Hydroureternephrosis due to loss of Sox9-regulated smooth muscle cell differentiation of the ureteric mesenchyme

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INTRODUCTION

Congenital abnormalities of the ureter occur frequently in human newborns, affecting up to 1% of the population. They are diagnosed in a prenatal screen during obstetric sonography, and anatomically manifest in dilatation of the ureter (hydronephrosis), caused by an obstructive uropathy. Disease entities include ureteropelvic and vesicoureteral junction obstruction, megaureter, duplex ureter or vesicoureteral reflux, all of which are the most prevalent. In combination or alone they frequently lead to hydronephrosis, dilatation of the calyces and renal pelvis, causing renal injury or scarring and ultimately require kidney transplantation. The underlying genetic causes of this heterogeneous group of anomalies as well as their etiologies are largely unknown despite extensive genetic studies in human populations (1). However, genetic analysis in the mouse has started to reveal some of the cellular and molecular processes directing normal ureter development as well as disease mechanisms leading to obstructive uropathies (2). Ureter development in the mouse starts at embryonic day (E) 10.5 when an epithelial bud emerges from the Wolfian duct and invades the metanephric blastema, a mass of mesenchymal cells at the level of the hind limb buds. Loss of glial cell line-derived neurotrophic factor (GDNF), the mesenchymal signal that triggers ureter outgrowth, leads to kidney and ureter agenesis, whereas spatial deregulation of

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GDNF expression or signaling results in emergence of multiple ureters from which eventually blind-ending hydroureters arise (3). From E11.5 on, the proximal intra-renal portion of the ureter undergoes branching morphogenesis and gives rise to the pelvis and the collecting duct system of the adult kidney. Concurrently with branching, the ureter tips induce the surrounding mesenchyme to aggregate and epithelialize into renal vesicles from which nephrons mature. The distal ureter segment that is positioned outside the metanephros kidney. Concurrently with branching, the ureter tips induce (3). From E11.5 on, the proximal intra-renal portion of multiple ureters from which eventually blind-ending hydroureters GDNF expression or signaling results in emergence of multiple ureters from which eventually blind-ending hydroureters (4). From E14.5 on, the ureteric mesenchyme gives rise to smooth muscle (SM), stromal and pacemaker cells, whereas the ureteric epithelium differentiates to a highly specialized type of stratified epithelium, the urothelium. Failure of smooth muscle cell (SMC) differentiation and organization disrupts the rigidity and peristaltic activity of the ureter tube, again leading to obstructive uropathy. Id2 (5), Calcineurin (6), Shh (7), Dllg1 (8), Tshz3 (9) and Bmp4 (10) are among the factors that have been implicated in the cytodifferentiation of the ureteric mesenchyme. Formation of compliant junctions with renal pelvis and the bladder is crucial for the proper function of the ureter and require careful integration of the renal peristaltic conduction system with the proximal ureter on one end and a patent insertion of the distal ureter into bladder on the other end. The molecular regulation of the former is less understood, but is suggested to be regulated by angiotensin (11) and Bmp4 signaling (10). The latter occurs in the mouse between E13.5 and E14.5 through a process called maturation and is under the control of Ret signaling (12).

Sox9 encodes a transcription factor with a conserved SRY-related high mobility group DNA-binding domain (13). Sox9 is a critical regulator of chondrogenesis but requirement in the development of many other organs has been reported (14). Homozygous loss of Sox9 in mice leads to early embryonic lethality due to cardiac defects (15). Heterozygous Sox9 mutants die perinatally and serve as a model for campomelic dysplasia (CMPD; OMIM 114290), a human skeletal malformation syndrome with a high incidence of XY sex reversal (16). CMPD patients are also frequently affected from urinary tract anomalies including hydrourere, hydropnephrosis, renal hypoplasia and renal cysts (17). However, it has remained unclear whether these defects are secondary to skeletal anomalies or whether they reflect a primary requirement of Sox9 in the development of the ureter and/or the kidney. Evidence for the latter may be represented by the specific expression of Sox9 in the ureteric epithelium and mesenchyme of the E12.5 metanephric kidney (18). Here, we present genetic evidence for a primary requirement for Sox9 in the development of the ureteric mesenchyme in the mouse. Conditional inactivation of Sox9 from the ureteric mesenchyme results in proximal hydrourere associated with hydropnephrosis. Analyses of the underlying molecular changes suggest that Sox9 is required downstream of and in conjunction with Tbx18 for the timely and coordinated cytodifferentiation of the proximal ureteric mesenchyme into SMCs. Furthermore, gain-of-function experiments reveal the necessity for temporal restriction of Sox9 expression in the ureteric mesenchyme.

