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Transcriptional Dysregulation in the Ureteric Bud Causes Multicystic Dysplastic Kidney by Branching Morphogenesis Defect

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Abstract

Purpose—The calcineurin-NFAT signaling pathway regulates the transcription of genes important for development. It is impacted by various genetic and environmental factors. We investigated the potential role of NFAT induced transcriptional dysregulation in the pathogenesis of congenital abnormalities of the kidneys and urinary tract.

Materials and Methods—A murine model of conditional NFATc1 activation in the ureteric bud was generated and examined for histopathological changes. Metanephroi were also cultured in vitro to analyze branching morphogenesis in real time.

Results—NFATc1 activation led to defects resembling multicystic dysplastic kidney. These mutants showed severe disorganization of branching morphogenesis characterized by decreased ureteric bud branching and the disconnection of ureteric bud derivatives from the main collecting system. The orphan ureteric bud derivatives may have continued to induce nephrogenesis and likely contributed to the subsequent formation of blunt ended filtration units and cysts. The ureter also showed irregularities consistent with impaired epithelial-mesenchymal interaction.

Conclusions—This study reveals the profound effects of NFAT signaling dysregulation on the ureteric bud and provides insight into the pathogenesis of multicystic dysplastic kidney. Our results suggest that the obstruction hypothesis and the bud theory may not be mutually exclusive to explain the pathogenesis of multicystic dysplastic kidney. Ureteric bud dysfunction such as that induced by NFAT activation can disrupt ureteric bud-metanephric mesenchyma interaction, causing primary defects in branching morphogenesis, subsequent dysplasia and cyst formation. Obstruction of the main collecting system can further enhance these defects, producing the pathological changes associated with multicystic dysplastic kidney.

Keywords

kidney; morphogenesis; NFAT transcription factors; etiology; organogenesis

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In nephrogenesis the UB undergoes many rounds of branching to form an elaborate tree-like structure after invading the MM. UB derivatives eventually give rise to the collecting system and the ureteric epithelium. Renal branching morphogenesis is a complex process of cell growth, proliferation, migration and remodeling.^{1,2} A number of genes with roles in UB branching morphogenesis are implicated in various CAKUTs (congenital abnormalities of the kidneys and urinary tract).²⁻⁴ Nonetheless, the upstream signaling pathways that control these developmental processes and the underlying genetic programs are not yet clearly defined.

MCDK is one of the most common urogenital defects in children worldwide, affecting 1/4,300 live births.⁵ MCDK is characterized by multiple noncommunicating thin-walled cysts surrounded by dysplastic parenchymal tissue, resulting in impaired kidney function. Despite its clinical significance the etiology and pathophysiology of MCDK remain poorly understood. Recent studies linked MCDK to genetic changes, infection and urinary tract obstruction as well as abnormal UB formation and its aberrant interaction with the MM.⁶⁻⁹

Previous studies showed that proper regulation of calcineurin-NFAT signaling is essential for the development and homeostasis of many organs, including urogenital organs, by regulating gene transcription in various cell types.¹⁰⁻¹³ In response to increases in intracellular Ca^{2+} the phosphatase calcineurin dephosphorylates cytoplasmic NFATc proteins, exposes the concealed nuclear localization sequences and triggers nuclear translocation for transcriptional regulation.^{14,15}

To further explore the role of this pathway in the urinary system we investigated the effects of NFAT activation on urinary system development by generating a murine model with conditional UB specific NFATc1 activation. In addition to revealing the effects of transcriptional dysregulation in UB on urinary tract development, our results also showed that a single genetic change in the UB can lead to obstructive and nonobstructive defects in the embryonic kidney and urinary tract, culminating in MCDK.

MATERIALS AND METHODS

Mouse Strains and Sample Collection

All animal studies in mice (*Mus musculus*) were approved by the Washington University School of Medicine institutional animal care and use committee in accordance with NIH (National Institutes of Health) guidelines. The *Hoxb7-Cre*, *ROSA^{rtTA}* and *TetO-NFATc1^{Nuc}* strains were described previously.¹⁶⁻¹⁹ The *ROSA^{lacZ}* allele was used to track cells with *NFATc1* activation. Polymerase chain reaction genotyping was done using primers and the GoTaq® PCR system (see table).

