Teashirt 3 is necessary for ureteral smooth muscle differentiation downstream of SHH and BMP4

Xavier Caubit1,*, Claire M. Lye2,*, Elise Martin1,*, Nathalie Coré1, David A. Long2, Christine Vola1, Dagan Jenkins3, Alistair N. Garratt4, Helen Skaer5, Adrian S. Woolf2,† and Laurent Fasano1,†

Ureteric contractions propel foetal urine from the kidney to the urinary bladder. Here, we show that mouse ureteric smooth muscle cell (SMC) precursors express the transcription factor teashirt 3 (TSHZ3), and that Tshz3-null mutant mice have congenital hydronephrosis without anatomical obstruction. Ex vivo, spontaneous contractions that occurred in proximal segments of wild-type embryonic ureter explants were absent in Tshz3 mutant ureters. In vivo, prior to the onset of hydronephrosis, mutant proximal ureters failed to express contractile SMC markers, whereas these molecules were detected in controls. Mutant embryonic ureters expressed Shh and Bmp4 transcripts as normal, with appropriate expression of Ptch1 and pSMAD1/5/8 in target SM precursors, whereas myocardin, a key regulator for SMC differentiation, was not expressed in Tshz3-null ureters. In wild-type embryonic renal tract explants, exogenous BMP4 upregulated Tshz3 and myocardin expression. More interestingly, in Tshz3 mutant renal tract explants, exogenous BMP4 did not improve the Tshz3 phenotype. Thus, Tshz3 is required for proximal ureteric SMC differentiation downstream of SHH and BMP4. Furthermore, the Tshz3 mutant mouse model of ‘functional’ urinary obstruction resembles congenital pelvi-ureteric junction obstruction, a common human malformation, suggesting that TSHZ, or related, gene variants may contribute to this disorder.

KEY WORDS: Gene targeting, Teashirt 3 (Tshz3), UPJO, Ureter, Smooth muscle

INTRODUCTION

Diverse viscera contain contractile smooth muscle cells (SMC) and SM pathology is associated with several diseases, including asthma, lung fibrosis and liver cirrhosis. Thus, studies that unravel the molecular mechanisms regulating visceral SMC differentiation are potentially of great importance for understanding human disease. In the urinary tract, SM is present in the renal pelvis, the ureter, the bladder and the urethra, and is central to the functions of these organs. As in other viscera, ureteric SM develops from local mesenchyme (Yu et al., 2002) and hence the mouse ureter represents an appropriate paradigm to elucidate mechanisms controlling visceral SMC differentiation.

A dilated renal pelvis, or hydronephrosis, occurs in 0.9-7.7% of human gestations, the exact incidence depending on the criteria used to define the upper normal limit of the pelvic diameter (Ek et al., 2007; Gunn et al., 1995; Ismaili et al., 2006). Although most such dilatations are transient normal variants, others persist after birth and thus represent congenital malformations. In this context, a common diagnosis is unilateral or, less often, bilateral pelvi-ureteric junction obstruction (PUJO), present in up to 0.3-0.4% of all babies (Ek et al., 2007). PUJO ureters are not anatomically blocked but have aberrant SM arrangement where the proximal ureter joins the renal pelvis (dell’Agnola et al., 1990; Zhang et al., 2000). Ureteric peristalsis propels urine from the renal pelvis towards the urinary bladder, and a failure of this activity causes ‘functional’ flow impairment leading to urinary tract dilatation and kidney damage (Mendelsohn, 2004). Peristalsis is propagated distally along the urinary tract by SM in the ureter coat. Therefore, failure in SM differentiation along the urinary outflow tract may be an important primary cause of functional obstruction and hydronephrosis.

Between mouse embryonic day (E) 10 and 11, the metanephric mesenchyme (MM) signals to the ureteric bud (UB) to promote its outgrowth from the mesonephric duct and entry into the MM. Thereafter, UB branching morphogenesis generates kidney collecting ducts, and UB branch tip signals induce MM cells to aggregate and undergo mesenchymal-to-epithelial transition, forming nephrons. Meanwhile the unbranched stalk of the UB outside the MM elongates to form the ureter tube epithelium, the ‘urothelium’, while surrounding mesenchymal cells also contribute to the ureter becoming the cells of the lamina propria, SM and connective tissue.

Reciprocal signalling between the epithelial and mesenchymal components of ureter is essential for correct ureter development (Airik and Kispert, 2007; Mendelsohn, 2006). UB stalk epithelia secrete sonic hedgehog (SHH), which has a proliferative effect on ureteric mesenchyme and induces bone morphogenetic protein 4 (BMP4) in peri-urothelial mesenchymal cells (Yu et al., 2002). Inactivation of Shh in the urinary tract delays ureteric SMC maturation and causes loss of stromal cells located between the urothelial and SM layers (Yu et al., 2002). BMP4 promotes the differentiation of ureteric mesenchyme into SMCs and also facilitates urothelial maturation (Brenner-Anantharam et al., 2007; Miyazaki et al., 2003; Raatikainen-Ahokas et al., 2000). In response to signals from ureteric mesenchyme, UB stalk epithelia mature into urothelia, and express uroplakin (UPK)-rich plaques on their apical surfaces to maintain the ‘water-tight’ properties of this epithelium (Jenkins and Woolf, 2007).
Recently, other mouse models confirmed that, unless mesenchymal cells surrounding the ureter stalk differentiate normally, congenital malformations of the urinary tract will arise. The transcription factor TBX18 is expressed in undifferentiated mesenchymal cells around ureter. In Tbx18−/− mice, absence of condensation and differentiation of ureteral mesenchymal cells into SMs results on congenital hydroureter (Airik et al., 2006). Deletion of a regulatory subunit of calcineurin, Cnbl (Ppp3r1 – Mouse Genome Informatics) in the mesenchyme of the developing urinary tract results in reduced proliferation in the SMCs, leading to defective postnatal pyeloureteral peristalsis and renal obstruction (Chang et al., 2004). Despite these insights, there is still a crucial need to develop new mouse models for congenital ureter malformations to help understand the mechanisms that underlie SM differentiation.