RESULTS

Expression of Sox9 during ureter development

Earlier work revealed expression of Sox9 in the ureter tips and the mesenchyme surrounding the distal ureter stalk of the developing mouse kidney at E12.5 (18). However, a detailed analysis of Sox9 expression during metanephric kidney development has not yet been reported. Whole mount and section RNA in situ hybridization analysis of E11.5 to E16.5 kidneys and ureters confirmed two independent expression domains of Sox9 during kidney organogenesis (Fig. 1). Throughout all stages analyzed, strong Sox9 expression was found in the tips of the ureteric epithelium (Fig. 1A–D). Mesenchymal expression, in contrast, was only transient. At E11.5, Sox9 expression was detected in a band of mesenchyme flanking the metanephrogenic mesenchyme on the medial side and a thin stripe of mesenchyme surrounding the Wolffian duct on the lateral side (Fig. 1A and E). At E12.5, mesenchymal expression was found in the ureter only, with a gradient from proximal to distal (Fig. 1B and F). Sox9 expression levels peaked in the ureteric mesenchyme at E13.5 (data not shown). Thereafter, Sox9 became quickly downregulated in this domain and was absent by E16.5 (Fig. 1C, D, G, H). Sox9 protein recapitulated the mRNA pattern, but protein expression was maintained slightly longer in the two compartments (Fig. 1I–L). The observed Sox9 expression pattern in the ureteric mesenchyme was strikingly similar to that of Tbx18, which we had reported on earlier (4). To determine the epistatic relationship between Tbx18 and Sox9, we examined the expression of Sox9 in Tbx18-deficient ureters at E11.5 and E12.5. We found loss of Sox9 expression from the Tbx18-deficient ureteric mesenchyme at both developmental stages (Fig. 1M and N), suggesting that Sox9 acts downstream of Tbx18 in the developing ureteric mesenchyme.

Conditional inactivation of Sox9 in the ureteric mesenchyme

Since homozygous loss of Sox9 causes embryonic lethality at E11.5 (19), we employed a conditional gene inactivation approach to address the function of Sox9 in the ureteric mesenchyme. Pax3-Cre transgenic mice that express Cre recombinase under the proximal 1.6 kb Pax3 promoter fragment (20), or Tbx18Cre-mice that have Cre inserted into the start codon of Tbx18 (21), were crossed with floxed Sox9 mice (22). Before that, the efficiency of Cre-mediated recombination was determined by mating Pax3-Cre or Tbx18Cre mice with Rosa26LacZ reporter mice. Analysis of β-galactosidase activity in Pax3-Cre+/Rosa26LacZ mice showed that recombination had occurred in the entire posterior trunk mesenchyme at E9.5 and in the intermediate mesoderm including the ureteric mesenchyme at E11.5 and E12.5 (Supplementary Material, Fig. S1A–C). In Tbx18Cre/+;
Rosa26LacZ/+ mice, β-galactosidase activity was observed in the nephrogenic cord at E10.5, and the prospective ureteric mesenchyme, a domain abutting the mesenchyme of the Wolffian duct and metanephric kidney at E11.5 (Supplementary Material, Fig. S1D–E) as expected from the Tbx18 expression pattern (4). Analysis at E12.5 confirmed that in both Cre lines, recombination occurred only in the mesenchymal compartment (Supplementary Material, Fig. S1C and F). To address the efficiency of Sox9 inactivation in the ureteric mesenchyme, we analyzed Sox9 expression at the mRNA and protein level in Pax3-Cre/+;Sox9flx/flx and Tbx18Cre+/+;Sox9flx/flx embryos (both genotypes are hereafter referred to as Sox9 mutants). Absence of Sox9 mRNA and Sox9 protein from the ureteric mesenchyme at E11.5 and E12.5, respectively, confirmed that both cre lines are equally well suited to study Sox9 function in this tissue (Supplementary Material, Fig. S1G–L).