β-Galactosidase Assay

We performed β-galactosidase staining as previously described.²⁰ Briefly, mouse embryos or tissues were collected from timed pregnant females and fixed in 0.2% glutaraldehyde for 15 to 30 minutes. After permeabilization with 10% Triton™ X-100 in phosphate buffer the embryos and tissues were stained in X-gal solution composed of 50 mM potassium

ferricyanide, 50 mM potassium ferrocyanide and 1 mg/ml X-gal, and then post-fixed with 4% paraformaldehyde.

Histological Analysis

Embryos or tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (7 μ m) were collected and stained with hematoxylin and eosin. Paraffin sections were immunostained as previously described.²¹

Metanephros Culture

Metanephroi from mutant and control E12.5 embryos were cultured in serum-free Dulbecco's modified Eagle's medium/F12 medium with ITS (insulin-transferrin-sodium selenite) and grown on Transwell® filters (0.4 μ m) in 6-well plates.²² Dox (2 μ g/ml) was added as indicated. Metanephroi were cultured at 37°C in 5% CO₂ for the indicated times and imaged with a 2000TE microscope (Nikon®) every 30 minutes for 2 days.

RESULTS

Activation of NFAT Signaling in UB Derivatives Leads to Renal Hypoplasia and Dysplasia

Previous studies showed that proper regulation of calcineurin-NFAT signaling is essential for the development and homeostasis of multiple organ systems.^{10–13} We examined the consequences of NFAT activation in the developing urinary system. We generated mice with conditional activation of NFATc1 in UB derived cells by incorporating previously described *Hoxb7-Cre*, which directs Cre expression in UB derivatives,¹⁹ *ROSA^{rtTA}*, which expresses rtTA from a strong ubiquitous promoter¹⁶ and a *TetO-NFATc1^{Nuc}* transgene (fig. 1, A and B).^{13,18} In Cre-positive cells *loxP* recombination leads to rtTA expression. In the presence of the tetracycline analogue Dox the Dox-rtTA complex binds to *TetO* in the *TetO-NFATc1^{Nuc}* transgene to induce transcription of NFATc1^{Nuc}, an active form of NFATc1, which translocates into the nucleus to regulate its targets independent of calcineurin activity (fig. 1, C).^{13,18} For simplicity mice with all 3 alleles (*Hoxb7-Cre*, *ROSA^{rtTA}* and *TetO-NFATc1^{Nuc}*) are referred to as mutants. Their littermates without the full set of 3 transgenes and, thus, without NFATc1 activation even in the presence of Dox, are referred to as controls.

All mutants were phenotypically normal in the absence of Dox, indicating the strict dependence of NFATc1 activation on Dox (fig. 1, D). In contrast, most mutants treated with Dox beginning at conception (E0) died within P1 and only a few survived 3 to 4 weeks postnatally. No embryonic lethality was observed in mutants treated with Dox but CAKUT developed in all treated mutants by P1. The spectrum of phenotypes ranged from renal hypoplasia and dysplasia to hydronephrosis and hydroureter (fig. 1, E and F). Mutant kidneys showed hypoplasia and dysplasia, and were distinctly characterized by cystic structures and a significant decrease in the number of glomeruli. They also lacked a well-defined renal pelvis (fig. 1, H to J). Histopathological changes in mutant kidneys resembled those in patients with MCDK. We also found irregularities in the ureteral epithelium by P1 in mutants, which may have impaired pyeloureteral peristalsis (fig. 2, A and B). In contrast, we found no developmental abnormality of the bladder or lower urinary tract.

Renal Hypoplasia and Dysplasia in Mutants Resulted from Primary Defect and Not Urinary Obstruction

Since urinary obstruction can affect nephrogenesis, it was not immediately clear whether hypoplasia/dysplasia in mutant kidneys results from urinary tract obstruction or a primary defect in UB epithelium caused by the dysregulation of NFATc1 target genes. However, the observation that all mutant kidneys showed hypoplasia/dysplasia but only some mutant kidneys had apparent hydronephrosis and hydroureter suggests that hypoplasia/dysplasia in these mice was not simply due to urinary obstruction.

