NOTE TO USERS

PAX2 is reactivated in urinary tract obstruction and partially protects collecting duct cells from programmed cell death.

By

Tiffany J. Cohen

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Department of Experimental Medicine
McGill University
Montreal, Quebec
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"Make visible what, without you, might perhaps never have been seen."

-Robert Bresson

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ABSTRACT

Obstruction of the urinary tract activates apoptotic pathways in collecting duct cells and leads to loss of renal parenchyma prior to surgical intervention. It has been suggested that development molecular programs may be reactivated to offset acute organ damage. One such molecule, *Pax-2*, is expressed throughout the fetal collecting duct and was recently shown to suppress apoptosis during kidney development.

We hypothesized that during urinary tract obstruction (UTO), *PAX-2* expression is reactivated in the mature kidney, partially suppressing apoptosis; and that mice with *PAX-2* mutations will have increased susceptibility to parenchymal damage during UTO.

Wildtype (C3H) and heterozygous *PAX-2* mutant mice (C3H/*PAX-2*^{1Neu}) underwent unilateral ureteral ligation or sham operation at six weeks of age. Kidneys were removed, weighed and assayed for Pax-2 reactivation following 5, 10 and 15 days of UTO. Kidneys were then screened for apoptosis by *TUNEL* staining, and the novel anti-cleaved spectrin assay.

Pax-2 protein expression fell to nearly undetectable levels in the first weeks of life but was sharply reactivated in collecting duct cells of wildtype, but not in *PAX-2*^{1Neu} mutant mice with 10 days of UTO. Wildtype mice with UTO had increased *TUNEL*, cleaved spectrin and reduced kidney weight after 10-15 days UTO. Mutant mice had exaggerated increases in *TUNEL* staining, cleaved spectrin and parenchymal loss in the obstructed kidney.

In conclusion, we have shown that *PAX-2* is highly expressed in the fetal kidney but is normally down regulated in the postnatal period; it is rapidly reactivated in collecting duct cells during UTO. Mice with genetically limited *PAX-2* expression have heightened susceptibility to apoptosis and renal parenchymal loss following acute UTO.

RÉSUMÉ

L'obstruction de la voie urinaire (OVU) active les cascades apoptotiques dans les cellules des ducts collecteurs rénaux. L'OVU résulte aussi dans la perte du parenchyme rénal. Il a été proposé que des programmes de développements moléculaires puissent être réactivés pour contrecarrer les dommages d'organes. *Pax-2* est exprimée dans le duct collecteur fœtal et a récemment été démontrée à supprimer l'apoptose pendant le développement du rein.

Nous avons voulu determiner si l'OVU réactive l'expression de *Pax-2* dans le rein adulte et supprime l'apoptose. Les animaux ayant une forme mutante de *Pax-2* seriont ainsi plus susceptibles à des dommages du parenchyme rénal suite à une OVU.

Des souris de types sauvages (C3H) et hétérozygotes pour *Pax-2* (C3H/*PAX-2*^{1Neu}) ont subi des obstructions unilatérales de l'uretère ou bien une opération contrôle à l'âge de six semaines. Les reins ont été disséqués et pesés afin d'être traités aux immunohistochimies de *Pax-2* à 5, 10, et 15 jours postopératoires. Par la suite, l'apoptose des reins a été détecté par *TUNEL* et par immunohistochimie de spectrine clivée, une méthode récemment décrite.

L'expression de *Pax-2* chute à des niveaux presque indécelable dans les premières semaines suivant la naissance. Cependant, elle est réactivée à 10 jours postopératoires dans les cellules normales des ducts collecteurs, mais non dans les cellules des souris mutantes *PAX-2*^{1Neu} avec OVU. Les souris de types sauvages avec OVU ont démontré plus de *TUNEL*, de spectrine clivées et une masse des reins plus légère après 10-15 jours postopératoires d'OVU. Les souris mutantes avaient beaucoup plus de *TUNEL*, de spectrine clivées et de perte parenchymale dans le rein obstrué.

En conclusion, la protéine *Pax-2* est exprimée dans le rein fœtal, mais disparaît normalement, après la période postnatale. Elle est rapidement réactivée dans les cellules des ducts collecteurs ayant une OVU. Les souris ayant génétiquement moins de *Pax-2* sont susceptibles à plus d'apoptose et de perte du parenchyme rénale suivant une OVU.

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ABBREVIATIONS

ANG	Angiotensin
bp	Base pair
CED	Cell death gene
cM	Centi Morgan
CNS	Central nervous system
DAB	3'3-Diaminobenzidine
DNA	Deoxyribonucleic acid
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
GDNF	Glial-cell-derived neutrophilic factor
GFR	Glomerular filtration rate
HCL	Hydrochloric acid
IGF	Insulin-like growth factor
kDa	Kilo Dalton
Krd	Kidney and retinal degeneration
MM	Metanephric mesenchyme
mRNA	Messenger ribonucleic acid
NTP	Nucleotide tri-phosphate
PAX	Paired box gene
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
RBF	Renal blood flow

RCC	Renal cell carcinoma
RCS	Renal-coloboma syndrome
RET	Rearranged during transfection
SDS	Sodium dodecyl sulphate
TGF-β	Transforming growth factor-beta
TNF-α	Tumor Necrosis Factor-alpha
TUNEL	Terminal dUTP nicked end labelling
UB	Ureteric Bud
UTO	Urinary Tract Obstruction
UTP	Uracil tri-phosphate
UUO	Unilateral Ureteral Obstruction
WT-1	*Wilms* tumor gene 1
Z-VAD-fmk	*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

LIST OF TABLES AND FIGURES

PUBLICATIONS ARISING FROM WORK COMPLETED FOR THESIS

ABSTRACT (Oral Presentation)
Pediatric Academic Societies' Annual Meeting, San Francisco, CA, (2004).

PAX-2 Is Reactivated in Urinary Tract Obstruction and Partially Protects Collecting Duct Cells From Programmed Cell Death.

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PAX-2 Is Reactivated in Urinary Tract Obstruction and Partially Protects Collecting Duct Cells From Programmed Cell Death.

1. INTRODUCTION

1.1 Urinary Tract Obstruction

Congenital obstructive nephropathy is a leading cause of chronic renal failure in infancy (Chevalier 1995). Acute urinary tract obstruction (UTO) is characterized by impaired renal growth and development and a reduction in nephron number (Warady 1997). Prolonged obstruction leads to tubular atrophy, and interstitial fibrosis, which are hallmarks of advanced obstructive nephropathy (Chevalier 1999). Many studies have demonstrated heterogeneity in the nephric response to acute or chronic kidney disease; hypertrophy occurs in some nephrons, while progressive atrophy is observed in others (Bonventre 1998; Cachat 2003).

1.1.1 Experimental models of UTO

Most of our understanding of UTO stems from experimental animal models. There is some discrepancy in these findings, and the lack of standardized animal models may be accountable. Moreover, the majority of reports focus on the animals after release of obstruction and may thus very well reflect the response to release of obstruction rather than functional characteristics of the obstructed kidney. Generally, UTO results in a decrease in renal blood flow (RBF) and glomerular filtration rate (GFR) in the ipsilateral kidney; and increased RBF to the intact opposite kidney (Chevalier 1998). However, the effects of UTO on RBF and GFR depend on the duration and degree of obstruction. Also seen in acute UTO are tubular atrophy, interstitial fibrosis, and an altered expression of growth factors (Liapis 2003). A number of circulating hormones and paracrine factors are also implicated in the renal vasoconstriction, including angiotensin (Chevalier 1999;

Liapis 2003). Although progress has been made in this rapidly expanding field, our knowledge of obstructive nephropathy is still limited.

1.1.2 Changes in renal morphology

The changes in renal morphology following UTO depend on the degree and duration of obstruction, as well as the time of onset. Interstitial fibrosis and progression in radial scarring developed in the kidney in response to increasing periods of obstruction. In adult models the gradual destruction or atrophy of the renal parenchyma was associated with increased hydronephrosis (Himmelstein 1990). Hydronephrotic atrophy leads to the destruction of all renal parenchymal tissue, leaving behind a thin-walled sac of watery fluid (Fink 1980). Conversely, the acutely obstructed kidney may increase its weight within hours of obstruction due to renal parenchymal edema (Moody 1977).

1.1.2.1 Interstitial fibrosis

The pathologic marker of irreversible renal injury is interstitial fibrosis (Chevalier 1999). Interstitial fibrosis involves an increased interstitial volume due to matrix deposition, fibroblast differentiation/proliferation, and monocyte infiltration (Klahr 2003). The amount of collagen present in the interstitium and tubular basement membranes is partially associated with the degree of irreversible kidney damage (Cachat 2003). Macrophage infiltration of the glomerular and tubulointerstitial compartments results in the release a variety of products that potentially could injure the glomerulus, including proteolytic enzymes (collagenase, elastase), reactive oxygen products, platelet-derived growth factor (PDGF), coagulation factors and platelet-activating factors (Nathan 1987).

1.1.2.2 Tubular Atrophy

Over time, chronic UTO can also lead to tubular atrophy. Tubular atrophy, most likely results from a progressive loss of tubular cells by (Lange-Sperandio 2002). Tubular atrophy in the obstructed kidney is manifest by a thickened or sometimes duplicated tubular basement membrane (Moller 1984).

Tubular atrophy and interstitial fibrosis are almost invariably associated (Cachat 2003). However, the relationship between these two phenomena is not well understood. Trans-differentiation of tubular epithelial cells into fibroblasts may explain their association (Strutz 1995). Alternatively, fibrosis may promote atrophy releasing cytokines that induce apoptosis, such as TNF-α and Fas-ligand; or by secreting an abnormal ECM (Gonzalez-Cuadrado 1994; Sugiyama 1995).

1.1.3 Changes in renal blood flow

The immediate hemodynamic response to UTO is variable, but complete obstruction of the ureter results ultimately in a progressive reduction in ipsilateral RBF (Clausen 1977). The reduction in RBF during acute UTO is thought to be caused by an intrarenal vasoactive mechanism (Klahr 1985). This mechanism involves an early and continuous vasoconstriction that results from the activation of the renin-angiotensin system, with elevated renal renin expression in the obstructed kidney (Chevalier 1985; Harris 1991; Cachat 2003).

Inhibition of angiotensin type II (ANG II) in the neonatal guinea pig with chronic UTO prevents the reduction in renal blood flow (Chevalier 1985). This activation of the renin-angiotensin system accounts for at least 50% of the interstitial fibrosis that accompanies chronic obstructive nephropathy (Cachat 2003). Predominance of the ANG II receptor in the neonatal rat contributes to the heightened tubular apoptosis that follows UTO (Chevalier 1999). The rennin-angiotensin system therefore plays a role not only in controlling renal blood flow, but also plays a role in initiating other characteristic features of obstructive nephropathy.

1.1.4 Changes in renal function

In adult animal models, pelvic pressure increased immediately in response to complete unilateral ureteral obstruction, and GFR decreased when the pelvic pressure exceeded 20 mmHg (Hvistendahl 1996). The changes in renal function occurred during the first 24 hours of UTO. In rats, GFR was reduced to 52% of baseline value at 4 hours, 23% at 12 hours, and 4% at 24 hours (Harris 1981). After 24 hours of UTO, the continued decrease in GFR of the obstructed kidney was associated with a compensatory GFR increase in the contralateral kidney (Harris 1981; Capelouto 1993). Redistribution of GFR from the surface nephrons to the deep nephrons was seen during UTO (Capelouto 1993). This corresponds with blood flow redistribution from the outer cortex to the inner cortex and outer medulla (Clausen 1977).

There are conflicting reports on the ipsilateral GFR changes. Navar (1970), shows a dramatic yet transitory increase in GFR following UTO, using dogs. In contrast, studies

in rats and pigs uniformly show an immediate reduction in ipsilateral GFR after onset of acute obstruction (Hvistendahl 1996); (Provoost 1981). These findings indicate that there are major variations in the reactive mechanisms among species.

1.1.5 Growth Factors

A range of genes including growth factors tightly controls how the kidney responds to acute obstructive nephropathy. Growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) have recently been shown to play a role in the development and progression of fibrotic and sclerotic changes in the obstructed kidney. It can be inferred that the increased production of growth factors following acute UTO serves as the kidney's inborn mechanism of self-repair.

1.1.5.1 PDGF

Administration of PDGF to rats was associated with enhanced development of fibrotic changes in the chronically obstructed kidney (Nathan 1987). In vitro experiments, using renal interstitial fibroblasts, showed that PDGF induces fibroblast proliferation (Knecht 1991). In an opossum model of complete urinary tract obstruction, it has been reported that PDGF mRNA correlates with the morphologic features of tubulointerstitial damage indicating that PDGF participates in this form of kidney damage (Liapis 1994).

1.1.5.2 TGF-β

TGF- β is a cytokine, which stimulates ECM synthesis and inhibits its degradation (Roberts 1992). Expression of TGF-β1 mRNA is increased in the obstructed kidney (Walton 1992). Recent evidence also suggests that the infiltrating macrophage may play a role in propagating initial glomerular injury to the development of glomerulosclerosis via TGF- β stimulating matrix accumulation (Diamond 1994). (Diamond 1995) proposed that the markedly increased expression of TGF- β1 following UTO induced a pro-fibrogenic state and set off a cascade of disruptive events including the up regulation of tissue inhibitors such as metalloproteinase. In addition, TGF- β1 may serve as a potential stimulus for the modulation of quiescent interstitial fibroblasts into myofibroblasts (Diamond 1995).

1.1.5.3 EGF

The decrease of EGF expression has been associated with the development of obstructive nephropathy (Walton 1992). In addition, the injection of EGF in 1-day-old rat pups reduces the fraction of apoptotic renal cells by 50% in less than 2 hours (Coles 1993), indicating that the reduced EGF in the obstructed kidney would contribute to increased apoptosis. Furthermore, EGF has been shown to have anti-proliferative effects in the developing kidney (Gattone 1992).

1.1.5.4 IGF

In the obstructed kidney, administration of insulin-like growth factor 1 (IGF-1) attenuates the development of fibrosis, tubular cystic change and caliceal dilation (Steinhardt 1995). Recovery from acute renal failure in rats has been shown to be accelerated by

recombinant human insulin-like growth factor-1 (rhIGF-1) (Ding 1993). Clinical trials with IGF-1 have demonstrated its safety and maintenance of GFR in postsurgical patients (Carmichael 2003).

1.1.6 Cell death and tissue loss

Abnormalities of cell number are a frequent feature of renal disorders. Cell death is a key factor in the regulation of cell number. Apoptosis is an active form of cell death that is modulated by extracellular lethal and survival signals. Both apoptosis of intrinsic renal cells and of infiltrating leukocytes may contribute to the pathogenesis of renal disease.

Apoptosis triggered by ischemia or endogenous mediators of damage may be the initial insult capable of causing nephropathy. Apoptosis may also contribute to the persistence of renal injury. Progression of renal disease may be a consequence of a persistently high apoptotic rate of renal parenchymal cells leading to glomerular or tubular atrophy. Alternatively, a low rate of fibroblast apoptosis may promote renal fibrosis.

Tubular cell apoptosis has been observed during ischemic, toxic, and obstructive acute renal failure (Chevalier 1996; Cachat 2003). Apoptosis may also occur in cells proliferating in a compensatory fashion after renal injury (Lange-Sperandio 2002; Cachat 2003). These cells would be more sensitive to absolute or relative deficits in survival factors. In this setting, apoptosis might contribute to the persistence or delayed recovery from acute renal failure.

Other studies have also examined *in vivo* apoptosis in experimental UTO. Santarosa found that bladder outflow obstruction in adult rabbits caused an increase of apoptosis (1994). (Chuang 2002) reported that apoptosis was up-regulated in smooth muscle of obstructed ureters in adult rats (2002). Several studies have also examined the effects of experimental UTO on the developing kidney. Matsell reported that fetal ureteric obstruction in the monkey, was associated with increased apoptosis in the tufts of developing glomeruli (2002a). Furthermore, Chevalier found that ureteric obstruction in neonatal rats caused apoptosis in some tubules (2000). Finally, using ureteric obstruction in the developing opossum, Liapis reported a complex picture with enhanced apoptosis and proliferation in the renal interstitium, and a predominantly apoptotic response in tubules (2000).

1.2 Apoptosis

1.2.1 Physiology of Apoptosis

'Apoptosis' is a term that was coined by Kerr in 1972 to describe a succession of morphological changes shared by dying cells in various biological systems. Apoptosis is now described as a genetically programmed, morphologically distinct form of cell death that is triggered by a variety of physiological and pathological stimuli (Earnshaw 1999). The most important morphological features of apoptosis are cell shrinkage, accompanied by transitory but aggressive blebbing from the surface, culminating in the severance of the cell into clusters of membrane-bound bodies (Wyllie 1980b). One hallmark pattern early in the process of cell death is inter-nucleosomal fragmentation of DNA into approximately 200 base-pair fragments (Wyllie 1980a; King 1998). Organellar integrity

and structure is typically preserved, but the nuclear chromatin undergoes a distinguishing condensation, which then generates very dense, cap-like, heterochromatic regions (Earnshaw 1999). The appearance of several characteristic cell surface molecules also seems to be altered, causing apoptotic cells to be immediately and specifically recognized and cleared by neighbouring cells or professional phagocytic cells (macrophages) before lysis (Orrenius 1995). Such clearance allows for massive cell turnover without induction of an inflammatory response (Orrenius 1995; King 1998).

