Ureteric Bud Apoptosis and Renal Hypoplasia in Transgenic PAX2-Bax Fetal Mice Mimics the Renal-Coloboma Syndrome

ALISON DZIARMAGA*, PATSY CLARK†, CHERIE STAYNER‡, JEAN PIERRE JULIEN§, ELENA TORBAN¶, PAUL GOODYER*†, and MICHAEL ECCLES*‡

*Department of Human Genetics, McGill University, Montreal, Quebec, Canada; †Department of Paediatrics, Montreal Children’s Hospital, McGill University, Montreal, Quebec, Canada; §Department of Pathology, University of Otago, Dunedin, New Zealand; ¶Centre for Research in Neuroscience, Montreal General Hospital, McGill University, Montreal, Quebec, Canada; and Department of Biochemistry, McGill University, Quebec, Canada.

Abstract. In humans, PAX2 haploinsufficiency causes renal-coloboma syndrome (RCS) involving eye abnormalities, renal hypoplasia, and renal failure in early life. The authors previously showed that heterozygous mutant Pax2 mice have smaller kidneys with fewer nephrons, associated with elevated apoptosis in the ureteric bud (UB). However, PAX2 may have a variety of developmental functions such as effects on cell fate and differentiation. To determine whether apoptosis alone is sufficient to cause a UB branching deficit, the authors targeted a pro-apoptotic gene (Bax) to the embryonic kidney under the control of human PAX2 regulatory elements. The exogenous PAX2 promoter directed Bax gene expression specifically to the developing kidney UB, eye, and mid/hindbrain. At E15.5 PAX2Promoter-Bax fetal mice exhibited renal hypoplasia, elevated UB apoptosis, and retinal defects, mimicking the phenotype observed in RCS. The kidneys of E15.5 PAX2Promoter-Bax fetal mice were 55% smaller than those of wild-type fetal mice, and they contained 70% of the normal level of UB branching. The data indicate that loss of Pax2 anti-apoptotic activity is sufficient to account for the reduced UB branching observed in RCS and suggest that elevated UB apoptosis may be a key process responsible for renal hypoplasia. The authors propose a morphogenic unit model in which cell survival influences the rate of UB branching and determines final nephron endowment.

In humans and mice, heterozygous PAX2 mutations cause kidney, eye, and central nervous system abnormalities, constituting a syndrome called renal-coloboma syndrome (RCS) (1–3). The kidney abnormalities in patients with RCS involve primary renal hypoplasia, which together with optic nerve colobomas represent the major presenting features of RCS (3). Mutations in PAX2 have also been identified in patients with isolated primary renal hypoplasia, including oligomeganephronia (renal hypoplasia with glomerular hypertrophy) (4,5). Analyses of renal biopsies from children with either RCS or oligomeganephronia show that renal hypoplasia is secondary to mechanisms leading to a paucity of nephrons (3–5). This observation points to a deficit in ureteric bud (UB) branching in patients carrying PAX2 mutations, as UB branching is a prerequisite for nephron formation. Although PAX2 mutations lead to renal hypoplasia, the mechanism by which reduced PAX2 dosage leads to a decreased extent of UB branching and lack of nephrons in patients with RCS is not clear.

Mammalian kidney development begins early in embryonic life, when the UB emerges from the nephric duct. UB growth is elicited by trophic signals (e.g., glial cell–derived neurotrophic factor [GDNF] and Wnt2b) from the adjacent mesenchyme, which activate specific receptors on the UB cell surface (6,7). As the UB penetrates the mesenchyme, it begins to arborize, inducing individual nephrons at the tip of each of its branches as it grows. Signals from each branch tip recruit adjacent mesenchymal cells to condense at its lateral aspect and undergo rapid phenotypic transformation into polarized epithelial cells of the nephron. Each emerging nephron fuses with the UB, allowing egress of filtered fluid. The extent of UB arborization, which has been achieved by the time nephrogenesis ends in the perinatal period, determines the final number of nephrons constituting the individual’s nephron endowment for life.