To further confirm this we examined embryonic kidneys from mutants at E14.5. Since significant urine production has not yet started at E14.5,²³ any hypoplasia or dysplasia found at or before this stage is unlikely to result from urinary tract obstruction. Although control UB end buds were properly developed and evenly distributed, mutant kidneys had few, irregular UB end buds (fig. 2, C and D). Furthermore, mutant kidneys already appeared hypoplastic with disorganized UB buds at E14.5 (fig. 2, D), indicating that renal hypoplasia/dysplasia at this stage is a primary defect rather than a secondary effect of obstruction. Additionally, the ureteral epithelium already showed many small outgrowths at E14.5 (fig. 2, D), which likely contributed to subsequent development of the disorganized ureteral epithelium found in mutants at P1 (fig. 2, A and B).

Activation of NFATc1 in the UB Results in Branching Morphogenesis Defects

To better understand the mechanisms by which the observed congenital renal defects develop in mutants we examined the effects of NFATc1 activation in the UB on branching morphogenesis by real-time imaging of in vitro cultures of metanephroi. We collected metanephroi from E12.5 embryos after treating pregnant females with Dox from E0 to E12.5. We then cultured 1 metanephros per embryo in medium containing Dox and the contralateral metanephros was cultured in the absence of Dox. The *Hoxb7-Cre* transgene used in our experiments co-expresses green fluorescence protein from an internal ribosome entry site (*IRES-EGFP*), allowing direct viewing of UB derivatives in living samples with a 2000TE confocal microscope (fig. 3).¹⁹ We used time lapse photography to visualize the progression of branching morphogenesis at 30-minute intervals during 48 hours.

Metanephroi of mutant embryos demonstrated reduced UB branching and noticeable expansion of UB stalks. Without further in vitro Dox treatment branching morphogenesis was delayed but it caught up to form a UB tree comparable to that of controls. In contrast, continuous NFATc1 activation by Dox led to severe UB patterning defects, which culminated in a dramatic reduction and disorganization in branching morphogenesis. Dox treatment had no effect on control metanephroi (supplementary video 1, <http://jurology.com/>, and fig. 3).

We further assessed these branching defects by performing whole mount β -galactosidase assay on metanephroi after the 48-hour culture period. Severe disorganization of UB branching in mutant metanephroi with continued in vitro Dox administration was strikingly apparent (fig. 4). In some areas UB derivatives became disconnected from the main UB tree in mutant metanephroi. This suggests that later in development these disconnected UB

derivatives likely contribute to the formation of micro-obstruction and cysts in the mutant kidney, as discussed.

Strict Control of NFATc1 Transcriptional Targets in the UB is Required for Branching Morphogenesis in Metanephric Kidney

Although NFATc1 activation in the UB clearly led to branching morphogenesis defects, it remained unclear whether this was a direct effect or the manifestation of earlier developmental abnormalities. To further delineate branching defect dynamics we performed another experiment in E12.5 metanephroi from females not treated with Dox. Control and mutant metanephroi were phenotypically indistinguishable at E12.5. Impaired branching morphogenesis was observed in mutants as early as 6 to 8 hours after Dox treatment. The decrease in branches and the disorganization of the tip stalk structure became obvious by 24 hours. Furthermore, in many UB branches in mutants the typical swelling ampullae or tips did not develop (supplementary video 2, <http://jurology.com/>, and fig. 5). This suggests that inappropriate NFATc1 activation results in the dysregulation of genes critical for establishing the distinction between UB tips and stalks.

We next treated pregnant females with Dox at E12.5 and collected metanephroi at E14.5. NFATc1 activation was sufficient to cause the development of dysplastic kidneys with severe UB branching defects (fig. 6). These results support our in vitro metanephros culture findings and demonstrate that impaired branching morphogenesis in mutants was not secondary to earlier developmental defects. These findings also strongly suggest that strict regulation of NFATc1 transcriptional targets in the UB is required for early branching morphogenesis and subsequent nephrogenesis.

