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ABSTRACT

Autosomal dominant polycystic kidney disease (PKD) is caused by mutations of the PKD1 or PKD2 genes. Cyst cells exhibit sustained expression of fetal genes such as PAX2. Normally, PAX2 is involved in kidney development and is rapidly downregulated after birth. Overactivity of the canonical β-catenin signaling pathway has also been linked to the formation of renal cysts. To determine whether β-catenin activity is linked to the level of PAX2 expression in vivo, we created a transgenic mouse overexpressing PAX2 in mature proximal tubules of the kidney. Here we report that the canonical β-catenin signaling activity is increased in mice bearing the targeted PAX2 transgene.

There is also evidence that non-canonical Wnt signaling may be involved in the development of renal cysts but this pathway is uncharacterized. We therefore studied the ontogeny of a downstream marker of this pathway (NFAT) and its localization in the developing kidney. Here we report that NFAT activity is high in early stages of kidney development and is rapidly downregulated at birth. The NFAT signal is diffuse and is expressed in both mesenchymal and epithelial cells of the developing kidney.
RESUMÉ

La formation de kystes aux reins est causée par une mutation dans le gène PKD1 ou PKD2. Les cellules formant les kystes expriment certains gènes qui sont normalement seulement exprimés au stage foetal, comme le facteur de transcription PAX2. Normalement, PAX2 est essentiel pour le développement des reins et disparaît rapidement après la naissance. La réactivation du cheminement Wnt/β-caténine canonique a aussi été liée au développement des kystes rénaux. Pour déterminer si l'activité de β-caténine est liée au niveau d'expression de PAX2, on a créé une souris transgénique exprimant PAX2 dans les tubules proximaux du rein. On rapporte que l'activité de β-caténine est élevée dans les reins des souris ayant un transgène ciblé de PAX2.

Il y a aussi des évidences que le cheminement de Wnt non-canonical est lié au développement des kystes rénaux. Cependant, ce cheminement est peu caractérisé. On a donc étudié l'ontogenèse d'une molécule de ce cheminement (NFAT) et sa localisation dans le développement du rein. On rapporte que l'activité de NFAT est élevée au commencement du développement des reins et diminue rapidement à la naissance. De plus, NFAT est actif dans les cellules mésenchymales et épithéliales pendant le développement des reins.
PRESENTATIONS ARISING FROM WORK COMPLETED FOR THESIS AND CO-AUTHOR CONTRIBUTIONS

PAX2 Activates the Canonical β-catenin Signaling Pathway in Tubular Cells During Renal Development.
*Manuscript in preparation*

The candidate was responsible for creating the rBAT/PAX2 transgenic mice and measuring renal β-catenin levels in these transgenic mice compared to their wildtype littermates.

Pax2 Expression in the Mature Proximal Tubules and the Development of Cystic Kidneys
Anne-Marie Patenaude, Diana Iglesias, Alison Dziarmaga and Paul Goodyer.
Poster presentation at the Reproductive and Developmental Axis Day, McGill University, April 2005.

PAX2 Expression in the Mature Proximal Tubules and the Development of Cystic Kidneys
Anne-Marie Patenaude and Paul Goodyer
Platform/Poster presentation to the 9th International Workshop on Developmental Nephrology, Adelaide, Australia, August 2004.

Sustained PAX2 Expression in Mature Proximal Tubules: Relationship to Cystic Kidney Disease
Anne-Marie Patenaude and Paul Goodyer
Oral presentation at the Graduate Student Research Day of the Department of Human Genetics, McGill University, May 2002.
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<tr>
<td>ADPKD</td>
<td>Autosomal Dominant Polycystic Kidney Disease</td>
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<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
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<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
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<td>ARPKD</td>
<td>Autosomal Recessive Polycystic Kidney Disease</td>
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<tr>
<td>Bcl-2</td>
<td>B cell Leukemia 2</td>
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<td>BMPs</td>
<td>Bone Morphogenetic Proteins</td>
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<td>Bp</td>
<td>Basepairs</td>
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<tr>
<td>CaCN</td>
<td>Calcineurin</td>
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<tr>
<td>CamKII</td>
<td>Calcium-calmodulin dependent Kinase II</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein Kinase 1</td>
</tr>
<tr>
<td>Cox-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>Daam1</td>
<td>Dishevelled Associated Activator of Morphogenesis 1</td>
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<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DKK</td>
<td>Dickkopf</td>
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<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DSH</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>E</td>
<td>Mouse embryonic day</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Fmi</td>
<td>Flamingo</td>
</tr>
<tr>
<td>FZ</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GATA</td>
<td>proteins that interact with conserved (T/A)GATA(G/A) motifs</td>
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<tr>
<td>GDNF</td>
<td>Glial Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
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<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
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<td>GTPases</td>
<td>Guanosine triphosphate hydrolysis</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-triphosphate</td>
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<tr>
<td>JNK</td>
<td>Jun-N-terminal Kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>Knpy</td>
<td>Knypek</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid enhancer binding protein</td>
</tr>
<tr>
<td>LTL</td>
<td><em>Lotus tetragonolobus</em> lectin</td>
</tr>
<tr>
<td>LRP5</td>
<td>LDL-Receptor-related Protein 5</td>
</tr>
<tr>
<td>LRP6</td>
<td>LDL-Receptor-related Protein 6</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>NLK</td>
<td>NEMO-Like Kinase</td>
</tr>
<tr>
<td>P</td>
<td>Post natal day</td>
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<tr>
<td>PAX2</td>
<td>Paired-box 2</td>
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<tr>
<td>PCP</td>
<td>Planar Cell Polarity</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformadehyde</td>
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<tr>
<td>PKD</td>
<td>Polycystic Kidney Disease</td>
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<tr>
<td>PKD1</td>
<td>Polycystic Kidney Disease gene 1</td>
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<tr>
<td>PKD2</td>
<td>Polycystic Kidney Disease gene 2</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>sFRP</td>
<td>Secreted Frizzled Protein</td>
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Stbm  Strabismus
TAK-1  Tat-Associated Kinase 1
TCF  T-Cell Factor
Wnt  Wingless
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
1. INTRODUCTION

1.1. Mammalian kidney development

The kidney develops through reciprocal interactions between the metanephric mesenchyme and the ureteric bud epithelium. These two tissues both derive from the intermediate mesoderm. The ureteric bud forms as an evagination from the mesonephric duct into the metanephric mesenchyme and each ureteric bud branch tips induces the mesenchymal cells to condense (Bates, 2000). In turn, the mesenchymal cells induce the ureteric bud to branch. The condensing mesenchymal cells aggregate and epithelialize to form renal vesicles. Each renal vesicle then undergoes morphogenesis to form the nephron, the functional unit of the kidney. At their distal ends, nephrons fuse with a branch of the ureteric bud to provide an outlet for fluid to enter the collecting system. The nephron consists of many segments: the renal corpuscle, the proximal convoluted and straight tubule, the loop of Henle, and the distal convoluted tubules (Yu et al, 2004). (Figure 1).