In Drosophila, renal (Malpighian) tubules (MPTs) are major excretory and osmoregulatory organs. They derive from two cell populations, ectodermal epithelial buds and surrounding mesenchymal mesoderm, and unexpected parallels exist between MPTs development and vertebrate nephrogenesis (Denholm et al., 2003). Several fly MPT genes such as Kr, cut, hibriss and Odd have vertebrate homologues (Gli3, Cux1, nephrin and Osr, respectively) implicated in kidney development (Sharma et al., 2004; Tena et al., 2007; Vanden Heuvel et al., 1996; Zhang et al., 2002). We have previously shown that stellate cells within MPT express two related zinc-finger transcription factors, teashirt (tsh) and tiptop (tio) (Denholm et al., 2003; Laugier et al., 2005). Furthermore, we found that the three mouse teashirt (Tshz) genes were functionally equivalent to Drosophila tsh in terms of rescuing homeotic and segment polarity phenotypes of a tsh-null mutant fly (Caubit et al., 2005; Manfrold et al., 2004).

Based on the above observations the Tsh/Tshz families, we suspected that they might be expressed in, and have roles in, mammalian renal tract development. Here, we show that mouse ureteric SMC precursors express Tshz3 and that Tshz3-null mutant mice have congenital hydroureter without anatomical obstruction. Ex vivo, the spontaneous contractions that occurred in proximal segments of wild-type embryonic ureteral explants were absent in Tshz3 mutant ureters. In vivo, prior to the onset of hydroureter, mutant proximal ureters failed to express contractile SMC markers, whereas these molecules were detected in controls. Mutant embryonic ureters expressed Shh and Bmp4 transcripts, as normal, with appropriate expression of Pch1 and pSMAD1/5/8 in target SM precursors, whereas myocardin, a key regulator for SMC differentiation (Wang and Olson, 2004), was not expressed in Tshz3 null ureters. In wild-type embryonic renal tract explants, exogenous BMP4 upregulated Tshz3 and myocardin expression. Thus, Tshz3 is required for proximal ureteric SMC differentiation downstream of Shh and BMP4.

MATERIALS AND METHODS

Gene targeting of the Tshz3 locus

Tshz3 genomic DNA fragments were isolated from a 129/Ola mouse genomic library. To generate the targeting construct, we used a vector (pKO) containing a neo-resistance gene derived from the pMC1NeoPolyo/A vector (Mansour et al., 1988) flanked in 5′ with a multiple cloning site and separated in 3′ from the HSV-tk cassette by Sall and BamHI sites. A 53 kb short arm, comprising the start of exon 2, was PCR generated and fused in frame with a lacZ/40-polyA cassette excised from pETL as a 4.25 kb BamHI fragment (Mombaerts et al., 1996). The 0.53 kb lacZ cassette was inserted 5′ to the neo gene and a 5.8 kb fragment 3′ of the Tshz3 gene was inserted between the neo and HSV-tk genes of pKO. E14 (129/Ola) ES cells were electroporated with 20 μg of targeting vector and cultured in presence of 300 μg/ml G418 for positive selection. Two days later, negative selection was applied using 2 μM gancyclovir. The neoycin sequence was used as a probe to check unique integration event. Correct recombination 5′ to the locus was controlled by PCR using a primer upstream to the 5′ homology region, (5′TTAACAAATAAGGCACCAGT3′) and a reverse primer in the lacZ gene (5′CCCTTTCGTATTACGCGAAG3′).

Generation of Tshz3-null mice

Animals were treated according to protocols approved by the French Ethical Committee. Male chimeras, generated after injection of Tshz3lox-ES cells into C57BL/6 blastocysts, were mated to C57BL/6 females. Offspring (n=249) were assayed for germline transmission of the Tshz3lox allele but no transmission was PCR detected. Male chimeras were then mated to CD1 females. F1 Tshz3lox- mice were intercrossed to obtain Tshz3lox/lox mutant mice. Alternatively, F1 Tshz3lox- mice were crossed to CD1 females to generate Tshz3lox/+, and mutant mice were analysed after six generations on the CD1 background. Genomic DNA was PCR-genotyped. Primers (5′GGAAGGGACTGGCGTCTATTG3′ and 5′CGATACCTGGA- AGCCGAGG3′) for the neo sequence amplified a 478 bp fragment from the recombinant allele, and primers for exon 2 (5′CGGAGCAT- CTGGACGCTATT3′ and 5′CTGATATCCTGGAAGAGTC3′) amplified a 630 bp fragment from the wild-type allele. Representative and reproducible morphology and gene/protein expression patterns based on four to 20 embryos for each genotype at each embryonic stage are shown.