DISCUSSION

Calcineurin-NFAT signaling pathway is essential for normal urinary tract development. Previous studies showed that the effects of alterations in calcineurin-NFAT signaling are cell type and context dependent.^{10–12,14,24} While these alterations cause no apparent disruption in many cell types, they can lead to profound molecular and cellular changes in other cell types, subsequently affecting organ development and function. Thus, we investigated the specific effects of NFAT activation in urinary system development by generating a murine model in which NFATc1 could be conditionally activated in the UB. Hypoplasia, MCDK and urothelial defects developed throughout embryogenesis in all mice with NFATc1 activation in UB derivatives.

Although MCDK is one of the most common forms of congenital urinary tract abnormalities, the underlying causes of MCDK in patients are largely unclear. There are at least 2 explanations for the etiology of kidney dysplasia. The obstruction hypothesis emphasizes the role of prenatal urinary tract obstruction and the bud theory emphasizes abnormal interaction between the UB and the MM.^{9,25–29} Prenatal obstruction is suspected to cause kidney dysplasia, including MCDK. However, the severity of prenatal obstruction does not always correlate well with renal dysplasia severity,^{9,25,26} indicating that other critical factors are also responsible.

To determine whether hypoplasia/dysplasia in mutants resulted from NFATc1 activation in UB derivatives or from embryonic urinary tract obstruction we examined mutant kidneys at E14.5 before significant urine production. Mutant kidneys were already hypoplastic/dysplastic at this time, suggesting that defects in the mutants at this stage more likely resulted from primary defects caused by dysregulation of NFATc1 targets in UB epithelium and not from urinary tract obstruction.

However, this did not exclude the possibility that later defects were caused or enhanced by embryonic urinary tract obstruction. In fact, the mutant ureteral epithelium became irregular with multiple abnormal sprouts at E14.5. Because effective transfer of urine through the ureter requires highly coordinated peristalsis, this urothelial irregularity may directly hamper the peristalsis machinery by altering ureteral structural and functional properties. The observation of irregularities in the ureteral epithelium, hydroureter and hydronephrosis suggests a later role of obstruction in the pathogenesis of the dysplastic phenotypes in the mutant kidneys.

In addition to obstruction, another explanation for the pathogenesis of kidney dysplasia is related to the bud theory, which suggests that abnormal interactions between the UB and the surrounding MM give rise to cystic dysplasia and obstruction.^{27–29} In our study in the mutants significant defects developed in branching morphogenesis, which required close coordination between the UB and the MM. Transcriptional dysregulation induced by NFAT activation in the UB derivatives likely disrupts such coordination, leading to branching morphogenesis defects, including abnormal end bud morphology, improper end bud and stalk distinction, and the separation of some UB derivatives from the developing collecting system. The aberrant and even disconnected end buds may still have the ability to induce nephron formation. However, without proper urine drainage to the main collecting duct system micro-obstructions could develop from nephron structures induced by the disconnected UB derivatives (figs. 3 to 5). This would lead to the formation of cysts analogous to those found in MCDKs.

Thus, the cysts in MCDK may derive from branching morphogenesis defects and micro-obstruction as a result of the aberrant UB function and UB-MM interactions. In fact, abnormal UB-MM interactions may even be the cause of obstruction in these mutants. The coordinated growth of invading UB and surrounding MM appeared to be affected in these mutants, as reflected in the irregular growth of the ureteral epithelium, likely contributing to the hydronephrosis and hydroureter that we found. The uncoupling of growth regulation of the UB epithelium and the underlying mesenchyma can also affect the normal development of ureteral smooth muscles essential for peristalsis and interfere with urine transfer.