1.2. Wnt signaling

1.2.1. Wnt genes

Many regulatory molecules are involved in forming the kidney. At present, more than 40 genes participate in rodent kidney development (Bates, 2000). Transcription factors, growth factors and their receptors, as well as proteoglycans and their biosynthetic enzymes are essential for normal kidney formation (Vainio et al, 2002). Wnts are a large family of secreted glycoproteins that regulate key morphogenetic steps during embryogenesis (Vainio et al, 2002). They are involved in cell differentiation, cell migration, cell polarity and cell proliferation (Pandur et al, 2002). There are five Wnt
Figure 1. Stages of kidney formation (Yu et al, 2004). Kidney formation starts by reciprocal interactions between the metanephric mesenchyme and cells of the ureteric buds. At E11.5, in the mouse, the mesenchymal cells condense and aggregate around the ureteric bud tips. At E12.5, these mesenchymal cells undergo epithelialization to form renal vesicles. At E13.5, tubulogenesis starts and will later form the nephron.
genes: Wnt2b, Wnt4, Wnt6, Wnt7b and Wnt11 expressed in the mammalian fetal kidney. Wnt4 is thought to be an important initiating signal for tubulogenesis. It is expressed in the condensed mesenchyme and in pretubular aggregates to form each s-shaped body (Stark et al 1994). Wnt4 knock-out mouse fails to undergo mesenchymal to epithelial transformation and tubules do not form despite initial ureteric bud outgrowth (Stark et al 1994). The metanephric mesenchyme expresses Wnt2b, which stimulates ureteric bud growth in vitro (Lin et al, 2001). The ureteric bud expresses Wnt7b at its trunk. Little is known about its functional importance (Kispert et al, 1996). The tips of the ureteric bud express Wnt11 at all stages of metanephric development (Kispert et al, 1996). In the absence of Wnt11, branching morphogenesis is abnormal and results in kidney hypoplasia. Thus, Wnt11 has been proposed to regulate ureteric branching, in part, by a positive autoregulatory feedback loop with mesenchymal GDNF signals (Majumdar et al, 2003). The ureteric bud also expresses Wnt6, but like Wnt7b, little is known about its role. Some groups postulated that Wnt6 triggers epithelial signals of tubulogenesis by activating the Wnt4 pathway (Itaranta et al, 2002) (figure 2). Moreover, Wnts activate more than one type of signaling pathway (Veeman et al, 2003). The best understood pathway is the canonical Wnt pathway which activates the nuclear functions of β-catenin. The non-canonical Wnt pathways do not utilize β-catenin as a signal transducer, but involve either intracellular release of calcium ions (Kuhl et al, 2000) or activation of small GTPases and Jun-N-terminal kinase (Habas et al, 2003).
Figure 2. Wnts expression in the developing kidney (Vainio, 2003). The ureteric bud (UB) expresses factors that induce the mesenchymal cells to condense (black arrow) and reciprocal signals from the mesenchyme induce ureteric bud branching (white arrow). The activation of Wnt4 is induced in the mesenchyme (IM) by signals from the UB and triggers nephron formation in the mesenchyme by auto induction (curved arrow). The ureteric bud expresses Wnt6 which can induce tubule formation, Wnt7b in the UB trunk and Wnt11 in the UB tips. Wnt2b is expressed in the perinephric mesenchyme (PM) and can promote its initiation by reciprocal signals.
1.2.2. Canonical Wnt signaling

The canonical Wnt pathway, also known as the Wnt/β-catenin pathway, is highly active in fetal kidney and ceases to signal in the perinatal period (Iglesias et al, 2004). Wnt2b, Wnt4 and Wnt7b activate the canonical β-catenin pathway in the kidney. In the absence of Wnt ligands, β-catenin is recruited into a complex for its degradation containing APC (Adenomatous polyposis coli) and Axin, which sequentially recruits CK1 (Casein kinase 1) then GSK3 (Glycogen synthase kinase 3) to phosphorylate β-catenin. This leads to β-catenin ubiquitination and proteasomal degradation (Moon et al, 2004). Therefore, when the signaling pathway is inactive, cells maintain low level of cytoplasmic and nuclear β-catenin. However, β-catenin can associate with cadherins at the plasma membrane and avoids degradation (Nelson et al, 2004). Wnts interact with secreted antagonists sFRP (secreted Frizzled protein) and DKK (Dickkopf) which prevent activation of the pathway (Moon et al, 2004). When Wnts surpass the capacity of inhibitors, they interact with the FZ (Frizzled) family of receptors which heterodimerize with either LRP5 or LRP6 co-receptors (LDL-receptor-related protein 5 and 6) (He et al, 2004). The binding of Wnt to FZ leads to the activation and recruitment of DSH (Dishevelled) to the membrane. Active DSH inhibits Axin and GSK3, which in turn reduces the phosphorylation and degradation of β-catenin (Tolwinski et al, 2004). Therefore, when the pathway is activated, it increases the stability of β-catenin in the cytoplasm permitting its accumulation in the nucleus. In the nucleus, β-catenin interacts with TCF and LEF family members to activate the transcription of target genes that influence cell proliferation, cell survival and cell fate (Moon et al, 2004) (Figure3).
1.2.3. Wnt/calcium signaling

Upon binding to certain members of the Frizzled family of Wnt receptors, some Wnts, for example Wnt4 and Wnt11 are able to elicit an intracellular release of calcium ions. Binding of these Wnts to their Frizzled receptor leads to an activation of heterotrimeric G-proteins and subsequent activation of phospholipase C by the G-protein beta/gamma (Slusarski et al, 1997). This enzyme cleaves PIP$_2$ (phosphatidylinositol-4,5-biphosphate) into IP$_3$ (inositol-1,4,5-triphosphate) and DAG (diacylglycerol). IP$_3$ releases from the membrane, binds to the IP$_3$ receptor which then locally releases calcium ions from intracellular stores. This activates calcium-sensitive proteins: PKC (protein kinase C) (Sheldahl et al, 1999), CamKII (calcium-calmodulin dependent kinase II) (Kuhl et al, 2000), and/or CaCN (calcineurin) (Saneyoshi et al, 2002) (Figure4).

1.2.3.1. Protein kinase C pathway

DAG activates PKC in a calcium-dependent manner and is a key enzyme in development, by regulating growth and differentiation (Kuhl, 2004). Wnts regulate PKC which has been linked to cell-cell adhesion and tissue separation during embryogenesis (Winklbauer et al, 2001), cell movements during gastrulation (Kinoshita et al, 2003) and cardiomyogenic differentiation (Koyanagi et al, 2005). Interestingly, activated PKC inhibits canonical Wnt signaling upstream of β-catenin by phosphorylation of Dishevelled (Kuhl et al, 2001). There is a definitive link between Wnt signaling and PKC but the details of this pathway are still poorly understood. In the kidney, PKC is involved in ureteric bud branching through activation of MTF (metanephros-derived tubulogenic factor) (Araki et al, 2003). Conversely, inhibition of PKC in the developing kidney
Figure 3. Canonical Wnt signaling (Moon et al, 2004). A) In the absence of Wnts, β-catenin is degraded and there is no expression of target genes. B) In the presence of Wnts, β-catenin accumulates in the cytoplasm and enters the nucleus, binds to TCF/LEF-family of transcription factors to activate expression of target genes.
interferes with nephron formation, induces growth arrest and apoptosis (Serlachius et al, 1997). In adult life, PKC has been suggested to have a role in functional regulation of the mature kidney (Ostlund et al, 1995).

1.2.3.2. Calcium-calmodulin dependent kinase II pathway

CamKII is a multifunctional protein kinase and is activated by binding of calcium/calmodulin (Kuhl, 2004). It has a role in the development of slow muscle fibers (Anakwe et al, 2003). Also, it has been shown to function during early ventral development in Xenopus (Kuhl et al, 2000) through activation of TAK-1 and NLK (Ishitani et al, 2003). Interestingly, NLK is an inhibitor of the Wnt/β-catenin pathway (Ishitani et al, 1999). Many transcription factors such as ELK-1 (Miranti et al, 1995) and cytoskeleton proteins (Braun et al, 1995) are downstream targets of CamKII. However, it is unclear if these proteins are also activated during Wnt signaling.

1.2.3.3. Calcineurin/NFAT pathway

Calcineurin activity is controlled by calcium/calmodulin and targets NFAT (nuclear factors of activated T cell). NFAT was first identified in T cells as a binding factor to the distal antigen receptor responsive element of the human IL-2 promoter (Shaw et al, 1988). The NFAT family is comprised of five members (NFAT1-5) by homology domains. However, NFAT5 is not regulated by calcium/calcineurin. In an inactive state, NFAT is phosphorylated and stays in the cytoplasm. When calcineurin gets
activated, it dephosphorylates NFAT, activating the transcription factor, and NFAT enters the nucleus to regulate target gene expression. NFAT binds to DNA as a monomer or a dimer but also binds to target genes in partnership with AP-1 (Jun/fos) (Macian et al, 2001) or GATA transcription factors (Molkentin et al, 1998). The NFAT binding site is a 9bp element, possessing the consensus sequence (A/T)GGAAA(A/N)(A/T/C)N (Macian et al, 2001). NFAT plays important roles in the control of gene expression during cell activation and differentiation and is well characterized in the immune system. However, there is now evidence that NFATs are expressed in many cell types and tissues. Studies show that the Wnt/calcium pathway activates calcineurin/NFAT and has a role in ventral development (Saneyoshi et al, 2002). The calcineurin/NFAT pathway also regulates the cell cycle machinery (Viola et al, 2005) and cell differentiation through activation of p21 in keratinocytes (Santini et al, 2001). NFAT activates growth signals such as BMPs in skeletal development (Tomita et al, 2002) and promotes angiogenesis through VEGF (Rafiee et al, 2004) and cox-2 (Hernandez et al, 2001), ultimately leading to heart development. NFATs also inhibit apoptosis by activating bcl-2 (Kawamura et al, 2004) and are promote tissue invasion and migration (Jauliac et al, 2002).