Sox9 inactivation results in hydroureter and hydronephrosis

To investigate the effect of conditional Sox9 inactivation on ureter development, we analyzed Pax3-Cre/+;Sox9flx/flx and Tbx18Cre+/+;Sox9flx/flx fetuses at E18.5. External and skeletal examination of resulting Sox9-mutant embryos using both Cre lines invariably revealed severe morphological defects that manifested in a shortened body axis, rudimentary limbs and tail truncations (Supplementary Material, Fig. S2A, B, K, L). Closer inspection of the urinary system showed the presence of a dilated ureter section next to the ureteropelvic junction (UPJ) (Fig. 2A–C). However, depending on the Cre line used and the Sox9 genotype, we observed a phenotypic range that varied both in severity and penetrance (Table 1 and Supplementary Material, Fig. S2C–J, M–T). Significantly, we did not observe a sex bias with respect to the ureter phenotype. Inactivation of only one Sox9 allele resulted primarily in unilateral hydroureter formation with 25% penetrance irrespective of the Cre line used (Table 1 and Supplementary Material, Fig. S2C–E, M–O). Ablation of both Sox9 alleles in Pax3-Cre/+;Sox9flx/flx embryos increased the penetrance up to 63% and bilateral hydroureter was 7-fold more frequent. We noted a strong enhancement of this phenotype in Tbx18Cre+/+;Sox9flx/flx mice, characterized by more distended and caudally progressing hydroureter formation with complete...
penetration. As we have previously shown that heterozygous Tbx18 mice do not display hydroureter (4) and that the Pax3-Cre and Tbx18Cre lines equally efficiently delete Sox9 from the ureteric mesenchyme at E11.5 (Supplementary Material, Fig. S1G and J), this result suggests that Tbx18 and Sox9 genetically interact during ureter development.

In contrast to Tbx18-deficient embryos, loss of Sox9 did not affect ureter length (Fig. 2B and C). Seventy-two percent of the Pax3-Cre+/+;Sox9floxflox embryos displayed a prominent bladder distention (Fig. 2B, Supplementary Material, Fig. S2F–I), which was the most penetrant urinary system anomaly observed with this genotype (Table 1). The bladder phenotype occurred only rarely in Pax3-Cre+/+;Sox9floxfloxflox embryos and it was never observed in Tbx18Cre++;Sox9floxflox mice indicating that it is not secondary to hydroureter formation in Pax3-Cre++;Sox9floxflox mice but results from an independent requirement for Sox9 in bladder and/or urethral development. Support for the latter can be derived from the normal histological appearance of the SMC layer of the dilated bladder in E18.5 Pax3-Cre++;Sox9floxflox embryos and the absence of Sox9 expression from the bladder mesenchyme in the wild-type (Supplementary Fig. S3 and data not shown).

Histological examination of E18.5 kidneys and ureters (Fig. 2D–L) revealed the occurrence of hydropnephrosis, dilatation of renal pelvis, calyx and collecting ducts associated with the strongest hydroureter cases (Fig. 2E and F). In Sox9 mutant proximal ureters, an extreme thinning of the ureteric wall due to increased hydrostatic pressure was apparent (Fig. 2H and I). Outside the dilated region, mutant ureters appeared grossly normal, presenting a pseudo-stratified epithelium and a multi-layered mesenchymal compartment (Fig. 2J–L). To resolve whether the hydroureter in Sox9-mutant embryos resulted from physical or functional obstruction, we analyzed the continuity of the ureteric lumen by injecting ink into the renal pelvis. In all genotypes, the ink readily passed into the bladder (Fig. 2M–O) excluding physical barrier formation as a cause for obstruction. To further analyze the nature of functional ureter impairment, we cultured E15.5 ureter explants for 4 days monitoring daily for peristaltic activity and contraction patterns. Whereas wild-type ureters elongated extensively in culture and initiated unidirectional peristaltic contractions (Fig. 2P), Pax3-Cre/+; Sox9floxflox mutant ureters showed only mediocre elongation and weak peristaltic activity (Fig. 2Q), and Tbx18Cre++; Sox9floxflox ureters failed to extend and perform peristaltic contractions. Notably, Tbx18Cre++; Sox9floxflox ureteric mesenchyme was not condensed but lost its cohesive structure and dispersed onto the culture membrane (Fig. 2R and data not shown). Taken together, loss of Sox9 causes functional ureter obstruction leading to dilatation of the proximal ureter and the renal pelvis. Enhancement of the hydroureter phenotype by Tbx18Cre+ compared with Pax3-Cre-mediated Sox9 inactivation suggests that Tbx18 and Sox9 operate in the same pathway during ureter development.

