cell population and was not seen in αSMA+/CA II+ intercalated cells of either obstructed or control kidneys. Increased proliferative events were also observed in forming peritubular collars surrounding obstructed collecting ducts (Figure 4.6).

4.2.7. Depletion of Intercalated Cells in Obstructed Collecting Ducts

Based upon these findings, the inner medullary collecting duct of the fetal kidney appears to be composed of at least two populations of epithelial cells. Similar to the adult kidney, the predominant population of cells is the AQP2-positive principal cell population comprising approximately 80% of the epithelium. These studies indicate that the remaining 20% of the fetal collecting duct is composed of αSMA+/CA II+ intercalated cells that may represent an immature intercalated cell population, a putative population of collecting duct epithelial progenitors, or a combination of the two.
Figure 4.6: Proliferation of the Obstructed Collecting Duct Epithelial Cells and Surrounding Interstitium. (A) Nuclear Ki-67 immunoreactivity in selected obstructed collecting ducts showing cellular proliferation in AQP2+ principal cells (arrowheads) and peritubular interstitium (arrow) compared to control collecting ducts. (B) Comparison of Ki67-IR in control and obstructed collecting ducts shows increased number of Ki67 +ve interstitial cells adjacent to moderately to severely dilated collecting ducts, but no significant increase in Ki67 +ve collecting duct epithelial cells. Results are expressed as the percentage of Ki67 +ve cells per number of epithelial cells comprising the circumference of the collecting duct. * p < 0.05 vs. control. Scale bar = 25 μm. Error bars = standard error of the mean. N = 3. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
CHAPTER 4

Given the unexpected expression of αSMA, and therefore of αSMA+/CA II+ intercalated cells in the collecting duct epithelium, and to explore the possibility that these cells are undergoing EMT, differences in the numbers of this subpopulation of cells between obstructed and control kidneys were compared. Importantly, in the obstructed ducts of the near term fetal kidneys we observed a decrease in the number of αSMA+/CA II+ intercalated cell epithelial cells when compared to both adjacent, unaffected ducts in the same kidney and to ducts of control and contralateral kidneys of comparable gestational age. This was confirmed by cell counts (Figure 4.7) which show that there was a significant depletion of this cell population from 18.2% to approximately 12% in mild and moderately obstructed ducts, and to 7.81% in severely affected ducts with completely formed peritubular collars. This depletion was not associated with epithelial cell apoptosis, as assayed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling analysis (TUNEL). These results reveal a loss of αSMA+/CA II+ intercalated cells in collecting ducts following obstruction that was statistically significant (p < 0.01) when compared with control collecting ducts.

4.2.8. Cell Migration Through the Collecting Duct Basement Membrane

Given the unexpected finding of αSMA-positive cells in the fetal collecting duct and their disappearance with injury, and to further support the possibility that the αSMA+/CA II+ intercalated cells were undergoing EMT, the ability of these cells to cross the collecting duct basement membrane was explored. As previously suggested (197,198), EMT includes the mesenchymal expansion of the interstitium
Figure 4.7: Depletion of Intercalated Cells in Obstructed Fetal Collecting Ducts. (A) In the epithelia of the obstructed, dilated collecting ducts there is a loss of CA II+ cells compared to control collecting ducts. (B) The number of CA II+ cells in the obstructed collecting ducts, regardless of severity, was significantly reduced when compared to control and contralateral collecting duct. * p < 0.01 versus control and contralateral. (C) TUNEL analysis of obstructed and control kidneys indicate infrequent apoptotic events (arrows) in the medulla and rarely in the collecting duct epithelium. Scale bar = (A) 50 μm, (C) 25μm. Error bars = standard error of the mean. N = 3. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
and the development of peritubular αSMA collars, and therefore requires the migration of the transitioning cell through the basement membrane and into the interstitial space. Of note in mild to moderately obstructed collecting ducts, there was an observable disruption in collagen IV (Coll IV) expression of the basement membrane, specifically at the site of αSMA collar formation. In addition to the loss of basement membrane integrity, co-localization experiments also demonstrated migration of αSMA+/CA II+ intercalated cells through the plane of the collecting duct basement membrane and into the αSMA-positive peritubular collars (Figure 4.8). Observation of these migratory events is uncommon in the near-term obstructed kidney, indicating that only a small fraction of total collecting duct epithelium is undergoing EMT at any given moment. Although persisting early in transition, expression of CA II and other epithelial characteristics including E-Cad appear to be lost soon after migration as these cells acquire their final myofibroblastic phenotype.

4.3. Discussion

In this study, we have used a clinically relevant model of fetal urinary tract obstruction to implicate medullary injury in progressive renal fibrosis and dysplasia. As described, significant medullary disruption occurs as a result of ureteric obstruction, with segmental alteration of the medullary architecture featuring dilation and cystic transformation of medullary collecting ducts, the formation of peritubular smooth muscle collars, and the expansion of the medullary interstitial compartment. These changes closely resemble the changes seen in
Figure 4.8: Disruption of Basement Membrane and Evidence of Cellular Migration in Obstructed Fetal Collecting Ducts. (A) In the normal collecting duct epithelial basement membrane, there is thin, linear, circumferential, and homogeneous Coll IV immunoreactivity (arrowhead). In the obstructed collecting duct, the basement membrane Coll IV immunoreactivity is diffuse and thickened, particularly in areas of peritubular αSMA expression (arrow), and is absent in other areas. (B) In the obstructed collecting duct, CA II immunoreactivity indicates migration of αSMA+/CA II+ cells (arrowheads) into interstitial space and the αSMA+ peritubular collar, and, as in (C) outside of the normal plane of the epithelial cell layer. (D) In the transitioning cell, E-Cad expression persists in the interepithelial junctions but becomes diffusely cytoplasmic suggesting disruption of the cadherin junction complex. Scale bar = (A, B, C, D) 25 μm. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
human fetal urinary tract obstruction and fetal renal dysplasia (123,193,199,200) and are highly reproducible in this model.

To date, studies of the effects of urinary tract obstruction have utilized, almost exclusively, postnatal models of the rodent kidney (92,107,144,198,201,202). These models focus exclusively on the effects on the proximal tubule and the role of EMT in these segments (203,204). In general, the response to injury is different in the fetus, neonate, and adult, with fibrosis being a major component of the latter (205). More specifically, while the fetal kidney’s response to obstruction includes the development of reactive peritubular collars and the expansion of the interstitial compartment, which may be harbingers of fibrosis, the effects may be more dramatic and include an interruption in normal nephrogenesis and growth of the kidney. Unfortunately, and perhaps due to the limitations of the models, there has been very little attention focused on the medulla and medullary collecting duct.

Given the extensive changes seen in the medulla following in utero urinary tract obstruction in both human and nonhuman primates, we explored the possibility that collecting duct EMT contributes to medullary interstitial fibrosis. To our knowledge, neither the ability of these cells to undergo EMT nor the contribution of the medullary collecting duct to fibrosis has been previously reported. In this study, the phenotypic conversion of collecting duct epithelial cells in a manner consistent with EMT was shown. The onset of this conversion is highlighted by the degradation of interepithelial E-Cad and diffuse cytoplasmic immunoreactivity that
demonstrates the injury-induced disassociation of adherens junctions (206).

Similarly, this conversion is highlighted by the de novo expression of VIM in the collecting duct epithelium adjacent to developing peritubular collars, the associated disruption of the tubular basement membrane, and evidence of αSMA+/ CA II+ collecting duct epithelial cell migration. As expected, evidence of cellular migration was infrequently documented given the short window of time during which it occurs and the likelihood that it occurs soon after obstruction. Finally, the phenotypic transition of collecting duct epithelium was also temporally and spatially associated with the formation of peritubular αSMA-positive collars, lending further support for the probable role of the collecting duct transition in this peritubular pathology.

Interestingly, the increase in basolateral VIM expression is seen not only in the migrating αSMA+/CA II+ intercalated cell population, but also in the AQP2-positive principal cell population. In migrating epithelial and collecting duct-derived peritubular collar cells, this VIM expression likely indicates the conversion of these cells to a myofibroblastic phenotype where VIM is critical for growth, motility and structural stiffness of fibroblasts (207,208). Interestingly, the expression of VIM has also been demonstrated to be of critical importance to the maintenance of endothelial cell structural integrity in response to mechanical stress (207) and to be regulated by changes in physiologic fluid flow (209). Therefore, the de novo expression of VIM in the non-migrating cells of the injured collecting duct epithelium, along with the formation of muscular collars not unlike those found in
arterioles, may represent an equivalent cellular adaptation to the reduced flow and increased luminal pressure resulting from urinary tract obstruction.

Unlike the proximal tubule, the collecting duct is composed of two distinct epithelial cell types, the principal and intercalated cells. Embryologically, these collecting duct cell lineages originate from a common ureteric duct-derived progenitor, but have specific and distinct differentiated functions and phenotypes. However, there is in vivo and in vitro evidence that collecting duct cells, and in particular type B intercalated cells, have the capacity to differentiate into the various cell types comprising the normal collecting duct epithelium (175,176). More recent evidence with gene knockout mice suggests the presence of an upstream collecting duct epithelial progenitor. The elimination of a crucial embryologic transcription factor Foxi1 was shown to result in a loss of distinct principal and intercalated cell phenotypes with clonal expansion of a hybrid, putative progenitor population with primitive functional characteristics of both cell types (210). Putative progenitors have also been identified in the collecting duct and throughout the kidney as BrdU-label retaining cells in pulse-chase studies (211-213), with the majority of the slowest cycling label retaining cells localized to the tubular epithelium and interstitium of the renal papilla (214,215). These label retaining cells have been implicated in organ repair following renal ischemia and unilateral urinary tract obstruction (216). These studies also suggest that while initially the number of label retaining cells increases, this population is ultimately depleted in the long-term indicating their activation and proliferation in response to injury.
In the current study, we identified a subpopulation of cells abundant in the inner medulla of the fetal monkey kidney that expresses diffuse αSMA and the intercalated cell marker, CA II. While previous studies with postnatal human, rabbit, and rat kidneys have suggested the complete absence of intercalated cells in inner medullary and papillary collecting ducts (178,179,190), our data shows that the normal fetal monkey kidney contains 18% αSMA+/CA II+ intercalated cells in the collecting ducts of the inner medulla. As acid-base regulation is not yet an essential function of fetal kidneys, the abundance of these cells suggests they are less likely to be differentiated intercalated cells, but may represent the collecting duct epithelial progenitors that are destined to become principal cells or intercalated cells as needed postnatally. In addition to retaining the putative ability to undergo EMT, these putative progenitors are morphologically distinct from their neighboring columnar epithelia and are non-proliferative. Although these cells did not proliferate in response to injury, we did observe an increase in proliferation in the principal cell and myofibroblast populations. These data suggest that the progenitors may commit to epithelial or myofibroblastic differentiation and then proliferate to expand these cell populations. Furthermore, these data also revealed that αSMA+/CA II+ intercalated cell numbers in the collecting duct of the obstructed kidney decreased from 18% to 8% in severely dilated, fully collared collecting ducts (generally representing 2 or less cells per duct). This decrease was not associated with an increase in epithelial apoptosis (Figure 4.7) as shown by TUNEL analysis. Intriguingly, many of the most severely injured ducts exhibit a complete depletion of
the αSMA+/CA II+ intercalated cell population. These results are consistent with the observation of the depletion of renal medullary progenitor cells in a transient renal ischemia model (214). It is possible that this depletion may impair the potential for further collecting duct cell renewal and repair following prolonged injury such as occurs with sustained obstruction, and could result in attenuation of the epithelium, epithelial apoptosis, and denuding of the basement membrane.

In conclusion, the findings presented here suggest that these cells may represent a progenitor population within the collecting duct epithelium that responds to tubular dilation and injury in two distinct ways: (1) by clonal expansion of principal cells to maintain epithelial integrity, and (2) by committing to a myofibroblastic phenotype through EMT and forming peritubular collars in response to increased luminal pressure (Figure 4.9). Commitment to these pathways appears to be at the cost of the progenitor population, which is significantly depleted following injury. This depletion may impact the postnatal formation of intercalated cells with not only functional consequences such as defects in bicarbonate and water re-absorption, but may result in a reduced capacity of the kidney to respond to injury. Loss of repair potential, in combination with the observed decrease in glomerular number observed with this disorder, may contribute to a predisposition to CKD in later life.