Apoptosis is responsible for cell death in development, atrophy induced by endocrine and other stimuli, normal tissue turnover, negative selection in the immune system, and a considerable amount of T-cell killing (Meier 2000). Apoptosis is a major factor in the kinetics of tumors, both growing and repressing. It also accounts for many of the cell deaths that follow exposure to cytotoxic compounds, hypoxia or viral infection. So how does it work?

1.2.2 Caspases

Molecular genetic studies on the nematode *Caenorhabditis elegans* led to the discovery of a set of proteins, widely represented by homologues in other species, that are responsible for turning the final commitment to death on or off. In the nematode these proteins include the products of the *ced3* and *ced4* genes (which initiate cell suicide), *ced9* (which prevents it) and a series of some seven genes involved in recognition and phagocytosis of the doomed cell (Meier 2000).

CED3 is the prototype of a family of around a dozen mammalian proteases, called caspases, because of the obligatory cysteine in their active site and their predilection for cutting adjacent to aspartate residues (Earnshaw 1999; Meier 2000). Over a dozen caspases have been identified in humans; about two thirds of these have been suggested to function in apoptosis (Thornberry 1998; Earnshaw 1999). Caspases are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes and hydra (Budihardjo 1999; Cikala 1999; Earnshaw 1999).

Once activated, caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases. Collectively, these scissions disrupt survival pathways and disassemble important architectural components of the cell, contributing to the stereotypic morphological and biochemical changes that characterize apoptotic cell death (Earnshaw 1999).

1.2.3 Caspase Inhibition

Because they bring about most of the visible changes that characterize apoptotic cell death, caspases can be thought of as the central executioners of the apoptotic pathway (Hengartner 2000). Using what is known about the specific sequences of the caspases, specific competitive inhibitors of apoptosis have been designed (Thornberry 1998). Many laboratories are currently using specific inhibitors of apoptosis in conditions where elevated apoptosis results in degeneration of tissue. Pharmaceutical companies are

particularly interested in the use of caspase inhibitors as a possible therapy in stroke, heart disease and renal disease.

1.2.3.1 Z-VAD-fmk

Z-VAD-fmk is a cell permeable, broad inhibitor of caspases, including caspases 1, 3, 4, and 7. Z-VAD-fmk was shown to reduce the severity of myocardial injury in animal models of myocardial infarction (Dumont 2001). The benefits of caspase inhibition have been observed has been widely used to successfully inhibit apoptosis *in vitro*.

Inhibition of caspase-mediated apoptosis with Z-VAD-fmk also has been shown to reduce brain injury and neuronal loss following cerebral ischemia in rat and mouse models (Jacobson 2002).

1.2.3.2 Endotoxin

Endotoxin, or bacterial lipopolysaccharide, depresses myocardial contractility in laboratory animals and is also responsible for cardiac dysfunction associated with human sepsis (Parillo 1985). Z-VAD-fmk, in conjunction with endotoxin, not only reduced caspase activities and nuclear apoptosis, but also completed prevented endotoxin-induced myocardial dysfunction (Fauvel 2001).

1.2.4 Apoptosis in development

Physiological cell death is an indispensable constituent of animal development, vital for establishment and maintenance of tissue architecture (Saxén 1987; Jacobson 1997).

During animal development, many structures are formed that are later removed by apoptosis (Jacobson 1997). This allows for greater flexibility, as primordial structures can be adapted for different functions at distinct stages of life or in different sexes. For example, in females, the Mullerian duct gives rise to the uterus and oviduct (Grobstein 1955; 1956). In males, the Mullerian duct it is not needed, and is consequently expunged (Vainio 2002).

During development, apoptosis is also used to eliminate structures remnant of ancestral incarnation. This is illustrated in early vertebrate development, where the pronephric kidney tubules arise from the nephrogenic mesenchyme (Rothenpieler 1993; Kuure 2000). Although these pronephric tubules form functioning kidneys in fish and in amphibian larvae (Saxén 1987), they are not active in mammals and degenerate.

Apoptosis also functions as part of a quality control and repair mechanism during development by compensating for many genetic or stochastic developmental errors (Jacobson 1997; Gartner 2000). Cells that have been programmed incorrectly are, effectively, cells that will go astray. These cells will fail to receive their appropriate trophic signals for survival and will consequently activate their innate auto-destruct mechanism (Hengartner 2000).

1.3 Kidney Development

1.3.1 The evolution of kidney development: a three-part phenomenon

The building of intricate organs during embryonic life is one of the most interesting and complex developmental processes. The morphogenesis of the mammalian kidney, with its characteristic shape and size, defined structural composition and specialized physiological properties, is the result of coordinated gene action that directs the developmental fate of cells participating in the process. The acquisition of different renal cell fates involves interplay of cell proliferation, differentiation, migration, growth, and death. The elaboration and culmination of these cellular ensembles requires precision in both a temporal and spatial manner.

The mammalian kidney has long served as an important model for studying the vertebrate excretory system (Saxén 1987; Lechner 1997; Schimmenti 1997; Davies 2000). The excretory systems of all vertebrates consist of filtration units that allow small molecules to pass from the blood out into the urinary space, specialized epithelia that selectively recover solutes and return them to the blood, and drainage ducts to move urine out of the body (Davies 1999; 2000). The functional unit of the vertebrate excretory system, the nephron, consists of: the vascular loop of the glomerulus, the capsule, the nephrocoel, the nephric duct, the nephrostome, and the tubule (Saxén 1987).

To understand the molecular development of the kidney, one must first be familiar with the morphologic events that take place during kidney development. The mammalian kidney represents an excellent example of ontogeny representing phylogeny with three sets of kidneys developing successively: the pronephros, the mesonephros and the metanephros (Saxén 1987; Kuure 2000). The more simple forms of the kidney (the

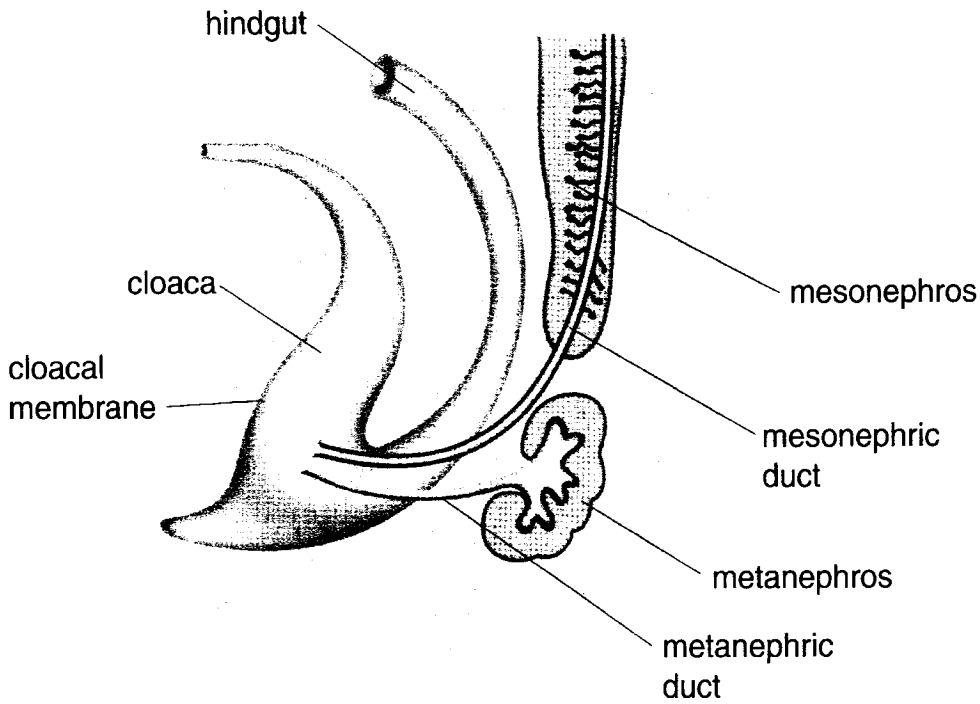
pronephroi and mesonephroi) are transient structures during higher vertebrate embryogenesis, but are fully exploited in lower vertebrates (Saxén 1987).

1.3.1.1 The pronephroi

The pronephros is analogous to the kidney of a primitive fish, and serves as the excretory organ in the larval stages of amphibians. In both human and mouse embryos, the pronephros appears in the cervical region around the third week of gestation in humans (E22) and at about E8 in mice. They do not undergo differentiation into any recognizable nephrons and have no significant excretory function (Robillard 1994). The pronephroi undergo regression in the fifth week of human gestation (Saxén 1987; Jacobson 1997).

1.3.1.2 The mesonephroi

The mesonephroi are the permanent kidneys in amphibians, and function briefly in mammals until their metanephroi are fully developed. The mesonephroi appear immediately caudal to the last of the pronephric tubules, and may actually partially overlap the most caudal part of the pronephros. The mesonephroi consist of vascularized glomeruli and distinct proximal tubules that drain into the mesonephric duct, which in turn, opens into the cloaca (Fig. 1) (Saxén 1987; Robillard 1994). The mesonephric tubules however, have no loop of Henle or differentiated distal tubular segments. The first mesonephric nephrons are seen around 24 to 25 days of gestation in humans (E24) and about E9.5 in mice. As with the pronephros, the earliest mesonephric nephrons start to degenerate (Robillard 1994).



Adapted from Hallgrimsson, 2003

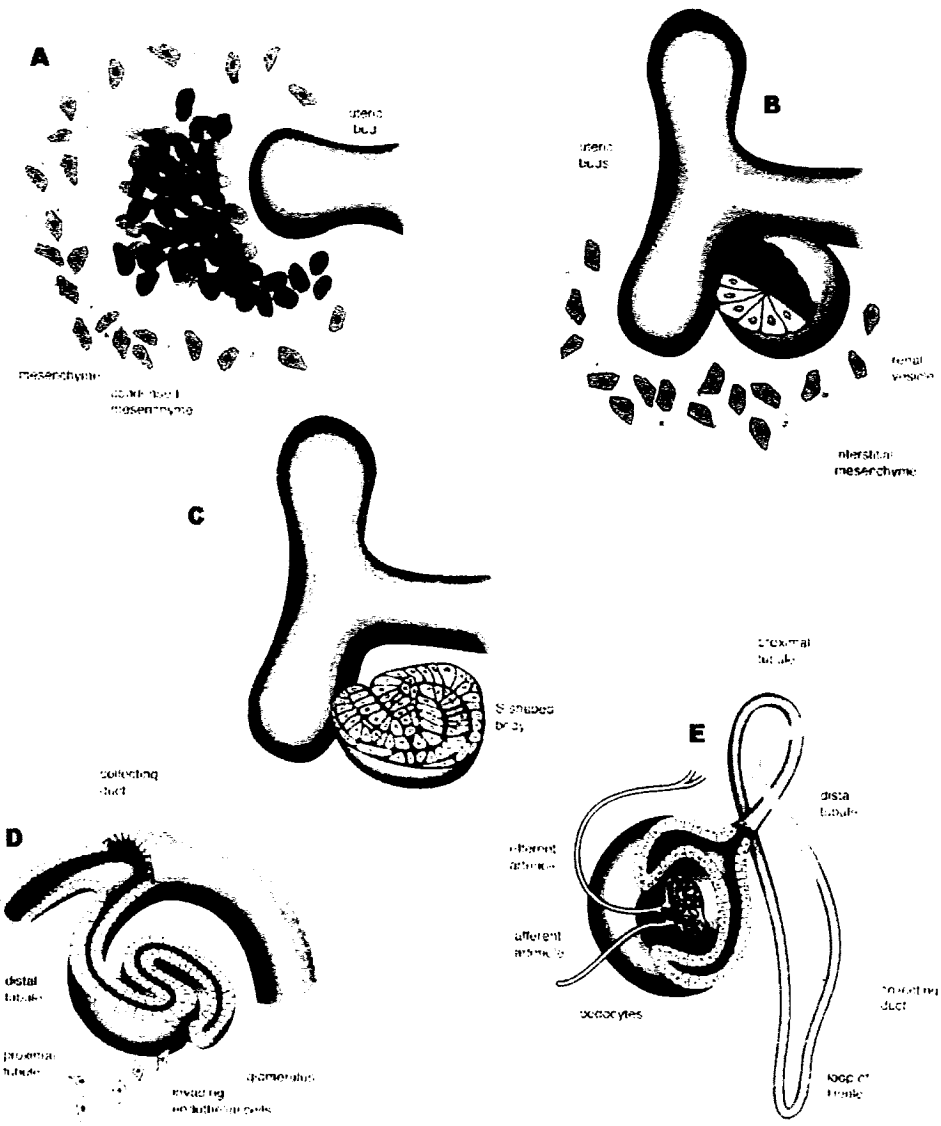
Figure 1: Development of the mammalian kidney. The mesonephroi function briefly in mammals until their metanephroi are fully developed. The mesonephroi appear immediately caudal to the last of the pronephric tubules, and may actually overlap the most caudal part of the pronephros.

1.3.1.3 The Metanephroi

The permanent mammalian kidneys, the metanephroi, begin to develop at E35-E37 in humans and E11 in mice, with the demarcation of the metanephric blastema in the caudal part of each intermediate mesoderm (Kreidberg 1993; Torres 1995). This blastema is a domain of perhaps a few thousand cells, and is usually known as the metanephric mesenchyme (MM). A small epithelial bud or diverticulum also known as the ureteric bud (UB) then emerges from the lower end of the Wolffian duct near its entry into the cloaca and future bladder (Lipschutz 1998; Cho 2003).

The metanephros forms as a result of a reciprocal epithelial-mesenchymal induction between the epithelial UB and the MM (Grobstein 1955; Saxén 1987; Cho 2003). Mesenchymal cells aggregate at the tips of the UB, and the UB induces the mesenchyme to condense (Fig. 2A). The mesenchyme in turn induces the UB to branch (Fig. 2B), as the condensing mesenchyme becomes polarized into a renal vesicle. The renal vesicleundergoes an epithelial conversion and elongates into an S-shaped body (Fig. 2C) (Grobstein 1956; Saxén 1987; Hammerman 1992; Cho 2003). The glomerulus is beginning to take shape and much of the epithelia now expresses specific markers for proximal or distal tubules (Fig. 2D). Proliferation of tubular epithelium generates a highly convoluted proximal and distal part. The glomerular tuft develops, as endothelial cells and podocytes come into contact to form the nephron. (Fig.2E).

The stalk of the UB forms the ureter and the cranial end forms the renal pelvis and collecting ducts (Saxén 1987; Lipschutz 1998). The collecting tubules merge with the



Adapted from Cho, 2003

Figure 2: Nephrogenesis. Mesenchymal cells aggregate at the tips of the UB, causing the mesenchyme to condense (A). The mesenchyme in turn induces the UB to branch (B). The condensing mesenchyme undergoes an epithelial conversion and elongates into an S-shaped body (C). The epithelia now express specific markers for proximal or distal tubules (D). The glomerulus develops, and the nephron forms (E).

mesenchyme-derived secretory nephrons and drain the urine into the renal pelvis (Saxén 1987; Sariola 1997). The urine is now free to travel down the ureter into the bladder where it is excreted.

1.3.2 Regulatory molecules in kidney development

For a candidate gene to be indisputably shown to be involved in organ development, three conditions are required. First, the gene must be expressed where organ development is taking place. Second, the gene has to be expressed at the time of organ development. Finally, if the gene is disrupted, normal organ development must not occur (Lipschutz 1998).

Of all the genes involved in kidney development, 11 have been shown by gene disruption studies to be necessary for the development of functional kidneys (Lipschutz 1998). The four that will be described below are those that cause defects in early kidney development.

1.3.2.1 *c-ret*

c-ret encodes a receptor tyrosine kinase, which is normally expressed in the developing nervous system. It is also found to be expressed in the mesonephric duct, and the UB of the developing kidney, but not in the MM (Pachnis 1993).

Mice with a *c-ret* null mutation die within one day of birth due to renal failure. Mutant kidney rudiments, if present, have reduced recognizable nephric elements, substandard

UB branching without formation of mature collecting ducts, and large regions of undifferentiated mesenchyme (Schuchardt 1994).

1.3.2.2 *GDNF*

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-β (TGF–β) superfamily (Durbec 1996; Trupp 1996), and was identified as the ligand for the *c-ret* receptor. *GDNF* is a target-derived guidance cue that ensures proper outgrowth and invasion of the UB into the MM (Brophy 2001). Mice with mutations in *GDNF* demonstrate defects akin to *c-ret* deficient mice, including kidney aplasia and dysplasia (Pichel 1996; Sanchez 1996).