This complex process is orchestrated by key developmental transcription factors. Among the earliest of these is PAX2, which is expressed in the nephric duct and uninduced mesenchyme before formation of the metanephros, throughout the arborizing UB and finally in the condensing mesenchyme after induction (8). With completion of nephrogenesis, expression of the renal PAX2 gene is rapidly downregulated. In 1996, a strain of mice (Pax21Neu) was identified with RCS due to a sponta-
neous Pax2 mutation identical to the most common mutation in humans with RCS (9). In fetal (Pax2^{1Neu +/−}) mutant mice, we showed that the renal hypoplasia is associated with reduced nephron number and elevated apoptosis in the UB epithelium (3,10). Furthermore, a direct role for Pax2 in survival of UB cells is supported by our observation that mMCD-3 collecting duct cells undergo apoptosis when transfected with an antisense Pax2 expression construct (10). In contrast, Pax2 null mutant mice have bilateral renal agenesis; although the caudal portion of the nephric duct initially forms, it then degenerates (9,11).

Although mutations in Pax2 are associated with elevated levels of UB apoptosis, it is not known whether apoptosis alone accounts for suboptimal UB arborization in RCS. Indeed, relatively high basal levels of apoptosis are known to occur accounts for suboptimal UB arborization in RCS. Indeed, relatively high basal levels of apoptosis are known to occur, although the significance of this apoptosis is not understood (12,13). Normally, apoptosis occurring in the UB is sufficient to achieve their developmental fate. It is, however, important to know whether apoptosis occurring in the UB is sufficient to directly cause renal hypoplasia or whether additional mesenchyme-related functional deficits are also required. Our hypothesis is that the enhanced susceptibility of UB cells to programmed cell death in Pax2 haploinsufficiency directly compromises the rate of UB arborization and has a direct role in causing renal hypoplasia.

To examine the latter hypothesis, we have targeted an apoptosis-inducing transgene to the developing UB of fetal kidneys. We show that transgenic fetal mice expressing the pro-apoptotic Pax2Promoter-Baxa transgene in embryonic kidney, eye, and brain under control of exogenous Pax2 regulatory elements have elevated UB apoptosis and renal hypoplasia due to reduced UB branching. These data demonstrate that enhanced UB cell apoptosis during renal development compromises UB arborization, mimicking the RCS phenotype caused by Pax2 mutations. We propose the existence of an elemental UB morphogenic unit with an intrinsic mechanism for timing iterative branching events. In this model, susceptibility of the UB to apoptosis regulates UB arborization during kidney development.

Materials and Methods

PAX2Promoter-Baxa Transgene Construction

An IRES-lacZ vector, generously provided by Dr. Daniel Dufort (McGill University, Montreal), was modified by removing one SpeI restriction site and inserting an oligonucleotide containing five unique restriction sites to facilitate cloning. A 4-kb AccI/NotI fragment containing 5′ flanking sequences of the human PAX2 gene (16) was ligated into compatible NarI and NotI sites in the modified IRESlacZ vector. A NotI/EcoRV fragment of Baxa cDNA was then ligated downstream from the promoter, using NotI and an end-filled SpeI site in the IRES vector. The GenBank accession number for the 4.2-kb Apal/NcoI fragment of the human PAX2 promoter and flanking sequences is AF515729.

Transient Transfections and Cell Culture

The activity and tissue specificity of a 4.2-kb upstream region of the PAX2 gene was analyzed in transient transfections of mMCD-3, HEK293, COS-7, and NIH 3T3 cell lines. Plasmid DNA preparation, cell culture, and transient transfections using FuGene 6 (Roche) were carried out as described (16), with modifications. Briefly, cells at 50 to 70% confluency were co-transfected with 0.4 μg of pRSV-β-gal and 0.8 μg of human PAX2Promoter-pGL2Basic. Cells were harvested 48 h after transfection, lysed in a passive lysis buffer (Promega), and assayed for firefly luciferase and β-gal (Galacto-Star, TROPIX). Transfections were performed in replicates of six on three separate occasions.