4.4. Conclusions

We have shown that the fetal primate model of urinary tract obstruction closely parallels that of congenital obstruction in the human fetus (5). Notably,
Figure 4.9: Paradigm of Progenitor Differentiation in Response to Fetal Urinary Tract Obstruction. With urinary tract obstruction, progenitor cells within the collecting duct epithelium may respond to tubular injury in a number of different ways, including proliferating and expanding the principal cell population to maintain epithelial integrity, and/or committing to a myofibroblastic phenotype through EMT and forming peritubular collars in response to increased intraluminal pressure. Reprinted from Butt et al., Kidney Int, 2007; 71(2), 936-944.
obstruction in the fetal primate induces a similar depletion of intercalated cells and principal cells in the medullary collecting duct that is associated with an increase in both tubular dilatation and smooth muscle collar formation. In addition, using the primate model, we have further described the remodeling and dedifferentiation of the collecting duct epithelium. We have shown that, following obstruction, the collecting duct epithelium undergoes a loss of epithelial markers and the associated gain of a mesenchymal phenotype. We have also highlighted evidence to support the role of collecting duct epithelial EMT in contributing to renal interstitial expansion and, putatively, the progression to a fibrotic end-point. We hypothesized that epithelial dedifferentiation serves two roles following obstruction: (1) to allow for proliferation to maintain the integrity of the epithelial barrier, and (2) to contribute to the repair process by promoting the formation of peritubular collars via EMT.
CHAPTER 5 OBSTRUCTION IN THE MOUSE KIDNEY

5.1. Introduction

In previous chapters, we have described the extent of collecting duct and medullary changes that occur following obstruction in the fetal human and primate kidney (1,2). We have also proposed a paradigm regarding the role of collecting duct injury and remodeling following obstructive injury. However, further investigation of the underlying mechanisms by which the collecting duct epithelium senses and responds to injurious stimuli requires the use of a model allowing experimental and genetic manipulation. The rodent UUO model is a well-described model of urinary tract obstruction in which many key features of the renal changes that follow obstruction have been characterized (reviewed in (112-114,116)), as described previously in section 2.2.3. However, since UUO elicits a dramatic fibrotic response, much of the published research focuses on this facet of renal injury following obstruction. As a result, most studies to date have focused on later time points in which the fibrotic response predominates. In addition, much of the remaining focus to date has been directed at cortical injury, specifically of the proximal convoluted tubule and glomerulus. As a result the current paradigm of renal injury following obstruction centers on the role of the proximal convoluted tubule in activating the RAS system (155,158-160). This activation has been shown to be a major mediator in the progress of downstream fibrosis following obstruction.
Other factors occurring in the early stages following obstruction have also been described including changes in tubular hydrodynamics, tubular dilatation, and medullary injury. Currently, our knowledge of the contributions of these factors and the potential cellular responses they invoke is lacking. The objectives of the work presented in this chapter are first to establish the mouse UUO model in our lab, second to characterize the injury and renal response following obstruction particularly at earlier time points and, finally, to focus on and characterize the degree of collecting duct injury and alterations following obstruction.

5.2. Results

5.2.1. General Features of UUO

Following surgical ligation, 6 – 8 week old male mice were allowed to recover and were then housed for 1, 2, 3, 6, or 9 days. Upon sacrifice and tissue collection, successful ligation of the ureter was confirmed by examination of the gross pathology of the kidney. As expected, obstruction of the ureter by surgical ligation resulted in the accumulation of urine with significant distension of the ureter proximal to the site of occlusion. Obstruction caused visible swelling and progressive hydronephrosis in the ligated kidneys. Examination of renal histology following obstruction revealed that the increase in urinary accumulation and progressive distension of the ureter extended through the renal pelvis and induced substantial distension of the urinary space of the single unipapillary kidney (Figure 5.1A). As a consequence of this back up of urine into the uretero-calyceal region,
**Figure 5.1: General Features of UUO.** Following urinary tract obstruction, the accumulation of urine proximal to the site of ligation causes (A) progressive distension of the ureter and calyx, and (B) associated compression of the renal outer medulla (OM) and cortex. IM = inner medulla.
progressive compression of the outer medullary, and to a lesser extent, cortical parenchyma was apparent (Figure 5.1.B).

The retrograde accumulation of urine, and the associated increases in luminal pressure, was observed within the tubules of the outer medulla and cortex following as little as 1 day of obstruction. In the outer medulla, the majority of these dilated tubules were collecting ducts (Figure 5.2). Following prolonged obstruction, the presence of dilated tubules of non-collecting duct origin was more pronounced. Similarly, the majority of dilated tubules in the obstructed cortex consisted of collecting ducts and distal convoluted tubules, with little or no observable change to the morphology or structure of the other cortical nephron segments early in obstruction, in particular the proximal convoluted tubules. At the later time points of 6 and 9 days post-obstruction, dilation of other tubular segments of non-collecting duct and –distal convoluted tubule origin became pronounced (Figure 5.2). These observations confirm that, in our hands, surgical ligation of the ureter results in experimentally relevant obstruction of urinary flow. These results are also consistent with the hypothesis that the effects of ureteric obstruction temporally proceed retrograde from the collecting duct and distal convoluted tubule, then to the descending loop of Henle and proximal convoluted tubule.

5.2.2. Apoptosis

One of the key features described in the early progression of obstructive injury is the onset of epithelial apoptosis, as described by others. Apoptotic cell death is
Figure 5.2: Tubular Dilatation Following UUO. Tubules of the distal nephron, including (A) the cortical distal convoluted tubule and collecting duct containing vATPase +ve intercalated cells (Green), and (B) medullary AQP2 +ve collecting ducts (Red) are preferentially dilated at 1 to 3 days post-obstruction, with other nephron segments exhibiting dilatation at later time points of 6 and 9 days post obstruction. Scale bar = 100 μm.
reported to rapidly impair tubular function and promote tubular atrophy. This phenomenon is best described during UUO in the rat (136,141,142,217-219). In our model, apoptotic events in control and in sham-operated kidneys of all time points were rare. The few events observed occurred in isolation and are indicative of the normal rate of cell turnover within the kidney. In the early-obstructed kidney, apoptotic events occurred primarily within segments of the distal nephron (Figure 5.3).

Following 1 to 3 days of obstruction, apoptosis was observed in peri-calyceal tissue of the outer medulla immediately adjacent to the expanded calyceal space and nearest the point of transition from papillary to calyceal lining. This peri-calyceal region (box in Figure 5.3) demonstrates substantial outer medullary compression with widespread cellular apoptosis and TUNEL-positive immunoreactive cellular debris in both collecting ducts and tubules of the loop of Henle. Notably, apoptosis remained an infrequent event within the ducts and tubules of other areas of the outer medulla, indicating that these epithelia remained largely intact and viable. Apoptotic events were also observed in the isolated, severely dilated collecting ducts and distal convoluted tubules of the cortex, particularly at the renal poles. Apoptotic events in the proximal convoluted tubule were infrequent and restricted to isolated juxtamedullary tubules entering the outer medulla. Little inner medulla apoptosis was observed, however individual cells within the transitional epithelial
Figure 5.3: Apoptosis Following UUO. Obstruction causes increased accumulation of urine in the calyx, causing (A) compression of the peri-calyceal region (box). This region undergoes dramatic apoptosis (TUNEL stain, Green) (vATPase, Red) while (B) apoptotic events remain rare elsewhere in the outer medulla and cortex. (C) Isolated, dilated cortical tubules and (D) juxtamedullary proximal convoluted tubules also exhibit apoptosis, particularly in the upper and lower poles of the kidney. Following 6 days of obstruction, apoptosis occurs in non-collecting duct segments of outer medulla (E) and is widespread in the proximal convoluted tubule (F). Pyknotic nuclei demonstrate tubular atrophy in the cortex (G). Scale bars = (B) 100 μm, inset 25 μm, (C-F) 25 μm, and (G) 100 μm.
lining of the papilla exhibited TUNEL-positive immunoreactivity indicative of apoptosis (Figure 5.3).

Following 6 days of obstruction, the trends of the earlier time points continued, with apoptosis in the dilated cortical collecting ducts and distal convoluted tubules, but attenuated apoptosis in the peri-calyceal outer medulla. Notably, apoptosis at this time point was observed in additional tubular segments in both the outer medulla and cortex. These segments lack vATPase, a marker of the collecting duct and distal convoluted tubule, and may represent the ascending or descending segments of the loop of Henle. In addition, at this time point tubular injury and the associated apoptotic events continued to impact the proximal segments of the nephron. Apoptosis within the proximal convoluted tubule epithelium was evident and associated with the onset of proximal convoluted tubule injury, including tubular dilatation and brush border flattening. Within the outer cortex, atrophic tubules and pyknotic TUNEL-positive nuclei suggest the occurrence of substantial cortical tubular epithelial cell death occurring between 3 and 6 days post-obstruction (Figure 5.3).

Following 9 days of obstruction the frequency of apoptotic events was dramatically reduced, though still present, in all compartments of the kidney. The presence of pyknotic TUNEL-positive nuclei in the cortical region persisted. Additionally, new regions of widespread parenchymal atrophy and apoptosis were apparent in the polar regions of the renal cortex (Figure 5.3). These regions are believed to contain
nephrons proximal to or “upstream” of the peri-calyceal outer medulla regions that were extensively injured early in the obstructive injury.

5.2.3. Interstitial Response

Obstructive injury of the kidney is associated with dramatic changes to the interstitial compartment in many published models. In our hands, interstitial expansion and tubular epithelial remodeling is a key feature of the early response to obstructive injury and occurs even before the onset of the interstitial fibrosis evident at later time points. Specifically, the accumulation of myofibroblasts expressing αSMA is as a hallmark feature of both repair and fibrosis. In normal and sham-operated kidneys of all time-points, αSMA immunoreactivity was restricted to the vasculature, calyceal lining, and a small population of fibroblasts located in the interstitium between the various tubules and ducts of the medulla and cortex (Figure 5.4).

Following UUO, expansion of the interstitial space and accumulation of αSMA-positive cells occurred rapidly in both the outer medulla and cortex. αSMA immunoreactivity in the outer medullary and cortical interstitium increased progressively with obstruction, but most prominently in the outer medulla. In control, sham and obstructed kidneys, αSMA immunoreactivity was not observed in any renal epithelium, and remained restricted to the interstitial compartment, vasculature or muscular layers of the calyx and ureter (Figure 5.4). Western blot analysis of tissue lysates demonstrated that the protein level of αSMA is elevated
Figure 5.4: Interstitial αSMA Accumulation. Interstitial αSMA (Red) expression increases progressively with prolonged obstruction, with more dramatic accumulation of interstitial αSMA in the outer medulla.
and increases over time in kidneys obstructed for 3 to 9 days (Figure 5.5A) and
when compared to either control or sham kidneys. However, these increases are
relatively modest considering the magnitude of the αSMA response observed with
immunohistochemistry.

To better describe the magnitude and localization of interstitial αSMA within
specific regions of the kidney, morphometric analysis was used to quantify the area
of interstitial αSMA immunoreactivity. Using a 99-point grid (11 vertical and 9
horizontal lines), counts were made from 3 kidneys per condition. 10 representative
outer medullary and cortical high power fields (40x objective) (examples in Figure
5.5B) per kidney were quantified with equal representation of inner and outer
tissue in both regions. Points lying on large vessels of the vascular system were not
counted. The results are expressed as the number of points per high power fields
that lie on αSMA-positive interstitium, and are representative of the overall area of
αSMA immunoreactivity (Figure 5.5C). At all time points, the medullary αSMA
counts were higher than those of the cortex. ANOVA results demonstrate a
significant affect of both obstruction and time on the area of αSMA
immunoreactivity. In both cortex and outer medulla, posthoc testing demonstrates
no differences between any of the sham groups, but a significant increase (p < 0.05)
in αSMA area after 2 days of obstruction before plateauing from 6 to 9 days. In the
outer medulla, αSMA area increased significantly (p < 0.05) from 2 to 6 days, with
an intermediate increase in αSMA area at 3 days obstruction. In the cortex, αSMA
A. Western Blotting

<table>
<thead>
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<th></th>
<th>Ctrl</th>
<th>Sham</th>
<th>Obs.</th>
</tr>
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<tbody>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>9 Days</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
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**Morphometry**

B. Control

![Image](image7.png)

Obstructed

![Image](image8.png)

C.

<table>
<thead>
<tr>
<th>Outer Medulla</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
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<td>35.4</td>
<td>15.3</td>
</tr>
<tr>
<td>39.1</td>
<td>24.3</td>
</tr>
<tr>
<td>39.3</td>
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Counts per High Power Field

- **Control**
- **Sham**
- **Obstructed**

**Figure 5.5: Magnitude of αSMA Accumulation.** (A) αSMA expression in kidney protein lysates increases at 3 and 9 days in obstructed versus control and sham animals. (B) Morphometric analysis of representative high power fields demonstrates (C) significant increases in αSMA area from 1-2 days in both outer medulla and cortex, with more gradual αSMA area increase from 2 to 6 days obstruction. In both the outer medulla and cortex, αSMA area reached a plateau from 6-9 days showing no significant differences. In the obstructed kidneys, all medullary counts were higher than their corresponding cortical counts, * = p < 0.05. Error Bars = standard deviation. N=3 animals. Scale bar = 25 μm.
area does not differ between 2 and 3 days, but increased significantly (p < 0.05) from 3 to 6 days. (Figure 5.5C). These results demonstrate that αSMA-positive cells rapidly accumulate in the renal interstitium following obstruction, and that this effect is more pronounced in the medulla than in the cortex.