1.3.2.3 *WT-1*

WT-1, Wilms tumor suppressor, is a transcription factor with zinc finger binding domains (Lipschutz 1998). Germline mutations in the tumor suppressor function of the *WT-1* gene result in the Wilms' childhood kidney tumor (Armstrong 1992; Lee 1999). *WT-1* can be detected in the developing mouse renal and gonadal structures and transiently in mesothelium around the heart and gut, ventral spinal cord, and brain (Armstrong 1992). In the developing kidney, *WT-1* is found in the mesonephric tubules, with subsequent expression in derivatives of the MM, but not in the mesonephric duct or UB (Armstrong 1992). *WT-1* knockout mice do not form UBs and have only transient MMs (Kreidberg 1993) (Sariola 1997). *WT-1* mutant mesonephric ducts are intact and can be stimulated by wildtype mesenchyme to sprout Ubs (Kreidberg 1993; Donovan 1999).

1.3.2.4 *Pax-2*

Pax-2 is a paired-box DNA binding protein expressed in developing mouse kidney and nervous tissues including the optic stalk, the midbrain-hindbrain junction, and the spinal cord (Mansouri 1996). In the kidney, *Pax-2* is detected in the caudal mesonephric duct, the UB, and later in mesenchymal condensates induced by the UB (Dressler 1996). As the condensates convert into nephric epithelia, *Pax-2* expression is suppressed (Dressler 1996; Ryan 1996). *Pax-2* null mutant mice do not develop kidneys (Torres 1995). Conversely, unregulated expression of *Pax-2* is associated with cystic, hyperproliferative mouse kidneys (Ostrom 2000). Several developmental roles for *Pax-2* have been identified. In the MM, *Pax-2* activates *GDNF* expression (Brophy 2001), but in the nephric duct it may influence cell fate specification (Bouchard 2002). *Pax-2* has also been shown to have an anti-apoptotic effect in the developing kidney, where heterozygous *Pax-2*^{1Neu} mice have sub optimal UB arborisation (Porteous 2000; Torban 2000). *Pax-2* will be discussed in detail in section 1.4.

1.3.3 Apoptosis in the developing kidney

It is clear that nephrogenesis is an active process of proliferation, restructuring via morphogenesis, and functional differentiation that is controlled by tightly regulated and specific renal gene expression (Orellana 1998). Nephrogenesis also relies heavily on programmed cell death (Coles 1993; Ostrom 2000; Torban 2000).

Many apoptotic bodies were found within the lumen of developing tubules (Ostrom 2000), and increased apoptotic gene expression corresponded to a decreased rate of UB

branching (Porteous 2000; Torban 2000). It was even discovered that human dysplastic kidneys are the result of aberrant temporal and spatial expression of apoptotic genes (Winyard 1996).

These findings insist that cell removal is just as important in renal development as cell "creation" or differentiation to the extent that too many unwanted cells may be just as critical as not enough "wanted" cells

1.4 *Pax-2*

Pax-2 is a member of the paired box (PAX) class of transcription factors, with an important role in embryonic development (Dressler 1993). PAX genes contain a highly conserved 'paired box' DNA-binding domain, and encode a DNA-binding domain, enabling PAX proteins to bind the promoters of specific genes to transcriptionally regulate their expression (Mansouri 1996). The PAX2 gene is located on human chromosome 10, and in mice it resides on chromosome 19. *Pax-2* is critically required during the development of the central nervous system, ear, eye, and genitourinary axis (Nornes 1990; Eccles 1992; Dressler 1993; Dahl 1997; Torban 2000).

1.4.1 *Pax-2* expression in development

Expression of *Pax-2* was identified in the CNS, specifically in the midbrain-hindbrain region and spinal cord (Nornes 1990; Goulding 1993; Alvarez-Bolado 1997). In the ear, *Pax-2* is expressed in primordial organs responsible for auditory functions, the cochlea and the spiral ganglion (Torres 1996). In the developing eye, *Pax-2* is found initially in

the optic stalk and then in the optic nerve, demarcating boundaries between optic nerve and prospective outer retinal layer (MacDonald 1995; Torres 1996). Early in the developing genitourinary system, *Pax-2* is seen in the nephrogenesis duct at the level of the pronephric and mesonephric tubules. Later, *Pax-2* is strongly detected in the Wolffian and Mullerian ducts (Torres 1995). *Pax-2* expression is most prominent in the UB as it emerges from the Wolffian duct and penetrates the MM (Dressler 1990; Torres 1995). As signals from the branching tips of the UB induce the MM, adjacent mesenchymal cells condense into vesicular clusters, which begin to express *Pax-2* (Dressler 1990; Eccles 1998). *Pax-2* is detected in early epithelial structures derived from the induced mesenchyme, the comma- and S-shaped bodies. However, as these structures differentiate into glomeruli and proximal portions of the emerging nephrons, *Pax-2* expression is abated. At birth, *Pax-2* mRNA is undetectable by *in situ* hybridization (Dressler 1990; Eccles 1992).

1.4.2 Role of *Pax-2* in kidney development

Several discrete roles for *Pax-2* during kidney development have been identified. In the MM, *Pax-2* activates *GDNF* expression (Brophy 2001), but in the nephric duct, it may influence cell fate specification (Bouchard 2002). And an anti-apoptotic function for *Pax-2* in the branching UB of the developing kidney was recently identified, where heterozygous *Pax-2*^{1Neu} mice have sub optimal UB arborisation associated with elevated apoptosis of UB cells (Porteous 2000; Torban 2000).

1.4.3 *Pax-2* mutant mouse models

There are currently three *Pax-2* mutant models that further elucidate the role of *Pax-2* in development of the genitourinary system: *Krd* mice (Keller 1994), *Pax-2* knockout mice (Torres 1995), and 1Neu mice (Favor 1996).

1.4.3.1 *Krd* strain

The *Krd* strain carries a large deletion (about 7 cM) in the distal region of chromosome 19 including the *Pax-2* locus (Keller 1994). Heterozygous *Krd* mice develop a high incidence of kidney defects (hypoplastic and cystic kidneys) as well as retinal abnormalities (reduced cell numbers in all nuclear layers of the retina). Homozygotes are lethal in the pre-implantation stage (Keller 1994). This early embryonic lethality of the *Krd* homozygotes most likely results from the disrupted functions of some of the estimated 400 genes within the 7cM deletion. Nonetheless, both renal and ocular phenotypes of the *Krd* mouse are strikingly similar to the other two *Pax-2* mutant models.

1.4.3.2 *Pax-2* knockout mice

The *Pax-2* knockout mouse (Torres et al, 1996) shows severe abnormalities in development of the meso- and metanephric mesenchyme in homozygous embryos that result in the absence of genital tracts and metanephric kidneys. Heterozygous mutants frequently showed reductions in kidney size.

1.4.3.3 *Pax-2*^{1Neu} mice

The *Pax-2*^{1Neu} mouse (Favor 1996) has an insertion of a single guanosine nucleotide on chromosome 19 in exon 2 of the *Pax-2* locus, causing a frameshift mutation, which results in a premature stop codon (Fig. 3). This single base pair insertion is identical to the mutation seen in humans with Renal Coloboma Syndrome (RCS). Heterozygous 1Neu mice, like humans with RCS, have renal hypoplasia as well as ocular colobomas, while homozygous 1Neu mice have complete renal agenesis, severe ocular and auditory anomalies, as well as loss of the mid- to hindbrain region, and die within hours of birth (Favor 1996).

All three abovementioned mouse models as well as the rare human condition, RCS, exhibit severe kidney abnormalities, suggesting that *Pax-2* plays a critical role in normal kidney development.

1.4.4 *Pax-2* and renal malformations

1.4.4.1 Renal cell carcinoma

Another kidney-derived tumor in which *Pax-2* expression has been observed is renal cell carcinoma (RCC), a common adult renal malignancy, which is thought to arise from the proximal tubule epithelium (Anglard 1992). A high proportion (73%) of RCC cell lines and primary RCC tumors express *Pax-2* (Gnarra 1995).

Pax-2 expression may be part of the intricate progression of RCC tumorigenesis by either of two mechanisms. *Pax-2* may become reactivated in differentiated tubular epithelial cells and may represent a proliferation stimulus. Alternatively, failure to suppress *Pax-2*

during development may predispose renal epithelial cells to additional genetic lesions in an involved oncogenic transformation pathway. Either process would be dependent on *Pax-2* expression in the context of mutations in regulatory genes (Gnarra 1995).

1.4.4.2 Renal coloboma syndrome

Renal coloboma syndrome (RCS) is an autosomal dominant disorder, which results from a mutation in the *Pax-2* gene (Sanyanusin 1995; Porteous 2000). There are many mutations of the *Pax-2* gene which result in RCS, with the most common of these mutations being the insertion of an 8^{th} guanosine on the 5' end of the paired box domain on chromosome 10q25 (Schimmenti 1997; Porteous 2000). RCS is characterized by a range of phenotypes, such as anomalies of the central nervous system (mid/hind-brain, spinal cord), anomalies of the excretory system, ocular anomalies and hearing loss (Sanyanusin 1995; Schimmenti 1997; Cunliffe 1998). The most common phenotypes associated with RCS are renal hypoplasia and optic nerve colobomas (Weaver 1988; Sanyanusin 1995; Schimmenti 1997). End-stage renal failure is common in RCS, but the mechanism by which *Pax-2* mutations lead to renal failure is unknown (Porteous et al, 2000).

1.4.4.3. 1 Neu mutation

Favor first characterized the 1Neu mouse in 1996. The 1Neu mutation consists of an insertion of an 8^{th} guanosine nucleotide on chromosome 19 (exon 2), causing a frameshift mutation that results in a premature stop codon. This single base pair insertion observed in the 1Neu mouse is identical to the mutation seen in humans with RCS; therefore, the

1Neu mouse serves as a perfect model of humans with RCS. Heterozygous 1Neu mice, like patients with RCS, have renal hypoplasia as well as ocular colobomas, while homozygous 1 Neu mice have complete renal agenesis, severe ocular and auditory anomalies, as well as loss of the mid- to hindbrain region, and die within hours of birth.

1.4.4.4 Wilms Tumor

Wilms tumor is a childhood embryonic neoplasm of the kidney in which *Pax-2*, and a number of other renal developmental genes, have been shown by *in situ* hybridization and immunohistochemistry to be misexpressed (Dressler 1992; Eccles 1992; Eccles 1995).

WT-1 is a tumor suppressor gene that is mutated in Wilms tumors. *Pax-2* and *WT-1* encode transcription factors expressed in patterns that overlap both spatially and temporally during fetal kidney development. It has been suggested that *WT-1* regulates *Pax-2* expression(Ryan 1996). In its mutated form, *WT-1* would be unable to normalize *Pax-2* expression, and this accounts for the over expression of *Pax-2* (Dressler 1996). Whether the persistent expression of *Pax-2* in Wilms tumor simply reflects the embryonic stage of development from whence the tumor tissue arose, or whether it signifies a direct or indirect involvement of persistent *Pax-2* expression in Wilms tumor, remains to be determined (Eccles 1998).

2. RESEARCH PROPOSAL

Apoptosis plays a crucial role in the evolution of renal malformations and in the progression of obstructive nephropathy (Chevalier 1999a). Elucidation of the mechanisms underlying renal apoptosis in these disorders may lead to improved management for patients who suffer from such malady.

It has been suggested that developmental paradigms may be recapitulated in order to restore or preserve mature organ function following acute organ damage (Liapis 2003). Many genes involved in urogenital development have been identified. One of these genes is *Pax-2*. During development, *PAX-2* protects cells from apoptosis. However, in the mature kidney, its expression is undetectable.

In this project, we hypothesize that *PAX-2* is reactivated in the mature, acutely obstructed kidney, helping to suppress apoptosis, and to abate renal parenchymal loss. If so, then the extent to which *PAX-2* is reactivated determines the amount of renal parenchymal loss, and animals with mutations in their *Pax-2* locus (1Neu) will have increased susceptibility to parenchymal damage during UTO.

In order to test this theory, we wish to develop a model of acute renal obstruction in which we can track re-expression of the *Pax-2* gene as well as levels of apoptosis and the integrity of the obstructed kidney.

3. MATERIALS AND METHODS

3.1 General

3.1.1 Breeding of Litters for *Pax-2*^{1Neu} Mutant and Wildtype Mice

On the day of breeding, wildtype (C3H) females were weighed to determine a "starting weight". The females were then crossed with heterozygous 1Neu males. Because 1 Neu mice do not form vaginal plugs, the weight of each of the females in the cage was montinored and this was used to establish pregnancy.

3.1.2 Genotyping of *Pax-2*^{1Neu} mice

To genotype 1NEU mice, a method was used which combines the use of Polymerase Chain Reaction (PCR) and digestion by the restriction enzyme, XcmI (New England Biolabs). Specially designed primers were used to create a restriction site for XcmI that would not otherwise have been present (Fig 3A). The sense and anti-sense primers used were 5' GTGTGAACCAGCTCGGGGGTG 3' and 5' GCCCAGGATTTTGCTGACACAGCC 3' respectively. The actual reaction was done using 10x PCR Buffer (Gibco-BRL, Burlington, ON), 50mM $MgCl_2$ (Gibco-BRL), 1μL each of the sense and anti-sense primers, 2.5mM dNTPs, 1 unit Taq Polymerase (Gibco-BRL), 0.25-0.75 μg of DNA and distilled water to reach a final volume of 20 μL. The cycle began at 95°C for 3 minutes, followed by 35 cycles of 10 seconds at 95°C, 10 seconds at 61°C and 20 seconds at 72°C, ending with an additional 3 minutes at 72°C and a 4°C soak. 3 μL of each of the amplified samples were digested for 1 hour at 37°C with 1 unit XcmI restriction enzyme

A

Wildtype Sequence (Exon2)
5'...**CCA**GCTCGGGGG**GGT**...3'
⇑

1NEU Mutation
↓
5'...**CCA**GCTCGGGGG**GG*G*T**...3'
⇑

primers create a mutation substituting
G for T to create the restriction site for XcmI
in the 1Neu but not wildtype sequence

XcmI Restriction Site:
↓
5'...**CCA**NNNNN NNNN**TGG**...3'
3'...GGTNNNN NNNNNACC...5'
↑

B

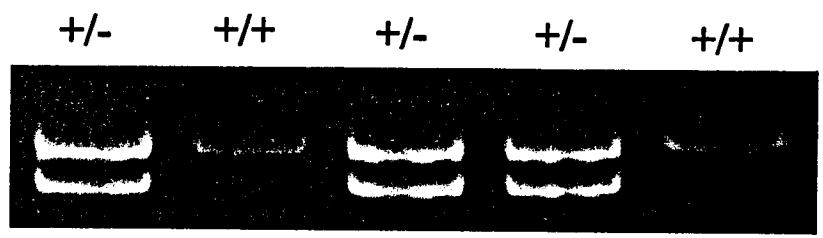


Figure 3. Genotyping Pax2^{1Neu} Mice. The genotyping of 1Neu mice was done by PCR amplification and restriction enzyme digest. Specially designed primers were used to produce a mutation, substituting G for T to create the recognition site for XcmI in the 1Neu but not wildtype sequence (A). Only the 1Neu will be cut, resulting in a product 15 bp shorter than the wildtype. The wildtype band is seen at 166bp, while the Pax2^{1Neu} mutant band is 151bp (B).

(NEB), 2 units NEB2 Buffer and distilled water to reach a final volume of 20 μL. The digested samples were then run on 10% polyacrylamide gels and visualized by ethidium bromide staining. Wildtype mice displayed one band at 166 base pairs, while heterozygous 1Neu mice showed two bands, one at 166 base pairs and another at 151 base pairs (Fig. 3B). Samples of DNA used for genotyping were obtained using the Wizard DNA Purification Kit (Promega).

3.2 Ontogeny of *Pax-2* expression in wildtype mice

3.2.1 Dissection of kidneys

C3H females were crossed with wildtype C3H males for this study. Once females gave birth, litters were allowed to mature to the desired stage at which time the pups would be sacrificed and dissected. The sacrificed pups were transported on ice to a sterile tissue culture area where their kidneys were removed with the aid of a dissecting microscope (Leica) and fine dissecting instruments. Once removed from the body, the kidneys were transferred with tweezers to a petri dish containing sterile PBS. This was repeated at weekly intervals beginning at week 0 of life, and ending at week 10.

