JCI The Journal of Clinical Investigation

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J Clin Invest. 2004;113(7):1051-1058. https://doi.org/10.1172/JCI20049.

Article **Development**

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Calcineurin is required in urinary tract mesenchyme for the development of the pyeloureteral peristaltic machinery

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Congenital obstructive nephropathy is the principal cause of renal failure in infants and children. The underlying molecular and cellular mechanisms of this disease, however, remain largely undetermined. We generated a mouse model of congenital obstructive nephropathy that resembles ureteropelvic junction obstruction in humans. In these mice, calcineurin function is removed by the selective deletion of *Cnb1* in the mesenchyme of the developing urinary tract using the Cre/lox system. This deletion results in reduced proliferation in the smooth muscle cells and other mesenchymal cells in the developing urinary tract. Compromised cell proliferation causes abnormal development of the renal pelvis and ureter, leading to defective pyeloureteral peristalsis, progressive renal obstruction, and, eventually, fatal renal failure. Our study demonstrates that calcineurin is an essential signaling molecule in urinary tract development and is required for normal proliferation of the urinary tract mesenchymal cells in a cell-autonomous manner. These studies also emphasize the importance of functional obstruction, resulting from developmental abnormality, in causing congenital obstructive nephropathy.

Introduction

Congenital obstructive nephropathy is the most frequent cause of renal failure in infants and children (1, 2). Antenatal screening detects fetal hydronephrosis in 1 out of 100 births, with about 20% being clinically significant (3). Ureteropelvic junction (UPJ) obstruction is found in 40–50% of these clinically significant cases, with an estimated incidence of 1 in 1,000–1,500 (3). Autopsies of human patients with obstructive nephropathy show that the defective urinary tracts have pathological changes in both smooth muscle (SM) arrangement and pyeloureteral innervation (4). However, these autopsies were taken from patients at advanced stages of the disease, making it difficult to determine the initial causative lesions. Experimental surgical animal models for obstructive nephropathy have been valuable in determining the pathological impacts of the obstruction, but less informative about the etiology (5). Congenital obstructive nephropathy has been described in animals with spontaneous mutations or targeted genetic modifications (5). Some of these mutations may interfere with pyeloureteral peristalsis, which is necessary for efficient urine transport from the kidney to the bladder (6). Indeed, defective peristalsis has been shown to cause hydronephrosis in mice (7). The underlying mechanisms in most cases, however, remain to be determined (5).

Calcineurin is a Ca^{2+}-dependent serine/threonine phosphatase composed of a regulatory subunit, CnB, and a catalytic subunit, CnA. CnA is encoded by three genetic loci and CnB by two. One of the CnB isoforms (*Cnb1*) is ubiquitously expressed, while the other (*Cnb2*) is testis-specific. The calcineurin/NFATc signaling pathway regulates early immune response, cardiovascular development, myocardial function, memory and learning, axonal guidance, and other processes (8–13). Calcineurin, activated by a sustained increase in intracellular calcium concentration, dephosphorylates the NFATc family of transcription factors, leading to their cytoplasmic-nuclear translocation. Once in the nucleus, NFATc proteins cooperate with their nuclear partners (NFATn) to form active transcriptional complexes on target genes (13, 14). The calcineurin inhibitors cyclosporin A (CsA) and FK506 block the NFAT-dependent transcription in lymphocytes that is necessary for immune response. Therefore, these inhibitors have been used widely as immunosuppressive drugs in organ transplantation to prevent acute rejection (15, 16).

The use of calcineurin inhibitors has been associated with acute and chronic damage to the kidney (17), which suggests an important role of calcineurin in kidney function. Calcineurin has also been shown to modulate channel functions and to transduce signals regulating transcription in various renal cell types (18–21). However, the specific function of calcineurin in the development of the urinary system has not been thoroughly investigated. We report here the generation of a genetic model of congenital obstructive nephropathy that specifically mimics UPJ obstruction in humans. Our study revealed an indispensable regulatory role of calcineurin in the development of the pyeloureteral peristaltic machinery. We further demonstrated that reduced cell proliferation in the developing pelvicoureteral mesenchyme due to deficient calcineurin signaling is an underlying mechanism of abnormal urinary tract development in these mice, which results in congenital obstructive nephropathy.

Nonstandard abbreviations used: calcineurin A (CnA); calcineurin B (CnB); cyclosporin A (CsA); embryonic day (E); flanked by *loxP* sites (floxed); postnatal day (P); smooth muscle (SM); α-smooth muscle actin (αSMA); SM cell (SMC); ureteric bud (UB); ureteropelvic junction (UPJ).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **113**:1051–1058 (2004). doi:10.1172/JCI200420049.

Methods

Immunostaining. Immunostaining with an anti-Cnb1 polyclonal antibody (1:20, rabbit; Upstate Group Inc., Charlottesville, Virginia, USA) was done on 7-μm paraffin sections. Briefly, paraffin sections were dewaxed and rehydrated to PBS. High-temperature antigen retrieval was done by two treatments of 0.1 mM EDTA in a conventional microwave oven. The slides were incubated with 100 mM glycine for 10 minutes and a blocking solution (2% inactivated normal goat serum and 10 mg/ml BSA) for 45 minutes at room temperature. The anti-Cnb1 antibody was used at 1:20 for 45 minutes. An Alexa Flour 566–conjugated goat anti-rabbit antibody (Molecular Probes Inc., Eugene, Oregon, USA) was used at 1:500 in 10 mg/ml BSA in PBS. Whole-mount staining was done as described previously (22). Other antibodies used were a monoclonal anti-neurofilament antibody (1:100, mouse; University of Iowa, Developmental Study Hybridoma Bank, Iowa City, Iowa, USA) and a monoclonal anti–α-smooth muscle actin (anti-αSMA) antibody (1:200, mouse; Sigma-Aldrich, St. Louis, Missouri, USA), and a polyclonal Ki67 antibody (1:300, rabbit; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom). Midline sagittal cryostat sections at 7 μm were used for the Ki67 immunostaining and the subsequent quantification. Only the mesenchymal cells were counted. Results were presented as number of Ki67-positive cells per 100 μm along the developing renal pelvic wall.