As with αSMA, reorganization and deposition of collagens is another key interstitial feature following obstruction. The deposition and accumulation of collagens I and III are frequently used as markers of fibrosis at later time points following obstruction (151,220-222). Coll IV, on the other hand, is the major component of the basement membrane. Our previous studies, and others in the published literature, have demonstrated remodeling and abnormal deposition of Coll IV to be a marker of epithelial injury and remodeling (223). In control and sham-operated kidneys, Coll IV was localized to the basement membranes surrounding all tubules and ducts of the outer medulla, cortex and vasculature. Obstruction resulted in the expansion of the cortical and medullary interstitial space by infiltrating cells, including αSMA-positive myofibroblasts, and in the disruption, expansion, and duplication of the basement membranes that surround the adjacent tubules or ducts. In addition, the expanded interstitial space exhibited increased Coll IV deposition that was more pronounced in the outer medulla than in the cortex and that corresponds to the area of αSMA accumulation (Figure 5.6). Together, the described changes in interstitial αSMA and Coll IV immunoreactivity highlight the rapid expansion and remodeling of the renal interstitium that is more pronounced in the outer medulla than in the cortex.
**Figure 5.6: Interstitial Collagen IV Accumulation.** Interstitial Coll IV (Red) expression increases progressively with prolonged obstruction, with more dramatic accumulation in the outer medulla than in the cortex. Scale bar = 25 μm.
5.2.4. Collecting Duct Epithelial Changes

In addition to changes to the interstitial compartment, we have previously shown that ureteric obstruction alters the development and differentiation of the collecting duct epithelium in both human and primate fetal kidneys. The collecting duct epithelium is composed of two cell types: AQP2-positive principal cells that comprise the majority of the collecting duct cells and vATPase-positive intercalated cells that comprise the remainder. In our mouse model of UUO, ureteric obstruction resulted in a progressive loss of AQP2 protein over time in medullary and cortical collecting duct epithelial cells, similar to our previous descriptions of fetal human and primate kidneys. In the murine kidney, the most significant depletion of principal cell AQP2 occurred within the inner strip of the outer medulla, and to a lesser extent in the inner medulla, while cortical collecting ducts exhibited relatively minor changes (Figure 5.7). Similarly, ureteric obstruction resulted in the loss of vATPase-positive intercalated cells (Figure 5.8). With increasing length of time of obstruction, the protein levels of AQP2 in kidney lysates decreased dramatically as determined by western blot analysis, while vATPase protein levels remained largely unchanged (Figure 5.9). Since other nephron segments including the distal convoluted tubule and the ascending portion of the loop of Henle also express vATPase, it is likely that western blot analysis is not sufficiently sensitive to reflect the relative changes in abundance demonstrated by immunohistochemistry.
Figure 5.7: Loss of Principal Cell AQP2 Expression. Progressive loss of AQP2 expression (Red) is observed over time with urinary obstruction in principal cells of the inner and outer medullary collecting duct, with the largest reduction in the inner stripe of the outer medulla, and little change in the cortical collecting duct. Scale bars = 100 μm.
**Figure 5.8: Loss of Intercalated Cells.** The number of vATPase +ve intercalated cells (Green) in collecting ducts and distal convoluted tubules declines following obstruction, with a dramatic loss of intercalated cells in inner medulla and outer medulla collecting ducts and a modest intercalated cell reduction in cortical collecting ducts and distal convoluted tubules. Scale bars = 100 μm.
A. AQP2

**Figure 5.9: Changes in Intercalated Cell and Principal Cell Protein Expression.** A significant reduction of (A) AQP2 expression but not (B) vATPase expression is observed by Western blot analysis of protein lysates of kidneys 3 and 9 days post-obstruction when compared to control and sham-operated kidneys. Error bars = standard deviation. N = 3 animals.
Alteration of E-Cad localization and its displacement from the cell membrane is another hallmark of epithelial injury. In the collecting duct epithelium of control and sham-operated kidneys, E-Cad localized to the basolateral membrane where it performs a crucial role in epithelial intercellular adhesion. As we have previously characterized in the obstructed fetal primate kidney, murine UUO resulted in the dislocation of E-Cad in the collecting duct epithelium. Following 1 to 3 days of obstruction, the localization of E-Cad at the lateral membrane remained intact, while its basal membrane expression was dramatically disrupted. Following 3 and 6 days of obstruction, cytoplasmic E-Cad immunoreactivity increased, with more diffuse localization at the lateral membrane. By 9 days of obstruction, medullary collecting duct epithelial cells lost their normal patterned structure, displaying amorphous morphology with diffuse and irregular lateral membrane E-Cad localization and an increased proportion of cytoplasmic E-Cad (Figure 5.10).

These results indicate that obstruction causes observable alterations in the structure and cellular composition of the collecting duct epithelium. The loss of the functional transporters AQP2 and vATPase in principal cells and intercalated cells respectively, in conjunction with the disruption of E-Cad localization, suggests that obstructive injury results in cellular dedifferentiation and promotes mesenchymal transformation of the collecting duct epithelium.
Figure 5.10: Disruption of E-Cad Localization. E-Cad (Green) localizes specifically to the principal cell basolateral membrane in control and sham-operated kidneys (bracket). Following 3 days of obstruction, E-Cad expression is lost at the basal membrane (arrowhead), while expression at the lateral membrane is more diffuse (arrow). Increased cytoplasmic E-Cad is observed following 6 days of obstruction (arrow). 9 days post-obstruction, collecting duct cells lose their regular epithelial patterning and become amorphous, with E-Cad poorly localized to the lateral membrane and becoming more cytoplasmic. Scale bar = 25 μm.
5.3. **Discussion**

The main objective of work presented in this chapter was to establish a murine UUO model of urinary tract obstruction in our lab. To that end, we have characterized and focused upon the morphological, histological, and functional changes occurring within the medulla and collecting duct epithelium that have been previously underreported. Our analyses of obstructed kidneys at several time points from 1 to 9 days provide insights into the underlying mechanisms and key steps in the progression of obstructive injury. These insights include evaluation of the magnitude of the physical stresses caused by urinary tract obstruction, the rapid onset and retrograde progression of the injury, as well as the scale of the medullary and collecting duct changes.

The significant physical stresses caused by the restriction of urinary flow can be readily appreciated by gross examination of the obstructed kidneys. Specifically, the progressive hydronephrosis and ureteric distension indicate the substantial volume of urine retained within the kidney. In addition, calyceal distension, compression of the medullary and cortical parenchyma, and tubular luminal expansion and dilatation demonstrate the significant pressure and mechanical stress associated with the restriction of urinary flow. Cellular injury is evident from the extent of cellular apoptosis, which correlates with the degree of tubular dilation, particularly in the peri-calyceal region of the outer medulla, where an increase calyceal volume and pressure results in compression of the adjacent renal tissue. This leads to
substantial tubular apoptosis and atrophy in these areas. Interestingly, these regions of the medulla correspond with those proximal nephrons of the poles of the cortex where substantial atrophy and apoptosis occurs during prolonged obstruction. These cortical regions have been also been shown to be irreversibly damaged following relief of urinary obstruction (223).

Our results also indicate the rapid onset of physical stress and associated cellular injury due to obstruction, manifested as dilatation of the renal tubules as early as 1-day post ligation. Not surprisingly the retrograde accumulation of urine impacts the collecting duct and distal convoluted tubule first. It is interesting that with the exception of the peri-calyceal region, cellular apoptosis correlates with the extent of tubular dilatation and also occurs primarily in the collecting duct and distal convoluted tubule during early obstruction. Surprisingly, proximal convoluted tubule cellular apoptosis and proximal convoluted tubule dilatation do not occur until 6 days post-obstruction. However, by this time significant cellular injury and interstitial reaction is demonstrable in other compartments and tubules of the kidney. It is therefore probable that the antecedent injury to the collecting duct and distal convoluted tubule has an important role in the initiation and mediation of the early response to injury.

The attenuation of apoptosis immediately prior to the onset of substantial fibrosis is also surprising. Given the chronic and persistent nature of the obstruction, it seems unlikely that cells at 9 days are experiencing less physical or mechanical strain than
at earlier time points. It is therefore interesting to speculate that apoptotic events may represent a necessary feature of the epithelial response and remodeling during obstruction, rather than the outcome of direct injury.

As observed during obstruction in the fetal primate (2), UUO is associated with reorganization and thickening of tubular basement membranes. Unlike the fetal primate, Coll IV remodeling and deposition appears much more extensive during murine UUO. Whether Coll IV remodeling and deposition reflects disruption of the basement membrane as part of the process of EMT, or as a response to injury whereby increased Coll IV reduces tubular compliance and susceptibility to injury remains unclear. Similarly, the significance of the dramatic recruitment of αSMA positive cells into the renal interstitium in early obstruction is also unclear. It is possible this early activation of putative αSMA-positive myofibroblasts as early as 2 days post-obstruction serves as an antecedent to the fibrosis seen with prolonged injury. However, it is likely that these cells also play a role in the early response to obstructive injury, and may reflect part of the normal acute repair response. As with the deposition of interstitial collagen, this response by αSMA expressing cells may also provide additional structural support by decreasing tubular compliance and subsequent injury.

As observed during obstruction in the fetal human and primate kidney, murine UUO also results in the remodeling and dedifferentiation of the collecting duct epithelium. Notably, both the expression of AQP2 in principal cells and the
abundance of vATPase-positive intercalated cells are reduced. Others in our lab have subsequently described the decrease in intercalated cells following murine UUO. Using the same UUO model Marc Solomon demonstrated that intercalated cell numbers decreased by 32-33% after 3 days and 45-61% after 14 days obstruction. In further agreement with the previously described epithelial changes observed in the obstructed primate kidney (2), structural changes in the collecting duct epithelia were apparent in our UUO kidneys. As in the fetal primate, obstruction causes the dislocation of E-Cad within the collecting duct epithelium. E-Cad remodeling is a hallmark feature of epithelial injury that generally precedes cellular depolarization, due to loss of the associated tight junctions, and dedifferentiation of epithelial cells (224). These steps are considered key in the acquisition of a less differentiated or mesenchymal phenotype that may be necessary to allow for sufficient epithelial plasticity to permit appropriate epithelial repair.

5.4. Conclusions

Thorough examination of obstructed postnatal mouse kidneys at several time points reveals many of the same features described previously in the fetal human and primate. Specifically, we highlight substantial medullary injury with significant remodeling of the medullary and cortical collecting duct that we feel has been underrepresented in the published work with this UUO model to date. We also believe that the induction of obstruction in the adult mouse via UUO provides a
suitable model for identifying possible mechanisms of collecting duct injury and repair.
CHAPTER 6 MECHANISMS OF INJURY AND REPAIR

6.1. Introduction

Urinary tract obstruction is a disorder that causes widespread and often irreversible damage to the kidney. When afflicting the fetal kidney, obstruction impairs the normal process of renal development and maturation resulting in renal dysplasia and fibrosis. Obstruction in the adult kidney induces rapid injury and, if prolonged, results in substantial parenchymal loss and fibrosis. Both dysplasia and fibrosis can have a negative impact on kidney function and, in severe cases involving both kidneys, result in renal insufficiency requiring dialysis or transplantation.

In the previous chapter, we showed that UUO in the adult mouse recapitulates many of the key features of renal collecting duct injury described in the fetal human and primate kidney. These results demonstrate that regardless of whether obstruction occurs in the adult or fetus, significant injury and remodeling of the medulla and collecting duct are conserved features of urinary tract obstruction (1,2). In the various models of obstruction examined by our lab, the collecting duct experiences dramatic dilatation that is associated with disruption of collecting duct epithelial function, cell adhesion, epithelial dedifferentiation, basement membrane remodeling, and accumulation of αSMA-positive cells in the adjacent interstitium. Furthermore, these changes occur in the absence of apparent physical tubular damage or widespread cell death.
Acute urinary tract obstruction causes an immediate rise in ureteric and intrarenal pressure (129,130). In characterizing the early stages of obstruction following murine UUO in Chapter 5, we described several of the physical manifestations of this increased intrarenal pressure including gross hydronephrosis, ureteric distension, compression of the medulla and cortex, and progressive retrograde dilatation of the distal nephron. This urinary pressure, and the resulting physical effects, is the predominant stimulus that affects the epithelium of the obstructed nephron segments. Several mechanisms have been suggested by which physical stimuli resulting from obstruction may be transduced by the epithelium including flow sensation by primary epithelial cilia (225,226) and sensation of mechanical strain through integrins located in focal adhesion complexes, among others (reviewed in [129]). However, there is growing interest in other putative mechanisms that may contribute to this transduction including the possible participation of mechanosensory members of the transient receptor potential (TRP) protein family.

6.1.1. Transient Receptor Potential Channels

The TRP superfamily of proteins is a collection of 28 known mammalian cation channels. TRPs are divided into six subfamilies based upon their structural homology including the canonical (TRPC1-7), melastatin (TRPM1-8), vanilloid (TRPV1-6), ankyrin (TRPA1), mucolipin (TRPML1-3) and polycystin (TRPP1-3) TRP subfamilies (227). Structurally, the TRP channels are formed of homotetramers, with each subunit containing 6 membrane-spanning domains (designated S1-6). Segments S1-S4 make up the peripheral portions of the channel complex, while
CHAPTER 6

segments S5 and S6 from the adjacent subunits combine to form the central cation-selective pore (228). Most TRPs are Ca\(^{2+}\) permeable, with the exceptions of TRPM4 and TRPM5, and contribute to activation of a wide range of Ca\(^{2+}\)-dependent cell functions ranging from gene transcription to cell death (227).

Recent interest in the TRPs stems from their demonstrated roles as cellular sensors of many types of noxious or physical stimuli including ligand binding, chemicals, temperature, cell swelling, endogenous and exogenous agonists, osmolarity, voltage, and mechanical strain. Increasingly, TRPs are being associated with a wide range of human diseases, and several TRP family members have been linked to renal diseases including diabetic nephropathy (TRPC1), glomerular disorders (TRPC6), hypomagnesaemia (TRPM6), idiopathic hypercalciuria and vitamin D-dependent rickets (TRPV5), hypertensive renal damage (TRPV1) and autosomal-dominant polycystic kidney disease (TRPP1 and TRPP2) (228). In the context of urinary tract obstruction, the putative mechanosensory roles of several members of the TRP family makes them intriguing candidates for sensing the physical manifestations of obstruction. Of particular interest is the vanilloid family member TRPV4, which is abundantly expressed in the kidney and demonstrates osmo- and mechano-sensory activity.

6.1.2. TRPV4

Like the TRP family in general, TRPV4 is activated by a wide range of stimuli including hypotonic swelling, chemical agonists, temperatures below 27°C, acidic pH and arachidonic acid metabolites (229). The activation of TRPV4 by these
various stimuli is not fully understood and is believed to occur by multiple mechanisms. Activation of TRPV4 in response to hypotonic cell swelling occurs indirectly following cell swelling activation of phospholipase A2, leading to downstream production of arachidonic acid (229). Activation of TRPV4 via temperature and phorbol esters uses a separate mechanism of activation that is dependent on specific amino acids in the S3 transmembrane domain. A voltage-dependent gating mechanism has also been proposed (230). Phosphorylation of TRPV4 by protein kinase C, protein kinase A and members of the Src kinase family appears to modulate channel sensitivity rather than activation (231,232), while trafficking and localization of TRPV4 are influenced by glycosylation and ubiquitinization (233,234).