1.2.4. Planar Cell Polarity signaling

The planar cell polarity pathway (PCP pathway) has a well characterized role in determining epithelial polarity in Drosophila (Gubb et al, 1982). In mammalian cells, polarity is a property shown by some epithelia to become polarized within the plane of the epithelium, along an axis perpendicular to the apical-basal axis of the cell (Fanto et al, 2004). This pathway involves the cadherin-related molecule flamingo (Fmi), the proteoglycan knypek (Kny) and the molecule strabismus (Stbm). Stbm interacts with Dsh
Figure 4. Wnt/Ca\(^{2+}\) signaling. Upon binding of particular Wnts to FZ receptor, there is release of intracellular calcium ions via G proteins and PLC. High intracellular calcium activates three calcium sensitive enzymes: PKC, CamKII and Calcineurin. When Calcineurin is activated, it dephosphorylates cytoplasmic NFAT which translocates in the nucleus where it activates the expression of target genes.
which then interacts with Daam1 (Huelsken et al, 2002). Downstream mammalian effectors such as the small GTPases Rac and Cdc42 become activated which in turn turn-on the c-Jun/NH$_2$-terminal kinase (JNK) signaling cascade (Noselli et al, 1999) (Figure 5). Interestingly, Stbm functions as an antagonist of the Wnt/β-catenin pathway but promotes phosphorylation of c-Jun- and AP-1-dependent transcription (Park et al, 2002). The PCP pathway is involved in convergent-extension movement during vertebrate gastrulation, reorganization of the cell surface/cell shape changes and organization of the actin cytoskeleton (Fanto et al, 2004).

1.3. Polycystic Kidney Disease

1.3.1. Wnt signaling and Polycystic Kidney Disease

The β-catenin signaling pathway is implicated/deregulated in many cancers (Morin, 1999). There is now evidence that Wnt signaling may also be involved in cystogenesis as transgenic mice over expressing β-catenin develop severe polycystic kidney disease (Saadi-Kheddouci et al, 2001). Similarly, transgenic mice over expressing c-myc, a downstream target of β-catenin, in renal epithelia also develop cystic kidneys (Trudel et al, 1991). Moreover, studies suggest that the polycystins, proteins encoded by the genes responsible for PKD, are linked to the β-catenin signaling pathway (Kim et al, 1999) and to the non-canonical Wnt signaling through PKC and JNK/AP-1 (Arnould et al, 1998) and through calcineurin/NFAT (Puri et al, 2004).
Figure 4. Planar cell polarity signaling. The molecule flamingo (Fmi), knypek (Kny) strabismus (Stbm) are involved and interact with Dsh. Dsh interact with Daam1, leading to the activation of small GTPases (Rac, cdc42, RhoA) which then activate JNK. JNK translocates in the nucleus and interact with AP-1 to activate the expression of target genes.
1.3.2. What is PKD?

Polycystic kidney disease is a genetic disease transmitted in an autosomal recessive (ARPKD) or an autosomal dominant (ADPKD) fashion. ADPKD is more prevalent than the recessive form and have an incidence of around one per 1000 in the population and accounts for about 10% of end-stage renal failure (Wu et al, 2000). It is a slowly progressive genetic disease with bilateral renal cysts formation (fluid-filled cavities lined by epithelium). Cysts arise as focal out pouch from any segment of the nephron and increase in size and number with time (Boletta et al, 2003). The disease results from mutations in either the PKD1 gene (85% of cases) or the PKD2 gene (most of the remainder) (Gabow, 1993). The PKD1 gene encodes the protein polycystin-1 and the PKD2 gene encodes polycystin-2. Recent evidence suggests a “two-hit” genetic model of cystogenesis in which an inherited mutation in the PKD gene is combined with a spontaneous somatic mutation (Quian et al 1996). Renal cysts are thought to be clonal, arising from a single parent cell that has randomly acquired the second genetic mutation during cell division. PKD1 and PKD2 are essential for the normal development of tubular epithelium of the kidney, liver and pancreas although their roles are unclear. PKD1 and PKD2 expressions are developmentally regulated with higher levels in embryonic and fetal kidneys compared to adult tissues (Chauvet et al, 2002). Homozygous null mutations of either genes in mice results in renal cysts formation beginning at E15, a period where tubules elongate and mature (Lu et al, 1997 and Wu et al, 1998). Polycystin-1 and polycystin-2 functions in the kidney are unknown but they could induce terminal epithelial differentiation and control the size of tubules (Boletta et al, 2003). Analysis of tissue from cysts indicates that cyst cells have abnormal expression of fetal genes.
compared to their surrounding normal cells (Wilson, 2004). Interestingly, ADPKD cells express high levels of the developmental transcription factor PAX2 (Wilson et al, 1995). PAX2 over expression has been reported in other cystic kidney diseases such as nephronophthisis (Murer et al, 2002) and multicystic/dysplastic kidneys (Winyard et al, 1996). Moreover, transgenic mice with unregulated over expression of PAX2 results in cystic dysplasia of the kidney (Dressler et al, 1993).

1.3.3. PAX2

PAX2 is a member of the “paired-box” family of homeotic genes (Sanyanusin et al, 1996) and is essential in the conversion of mesenchymal to epithelial cells (Rothenpieler et al 1993). Heterozygous PAX2 mutations cause renal hypoplasia and homozygous PAX2 inactivation results in absence of kidney formation (Favor et al, 1996, Torres et al, 1995). PAX2 is expressed in the induced mesenchyme and the ureteric bud. It is thought to stimulate growth by activating some particular genes, such as GDNF (Brophy et al, 2001) and suppressing apoptosis (Porteous et al, 2000). However, PAX2 is rapidly down regulated postnatally and is barely detectable in the mature kidney (Dressler et al, 1992). Interestingly, PAX2 may directly activate the expression of Wnt4 in renal tubular cells of mesenchymal origin (Chu et al, 2004). PAX2 and Wnt signaling could therefore be implicated in formation of renal cysts.
2. RESEARCH PROPOSAL

There is evidence that failure to downregulate developmental genes is involved in polycystic kidney disease. Since PAX2 is overexpressed in PKD cyst cells, we hypothesize that polycystin-dependent signals might normally suppress PAX2 during development. This failure of polycystin suppression of PAX2 might enhance Wnt signaling activity in cyst cells.

To determine the effect of sustained high-level PAX2 on renal β-catenin signaling, we generated a transgenic mouse line bearing the full-length hPAX2 cDNA linked to a β-galactosidase reporter in an IRES vector under the transcriptional control of the human cystinuria gene (rBAT) promoter. This construct was intended to target PAX2 expression to the mature postnatal proximal tubules.

To determine if non-canonical Wnt signaling is also involved in cyst formation, we studied the ontogeny and localization of NFAT activity in the kidneys of transgenic mice bearing a luciferase reporter under the control of nine NFAT binding sites. Little is known about this signaling pathway in the kidney. Thus, these preliminary experiments will be useful for later studies involving this signaling pathway in kidney diseases.
3. MATERIALS AND METHODS

3.1. Transient transfection assay

3.1.1. Cell lines

OK (opossum proximal tubules) cells, JTC12 (monkey proximal tubule) cells, HEK293 (human embryonic kidney) cells and COS-7 (monkey fibroblasts) were all cultured in Dulbecco’s modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured in a 37°C incubator supplemented with 5% CO₂.

3.1.2. Tissue specificity of the rBAT promoter

rBAT-luciferase activity was compared in JTC12, HEK293 and COS-7 cells. All cells were seeded in 6-well tissue culture dishes 24h before transfection. To ensure tissue specificity of the proximal tubule-specific promoter of the SLC3A1 cystinuria gene (rBAT), cells were transfected with 0.8μg rBAT promoter into a luciferase reporter vector (pGL3 basic) (Promega, Madison, WI) and 0.2μg pCMV-β-galactosidase plasmid (BD Biosciences, Bedford, MA) to permit normalization of transfection efficiencies. Transfections were performed using Fugene6 reagent (Roche, Mannheim, Germany) following procedures recommended by the manufacturer. 48h after transfections, β-galactosidase activity was assayed using Galacto-Light (Tropix Inc., Bedford, MA) and firefly luciferase was assayed using luciferin (Promega, Madison, WI). Both measurements were quantified in a luminometer (EG&G Berthold microplate
luminometer LB 96 V). Activity was expressed as (relative light) units of luciferase / units of β-galactosidase.