Immunoprobinding and in situ hybridisation

Tissues were fixed in 4% paraformaldehyde. Paraffin-embedded sections (5–10 μm) were stained with Haematoxylin and Eosin or Masson’s trichrome. X-Gal staining was performed as described (Relaix et al., 2004). Immunostaining was performed either on 14 μm cryosections of tissues or on paraffin-embedded sections after quenching endogenous peroxidase and antigen retrieval followed by reaction with secondary antibodies (details available on request). Whole explants were blocked in 5% goat serum/PBS/0.3% Triton X-100 then incubated with mouse anti-smooth muscle α actin (SMA; Sigma, clone 1A4; 1/1000) and rabbit anti-E-cadherin (G. Rougon, IBDML-France; 1/500) antibodies. Guinea-pig anti-TSHZ3 antibody (1/5000) was raised against mouse amino acids 557-664, cloned as a His-tagged fusion protein in pET14b (Novagen) and produced by A. Garratt’s laboratory. Other primary antibodies were: rabbit anti-β-galactosidase (Cappel; 1/1000); rabbit anti-Pax2 (Zymed; 1/50); mouse anti-proliferating cell nuclear antigen (PCNA; BD Pharmingen; 1/200); rabbit anti-pSMAD1/5/8 (Cell Signaling; 1/200); rabbit anti-retinaldehyde dehydrogenase 2 (RALDH2; 1/2000) (P. McCaffery, University of Aberdeen, UK); goat anti-myocardin (se-21559; Santa-Cruz; 1/200); rabbit anti-aquaporin 2 (Chromicon; 1/400); sheep anti-uroomodulin (Biodesign, AMS Biotechnology Distribution; 1/500); rabbit anti-SM myosin heavy chain (SMMHC; anti-MHC204/200; 1/500; M. Conti and R. Adelstein, Laboratory of Molecular Cardiology, Bethesda, USA) (Kelley et al., 1991); rabbit anti-SM protein alpha 22kDA (SM22a; 1/1000; M. Gimona, Austrian Academy of Sciences, Salzburg, Austria); and rabbit antiseraum to total UPK-1 (1/100; T. T. Sun, New York School of Medicine, USA). Apoptotic cells were detected using the In Situ Death Detection Kit (Roche). For each sample, the number of apoptotic cells, and the total number of cells were counted for each of the following cell populations in the proximal ureter: urothelium, aggregated mesenchyme and loose mesenchyme.

India ink injection was injected into renal pelvis as described previously (Airik et al., 2006). In situ hybridisation using digoxigenin-labelled or radioactive probes was performed on sections as described (Caubit et al., 2005). Probes used were: Bmp4 (B. Hogan, Duke University, Durham, USA); Pch1 (M. Scott, HHMI, Stanford University School of Medicine, USA); Shh (D. Epstein, University of Pennsylvania School of Medicine, USA); Smad and Myocd (E. Oslon, University of Texas Southwestern Medical Center, Dallas, USA); foxd1 (A. Kispert, Medizinische Hochschule Hannover, Germany); and Raldh2 (J. Xavier-Neto, HC.FMUSP, São Paulo-SP, Brazil).

Embryonic ureter culture and video microscopy

E15.5 ureters were dissected and explanted onto platforms (Millipore; pore size 0.4 μm) and cultured in defined, serum-free media, as described for embryonic mouse urinary bladders (Burgu et al., 2006). The timelapse imaging is detailed in the movie legends (see supplementary material). Images were analysed using Metamorph software.
Metanephric explant cultures

Kidney rudiments were isolated and cultured as described (Brenner-Anantharam et al., 2007). Explants, each consisting of a metanephros and an attached ureter, were isolated from E12.5 mouse embryos and cultured for 96 hours. For real-time quantitative PCR (qPCR) analyses, explants were isolated from E13.5 mouse embryos and cultured for 72 hours. In some cultures, 100 ng/ml recombinant BMP4 (R&D Systems) was added to the culture medium. In all cases, medium was changed daily. RNA was isolated using TRIzol Reagent (Bioline) according to the manufacturer’s instructions. Total RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories). Real-time PCR was performed on a PTC-200 DNA Engine Opticon System (Bio-Rad Laboratories) using iQ SYBR Green Supermix (Bio-Rad Laboratories). Primer sequences used for Sybr RT-PCR are as follows: Tshz3, 5′-geggegeagcctatgtgtctg and 3′-tgctagctgctactgctgctgctgctg; Myocd, 5′-cagggctagccgagctg and 3′-aatgtgcctgagcagcag; Id3, 5′-aggtgccagggatatctg and 3′-tcagctgctggtagcggag. Results were normalised to Hprt expression.