Activation of TRPV4 impacts epithelial function and has been shown to increase paracellular permeability of mammary cells through the disruption of tight junctions (235), and thereby regulates skin barrier function (236). In response to physical and chemical cues, TRPV4 regulates ciliary beat frequency in pulmonary epithelium (237,238). TRPV4 has also been linked to diverse cellular responses ranging from the induction of physical sensation-mediated beta cell death in the pancreas (239), to the regulation of osteoclast differentiation (240). Accumulating evidence supports a role for TRPV4-mediated mechanosensation, although this function is not well described. In the bladder urothelium, stretch-induced TRPV4 activation has been implicated in the sensation of bladder distension (241). The response of TRPV4 to mechanical stress is best described in endothelial cells where
flow-mediated dilation and cellular stretch serve to regulate vascular tone (242-245), promote endothelial cell proliferation (246), and mediate strain-induced remodeling of the endothelial cytoskeleton and cell organization (237).

In the kidney, TRPV4 is localized to the water-impermeant segments of the nephron including the ascending loop of Henle, distal convoluted tubule, and collecting duct. In the latter segment, TRPV4 is expressed in both principal and intercalated cells, with moderate and strong immunoreactivity respectively (247). Little is known about the function of TRPV4 in the kidney beyond its presumed osmoregulatory functions. However, a limited number of studies have explored the role of TRPV4-mediated mechanosensation in the collecting duct. In the cortical collecting duct, and in the M-1 cortical collecting duct cell line, TRPV4 is activated in response to flow/fluid shear stress (248,249). In cell culture models using Madin-Darby canine kidney cells, TRPV4 has also been described in the apical membrane where it associates with TPRPP2 (polycystin-2) (250), however the relevance of this association in vivo where basolateral TRPV4 localization is reported is unknown. TRPV4 and TRPP2 complexes associated with the primary cilia of the collecting duct epithelia have been proposed to form a polymodal flow sensor. Intriguingly, systemic administration of the specific TRPV4 agonist GSK101690A in a rat model of polycystic kidney disease was found to reduce renal cysts by 28.4% and attenuate fibrosis by 58.3% (251). The similarities in outcomes of polycystic kidney disease and urinary tract obstruction suggest that TRPV4 may serve a role in the sensation of altered urinary flow or pressure-mediated stretch following obstruction.
6.1.3.  **TRPV4 Deficient Mice**

The availability of mice deficient for the TRPV4 gene provides an excellent opportunity to mechanistically examine the contribution of TRPV4 to the renal response following urinary tract obstruction. TRPV4 knockout (Trpv4<sup>−/−</sup>) mice were generated in the lab of Dr. Makato Suzuki by insertion of a phosphoglycerine kinase-neomycin (PGK-neo) cassette into the fourth exon (252,253). Briefly, this insertion was achieved through generation of a targeting vector containing the PGK-neo cassette with flanking arms of homologous sequence to the site of desired insertion (254). After electroporation into embryonic stems cells, colonies were screened to select for those that underwent homologous recombination to incorporate the PGK-neo cassette. These clones were then injected into C57/B6 embryos to generate chimeric mice. Chimeric males were bred to C57/B6 mice to produce heterozygous offspring, which were subsequently crossed to generate mice homozygous for the TRPV4 disruption. Disruption of Trpv4 expression was confirmed by Southern blot, polymerase chain reaction (PCR), western blotting, and histological staining.

Mice lacking TRPV4 exhibit no outward phenotype and display normal appearance, growth, size, temperature, fertility, and behavior. Histologically, no renal or hepatic abnormalities were noted in Trpv4<sup>−/−</sup> mice (250,253). In TRPV4 deficient mice no alterations of the electrolyte concentrations of sodium, potassium, calcium and chloride were observed in the serum. However, disruption of TRPV4 was found to impair pressure sensation and alter systemic regulation of serum osmolality in
response to salt challenge (252,253,255). In addition, it is suggested that one of TRPV4’s functions in vivo is the regulation of cell volume changes in response to osmotic changes. The putative role of TRPV4 during kidney injury, specifically in the form of urinary tract obstruction, remains to be demonstrated.

We believe the epithelial and interstitial alterations that follow urinary tract obstruction, as described above, are not merely a side-effect of injury but represent an adaptive response initiated by the collecting duct epithelium following the sensation of injury. We hypothesize that TRPV4, with its reported mechanosensitive properties and its expression in the kidney collecting duct, may serve the role of sensing tubular obstruction of urine flow, transducing this stimulus into a downstream cellular response, thereby modulating the epithelial repair and fibrotic response.

6.2. Results

6.2.1. TRPV4 Expression In Vivo and In Vitro

After a literature search identified a number of Trps reportedly expressed in the kidney, we sought to confirm the gene expression of several Trps in our mouse model and identify those expressed in our collecting duct-derived mIMCD3 cell line, to be used in experiments to be described. Using custom designed mRNA specific primers, selected Trps were PCR amplified from cDNA pools from isolated mouse whole kidney tissue or the mouse inner medullary collecting duct (mIMCD3) cell
line. From this primer set, most Trps are expressed in both the whole kidney and mIMCD3 cells, with only Trpv1 and Trpv6 absent from the latter (Figure 6.1A).

We chose TRPV4 for additional study based upon its high level of expression in the kidney, its reported mechanosensory role, and its reported role in stretch- and strain-mediated function (as described in section 6.1.2). To confirm its expression in our mouse and cell models, TRPV4 was localized in both frozen tissue sections and mIMCD3 monolayers using a polyclonal anti-TRPV4 antibody from Alomone Labs using fluorescent immunohistochemistry. In the mouse kidney, TRPV4 displayed strong immunoreactivity in AQP2-positive collecting ducts of both the medulla and cortex. Within these ducts, TRPV4 was not restricted solely to AQP2-positive principal cells, but was also observed in intercalated cells (Figure 6.1B). These findings generated with this commercially available polyclonal anti-TRPV4 antibody correspond with those previously published (247). Notably, mIMCD3 cells cultured on coverslips or chamber slides do not express TRPV4 nor AQP2, likely due to the poor polarization and epithelialization of cells cultured on solid substrates. However, to promote cell polarity and a representative differentiated collecting duct epithelial cell layer, mIMCD3 cells were grown on hanging cell culture supports. Under these conditions, immunoreactivity for both TRPV4 and AQP2 was observed in mIMCD3 cells, with AQP2 expressed in apical vesicles and TRPV4 localized to the lateral membrane of moderate to high density cell monolayers (Figure 6.1C).
A. TRP mRNA Expression

![TRP mRNA Expression Graph]

**In vivo**
Whole Mouse Kidney

**In vitro**
Mouse IMCD3 Cell line

Size (bp)

TRPV4 Localization

![TRPV4 Localization Diagram]

B. **In vivo** (Mouse)

TRPV4
AQP2

![In vivo Images]

C. **In vitro** (IMCD3)

TRPV4
AQP2

**Figure 6.1: TRPV4 Expression In Vivo and In Vitro.** (A) mRNA expression of selected TRPs in the mouse kidney and IMCD3 cell line. (B) TRPV4 colocalizes (yellow) to AQP2-positive collecting ducts in both the cortex and medulla of the mouse kidney. Insets show opposing halves of overlapping images demonstrating TRPV4 and AQP2 colocalization in contiguous portions of the collecting duct. (C) In IMCD3 cells cultured on hanging cell culture inserts, TRPV4 protein is expressed at the lateral membrane, while AQP2 localizes to apical vesicles.
6.2.2. *Cellular Localization of TRPV4 In Vivo and In Vitro*

Using confocal microscopy, the cellular sub-localization of TRPV4 within the collecting duct epithelium was further investigated in both kidney tissue and in the mIMCD3 cell line. TRPV4 localized to the basolateral membrane of epithelial cells both *in vivo* and *in vitro*. Further immunohistochemistry studies demonstrated colocalization of TRPV4 with the basolateral adherens junction proteins E-Cad and β Catenin (βCat) at the basolateral membrane. In mIMCD3 cells, E-Cad and TRPV4 co-localized in all cells (Figure 6.2). E-Cad:βCat and TRPV4:βCat co-localized in most cells, while βCat localized in some regions of the monolayer in the absence of significant E-Cad or TRPV4 immunoreactivity. These regions (asterisk in Figure 6.2B) represent lower density cells with less mature adherens junctions and likely reflect the association of βCat with other members of the cadherin family.

As TRPV4, βCat and E-Cad were observed to colocalize to the basolateral membrane, we examined whether these proteins interacted to form actual protein complexes. Following immunoprecipitation of protein lysates from mouse kidney and mIMCD3 cells with a polyclonal anti-TRPV4 antibody, both E-Cad and βCat were detected (Figure 6.2C). These results confirmed the presence of bound complexes of TRPV4, E-Cad and βCat and were further supported by the reciprocal immunoprecipitation using anti-βCat antibody “pull down” and probing for E-Cad and TRPV4. Furthermore, the association of mechanosensitive TRPV4 with these adherens junction proteins in our kidneys and kidney cell lines suggests an intriguing
Confocal co-localization

A. In vivo (Mouse)

B. In vitro (mIMCD3)

C. Immunoprecipitation

Figure 6.2: TRPV4 Co-localization In Vivo and In Vitro. (A) TRPV4 protein expression (Green) is localized to basolateral membranes of the collecting duct epithelial cells, and colocalizes with E-Cad (Red), which also colocalizes with βCat (Red). (B) Expression of TRPV4, E-Cad, and β Cat in mIMCD3 cells cultured on hanging cell culture supports. In the cell monolayer TRPV4 and E-Cad colocalize in all cells, while βCat expression is seen in areas where TRPV4 and E-Cad expression are absent (asterisk), reflecting βCat’s ability to bind other members of the Cadherin family. (C) Immunoprecipitation studies using an anti-TRPV4 “pull-down” show co-precipitation of E-Cad and βCat in mouse kidney and mIMCD3 protein lysates, confirming the formation of complexes of these 3 proteins.
mechanism whereby TRPV4 activation may influence the stability and degradation of E-Cad-mediated cell adhesion, as observed during obstructive injury.

6.2.3. **TRPV4 During Urinary Tract Obstruction**

While the preceding experiments demonstrated the expression of TRPV4 in the normal mouse kidney, the effect of renal injury on TRPV4 expression and localization is unknown. We therefore examined the impact of murine UUO on TRPV4 distribution. By fluorescent immunohistochemistry, control and sham kidney tissue sections expressed TRPV4 throughout the collecting duct, and with both basolateral membrane and diffuse cytoplasmic localization. Following obstruction, basolateral TRPV4 localization became more distinct while cytoplasmic immunoreactivity decreased (Figure 6.3A).

To examine gene expression of *Trpv4* following obstruction, mRNA isolated from LCM-captured inner medulla and outer medulla tissue was analyzed by quantitative PCR. This technique allowed for region-specific analysis of gene expression in obstructed and sham kidneys. Following 3 and 9 days of obstruction, in the inner medulla, *Trpv4* mRNA expression was markedly reduced by 63 – 67% (Figure 6.3B). In contrast, outer medullary *Trpv4* gene expression was increased by 26 to 28% over the same period. In kidney protein lysates, TRPV4 protein expression exhibited a response intermediate to the inner medulla and outer medulla gene expression
A. Localization

B. TRPV4 mRNA Levels

C. TRPV4 Levels

Figure 6.3: TRPV4 During Obstruction. (A) TRPV4 protein localizes to the basolateral membrane of the collecting duct cell with diffuse cytoplasmic expression in control and sham-operated kidneys. Following obstruction, basolateral expression of TRPV4 in both the inner and outer medulla collecting duct cells intensifies, while cytoplasmic TRPV4 expression is reduced. (B) qPCR analysis of LCM-captured inner medulla or outer medulla tissue indicates significantly reduced gene expression of TRPV4 in the inner medulla, with modest increases in the outer medulla, at 3 and 9 days post-obstruction. (C) In kidney lysates, TRPV4 protein levels show mild reduction at 3 days, with no change at 9 days. Error bars = standard deviation. N = 3 animals. Scale bar = 25 μm.
changes, with a reduction of 24% at 3 days post-obstruction, but no change after 9
days obstruction (Figure 6.3C). Obstruction therefore appears to have a modest
impact on \textit{Trpv4} expression, resulting in an apparent decrease, with the most
evident effects observed in the inner medulla. In the collecting duct, injury altered
the subcellular localization of TRPV4, reducing its cytoplasmic immunoreactivity
while maintaining localization to the basolateral membrane.

\textbf{6.2.4. Activation of TRPV4}

To investigate the functional role of TRPV4 \textit{in vitro}, we developed a modified cell
culture model using our mIMCD3 cell line cultured on hanging cell culture supports.
This method was necessary to allow appropriate expression of proteins including
AQP2 and TRPV4 (discussed in section 6.3). TRPV4 activity was stimulated using the
TRPV4-specific agonist GSK101690A and inhibited using the TRPV-family inhibitor
ruthenium red (RR) (242,243). To determine appropriate agonist dosing, the time
and dose responses were examined using treatments of 5nM, 20nM, 50nM, and
100nM GSK101690A for 24, 48, or 72 hours. Treatment with 100nM GSK101690A
resulted in cell toxicity, while lower doses resulted in dose-dependent alterations in
monolayer organization and cell morphology. These effects were limited to localized
regions at the lower doses of 5nM and 20nM GSK101690A, while 50nM treatment
induced widespread morphological changes without significantly impairing cell
growth. Changes in cell morphology were observed following 24 hours of
stimulation, and were more pronounced at 48 and 72 hours. The maximal response
was observed after 72 hours of treatment with 50nM GSK101690A, and these
conditions were used for the subsequent morphological and protein studies (Figure 6.4A). For cell fractionation studies, 50nM GSK101690A treatment was used for 5 minutes, 1 hour, 6 hours, and 24 hours.