Generation of Transgenic Mice

XhoI linearized plasmid DNA from the PAX2Promoter-Baxa construct was diluted to 2 ng/μl and microinjected into the male pronuclei of C57B/6 J C3H fertilized eggs. Injected eggs were then transferred into the oviducts of pseudopregnant females. Transgenic fetal mice were identified by PCR amplification of the IRES region from DNA obtained from tails (animals which came to term) or hind limb (embryos). Transgenic mice were viable and fertile. PCR primers used for genotyping transgenic mice were, Bax3F: 5'-TAATCTTGGAAGTCTCCATCCG-3′, and IRES3R: 5′-CAGATCGAGTCCATAAATG-3′, which amplified a 500-bp fragment from the PAX2Promoter-Baxa construct.

β-Galactosidase Detection

Fetal age was determined by timed pregnancy and morphologic analysis according to Kaufman’s Atlas of Mouse Development. Whole-mount histochemistry for β-galactosidase was carried out as described (17); embryos were stained overnight. After whole-mount staining, embryos were washed and fixed in 4% paraformaldehyde, dehydrated in ethanol, embedded in paraffin wax, and sectioned at 7 μm.

Maximal Kidney Cross-Sectional Area Determination, Glomerular Counting, and TUNEL Staining to Determine the Number of Cells Undergoing Apoptosis

The cross-sectional area was determined in kidney sections showing the maximal cross-sectional area in comparison to adjacent sections. To do this, whole embryos were paraffin-embedded in the same orientation before sagittal serial sectioning. Sections were stained with nuclear red, and the section containing the maximal cross-sectional area was measured for each kidney (n = 9 wild-type kidneys; n = 10 Bax transgenic kidneys). Photographs of each section showing the maximal cross-sectional area were taken at 4×.

For determination of the number of glomeruli in wild-type and Bax transgenic kidneys, the glomeruli, S-shaped bodies, and comma-shaped bodies were counted in sections with the maximal cross-sectional area (n = 8 kidneys from 5 wild-type fetal mice; and n = 10 kidneys from 6 Bax transgenic fetal mice). The sections used for the glomerular counts were the same sections as those used to determine the maximal cross-sectional area above. In addition, glomeruli were counted with the same methodology in one kidney each from two
additional PAX2Promoter-Bax transgenic animals, yielding counts of 13 and 13 glomeruli, respectively.

For determination of the number of cells undergoing apoptosis in wild-type and Bax transgenic kidneys, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining was performed as described (3). The number of cells that stained positive for TUNEL in the UB, which was observed to contain the majority of TUNEL-positive staining, were counted. Two sections from each kidney, near to the center of the kidney, were used for counting (n = 5 kidneys from 4 wild-type fetal mice; n = 5 kidneys from 4 Bax transgenic fetal mice). In two of the PAX2Promoter-Bax transgenic fetal mice, there was only one kidney present. The area in mm² occupied by the UB in the sections was measured, and the data were expressed as a ratio of TUNEL-positive apoptotic cells/mm².

Bax Immunostaining and Western Blot

Embryonic 7-μm mouse sections were deparaffinized, rehydrated, and boiled twice for 5 min in 10 mM citrate buffer. Endogenous peroxidase activity was quenched using 3% H₂O₂ (in methanol) for 15 min at room temperature. After 30-min incubation with blocking serum, sections were incubated with primary, polyclonal rabbit anti-Bax antibody (Santa Cruz Biotechnology) at 4°C overnight, followed by staining using a Vectastain ABC universal kit (Vector Laboratories) as described by the manufacturer, and incubation with DAB (3). Sections were counterstained in 0.5% methyl green for 1 min each, dehydrated, and mounted with Permount.