RESULTS

Tshz3 is expressed in mesenchymal populations of the developing urinary tract

Using an anti-TSHZ3 antibody, we looked at the spatial distribution of the TSHZ3 protein in developing metanephros (Fig. 1). The expression patterns were confirmed by in situ hybridisation analyses (not shown). At E11.5 TSHZ3-expressing cells were noted around the UB stalk (Fig. 1A). At E12.5, TSHZ3 was expressed in mesenchymal cells along and around the UB stalk, and was absent from the UB epithelium that itself expressed PAX2 (Schedl, 2007) (Fig. 1B). At E12.75, in addition to the expression in the nascent ureteric mesenchyme, TSHZ3 was detected in scattered cells within the metanephric medullary stroma (Fig. 1C). At E15.5, TSHZ3 expression was noted in mesenchymal cells of the ureter and renal pelvis, and in renal medullary stroma; the outer rim of nephrogenic mesenchyme was negative (Fig. 1D). At this stage, transverse sections of the ureter revealed that TSHZ3 was detected in mesenchymal cells condensed around urothelia, and also in loosely organised cells with cell bodies arranged tangentially (Fig. 1E). In the E15.5 bladder, TSHZ3 was detected in rare mesenchymal cells adjacent to the epithelium and in the peripheral mesenchyme where SM starts to differentiate (Fig. 1F) (Li et al., 2006). At E18.5, TSHZ3 expression was maintained in ureteric mesenchymal cells, including those directly subjacent to the ureteral epithelium, where stromal cells are located (Fig. 1G). In the E18.5 bladder, TSHZ3 was found in the (submucosal) loose connective tissue adjacent to the epithelium and in the detrusor SM layer (Fig. 1H). These expression patterns are consistent with the hypothesis that the TSHZ3 transcription factor plays roles in renal tract development.

Generation of mice containing a null mutation of Tshz3

To address the issue of the functional contribution of Tshz3 in the developing renal tract, we generated mice homozygous for a null mutation in the Tshz3 gene (Tshz3lacZ/lacZ) (Fig. 2A-D). Chimeras were mated to CD1 females and seven chimeras achieved germline transmission. Crossing Tshz3lacZ/lacZ parents failed to produce viable null mutant offspring but genotyping E18.5 litters showed that homozygous mutants (Tshz3lacZ/lacZ) developed at the expected Mendelian ratio. E18.5 Tshz3lacZ/lacZ embryos obtained after Caesarian section became cyanotic and died within 1 hour. They showed no external anatomical differences from the wild types, but they did not move spontaneously and reacted only to strong stimuli. Tshz3lacZ/lacZ lungs sunk, whereas control lungs floated on water (data not shown). Thus, Tshz3lacZ/lacZ animals die because of an inability to breathe. The rapid death is unlikely to be associated with renal tract defects described below because even severe kidney excretory failure is not fatal for at least 1 day. Instead, it is likely that Tshz3 is also necessary for respiration and this is the focus of a separate investigation.

Using comparative analysis on whole mounts and tissues sections, we confirmed that the expression of the Tshz3lacZ allele recapitulated the expression pattern of endogenous Tshz3, providing evidence that the Tshz3 gene was correctly targeted. Part of this analysis is illustrated in Fig. 2, where TSHZ3 and β-galactosidase (β-gal) were detected in periureteral mesenchymal cells and nuclei (DAPI, blue) at E15.5 in metanephros (D), ureter (E) and bladder (F), and at E18.5 in ureter (G) and the bladder (H). Broken line in E separates the submucosal layer and the detrusor. d, detrusor SM; e, epithelium; mm, mesenchymal mesenchyme; mms, metanephric medullary stroma; pUB, proximal ureteric bud; sl, submucosal layer; UBs, ureteric bud stalk.

Inactivation of Tshz3 causes hydroureter and SMCs malformation

Mutant urinary tracts displayed a prominent proximal hydroureter and the kidneys were markedly hydronephrotic (Fig. 3B,C); a fully penetrant bilateral phenotype evident from E16.5 that affects both sexes. In heterozygous embryos, rare cases (4/80) of unilateral hydroureter occurred. Histological analysis confirmed dilation of the renal pelvis in null mutants (Fig. 3D,E) and showed that the dilated proximal ureters were thin-walled (Fig. 3J). In wild-type ureters,
the multilayered epithelium was surrounded by condensed mesenchymal cells that differentiated into multiple SM layers (Fig. 3F,G). Close inspection of Tshz3lacZ/lacZ mesenchymal cells that differentiated into multiple SM layers (Fig. 3H,K). The multilayered epithelium was surrounded by condensed stromal genes (Geleff et al., 1997) in the deeper cortex; transcripts of cortical several layers of podoplanin-expressing glomeruli (Breiteneder-observed condensing nephrogenic MM in the outer cortex and material). We then assessed the expression patterns of several were similar to wild type (see Fig. S1 in the supplementary material). However, at E16, as hydroureter developed, peri-urothelial cells appeared less organised compared with wild-type ureters (Fig. 3L-O).

Other than being hydronephrotic, E18.5 null mutant kidneys were similar to wild type (see Fig. S1 in the supplementary material). We then assessed the expression patterns of several genes expressed in subsets of cells in the mammalian kidney. We observed condensing nephrogenic MM in the outer cortex and several layers of podoplanin-expressing glomeruli (Breiteneder-Geleff et al., 1997) in the deeper cortex; transcripts of cortical stromal genes Foxd1 and Raldh2 (Schmidt-Ott et al., 2006) were normally expressed, as was the Pax2 transcription factor, which was detected in nuclei of MM condensations and in UB branch tips (Winyard et al., 1996). Within the medulla of the E18.5 kidney, similar expression patterns in wild types and mutants were detected for SMAA, which is normally transiently expressed in interstitial cells (Chung and Chevalier, 1996), for the collecting duct markers Pax2 (Winyard et al., 1996) and aquaporin 2 (Marpes et al., 1995), and for uromodulin, a marker of the thick limb of the ascending loop of Henle (Hoyer et al., 1974). The morphology and histology of the bladder were not affected in Tshz3lacZ/lacZ (see Fig. S2A,B in the supplementary material).