3.1.3. Transgene functionality by x-gal staining

To ensure the transgene was functional, JTC12 (monkey proximal tubule) cells and OK (opossum proximal tubule) cells were transiently transfected with the transgenic construct using Fugene6 reagent (Roche, Mannheim, Germany). Cells were fixed in cultured well at room temperature for 10 minutes in PBS containing 0.2% glutaraldehyde and 1% formaldehyde, rinsed in PBS and stained in the dark for 48 hours at 37°C in PBS with 2mM MgCl₂, 0.02% NP-40, 0.01% Deoxycholate, 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5mM potassium ferricyanide and 5mM potassium ferrocyanide. β-galactosidase converts x-gal and produce a blue reaction. After the reaction, the presence of blue cells indicates β-galactosidase of the transgene is active. Images were visualized with a Zeiss microscope at 10X objective.

3.2. Western immunoblotting

Proteins were isolated from OK (opossum proximal tubules) or JTC12 (monkey proximal tubules) transfected cells, from kidneys of 13-28 weeks old rBAT/PAX2 mice and control littermates using a lysis buffer consisting of 8M urea, 0.1mM EDTA, 4% SDS and 40mM Tris-HCL pH6.8. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL), following the manufacturer's instructions. Thirty micrograms of total protein lysate were diluted in 62.5mM Tris-HCL pH6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.004% bromophenol blue. Samples were
boiled for 5 minutes and subjected to SDS-polyacrylamide gel electrophoresis using a
10% separating gel. Samples were transferred to a nitrocellulose Hybond-ECL membrane
(Amersham Biosciences, Little Chalfont, England), treated with blocking buffer (5% dry
milk in PBS-tween 0.1%) for 1 hour, washed in buffer (PBS-Tween 0.1%) and probed
with rabbit polyclonal anti-PAX2 (1:250) (Zymed, San Francisco, CA) and rabbit
polyclonal anti-total-β-catenin (1:1000) (Cell Signaling, Beverly, MA). Membranes were
washed and probed with the secondary antibody peroxidase-conjugated anti-rabbit IgG
(1:1000) (Perkin Elmer life science, Boston, MA). Membranes were washed and exposed.
Membranes were then stripped in distilled water with 62.5mM Tris-HCl pH6.7,
2% SDS, 100mM 2-mercaptoethanol and probed with mouse monoclonal anti-actin
(1:5000) (Oncogene, San Diego, CA) to ensure equal loading. Membranes were washed
and probed with peroxidase-conjugated anti-mouse IgG (1:2000) (Calbiochem, San
Diego, CA). All washes were carried at room temperature 3 x 10 minutes and incubations
with antibodies were all performed for 1 hour at room temperature. All membranes were
treated with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech,
Little Chalfont, England) and exposed to autoradiography film. The pixel density of
protein bands identified by immunoblotting was adjusted for the density of a control
protein (actin) using the MCID-M5 4.0 image analysis software.
3.3. Creating rBAT/PAX2 transgenic mice

3.3.1. Cloning the rBAT promoter into the IRES vector

3.3.1.1. Preparation of the rBAT promoter

First, the rBAT promoter was cloned into an IRES (Internal Ribosome Entry Site) expression vector containing a LacZ reporter (provided by Dr. D.Dufort). A SacII restriction site was inserted at the 5' end of rBAT in pGL3 basic using primers:

 Sense: 5'-ACACACACCGCGGCTTGGAGACAGAGTCTCC-3'
 Antisense: 5'-TCTTCCAGCGGATAGAATGG-3'

The reaction was done using 1X DNA polymerase pfu buffer (Invitrogen, Carlsbad, CA), 0.2mM dNTPs, 100ng/μl each of the sense and antisense primers, 2.5U DNA polymerase pfu and 100ng/μl template DNA. The cycle began with a "hotstart" at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 1 minute at 72°C, ending with an additional 10 minutes at 72°C and a 4°C soak. The amplified PCR product was cleaned using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The PCR product was digested with 1 unit NarI restriction enzyme (NEB, Beverly, MA) in 1X T4 polymerase buffer (Invitrogen, Carlsbad, CA), for 2h30 at 37°C. The reaction was inactivated at 65°C for 20 minutes. To blunt end the NarI site, 1 unit T4 polymerase (Invitrogen, Carlsbad, CA), and 0.2mM dNTPs were added to the NarI digest cocktail and incubated for 20 minutes at 11°C. The reaction was stopped at 65°C for 15 minutes. Then, 1 unit of SacII restriction enzyme (NEB, Beverly, MA) was added to the NarI blunt cocktail. The digest was carried at 37°C for 3 hours and heat inactivated for 20 minutes at 65°C.
3.3.1.2. Preparation of the IRES vector

The IRES vector was digested with 1 unit NotI enzyme (NEB, Beverly, MA) in 1X T4 polymerase buffer (Invitrogen, Carlsbad, CA), for 3 hours at 37°C and heat inactivated for 20 minutes at 65°C. The NotI site was blunted by adding 1 unit T4 DNA polymerase (Invitrogen, Carlsbad, CA) with 0.2mM dNTPs for 20 minutes at 11°C and the reaction was stopped by heating to 65°C for 15 minutes. 1 unit SacII enzyme (NEB, Beverly, MA) was added for 3 hours at 37°C and heat inactivated for 20 minutes at 65°C.

3.3.1.3. Ligation of the rBAT promoter into the IRES vector

Both the rBAT promoter and the IRES vector were cleaned using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). In gel ligation was performed using 0.5μg IRES vector and 5μg rBAT insert that were runed on 1% low melting point agarose. Each DNA gel piece was isolated in a separate tube and melted at 70°C for 5 minutes. 5μl rBAT DNA and 5μl IRES DNA were mixed together and put back at 70°C for 5 minutes followed by 1 minute at 37°C. The ligation mix: 2X T4 ligase buffer (Invitrogen, Carlsbad, CA), and 1 unit T4 DNA ligase (Invitrogen, Carlsbad, CA), was heated to 37°C for 1 minute, added to the DNA tube and incubated overnight at 15°C. The reaction tube containing the ligation was then incubated for 5 minutes at 70°C and transferred to 37°C for 1 minute.

3.3.1.4. Cell transformation and colony analysis

50μl DS5α competent cells were transformed with 5μl ligated DNA by incubation on ice for 30 minutes, followed by 30 seconds at 42°C and back on ice. 250μl Luria Broth
(LB) was added to the cell transformation tube, spread on LB-ampicillin agar plates and incubated overnight at 37°C. Colonies were analyzed by PCR using primers:

**Sense:** 5'-ATTAACCCTCACTAAAGGGA-3'

**Antisense:** 5'-TCCAACTCACAACGTGGC-3'

The reaction was done using 1X PCR buffer (Invitrogen), 1.5mM MgCl₂, 0.2mM dNTPs, 0.4μM each of the sense and antisense primers, 1 unit Taq polymerase (Invitrogen, Carlsbad, CA), and 10μl of each colony diluted in distilled water. The PCR program is the same as section 3.3.1.1. Products were run on 2% agarose gel. Bands at 0.1kb correspond to IRES vector alone (no rBAT insert ligation) and bands at 0.9kb correspond to IRES vector with rBAT insert (Figure 6A).

### 3.3.2. Cloning the PAX2 cDNA into the rBAT-IRES vector

#### 3.3.2.1. Preparation of PAX2 and rBAT-IRES

A SpeI restriction site was inserted at the 5' end of the PAX2 cDNA in pBS II SK⁻ (provided by Dr. Eccles) using primers:

**Sense:** 5'-ACACACACACTAGTATGGATATGCACTGCAAAGC-3'

**Antisense:** 5'-AATTAACCCTCACTAAAGGG-3'

The PCR reaction conditions and protocol were the same as section 3.3.1.1. The amplified product was cleaned using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The PAX2 cDNA and the rBAT-IRES vector were both digested in 1X REACT4 buffer (Invitrogen, Carlsbad, CA), with 1 unit SpeI (Invitrogen, Carlsbad, CA), for 1 hour at 37°C and heat inactivated for 10 minutes at 65°C.
3.3.2.2. Ligation of the PAX2 cDNA into the rBAT-IRES vector

See section 3.3.1.3.