Histology and β–gal assays. For standard histological evaluation, samples fixed in 10% formalin or 4% paraformaldehyde were washed, dehydrated, and embedded in paraffin. Seven-micron sections were collected and stained with H&E. β-Gal assays on whole-mount preparations and cryostat sections were performed as described previously (23) with minor modifications. Briefly, organs were fixed in 0.2% glutaraldehyde in pH 7.4 phosphate buffer with 100 mM $MgCl_2$ for 18-25 minutes (according to size) at 4°C. Samples were washed and incubated in 15% sucrose in PBS for 1 hour at 4°C. This was followed by incubation with 30% sucrose in PBS overnight at 4°C. Samples were embedded in OCT and cut at 5 μm. The sections were refixed in 0.2% glutaraldehyde for 5 minutes, washed, and stained in *LacZ* staining buffer (pH 7.4, 0.5 mg/ml X-gal) at room temperature (about 25°C) for 6 hours. Finally, the sections were counterstained by nuclear fast red. For harvesting of embryos, the age of embryos was determined by conventional postcoital date, confirmed by ultrasonography whenever possible (24).

Imaging the urinary system. Still images were taken under a Nikon MZ-1500 stereomicroscope (Nikon, Melville, New York, USA) with a MicroPublisher digital imaging system (QImaging, Burnaby, British Columbia, Canada). For video capturing of the pyeloureteral peristalsis, we dissected out the urinary systems in PBS. The samples were allowed to rest at 37°C in DMEM with 10% FCS for about 15 minutes before video capturing began with the MicroPublisher imaging system driven by StreamPix software (NorPix Inc., Montreal, Quebec, Canada).

Corrosive casting. Corrosive casting of the urinary tract was done with a Batson's plastic replica and corrosion kit (Polysciences Inc., Warrington, Pennsylvania, USA). The casting polymers were prepared immediately before the procedure, following the manufacturer's instructions, and injected into the pelvicaliceal space using gauge 30 needles and 0.3-ml tuberculosis-injection syringes. Gentle pressure was applied to the syringe until the blue casting polymers reached the bladder. The tissues were left at 4°C overnight before being placed in maceration solution at 50°C to corrode for 6–8 hours.

All animal studies were approved by the Animal Study Committee at Washington University School of Medicine and Stanford University School of Medicine.

Results

$Pax3Cre^{T/+};Cnb1^{F/F\ or\ F/Δ}$ mice develop postnatal congenital obstructive nephropathy. Since both CnA and CnB are indispensable for the phosphatase activity of calcineurin, removal of *Cnb1*, the only isoform in non-testis tissues, is sufficient to inactivate calcineurin in these sites. We thus generated a floxed-*Cnb1* allele ($Cnb1^F$) in which three exons of *Cnb1* were flanked by *loxP* sites (25). A recombined germ-line allele ($Cnb1^Δ$) was generated by combination of the $Cnb1^F$ allele with a *β-actin-Cre* transgene. The $Cnb1^{Δ/Δ}$ mice, phenotypically indistinguishable from mice homozygous for the *Cnb1** loss-of-function allele (22), died at embryonic day 11 (E11) because of defective vascular development. No abnormality has ever been found in mice that are $Cnb1^{F/+}$, $Cnb1^{F/F}$, $Cnb1^{Δ/+}$, or $Cnb1^{F/Δ}$. The *Pax3Cre* transgene ($Pax3Cre^T$) has been shown to direct recombination of *loxP* sites in neural crest cells and other tissues (26). We introduced $Pax3Cre^T$ into mice carrying $Cnb1^F$ (or $Cnb1^Δ$) (Figure 1) to generate $Pax3Cre^{T/+};Cnb1^{F/F}$ or $Pax3Cre^{T/+};Cnb1^{F/Δ}$ mice. These mice are phenotypically identical and are collectively referred to as $Pax3Cre^{T/+};Cnb1^{F/F\ or\ F/Δ}$, or simply "mutants" in this report. Littermates retaining at least one functional *Cnb1* allele in every cell were used as controls.

The $Pax3Cre^{T/+};Cnb1^{F/F\ or\ F/Δ}$ mice were born at normal weight and at the expected mendelian ratio but failed to thrive and died within 3 weeks after birth. While the urinary systems in most postnatal day 1 (P1) mutants appeared outwardly normal (Figure 2, A and B), all mutants at P5 had hydronephrosis (Figure 2, C and D). Hydronephrosis became progressively more severe at P12 (Figure 2, E and F). In most cases, the dilatation of the urinary tract occurred at the level of the UPJ, phenotypically resembling the UPJ obstructive nephropathy in humans. We did not observe any correlation between the bladder volume and the genotypes of the mice. Histopathological examination revealed no signs of obstruction before birth at E18.5–E19.5 (Figure 2, G and H), but some of

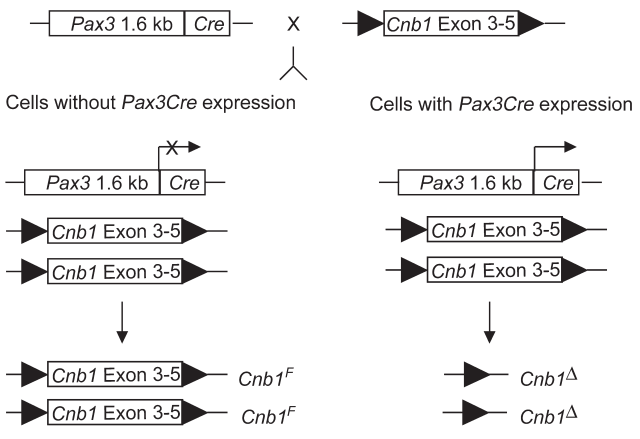

Figure 1
Deletion of calcineurin in cells expressing *Pax3Cre*. We generated mice carrying both the floxed-*Cnb1* allele and the *Pax3Cre* transgene. In cells without *Pax3Cre* expression, the floxed-*Cnb1* allele remained intact and functional. In cells with *Pax3Cre* expression, recombination occurred between the two *loxP* sites, leading to the deletion of exons 3–5 of *Cnb1* and the inactivation of the gene.