To determine the onset of urinary tract malformations in Tshz3lacZ/lacZ, we harvested embryos from timed mating and analysed urinary tract histology. From E12.5, we observed that the mesenchymal progenitors condensed as normal around the ureteric epithelium until E15.5 (data not shown) (see Fig. S2G,H in the supplementary material). However, at E16, as hydroureter developed, peri-urothelial cells appeared less organised compared with wild-type ureters (Fig. 3L-O).

Analysis of E18.5 mutant urinary tracts by India ink injection revealed no sign of physical obstruction: ink flowed down the ureter into the bladder, suggesting that hydroureter and hydronephrosis were caused by functional, rather than by anatomical, obstruction (Fig. 3P-R). Furthermore, because the structural organisation of ureteric muscle is lost in Tshz3lacZ/lacZ mutant (-/-) ureter; TSHZ3 is not detected in mutant ureter. Co-immunostaining of TSHZ3 and β-gal (right panel) on a section from E14.5 Tshz3lacZ/+ heterozygote ureter.

**Fig. 2. Targeted disruption of Tshz3.** (A) Gene targeting strategy. The mouse Tshz3 locus consists of two exons and one intron, and spans more than 75 kb; the first and second exons encode, respectively, 13 and 1068 amino acids. The in-frame insertion of the lacZ-coding sequence (hatched box) and the neomycin expression cassette (pTK-neo; black box) into exon 2 (ex2; light dotted box) resulted in complete deletion of the zinc-finger motifs and created an XbaI digest size difference between wild-type and targeted loci. Restriction sites are abbreviated as follows: B, BamHI; E, EcoRI; S, SalI, N, Nol1, X, XbaI. (B) Southern blot analysis of DNA from wild-type (+/+), heterozygous (+/-), and homozygous null (−/−) ES cells clones using XbaI-digested genomic DNA and a 3′ external probe (3′ probe; white box in A). (C) Appropriate recombination 5′ to the locus was confirmed by PCR with primers shown in A (short arrows). (D) PCR-based genotyping of wild-type (+/+), heterozygous (+/-) and homozygous null (−/−) embryos. (E) Immunostaining of TSHZ3 (left panel) on sections from E15.5 wild-type (+/+), and Tshz3allele (+/−) ES cell clones using XbaI-digested genomic DNA and a 3′ external probe (3′ probe; white box in A). (F) Appropriate recombination 5′ to the locus was confirmed by PCR with primers shown in A (short arrows).
also grew, initiated contractions at a similar proximal/distal level and propagated a pulse-wave distally. Significantly, however, null mutant proximal ureters completely failed to contract (Fig. 4D; see Movie 2 in the supplementary material). This result strongly supports the hypothesis that abnormal peristalsis of the proximal part of the ureter occurs in vivo, causing a functional obstruction that leads to hydronephrosis and hydroureter.

**Tshz3 is expressed in differentiating SMCs**

To better characterise the Tshz3-positive cell population, we performed double immunostaining for β-gal (to report the expression of Tshz3) and SMAA, a marker of SMCs. In wild types, strong expression of SMAA has been reported at E15.5 in the condensed mesenchymal cells of the proximal ureter (Yu et al., 2002). In E15.5 heterozygous proximal ureters, we observed SMAA/β-gal double-positive cells in the condensed mesenchymal layer adjacent to the epithelium (Fig. 5B). In addition, β-gal was detected in loose mesenchymal cells excluded from the SMAA-positive layer, indicating that Tshz3 was expressed both in undifferentiated mesenchymal cells far from the urothelium and in differentiating SMCs. At E18.5, β-gal expression was sustained in SMCs and in fewer mesenchymal cells at the periphery (Fig. 5D). From E18.5 onwards, we also detected β-gal expression within cells directly adjacent to the urothelium that were not positive for SMAA (Fig. 5D,F). These cells are thought to be progenitors of ureteral connective tissue and express Raldh2 (Mahoney et al., 2006). At E18.5, cells double-positive for RALDH2 and β-gal activity were detected adjacent to the epithelium, suggesting that Tshz3 marks progenitors of ureteral connective tissue (Fig. 5G). Together, these data confirmed that Tshz3 is expressed in the SM of the developing ureter, and in other ureteric mesenchymal cell populations.