**Smooth muscle cell deficiency in Sox9+/− ureters**

Reduction in peristaltic activity of Sox9-deficient ureters and hydroureter formation may be caused by structural or functional insufficiency of the SMC layer of the ureter. To test this hypothesis, we investigated differentiation of the ureteric mesenchyme at E18.5 by analysis of expression of a panel of marker genes including the SMC regulator myocardin (Myocd), as well as SM structural components cardiac troponin T2 (Tnnt2), transgelin (Tagln formerly Sm22a) and SM myosin heavy chain peptide 11 (Myh11). Expression of all of these genes was reduced in the proximal region of Sox9-deficient ureters derived from both Cre lines (Fig. 3A–L), whereas their expression was unaltered in the unaffected distal region. The grade of reduction correlated with the severity of the proximal ureter dilatation, being higher for Tbx18Cre+/-mediated than Pax3-Cre-mediated Sox9 inactivation. Deficiency of SMC differentiation was confirmed by antibody staining for Acta2 (actin, alpha 2, SM, aorta) and Myh11 at this stage (Fig. 3M–R). To further define the relation between SMC defects and the hydroureter phenotype of Sox9 mutants, we explanted ureters at E14.5 and E15.5, that is, before onset of urine production. Ureters cultured for 4–6 days exhibited a severely diminished mesenchymal coating and a reduced expression of SMC marker genes Myocd, Tnnt2 and Tagln proving that SMC defects occur independently of increased mechanical pressure in the proximal Sox9-deficient ureter (Supplementary Material, Fig. S4). Analysis of urothelial differentiation by immunohistochemistry against pan-Uroplakins showed that the urothelium matured normally in Sox9-deficient ureters (Fig. 3S–U). Taken together, these data suggest that deficiency in SM function underlies the reduction in peristaltic activity and hydroureter formation in Sox9-mutant mice.

**Late onset of ureter and kidney defects in Sox9-mutant embryos**

To define both the onset and the progression of ureter malformations in Sox9-mutant embryos, we analyzed ureters and

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**Table 1. Distribution and penetrance of urinary system defects in Sox9-mutant embryos at E18.5**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Unilateral hydroureter (%)</th>
<th>Bilateral hydroureter (%)</th>
<th>Bladder distension (%)</th>
<th>Penetrance (%)</th>
<th>Number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax3-Cre/+;Sox9floxflox</td>
<td>34</td>
<td>28</td>
<td>72</td>
<td>63/94*</td>
<td>32/129</td>
</tr>
<tr>
<td>Pax3-Cre/+;Sox9floxflox</td>
<td>25</td>
<td>4</td>
<td>5</td>
<td>30/32*</td>
<td>29/129</td>
</tr>
<tr>
<td>wt;Sox9floxflox</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>68/129</td>
</tr>
<tr>
<td>Tbx18Cre++;Sox9floxflox</td>
<td>6</td>
<td>94</td>
<td>–</td>
<td>100</td>
<td>20/71</td>
</tr>
<tr>
<td>Tbx18Cre++;Sox9floxflox</td>
<td>24</td>
<td>14</td>
<td>–</td>
<td>38</td>
<td>21/71</td>
</tr>
<tr>
<td>Tbx18Cre++;Sox9floxflox,Tbx18Cre++;Sox9floxflox</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>30/71</td>
</tr>
</tbody>
</table>

*Cumulative penetrance of hydroureter and bladder distension phenotypes.
and subsequently of Tnnt2 to distal wave. It is preceded by expression of teric mesenchyme starts at E15.5 and proceeds in a proximal Myocd E14.5 in the proximal ureter region. Expression of (around E15.5) (Fig.4A’–F’). SM differentiation of the ureteric mesenchyme (EM) genes during organogenesis, we studied whether it similarly controls the integrity of the ECM in developing ureters at E12.5. Indeed, collagen type II alpha 1 (Col2a1) expression was absent (Fig. 5A, E, I) and the expression of other ECM genes, not previously related to Sox9 function, versican (Vcan) and thrombospondin 1 (Thbs1) were strongly downregulated in the Sox9-deficient ureteric mesenchyme (Fig. 5B, F, J and C, G, K). Furthermore, expression of the gene encoding the cytosolic enzyme carbonic anhydrase 3 (Car3) was lost from the Sox9+/− ureters (Fig. 5D, H, L). Since Sox9 and these genes are co-expressed in the inner ring of condensed ureteric mesenchymal cells, their loss may reflect a cell-autonomous requirement for Sox9 and may contribute to the observed defects in SMC differentiation.