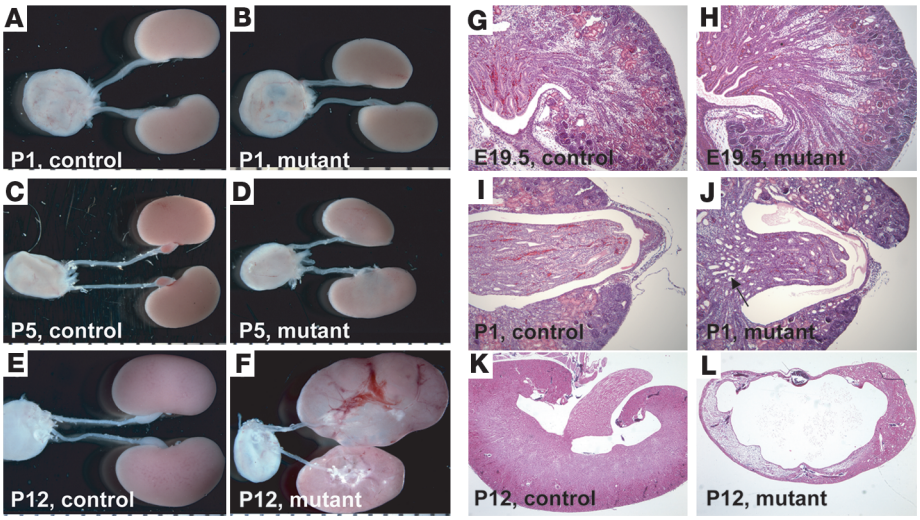

Figure 2
Conditional deletion of *Cnb1* resulted in congenital obstructive nephropathy. (**A**–**F**) Urinary systems from controls (**A**, **C**, and **E**) and their littermate mutants (**B**, **D**, and **F**). Pictures were taken under different magnifications according to their size. The unit on the ruler is 1 mm. (**G**–**L**) H&E-stained paraffin sections from the controls (**G**, **I**, and **K**) and mutants (**H**, **J**, and **L**). Arrow in **J** points to a mildly dilated collecting tubule.

the late P1 mutants started to show evidence of obstruction with mild tubular dilatation (Figure 2, I and J). At P12, kidneys in the mutants became hydronephrotic with severe parenchymal atrophy and massive nephron loss, accompanied by interstitial fibrosis and severe dilatation of the tubular and pelvicaliceal space (Figure 2, K and L). Renal function was severely compromised at this stage, as evidenced by the high blood urea nitrogen levels in the mutants (323 ± 105.8 mg/dl, vs. 28 ± 5.7 mg/dl in the controls).

In the urinary system, the deletion of Cnb1 occurs specifically in the metanephric mesenchyme and ureteric mesenchyme. The renal defects in these mice prompted us to examine the state of *Cnb1* deletion in their urinary systems. Conversion of the $Cnb1^{F}$ allele to the $Cnb1^{\Delta}$ allele was detected in the urinary system of the $Pax3Cre^{T/+};Cnb1^{F/F\,or\,F/\Delta}$ mice by PCR (data not shown). We further detected a decrease of *Cnb1* transcripts in the mutant kidney and ureter by RT-PCR (Figure 3A). To determine which cell types express the *Pax3Cre* transgene, we crossed the $Pax3Cre^{T/+}$ mice with *ROSA26RLacZ* (*ROSA*) mice, in which Cre-expressing cells are marked by *LacZ* (23). Extensive *LacZ* expression was observed in the urinary systems from the $Pax3Cre^{T/+};ROSA^{T/+}$, but not the $Pax3Cre^{+/+};ROSA^{T/+}$, newborns (Figure 3B). The structures derived from the metanephric mesenchyme, including the glomeruli and the proximal and distal tubules, expressed *LacZ* in the $Pax3Cre^{T/+};ROSA^{T/+}$ samples (Figure 3, C and D). The structures derived from the ureteric bud (UB), such as the collecting duct system and the urothelium lining the calyx and the pelvis, did not show *LacZ* expression (Figure 3, C and D). In the developing UPJ area, *LacZ* expression was absent in the urothelium but present in the mesenchymal derivatives, where layers of smooth muscle cells (SMCs) appeared (Figure 3, E and F). *LacZ* expression was also detected in the SM layers of the ureter but not in its transitional epithelium (Figure 3G). Since *LacZ* expression is indicative of the recombination of a floxed locus, the *Cnb1* gene would undergo recombination in the same cells in $Pax3Cre^{T/+};Cnb1^{F/F\ or\ F/\Delta}$ mice. This was confirmed by direct immunostaining with an anti-Cnb1 antibody. While Cnb1 protein was detected in every cell of the urinary system in the controls (Figure 3, H and J, and data not shown), it was absent from the mesenchymal derivatives in the mutants. Normal levels of Cnb1 protein expression were observed in the UB-derived collecting duct system and the urothelium in the mutants (Figure 3, I and K).