Western blot analysis to detect Bax protein was carried out using a polyclonal rabbit anti-Bax antibody (Santa Cruz Biotechnology), followed by detection with a peroxidase goat anti-rabbit IgG (Perkin Elmer). To detect actin in Western blots, a monoclonal mouse anti-actin antibody (Oncogene) was used, followed by detection with a peroxidase goat anti-mouse IgM (Calbiochem). The secondary antibody-peroxidase conjugate was detected using chemiluminescence as described by the manufacturer (Calbiochem).

Dolichos Biflorus Agglutinin (DBA) Staining

For counting of UB branch tips, E15.5 kidneys were microdissected from transgenic fetal mice. Each kidney was fixed in 200 μl of 4% formaldehyde in phosphate-buffered saline (PBS) for 48 h. The fixative was removed, and the kidneys were washed 4 × 10 min at room temperature in 500 μl of PBS-T (PBS + 1% Triton X-100). After washing, kidneys were incubated in 200 μl of Dolichos Biflorus Agglutinin-FITC (DBA-FITC) in PBS (1:100 dilution) overnight at 4°C, as described (18). The DBA-FITC was removed, and kidneys were washed for 10 min at room temperature in 500 μl of PBS-T. Kidneys were then incubated at 4°C overnight in PBS-T or until ready to photograph. For photographing, whole kidneys were placed on a slide under a cover slip with no mounting solution. Images were obtained, and the total number of UB terminal ends per kidney was counted directly on the computer screen.

Results

Generation of PAX2Promoter-Bax Transgenic Fetal Mice

A 4-kb DNA fragment containing the human PAX2 promoter (16) was cloned into a promoterless expression vector containing murine Baxa cDNA and used to generate PAX2Promoter-Bax transgenic mice. This expression construct also carried a lacZ reporter gene flanked by a 5' internal ribosome entry site (IRES) sequence, allowing independent translation of β-galactosidase under the transcriptional control of the PAX2 promoter (Figure 1A). In cell culture transfection experiments, the 4.2-kb hPAX2 promoter was able to drive high levels of reporter gene expression in specific kidney epithelial cell lines, particularly murine inner medullary collecting duct cells (mIMCD-3) (Figure 1B). High conservation of sequence identity between the human PAX2 promoter and the murine PAX2 promoter was observed between 3290 and 4158 (19) and also in a 400-bp region, which contained 89% identity between positions 383 and 782 (Figure 1A). In mice, the 400-bp sequence had previously been shown to direct expression of PAX2 in the Wolffian duct, UB, and collecting ducts during development (17). Transgenic mice were generated by pronuclear injection and germline integration of the PAX2Promoter-Bax construct (Figure 1C). Eleven PAX2Promoter-Bax transgenic embryos were analyzed at E13.5 or E15.5. In addition, six founder PAX2Promoter-Bax transgenic mice were born. No significant skewing of Mendelian ratios was observed in 45 offspring from three of these founders, suggesting that there is little or no embryonic lethality as a result of the presence of the transgene.
Expression of $\beta$-Galactosidase Reporter and Bax$\alpha$ during Kidney Development in PAX2Promoter-Bax Transgenic Mice

The murine Pax2 gene is expressed in the developing midbrain and hindbrain, eye, ear, and fetal urogenital tract, including Wolffian and Mullerian ducts, UB, collecting ducts, and condensing mesenchyme of fetal kidney (8,20). To determine whether the PAX2Promoter-Bax transgene was expressed in the correct Pax2-specific pattern, we analyzed expression of the lacZ reporter gene in transgenic embryos. Strong $\beta$-galactosidase staining was identified in the kidneys and mid/hindbrain of wholemount E15.5 embryos (Figure 2A). A more detailed analysis of tissue-specificity and temporal regulation of the transgene showed that the transgene was expressed in the developing urogenital tract, including Wolffian duct, UB, and collecting ducts, and in the developing eye of E15.5 embryos (Figure 2, B through D). High levels of expression of the transgene were observed in the fetal hindbrain, eyes, and collecting ducts, mimicking the pattern of strongest endogenous Pax2 expression (8,20).