3.2.2 *Pax-2* western blot

Kidneys were thoroughly lysed using an abrasive western lysis buffer consisting of 62.5 mM Tris pH 6.8, 10% Glycerol, 6 M Urea, 2% SDS and 5% β–mercaptoethanol. The samples were then heated to 65 ^{o}C for 15 minutes and the loading dye (to 1X) was added. Once the loading dye was added, the samples were boiled at 100 ^{o}C for 5 minutes, spun down briefly in a microcentrifuge and loaded on a polyacrylamide gel consisting of two

layers; a top (stacking) layer (4%) and a lower (separating) layer (10%). The gel was run at 100V for approximately 2 hours followed by a 1-hour transfer at 100V. Once the transfer of protein from gel to membrane was complete, the membrane was blocked by incubating in PBS-Tween (0.1%) + 5% milk at room temperature for 2 hours. Next, the blot was probed with *Pax-2* antibody (Zymed) (1/250) for 2 hours, followed by 2 washes of 10 minutes each with PBS-Tween (0.1%). The blot was then probed with a secondary antibody (1/1000 Anti-Rabbit) and washed 3x 5 minutes with PBS-Tween (0.1%). The final step was to develop the blot by immersing it in an enhanced chemiluminescence (ECL) developing solution (Amersham Biosciences) and immediately exposing it to film. A band appearing at 42 kDa represents *Pax-2*.

The blots were then stripped using 62.5 mM Tris pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol, and distilled water, heated to 50°C.A β–actin western blot was performed on the same membrane. The 41.73 kDa bands that appear represent the amount of β–actin protein in our samples, and serves to normalize the *Pax-2* western blot results.

3.3 Mouse model for UTO

Both C3H wildtype and 1Neu mice were anaesthetized by inhaling 0.5-1 L of oxygen/1% isoflurane for the duration of the operative procedure. A paramedial incision was made in the upper left abdomen. By medially reflecting the left colon, the mid ureter was exposed. The ureter was ligated with 5.0 surgical silk, and was then tucked back into the abdominal cavity. The muscle of the anterior abdominal wall was sutured with Vicryl 5.0

(Ethicon), and the skin was stitched with stainless steel wire. To minimize pain, 0.05mg/kg of buprenorphine (0.5cc of 0.005mg/cc suspension) was applied subcutaneously to the sealed abdomen every 6-8 hours.

Both Wildtype and mutant mice were sacrificed following zero, five, ten, or 15 days of obstruction by placing the mice in a CO_2 chamber. Both right and left kidneys were excised for further study.

Please see McGill University Animal Use Protocol #4530 for further information, appended in section 6.

3.4 Pax-2 expression following UTO

3.4.1 *Pax-2* immunohistochemistry

The whole kidneys were were stored and embedded in blocks of paraffin. Kidneys were sectioned 5 μm thick using a microtome (Leica).

Tissue sections were heated to 60°C for 1 hour. They were then deparaffinized by treating 2x 5 minutes with histology grade Xylene (Fisher Scientific) and rehydrated by soaking 2x 5 minutes in 100% ethanol, 2x 5 minutes in 95% ethanol, 3 minutes in 90% ethanol, 3 minutes in 70% ethanol, 3 minutes in 50% ethanol, and finally 3 minutes in distilled water. Next, endogenous peroxidases were blocked by incubating the sections in 3% H_2O_2 for 30 minutes at room temperature. The tissue sections were then incubated in diluted normal blocking serum (Vectastain Universal Kit) for 20 minutes. The sections

were incubated for 30 minutes with primary antibody (*Pax-2* from Zymed) diluted in buffer (1/50 dilution) at 37 °C. The sections were washed in buffer, and then incubated for 30 minutes in diluted biotinylated secondary antibody solution (Vectastain Universal Kit) at 37 °C. The sections were then incubated with Vectastain, incubated with DAB peroxidase substrate kit (Vector Laboratories), and counterstained with Methyl Green dye. The sections were then dehydrated and mounted using Permount (Fisher Scientific).

3.4.2 *Pax-2* western blot analysis

For *Pax-2* western blot protocol, see section 3.2.2.

Western blots were quantified using the *MCID-M5 4.0* image analysis software. This software processes the spatial measures for the number of pixels contained in each band of the Western Blots. Intensities from the *Pax-2* bands were divided by the β-actin band intensities, resulting in a normalized "intensity" for *Pax-2* expression.

3.5 Anti-Spectrin p120 neoepitope Expression Following UTO

The anti-Spectrin p120 neoepitope antibody was obtained from Merck-Frosst (SVEALIK©-KLH; 24mg/ml. MF# G0021805K; aff, pur.). This antibody was used to detect apoptosis by the caspase-3 produced alpha II spectrin 120kDa breakdown product. Caspase-3 is specific to apoptosis, and not necrosis, and therefore, is a measure of the apoptosis.

Tissue sections were heated to 60°C for 1 hour. They were then deparaffinized by treating 2x 5 minutes with histology grade Xylene (Fisher Scientific) and rehydrated by soaking 2x 5 minutes in 100% ethanol, 2x 5 minutes in 95% ethanol, 3 minutes in 90% ethanol, 3 minutes in 70% ethanol, 3 minutes in 50% ethanol, and finally 3x 5 minutes in PBS + Triton (0.03%). The sections were then digested with Trypsin (0.05%) and incubated at 37°C. The sections were washed with PBS + Triton (0.03%) and endogenous peroxidases were blocked by incubating the sections in 3% H_2O_2 for 30 minutes at room temperature. The tissue sections were then incubated in diluted normal blocking serum (Vectastain Universal Kit) for 20 minutes. The sections were incubated for 30 minutes in primary antiserum (spectrin p120 antibody) diluted in buffer (1/100 dilution) overnight at room temperature. The sections were washed in buffer, and then incubated for 30 minutes in diluted biotinylated secondary antibody solution (Vectastain Universal Kit) at 37 °C. The sections were then incubated with Vectastain, incubated with DAB peroxidase substrate kit (Vector Laboratories), and counterstained with Methyl Green dye. The sections were then dehydrated and mounted using Permount (Fisher Scientific).

3.6 *TUNEL* labelling

The in situ cell death detection kit (Roche Diagnostics) was used to detect apoptosis by terminal dUTP nicked end labelling (TUNEL) in serial sagittal sections of paraffin-embedded wildtype and $Pax2^{1Neu}$ mutant kidneys.

Tissue sections were heated to 60°C for 1 hour, deparaffinized, and rehydrated. They were then treated with 15 mg/ml Proteinase K in 10 μM Tris/HCL pH 7.5 for 20 minutes

at room temperature. Next endogenous peroxidases were blocked by incubating the sections in 2% H_2O_2 for 20 minutes at room temperature. The tissue sections were then incubated in labelling solution for 85 minutes at 37°C and washed 3 x 3 minutes with PBS. Next the sections were incubated with the peroxidase converter for 60 minutes at room temperature. Following 3 washes of 3 minutes each with PBS, the tissue sections were incubated for 5 minutes at room temperature with DAB substrate solution (Vector Laboratories), counterstained with methyl green and mounted using Permount (Fisher Scientific).

3.7 Renal Parenchymal Loss Following UTO

3.7.1 Kidney Weights

Sutures were removed from excised obstructed kidneys, and both right and left kidneys were drained of fluid, so that only dry weight was recorded. Kidneys were weighed with a digital scale (Leica), and dry weights were recorded to the nearest hundredth of a gram.

3.7.2 Statistics

Using the *InStat* computer program, a Mann-Whitney test was performed to compare the median weights of wildtype and mutant kidney weights for each interval of obstruction (0, 5, 10, and 15 days).

The values entered into the program were not raw data, but rather Left Kidney Weight divided by Right Kidney Weight to normalize for the fact that 1Neu animals have smaller kidneys.

4. RESULTS

4.1 Ontogeny of *Pax-2* expression in wildtype mice

Pax-2, the developmental transcription factor is still expressed postnatally in the wildtype mouse kidney as detected by western blot analysis. For every time point, four mice were assayed, and the staining pattern was consistent in all four animals. By two weeks of age, levels of Pax-2 protein expression begin to weaken, and by six weeks of age, Pax-2 protein expression is barely detectable in the mature wildtype mouse kidney (Fig. 4).

4.2 Pax-2 is re-expressed following UTO in wildtype mice

Following UTO in the six week old wildtype C3H mouse, *Pax-2* protein is detected in the collecting duct cells of the obstructed kidney, and not in the collecting duct cells of the contralateral kidney, as shown by immunohistochemisty (Fig. 5).

Pax-2 protein levels were then measured by western blot and quantified. Each point represents the mean of four individual animals. Expression levels in the obstructed kidney increased as time of obstruction increased, and by 15 days of unilateral ureteral obstruction, *Pax-2* protein expression in the obstructed kidney neared fetal levels (Fig.6). This sharp Pax-2 protein reactivation was not seen in the contralateral kidney.

4.3 Apoptosis following UTO in wildtype mice

The appearance of apoptosis was then tracked by *TUNEL* assay and by the novel anti-spectrin antibody, and both methods revealed an increase in apoptosis in the collecting

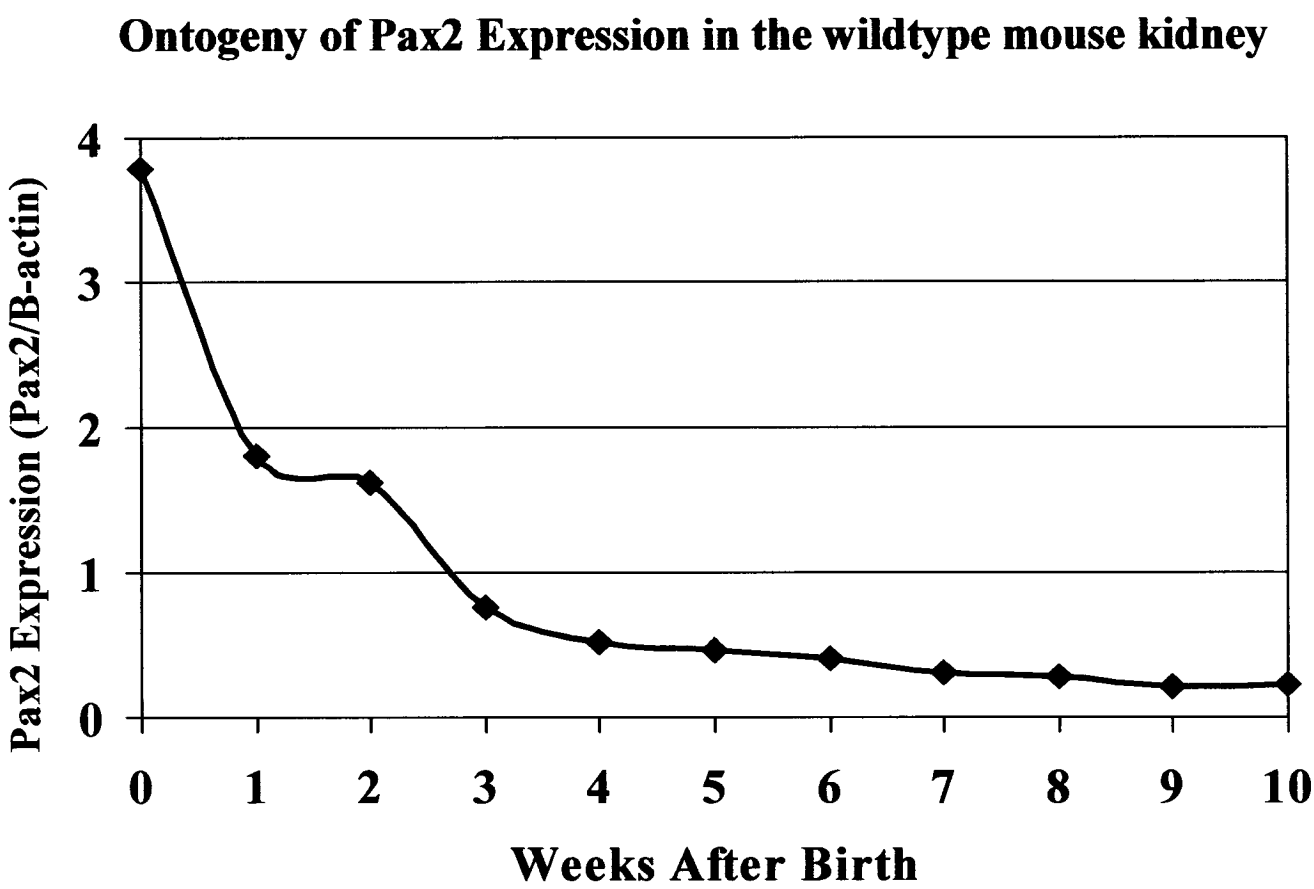
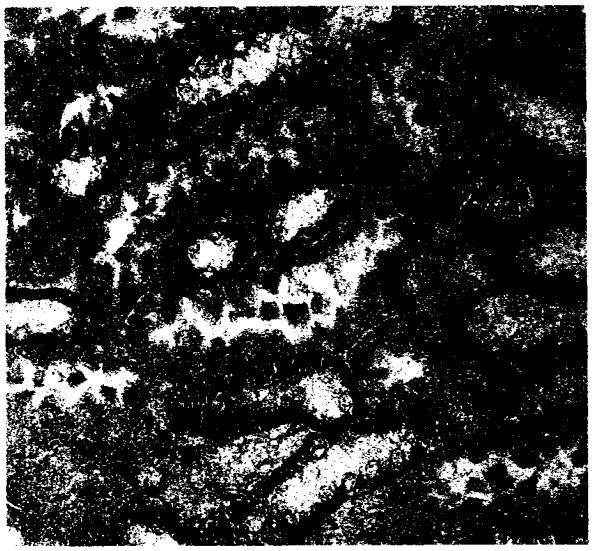
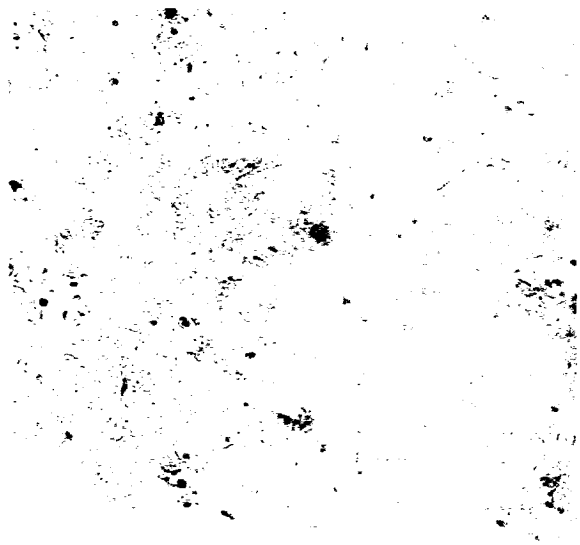


Figure 4: Ontogeny of Pax2 expression in the postnatal mouse kidney. Pax2, the developmental transcription factor is still expressed postnatally in the wildtype mouse kidney as detected by western blot analysis. By two weeks of age, levels of Pax2 protein expression begin to fall, and by six weeks of age, Pax2 protein expression is barely detectable in the mature wildtype mouse kidney. Each point represents the mean of 4 individual animals.



Obstructed Kidney



Contralateral Kidney

Figure 5: Pax2 expression in collecting duct cells. Using immunohistochemistry, Pax2 protein is detected in the collecting duct cells of the obstructed kidney, and not in the contralateral kidney, following 10 days of UTO.