**Prolonged Sox9 expression in the ureteric mesenchyme causes cellular disorganization and increased deposition of ECM components**

Expression of Sox9 in the ureteric mesenchyme is downregulated shortly before the onset of SMC differentiation. To address the functional significance of this regulation and further analyze Sox9-regulated pathways, we prolonged Sox9 expression in the ureteric mesenchyme beyond E14.5 using a conditional Cre/loxP-based Sox9 misexpression approach. To this end, we generated an HprtSox9-allele by integrating a bicistronic transgene-cassette containing the mouse Sox9 ORF followed by IRES–GFP into the ubiquitously expressed Hypoxanthine guanine phosphoribosyl transferase (Hprt) locus (Supplementary Material, Fig. S8). To activate transgene expression, we used the Tbx18Cre mouse line (21). In male embryos, the transgene is expressed in a uniform manner, whereas random X-chromosome inactivation in females causes mosaicism. In contrast to the wild-type attached kidneys from E12.5 to E16.5. On the morphological level, differences between wild-type and Sox9-mutant urogenital systems were not apparent at E12.5 and at E14.5 (data not shown). Histological stainings of ureter sections at E12.5, E13.5 and E14.5 showed an inner layer of highly condensed cells and an outer layer of loose mesenchyme indistinguishable from the wild-type at these stages (Fig. 4A–F and Supplementary Material, Fig. S5A–I). Condensed cells appeared morphologically normal and were found in numbers identical to that of the control at E14.5 (Fig. 4A–F and Supplementary Material, Fig. S5J–L), indicating that initial condensation of the prospective ureteric mesenchyme around the ureter stalk was not affected by the loss of Sox9. Dilation of the proximal ureter and the pelvicaliceal space was first apparent at E16.5, i.e. shortly after onset of urine production in the kidney (around E15.5) (Fig. 4A’–F’). SM differentiation of the ureteric mesenchyme starts at E15.5 and proceeds in a proximal to distal wave. It is preceded by expression of Myocd at E14.5 in the proximal ureter region. Expression of Myocd, and subsequently of Tnnt2, Tagln and Myh11, was significantly reduced at E14.5 to E16.5 (Fig. 4G–R, G’–R’) indicating that activation of the SMC differentiation program is perturbed by the loss of Sox9.

**Changes of the extracellular matrix composition in Sox9-deficient ureters**

To address the underlying molecular causes of the SMC differentiation defect in Sox9-deficient ureters, we analyzed a panel of genes that have been associated with ureteric mesenchyme development. Secreted frizzled-related protein 2 (Sfrp2) and Tcf21 (Pod1) are markers for this tissue (4) without functional assignment to date, whereas Shh (7), Bmp4 (10), Tbx18 (4) and Tshz3 (9) are essential regulators of ureter development and control various aspects of SMC differentiation. Expression of all these genes was unchanged in Sox9-deficient ureters (Supplementary Material, Fig. S6). Furthermore, expression of Bmp-signaling mediators P-Smad1/5/8 and P-p38 was unaltered (Supplementary Material, Fig. S5), suggesting that SMC defects in Sox9-deficient ureters are not due to reduced expression of these factors. We were also unable to detect changes in cell proliferation or apoptosis that could underlie or be associated with hydroureter formation (Supplementary Material, Fig. S7).

Because Sox9 regulates the expression of several extracellular matrix (ECM) genes during organogenesis, we studied whether it similarly controls the integrity of the ECM in developing ureters at E12.5. Indeed, collagen type II alpha 1 (Col2a1) expression was absent (Fig. 5A, E, I) and the expression of other ECM genes, not previously related to Sox9 function, versican (Vcan) and thrombospondin 1 (Thbs1) were strongly downregulated in the Sox9-deficient ureteric mesenchyme (Fig. 5B, F, J and C, G, K). Furthermore, expression of the gene encoding the cytosolic enzyme carbonic anhydrase 3 (Car3) was lost from the Sox9+/− ureters (Fig. 5D, H, L). Since Sox9 and these genes are co-expressed in the inner ring of condensed ureteric mesenchymal cells, their loss may reflect a cell-autonomous requirement for Sox9 and may contribute to the observed defects in SMC differentiation.