CONCLUSIONS

This study reveals the profound effects of dysregulation of NFAT signaling in the UB and provides insight into MCDK pathogenesis. These results suggest that the obstruction hypothesis and the bud theory may not be mutually exclusive for explaining MCDK pathogenesis. Dysfunction in the UB such as that induced by NFAT activation can disrupt UB-MM interaction, causing primary defects in branching morphogenesis, subsequent

dysplasia and cyst formation partially due to micro-obstruction independent of obstruction in the main collecting system. In addition to these micro-obstructions, prenatal obstruction of the main collecting system can further enhance these defects in the kidney, producing the pathological changes associated with MCDK.

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Abbreviations and Acronyms

Dox	doxycycline
E	embryonic day
MCDK	multicystic dysplastic kidney
MM	metanephric mesenchyme
NFAT	nuclear factor of activated T cells
P	postnatal day
rtTA	reverse tetracycline-controlled transactivator
TetO	tetracycline-response operon promoter element
UB	ureteric bud

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Figure 1.

Conditional NFATc1 activation in UB derivatives led to renal hypoplasia/dysplasia. *ROSA-lacZ* reporter reveals *Hoxb7-Cre* transgene expression in wolffian duct (arrow) and UB (triangle) at E11.5 (A) and E12.5 (B). Cre limited rtTA production to wolffian duct and UB (C). Dox-rtTA complex binding to *TetO* led to NFATc1^{Nuc} production. Newborn mice were treated with Dox starting on E0 (D to F). Open triangle indicates male reproductive ducts. Black triangles indicate kidneys (E and F). Arrow indicates ureter (E). Arrow indicates hydronephrosis (F). Newborn shows clear division of renal cortex, outer medulla, inner medulla, papilla and pelvis (G). Hypoplasia/dysplasia (H). Higher magnification reveals poor renal cortex-medulla division, no pelvis, few glomeruli and areas with cystic appearance (I and J).

Figure 2.

Abnormalities in ureteral epithelium and renal hypoplasia/dysplasia developed before urine production onset. Ureter proximal portion in newborn (A). Littermate with apparent ureteral epithelium irregularity due to NFATc1 activation (B). Dotted lines indicate overall shape of ureter since β -galactosidase activity reveals only epithelial cells (A and B). E14.5 kidney and ureter (C and D). Mutant was treated with Dox starting on E0. Note many small protrusions arising from ureteral epithelium (D). White arrows indicate UB end buds. Black arrows indicate ureteral epithelium.

Figure 3. NFATc1 activation in UB in vivo and in vitro affected branching morphogenesis in metanephroi from E12.5 embryos from pregnant females treated with Dox since E0. +Dox, 2 μ g/ml Dox.

Figure 4.

Aberrant UB branching induced by NFATc1 activation in metanephroi from E12.5 embryos from pregnant females treated with Dox from E0 to E12.5 and cultured for 48 hours in Dox presence or absence (A to D). β -galactosidase assay reveals *Hoxb7-Cre* positive cells with NFATc1 activation in UB derivatives. U, ureter. Arrow indicates long unbranched extension (D). Arrowhead indicates expanded stalk region (D). Note some isolated UB derivatives with no apparent connection to remaining UB tree (D).

Figure 5. NFATc1 activation in UB led to branching defects within 12 hours in metanephroi from E12.5 embryos from untreated female. +Dox, cultured with 2 μ g/ml Dox.

Figure 6. NFATc1 activation from E12.5 to E14.5 was sufficient to cause branching and ureteral epithelial defects in vivo in kidney and ureter of E14.5 control and mutant treated with Dox from E12.5 (A and B). Arrow indicates UB end buds.

Table

PCR genotyping

Primer	Sequences (5'-3')	Product Size (bp)
FCCreF1	TCGATGCAACGAGTGATGAG	420
FCCreR1	TCCATGAGTGAACGAACCTG	
ROSA5	AGTTCTCTGCTGCCTCCTG	
RTTA3	AAGACCGCGAAGAGTTTGTC	Wild-type 322, transgene 250
ROSA3	CGAGGCGGATACAAGCAATA	
TnF1	AAGAAGATGGTCCTGTCTGG	351
TnR1	GTAGTCTGGTACGTCGTAC	