The effect of GSK101690A treatment on cell morphology was examined by fluorescent immunohistochemistry following removal and staining of the membrane from hanging cell culture inserts. mIMCD3 monolayers normally exhibit regularly patterned cobblestone morphology upon reaching moderate to high density with colocalization of E-Cad and βCat at the intercellular junctions. Following GSK101690A treatment for 72 hours, mIMCD3 cells exhibit a substantially altered cellular morphology. Treated cells display a spindle-shaped or fusiform morphology and organize into aligned, ridge-like structures. Notably, these changes resemble similar changes following treatment of mIMCD3 cells with TGFβ-1 (4). Furthermore, GSK101690A treatment also disrupts the association of E-Cad and βCat, resulting in disruption of the complexes and dislocation of βCat to the cellular cytoplasm (Figure 6.4A). Co-treatment of cells with 50nM GSK101690A and 10μM RR restored normal morphology.

To investigate the effects of TRPV4 activation on the cell protein levels of TRPV4, E-Cad, and βCat, protein lysates were collected from control, GSK101690A, GSK101690A and RR, and RR-only treated cells. Following 72-hours of treatment with 50nM GSK101690A, the relative levels of βCat, E-Cad and TRPV4 decreased by 37%, 44%, and 64% respectively. Co-incubation of 50nM GSK101690A with 10μM
A. Morphology

Control

Agonist
50 nM GSK10167090A

B. Protein Levels

Figure 6.4: TRPV4 Activation In Vitro. (A) Stimulation of mlMCD3 cells cultured on hanging cell culture supports with GSK101690A alters monolayer morphology (arrows) and disrupts E-Cad/βCat colocalization. (B) GSK101690A stimulation also significantly reduces E-Cad, βCat and TRPV4 protein expression in mlMCD3 cells as demonstrated by Western blot analysis of cell lysates, while co-incubation with the TRPV antagonist RR, or treatment with RR alone, restores E-Cad and βCat expression and partially restores TRPV4 expression. Scale bar = 25 μm. Error bars = standard deviation. N = 3 experiments.
RR or treatment with 10μM RR alone restored E-Cad and βCat protein to control levels. TRPV4 protein levels were partially restored following co-incubation of 50nM GSK101690A with 10μM RR or treatment with 10μM RR alone suggesting that RR negatively influences TRPV4 expression (Figure 6.4B). These findings indicate that the activation of TRPV4 dramatically impacts its own expression, and that of both E-Cad and βCat.

In addition to serving a crucial role in the maintenance of cell-cell adhesion, the association of E-Cad and βCat also has implications on epithelial function. Following disassociation of adherens junctions, free cytoplasmic βCat can, under appropriate conditions, translocate to the nucleus and activate the transcription of a host of genes associated with EMT and fibrosis. Recently, reports describing the correlation of nuclear E-Cad with invasiveness and metastasis in several types of cancer (256-261), have raised questions of a possible transcriptional modulating role following its translocation.

Therefore, to investigate whether TRPV4 activation results in nuclear accumulation of either E-Cad or βCat, cell fractionation experiments were performed on cells stimulated with 50nM GSK101690A for 5 minutes, 1 hour, 6 hours and 24 hours and compared to untreated cells. Both E-Cad and βCat are detected at constant levels in the cytoplasmic fractions at all time points. In the nuclear fraction, both βCat and E-Cad increased following stimulation for 5 minutes (7.5-fold and 4.4-fold
respectively) and 1 hour (5.75-fold and 2.45-fold respectively) (Figure 6.5). These results demonstrate that TRPV4 activation induces rapid βCat and E-Cad translocation to the nucleus, and provides an intriguing link between activation of the mechanosensitive TRPV4 channel and potential downstream signaling and transcriptional activity within the collecting duct epithelial cell.

6.2.5. Obstruction in TRPV4 Deficient Mice

This section describes ongoing experiments and includes preliminary observations detailing the effect of UUO in the Trpv4⁻/⁻ mouse.

To further confirm the role of TRPV4 activation in the sensation and transduction of obstructive injury, we performed the UUO procedure in our Trpv4⁻/⁻ mice. While experimentation and analysis of the results of these procedures is ongoing, preliminary qualitative results provide some intriguing initial insights into the effect of TRPV4 deficiency following obstruction. Surgical ligation of Trpv4⁻/⁻ mice was performed as previously described in our wild type mice, and kidneys were collected following 2 and 6 days of obstruction.

Obstructed kidneys from Trpv4⁻/⁻ mice exhibited the characteristic gross pathology of UUO including hydronephrosis, ureteric distention and calyceal expansion. In sham-operated animals, Trpv4⁻/⁻ kidneys displayed increased αSMA immunoreactivity in their outer medullary interstitium compared to wild type mice.
Figure 6.5: Nuclear Translocation Following TRPV4 Activation. Treatment of mIMCD3 cells with GSK101690A causes rapid translocation of both βCat and E-Cad to the nuclear compartment, as demonstrated by Western blot analysis of protein obtained after cell fractionation. Vinc and Creb are used as cytoplasmic and nuclear controls respectively. Creb was not detected in cytoplasmic fractions. Error bars = standard deviation. N = 3 experiments.
No other differences were noted between the TRPV4 deficient and wild type sham kidneys.

Fluorescent immunohistochemical analysis of 2 and 6 day obstructed kidneys from Trpv4−/− mice revealed a loss of AQP2–positive principal cells and vATPase-positive intercalated cells similar to wild type mice. The basolateral localization of E-Cad and βCat was observed in TRPV4 deficient mice despite the lack of TRPV4 but additional analysis with confocal microscopy is required to determine if the association of E-Cad and βCat differs from wild type mice.

Obstructed kidneys from Trpv4−/− mice do display some intriguing differences from age-matched wild type obstructed kidneys. Trpv4−/− kidneys demonstrated increased collecting duct and distal convoluted tubule dilatation post-obstruction. Obstructed Trpv4−/− kidneys also demonstrated increased interstitial αSMA expression surrounding the intralobular vessels and dilated tubules in the cortex. Additionally, and in contrast to the findings in the renal cortex, medullary interstitial αSMA immunoreactivity was mildly decreased. Using morphometric analysis as described in Chapter 5, the area of αSMA immunoreactivity in obstructed Trpv4−/− kidneys, while reduced when compared to obstructed wild type mice was not statistically significant (P=0.055) at 2 days post-obstruction (Figure 6.6). Further analysis of additional Trpv4−/− kidneys and quantification of later time points will clarify the potential changes in interstitial αSMA. Interestingly after 6 days of obstruction, Trpv4−/− kidneys exhibited the almost complete absence of apoptosis in
**Tubular Dilatation**

**A.**

Cortex (vATPase)

**B.**

Medulla (AQP2)

**C. Interstitial αSMA**

Cortex (αSMA)

**Figure 6.6: Obstruction in Trpv4/- Mice.** UUO causes more severe dilatation of (A) cortical distal convoluted tubule and collecting ducts (vATPase, Green) and of (B) medullary AQP2 +ve collecting ducts (red) in Trpv4/- mice than in wild type obstructed animals. (C) Interstitial αSMA expression surrounding dilated cortical distal convoluted tubules and collecting ducts was increased in obstructed Trpv4/- mice when compared to age-matched obstructed wild type mice. Scale bar = 100 μm. Day 2: N= 3, Day 6: N=2 experiments.
both the collecting ducts and distal convoluted tubules, and less severe cortical atrophy when compared to obstructed wild type kidneys (Figure 6.7).

Taken together, we believe these results suggest that TRPV4 deficiency attenuates collecting duct and distal convoluted tubule sensation of obstructive stimuli and dampens the downstream cellular responses to obstruction, including apoptosis and potentially the transduction of profibrotic signals. The apparent increased tubular dilatation in the obstructed Trpv4−/− kidneys also suggests that absence of TRPV4 in the intercellular junctions renders the epithelium more compliant and susceptible to cystic dilatation. Further investigation is required to confirm these preliminary findings.

6.3. Discussion

In the work presented in this chapter, we have investigated the expression, localization and role of TRPV4 in vivo in wild type and experimental mice and in vitro in a relevant kidney collecting duct cell line. Our results confirm previously published findings regarding the localization of TRPV4 to the distal nephron (247), with abundant expression throughout the collecting duct.

To examine the expression of TRPV4 in vitro we used the well-characterized mouse mIMCD3 cell line. This cell line is a widely used, immortalized cell line isolated from the terminal portion of the inner medullary collecting duct of the SV40 transgenic
**Figure 6.7: Apoptosis in Trpv4-/- Mice Following UUO.** After 6 days of obstruction, Trpv4-/- mouse kidneys display a decrease in CD epithelial cell apoptosis, most notably in (A) dilated cortical distal convoluted tubules (arrow) and (B) dilated cortical collecting ducts (arrowhead) when compared to corresponding obstructed wild type kidneys. (TUNEL stain, Red) (vATPase, Green). Scale bar = 25 μm. Day 2: N= 3, Day 6: N=2 experiments.
mouse (262). These cells have been characterized as principal cells based upon their functional characteristics including sodium flux, growth factor responsiveness and osmotic tolerances. This identification agrees with the composition of the terminal inner medullary collecting duct, which is comprised solely of principal cells. However, in working with this cell line and others, we have observed that epithelial cells cultured using standard methods on plastic or glass surfaces exhibit impaired localization of key proteins and surface markers. In particular, mIMCD3 cells cultured on solid substrates fail to express the primary principal cell marker, AQP2. To restore this functionality, some studies have used transfection systems to overexpress AQP2 in these cells (263). Abnormal cell polarization is believed to be a common feature of culturing epithelial cells using standard methods. In vivo, epithelial cells reside on a basement membrane with aqueous environments on both their apical and basolateral sides. Culture in systems that provide an aqueous environment on one side, and a solid substrate on the other may impede the establishment of this polarized epithelial phenotype. In these settings, epithelial cells may be unable to establish apical/basal polarity leading to impaired trafficking and localization of proteins to their appropriate membrane location.

To better recreate the endogenous in vivo environment, we cultured mIMCD3 cells on hanging cell culture inserts. These inserts have separate apical and basolateral media chambers divided by a porous polyester membrane. When grown under these conditions, mIMCD3 cells exhibit a more differentiated epithelial phenotype and express AQP2 appropriately in apical vesicles. Furthermore, in cells grown on
inserts, TRPV4 expression becomes apparent, with diffuse cytoplasmic immunoreactivity observed in low-density cells, and basolateral localization observed at moderate to high cell density. The strengths of this cell culture model are that it permits appropriate epithelial differentiation and allows appropriate expression of TRPV4 in a fashion that parallels that observed \textit{in vivo}. Furthermore, it allows the direct study of the role of TPRV4 using the TRPV4-specific agonist GSK101690A and the TRPV-family antagonist RR.

\textit{In vivo} and \textit{in vitro}, collecting duct cells demonstrate basolateral localization of TRPV4. Confocal microscopy demonstrates that TRPV4 colocalizes with E-Cad and βCat in the basolateral membrane. Immunoprecipitation experiments have demonstrated that TRPV4, βCat and E-Cad do indeed interact to form a membrane complex. Recent studies confirm this relationship in other cells types including the bladder urothelium (264) and keratinocytes (236). Evidence suggests that complex formation may occur via direct interaction of the cytoplasmic N-terminal domain of TRPV4 and the armadillo repeat region of βCat (236). Alternately, linkage to βCat and E-Cad via αCatenin has been proposed (264). No evidence exists for a direct interaction of TRPV4 and E-Cad, suggesting co-precipitation of E-Cad occurs via its association with βCat.

As described previously, E-Cad and βCat play an important role in epithelial differentiation, and E-Cad disruption has been demonstrated to be a key feature in epithelial injury following obstruction. Colocalization and complexing of TRPV4
with adherens junction proteins raises the intriguing possibility of direct interaction, and thus a possible link between TRPV4 activation and E-Cad-mediated adherens junction disruption. The function of E-Cad is dependent upon Ca$^{2+}$ for both its structure and interaction with E-Cad from adjacent cells. TRPV4, as a cation channel with Ca$^{2+}$ selectivity, may play an important role in E-Cad-mediated adhesion via Ca$^{2+}$ availability. Sokabe et al. have shown that TRPV4 influences the formation and tightness of cell-cell junctions, with TRPV4 deficiency altering or delaying epithelial maturation (236). They hypothesize that TRPV4 may regulate cell-cell junction formation by serving as a scaffold for E-Cad:βCat association, or by modulating Ca$^{2+}$ availability to enhance junction formation. In obstruction, mechanosensation of physical strain by TRPV4 may reverse this process, contributing to E-Cad dislocation and translocation of βCat to the nucleus.

Our in vivo and in vitro results support this hypothesis. In vivo, we have described the disruption of E-Cad localization. In vitro, we have shown that TRPV4 activation results in altered cellular morphology consistent with changes observed following TGFβ-1 stimulation (4). TRPV4 activation also alters the protein abundance and colocalization of E-Cad and βCat, and promotes translocation of both proteins to the nucleus. These findings directly link TRPV4 with a putative mechanism for promoting downstream transcriptional regulation and activation of response genes.

Finally, preliminary observations in Trpv4$^{-/-}$ mice following obstruction further support this hypothesis, and suggest that activation of TRPV4 in wild type mice may
promote epithelial apoptosis and downstream profibrotic events. Further
investigation into the effects of TRPV4 disruption in vivo will aid in identifying
additional downstream effects of this signaling cascade on the epithelial response to
obstructive injury and subsequent fibrosis.