3.3.2.3. Cell transformation and colony analysis

See section 3.3.1.4. Bands at 0.9kb correspond to rBAT-IRES vector alone (no PAX2 insert ligation) and bands at 2.1kb correspond to rBAT-IRES vector with the PAX2 cDNA insert (Figure 6B). Colonies having the PAX2 insert were then digested to select those with the sense orientation using 1X NEbuffer 4 (NEB, Beverly, MA), 1 unit NaeI (NEB, Beverly, MA) and 1 unit SacII (NEB, Beverly, MA) for 2 hours at 37°C. Bands at 1 kb represent PAX2 in sense orientation and bands at 2kb are PAX2 in antisense orientation.

3.3.3. Transgene injection into fertilized oocytes

The transgene (rBAT-PAX2-IRES-LacZ-SV40polyA) was excised from the vector with 1 unit SacII (NEB, Beverly, MA), 1 unit XhoI (Invitrogen, Carlsbad, CA), in 1X REACT4 buffer (Invitrogen, Carlsbad, CA), for 1 hour at 37°C and cleaned using the Qiaquick gel extraction kit (Quiagen, Hilden, Germany). The transgene was injected into pronuclei of 1 cell fertilized mouse embryos (n=30) from C3H females and C57BL/6 males at the McGill core transgenic mouse service.

3.4. Southern Blot

Transgenic founders were identified by Southern blot of mouse tail genomic DNA, isolated using the Wizard DNA Purification Kit (Promega, Madison, WI), with a 3.5 kb HindIII-EcoRI fragment of the transgene spanning part of the IRES and LacZ.
Figure 6. Preparation of the transgenic construct. A) Cloning the rBAT promoter into the IRES vector. Bands at 0.9kb represent amplification of the IRES vector multiple cloning site where the rBAT promoter got inserted. B) Cloning the PAX2 cDNA into the rBAT/IRES vector. Bands at 2.1kb represent amplification of the IRES multiple cloning site with the rBAT promoter and the inserted PAX2 cDNA. The band at 0.9kb stands for no ligation of PAX2; only the rBAT promoter was amplified.
sequence. To prepare the probe, 20μg transgene DNA was digested in 1X NE4 buffer (NEB, Beverly, MA) with 1 unit EcoRI (NEB, Beverly, MA) and 1 unit HindIII (NEB, Beverly, MA) for 2 hours at 37°C and cleaned using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). 10ng/μl probe DNA was denatured at 100°C for 5 minutes and cooled on ice for 5 minutes. The probe was labeled with alkaline phosphatase following the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, England). 10μg of each sample of genomic DNA was digested in 1X NE2 buffer (NEB, Beverly, MA) with 1 unit BamHI (NEB, Beverly, MA) for 5 hours at 37°C and run on a 1% agarose gel overnight at 20V. After electrophoresis, the DNA was denatured for 45 minutes in 1.5M NaCl and 0.5M NaOH, rinsed in distilled water and neutralized for 30 minutes in 1M Tris pH7.4 and 1.5M NaCl. The DNA was transferred on Hybond N+ membrane (Amersham Pharmacia Biotech, Little Chalfont, England) in 20X SSC buffer for 48 hours. The membrane was then soaked in 6X SSC for 5 minutes and the DNA was fixed on the membrane by baking for 1 hour at 80°C in a vacuum oven. The DNA was hybridized with the labeled probe at 60°C overnight in AlkPhos Direct hybridization buffer (Amersham Pharmacia Biotech, Little Chalfont, England) supplemented with 0.5M NaCl and 4% blocking reagent (Amersham Pharmacia Biotech, Little Chalfont, England). The membrane was washed 2 x 10 minutes at 60°C in 2M urea, 0.1% SDS, 50mM Na Phosphate pH7.0, 150mM NaCl, 1mM MgCl$_2$ and 0.2% blocking reagent (Amersham Pharmacia Biotech, Little Chalfont, England). The membrane was then washed 2 x 10 minutes at room temperature in 50mM Tris base, 0.1M NaCl and 2mM MgCl$_2$. The membrane was treated with CDP-Star detection reagent (Amersham Pharmacia Biotech,
Little Chalfont, England) for 5 minutes and exposed to autoradiography film for 1 hour. Founders carrying the transgene have a band at 3.5kb (Figure 7).

3.5. rBAT/PAX2 genotyping

Transgenic offspring were obtained by breeding founder transgenic mice to non transgenic C3B6 or by backcross breeding to C3H mice. Genomic DNA was isolated using the Wizard DNA Purification Kit (Promega, Madison, WI). Transgenic mice were identified by PCR amplifying a 250bp fragment of the IRES sequence (Figure 8) using primers:

Sense: 5'-CTGGCCCTGTCTTTCTTGA-3'
Antisense: 5'-TCCAACTCACAAACGTGCGC-3'

The reaction was done using 1X PCR buffer (Invitrogen, Carlsbad, CA), 2.0mM MgCl₂, 0.2mM dNTPs, 0.4μM each of the sense and antisense primers, 1 unit Taq polymerase (Invitrogen, Carlsbad, CA). The PCR conditions were 95°C for 5 minutes, 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by an additional 10 minutes at 72°C and a 4°C soak. PCR products were run on a 2% agarose gel for 30 minutes.

3.6. Detection of lacZ activity

Adult kidneys from transgenic and non transgenic mice were removed and prefix in PBS containing 2mM MgCl₂, 0.02% NP-40, 0.01% Deoxycholate, 1% formaldehyde and 0.2% glutaraldehyde for 1 hour at room temperature. Kidneys were then rinsed in washing
Figure 7. Southern blot to identify transgenic founders bearing the rBAT/PAX2 transgene. Genomic DNA was digested, run on a gel and transferred to membrane. The membrane was hybridized with a 3.5kb probe spanning part of the IRES and LacZ sequence. Two transgenic founders (TG) were detected by the presence of a band at 3.5kb representing the transgene compared to no transgene detection in wildtype littermates (WT).
Figure 8. rBAT/PAX2 genotyping. Primers were designed to amplify a 250bp sequence of the IRES region from genomic DNA. Mice carrying the rBAT/PAX2 transgene (TG) get amplification of the IRES sequence but not their wildtype littermates (WT).
buffer (PBS with 2mM MgCl₂, 0.02% NP-40, 0.01% Deoxycholate) and stained in the dark overnight in washing buffer supplemented with 1mg/ml X-gal, 5mM potassium ferricyanide and 5mM potassium ferrocyanide. After staining, kidneys were washed in PBS, post-fixed in 4% formalin at 4°C overnight, dehydrated through graded alcohols, embedded in paraffin and sectioned (Leica microtome) at 7μm. Sections were rehydrated through graded alcohols, counterstained with nuclear red, dehydrated and mounted using Permount (Fisher, Pittsburgh, PA). Images were visualized with a Zeiss microscope.

3.7. β-galactosidase immunofluorescence

Adult kidneys from transgenic and wildtype mice were embedded in O.C.T. (Sakura, Tokyo, Japan) and sectioned (Leica cryostat) at 14μm. Kidney sections were fixed in acetone for 20 minutes at room temperature, washed in PBS 3 x 3 minutes, blocked in normal serum for 1h at room temperature and incubated with rabbit anti-β-galactosidase (1:250) (Chemicon International, Temecula, CA) diluted in normal serum overnight at 4°C. Sections were washed 3 x 3 minutes in PBS and incubated with the secondary antibody: Rhodamine anti-rabbit IgG (1:50) (Chemicon International, Temecula, CA) diluted in normal serum for 1 hour at room temperature and washed in PBS 3 x 3 minutes. Sections were then incubated with fluorescein-*Lotus Tetragonolobus* lectin (1:200) (Vector Laboratories, Burlingame, CA), specific to proximal tubules, diluted in 10mM Hepes pH 7.5 and 0.15M NaCl, at 4°C overnight. Sections were mounted using Gel Mount™ Aqueous (Sigma, St-Louis, MO). Images were visualized by fluorescence using a Zeiss microscope.
3.8. **Hematoxylin and eosin staining**

Kidneys were fixed in 4% PFA overnight at 4°C, embedded in paraffin and sectioned at 7μm thickness (Leica microtome). Kidney sections were deparaffinized at 58°C for 1 hour, followed in xylene for 2 x 5 minutes and rehydrated through graded ethanols. Sections were stained for 1 minute in hematoxylin, rinsed in H₂O, stained in eosin and rinsed in H₂O. Sections were then dehydrated through graded ethanols, cleared in xylene and mounted with permount (Fisher, Pittsburgh, PA). Images were visualized by light microscopy using a Zeiss microscope.