The transgenic expression pattern (UB and collecting duct) was confirmed by immunohistochemical staining of E15.5 transgenic and wild-type fetal mice with an anti-Bax antibody. Elevated Bax$\alpha$ expression was detected in the UB and collecting ducts of E15.5 transgenic fetal mice (Figure 2F). Endogenous Bax$\alpha$ expression was present in the UB and collecting ducts in E15.5 wild-type kidneys but was at a lower level in wild-type kidneys than in the PAX2Promoter-Bax transgenic fetal mice, as shown by immunohistochemistry (Figure 2G). When mIMCD-3 cells transfected with the PAX2Promoter-Bax transgene were analyzed by Western blot using the anti-Bax antibody, there was a greater level of Bax expression in the transfected cells than in nontransfected cells (Figure 3), again suggesting that the Bax transgene was relatively strongly expressed under the control of the PAX2 promoter.

Apoptosis in PAX2Promoter-Bax Transgenic Kidneys

As there were high levels of expression of the pro-apoptotic Bax$\alpha$ gene in the UB of PAX2Promoter-Bax transgenic fetal mice, we analyzed the pattern and number of cells undergoing apoptosis in the fetal kidneys of transgenic and wild-type mice. In transgenic E15.5 kidneys, high levels of apoptosis were evident in UB and collecting duct epithelium, whereas apoptosis levels in the UB and collecting ducts of wild-type fetal mice were very low (Figure 4, A and B). The number of apoptotic cells per mm$^2$ in the transgenic fetal kidney ureteric bud epithelia was more than tenfold higher than that in wild-type kidneys (Figure 4C). By eye, apoptosis levels did not appear to be elevated in other regions of the kidneys of PAX2Promoter-Bax transgenic fetal mice, including in the condensing mesenchyme or glomeruli.

Histologic Analyses of Kidney Formation in PAX2Promoter-Bax Transgenic Fetal Mice

The kidneys of E15.5 transgenic fetal mice ($n = 10$) were 55% ($\pm$ 4% SEM) smaller in maximal cross-sectional area than those of wild-type fetal mice ($n = 9$) (Figure 5). The
reduction in kidney size was accompanied by the presence of fewer epithelial structures, including fewer glomeruli in sections of the PAX2Promoter-Bax transgenic kidneys compared with wild-type kidneys (Figure 6, A and B, arrows). Except for this, the glomeruli and UB in the Bax transgenic kidneys were approximately the same size as in wild-type fetal mice, albeit not as well developed (Figure 6, C and D). Quantifying the number of glomeruli present in the kidney sections showed that there were significantly fewer glomeruli in all sections from PAX2Promoter-Bax transgenic fetal mice compared with wild-type fetal mice (Figure 6E). However, despite the smaller kidney size in the PAX2Promoter-Bax transgenic animals, body lengths measured from crown to rump in whole fetal sections on slides, which were from the same identical sections in which the maximal cross-sectional kidney areas were determined, were not significantly different in transgenic and wild-type animals. Five wild-type animals had an average crown-rump body length of $1.27 \pm 0.14$ mm, whereas 11 PAX2Promoter-Bax transgenic animals had an average crown-rump body length of $1.20 \pm 0.13$ mm. The overall appearance of the wild-type and transgenic fetal sections on the slides was indistinguishable.

The reduction in the number of glomeruli in the Bax transgenic kidneys was confirmed by observing a reduction in the extent of UB branching in dolichos bifluoros agglutinin (DBA)-stained PAX2Promoter-Bax E15.5 fetal kidneys compared with wild-type kidneys (Figure 7, A through D). However, despite the smaller kidney size in the PAX2Promoter-Bax transgenic animals, body lengths measured from crown to rump in whole fetal sections on slides, which were from the same identical sections in which the maximal cross-sectional kidney areas were determined, were not significantly different in transgenic and wild-type animals. Five wild-type animals had an average crown-rump body length of $1.27 \pm 0.14$ mm, whereas 11 PAX2Promoter-Bax transgenic animals had an average crown-rump body length of $1.20 \pm 0.13$ mm. The overall appearance of the wild-type and transgenic fetal sections on the slides was indistinguishable.