The mutants have defective pyeloureteral peristalsis. The excised pyeloureteral complexes retain the ability to spontaneously contract with characteristics similar to those observed in vivo (6), allowing the study of potential defects in pyeloureteral peristalsis by video recording in vitro. Normal pyeloureteral peristalsis was initiated by the rhythmic contraction of the pelvis, immediately followed by an extension of the proximal ureter very close to the UPJ. This proximal region of the ureter retracted, and the peristaltic wave was propagated down the length of the ureter to the bladder (Figure 4, A–E; and see Supplemental Video 1; supplemental material available at http://www.jci.org/cgi/content/full/113/6/1050/DC1. The mutant ureters, however, had periodic movement resembling a rolling and pulling motion instead of a strong peristaltic wave (Figure 4, F–J; and see Supplemental Video 2). The contraction of the utmost proximal ureter was minimal compared with that of the control samples. We believe that the abnormal pyeloureteral peristalsis is responsible for a functional obstruction that leads to hydronephrosis. Although effective peristalsis is lacking, the pacemaking mechanism in the mutant appears to be largely unaffected.

Abnormal pyeloureteral peristalsis results from defective development of the renal pelvis and ureter. The defective pyeloureteral peristalsis led us to examine the peristaltic machinery along the urinary tract. The renal pelvis is a muscular structure that connects the collecting duct system to the ureter. By P5, the pelvis in the controls protruded out of the kidney, forming a funnel-shaped muscular extension that connected to the ureter at the UPJ situated outside of the kidney (Figure 5A). In the mutant littermates, however, the funnel-shaped extension of the renal pelvis failed to develop, resulting in the retention of the UPJ in the hilum (Figure 5B). The renal pelvis has SM layers to provide adequate structural support and contractibility. Immunostaining with an anti-αSMA antibody showed that the funnel-shaped extension began to take shape right after birth in the controls (Figure 5C), while this extension was undetectable in the mutants (Figure 5D). At P5, the control renal pelvis had a well-formed extension surrounding the papilla, while the same structure was lacking in the mutants (Figure 5, E and F). Similar observations were made when antibodies against desmin, calponin, caldesmon, and SM myosin heavy chains 1 and 2 were used, confirming that the defect was indeed in the SM layers (data not shown). The renal pelvis is an integral part of the pyeloureteral machinery. Defective renal

pelvic development in the mutant appeared to have caused abnormal pyeloureteral peristalsis.

To study the lumen of the urinary tract, we made replica molds of the interior of the urinary tract by using the corrosive-casting technique. The casting polymers injected into the pelvicaliceal space flowed through the urinary path to the bladder (Figure 6, A and B). The urinary tracts appeared to be free of physical blockage in the mutants, though apparent irregularities in lumen size and surface were demonstrated by the casts (Figure 6B). Disorganization of the SM layers occurred in the absence of urinary tract infection, the latter of which was determined by urinalysis (Figure 6, C–H, and data not shown). Despite the abnormal ureteric wall (Figure 6, A–H), severe hydroureters occurred in only about 12% of the mutants (Figure 6, I and J). This was probably because the obstruction of the upstream renal pelvis reduced the downstream hydrostatic pressure in the ureters, thereby masking the full manifestation of ureteric obstruction and hydroureter formation.

Deletion of Cnb1 inhibits the proliferation of the mesenchymal cells along the urinary tract. Immediately after birth, the mesenchymal cells in the developing renal pelvic wall were highly proliferative, as evidenced by Ki67 staining, which detects a nuclear antigen in proliferating cells. Proliferation in the urothelium was largely restricted to the innermost layer directly adjacent to the mesenchymal cells (Figure 7, A and B). The proliferation of cells in the urothelium was not affected in the mutants (Figure 7, C and D). This is not surprising, since *Cnb1* expression persisted in the mutant urothelium. When only the mesenchyme-derived cells are counted, the controls had 9.22 ± 1.94 Ki67-positive cells per 100 μm along the renal pelvic wall, while the mutants had 2.63 ± 1.04 Ki67-positive cells per 100 μm (Figure 7E). The mutants had significantly fewer proliferating mesenchymal cells that lacked *Cnb1* ($P < 0.01$ by *t* test). This observation indicates that calcineurin is required, in a cell-autonomous manner, for the normal proliferation of the mesenchymal cells in the urinary tract and that defects in the maintenance of normal proliferation in the developing structures along the urinary tract can cause abnormality in the formation of the pyeloureteral peristaltic machinery. There was no significant increase in the number of apoptotic cells in the developing mutant pelvis before the onset of obstructive nephropathy (data not shown).

There is no apparent defect in urinary innervation. Since *Pax3Cre* is also expressed in the neural crest cells (26), which give rise to diverse structures including the peripheral nervous system, we investigated possible defects in the innervation of the mutant urinary system. Whole-mount preparations of newborn mutant urinary systems stained with an anti-neurofilament antibody revealed no obvious defects in innervation regarding the density and distribution of the major nerve fibers (data not shown). Immunostaining using the same antibody on ureter sections revealed similar numbers of nerve branches per section in control (13.61 ± 2.63) and mutant (13.97 ± 2.20) samples (Figure 8, A–E). In addition, the acetylcholinesterase histology revealed similar cholinergic innervation of the urinary system and colon in control and mutant mice (data not shown). The absence of innervation defect, taken together with abnormal mesenchymal proliferation and SM defects in the mutant mice, indicates that the abnormal peristalsis that leads to functional obstruction is myogenic in nature.