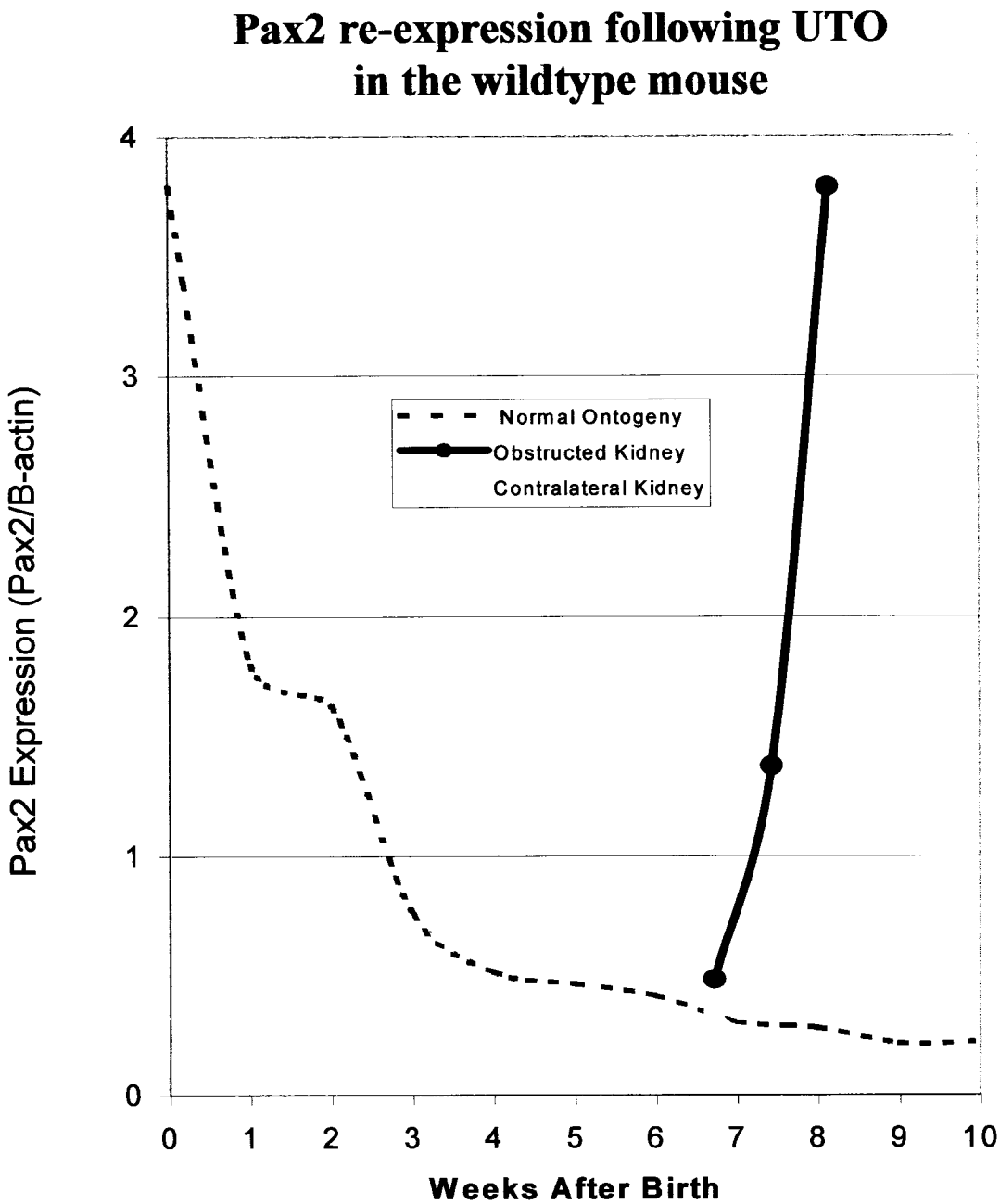


Figure 6: Pax2 re-expression following UTO in wildtype mice. In the obstructed kidney, Pax2 protein levels increased as time of obstruction increased. By 15 days of UTO, Pax2 protein expression in the obstructed kidney neared fetal levels. The sharp reactivation of Pax2 protein was not seen in the contralateral kidney. Each point represents the mean of 4 individual animals.

duct cells of obstructed kidneys (Fig. 7). Ten different mice were assayed, and the staining patterns were consistent in all animals. The heightened apoptosis is seen as early as five days post-obstruction (Fig. 7A, D), and is most prominent by ten days of obstruction (Fig. 6B, E). At 15 days of obstruction, spectrin staining was detectable, but not as strong as 10 days of UTO (Fig. 7C). Likewise, apoptotic cells are visible, but are not as abundant as they were at 10 days of UTO (Fig. 7F).

4.4 Renal Parenchyma Loss Following UTO in wildtype mice

There is significant renal parenchymal loss in the obstructed kidney of wildtype mice compared to the contralateral kidney following fifteen days of obstruction (Fig. 8).

4.5 *Pax-2* expression in wildtype versus 1Neu mice following UTO

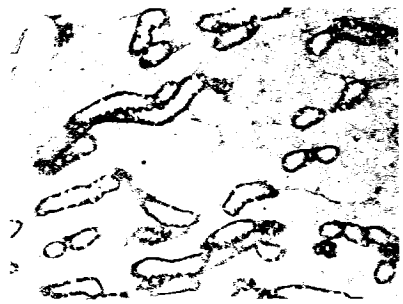
Following UTO in 1Neu animals, there was an increase of *Pax-2* protein as detected by Western blot and by immunohistochemistry (data not shown), though it was not as dramatic as seen as the increase in wildtype mice (Fig. 9A,B).

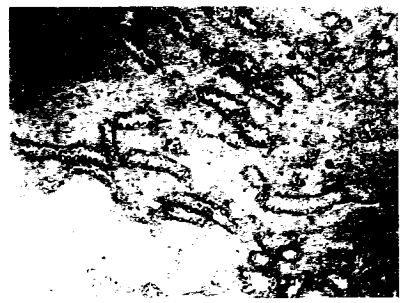
4.6 Apoptosis in wildtype versus 1Neu mice following UTO

Following UTO, there was a greater increase of apoptosis in 1Neu mice than in their wildtype littermates as detected by TUNEL assay (Fig. 10 A,C) and by anti-spectrin p120 neoepitope antibody (Fig. 10B,D).

4.7 Renal Parenchyma Loss in wildtype versus 1Neu mice following UTO

Anti-Spectrin p120 neoepitope IHC





(A) 5 days UTO (B) 10 days UTO (C) 15 days UTO

TUNEL

(D) 5 days UTO (E) 10 days UTO (F) 15 days UTO

Figure 7: Apoptosis following UTO in wildtype mice. Heightened apoptosis is seen as early as five days post-obstruction (A, D), and is most prominent by ten days of obstruction (B, E). At 15 days of obstruction, spectrin staining was detectable, but was not as strong as it was on day 10 of UTO (C). Likewise, TUNEL staining reveals apoptotic bodies at day 15, but not as many as were seen at day 10 of UTO (F). Ten mice were assayed for apoptosis, and staining patterns were consistent in each animal.

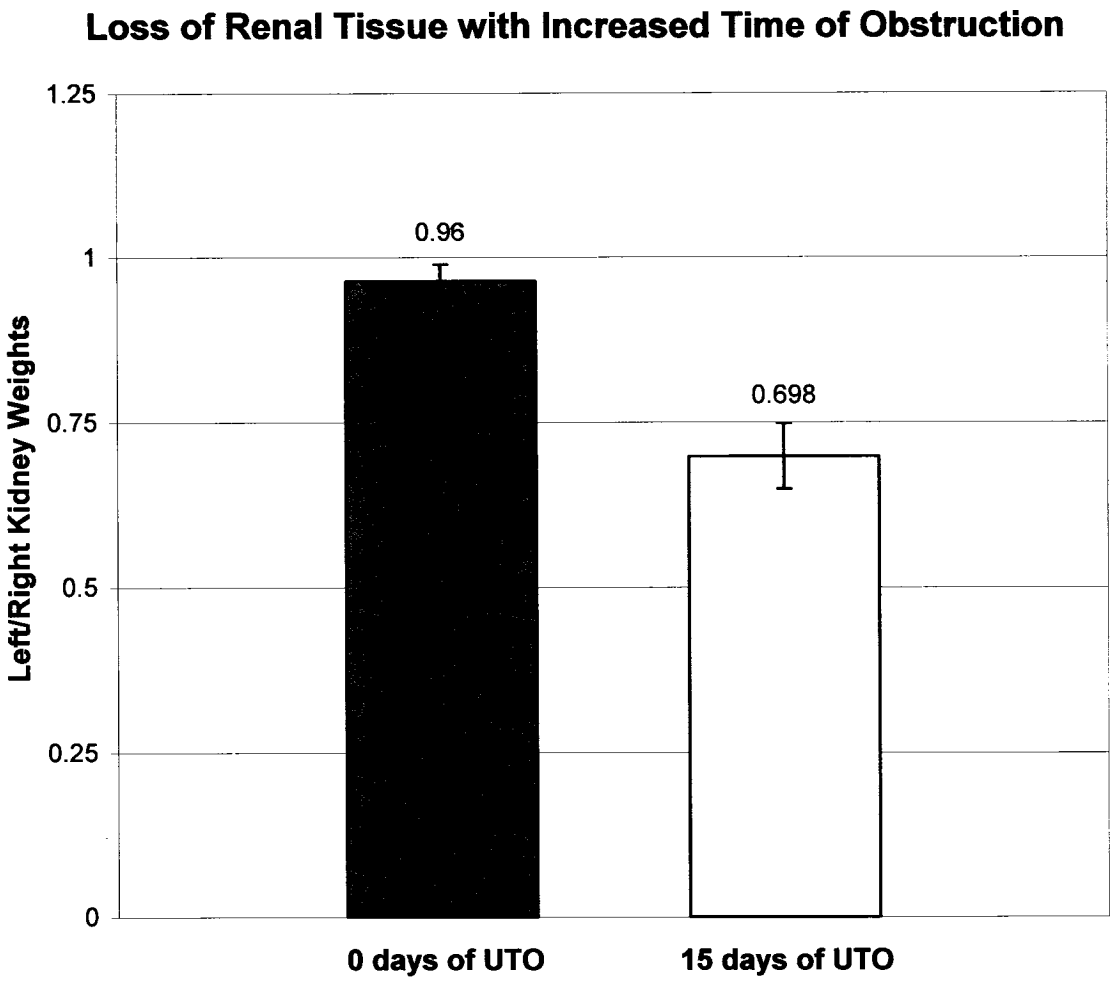
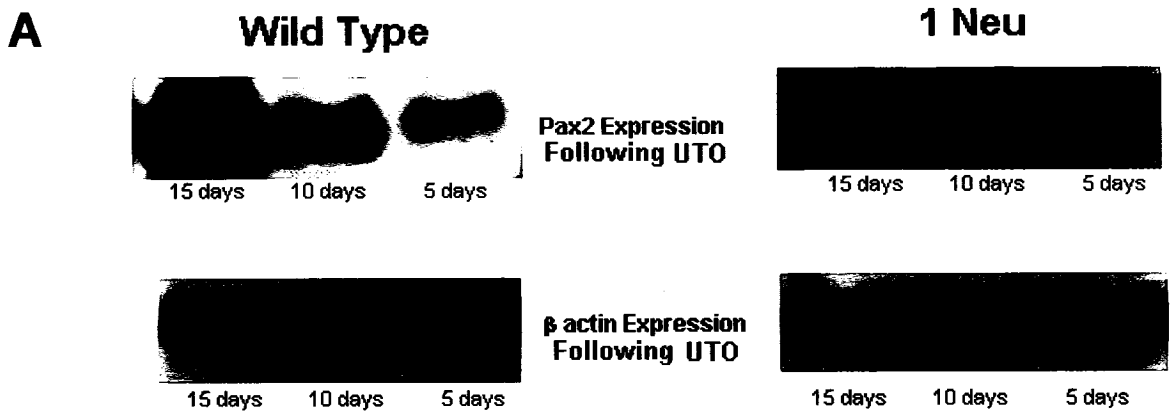


Figure 8: Loss of renal parenchyma following UTO in wildtype mice. There is significant renal parenchymal loss (27.61% loss) in the obstructed kidney of wildtype mice compared to the contralateral kidney following fifteen days of obstruction ($p = 0.002$).



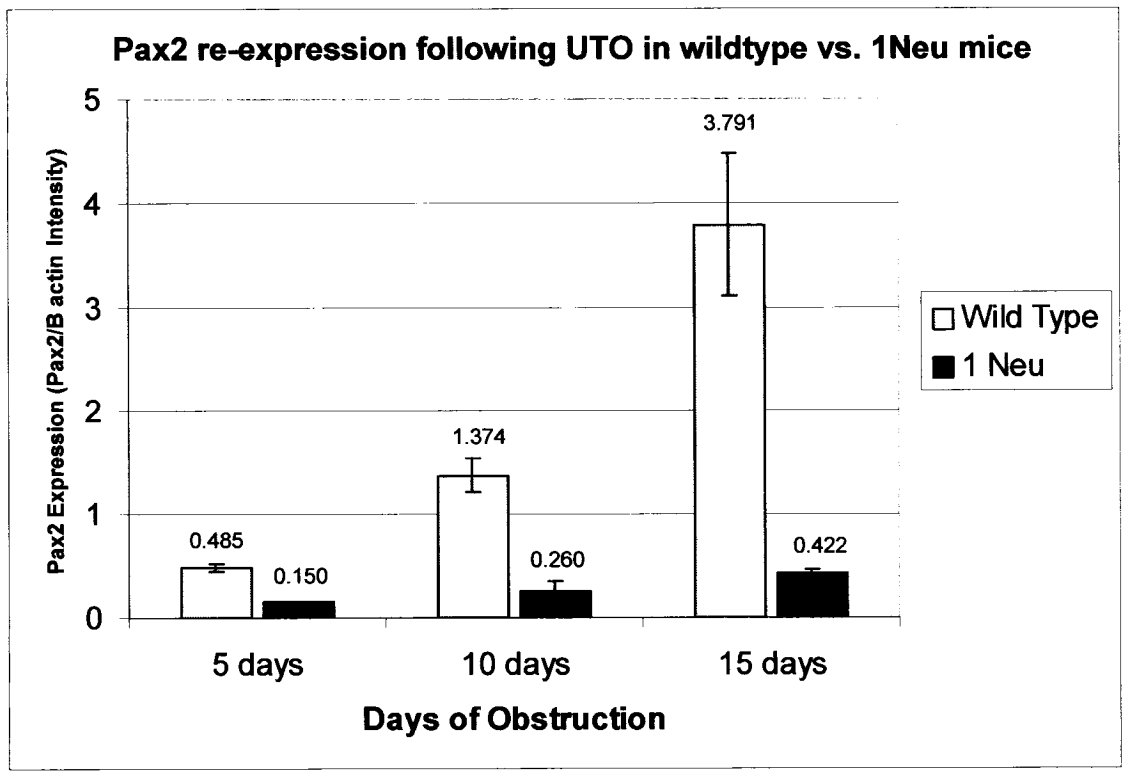


Figure 9: Pax2 expression in wildtype versus 1Neu mice following UTO. Western blot analysis of Pax2 protein expression following 5, 10, and 15 days of UTO (A). Following 15 days of UTO, 1Neu mice had a 181% increase of Pax2 protein from basal levels, while wildtype mice had a 682% increase ($p= 0.001$) (B).

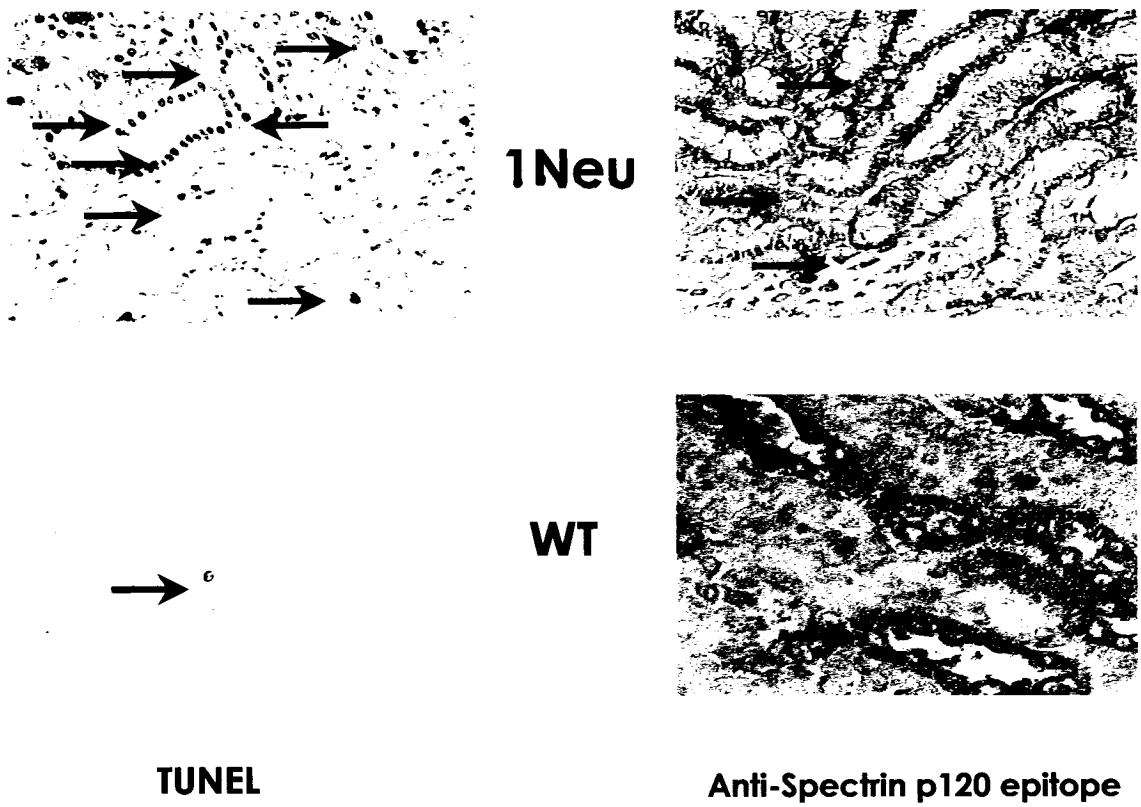
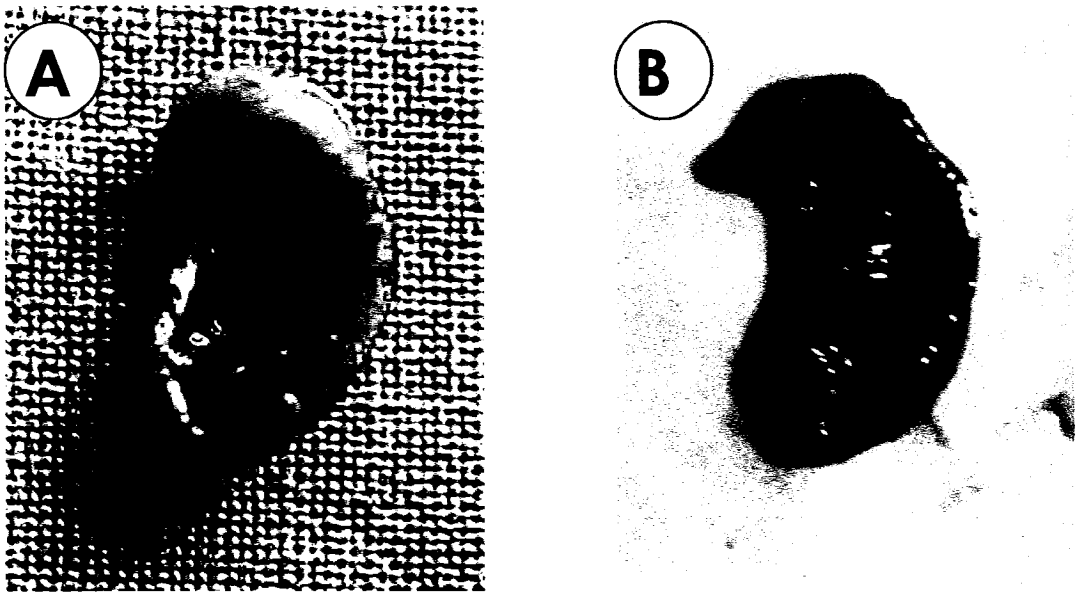


Figure 10: Heightened apoptosis in 1Neu mice following UTO. Following 10 days of UTO, there was a greater increase of apoptosis in 1Neu mice than in their wildtype littermates as detected by TUNEL assay (Fig. 10 A, C) and by anti-spectrin p120 neoepitope antibody (Fig. 10B, D).