**Tshz3 deficiency causes perturbed development of ureteric SMC**

To test whether the hydronephrosis and hydroureter observed in the Tshz3 mutant were caused by abnormalities in the SM, we examined SMCs development in Tshz3lacZ/lacZ ureters. A hallmark of SMCs differentiation is the elevated expression of SMC-selective differentiation marker genes, including Smaa, Smhc and Sm22a (Owens, 1995). The differentiation of SM in the mouse ureter and the pelvis has been documented to occur in a proximodistal wave (McHugh, 1995; Yu et al., 2002). In wild-type embryos, SMAA is first detected at E14.5 in few cells within aggregated mesenchyme of the proximal ureter and the nascent renal pelvis (data not shown).
Examination of proximal ureters of E15.5 Tshz3lacZ/lacZ mutant embryos revealed almost absent expression of SMC contractile proteins, including SMAA, SMMHC and SM22 (Fig. 6D-F) versus controls embryos (Fig. 6A-C). In vivo, in vascular SMC, the expression of genes encoding SMAA, SMMHC and SM22 depends on myocardin (Huang et al., 2008; Li et al., 2003). At E15.5, wild-type ureteric mesenchymal cells co-expressed MYOCD and SMAA proteins (Fig. 6G), and in situ hybridisation revealed Myocd expression in ureteral mesenchymal cells (Fig. 6H). By contrast, expression of Myocd was deficient in Tshz3lacZ/lacZ proximal ureters (Fig. 6K), correlating with absent expression of Smaa transcripts (Fig. 6I,L) and MYOCD and SMAA proteins (Fig. 6J). Crucially, at this timepoint, urinary tract dilatation was not yet present, so the observed downregulation of SMC marker expression in the mutant could not be a secondary effect resulting from hydroureter-related distortion. In wild types at E17.5, SMAA immunostaining revealed that proximal ureteric SMC layers became more organised and thicker (Fig. 6M). By contrast, in Tshz3lacZ/lacZ mutants, we observed that SMAA expression was lost in the proximal-most ureter (Fig. 6O) where the dilatation was prominent. Similar aberrant expression patterns were noted with SMMHC and SM22 (data not shown). However, in the distal-most part, where the diameter of the Tshz3lacZ/lacZ ureter was normal, SMAA was detected in the mesenchymal cells although at a lower level than in wild types (Fig. 6N,P), perhaps correlating with an apparently slower distal propagation of contraction wave ex vivo (note the longer contraction at the ‘red’ level in the mutant versus the wild-type ureter depicted in Fig. 4C,D). In Tshz3lacZ/lacZ bladders, SMA was normally expressed (see Fig. S2C-F in the supplementary material). Taken together, these data are consistent with the idea that TSHZ3 regulates expression of Myocd in visceral tissue, and that, as in vascular SMCs, expression of SM-specific genes in ureteric SMCs depends on Myocd.

Next, we determined whether the failure of SMC development might reflect defects in proliferation and/or apoptosis of periureteric cells (see Fig. S3 and Table S1 in the supplementary material). As assessed by PCNA immunostaining (see Fig. S3A,B in the supplementary material), we recorded no significant difference in proliferation between wild-type and mutant proximal ureters. As assessed by TUNEL analyses, apoptosis was never detected in aggregated cells around the urothelium in either genotype, whereas...
there was a similar, low prevalence in urothelia and also mesenchymal cells outside the aggregated mesenchymal layer. Furthermore, comparison of expression between Tshz3lacZ/+ and Tshz3lacZ/lacZ ureters revealed similar numbers of β-gal-positive cells in both genotypes (see Fig. S3C,D in the supplementary material). Thus, Tshz3 was not essential for SMC precursor proliferation or survival.

Because we found that Tshz3 is expressed in RALDH2-positive cells, we investigated whether Tshz3 was also involved in differentiation of these cells. At E18.5, RALDH2-positive cells were present in the dilated part of the Tshz3 mutant ureter, but were scattered and did not form a continuous layer, probably as a consequence of the dilation caused by the hydrourerter phenotype (Fig. 6Q,S). Distal to the dilated part, RALDH2-positive cells form a continuous layer as in wild-type ureter (not shown). In conclusion, loss of Tshz3 does not affect the differentiation of the ureteric stromal cells, although their distribution is compromised.

Recent studies suggest that a signal from the ureteric mesenchyme to the ureteric epithelium participates in the differentiation of the urothelium (Airik et al., 2006). To investigate whether loss of Tshz3 in the ureteric mesenchyme would compromise differentiation of the urothelium, we analysed UPK expression in E18.5 Tshz3lacZ/lacZ proximal ureters. Expression in both mutant and wild-type urothelium indicated that, despite failed SMCs differentiation, urothelia matured normally (Fig. 6R,T).

Tshz3 is downstream to SHH and BMP4 with regard to ureteric SMC differentiation

SHH signalling plays an essential role in ureteric SMC development by promoting proliferation of ureteric mesenchymal cells and inducing them to secrete BMP4; BMP4 in turn promotes SMC differentiation and Bmp4+/− mice exhibit hydrourter (Brenner-Anantharam et al., 2007; Miyazaki et al., 2000; Yu et al., 2002). We sought evidence for SHH signalling by in situ hybridisation for Shh, Ptc1 and Bmp4 in Tshz3lacZ/lacZ proximal ureters. Shh was expressed by E15.5 urothelia of both wild type and Tshz3lacZ/lacZ, and Ptc1 and Bmp4 were expressed in peri-urothelial cells in both genotypes (Fig. 7A-F). In addition, we analysed the phosphorylation of SMAD proteins considered as mediators of BMP signal transduction (Massague et al., 2005). At E14.5, similar levels of nuclear pSMAD1/5/8 protein were detected in periureteral mesenchymal cells in wild-type and Tshz3 mutant proximal ureters (Fig. 7G,H). Taken
together, these results indicate that Tshz3 mutant mesenchyme was directly responding to SHH and BMP4 signalling, in the same way as wild-type cells, even though they failed to form SMC.