6.4. Conclusion

These findings support a role for TRPV4 in the sensation and transduction of injury
due to urinary flow obstruction. We have demonstrated that TRPV4 is expressed in
the basolateral compartment of collecting duct epithelial cells both in vivo and in
vitro and that in its subcellular localization, TRPV4 forms protein complexes with E-
Cad and βCat. In vitro, we have confirmed that TRPV4 activation influences the
expression and cellular localization of both E-Cad and βCat, promoting the
translocation of both proteins into the nucleus. We believe that nuclear
translocation of βCat, and possibly E-Cad, may prove to be a key step in the
transduction of physical injury into a downstream change in gene transcription,
thereby invoking a cellular response to urinary tract obstruction.
CHAPTER 7 GENERAL DISCUSSION AND CONCLUSIONS

7.1. **Summary**

Urinary tract obstruction is a major cause of morbidity and mortality worldwide, especially in young boys. Chronic obstruction leads to substantial renal injury and to progressive kidney fibrosis. When occurring *in utero*, chronic obstruction has the added impact of derailing normal organogenesis causing impaired renal development and dysplasia. Both of these outcomes negatively impact kidney function.

Our current understanding of the pathophysiology of obstructive injury centers largely on the events promoting fibrosis. However, fibrosis is a late event in the progression of renal injury following obstruction. Our understanding of the early events following urinary tract obstruction is incomplete. The predominant existing paradigm of obstructive injury focuses predominantly on the role of the proximal convoluted tubule in the initiation and propagation of the kidney’s response to obstruction. Hemodynamic stress upon the proximal convoluted tubule epithelium activates the intrarenal RAS system while decreased renal blood flow promotes tissue hypoxia (112,114,132,155,158-160). These factors are believed to drive inflammation and induce tubular apoptosis and atrophy that ultimately initiates the progression of fibrosis.
However, several key factors are underappreciated in this paradigm; in particular the contributions of the early physical manifestations of obstruction, such as the accumulation of urine and the increase in intrarenal pressure (129,130). Furthermore, the potential contributions of other segments of the nephron have been largely ignored. To improve our understanding of the pathophysiology of the progression of obstructive injury, a broader understanding of the contribution of these early events and physical stresses on other tubular segments is required.

7.2. **Objective and Scope of Thesis**

The objective of the work presented in this thesis was to investigate the role of collecting duct injury and remodeling following urinary tract obstruction. Interest in the collecting duct originates from observations in clinical cases of human fetal obstruction where collecting duct pathology is prominent, particularly in the kidney medulla. Urinary obstruction in the fetal non-human primate confirms the relevance of these observations, and provides a useful model to investigate the injury further.

We have investigated and characterized three different consequences of obstruction. First, we have studied kidneys from pathological specimens from fetuses and young infants suffering from urinary tract obstruction. In normal human fetal kidneys, we have described the ontogeny of collecting duct intercalated cell maturation and characterized the remodeling that occurs following obstruction. Second, we have used the fetal primate model, which closely recapitulates the pathology of fetal human obstruction, to recreate and study the collecting duct
injury, cellular dedifferentiation, and associated interstitial reaction in the obstructed renal medulla. Third, we have established and studied a murine UUO model in our lab, and characterized the salient features of medullary and collecting duct injury. Finally, we used this model and adapted the mIMCD3 cell model to examine the mechanisms by which the collecting duct epithelium senses and responds to obstructive injury.

7.3. **Relevance of Findings**

7.3.1. **Role of the Collecting Duct in Obstruction**

Collecting duct epithelial injury and the response to this injury was a common finding in the kidneys of human fetuses and infants with the clinical manifestations of urinary tract obstruction and in the two relevant animal models studied. In particular, the fetal primate model provides the best definition and resolution of the collecting duct and interstitial changes that result following fetal urinary tract obstruction. In this model, segmental dilatation of the collecting duct system was observed, with the earliest and most widespread changes affecting collecting ducts of the medulla. As described in Chapter 4, dilated collecting ducts exhibited characteristic thinning of their epithelia, loss of epithelial markers including E-Cad, gain of mesenchymal markers such as VIM, and disruption and remodeling of their adjacent basement membranes (2). Notably, these collecting duct changes are directly associated with adjacent interstitial expansion and formation of αSMA-positive peritubular collars. These changes closely parallel those observed during fetal obstruction in humans (5). In obstructed kidneys of both the human and
primate, we have also shown that these changes were associated with impaired
development or dedifferentiation of the collecting duct epithelium (2,5,6). These
responses likely represent a direct reaction of the epithelium to dilatation, to
luminal pressure, and possibly to epithelial cell stretch induced by urinary tract
obstruction. In the postnatal mouse, the same key features were observed, with the
primary differences being the rapid onset of the obstructive injury and the
widespread nature of the interstitial αSMA accumulation. The murine UUO model
also exhibited a greater involvement of the proximal nephron segments. This likely
reflects the difference between the partial urinary obstruction present in the fetal
human and primate, versus the complete obstruction of urinary flow that follows
surgical ureteric ligation.

The primary function of any epithelial cell is to maintain its crucial barrier function
regardless of secondary transport, sensory, secretory or mechanical functions
(265,266). Barrier function is essential to maintaining the separation of the noxious
external environment from the delicately balanced and finely tuned internal milieu.
In this light, we have previously proposed a paradigm for the collecting duct
epithelial response to injury that centered on this crucial aspect of collecting duct
epithelial biology (Figure 4.9) (2). This paradigm suggests that the many features
described herein are not simply effects of obstructive injury, but rather represent a
specific and active response to injury. This response is aimed at preventing the loss
of barrier function caused by severe tubular injury, cellular atrophy and death, and
denudation of the basement membrane. In this paradigm, collecting duct epithelial
cells respond to obstructive injury by committing either to maintenance of the epithelium via proliferation, or to the promotion of interstitial changes aimed at mitigating further pressure or stretch-induced injury.

One way in which collecting duct epithelial cells may contribute to the interstitial reaction following obstruction is via EMT. EMT is fundamental process that is crucial in embryonic development and cancer metastasis. During EMT, epithelial cells lose their epithelial adhesion, gain the expression of de novo mesenchymal markers, disrupt their adjacent basement membrane and migrate into the interstitium (149,150,267). In the kidney, EMT is widely reported as a feature of the epithelial response to many profibrotic stimuli and following urinary tract obstruction (198,268). These studies suggest that EMT of renal epithelial cells into interstitial mesenchymal cells contributes to the accumulation of the key fibrotic effector cell, the αSMA-positive myofibroblast. Early cell-tracing studies have provided support for this process in the progression of obstructive injury (269). However, more recent lineage studies question the contribution of epithelial cells to the myofibroblast population and refute the occurrence of EMT in the renal epithelia (270,271). This debate continues to be heated between the proponents of both sides of the EMT issue (reviewed in (239,241,244,250)). It appears obvious that much of the debate surrounding EMT lies with its definition (272). Classically, EMT is assumed to represent the complete conversion of an epithelial cell into a migratory mesenchymal one. In this context, the occurrence of EMT in vivo may be
exceedingly rare. However, if a more inclusive definition of EMT is considered, it can be understood that EMT need not be a complete process.

Evidence of epithelial dedifferentiation in obstructive injury indicates that epithelial cells can progress to intermediate stages between the epithelial and mesenchymal phenotype during the process of epithelial repair and remodeling. The term “epithelial-mesenchymal-epithelial transition” has been proposed to describe this phenomenon (224). This paradigm asserts that de-differentiation of epithelial cells is an integral step in the repair of injured tubular epithelia and is followed by re-epithelialization to reconstitute the intact epithelial layer. In this context, classical EMT would still occur under conditions where injury persists or repair becomes dysregulated (224). While it remains unclear if EMT is an ultimate end-point of this progression in vivo, it is important to note that EMT is not the only mechanism by which epithelial injury can propagate an interstitial response. Following injury and activation, epithelial cells may modulate the interstitial reaction through the production of cytokines and growth factors.

In the work presented in this thesis, I have characterized the response of the collecting duct epithelium using several models of obstruction and suggested a role for epithelial sensation in the modulation of the subsequent injury and repair response. This process clearly involves the loss of differentiated collecting duct epithelial features including the expression of key transport proteins including AQP2 and vATPase, as well as fundamental epithelial adhesion proteins such as E-
Cad. We believe these changes reflect the acquisition or maintenance of a less differentially epithelium, or epithelial-mesenchymal-epithelial transition, a process aimed at maintaining and repairing the injured tubular epithelium, as highlighted in the paradigm above.

7.3.2. Mechanisms of Collecting Duct Injury and Response

Having characterized the common features of obstruction on the collecting duct epithelium, I investigated a putative mechanism linking the initial physical stress to a downstream epithelial response. I used the murine UUO model, in conjunction with a collecting duct-derived cell culture model, to examine the role of TRPV4 during urinary tract obstruction. TRPV4 is a mechanosensitive Ca\textsuperscript{2+} channel that is linked to strain- and stress-mediated cell responses (237,238,241-245) I have demonstrated that this channel protein forms complexes with E-Cad and βCat at the site of adherens junctions on the basolateral membrane. Through in vitro studies, I have demonstrated that TRPV4 activation influences the expression, localization and nuclear translocation of both of these key adherens junction proteins.

The disruption of E-Cad-associated adherens junctions is a common feature of epithelial injury (273-275). In this thesis, I have demonstrated that E-Cad disruption and dislocation is a key feature of obstruction in the fetal primate (2) and in the adult mouse kidney. Work performed by others in our lab and in which I have collaborated has also demonstrated disruption and altered localization of E-Cad
following obstruction in human fetal kidneys (5) and in the mIMCD3 cell model following TGFβ-1 stimulation (4).

The stability of adherens junctions has been shown to be integral in determining the structure and function of epithelia and plays a vital role in cell-cell adhesion and epithelial polarization (276-278). In mature epithelial cells, such as those of the collecting duct, adherens junctions are complexes composed of E-Cad in association with α and β catenins. The direct interaction of E-Cad and βCat promotes intercellular E-Cad-mediated adhesion, while α catenin links these complexes to the actin cytoskeleton.

The appropriate maintenance of the stability of E-Cad-based adherens junctions also serves an important secondary role in sequestering βCat expression to these complexes (278,279). The regulation and localization of βCat plays a crucial role in determining epithelial differentiation and structure. Under normal conditions, free cytoplasmic βCat is rapidly degraded. However, in the presence of secondary signals including WNT and TGFβ signaling, inhibition of the degradation machinery allows for the accumulation of βCat in the cytoplasm and its subsequent translocation to the nucleus. In the nuclear compartment, βCat interacts with members of the LEF/TCF family of transcription factors (233) and promotes the expression of a host of target genes that have significant implications on epithelial cell function. The up regulation of several factors including the SNAILs can negatively regulate E-Cad expression and reinforce βCat signaling (280,281). As discussed below, many other
activated genes have described roles in the wound healing, EMT, and fibrotic responses.

The activation of βCat signaling and transcriptional control is associated with epithelial injury following urinary tract obstruction (234). Following epithelial injury, βCat activation leads to the expression of profibrotic factors including c-myc, twist, LEF, and fibronectin, and to the induction of fibrosis (282). βCat accumulation also occurs following stimulation with TGFβ, a key fibrotic mediator observed during obstruction (283). The stabilization of βCat promotes the expression of profibrotic factors including SNAIL, fibronectin and plasminogen activator inhibitor-1 (130). βCat translocation is also linked to the promotion of cellular phenotypic transition or EMT, and studies demonstrate the role βCat/LEF-mediated signaling in the promotion of EMT (284) and expression of profibrotic target genes including VIM, collagens I and III, fibronectin, plasminogen activator inhibitor-1, fibroblast specific protein 1, SNAIL 1 and 2, MMPs, and αSMA (130,236,282,285,286). Intriguingly, the blockade of βCat signaling dramatically inhibits the production of these factors (282,287). In addition, βCat blockade attenuates UUO-induced fibrosis and down-regulates the expression of fibronectin, collagen I, collagen III, plasminogen activator inhibitor-1, fibroblast specific protein 1, SNAILs, and αSMA accumulation. These findings highlight the central role of βCat in the epithelial injury following obstruction.
\[ \text{\textbeta} \text{Cat activity is also gaining importance in the field of kidney development. It has been shown to be important for both MM induction (288) and ureteric bud branching morphogenesis (289). \textbeta\text{Cat deficiency impairs the expression of key ureteric bud development genes including LIM1, PAX2, RET and WNT11 leading to renal aplasia or dysplasia (289,290). Stabilization of \textbeta\text{Cat also promotes aplasia or dysplasia (256). These results suggest that \textbeta\text{Cat is essential for the maintenance of ureteric bud cells in a progenitor state (290). It also highlights a key role for \textbeta\text{Cat on ureteric bud/collecting duct development with loss of \textbeta\text{Cat promoting premature ureteric bud differentiation while \textbeta\text{Cat over activity inhibits differentiation.} }\] 

One of the central issues in the study of urinary tract obstruction is the differing outcome associated with adult and fetal obstruction. In adult obstruction, the primary outcome of chronic disease is fibrosis. However, in the fetus, obstruction causes a combination of renal fibrosis and dysplasia. It is unclear whether these two outcomes share a common mechanism of induction and whether fibrosis and dysplasia represent two completely independent disease processes, or are in fact divergent manifestations of the same underlying mechanistic process.

The increasingly well-defined role of \textbeta\text{Cat in both the development of the kidney and in epithelial injury provides an intriguing link between the pathogenesis of fetal and adult obstruction. Furthermore, the role of TRPV4 described in this thesis suggests a possible unifying mechanism in both the fetus and adult by which physical stresses caused by obstruction can be sensed and transduced into a
reparative or fibrotic response. Through its activation in response to mechanical stimuli and through its modulating effects on the association of the adherens junction proteins E-Cad and βCat, TRPV4 could initiate downstream βCat signaling that elicits the differing responses observed during adult and fetal obstruction. In the adult, βCat mediated signaling promotes the dedifferentiation of the collecting duct epithelial while driving the expression of key profibrotic factors. In the fetus, βCat modulates the balance of ureteric bud and MM progenitor function versus differentiation, resulting in impaired branching morphogenesis, attenuated MM induction and the progression to renal dysplasia.