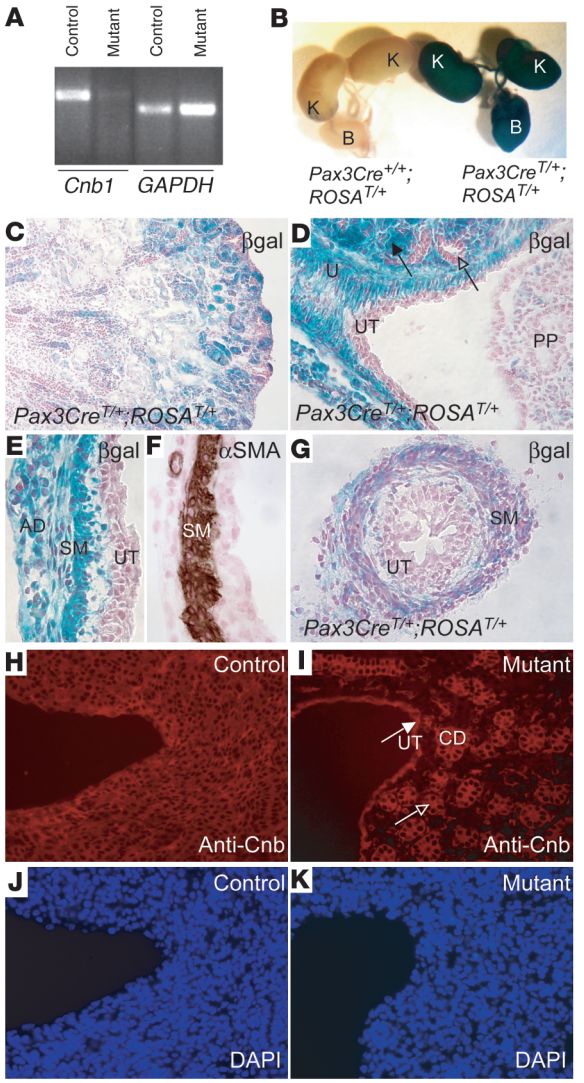

Figure 3

Pax3Cre directs *Cnb1* deletion in the metanephric and the ureteric mesenchyme. (**A**) RT-PCR shows the expression of *Cnb1* in the kidney and ureter of the mutant (*Pax3Cre*$^{T/+}$;*Cnb1*$^{F/Δ}$) is reduced compared with that in the control (*Pax3Cre*$^{T/+}$;*Cnb1*$^{F/+}$). *GAPDH* indicates equal loading. One hundred nanograms of total RNA was used in each lane. (**B**) Extensive Cre expression (revealed by *lacZ* expression; blue) was found in the kidney (K), ureter, and bladder (B) of the *Pax3Cre*$^{T/+}$;*ROSA*$^{T/+}$ but not the *Pax3Cre*$^{+/+}$;*ROSA*$^{T/+}$ mice. (**C**–**E** and **G**) Samples from P1 *Pax3Cre*$^{T/+}$;*ROSA*$^{T/+}$ mice. *LacZ* expression is evident in the glomeruli and tubules that are derived from the metanephric mesenchyme but not in the collecting duct system originated from the UB (**C**, ×20). The filled arrow in **D** points to the developing glomeruli. The open arrow points to one of the UB branches. In the developing renal pelvis, the SM layers (SM) and the adventitia (AD) express *LacZ*, while the UB-derived urothelium (UT) remains *LacZ* negative (**D**, ×40, and **E**, ×60). U, ureter; PP, papilla. The SM layers in the developing renal pelvic wall are illustrated by αSMA staining on a wild-type newborn sample (**F**, ×60). *LacZ* is also selectively expressed in the SM layers in the ureter but not in the urothelium (**G**, ×60). (**H**–**K**) Cnb1 protein can be detected by immunostaining in every cell in the control (**H** and **J**) but is absent in metanephric mesenchyme– and ureteric mesenchyme–derived structures in the mutant littermates (**I** and **K**). Cnb1 proteins remain in the UB-derived collecting duct (CD) system (open arrow) and the urothelium (filled arrow).

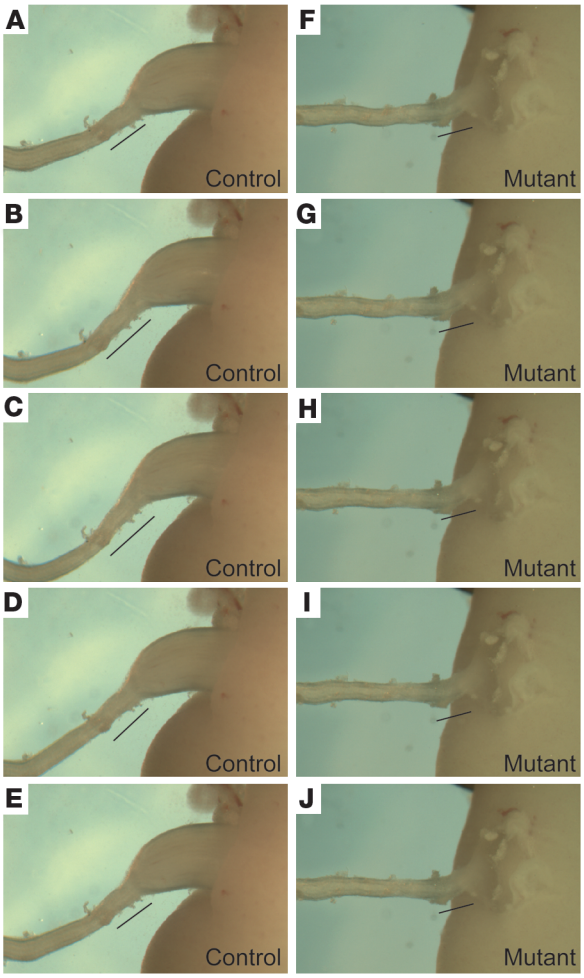

Figure 4
Defective pyeloureteral peristalsis in the mutant mice. These are a series of images taken at 2-second intervals during a peristaltic cycle in samples from a control (**A**–**E**) and a mutant littermate (**F**–**J**) at P5. The black bars indicate the change of length in the most proximal segment of the ureter.