To investigate whether addition of BMP4 could restore SMAA in absence of TSHZ3, we cultured renal tract rudiments with, or without, exogenous BMP4 protein (Fig. 8A). In wild types without added BMP4, both the proximal and distal ureter robustly expressed SMAA. Tshz3lacZ/lacZ explants cultured without exogenous BMP4 displayed little SMAA immunoreactivity in the proximal ureter, although some was expressed distally; furthermore, exogenous BMP4 did not ‘rescue’ SMAA expression (Fig. 8A). In other experiments, we tested whether exogenous BMP4 might alter the levels of transcripts for Tshz3, Myocd, and also Id3, a gene known to be upregulated by BMP4 (Shepherd et al., 2008) and expressed in embryonic renal tracts (Jen et al., 1996). As assessed by qPCR, expression of all three genes was significantly upregulated after exposure of explants to BMP4 (Fig. 8B).

**DISCUSSION**

We have shown that Tshz3 is expressed as early as E11.5 in the mesenchyme around the UB stalk, but that its function appears to be dispensable for ureter development until E14.5, when SMCs differentiation starts. Indeed, in the absence of TSHZ3, early events, such as proliferation and condensation of the undifferentiated mesenchymal cells around the distal UB, occur properly. However, the differentiation program triggered by the activation of SM factors, such as SMAA or SM22A, is impeded. Furthermore, our ex vivo data support the contention that hydronephrosis results from functional urinary flow impairment caused by defective ureteric contractility. Studies of (non-ureteric) mesenchymal cells suggest that SM differentiation depends on the ability of serum response factor to recruit its transcriptional co-activator myocardin (Pipes et al., 2006). We show that myocardin is expressed in wild-type ureteric SMC, validating the previously reported Myocd upregulation during mouse ureteric maturation (Mitchell et al., 2006). Our findings that, in the Tshz3 mutant, expression of myocardin is lost in SM precursor cells enables us to propose that TSHZ3 plays an important role in the induction of transcriptional programs that regulate SMCs differentiation.

**Tshz3 and radial patterning**

During ureter morphogenesis, Tshz3 was detected in the undifferentiated mesenchymal cells that contribute to the SM, the adventitia and the stromal layers. So far, very few genes have been implicated in the reciprocal signals that trigger the specification, proliferation and differentiation of epithelial and mesenchymal compartments. However, Shh, Bmp4 and Tbx18 appear to have crucial roles in the development of both compartments. SHH signalling is also required for establishing and/or maintaining the stromal cells, a mesenchymal cell population of undefined origin (Mahoney et al., 2006; Yu et al., 2002). In Tshz3 mutant ureters, expression of RALDH2 indicates
that differentiation of stromal cells occurs properly, suggesting that the sub-epithelial mesenchymal cells are SHH-responsive. Analysis of the Tshz3ΔlacZ,lacZ mutant ureters indicates that differentiation of the epithelium also occurs normally, and suggests that this cannot depend on the differentiation of the SMCs themselves, but rather on earlier events, as has been suggested by Bmp4 loss of function analyses (Brenner-Anantharam et al., 2007). Our data also show that TSHZ3 is dispensable for recruitment and condensation steps (see Fig. S2G,H in the supplementary material) before SM differentiation itself (Raatikainen-Ahokas et al., 2000). Therefore, the Tshz3 mutant provides a unique genetic tool in which stromal and urothelial differentiation, and also mesenchymal recruitment and condensation, are uncoupled from differentiation of SMCs.

In vivo, urothelial Shh was expressed as normal in mutants, which had overtly responsive adjacent mesenchymal cells, as shown by Ptc1 expression and indices of proliferation in the latter compartment. Furthermore, BMP4 signalling was initiated as normal in Tshz3 mutant ureters, as assessed by expression of Bmp4 and pSMAD1/5/8 in cells around ureteric urothelia. Nevertheless, Tshz3-null mutation specifically impaired the differentiation of SMC progenitors in proximal ureters in vivo, and, ex vivo, exogenous BMP4 treatment did not rescue Tshz3 mutant proximal ureter SMC differentiation, as assessed by SMAA expression. Before E12.5, when BMP4 signalling is essential for SMC differentiation (Brenner-Anantharam et al., 2007), TSHZ3 was expressed by SM progenitors and we showed that, in renal tract explants, Tshz3 expression was enhanced upon BMP4 treatment. These results support the idea that Tshz3 is downstream of BMP4 and might even be a direct target of BMP4 signalling.

Several studies suggest that BMP4 is not the only signal necessary for ureteric SM differentiation and it is possible that TSHZ3 is required for these other signals (Airik and Kispert, 2007; Chang et al., 2004; Miyazaki et al., 2003; Yu et al., 2002). The molecular mechanisms whereby developmental signals modulate the regulatory network for SM gene transcription warrants further studies. In vivo, loss of Tshz3 leads to the absence of myocardin and in vitro BMP4 stimulate Tshz3 and Myocd expression. This study suggests that Tshz3 could serve as a central transcriptional factor that integrates BMP4 signalling into the transcriptional regulatory network by controlling the expression of myocardin, a key factor for SM differentiation.