3.9. **NFAT-luciferase transgenic mice**

The NFAT-luciferase transgenic mice, in the FVBN background, were provided by Dr. Molkentin from the Cincinnati's Children Hospital. These mice have nine copies of an NFAT binding site from the IL-4 promoter (5’-TGGAAAATT-3’) positioned 5’ to a minimal promoter from the α-myosin heavy chain gene, upstream of the luciferase reporter in pGL3 Basic.

3.9.1. **Breeding and timing of litters**

On the day of breeding, wildtype CD-1 females were weight and crossed to a NFAT-luciferase male. On the following day, the male was removed from the cage of the females and the weight of each female was monitored and used to determine pregnancy.
3.9.2. NFAT-luciferase genotyping

Genomic DNA was isolated using the Wizard DNA Purification Kit (Promega, Madison, WI). Transgenic mice were identified by PCR amplifying a 500bp fragment of the luciferase sequence (Figure 9) using primers:

Sense: 5'-ATCCGCTGGAGATGGAACCG-3'
Antisense: 5'-AGATGAGATGTGACGAACGTG-3'

The reaction was done using 1X PCR buffer (Invitrogen, Carlsbad, CA), 2.0mM MgCl₂, 0.2mM dNTPs, 0.4μM each of the sense and antisense primers, 1 unit Taq polymerase (Invitrogen, Carlsbad, CA). The PCR conditions were 96°C for 5 minutes, 30 cycles at 96°C for 25 seconds, 58°C for 25 seconds and 72°C for 1 minute, followed by an additional 10 minutes at 72°C and a 4°C soak. PCR products were run on a 1% agarose gel for 30 minutes.

3.9.3. Detection of NFAT activity by luciferase immunostaining

Kidneys were fixed in 4% PFA overnight at 4°C, embedded in paraffin and sectioned at 5μm thickness (Leica microtome). Kidney sections were deparaffinized at 58°C for 30 minutes, followed in xylene for 2 x 5 minutes and rehydrated through graded ethanols. Sections were boiled in 0.1M citrate buffer (pH 6.0) for antigen retrieval and then incubated in 3% H₂O₂ to blocked endogenous peroxidase activity. Slides were washed in PBS, blocked in horse serum (Vector Laboratories, Burlingame, CA) for 3 hours at room temperature and incubated overnight at 4°C with rabbit anti- luciferase antibody (1:80)
Figure 9. **NFAT-luciferase genotyping.** Primers amplify a 500bp sequence from the luciferase gene. The NFAT-luciferase amplicon is identified in genomic DNA from transgenic mice (TG) but not from their wildtype littermates (WT).
(Cortex Biochem, San Leandro, CA). After washes in PBS, immunoreactivity was detected using the universal IgG secondary antibody (Vector Laboratories, Burlingame, CA), Vectastain ABC kit (Vector Laboratories, Burlingame, CA), following the manufacturer's instructions and DAB (Vector Laboratories, Burlingame, CA) as a chromogen, according to the manufacturer's protocol. Sections were then counterstained in Gill's hematoxylin for 1 minute, rinsed in water and dehydrated through graded ethanols and mounted with permount (Fisher, Pittsburgh, PA). Images were visualized by light microscopy using a Zeiss microscope.

3.9.4. Luciferase assays

Kidneys were lysed in 1X Passive Lysis Buffer (Promega, Madison, WI) with 1mM DTT, 0.2mM PMSF and 5ug/ml leupeptin, homogenized and centrifuged for 8 minutes at 18000g. Firefly luciferase activity was determined using 100ul of the clarified lysates with addition of 100ul of 1X luciferin (Promega, Madison, WI) in 0.1M tris pH 8.0, with parameters of 2 seconds delay, 2 seconds integration. Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL) following the manufacturer's instructions. The luminescence for each of the protein sample was calculated as arbitrary units of light per microgram of protein.

3.10. Data analysis

Mean differences were examined by two-tailed Student's t-test. Significance was taken at a value of P<0.05.
4. RESULTS

4.1. The rBAT promoter is specific to proximal tubule cells in vitro

The onset of renal rBAT expression is detected in late embryonic stages; levels are still low in early postnatal life, increase rapidly at the end of lactation and are high in mature postnatal proximal tubules (Furriols et al, 1993). To ensure the 0.8kb segment of the 5’ flanking sequence upstream of the human SLC3A1 (cystinuria) gene (rBAT) targeted gene expression specifically, the 0.8kb rBAT promoter-luciferase reporter was transfected into various cell lines and standardized for transfection efficiencies with beta-galactosidase. The 0.8kb rBAT promoter drives high-level expression of luciferase in JTC12 cells (derived from monkey proximal tubule) but not in monkey fibroblasts (COS-7) nor in human embryonic kidney cells (HEK293) (p<0.005) (Figure 10).

4.2. rBAT/PAX2 transgene expresses PAX2 and β-galactosidase proteins in vitro

The transgenic construct consists of the full-length hPAX2 cDNA linked to a β-galactosidase reporter in an IRES (internal ribosomal entry site) vector under the transcriptional control of the 0.8kb human cystinuria gene (rBAT) promoter (Figure 11A). The IRES sequence permits the translation of two proteins from the same mRNA. Thus both PAX2 and β-galactosidase proteins should be expressed. To ensure the transgene was functional, JTC12 (monkey proximal tubule) cells were transiently transfected with the transgenic construct and stained with X-gal. β-galactosidase activity was detected in about 1% of cells (Figure 11B). No X-gal staining was present in control cells in which transfection of the plasmid were omitted. Therefore, the small amount of positive cells was concluded to be a result of the transfected plasmid expressing
Figure 10. The rBAT promoter is specific to proximal tubule cells in vitro. JTC12 (monkey proximal tubule) cells, KEK293 (human embryonic kidney) cells and COS7 (monkey fibroblasts) were transfected with a luciferase reporter driven by the rBAT promoter (N=3). Transfection efficiencies were standardized for β-galactosidase activity. Luciferase activity was selective for proximal tubule cells (p<0.005).
β-galactosidase. The poor transfection efficiency is due to the JTC12 cells being poorly
transfectable. OK (opossum proximal tubule) cells were also transiently transfected with
the transgenic construct and proteins were isolated to assess the levels of PAX2
expression by western immunoblots and standardized for endogenous actin. Transfected
cells had about 2 fold more PAX2 protein than non-transfected cells (p=0.01) (Figure
11C).

4.3. rBAT/PAX2 transgene expression is targeted to proximal tubules in vivo

β-galactosidase activity was assessed in adult mouse kidneys to ensure the
transgene showed tissue specific expression. Selective β-galactosidase activity was
identified in the mature proximal (LTL-positive) tubules of 4 months old transgenic mice
by immunofluorescent staining for β-galactosidase and by x-gal staining for β-
galactosidase activity (Figure 12A, B, C). The transgene was successfully targeted to
mature proximal tubules.