7.4. **Strengths and Limitations**

The key strength of the work described in this thesis is the models that have been used for investigation. The opportunity to work with human clinical samples is a privilege, and greatly increases the translational importance of the work. Careful observations in and descriptions of the kidneys of children and fetuses with the clinical manifestations of urinary tract obstruction have resulted in a focus on relevant features in other experimental animal models. Similarly, study of the fetal primate model of obstruction has allowed for analysis in a controlled model that replicates the clinical disease. Observations in the fetal human and in the non-human primate models of obstruction have allowed for subsequent investigation of the murine UUO model with fresh insight and perspective. Moreover, the investigation of obstructive injury in all three models is a truly unique opportunity
to identify the common and conserved features of obstruction and has hopefully afforded unique insight into the pathophysiology of urinary tract obstruction.

While studies using fetal human and primate kidneys are incredibly informative of the consequences, pathogenesis and progression of obstruction, they are by their very nature limited to descriptive analyses rather than mechanistic research investigating the contribution of potentially key mediators or target proteins. This obstacle was overcome by using a relevant cell culture model, as well as a murine UUO model in conjunction with transgenic mice. These models have allowed for investigation of the role of TRPV4 during obstruction.

7.5. **Future Work**

As of the writing of this thesis, additional studies are ongoing to further explore the role of TRPV4 in kidney injury and repair following obstruction. These studies include additional investigation of the importance of the interactions of TRPV4, E-Cad and βCat via quantifiable confocal microscopy, and further characterization and quantification of the effects of UUO in the Trpv4⁻/⁻ kidney.

The role of the collecting duct following renal injury remains an under reported feature of the progression of chronic renal disease. Recently, several studies describing the contribution of the collecting duct to fibrosis (291), inflammation (292), and repair (293,294) indicates an increasing interest in this area of study. I
hope that the work described in this thesis will positively impact future research on two levels.

First, we have demonstrated a substantial role for collecting duct injury in the early response to obstructive injury. However, further work is required to examine the downstream effects of collecting duct-mediated responses including the promotion of interstitial fibrosis via the recruitment of inflammatory cells and myofibroblasts. In addition, further characterization of key steps in collecting duct epithelial injury may identify additional putative biomarker proteins allowing for a more accurate and less invasive diagnosis of renal injury due to obstruction, potentially antenatally in affected fetuses.

Second, in investigating the role of TRPV4 in the collecting duct, we have highlighted a possible mechanism linking the sensation of physical stimuli with downstream repair during urinary tract obstruction. Additional work will serve to further elucidate this role. Of specific interest, in vitro studies using a cell stretching apparatus would serve to confirm the strain-dependent activation of TRPV4 in collecting duct cells, and provide a platform for examining the calcium signaling events that result. In addition, the generation of lineage-specific TRPV4 knockout mice would allow further in vivo examination of the role of TRPV4 in the collecting duct. Lastly, modulation of TRPV4 activity during disease may provide opportunities for therapeutic interventions. Gradilone et al. demonstrated that daily low-dose systemic application of the TRPV4 agonist GSK101690A (0.1 mg/kg) substantially
reduced cystogenesis in a rat model of polycystic kidney disease (251). These studies, combined with the work described in this thesis, suggest the need for similar in vivo agonist treatment experiments during obstruction. As TRPV4 deficiency results in worsened injury to the distal nephron after obstruction, it would be intriguing to determine if systemic activation of TRPV4 can attenuate epithelial injury and fibrosis. These studies would have obvious therapeutic implications for kidney injury due to childhood urinary tract obstruction.
CHAPTER 8 MATERIALS AND METHODS

8.1. General Methods

8.1.1. Paraffin Embedding and Sectioning

Collected or dissected kidneys were immediately fixed in 4% paraformaldehyde overnight, dehydrated through gradedethanols before transfer to toluene. Wax perfusion was achieved via a 1:1 mix of toluene wax. The tissue was then oriented in cassettes and embedded in wax overnight. Sections of each paraffin embedded tissue were sectioned with a microtome (5-10 µm), mounted on superfrost slides (Fisher Scientific), and baked overnight at 55°C. Sections were then cooled to room temperature and stored with desiccant. For fluorescent immunohistochemistry, tissue sections were deparaffinized in xylene and rehydrated by passage through graded ethanol. Slides were then subjected to 40 minutes of heat-induced epitope retrieval (HEIR) in 10 mM citrate buffer (pH 6) pre-heated in a domestic food steamer. Following HEIR, the sections were cooled for 40 minutes and incubated at room temperature. Blocking and immunohistochemistry were performed as per section 8.1.2.

8.1.2. Fluorescent Immunohistochemistry

Following hydration in PBS, deparaffinized tissue sections, fixed frozen sections, or fixed hanging cell culture inserts were blocked for one hour in buffer containing 0.1M PBS solution containing 1% bovine serum albumin (BSA), 0.1% cold fish-skin
gelatin, 0.1% Triton X-100, 0.05% tween-20, 0.5% sodium azide and 2% of either goat or horse normal serum. Excess blocking buffer was shaken off of the sections and the primary antibodies, diluted in 0.1M PBS containing 1% BSA, 0.1% cold fish skin gelatin, and 0.5% sodium azide were added immediately and incubated overnight at 4°C. Next, sections were incubated for 1 hour with fluorescently conjugated secondary antibodies diluted in 0.1M PBS containing 0.5% sodium azide. The secondary antibodies used and their dilutions are as follows: goat anti-mouse-IgG (H+L) AlexaFluor-350 and -488 conjugates (1:50 and 1:100 respectively), goat anti-rabbit-IgG (H+L) AlexaFluor-350 and -568 conjugates (1:50 and 1:100 respectively), and donkey anti-goat-IgG (H+L) AlexaFluor-568 (1:100)(Invitrogen). For 3-colour staining, an additional 1 hour incubation with mouse anti-αSMA-FITC conjugate (Sigma, 1:50) was performed following incubation with the relevant secondary antibodies. Nuclei were stained for 5 minutes with DAPI dilactate (1:36, Invitrogen) prior to mounting with Prolong Gold mounting media with antifade (Invitrogen), or mounted with Prolong Gold+ DAPI mounting media with antifade (Invitrogen). Immunofluorescent microscopy was performed on an epifluorescence microscope (Leica). Images were captured with a Retiga 1300i camera with RGB filter wheel (QImaging) and were processed and merged using Openlab software (Improvision). Confocal analysis was performed on a Leica DMIRE2 inverted microscope with SP2 AOBS laser scanning head.

8.1.3. RNA Extraction and RT-PCR

Total RNA was isolated from kidney samples containing cortex or medulla of control, obstructed, and contralateral kidneys using the RNAeasy Mini Kit protocol
with on-column DNAase treatment (Qiagen). cDNA pools were generated using
iScript reverse transcriptase (Biorad) and was immediately analyzed or stored at
≤20°C until use.

**8.1.4. Quantitative PCR**

Real-time quantitative PCR was performed on a 7000 Sequence Detection System
(Life Technologies) using custom designed primers (Table 8.1). All reactions had a
final volume of 25 μL consisting of 2.5 μL of cDNA and 20 μL of 2X TaqMan Universal
PCR MasterMix (Life Technologies) containing PCR buffer, dNTP, and Mg²⁺. All
reactions were run in triplicate using 96-well Optical Reaction Plates (Life
Technologies) under identical amplification conditions: 50°C for 2 min, 95°C for 10
min, 45 cycles of 95°C for 15 s, and 60°C for 1 min. Differences in the expression
levels of the target genes were determined as described in the User’s Bulletin #2
(Life Technologies). Normalization of RNA quantity between samples was accounted
for using the expression of the housekeeping gene Cyclophilin A (295) (primate) or
β Actin (mouse). The fold difference of each gene between samples was then
determined relative to control kidneys.

**8.1.5. Immunoblotting**

Normalized protein lysates were mixed with 5x sample buffer and electrophoresed
on a 10% gel using SDS-PAGE. Following transfer to PVDF membrane (Biorad), and
blocking with 5% BSA, blots were incubated with primary antibodies in TBST
containing 5% BSA for 1 hour at room temperature or at 4°C overnight. Blots were
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (Probe reporter dye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Cadherin</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5'-CAGTGACCAACGATGCATT-3'</td>
</tr>
<tr>
<td>MGB Probe</td>
<td>5'-(6FAM)-AAACAGCAAAGGC-3'</td>
</tr>
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<td>Reverse Primer</td>
<td>5'-GCTTGCCCTCAAAATCCA-3'</td>
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<tr>
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</tr>
<tr>
<td>Probe</td>
<td>5'-(6FAM)-TCCGGGAAATT-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-TTCTCGGCTTCTCTCTGTGAA-3'</td>
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<tr>
<td>Pan-TGFβ</td>
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<td>5'-GCCCACTGCTCCGTCGACA-3'</td>
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<td>5'-(VIC)-CAAAGATAAACACTGCAAGT-3'</td>
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<td>MGB Probe</td>
<td>5'-(VIC)-CTCCTCGAGCTTTTGG-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-CTGCTGTCTTTTGGAAACCTTGCTT-3'</td>
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</tbody>
</table>

Table 8.1: Custom designed, *Macaca mulatta*-specific probe and primer sets. Probes are MGB Probes™ from ABI and are dual-labeled with a non-fluorescent quencher and either 6FAM or VIC reporter dyes for use with 7000 Sequence Detection System (ABI). Adapted from Butt et al., *Kidney Int, 2007; 71*(2), 936-944.
incubated for 1 hour at room temperature with HRP conjugated anti- rabbit or - mouse secondary antibodies (Cell Signaling), treated with ECL solution and exposed on radiography film. Developed films were scanned on a commercial desktop scanner and analyzed via Image J software (NIH).

8.2. Chapter 3: Human Fetal and Postnatal Kidney Analyses

8.2.1. Sample Collection

All samples were collected in accordance within the ethical guidelines of the University of British Columbia and the Vall D’hebron University Hospital. Normal kidneys were collected at 8-10 weeks (n=2), 18 weeks (n=3), 26-29 weeks (n=3), and 36 weeks (n=1) gestation, and postnatally (n=2) at 12 months of age. All normal kidneys were obtained from fetuses with no documented abnormalities. Kidneys obstructed during fetal development were collected at 18 weeks (n=2), and 36 weeks (n=2) gestation, and postnatally (n=1) at 16 months of age. All obstructed kidneys were the result of bladder outlet obstruction with no other noted anomalies. The postnatal kidney was surgically unobstructed prior to collection. All obstructed kidneys exhibited hallmarks of renal dysplasia including glomerular and tubular cysts, associated interstitial expansion, peritubular collar formation, and medullary hypoplasia and would be considered severe according to our previously described scoring system (2,99).
8.2.2. Cell Counts

In control kidneys, collecting ducts from representative microscope fields were counted. In obstructed kidneys, where the severity of injury varies widely throughout the kidney, all medullary collecting ducts were counted and compared with the pooled results from the normal inner and outer medullae. In each analysis, the total number of epithelial cells per collecting duct was determined by counting the number of DAPI stained nuclei per duct. The abundance of intercalated cells and intercalated cell subtypes was determined by counting the number of cells expressing the relevant markers (Figure 3.1) per duct. The abundance of all intercalated cells in a region (total intercalated cells) is expressed as the percentage of collecting duct cells per duct while the abundance of individual intercalated cell subtypes is expressed as the percentage of intercalated cells per duct.

8.2.3. Antibodies

Primary antibodies used for fluorescent immunohistochemistry are as follows: vATPase (Santa Cruz), RhCG (Abnova), pendrin (MBL), AQP2 (Santa Cruz) and anion exchanger 1 (International Blood Group Reference Laboratory). For some experiments, Alexa488 was conjugated to the mouse-derived RhCG primary antibody to allow co-localization with the mouse-derived pendrin antibody using a monoclonal antibody labeling kit (Invitrogen).
8.2.4. Statistical Analysis

Standard deviations are listed for values of intercalated cells and intercalated cell subtypes as a percentage of collecting duct. Error bars represent standard error of the mean.

8.3. Chapter 4: Non-Human Primate Kidney Analyses

8.3.1. Non-Human Primate Model of Fetal Urinary Tract Obstruction

All animal procedures conformed to the requirements of the Animal Welfare Act (USA) and all protocols were approved by the Institutional Animal Care and Use Committee at the University of California at Davis and the Committee on Animal Care at University of British Columbia prior to implementation. Using an ultrasound guided non-surgical technique, as previously described (99), unilateral obstruction was performed in fetal monkeys by the injection of alginate spheres at 70 days gestation. Gravid adult rhesus monkeys were selected for this study (N=3). Fetuses were sonographically evaluated to confirm normal growth and development prior to performing the obstruction. The dams were administered ketamine hydrochloride (10 mg/kg) for these and subsequent ultrasound examinations. Patented, custom-designed alginate spheres shown to be highly efficient in inducing a physiologic obstruction with no evidence of toxicity were used for these studies (99). The animals were euthanized near term at 150 days gestation (term ~165 ± 10 days). Kidneys were collected, frozen, fixed, and stored as previously described at the time of tissue harvest. Representative sections of the right and left kidneys were processed for immunohistochemistry, placed in OCT, snap frozen for
future mRNA studies, and immersed in 10% buffered formalin for histopathology. Comparable studies were conducted in control specimens of similar gestational age (N=3).