Discussion

We have generated mice with homozygous *Cnb1* deletion in *Pax3Cre*-expressing cells and their derivatives. These mice developed postnatal obstructive nephropathy resembling human congenital UPJ obstruction. Our studies indicate that these mutants have defective pyeloureteral peristalsis resulting in inefficient urine transfer and a functional obstruction. The defective peristalsis is caused by the underdevelopment of the renal pelvis and, additionally, by the abnormalities in the SM layers and other cells in the ureteric wall. During the neonatal period, the kidney becomes the only organ for nitrogenous waste removal and increases its urine production about 50-fold. The early postnatal onset of the obstructive nephropathy coincides with the time when the pelvis is being developed and is needed to handle the increased urine flow (7). Since general deletion of *Cnb1* results in embryonic lethality, it is unlikely that *Cnb1* germ-line null mutations cause the majority of the UPJ obstruction in humans. However, the *Cnb1* mutants reported here must have disruption in certain biological processes common to the pathogenesis of congenital obstructive nephropathy. In addition, these mutants have defined genetic modifications, and their congenital obstructive nephropathy is inheritable and consistent. Hence, these mice can serve as a model for the study of the etiology and pathology of, and potential diagnostic markers and therapeutic intervention for, congenital obstructive nephropathy, particularly congenital UPJ obstruction. The deletion of calcineurin in components of the nephrons may also affect the development and function of the nephrons. Potential perinatal changes in the nephrons before the onset of hydronephrosis are under investigation. Primary defects of the nephron in these mutants may be more difficult to study postnatally because of the severe pathological changes resulting from the early-onset urinary tract obstruction.

Spontaneous peristalsis in the isolated or denervated pyeloureteral complexes retains characteristics similar to those observed in vivo and is not blocked by tetrodotoxin or other neuronal blockers (6). In renal transplantation, donor kidney and ureter are surgically ligated to the recipient bladder. Effective peristalsis occurs shortly after surgery with no evidence of immediate reinnervation (6). These observations suggest that ureteral motility is largely a myogenic process (6), though the innervating nerves may have a modulatory role (6, 27). We have not detected any developmental defects (Figure 8) in pyeloureteral innervation in the mutant mice, but potential extrarenal neuronal defects and subtle neuronal involvement in the observed obstructive nephropathy require further investigation.

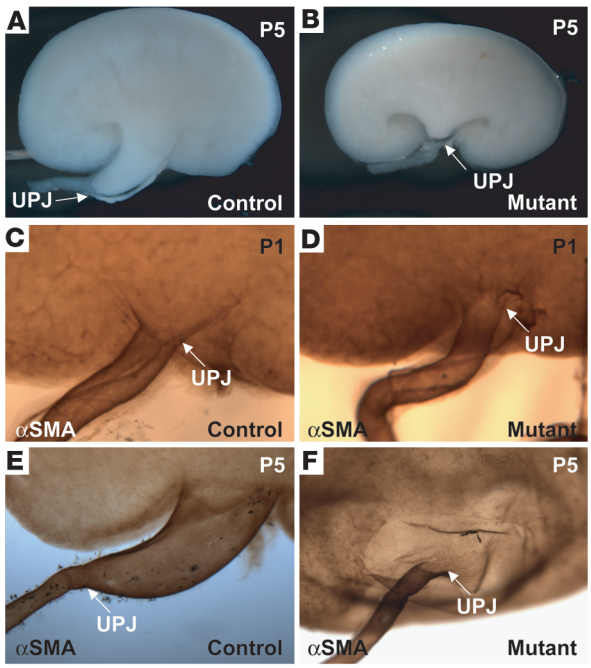

Figure 5
Samples from controls (*Pax3Cre*$^{T/+}$;*Cnb1*$^{F/+}$) (**A**, **C**, and **E**); and samples from their mutant littermates (*Pax3Cre*$^{T/+}$;*Cnb1*$^{F/\Delta}$) (**B**, **D**, and **F**). Arrows in each panel indicate the locations of the UPJs. (**A** and **B**) Hemisected kidneys. (**C**–**F**) UPJ areas immunostained with an αSMA antibody at P1 (**C** and **D**) and P5 (**E** and **F**) showing the developing renal pelvic extension in the controls (**C** and **E**) and the underdevelopment of such an extension in the same area in the mutants (**D** and **F**).