4.4. Canonical β-catenin signaling activity is increased in mice bearing the targeted
PAX2 transgene.

Normally, PAX2 protein is expressed at high levels in fetal kidney development,
but is down regulated in the mature kidney (Dressler et al, 1992). Canonical Wnt
signaling activity is also high in fetal kidney but falls dramatically in the perinatal period
(Iglesias et al, 2004). To assess the effect of sustained expression of PAX2 on renal β-
catenin signaling, transgenic mice bearing a transgene targeting PAX2 expression to the
mature postnatal proximal tubules were generated. PAX2 protein levels in whole kidney
Figure 11. rBAT/PAX2 transgene is functional *in vitro*. A) Schematic of the reporter construct used to generate rBAT/PAX2 transgenic mice. The transgenic construct was first tested by performing transient transfections in JTC12 (monkey proximal tubule) cells and was shown to express both β-galactosidase (N=3) and PAX2 proteins (N=3). B) Transfected cells were fixed and stained with X-gal. A few cells reacted and stained blue (arrows). C) PAX2 protein levels from cultured OK (opossum proximal tubule) cells were quantified by western immunoblots. PAX2/actin band intensity in transfected cells was about twice that from non transfected control cells (p=0.01).
Figure 12. rBAT/PAX2 transgene expression is targeted to proximal tubules in vivo. A) Transgene expression in rBAT/PAX2 adult kidney detected with a rhodamine-tagged (red) anti-β-galactosidase antibody. B) Co-localization with fluorescein-tagged (green) *Lotus tetragonolobus* lectin confirms that the transgene is expressed in proximal tubules. C) LacZ transgene expression in proximal tubules (PT) of rBAT/PAX2 adult kidney detected by X-gal staining. Images were photographed at 200X (immunohistochemistry) or 400X (immunofluorescence).
extracts of adult (17-28 weeks) mice were determined on Western immunoblots and normalized for endogenous actin. PAX2 levels in the rBAT/PAX2 transgenic mice were 2 times that in wildtype littermates (p=0.0003) (Figure 13). The same extracts were assessed for total β-catenin level; Western immunoblots were probed with an antibody specific for β-catenin and normalized for intensity of the actin band. Paralleling the increased level of PAX2, β-catenin levels in mature transgenic kidneys were 1.7 times that in wildtype controls (p=0.0002) (Figure 13). No cysts were observed in adult rBAT/PAX2 transgenic mice up to 1 ½ years old; kidneys were phenotypically normal (Figure 14).

4.5. Ontogeny of NFAT activity in the kidney

Canonical Wnt signaling activity is high in early stages of kidney development but become restricted to the tips of the ureteric buds by E18 and is progressively downregulated (Iglesias et al, 2004). Zhong et al (2001) compared AP-1-dependent luciferase activity in various tissue extracts of different aged mice. They found that AP-1 activity, in most organs, decreases with age. In the kidney, it decreases sharply from 1 day- to 3 months-old mice. Therefore, the level of AP-1 activity, reflecting activity of the planar cell polarity pathway (non canonical Wnt signaling) is also high in fetal life. Little is known about the Wnt/Ca\(^{2+}\) signaling in renal tissue. Because the NFAT transcription factor is known to be activated by Wnt/Ca\(^{2+}\) signaling, we used a transgenic mouse line bearing a luciferase reporter under nine NFAT binding sites (Wilkins et al, 2004). Wilkins et al (2004) used these transgenic mice to clarify the controversy about calcineurin-NFAT signaling in maintaining or initiating heart hypertrophy. Interestingly,
Figure 13. Expression of β-catenin is up regulated in rBAT/PAX2 transgenic mice. Lysates of whole kidneys (N=4) were analyzed by Western immunoblots for PAX2 and β-catenin. PAX2/actin band intensity was increased (160% of wildtype controls) in rBAT/PAX2 transgenic mouse kidney (p=0.0003). Similarly, total β-catenin expression was also increased (145% of wildtype controls) in rBAT/PAX2 mouse kidneys (p=0.0002).
Figure 14. rBAT/PAX2 transgenic mice have no cystic phenotype. A) Kidneys from 1 ½ years old transgenic mouse were stained with hematoxylin and eosin to study the renal morphology. No renal cysts were observed in adult rBAT/PAX2 transgenic mice. The kidneys were phenotypically normal compared to wildtype mice B). Images were photographed at 200X.
NFAT-luciferase activity was the highest during developmental maturation and decreased with age. In our study, NFAT transcriptional activity was studied during embryonic and perinatal kidneys by measuring luciferase activity in tissue extracts. NFAT activity is the highest in early embryonic stages (E14.5), it decreases markedly at E16.5-E18.5 and it decreases progressively in the perinatal period. There was no detectable activity in the adult kidney (Figure 15).

4.6 Tissue distribution of NFAT-luciferase activity in the kidney

To define the site of action of NFAT in the kidney, immunostaining against luciferase was performed in NFAT-luciferase transgenic mice. In embryonic kidneys, the signal is faint and diffuse. This signal was considered positive because it is absent from negative controls where the primary antibody was omitted (Figure 16I) and it is also absent in postnatal kidneys (Figure 16G). At E14.5, active NFAT is expressed in mesenchymal cells (Figure 16A), in condensed mesenchyme and renal vesicles (Figure 16B), in the ureteric bud and s-shaped bodies (Figure 16C), and newly formed glomerulus (Figure 16D). From E16.5 to E18.5, the signal is apparent in mesenchymal cells, mature tubules and faintly in glomerulus (Figure 16E). At P1, NFAT activity is undetectable in mouse kidneys (Figure 16F) as well as in kidneys of adult mice (Figure 16G).
Figure 15. Ontogeny of NFAT activity in the kidney. Lysates of whole kidneys were analyzed by measuring luciferase activity and standardized for the amount of proteins in each sample. Luciferase activity is the highest at E14.5, decreases modestly at E16.5-E18.5. From P1 to P21, luciferase activity continues to decrease with time. At 2 months old, no luciferase activity can be detected in kidneys from NFAT-luciferase transgenic mice.
Figure 16. Localization of active NFAT in the kidney. Immunohistochemistry against luciferase was performed in kidneys of NFAT-luciferase transgenic mice. A) At E14.5, NFAT is active in the metanephric mesenchyme (MM). B) It is also present in the condensed mesenchyme (CM) and renal vesicles (arrow) at E14.5. C) At E14.5, NFAT is also in the ureteric buds (UB) and the s-shaped bodies (S). D) Active NFAT is also in newly formed glomerulus (GL) at E14.5. E) At E16.5, active NFAT is detected in the metanephric mesenchyme (MM), glomerulus (GL) and mature tubules (*). F) At P1, NFAT is not detected mouse kidneys. G) NFAT is also absent in kidneys of 2 months old mice. H) Negative control-omission of primary antibody in kidneys of adult mice and I) in embryonic kidneys. Images were photographed at 200X (E16.5, P1, adult) or 400X (E14.5).
5. DISCUSSION

PAX2 increases β-catenin signaling

Since PAX2 is overexpressed in PKD cells, we hypothesized that it might be activating a pathway driving cystogenesis (Wilson et al, 1995). During normal development, PAX2 is strongly expressed in the ureteric bud and s-shaped body (the precursor of the proximal tubule). However, PAX2 is rapidly down-regulated postnatally (Dressler et al, 1992). Recent data from our laboratory show that β-catenin signaling is also present in the ureteric buds and s-shaped bodies. This is interesting because transgenic mice overexpressing beta-catenin develop PKD (Saadi-Kheddouci et al, 2001). Therefore, we hypothesized that PAX2 might activate the canonical Wnt/β-catenin pathway. To test this hypothesis, we created a transgenic mouse with PAX2 expression targeted to mature proximal tubules using the rBAT promoter. Indeed, our results show that renal β-catenin levels were significantly increased in these transgenic mice compared to wildtype littermates.

When the β-catenin signaling pathway is activated by binding of Wnt proteins to their cognate frizzled receptor, β-catenin degradation is blocked and this leads to β-catenin accumulation in the cell. Our data suggest that activity of the canonical pathway correlates with the level of PAX2 expression. The mechanism by which Pax2 activates canonical Wnt signaling is unknown, but could involve effects of PAX2 on expression of Wnt genes. Interestingly, our lab has recently demonstrated direct transcriptional activation of Wnt4 by PAX2 in the s-shaped body. Moreover, Wnt4 has been shown to activate the β-catenin pathway. These findings might account for our observations in the
rBAT/PAX2 transgenic mouse. Alternatively, PAX2 could activate any one of the genes involved in the Wnt signaling pathway, including β-catenin itself. Recent studies in our laboratory have examined the effects of PAX2 on the β-catenin pathway in cultured cells lacking Wnt4. The cells were transfected with a reporter vector containing the luciferase gene under the control of a β-catenin/TCF-responsive promoter. Luciferase activity was significantly increased by co-transfection with PAX2, suggesting an effect on the pathway in the absence of Wnt4. Further studies will be needed to unravel the complex mechanism by which PAX2 effects canonical Wnt signaling.