Figure 3. Western blot analysis of Bax expression in mIMCD-3 cells transfected with the PAX2Promoter-Baxa construct. Protein lysates from mIMCD-3 cells that were either nontransfected or transfected with the PAX2Promoter-Baxa construct were analyzed by Western blot using a Bax antibody. A 23-kD Bax protein was observed in both nontransfected and PAX2Promoter-Baxa–transfected cells, but the band observed in PAX2Promoter-Baxa–transfected cells was stronger than in the nontransfected cells, indicating higher Bax expression in the transfected cells. Western blots with an anti-actin antibody were used as a control for protein loading.

Figure 4. Apoptosis in wild-type and PAX2Promoter-Bax fetal kidneys. (A and B) Apoptosis, detected in kidney sections by TUNEL staining (arrows), was greatly enhanced in the UB and collecting ducts of E15.5 PAX2Promoter-Baxa transgenic fetal mice (B), compared with wild-type kidneys of the same age (A). Scale bar, 100 µm. (C) Quantitation of the number of cells undergoing apoptosis in Bax transgenic and wild-type kidneys revealed that there was significantly more apoptosis in the UB of the PAX2Promoter-Bax transgenic kidneys than in the wild-type kidneys, P < 0.0001.

Figure 5. Kidneys from PAX2Promoter-Bax transgenic fetal mice were smaller than those from wild-type fetal mice. The maximal cross-sectional area (mm²) of E15.5 PAX2Promoter-Baxa transgenic kidneys was 45% of that in wild-type fetal mice, P < 0.0001 (SEM).

Discussion

The RCS is a rare multisystem developmental disorder, presenting with optic nerve colobomas and renal abnormalities, caused by PAX2 mutations (1,2). Renal hypoplasia (reduced nephron number), leading to renal failure in approximately 70% of RCS patients is an important feature of this disease (1–3). During kidney development, rapid growth and iterative branching of the ureteric tree determines the number of
nephrons formed, which constitute final nephron endowment for life. The UB cell lineage is highly protected from programmed cell death, whereas apoptosis is widespread in the metanephric mesenchyme, where it is part of normal morphogenesis (3,12,13). This study demonstrates the effect of elevated levels of UB apoptosis on kidney development and shows that elevated UB apoptosis alone is sufficient to compromise the rate of new nephron formation and mimic the RCS phenotype.

Excessive levels of randomly occurring programmed cell death in fetal kidney cells might lead to small kidneys, but not necessarily to reduced nephron number. However, our studies show that apoptosis targeted to Pax2-expressing cells caused small kidneys with a reduced number of UB branches. We propose the existence of a UB “morphogenic unit,” which is

Figure 6. Reduction in the number of glomeruli and terminal UB branch ends in the kidneys of PAX2Promoter-Baxa transgenic fetal mice compared with wild-type fetal mice. (A and B) Tissue sections from wild-type kidneys (A) and PAX2Promoter-Baxa transgenic kidneys stained with nuclear red (B) showed differences in kidney size and glomerular number, with reduced numbers of glomeruli being present in the transgenic kidneys (arrows). (C and D) Tissue sections of wild-type (C) and PAX2Promoter-Baxa transgenic (D) kidneys at higher magnification showed that the glomeruli (G) and ureteric buds (U) were approximately similar in size and shape but that there were fewer such structures in the PAX2Promoter-Baxa transgenic than in the wild-type kidneys. Scale bar, 100 μm. (E) There were significantly fewer glomeruli in PAX2Promoter-Baxa transgenic kidneys (n = 10) than in wild-type kidneys (n = 8); P < 0.0001.