As seen in Figure 11A, there is scalloping of the medullary region in the wildtype kidney following 15 days UTO. The kidneys of the 1 Neu mouse following 15 days of UTO have enhanced medullary scalloping (Fig. 11B). Figure 11C reveals a greater renal parenchymal loss in the 1Neu mice than in their wildtype littermates following 15 days UTO. Left (obstructed) kidney weights were divided by right (contralateral) kidney weights to normalize for the variation of renal mass among wildtype mice, and between widtype mice and 1Neu mice. There is a 24% greater discrepancy between right and left kidney weights in 1Neu animals (0.532) than in their wildtype littermates (0.698) ($p = 0.04$).


A
B
WildType
1 Neu

C

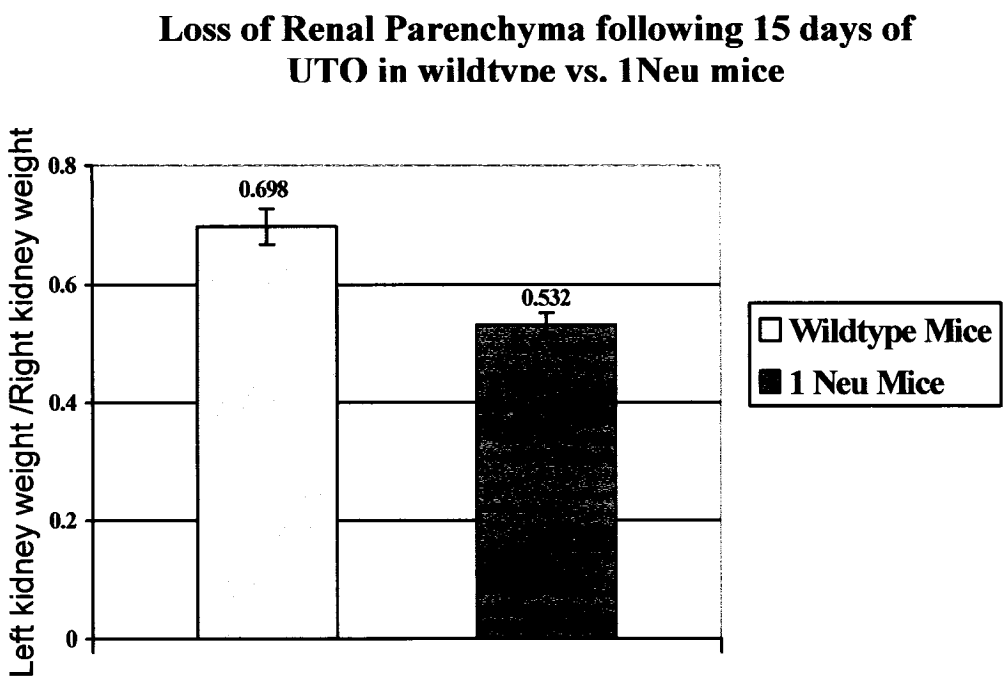

Loss of Renal Parenchyma following 15 days of UTO in wildtype vs. 1Neu mice
Left kidney weight /Right kidney weight
0.8
0.6
0.4
0.2
0
0.698
0.532
Wildtype Mice
1 Neu Mice

Figure 11: Greater loss of renal parenchyma in 1Neu mice following UTO. Scalloping of the medullary region in the wildtype kidney following 15 days UTO (A). Enhanced medullary scalloping in the 1Neu kidney following 15 days UTO (B). (C) There is a 24% greater discrepancy between right and left kidney weights in 1Neu animals (0.532) than in their wildtype littermates (0.698) following 15 days UTO ($p= 0.04$).

5. DISCUSSION

Apoptosis is well-described in Urinary Tract Obstruction (Matsell 2002a; Matsell 2002b) (Chevalier 2000; Chuang 2002) and seems to occur primarily in collecting duct cells (Cachat 2003).

During kidney development, collecting duct cells are reasonably protected from apoptosis by expression of various growth factors (e.g. *GDNF*) and transcription factor genes (e.g. *Pax-2*). The latter is especially critical for optimal nephrogenesis. Previous studies have shown that heterozygous PAX2 mutant mice undergo increased collecting duct cell apoptosis during fetal life and that this limits the rate of branching morphogenesis (Porteous 2000; Torban 2000).

Silverstein recently examined renal gene expression patterns during experimental UTO in rats and noted reactivation of various "fetal" genes (2003). We hypothesized that *Pax-2* might be among the genes reactivated during UTO and that this might serve to limit the apoptotic damage.

To elucidate these events, we investigated the relationship between apoptosis and *Pax-2* expression at varying time intervals following UTO in the mouse. In view of the value of mutant mouse strains to define mechanisms of obstructive nephropathy, mice expressing two copies of the Pax2 gene (wild-type) were compared with heterozygous animals that expressed only a single functional copy of Pax2 (1Neu).

Ontogeny of *Pax-2* expression in wildtype mice

The novel observations we have made regarding the effects of UTO on Pax-2 expression and apoptosis should be taken in the context of an adult organ. This is underlined in our ontogeny of *Pax-2*, which showed minimal expression of *Pax-2* protein past 6 weeks of age, but elevated levels of *Pax-2* expression during the first few weeks of life in the mouse. These findings are consistent with *Pax-2*'s role in renal development, where in the mouse, most nephrons are formed postnatally (Chevalier 1999).

UTO Model

By interfering with renal growth and development, congenital urinary tract obstruction constitutes one of the most important causes of renal failure in infants and children. Obstructive nephropathy is also a significant cause of renal insufficiency in the adult. Unilateral Ureteral Obstruction in the rodent is an established model of human obstructive nehropathy (Chevalier 1999a; Chevalier 1999b).

Pax-2 is re-expressed following UTO in wildtype mice

Our studies demonstrate that *Pax-2*, a developmental transcription factor is re-expressed in acute adult UTO. The increased expression of this gene after acute adult UTO mirrors that of several other genes in this condition, including EGF, rennin, clusterin and TGF-β (Chevalier 1996).

Pax-2 expression in wildtype versus 1Neu mice following UTO

In 1Neu mice, Pax-2 reactivation following UTO was apparent, but minimal. Because these mice carry only one functional copy of the PAX2 gene, this was expected.

During development, 1Neu mice have increased apoptosis in the cells of the ureteric bud, and this results in the 1Neu mice having smaller kidneys at birth with reduced ureteric bud branching. Following UTO, it seems that this developmental episode recurs, as 1Neu animals are compromised once again, with only one competent copy of the PAX2 gene.

Apoptosis following UTO in wildtype mice

Programmed cell death is increased with ongoing unilateral UTO, based on TUNEL labelling and anti-spectrin p120 neoepitope staining. The Contralateral kidney did not show this dramatic increase in apoptosis. There is much debate over the accuracy of TUNEL labelling, and its exclusivity for measuring apoptosis, and this was the reason for carrying out a second assay for apoptosis.

In this study, we used a novel anti-spectrin antibody, which recognizes an epitope of Spectrin (p120) that is revealed only with apoptotic cleavage. This second marker of apoptosis confirms the TUNEL results.

Our waves of apoptosis are detectable at day 5, but become maximal at day 10, with resultant evidence of tubular atrophy and parenchymal loss by day 10-15. These findings correspond to those found in the literature (Cachat 2003).

Apoptosis in wildtype versus 1Neu mice following UTO

The *Pax-2*^{1Neu} mouse's heightened susceptibility to apoptosis in UTO echos what was seen in development. In the developing kidney, *Pax-2*^{1Neu} mice have suboptimal branching of their ureteric buds, associated with collecting duct apoptosis (Porteous 2000).

Renal Parenchyma Loss in wildtype versus 1Neu mice following UTO

It was our hypothesis that if Pax-2 reactivation during acute UTO partially protected against apoptosis, then 1Neu mice, with only one functional copy of the PAX2 gene, would have more apoptosis and greater renal parenchymal loss.

Along with increased apoptosis, greater parenchymal loss was seen in the 1Neu mouse than in the wildtype mouse during UTO. This confirms the partially protective function of *Pax-2* in response to obstructive injury.

Conceivably, in humans there is variation in the degree to which Pax-2 or other anti-apoptotic genes are re-expressed, in which case there might be variation in the extent of apoptotic damage from individual to individual.

Possible mechanisms by which UTO leads to altered Pax-2 expression

One possibility is the increased pressure within the fetal urinary tract in UTO. Perhaps this physical stress, through increased axial strain, triggers the aberration of cell biology and the re-expression of *Pax-2*. There have been several such studies on detrusor and

other cells harvested from adults. Cachat reported that mechanical strech leads to increased apoptosis of both vascular smooth muscle and renal epithelial cells (2003)

A second possibility is the inflammatory process that occurs in UTO. Perhaps *Pax-2* contributes to curb the interstitial inflammatory process that occurs in chronic neonatal UTO by asserting its role in cell viability by regulating apoptosis. Silverstein revealed changes in protein abundance for immune modulator genes, but that the activation of these genes was not statistically significant (2003). Elucidation of specific immune modulators genes and their effects on *Pax-2* may lead to new insight in pathogenesis and management of congenital obstructive nephropathy.

Implications

As we begin to understand the molecular events responsible for these changes it will be important to define their relative roles in compromising postnatal renal function. Not only will the timing of release of obstruction be important in future intervention in these processes but also strategies to restore normal developmental gene and protein patterns in the developing kidney.

Several discrete developmental roles for Pax-2 have been identified. In the MM, Pax-2 activates GDNF expression (Brophy 2001), but in the nephric duct it may influence cell fate specification (Bouchard 2002). An anti-apoptotic function for Pax-2 in the branching ureteric bud (UB) of developing kidney was recently identified, where heterozygous Pax-2^{1Neu} mice have suboptimal UB arborisation associated with elevated apoptosis of UB

cells (Porteous 2000; Torban 2000). Our studies have confirmed the partially protective function of Pax-2 in response to obstructive injury. Conceivably, in humans there is variation in the degree to which Pax-2 or other anti-apoptotic genes are re-expressed, in which case there might be variation in the extent of apoptotic damage from individual to individual.

Novel future approaches to interfere with renal injury resulting from acute UTO may include treatment with growth factors or antioxidants. Alternative approaches may involve gene therapy and the inhibition of the intracellular regulatory switches. Increasing endogenous levels of *Pax-2* (or other anti-apoptotic genes) could significantly reduce renal parenchymal loss following acute UTO.

Furthermore, we speculate that, caspase-inhibitory drugs (such as those currently in development to limit tissue damage in stroke and heart attack), when endogenous protective mechanisms are inadequate.

Conclusions

Studies have previously confirmed some of the animal data as being relevant to human disease. For example, apoptosis and related genes are dysregulated in human dysplastic kidneys. Experimental animal models that closely simulate human development and disease will, thus, be essential for exploring these issues, and for assessing novel fetal therapies for future human application.

Our overall knowledge of gene expression in the human obstructed kidney is rudimentary. Nonetheless, the advent of strategies geared towards aversion or reversion of key cellular apoptotic events will undoubtedly be significant in maintaining function in or preventing end stage renal disease.

The challenge now will be to define the genetic regulatory cascades altered by urinary tract obstruction and the pathways that link them. This exciting area of research will lead to potential disease modifiers and have profound therapeutic implications.

6. APPENDIX

c) Description of Animals

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Supplier/Source	Charles River	Charles River	MCH	MGH and MCH	MGH and MCH	MGH and MCH
Strain	C3H	CD1	CD1/1Neu	C3H/1Neu	C3H/pax2pro/ Bax	C3H/pax2pro/ Bcl-2
Sex	M/F	M/F	M/F	M/F	M/F	M/F
Age/Wt	3-12 months	3-12 months	0-18 months	0-18 months	0-18 months	0-18 months
# To be purchased	20	8	0	0	0	0
# Produced by in-house breeding	0	0	120	250	200	200
# Other (e.g.field studies)	0	0	0	0	0	0
#needed at one time	10	3	8	8	8	8
# per cage	3-5	3-5	3-5	3-5	3-5	3-5
TOTAL# /YEAR	100	20	120	250	200	200

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

7. Justification of Animal Usage

a) Please justify the number of animals requested for each species described in the table 6c above, BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT. Include information on experimental and control groups, # per group, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The numbers of animals are for one year only, not the length of funding. Use the table below when applicable (space will expand as needed).

We have successfully produced 2 different transgenic mouse strains expressing Bax and Bcl-2 genes only in tissues which normally express PAX2 during development (by using a PAX2 promoter). We are currently completing the studies of these mice which are unique in the world. The numbers above reflect our estimate of the minimal safe number of mice to maintain a small colony until studies are complete - given the fact that the occasional Bax animal seems to be relatively infertile, given the occasional skewed representation of sex and genotypes within a given litter and given the fact that we will sacrifice pregnant mothers at various stages to obtain fetal kidneys for study. These number also take into account the fact that only half of the progeny from any given litter will be positive for the transgene, therefore the numbers include the entire litter, from which the wildtype will be euthanized upon results of the genotyping. Similarly, we must maintain the 1Neu colony for interbreeding with C3H/pax2pro/Bcl2 mice to complete our funded studies - the McGill 1Neu colony is one of four colonies in the world. Also, the maintanance of the 1Neu mouse colony will be required to provide Dr. Roman Jednak with animals for his obstruction study (protocol #4530). Both the completion of the C3H/pax2/Bcl2/1Neu study and animals provided for Dr. Jednak's obstruction study reflect the large number of C3H/1Neu animals required.

Test Agents or Procedures e.g. 2 Drugs	# of Animals and Species Per Group e.g. 6 rats	Dosage and/or Route of Administration e.g. .03, .05 mg/kg – IM, IP (4 variables)	# of endpoints e.g. 1, 7, 10 days (3 variables)	Other variables (i.e. sex, weight, genotypes,etc.) e.g. Male, Female groups (2 variables)	Total number of animals per year e.g. 2 x 6 x 4 x 3 x 2 = 288

*** For the above table, enter the first agent/procedure, press 'enter', then the 2nd agent... complete the first column, then the 2nd, then the 3rd...**

Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).

Since we study the development of the kidney as well as developmental defects affecting the kidney, it is imperative that we use an vertebrate animal model since most cell cultures cannot provide us with the useful details of organ development.

AUP Renewal Application – Addendum to page 3 – Item 6c
Paul Goodyer (PI) April/2003
Project: Effect of PAX genes on kidney development and function

	Strain 7
Species	Mouse
Supplier	MGH and MCH
Strain	C3H/PAX2pro/Bcl2/1Neu
Sex	M/F
Age/Wt	0-18 months
# Produced by	200
in-house breeding	0
# needed at one time	8
#per cage	3-5
TOTAL/Year	200

c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

All experiments involve mice bearing a mutant PAX2 gene (comparable to human children with small kidneys and the same mutation) or mice bearing transgenes which modify the extent of kidney cell apoptosis during development. 1Neu mice have proven to be a perfect model of the human Renal-Coloboma-Syndrome. allowing us to work out the cause of renal hypoplasia that can't be studied antenatally in humans. The transgenic animals are unique tools that we generated in order to further study the role of apoptosis during kidney development. These models will help delineate the molecular mechanisms of renal hypoplasia.