β-catenin signaling and cystogenesis

During development, canonical Wnt signaling activates target genes involved in cell growth. One of the β-catenin target gene is c-myc (He et al, 1998). Similarly to β-catenin overexpressing transgenic mice, overexpression of c-myc in the kidney also produce a cystic phenotype (Trudel et al, 1991). Therefore, we hypothesized that our rBAT/PAX2 transgenic mice might express excess β-catenin and develop cysts. However, their kidneys were phenotypically normal. In PKD, cysts arise predominantly in the collecting duct, but do arise in proximal tubules. Overexpression of β-catenin at sites other than the proximal tubule may be required for cystogenesis. Alternatively, canonical Wnt signaling promotes cystogenesis but may not be sufficient. Other steps or signaling pathways are needed. It seems likely, however, that we may not have seen cysts because not much β-catenin overexpression was produced. PAX2 levels were modestly increased and so were β-catenin levels. Species differences or absence of additional enhancer elements upstream of the 5’ 0.8kb fragment may account for the modest level of
transgene expression. Moreover, since rBAT expression starts in late embryonic stages of kidney development and only increases postnatally, β-catenin overexpression at an earlier stage of development (before the appearance of rBAT in maturing proximal tubule) may be required for the development of renal cysts.

Calcineurin/NFAT signaling in kidney development

Not much is known about NFAT activity in the kidney. However, Puri et al (2004) recently demonstrated that PC-1 activates the calcineurin/NFAT signaling pathway. Therefore, we hypothesized that NFAT is an important signaling molecule in kidney development. To test this hypothesis, we studied the ontogeny and localization of NFAT activity in transgenic mice bearing a luciferase reporter under the control of nine NFAT binding sites. Our data demonstrate that calcineurin/NFAT signaling is highly active in early stages of kidney formation with the most activity at E14.5. In the perinatal period, there is a remarkable down regulation and no detectable activity in the adult kidney. Moreover, our results show that NFAT is active in both mesenchymal and tubular epithelial cells of the developing kidney. It is retained weakly in early tubular and glomerular structures of the embryonic kidney. However, NFAT activity is absent in all structures of the adult kidney. Our data thus suggest that NFAT plays important roles during kidney development. Our findings differ from Puri et al (2004). They used a NFAT antibody that recognizes all the NFAT family members, even NFAT5 that is not activated by calcineurin, and that recognizes both the cytoplasmic and nuclear form. They found no staining in mesenchymal cells and found staining in tubular epithelial cells within the cortex of adult kidneys. In our experiment, we can only detect transgene
expression, representing the active form of NFAT, in our NFAT-luciferase transgenic mouse, thus making our results more valid.

Non-canonical Wnt signaling is important in development and has been shown to activate NFAT (Saneyoshi et al, 2002). Wnt11 is expressed at all stages of kidney development, at the tips of the ureteric buds, and is part of the non-canonical Wnt signaling pathway (Kispert et al, 1996). At the tips of the ureteric buds, this pathway could activate NFAT. Interestingly, Puri et al (2004) showed that PC-1 activates calcineurin/NFAT signaling. Thus, NFAT gets activated when PC-1 starts to be expressed, at E15.5. Possibly, at E15.5, PC-1 only or both signaling pathway activates NFAT: non-canonical Wnt signaling and PC-1. The mechanism by which NFAT gets activated in mesenchymal cells is unknown. Possibly by Wnt4 or even Wnt2b, depending on the combination of frizzled receptors. Further studies will be needed to determine all the mechanisms by which NFAT gets activated in the developing kidney.

Taking together our findings that NFAT is active in early stages of kidney formation and that it is expressed in both mesenchymal and epithelial cells of the developing kidney, it is probably not involved in tubular differentiation as suggested by Puri et al (2004). The calcineurin/NFAT signaling pathway is most likely involved in cell movement and in mesenchymal to epithelial cell transition of the developing kidney. In bone formation, NFAT has been shown to activate BMPs and FGFs (Tomita et al, 2002, Reinhold et al, 2004). In the kidney, BMPs are widely expressed throughout development. Therefore, renal NFAT could activate these same growth factors. More studies will clarify the downstream targets of NFAT in the developing kidney.
Calcineurin/NFAT signaling and cystogenesis

Many studies demonstrated that failure to down regulate developmental genes is involved in PKD. Only a few studies were performed about NFAT target genes in the kidney but COX-2 and MMPs were shown to be regulated by NFAT (Sugimoto et al, 2001, Alfonso-Jaume et al, 2004). Noteworthy, these proteins are known to be involved in several cancers (Vihinen et al, 2005, Chun et al, 2004). This is interesting because the β-catenin signaling pathway is implicated in many cancers and PKD develop in mice overexpressing it. Since NFAT target genes are implicated in cancers, calcineurin/NFAT signaling may be reactivated in PKD. The next step will be to determine if in PKD mice models, NFAT is reactivated after the stage that it is normally down regulated. It would also be of interest to determine if NFAT over expression in mature kidneys would produce a cystic phenotype.

Conclusions

PAX2 expression in mature proximal tubules leads to sustain activity of renal β-catenin. However, because of the modest level of PAX2 in our transgenic mice and the absence of renal cysts, we cannot conclude that PAX2 and/or β-catenin are sufficient, by themselves, to cause PKD. The calcineurin/NFAT signaling pathway is highly active in early stages of kidney formation and localized in both mesenchymal and epithelial cells. Further experiments will clarity the functions of this signaling pathway in kidney development.
6. REFERENCES


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Calcineurin inhibitors block dorsal-side signaling that affect late-stage development of the heart, kidney, liver, gut and somitic tissue during *Xenopus* embryogenesis. *Develop. Growth Differ.* 46:139-152.


September 23, 2003

The McGill University Animal Care Committee certifies that
Anne-Marie Patenaude has successfully completed a
Mouse Methodology Workshop on September 3, 2003.

The training included the following procedures:

- Handling and restraint
- Injections: subcutaneous, intramuscular, intraperitoneal, intravenous *
- Gavage (tube feeding)
- Blood collection: saphenous
- Determination of anaesthetic depth
- Euthanasia by cervical dislocation

* Intravenous injection has only been demonstrated, for certification of this procedure, a special session is needed

Certification is valid for 5 years, starting on the date of the workshop.

Suzanne Smith
Research Ethics Officer for Animal Studies
animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training
July 22, 2004

The McGill University Animal Care Committee certifies that

Anne-Marie Patenaude has successfully completed the

Basic Level

of the

The Theory Training Course on Animal Use for Research and Teaching

on


The training includes the following topics:

• Basic Level: Regulations & Procedures, Ethics, Basic Animal Care, Occupational Health & Safety

Please note that this certificate does NOT include practical training, which is obtained by successfully completing an Animal Methodology Workshop where another certificate is issued.

Certification is valid for 5 years, starting on the date indicated above.

Deanna Collin
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training
Le centre universitaire de santé McGill (CUSM)  
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Service de radioprotection  
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Ceci certifie que:  
This is to certify that: 

Anne-Marie Patenaude 

A réussi avec succès le cours en théorie et procédures de radioprotection en laboratoires  
Has successfully completed a basic theory and procedures radiation safety course for 
laboratories: 

27 October, 2003 

Tristan Welp  
Service de la Radioprotection  
Radiation Protection Service
Guidelines for completing the form are available at www.mcgill.ca/rgo/animal.

McGill University
Animal Use Protocol – Research

Title: PKD1, PAX2 and Wnts in polycystic kidney disease
(must match the title of the funding source application)

☐ New Application  ☑ Renewal of Protocol # 4783  ☐ Pilot  Category (see section 1): D -

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3. Funding Source:

External ☑  Internal ☐
Peer Reviewed: YES ☑ NO**
Status: Awarded ☑ Pending ☐
Funding period: 2003-2006

For Office Use Only:

Funding Source:

Source(s): CIHR
Peer Reviewed: YES ☑ NO**
Status: Awarded ☑ Pending ☐
Funding period:

Investigator’s Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee’s approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator’s signature: 

Date: 17/Aug/04

Chair, Facility Animal Care Committee:

Date: Sept. 21/04

University Veterinarian:

Date: Sept. 21/2004

Chair, Ethics Subcommittee (as per UACC policy):

Date: Jan. 28/04

Approved Animal Use
Beginning: Sept 1, 2004
Ending: Aug 31, 2005

☐ This protocol has been approved with the modifications noted in Section 13.

December 2003