Figure 7. The number of UB terminal ends in E15.5 PAX2Promoter-Baxa transgenic kidneys is reduced. Fetal E15.5 kidneys were stained with DBA to assess branching morphogenesis. (A and C) Terminal ends in wild-type DBA-stained kidneys (arrows); (B and D) terminal ends in PAX2Promoter-Baxa transgenic DBA-stained kidneys (arrows). Note that the wild-type kidneys are slightly bigger and have more branches compared with the transgenic kidneys. (E) The number of UB branch terminal ends in E15.5 PAX2Promoter-Baxa transgenic kidneys as detected by DBA staining was 70% of that in wild-type fetal mice, P < 0.0001 (SEM).
affected by PAX2 mutations in RCS as illustrated by our model of UB branching morphogenesis. In this model, each UB branching event is accompanied by local interactions with mesenchyme, suppressing further UB branching (Figure 8). As each new branch lengthens, the specialized UB tips grow beyond a putative zone where UB branching is inhibited, allowing the next cycle of branching to occur. In this model, stochastic depletion of cells during extension of the UB branch would slow its linear growth rate, delaying escape from the inhibitory zone and delaying the next round of branching. Assuming that there is a finite temporal window for UB branching during renal organogenesis, the balance of survival and pro-apoptotic signals would act as a timing mechanism for the rate of UB arborization during fetal life and ultimately determine final nephron number (Figure 8).

Many factors are involved in UB branching morphogenesis, including growth-signaling pathways (21). For example, the GDNF gene encodes a ligand for the RET receptor expressed on the surface of UB cells; both GDNF and RET are required for UB branching, growth, and cell survival (22–24), and heterozygous GDNF mutations are associated with renal hypoplasia (25). The collective influence of these genes on apoptosis levels would be expected to directly affect UB branching morphogenesis and nephron number. Therefore, differences between individuals in the basal level of UB apoptosis during development might help to explain why the complement of human nephrons varies from 300,000 to 1,100,000 in the general population, although there may also be other factors that contribute to this variation (reviewed in reference 26).

Further support for the morphogenic unit model arises from our observation that, in contrast to the effects of excessive apoptosis on nephron number, inhibition of apoptosis in wild-type and (Pax2<sup>1Neu</sup>+/-) mutant fetal kidneys leads to increased UB branching and increased nephron number. Ex vivo treatment of either wild-type or (Pax2<sup>1Neu</sup>+/-) mutant fetal kidneys for 2 d with the caspase inhibitor Z-VAD-fmk, increased UB branching in DBA-stained kidney explants compared with untreated explants (Clark et al., unpublished observation). Taken together, these results suggest that the hypoplastic renal phenotype in RCS is determined primarily by loss of Pax2 anti-apoptotic function in UB cells.

As well as its role in kidney development, Pax2-mediated cell survival may also be important for the development of other organs, including the eye. Expression of the PAX2Promoter-Bax transgene in embryonic retina was associated with an immature stage of eye development, with attachment of the inner layer of the optic cup to the posterior lens cells, and poor development of the vitreous body (Figure 2, D and E). These data suggest that there may be a role for Pax2-mediated cell survival in eye development.

In conclusion, we have examined the role of apoptosis in organ patterning by targeting Bax expression to specific cell lineages during development. The studies presented here illustrate a functional relationship between apoptosis, patterning in the UB, and branching morphogenesis, whereby increased susceptibility to apoptosis in the UB lineage during fetal kidney development is sufficient to recapitulate the nephron deficit of RCS. Our results predict that potential therapies, involving inhibition of apoptosis during development may be used to treat RCS, primary renal hypoplasia, if detected in utero. Consequently, nephron rescue therapies, if directed to the developing kidney, might fully restore the normal nephron complement in affected patients.

**Acknowledgments**

The authors thank Dr. Daniel Dufort for the IRES-LacZ expression vector, Dr. Gordon Shore for Bax cDNA and anti-Bax antibody, and Dr. Cynthia Goodyer and Dr. Fernanda Da Silva-Tatley for advice on cloning and discussions. Funding for this work was from the Canadian CIHR, the Cancer Society and the Health Research Council of New Zealand. ME was supported by a James Cook Fellowship of the Royal Society of New Zealand.

**References**