8. Animal Husbandry and Care

a) **Special cages** NO ☒ YES ☐ **Specify:**

Special diet NO ☒ YES ☐ **Specify:**

Special handling NO ☐ YES ☒ **Specify:** timed breeding

b) **Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?**

NO ☒ YES ☐ **Specify:**

c)

Multiple institution facility housing: NO ☐ YES ☒

Indicate all facilities where animals will be housed: **Building:** MGH **Room No:** 13th floor FS17.2

Indicate area(s) where animal use procedures will be conducted: **Building:** MCH. Place Toulon **Room No:** animal room #3

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
All animals will be directly transported by new, autoclaved, filtered boxes, picked up and directly deliverd by students using air conditioned vehicles from the MGH directly to the MCH after approval has been obtained from the MCH place toulon animal facility. The MCH facility supervisor will be advised of incoming animals 1-2 days in advance.

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection (UACC#1)	☐	Production of Monoclonal Antibodies (UACC#7)	☐
Anaesthesia (rodents) (UACC#2)	☐	Production of Polyclonal Antibodies(UACC#8)	☐
Analgesia (rodents/larger species) (UACC#3)	☐	Collection of Amphibian Oocytes (UACC#9)	☐
Breeding (transgenics/knockouts) (UACC#4)	☒	Rodent Surgery (UACC#10)	☐
Transgenic Generation (UACC#5)	☐	Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)	☐
Knockout/in Generation (UACC#6)	☐	Stereotaxic Survival Surgery in Rodents (UACC#12)	☐

10. Description of Procedures

a) **FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES IN THE ORDER IN WHICH THEY WILL BE PERFORMED - surgical procedures, immunizations, behavioural tests,**

immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED. Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.

To maintain minimal colonies of 1Neu . Bax and Bcl-2 strains mentioned above. we will dock tails of pups at no more than 3 weeks; tail tissues will be used for genotyping each animal. For all strains above, we will breed one male with 2-3 females overnight in a single cage, checking for plugs and separating the animals the following morning. Preganacy will be detected by inspection and monitoring weight. For C3H/1Neu. CD1/1Neu. C3H/pax2pro/Bax. C3H/pax2/bcl2 C3H/pax2/Bcl2/1Neu strains. pregnant females and their fetuses will be sacrificed between the gestational ages of E11 and E19 in a CO2 chamber and fetal kidneys will be excised for tissue analysis. In experiments, involving the the supply of mice for Dr. Jednak. C3H/1Neu females will be allowed to come to term. pups will be weaned and genotyped at 3 weeks of age as described above. Once the genotype has been confirmed. Dr. Jednak will be responsible for the transportation of the mice to the Children's Hospital animal facility.

b) Field Studies – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.

Method of capture/restraint, duration of captivity. potential injury/mortality. monitoring frequency:

Transportation and /or housing of animals in the field:

Special handling required:

Capture of non-target species. potential injury/mortality:

Will captured animals be released at or near the capture site YES ☐ NO ☐
If not, specify if they will be relocated to other locations and/or populations.

Describe any potential ecological disruption this study may cause:

It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

c) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. Table will expand as needed. (*complete 1st column pressing 'enter' after each species, then 2nd column...)

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency

d) Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed). (*complete 1st column pressing 'enter' after each species, then 2nd column...)

Species	Agent	Dosage (mg/kg)	Total volume (ml) per administration	Route	Frequency

e) Endpoints : 1) Experimental – for each experimental group indicate survival time .
2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).

1. Experimental: sacrifice 1Neu and transgenic pregnant females once fetuses have reached desired gestational age (E11-E19), sacrifice transgenics after 6 months, 9 months and 1 year to examine kidney tissues. survival of pregnant 1Neu female for the production of 1Neu pups
2. Clinical: animals are observed once/day and monitored bi weekly for food and water intake. Animals are also weighed once a week. Clinical endpoints leading to euthanasia would include weight loss, lack of food or water intake. loss of excessive hair. injury from fighting and behavioral abnormalities.

Specify person(s) who will be responsible for animal monitoring and post-operative care
Name: Tatiana Vassilieva **Phone#:** 412-4400 ext. 22953

f) Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical method of euthanasia without prior use of anaesthesia (justify here):

CO_2 chamber as recommended by CCAC

Specify Species

	☐ anaesthetic overdose, list agent/dose/route:
	☐ exsanguination with anaesthesia, list agent/dose/route:
	☐ decapitation without anaesthesia ☐ decapitation with anaesthesia,list agent/dose/route:
	☐ cervical dislocation
Mouse	☒ CO_2 chamber
	☐ other (specify)
	☐ not applicable (explain)

11. Category of Invasiveness: B ☐ C ☐ D ☒ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.*

Category C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.*

Category D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant. application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).*

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

a) Indicate which of the following will be used in animals:
☐Toxic chemicals ☐Radioisotopes ☐Carcinogens ☐Infectious agents ☐Transplantable tumours

b) Complete the following table for each agent to be used (use additional page as required).

Agent			
age			
Route of administration			
Frequency of administration			
Duration of administration			

Number of animals involved			
Survival time after administration			

c) After administration the animals will be housed in:
- ☐ the animal care facility
- ☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

d) Describe potential health risk (s) to humans or animals:
none

e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:
N/A

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

Standard Operating Procedure #UACC-4 April 2002 form

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s): *(can refer to column number from section 6c of main protocol)*

Mouse

Background strain:

C3H

DNA construct injected:

Mouse Bax cDNA under control of the PAX2 promoter

Gene locus removed:

2.2 Supplier: *Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.*

1. **Commercial:**

2. **Academic:** Transgenics produced with J-P Julien at Montreal General animal facility (transgenic service)

2.3 Phenotype: *Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.*

1. **Heterozygotes**: Bax transgenic mice are phenotypically comparable to PAX2 heterozygotes with mild renal hypoplasia, but no evidence of distress, such as weight loss, dehydration or behavioral abnormalities

2. **Homozygotes**: Since we are working with transegenics, we are not breeding animals to generate the transgene to be present in a homozygote fashion

2.4 Breeding: *Pregnant females will be caged individually near the time of the expected birth and the date and number of pups born marked. (For identification of pups: see below)*

Pregnant females will be caged individually near the time of the expected birth and the date and number of pups born marked. Prior to 3 weeks of age, the pups will be weaned and ear tagged for identification and a portion of the tail (0.5 cm max.)will be docked for DNA analyses.

Describe number of breeding pairs required: Small colonies will be maintained of C3H/pax2pro/Bax as described in the renewal application. 3-6 breeding pais will be used at one time in order to generate the animals that are required.

2.5 There are changes to this SOP indicated in the AUP form: ☐ YES ☒ NO

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

Standard Operating Procedure #UACC-4 April 2002 form

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s): *(can refer to column number from section 6c of main protocol)*

Mouse

Background strain:

C3H/1Neu

DNA construct injected:

Human Bcl-2 cDNA under control of the PAX2 promoter

Gene locus removed:

2.2 Supplier: *Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.*

1. **Commercial:**

2. **Academic:** Transgenics produced with J-P Julien at Montreal General animal facility (transgenic service)

2.3 Phenotype: *Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.*

1. **Heterozygotes**: we are currently still investigating these mice and are not ready to comment of any phenotypic abnormalities however, so far there is no evidence of distress, such as weight loss, dehydration or behavioral abnormalities

2. **Homozygotes**: Pax2 homozygotes are lethal and continue to be so, even with the expression of the Bcl-2 trasngene under the control of the PAX2 promoter.

2.4 Breeding: *Pregnant females will be caged individually near the time of the expected birth and the date and number of pups born marked. (For identification of pups: see below)*

Pregnant females will be caged individually near the time of the expected birth and the date and number of pups born marked. Prior to 3 weeks of age, the pups will be weaned and ear tagged for identification and a portion of the tail (0.5 cm max.)will be docked for DNA analyses.

Describe number of breeding pairs required: Small colonies will be maintained of C3H/pax2pro/Bcl2/1Neu as described in the renewal application. 3-6 breeding pais will be used at one time in order to generate the animals that are required.

2.5 There are changes to this SOP indicated in the AUP form: ☐ YES ☒ NO

MCGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

Standard Operating Procedure #UACC-4

April 2002 form

TRANSGENIC OR KNOCKOUT / IN MOUSE BREEDING

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form (AUP). Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (pages up to signature only) must be attached to the AUP form. The relevant SOP number must be referred to in section 9 of the AUP.

2. INFORMATION REQUIRED

2.1 Species/strain(s): *(can refer to column number from section 6c of main protocol)*

Mouse

Background strain:

C3H and CD1

DNA construct injected:

Gene locus removed:

The 1Neu mouse was discovered due to a spontaneous mutation, which was an insertion of a single nucleotide "G" in exon 2 of the PAX2 locus. This causes a frameshift mutation which results in a truncated protein.

2.2 Supplier: *Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.*

1. **Commercial:**

2. **Academic:** These animals were first provided from collaborators in Germany 6 years ago and have since been rederived here at the MCH-RI animal facility.

2.3 Phenotype: *Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.*

1. **Heterozygotes**: PAX2 heterozygotes present with mild renal hypoplasia, and on occasion ocular colobomas however they don't seem to present with anyevidence of distress, such as weight loss, dehydration or behavioral abnormalities

2. **Homozygotes**: homozygotes are embryonic lethal

2.4 Breeding: *Pregnant females will be caged individually near the time of the expected birth and the date and number of pups born marked. (For identification of pups: see below)*

Pregnant females will be caged individually near the time of the expected birth and the date and number of pups born marked. Prior to 3 weeks of age, the pups will be weaned and ear tagged for identification and a portion of the tail (0.5 cm max.)will be docked for DNA analyses.

Describe number of breeding pairs required: Small colonies will be maintained of CD1/1Neu, C3H/1Neu, as described in the renewal application. 3-6 breeding pais will be used at one time in order to generate the animals that are required.

2.5 There are changes to this SOP indicated in the AUP form: ☐ YES ☑ NO

7. REFERENCES

Alvarez-Bolado, G., Schwarz, M., Gruss, P. (1997). "PAX2 in the chiasm." Cell and Tissue Research **290**: 197-200.

Anglard, P., Trahan, E, Liu, S, Latif, F, Merino, MJ, Lerman, MI, Zbar, B, Linehan, WM. (1992). "Molecular and cellular characterization of human renal cell carcinoma lines." Cancer Research **52**: 348-356.

Armstrong, J. F., Pritchard-Jones, K., Bickmore, W.A., Hastie, N.D., Bard, J.B. (1992). "The expression of the Wilm's tumor gene, WT1, in the developing mammalian embryo." Mechanisms of Development **40**: 85-97.

Bonventre, J., Brezis, M, Siegel, N., et al. (1998). "Acute renal failure: Relative importance of proximal vs. distal tubular injury." American Journal of Physiology **275**: F623-F632.

Bouchard, M., Souabni, A., Mandler, M., Neubuser, A., Busslinger, M. (2002). "Nephric lineage specification by Pax2 and Pax8." Genes and Development **16**: 2958-2970.

Brophy, P. D., Ostrom, L., Lang, K.M., Dressler, G.R. (2001). "Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene." Development **128**: 4747-4756.

Budihardjo, I., Oliver, H, Lutter, M, Luo, X, Wang, X. (1999). "Biochemical pathways of caspase activation during apoptosis." Annu Rev Cell Dev Biol **15**(269-290).

Cachat, F., Lange-Sperandio, B., Chang, A.Y., Kiley, S.C., Thornhill, B.A., Forbes, M.S., Chevalier, R.L. (2003). "Ureteral obstruction in neonatal mice elicits segment-specific tubular cell responses leading to nephron loss." Kidney International **63**: 564-575.

Capelouto, C., Saltzman, B. (1993). "The pathophysiology of ureteral obstruction." Journal of Endourology **7**: 93.

Carmichael, P., Carmichael, AR (2003). "Acute renal failure in the surgical setting." ANZ Journal of Surgery **73**: 144-153.

Chevalier, R., Gomez, RA (1999). Obstructive Uropathy: Pathophysiology. Pediatric Nephrology, 4th ed. T. M. Barratt, Avner, E.D, Harmon, WE,. Baltimore, Lippincott Williams and Wilkins. **Chapter 54:** 873-885.

Chevalier, R. L. (1995). "Effects of ureteral obstruction on renal growth." Seminars in Nephrology **15**: 353-360.

Chevalier, R. L. (1998). "Pathophysiology of obstructive nephropathy in the newborn." Seminars in Nephrology **18**(6): 598-593.

Chevalier, R. L. (1999). "Molecular and cellular pathophysiology of obstructive nephropathy." Pediatric Nephrology **13**: 612-619.

Chevalier, R. L., Chung, K.H., Smith, C.D., et al. (1996). "Renal apoptosis and clusterin following chronic ureteral obstruction." Journal of Urology **155**: 1139-1144.

Chevalier, R. L., Peach, MJ. (1985). "Hemodynamic effects of enalapril on neonatal chronic partial ureteral obstruction." Kidney International **28**: 891-898.

Chevalier, R. L., Smith, CD., Wolstenholme, J., Krajewski, S., Reed, J.C. (2000). "Chronic ureteral obstruction in the rat suppresses renal tubular Bcl-2 and stimulates apoptosis." Experimental Nephrology **8**: 115-122.

Cho, E., Dressler, GR. (2003). Formation and Development of Nephrons. The Kidney: From Normal Development to Congenital Disease. P. Vize, Woolf, AS, Bard, JBL. San Diego, Elsevier Science. **Chapter 14:** 195-210.

Chuang, Y., Chuang, WL., Huang, SP., Huang, CH. (2002). "Over-expression of apoptosis-related proteins contributes to muscular damage in the obstructed ureter of the rat." Br J Urol Int. **89**: 106-112.

Cikala, M., Wilm, B, Hobmayer, E, Bottger, A, David, CN. (1999). "Identification of caspases and apoptosis in the simple metazoan Hydra." Curr Biol **9**: 959-962.

Clausen, G., Hope, A. (1977). "Intrarenal distribution of blood flow and glomerular filtration during chronic unilateral obstruction." Acta Physiol Scand **100**: 22.

Coles, H., Burne, JF., Raff, MC. (1993). "Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor." Development **118**: 777.

Cunliffe, H., McNoe, LA, Ward, TA, Devriendt, K, Eccles, MR. (1998). "The prevalence of PAX-2 mutations in patients with isolated colobomas or colobomas associated with urogenital anomalies." Journal of Medical Genetics **35**: 806-812.

Dahl, E., Koseki, H, Balling, R. (1997). "Pax genes and organogenesis." Bioessays **19**: 755-764.

Davies, J. A. (2000). "A Brief Introduction to Kidney Development." http://golgi.ana.ed.ac.uk/tutork.html.

Davies, J. A., Perera, A.D., Walker, C.L. (1999). "Mechanisms of epithelial development and neoplasia in the metanephric kidney." International Journal of Developmental Biology **43**: 473-478.

Diamond, J., Kees-Folts, D., Ding, G., Frye, JE., Restrepo, NC. (1994). "Macrophages, monocyte chemoattractant peptide-1, and TGF-beta 1 in experimental hydronephrosis." American Journal of Physiology **266**: F926.

Diamond, J., van-Goor, H., Ding, G., Engelmyer, E. (1995). "Myofibroblasts in experimental hudronephrosis." American Journal of Pathology **146**: 121.

Ding, H., Kopple, JD, Cohen, A, Hirschberg, R. (1993). "Recombinant human insulin-like growth factor-1 accelerates recovery and reduces catabolism in rats with ischemic acute renal failure." Journal of Clinical Investigation **91**: 2281-2287.

Donovan, M., Natoli, T., Sainio, K., Amstutz, A., Jaenisch, R., Sariola, H., Kreidberg, J. (1999). "Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud." Developmental Genetics **24**: 252-262.

Dressler, G. R. (1996). "Pax-2, kidney development, and oncogenesis." Medical Pediatric Oncology **27**: 440-444.

Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H.O., Gruss, P. (1990). "Pax2, a new murine paired-box containing gene and its expression in the developinng excretory system." Development **109**: 787-795.

Dressler, G. R., Douglass, EC. (1992). "Pax-2 is a DNA-binding protein expressed in embryonic kidney and WIlms tumor." Proc. Natl., Acad. Sci. USA **89**: 1179-1183.

Dressler, G. R., Wilkinson, J.E., Rothenpieler, U.W., Patterson, L.T., Williams-Simons, L., Westphal, H. (1993). "Deregulation of Pax2 expression in transgenic mice generates severe kidney abnormalities." Nature **362**: 65-67.