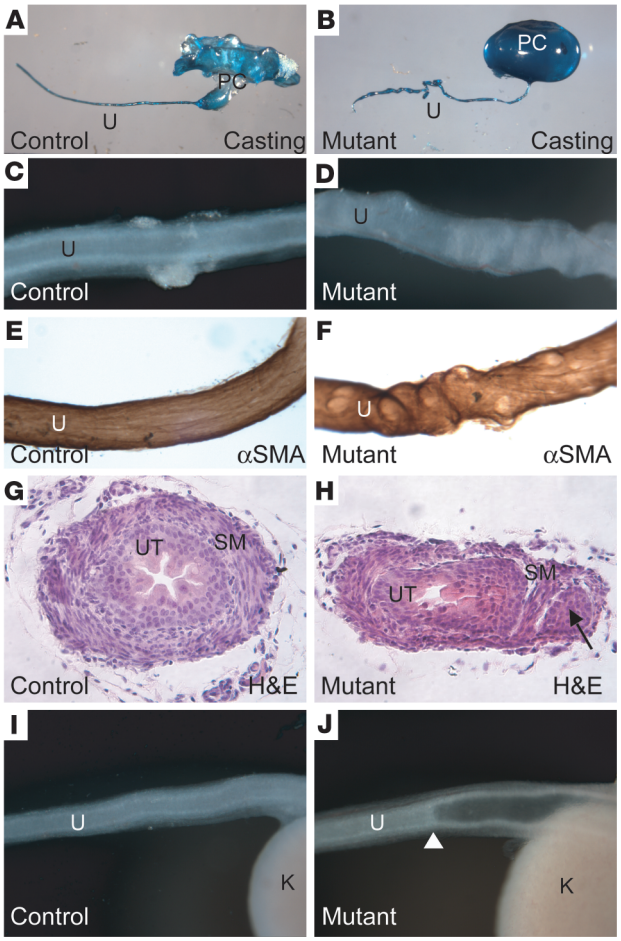

Figure 6
A defective ureteric wall may contribute to the defective peristalsis. (**A** and **B**) Replica moldings of the urinary tracts (from the pelvis to the distal end of the ureter) of the control (**A**) and the mutant (**B**) littermates at P5. (**C** and **D**) The ureteric wall is straight and smooth in the control (**C**) but irregular in the mutant (**D**). (**E**–**H**) The SM layers are disorganized in the mutants (**F** and **H**) but not the controls (**E** and **G**) at P5. Arrow in **H** points to the epithelial cells in the lumen of the cyst-like structure. (**I** and **J**) The mutant shown in **J** has hydroureter marked by the white triangle. Hydroureter has never been observed in the controls (**I**). PC, pelvicaliceal space.

We have previously shown that calcineurin is indispensable for the formation of VSMCs in mice carrying a mutant allele of *Cnb1* (22). In this study, we have found SM defects along the urinary tract in mice lacking *Cnb1* in the urinary tract mesenchyme. In addition, we have illustrated that the inactivation of calcineurin in the mesenchymal derivatives of the developing renal pelvic wall leads to reduced proliferation of these mesenchymal cells, including cells forming the muscular layer of the pelvis. The defective proliferation in these cells may account for at least some of the SM defects observed in the urinary tract and the failure in the formation of the funnel-shaped extension of the renal pelvis. The observation of reduced proliferation only in the mesenchymal derivatives lacking *Cnb1* but not in the urinary epithelial cells (which retain *Cnb1* function) (Figure 7) suggests that the requirement of *Cnb1* in cell proliferation is cell-autonomous. The fact that severe renal hypoplasia did not occur in mutants with extensive *Cnb1* deletion in the urinary mesenchyme indicates that *Cnb1* is essential in specific cell types, including the SMCs or their progenitors. In these cells, calcineurin may be required for the expression of receptors that sense local morphogenic signals, or for the signal transduction from such receptors. Alternatively, calcineurin may

CsA is fetotoxic in a number of animal studies (28) but has not been shown to be a major human teratogen (29). CsA is thus used in pregnant patients for its graft and life-saving immunosuppressive effects despite the fact that it induces intrauterine growth retardation in over half of the pregnancies after renal and liver transplants (30). Short-term studies did not reveal any adverse effect of in utero exposure to CsA on renal function of the children born to female transplant recipients (31). Our studies point to an important role of calcineurin in the development of the urinary tract. Since the CsA–cyclophilin A complex but not CsA itself inhibits calcineurin, a high calcineurin-to-cyclophilin ratio in the essential cells during critical developmental stages could have prevented overt CsA damage to the developing urinary system at the therapeutic dosage. But since long-term studies are lacking, the possibility exists that calcineurin inhibitors can have subtle effects on the development of the human urinary system and that clinical symptoms may take years to develop.

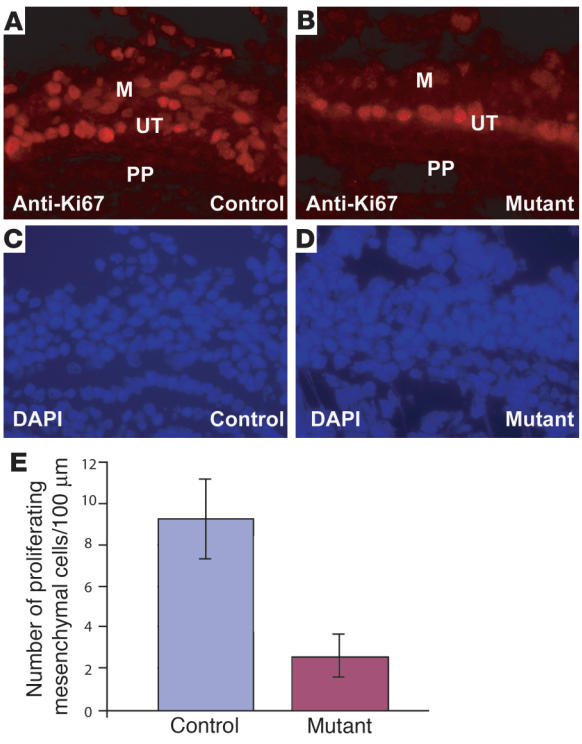

Figure 7
Calcineurin regulates cell proliferation in the developing renal pelvic wall. (**A** and **B**) Ki67 staining of the developing renal pelvic wall of control (**A**) and mutant (**B**) samples. (**C** and **D**) DAPI images of **A** and **B**, respectively. (**E**) The controls have significantly more proliferating mesenchymal cells along the developing renal pelvic wall. M, mesenchymal derivatives.