8.3.2. **Quantification of Intercalated Cell Abundance**

The percentage of intercalated cells was determined by manually counting the number of αSMA+/CA II+ cells versus total collecting duct cells in several separate fields from control, contralateral and obstructed kidneys. Obstructed collecting ducts were classified into mild (collar less than 50% formed), moderate (collars 50 - 99% formed) and severe (fully formed collars) based upon the degree of peritubular collar formation.

8.3.3. **Antibodies**

The primary antibodies used for fluorescent immunohistochemistry and their dilutions are as follows: mouse anti-VIM (Sigma); rabbit anti-Cytokeratin and mouse anti-Ki67 (Dako); anti-E-Cad (Neomarkers); anti-Coll IV (Fitzgerald); and goat anti-AQP2 and anti-CA II (Santa Cruz).

8.3.4. **Statistical Analysis**

Separate quantitative PCR reactions were run for each sample. Results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student’s t-test. A p-value of < 0.05 was considered statistically significant. For comparisons of αSMA+/CA II+ intercalated cell numbers between obstructed and control kidneys, statistical significance was determined by applying a two-tailed unequal variance t-test. Results for each classification of obstructed
ducts and for all obstructed ducts when pooled was significant to $p < 0.05$ when compared with controls.

### 8.4. Chapter 5 and 6: Murine UUO Model Analyses

#### 8.4.1. Animals and Surgical UUO

All animal protocols were approved by the Committee on Animal Care at University of British Columbia prior to implementation. Wild type C57B6 mice were acquired as needed from Jackson Labs at 5 weeks of age and housed locally for a minimum of one week to allow for acclimatization. Trpv4−/− mice were acquired with permission of Riken Bioresource Center and breeding pair was kindly provided by Dr. D. X. Zhang and housed in a local breeding colony. Mice aged 6 to 8 weeks were used in all UUO experiments. For wild type UUO surgeries, only male mice were used. For UUO of Trpv4−/− mice, both males and females were used. For induction of surgical obstruction, mice were anesthetized with 2-3% isoflurane, and administered pre-operative medications and isotonic saline. Following a flank incision and entry into the peritoneum, the right ureter was isolated and either ligated with a 5-0 Prolene suture or sham manipulated. Following closure, mice were monitored until fully recovered. For all analyses, 3 mice were used per time point ($N=3$).

#### 8.4.2. Sample Collection

Mice were euthanized at 1, 2, 3, 6, 9 days post-surgery and control, sham, obstructed, and contralateral kidneys collected. Kidneys were either paraformaldehyde fixed and paraffin-embedded as in section 8.1.2, or embedded in
OCT (Tissue-Tek) and flash frozen in isopentane cooled in dry-ice. Frozen tissues were sectioned using a cryostat (Leica), fixed at -20°C in 50:50 methanol/acetone and stained as per section 8.1.2.

8.4.3. Morphometric Analysis of \( \alpha \)SMA Area

Morphometric measurements were made using sections stained for \( \alpha \)SMA via fluorescent immunohistochemistry as per section 8.1.2. 10 representative 40x high power fields were captured from both cortex and outer medulla (with equal representation of the inner and outer portions of each region. Using a 99-point grid in Adobe Photoshop (11 vertical and 9 horizontal lines) (Figure 5.5B), points the fell on \( \alpha \)SMA-positive area were counted from 3 kidneys for each time point and experiment condition (control, sham, obstructed). Results are expressed as the number of \( \alpha \)SMA-positive points per high power field. Results were compared using a standard two-tailed t-test, and expressed with their standard deviations.

8.4.4. Analysis of Apoptosis via TUNEL

TUNEL analysis was performed using the Apoptag-FITC kit (Millipore) as directed. Following TUNEL stain, increased sensitivity and prolonged fluorescence was achieved by incubating slides with dilute anti-sheep AlexaFluor488 (1:1000, Invitrogen). Slides were counterstained with rabbit anti-vATPase (1:100, Santa Cruz).

8.4.5. Immunoprecipitation

Using either rabbit anti-TRPV4 or mouse anti-\( \beta \)Cat antibodies, TRPV4 or \( \beta \)Cat respectively was precipitated from lysates using protein-A sepharose beads and
collected by centrifugation. Precipitates were resuspended in sample buffer and analyzed for co-precipitation of TRPV4, βCat or E-Cad via immunoblotting.

8.4.6. Immunoblotting

Protein lysates for immunoblotting were made from 150 μm trim sections from flash frozen kidneys collected in protein lysis buffer containing 50:50 TPER Buffer (Thermo, Rockford, IL)/SDS buffer containing protease inhibitors (Roche, Quebec). Protein lysates were quantified using a NanoDrop spectrophotometer (NanoDrop) and immunoblotted as described in section 8.1.5.

8.4.7. Polymerase Chain Reaction

Mouse cDNA pools were generated as per section 8.1.3 and TRP expression analyzed via PCR amplification using primers custom designed using Primer3 (Table 8.2). The resulting samples were run on a 1% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

8.4.8. LCM, quantitative PCR and TRPV4 Gene Expression

Multiple frozen sections of sham and obstructed kidneys were sectioned onto PEN membrane frame slides (Life Technologies). For each time point and condition, multiple regions of inner medulla or outer medulla were collected on the same LCM cap and lysed in RLT buffer containing β-mercaptoethanol. RNA extraction and quantitative PCR were performed as per sections 8.1.3 and 8.1.4 respectively. The Taqman TRPV4 probe (#Mm00499025_m1) and primer set was used and normalized to (4351309) Taqman GAPDH endogenous control kit.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (Probe reporter dye)</th>
<th>Amplicon</th>
</tr>
</thead>
</table>
| TRPM3 | Forward Primer 5'-ACTCAAGCAAGCTCTCGGG-3'  
Reverse Primer 5'-CAGTTGTCTCGGAGGTCA-3' | 346 bp  |
| TRPM4 | Forward Primer 5'-GAGAGGTATGACCCGAAA-3'  
Reverse Primer 5'-CTCCCAACAGAGCTCTCAGC-3' | 481 bp  |
| TRPM6 | Forward Primer 5'-TGACCAGTTGAATGCAGAGC-3'  
Reverse Primer 5'-AGCTGGATTGGTGAACCGG-3' | 378 bp  |
| TRPM7 | Forward Primer 5'-GCAAAACATGTGTGAGTC-3'  
Reverse Primer 5'-CTCAGTCTGACTTCGCCC-3' | 248 bp  |
| TRPP1 | Forward Primer 5'-CTATCCAGGCAAACCTGT-3'  
Reverse Primer 5'-TGCAGGAGGCTTGAGAGA-3' | 577 bp  |
| TRPP2 | Forward Primer 5'-GTGGTTGCGAACACTGAATT-3'  
Reverse Primer 5'-TCTCCAGCTTGACACAGC-3' | 412 bp  |
| TRPV1 | Forward Primer 5'-GCAGTCTCAAAGACCCAGA-3'  
Reverse Primer 5'-CTTCAGTGCGGGGTGAAT-3' | 535 bp  |
| TRPV4 | Forward Primer 5'-ACAGGAAGCGCTTGACTGAT-3'  
Reverse Primer 5'-GTTGTTCTCGGGTGTTGT-3' | 491 bp  |
| TRPV6 | Forward Primer 5'-GGGTGAATGTGATGCA-3'  
Reverse Primer 5'-TTAACAGTGAGCAGTGCGG-3' | 484 bp  |

**Table 8.2: Custom designed, mouse-specific TRP primers.** Using Primer3, mRNA specific primers were generated to examine the gene expression of selected TRPs in the kidney and in mIMCD3 cells.
8.4.9. Antibodies

Primary antibodies used: (1) Immunohistochemistry - goat anti-AQP2 (Santa Cruz); rabbit anti-AQP2 (Sigma); rabbit anti-vATPase (Santa Cruz); mouse anti-β Catenin (BD); mouse anti-E-Cadherin (BD); mouse anti-E-Cadherin Alexa555 conjugate (BD); mouse anti-α Smooth Muscle Actin (Sigma); rabbit anti-Coll IV (Fitzgerald Industries); rabbit anti-TRPV4 (Alomone Labs); (2) Immunoblotting - rabbit anti-AQP2 (Sigma), rabbit anti-vATPase (Santa Cruz), mouse anti-β Catenin (BD), mouse anti-E-Cadherin (BD), mouse anti-α Smooth Muscle Actin (Sigma), rabbit anti-TRPV4 (Alomone Labs), rabbit anti-Creb (Cell Signaling), mouse anti-vinculin (Upstate); (3) Immunoprecipitation - mouse anti-β Catenin (BD), rabbit anti-TRPV4 (Alomone Labs).

Secondary antibodies used: (1) Immunohistochemistry - Alexa-488, 568 or 633 conjugated antibodies against mouse, rabbit or goat (Invitrogen) (2) Immunoblotting - HRP conjugated anti-rabbit or anti-mouse (Cell Signaling).

8.4.10. Statistical Analysis

For each experiments, 3 animals were used (N=3). For densitometry, results are presented as the mean of the values for 3 animals ± standard deviation. The results were compared using a standard two-tailed t-test (p < 0.01), and expressed with their standard deviations. For morphometric analysis, 10 representative fields from both the cortex and medulla were scored. Mean values for each kidney were compared via 2-way ANOVA and Tukey multiple-group comparison (p < 0.05).
8.5. **Chapter 6: In Vitro Cell Culture Model Analyses**

8.5.1. **Cell Line and Culturing**

Immortalized mouse mIMCD3 cells (ATCC) were cultured in DMEM/F12 media supplemented with 10% FBS and 100U/m Penicillin and Streptomycin. For experimentation, mIMCD3 cells were passaged onto hanging cell culture supports (Millipore and Corning). For IHC and protein expression analyses, cells were plated on hanging inserts at 7000 cells/cm², cultured 24 hours prior to treatment.

8.5.2. **Agonist and Antagonist Stimulation**

For IHC analysis, and to determine the time- and dose- response of TRPV4 activation, cells were stimulated with the TRPV4-specific agonists 4αPDD and GSK101690A and with the TRPV family antagonist Ruthenium Red (235,241-243). Agonist stimulations were performed in the presence of 10% FBS, as stimulation in the absence of FBS resulted in poor cell survival. Cells were stimulated at concentrations of 1, 10, or 50 μM 4α-PDD, or 5, 20, 50 or 100 nM GSK1016790A solubilized in DMSO for 24, 48, or 72 hours. Similar effects were noted at both at the 10μM - 50μM 4A-PDD range and the 50nM GSK101690A stimulations. The effect of the DMSO vehicle was examined, with effects apparent at the final vehicle concentrations of the 50μM 4α-PDD treatment (5% DMSO). GSK101690A treatments have lower vehicle concentrations of 0.05-0.1% at the highest doses used, and no vehicle effects were observed at these vehicle concentrations. For this reason, and due to its greater potency, the GSK101690A agonist was used for all
subsequent stimulations. Co-treatment of cells with GSK101690A and 10 μM RR, or treatment with RR alone restored normal cellular morphology. Following treatment, hanging inserts were rinsed in ice cold PBS and fixed in methanol overnight at -20°C. Membranes were washed in ice-cold acetone for 1 minute, air-dried and stored at 4°C until needed. For staining, membranes were removed from the hanging inserts, washed in PBS and stained (as per section 8.1.2).

For protein expression analyses, cells were plated and cultured and stimulated as above with 50nM GSK101690A, 50nM GSK101690A with 10μM RR, or with 10μM RR alone for 72 hours. Cell lysates were collected in RIPA buffer supplemented with protease inhibitors (Roche) and analyses by immunoblotting as described in section 8.1.5.

8.5.3. Cell Fractionation

To investigate the expression of proteins in either the cytoplasmic or nuclear compartments, cell fractionation was performed on mIMCD3 cells plated at 50,000 cells/cm², cultured for 24 hours, and stimulated with 50nM GSK101690A for 5 minutes, 1 hour, 6 hours, or 24 hours. Cells were collected in 200 μl of cold PBS using a cell scraper and pelleted by centrifugation. Cytoplasmic fractions were obtained from lysate supernatants following incubation and centrifugation in Buffer A (10mM HEPES, 1.5mM MgCl₂, 10 mM KCl, 1mM EDTA, 0.1% NP40). The remaining nuclear pellet was then washed and repelleted in Buffer B (10mM HEPES, 2mM MgCl₂, 0.1% EDTA, 0.1% EGTA) three times. Following disruption in Buffer C (20mM
HEPES, 0.42 M NaCl, 1.5 mM MgCl₂, 20 % glycerol, 0.1% EDTA, 0.1% EGTA), lysates were centrifuged and the supernatant collected for the nuclear fraction. All buffers contained protease inhibitors (Roche). Cell fractions were normalized using a BCA assay (Pierce) and analyzed via immunoblotting as per section 8.1.5.

8.5.4. Antibodies

Primary antibodies used: (1) Immunohistochemistry - rabbit anti-AQP2 (Sigma); mouse anti-β Catenin (BD); mouse anti-E-Cadherin (BD); mouse anti-E-Cadherin Alexa555 conjugate (BD); rabbit anti-TRPV4 (Alomone Labs); (2) Immunoblotting - mouse anti-β Catenin (BD), mouse anti-E-Cadherin (BD), anti-TRPV4 (Alomone Labs), rabbit anti-Creb (Cell Signaling), mouse anti-vinculin (Upstate); (3) Immunoprecipitation - mouse anti-β Catenin (BD), rabbit anti-TRPV4 (Alomone Labs).

Secondary antibodies used: (1) Immunohistochemistry - Alexa-488, 568 or 633 conjugated antibodies against mouse, rabbit or goat (Invitrogen) (2) Immunoblotting - HRP conjugated anti-rabbit or anti-mouse (Cell Signaling).

8.5.5. Statistical Analysis

For all experiments, 3 replicates were used (N=3). For densitometry and cell fractionation of lysates, results are presented as the mean of the values for 3 replicates ± standard deviation.
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