Dumont, E., Reutenlingsperger, CP, Smits, JF, Daemen, MJ, Doevendans, PA, Wellens, HJ, Hofstra, L. (2001). "Real time imaging of apoptotic cell-membrane changes at the single-cell level in the beating murine heart." Nature Medicine **7**: 1352-1355.

Durbec, P., Marcos-Gutierrez, C.V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M, et al. (1996). "GDNF signaling through the Ret receptor tyrosine kinase." Nature **381**: 789-793.

Earnshaw, W., Martins, LM, Kaufman, SH. (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochemistry **68**: 383-424.

Eccles, M. R. (1998). "The role of PAX2 in normal and abnormal development of the urinary tract." Pediatric Nephrology **12**: 712-720.

Eccles, M. R., Wallis, LJ, Fidler, AE, Spurr, NK, Goodfellow, PJ, Reeve, AE. (1992). "Expression of the PAX2 gene in human fetal kidney and Wilms tumor." Cell Growth and Differentiation **3**: 279-289.

Eccles, M. R., Yun, K, Reeve, AE, Fidler, AE. (1995). "Comparative in situ hybridization analysis of PAX2, PAX8 and WT1 gene transcription in human fetal kidney and Wilms tumors." American Journal of Pathology **146**: 40-45.

Fauvel, H., Marchetti, P., Chopin, C., Formstecher, P, Neviere, R. (2001). "Differential effects of caspase inhibitors on endotoxin-induced myocardial dysfunction and heart apoptosis." Am J Heart Circ Physiol **280**: H1608-H1614.

Favor, J., Sandulache, R., Neuhauser-Klaus, A., Pretsch, W., Chatterjee, B., Senft, E., Wurst, W., Blanquet, V., Grimes, P., Sporle, R. (1996). "The mouse Pax21Neu mutation is identical to a human PAX2 mutation in a family with renal coloboma syndrome and results in developmental defects of the brain, ear, eye and kidney." Proc. Natl., Acad. Sci. USA **93**: 13870-13875.

Fink, R. I., Caridis, D.T., Chmiel, R., Ryan, G. (1980). "Renal impairment and its reversibility following variable periods of complete ureteric obstruction." Aust NZ Journal of Surgery **50**: 77.

Gartner, A., Milstein, S., Ahmed, S., Hodgkin, J, Hengartner, MO. (2000). "A conserved checkpoint pathway mediates DNA damage-induced apoptosis and cell cycle arrest in C. elegans." Mol. Cell **5**: 435-443.

Gattone, V., Sherman, DA., Hinton, DA., Niu, FW., Topham, RT., Klein, RM. (1992). "Epidermal growth factor in the neonatal mouse salivary gland and kidney." Bio Neonate **61**(54).

Gnarra, J., Dressler, GR (1995). "Expression of Pax-2 in Human Renal Cell Carcinoma and Growth Inhibition by Antisense Oligonucleotides." Cancer Research **55**: 4092-4098.

Gonzalez-Cuadrado, S., Ortiz, A., Karp, S., Li, S., Cizman, B., Lopez-Armada, MJ., Madaio, M., Egido, J., Neilson, EG. (1994). "The Fas ligand (FasL)-Fas (CD95) system in the kidney." J Am Soc Nephrol **5**: 748.

Goulding, M., Lumsden, A, and Gruss, P. (1993). "Signals from the notochord and floor plate regulate the region specific expression of two Pax genes in the developing spinal cord." Development **117**: 1001-1016.

Grobstein, C. (1955). "Inductive interactions in the development of the mouse metanephros." Journal of Experimental Zoology **130**: 319-340.

Grobstein, C. (1956). "Trans-filter induction of tubules in mouse metanephrogenic mesenchyme." Experimental Cell Research **10**: 424-440.

Hammerman, M. R., Rogers, S.A., Ryan, G. (1992). "Growth factors and metanephrogenesis." American Journal of Physiology **262**: F523-F531.

Harris, K., Yanagisawa, H., Schreiner, GF., Klahr, S. (1991). "Evidence for two distinct and functionally important sites of enhanced thromboxane production after bilateral ureteral obstruction in the rat." Clin Sci (Colch) **81**: 209.

Harris, R., Gill, JM. (1981). "Changes in glomerular filtration rate during complete ureteral obstruction in rats." Kidney International **19**: 603.

Hengartner, M. (2000). "The biochemistry of apoptosis." Nature **407**: 770-776.

Himmelstein, S. I., Coffman, T.M., Yarger, W.E., Klotman, P.E. (1990). "Atrial natriuretic peptide-induced changes in renal prostacyclin production in ureteral obstruction." American Journal of Physiology **258**: F281.

Hvistendahl, J., Pedersen, TS., Jorgensen, HH., Rehling, M., Frokiaer, J. (1996). "Renal hemodynamic response to gradated ureter obstruction in the pig." Nephron **74**: 168.

Jacobson, M., Bergeron, L. (2002). Cell death in the nervous system. Apoptosis: the molecular biology of programmed cell death. M. Jacobson, McCarthy, N. Oxford, Oxford University Press.

Jacobson, M. D., Weil, M., Raff, M.C. (1997). "Programmed cell death in animal development." Cell **88**: 347-354.

Keller, S., Jones, JM, Boyle, A, Barrow, LL, Killen, PD, Green, DG, Kapousta, NV, Hitchcock, PF, Swank, RT, Meisler, MH. (1994). "Kidney and retinal defects (Krd), a transgenic-induced mutation with a deletion of mouse chromosome 19 that includes the Pax2 locus." Genomics **23**: 309-320.

King, K., Cidlowski, JA. (1998). "Cell cycle regulation and apoptosis." Annu Rev Physiology **60**(601-617).

Klahr, S., Ishidoya, S., Morrissey, J. (1985). "Role of angiotensin II in the tubulointerstitial fibrosis of obstructive nephropathy." American Journal of Kidney Disease **26**: 141.

Klahr, S., Morrissey, J. (2003). "Obstructive nephropathy and renal fibrosis: The role of bone morphogenic protein-7 and hepatocyte growth factor." Kidney International (Supplement) **87**: s105-12.

Knecht, A., Fine, LG., Kleinman, KS., Rodemann, HP., Muller, GA., Woo, DD., Norman, JT. (1991). "Fibroblasts of rabbit kidney in culture. II. Paracrine stimulation of papillary fibroblasts by PDGF." American Journal of Physiology **261**: F292.

Kreidberg, J. A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., Jaenisch, R. (1993). "WT1 is required for early kidney development." Cell **74**: 679-691.

Kuure, S., Vuolteenaho, R., Vainio, S. (2000). "Kidney morphogenesis: cellular and molecular regulation." Mechanisms of Development **92**: 31-45.

Lange-Sperandio, B., Cachat, F., Thornhill, BA, Chevalier, RL. (2002). "Selectins mediate macrophage infiltration in obstructive nephropathy in newborn mice." Kidney International **61**: 516-524.

Lechner, M., Dressler, GR (1997). "The molecular basis of embryonic kidney development." Mechanisms of Development **62**: 105-120.

Lee, S., Huang, K, Palmer, R., Truong, VB, Herzlinger, D, Kolquist, KA, Wong, J, Paulding, C., Yoon, SK, Gerald, W, Oliner, JD, Haber, DA. (1999). "The Wilms tumor suppressor WT-1 encodes a transcriptional activator of amphiregulin." Cell **98**: 663-673.

Liapis, H. (2003). "Biology of congenital obstructive nephropathy." Nephron Experimental Nephrology **93**: 87-91.

Liapis, H., Nag, M., Steinhardt, G. (1994). "Effects of experimental ureteral obstruction of platelet-derived growth factor-A and type I procollagen expression in fetal metanephric kidneys." Pediatric Nephrology **8**: 548.

Liapis, H., Yu, H., Steinddhardt, GF. (2000). "Cell proliferation, apoptosis, Bcl-2 and Bax expression in obstructed opossum early metanephroi." Journal of Urology **164**: 511-517.

Lipschutz, J. H. (1998). "Molecular Development of the Kidney: A review of the results of gene disruption studies." American Journal of Kidney Diseases **31**: 383-397.

MacDonald, R., Barth, KA, Xu, Q, Holder, N, Mikkola, I, Wilson, SW (1995). "Midline signalling is required for Pax gene regulation and patterning of the eyes." Development **121**: 3267-3278.

Mansouri, A., Hallonet, M., Gruss, P. (1996). "Pax genes and their roles in cell differentiation and development." Current Opinions in Cell Biology **8**: 851-857.

Matsell, D. G., Tarantal, A.F. (2002). "Altered primate glomerular development due to in utero urinary tract obstruction." Kidney International **61**: 1263-1269.

Matsell, D. G., Tarantal, A.F. (2002). "Experimental models of fetal obstructive nephropathy." Pediatric Nephrology **17**: 470-476.

Meier, P., Finch, A., Evan, G. (2000). "Apoptosis in development." Nature **407**: 796-801.

Moller, J., Skriver, E, Olsen, S., Maunsbach, AB. (1984). "Ultrastructural analysis of human proximal tubules and cortical interstitium in chronic renal disease (hydronephrosis." Virchows Arch A **402**: 209-237.

Moody, T. E., Vaughan, E.D., Gillenwater, J.Y. (1977). "Comparison of the renal hemodynamic response to unilateral and bilateral ureteral occlusion." Investigations in Urology **14**: 455.

Nathan, C. (1987). "Secretory products of macrophages." Journal of Clinical Investigation **79**: 319.

Navar, I., Baer, PG (1970). "Renal autoregulatory and glomerular filtration responses to gradated ureteral obstruction." Nephron **7**: 301.

Nornes, H., Dressler, GR, Knapik, EW, Deutsch, U, Gruss, P. (1990). "Spatially and temporally restricted expression of Pax2 during murine neurogenesis." Development **109**: 797-809.

Orellana, S. A., Avner, E.D. (1998). "Cell and molecular biology of kidney development." Seminars in Nephrology **18**: 233-243.

Orrenius, S. (1995). "Apoptosis: molecular mechanisms and implications for human disease." Journal of Int Med **237**: 529-536.

Ostrom, L., Tang, M.-J., Gruss, P., Dressler, G.R. (2000). "Reduced Pax2 gene dosage increases apoptosis and slows the progression of renal cystic disease." Developmental Biology **219**: 250-258.

Pachnis, V., Mankoo, B., Costantini, F. (1993). "Expression of the c-ret proto-oncogene during mouse embryogenesis." Development **119**: 1005-1017.

Parillo, J., Burch, C, Shelhamer, JH, Parker, MM, Natanson, C, Schuette, W. (1985). "A circulating myocardial depressant substance in humans with septic shock: septic shock patients with a reduced ejection fraction have a circulating factor that depresses in vitro myocardial cell performance." Clinical Invest. **76**: 1539-1553.

Pichel, J. G., Shen, L., Sheng, H.Z., Granholm, A.C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., Sariola, H., Westphal, H. (1996). "Defects in enteric innervation and kidney development in mice lacking GDNF." Nature **382**: 73-76.

Porteous, S., Torban, E., Cho, N.-P., Cunliffe, H., Chua, L., McNoe, L., Ward, T., Souzaa, C., Gus, P., Giugliani, R., Sato, T., Yun, K., Favor, J., Sicotte, M., Goodyer, P., Eccles, M. (2000). "Primary renal hypoplasia in human and mice with PAX2 mutations: evidence of increased apoptosis in fetal kidneys of Pax21Neu +/- mutant mice." Human Molecular Genetics **9**: 1-11.

Provoost, A., Molenaar, JC. (1981). "Renal function during and after a temporary complete unilateral ureter obstruction in rats." Investigations in Urology **18**: 242.

Roberts, A., McCune, BK., Sporn, MB. (1992). "TGF-B regulation of extracellular matrix." Kidney International **41**: 557.

Rothenpieler, U. W., Dressler, G.R. (1993). "Pax2 is required for mesenchyme-to-epithelium conversion during kidney development." Development **119**: 711-720.

Ryan, G., Steele-Perkins, V., Morris, J.F., Rauscher, F.J., Dressler, G.R. (1996). "Repression of Pax-2 by WT1 during normal kidney development." Development **121**: 867-875.

Sanchez, M. p., Silos-Santiago, I., Frisen, J., He, B., Lira, S.A., Barbacid, M. (1996). "Renal agenesis and the absence of enteric neurons in mice lacking GDNF." Nature **382**: 70-73.

Santarosa, R., Colombel, MC., Kaplan, S., Monson, F., Levin, RM., Buttyan, R. (1994). "Hyperplasia and apoptosis; opposing cellular processes that regulate the response of the rabbit bladder to transient outlet obstruction." Lab Invest. **70**: 503-510.

Sanyanusin, P., McNoe, L.A., Sullivan, M.J., Weaver, R.G., Eccles, M.R. (1995). "Mutation of PAX2 in two siblings with renal-coloboma syndrome." Human Molecular Genetics **4**: 2183-2184.

Sariola, H., Sainio, K. (1997). "The tip-top branching ureter." Current Opinions in Cell Biology **9**: 877-884.

Saxén, L. (1987). Organogenesis of the Kidney. Cambridge University Press. Cambridge.

Schimmenti, L. A., Cunliffe, H.E., McNoe, L.A., Ward, T.A., French, M.C., Shim, H.H., Zhang, Y.-H., Proesmans, W., Leys, A., Byerly, K.A., Braddock, S.R., Masuno, M., Imaizumi, K., Devriendt, K., Eccles, M.R. (1997). "Further delineation of renal-coloboma syndrome in patients with remarkable variability of phenotype and identical PAX2 mutations." American Journal of Human Genetics **60**: 869-878.

Schuchardt, A., D;Agati, V., Larsson-Blomberg, L., Costantini, F., Pachnis, V. (1994). "Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret." Nature **367**: 380-383.

Silverstein, D. M., Travis, B.R., Thornhill, B.A., Schurr, J.S., Kolls, J.K., Leung, J.C., Chevalier, R.L. (2003). "Altered expression of immune modulator and structural genes in neonatal unilateral ureteral obstruction." Kidney International **64**: 25-35.

Steinhardt, G., Liapis, H., Phillips, B., Vogler, G., Nag, M., Yoon, KW. (1995). "Insulin-like growth factor improves renal architecture of fetal kidneys with complete ureteral obstruction." Journal of Urology **154**(690).

Strutz, F., Okada,H, Lo, CW., Danoff, TD., Carone, R.L., . Tomaszewski, JE, Neilson, EG. (1995). " Identification and characterization of a new fibroblast specific marker: FSP-1." Journal of Cell Biology **130**: 393-406.

Sugiyama, H., Kashihara, N., Sekikawa, T., Maeshima, Y., Okamoto, K., Kanao, K., Makino, H., Ota, Z. (1995). "Cell-matrix interactions modulate mesangial cell apoptosis." J Am Soc Nephrol **6**: 912.

Thornberry, N., Lazebnik, Y. (1998). "Caspases: enemies within." Science **281**: 1312-1316.

Torban, E., Eccles, M.R., Favor, J., Goodyer, P.R. (2000). "PAX2 suppresses apoptosis in renal collecting duct cells." American Journal of Pathology **157**: 833-842.

Torres, M., Gomez-Pardo, E., Dressler, G.R., Gruss, P. (1995). "Pax-2 controls multiple steps of urogenital development." Development **121**: 4057-4065.

Torres, M., Gomez-Pardo, E., Dressler, G.R., Gruss, P. (1996). "Pax2 contributes to inner ear patterning and optic nerve trajectory." Development **122**: 3381-3391.

Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A.S., Sieber, B.A.,Grigoriou, M., Kilkenny, C., Salazar-Grueso, E., Pachnis, V., Arumae, U. (1996). "Functional receptor for GDNF encoded by the c-ret proto-oncogene." Nature **381**: 785-789.

Vainio, S., Lin, Y. (2002). "Coordinating early kidney development: Lessons from gene targeting." Nature Genetics **3**: 533-543.

Walton, G., Buttyan, R., Garcia-Montes, E., Olsson, CA., Hensle, TW., Sawczuk, IS. (1992). "Renal growth factor expression during the early phase of experimental hydronephrosis." Journal of Urology **148**: 510.

Warady, B. A. (1997). "Renal transplantation, chronic dialysis, and chronic renal insufficiency in children and adolescents." Pediatric Nephrology **11**: 49-64.

Weaver, R., Cashwell, LF, Lorentz, W, Whiteman, D, Geisinger, KR, Ball, M (1988). "Optic nerve coloboma associated with renal disease." American Journal of Medical Genetics **29**: 597-605.

Winyard, P. J. D., Risdon, A., Sams, V., R., Dressler, G.R., Woolf, A.S. (1996). "The PAX2 transcription factor is expressed in cystic and hyperproliferative dysplastic epithelia in Human Kidney Malformations." Journal of Clinical Investigation **98**: 451-459.

Wyllie, A. (1980). "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation." Nature **284**: 555-556.

Wyllie, A., Kerr, JFR, Currie, AR. (1980). "Cell death: the significance of apoptosis." Int Rev Cytol **68**: 251-306.