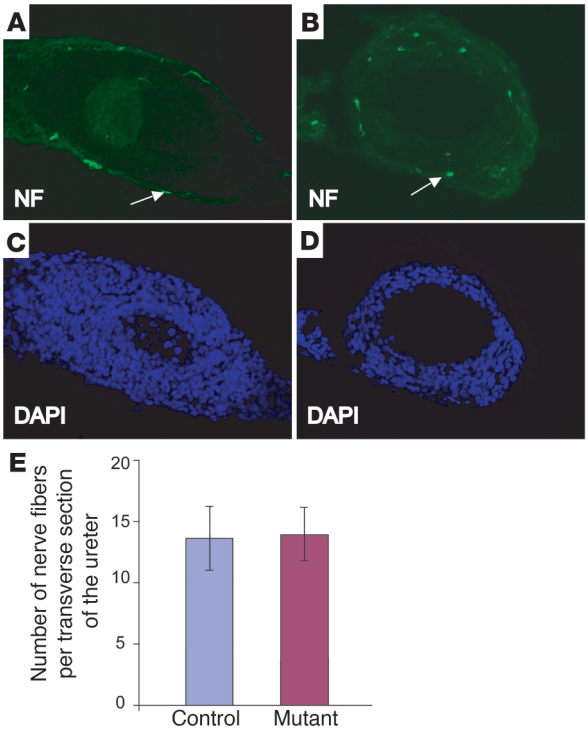

Figure 8
No developmental defects in the innervation of the urinary system in the mutants. (**A** and **B**) Immunostaining with an anti-neurofilament (NF) antibody revealed similar numbers of nerve fibers per transverse section between the control (**A**) and its littermate mutant at P1. (**C** and **D**) DAPI-stained sections from **A** and **B**, revealing the nuclei of the cells. (**E**) By counting only the nerve fibers occupying more than one-tenth the size of a nucleus of an average SMC, we found that the average numbers of nerve fibers are similar between the control samples and the mutant samples.

be required for the production of secreted autocrine growth factors, similar to its function in lymphocytes (13). Consistent with this hypothesis, the calcineurin/NFAT pathway was shown to be required for VLDL-induced SMC proliferation (32).

The *Pax3Cre* transgene is unexpectedly expressed in the mesenchyme along the urinary tract, but it is not expressed in the UB and its derivatives. This suggests that the *Pax3Cre* expression occurs in a subdomain of the intermediate mesoderm-derived urogenital ridge after it has separated from the progenitors of the UB. Reciprocal signaling between the UB and metanephric mesenchyme is the fundamental process in the development of the kidney (33). The development of the wall along the pelvis and the lower urinary tract is less studied, but similar epithelial/mesenchymal interactions appear to again play a critical role (34–36). The ureteric mesenchyme appears to emerge with the UB. But it is not clear whether the UB also recruits cells from the metanephric blastema to form the ureteric wall. Our unexpected finding makes the *Pax3Cre* transgene a useful tool in lineage studies and in directing gene deletion specifically in the mesenchyme along the urinary tract to dissect the signaling events. Since ectopic expression domains can occur due to local environment of the chromosomal insertion site of any transgene, the *Pax3Cre* expression may or may not represent the endogenous expression of *Pax3*.

The phenotypic similarity between these mutants and mice deficient in genes in the renin-angiotensin axis (37) presents an intriguing possibility that the role of angiotensin II and angiotensin II receptors in renal pelvis development is mediated by calcineurin (Figure 9). The interruption of Shh signaling in the UB also led to defective SM development and obstructive nephropathy (36). Shh in the UB communicates with its receptor Ptch in the mesenchyme to regulate BMP4 for the normal proliferation and patterning of the SM layers (36). We found that all four NFATc genes are expressed in the developing kidney (data not shown). The NFATc genes are likely to be major effectors of calcineurin function, as they are in the development of other organ systems (22). Interestingly, calcineurin/NFAT signaling has been shown to regulate BMP signaling in chondrogenesis (38). Directly and indirectly, all these signaling pathways appear to cooperate to regulate morphogenic events in urinary tract development (Fig-

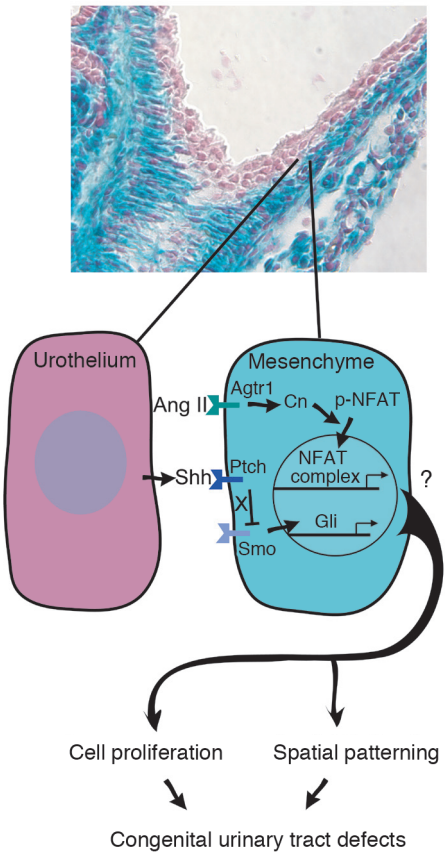

Figure 9
A model for the role of calcineurin (Cn) in the signaling events during urinary tract development. Deletion of *Cnb1* in the mesenchyme along the urinary tract results in abnormal development of the renal pelvis and ureter by disrupting the proliferation of the mesenchymal cells and the development of the peristaltic machinery. Deletion of *Shh* in the urothelium (36) and deletion of *Agtr1* and other genes in the renin-angiotensin axis (7, 37) also result in congenital obstructive phenotypes suggesting the possibility of interactions between these pathways. Ptch repression of Smo is relieved upon Shh binding, leading to the activation of Gli. p-NFAT, phosphorylated NFAT.

ure 9). Details of the interaction between these signaling pathways, however, remain to be investigated.

Acknowledgments

We are grateful to Lei Chen for excellent technical help with the generation of the floxed-*Cnb1* mice. We thank Helen Liapis, Jeremiah Morrissey, Jeffrey Miner, and Marc Hammerman for their valuable suggestions and comments. We thank Robert Heuckeroth for help with the acetylcholinesterase histology. This work was supported in part by funding from the Renal Division, Department of Internal Medicine at Washington University School of Medicine (to F. Chen) and from Howard Hughes Medical Institute (HHMI; to G.R. Crabtree), and by an NIH grant to F. Chen (R21 DK64816), an HHMI physician-scientist fellowship to C.-P. Chang, and an NIH grant to C.-P. Chang and G.R. Crabtree (R21 HL72092-01).

Received for publication September 16, 2003, and accepted in revised form January 6, 2004.

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