**Regionalisation of the ureter**

Tshz3 is evenly expressed in the mesenchyme along the entire proximodistal axis of the ureter. However, in the absence of Tshz3, SM markers were strongly downregulated in the proximal ureter and SM failed to develop, whereas in the distal part of the ureter, weak expression of SM proteins appears sufficient to produce functional SM. Therefore, our data show that the proximal and distal parts of the ureter respond differently to the absence of Tshz3, and hence show that the ureter is regionalised along its length. Temporal regionalisation of the ureter along the proximodistal axis is supported by the observation that SM differentiation occurs in a wave from the kidney to the bladder (Yu et al., 2002). However, no mesenchymal transcription factors differentially expressed along the ureter have been found, which could support a spatial regionalisation of this structure. According to its broad expression, it is unlikely that TSHZ3 alone differentially controls gene expression in a proximodistal gradient but it might well act as a co-factor of an essential regionalised factor or be recruited by a signalling pathway that acts locally. Therefore, our Tshz3-null mutant constitutes an excellent tool for the identification of such regionalised factors.

**Tshz3 mutant is a model for functional obstruction linked to SM impairment**

The lack of Tshz3 is associated with bilateral hydrenephrosis and proximal hydroureter, with an onset before birth. This mouse phenotype is reminiscent of human congenital PUJO, a common birth defect that is sometimes associated with significant kidney damage caused by urinary flow impairment (Decramer et al., 2006; Gunn et al., 1995; Imai et al., 2006). PUJO ureters are not anatomically blocked, but have aberrantly arranged SM in the region where the proximal ureter joins the renal pelvis (dell’Agnola et al., 1990; Zhang et al., 2000). In an intriguing parallel, we found that in Tshz3ΔlacZ,lacZ mice, SM differentiation was impaired in the proximal ureter. Thus, we postulate that the gross phenotypes of both Tshz3ΔlacZ,lacZ mice and human PUJO result from a failure of peristalsis in the region of the proximal ureter, which leads to ‘functional’ urine flow impairment. Recently, other mouse models for congenital hydrenephrosis with defects in SM have been generated. Mutants for Shh and Tbx18 affect both urothelium and SM. Loss of Dhh1 perturbs the orientation of the SMCs, causes a slight delay in SMC maturation, and causes stromal cell defects (Mahoney et al., 2006). Finally, inactivation of Cnb1, as well as A1t, causes obstruction after birth by affecting postnatal proliferation and maturation of pelvic SMCs (Chang et al., 2004; Miyazaki et al., 1998). The Tshz3 mutant will be useful for analysing prenatal functional kidney obstruction that results from incomplete SMC differentiation in the absence developmental defects of other ureteric cell populations. Mouse models have guided candidate gene screens for identification of mutations causing human urinary tract malformations (Jenkins and Woolf, 2007; Lu et al., 2007). We suggest that TSHZ3 should be examined as a candidate for congenital PUJO and related disorders, such as multicystic dysplastic kidney, a disorder characterised by severely disorganised ureteric and renal pelvic morphogenesis (Woolf et al., 2004).

The work was supported by the Biotechnology and Biological Sciences Research Council, the British Council, The Company of Biologists, EJope Alliance Program, the Association Française contre les Myopathies and Kids Kidney Research. We thank J. P. Chauvin for assistance with transmission electronic microscopy experiments, D. Rampley for performing Masson's trichrome staining and K. Mesbah for advice on immunohistochemistry. We thank J. Davies, A. Dimovski, Z. Guciev, T. Lecuit and V. Tasic for helpful discussions.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/19/3301/DC1

**References**


Table S1. Proliferation and apoptosis in embryonic proximal ureters

<table>
<thead>
<tr>
<th></th>
<th>E15 (+/+)</th>
<th>E15 (–/–)</th>
<th>E16 (+/+)</th>
<th>E16 (–/–)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urothelia</strong></td>
<td>13.2±4.3</td>
<td>9.92±6.0</td>
<td>11.7±4.8</td>
<td>6.3±0.62</td>
</tr>
<tr>
<td><strong>Aggregated</strong></td>
<td>7.9±3.8</td>
<td>4.3±1.9</td>
<td>2.1±1.5</td>
<td>2.3±0.64</td>
</tr>
<tr>
<td><strong>mesenchyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Loose mesenchyme</strong></td>
<td>9.5±1.3</td>
<td>11.3±2.9</td>
<td>6.4±3.3</td>
<td>4.9±1.3</td>
</tr>
<tr>
<td><strong>Apoptosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urothelia</strong></td>
<td>0.47±0.33</td>
<td>0.09±0.09</td>
<td>0.42±0.42</td>
<td>0.09±0.09</td>
</tr>
<tr>
<td><strong>Aggregated</strong></td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td><strong>mesenchyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Loose mesenchyme</strong></td>
<td>0.12±0.07</td>
<td>0.16±0.16</td>
<td>0.00±0.00</td>
<td>0.05±0.05</td>
</tr>
</tbody>
</table>

Point prevalence of proliferation (% PCNA-positive cells) and apoptosis (% TUNEL-positive cells) showed that the percentage point prevalence of positive nuclei in urothelia, condensed mesenchyme (containing presumptive SMCs) near the urothelium and looser mesenchyme outside this zone, was not significantly different in wild-type versus null mutant proximal ureters at both E15.5 (before the onset of hydronephrosis) and also at E16.5 (when hydronephrosis was present). Data are expressed as mean±s.e.m. with n=3 or 4 for each experimental group.