**Figure 3.** Defects in SMC differentiation in Sox9-deficient ureters at E18.5. (A–R) Molecular characterization of ureters for expression of SM marker genes by RNA in situ hybridization analysis (A–L) and immunohistochemistry for SM proteins on transverse section through the proximal ureter sections. SMC but not urothelial differentiation is severely compromised in Sox9-deficient ureters. Genotypes and markers are as indicated. ue, ureteric epithelium; um, ureteric mesenchyme.
situation, Tbx18Cre+/+;HprtSox9/y (male) fetuses expressed Sox9 both at the mRNA and protein level in the ureteric mesenchyme at E18.5 confirming the suitability of the approach (Fig. 6A–D). All transgenic mice appeared morphologically unaffected and exhibited a grossly normal urogenital system (Supplementary Material, Fig. S9A and B). Histological analyses uncovered a disorganized SM layer that was, however, not associated with hydroureter formation. SMCs appeared more rounded and were separated by excessive deposition of glycosaminoglycans/mucopolysaccharides (Fig. 6E–H). Detection of the SM proteins Acta2, Myh11 and Tagln revealed normal cytodifferentiation (Fig. 6I–P), but confirmed severe disorganization of the SM layer.

In order to assess cytodifferentiation and the integrity of the ECM in the ureteric mesenchyme of Sox9 transgenic mice, we analyzed the expression of a variety of molecular markers. Car3 expression was, in agreement with its downregulation in Sox9 mutants, upregulated in Tbx18Cre+;HprtSox9/Y mice suggesting a direct transcriptional regulation by Sox9 (Fig. 6Q and R). Sfrp2, which was implicated in procollagen processing (23), was strongly downregulated supporting the notion of ECM alterations caused by constitutive Sox9 overexpression (Fig. 6 S and T). We failed to detect upregulation of the ECM genes Col1a1, Col2a1, Col9a1 or Acan (Supplementary Material, Fig S9C–J) that are transcriptionally activated by Sox9 in other tissue contexts. Expression of the ECM gene Vcan that was not found in the wild-type at this stage, and Thbs1 were markedly induced by ectopic Sox9 (Fig. 6U–X).

The disorganization of the SM layer suggested that also fibroblast layers in the ureter might also be affected. However, normal expression of Dermatopontin (Dpt) and Raldh2 that mark adventitial and lamina propria fibroblasts, respectively, demonstrated a normal concentric pattern of ureteric tissues (Supplementary Material, Fig. S9K–N).

Together, these results demonstrate that prolonged Sox9 expression affects normal ECM deposition in the ureteric mesenchyme, which could be the cause for the dispersion of SMCs in Tbx18Cre+/+;HprtSox9/Y mice. This finding complements the loss-of-function data and strongly argues that transient Sox9 expression in the early ureteric mesenchyme regulates ECM composition as a prerequisite for subsequent SMC differentiation.

Figure 4. Onset and progression of SMC differentiation is delayed in Sox9-deficient ureters. (A–F, A’–F’) Histological analysis by hematoxylin and eosin staining of sagittal kidney sections (A–C, A’–C’) and of transverse sections of the proximal ureter (D–F, D’–F’) at E14.5 and E16.5. Hydroureter/hydronephrosis in Sox9-deficient ureters occurs after onset of urine production at E16.5. (G–R, G’–R’) In situ hybridization analysis of SM marker gene expression in transverse sections through the proximal ureter region reveals a failure to initiate SMC differentiation at E14.5 (G–R) and compromised SMC differentiation at E16.5 (G’–R’). Markers, stages and genotypes are as indicated: k, kidney; p, pelvis; u, ureter; ue, ureteric epithelium; um, ureteric mesenchyme.
A primary requirement for *Sox9* in the differentiation of the ureteric mesenchyme

CMPD, a semi-lethal disorder that primarily affects the skeletal system, is caused by heterozygous mutations in and around *SOX9*. CMPD patients are also frequently affected by defects of the urinary tract including hydroureternephrosis, renal hypoplasia and renal cysts (17). Our analysis of conditional *Sox9*-mutant mice suggests that hydroureternephrosis in CMPD patients is not secondary to skeletal defects but is due to a primary requirement for *Sox9* in the differentiation of the ureteric mesenchyme. Notably, heterozygosity of *Sox9* in mice results in variably penetrant, unilateral hydroureter formation and therefore mimics the urinary phenotype of CMPD patients. Ureter dilation in *Sox9* mutants occurred mostly at the level of the UPJ, phenotypically resembling the UPJ obstructive nephropathy in humans, the most common clinical form of obstructive nephropathy. Biopsies of human patients with obstructive nephropathy show that the defective urinary tracts have pathological changes in both SMC arrangement and pyeloureteral innervation (24), and our analysis supports the notion that abnormal SM structure and function are causative in this disease. *Sox9*-deficient ureters show a delayed onset of SMC differentiation, reduced expression of SM structural genes and defects in myofilament structure. Peristalsis was severely compromised accounting for hydroureter formation shortly after onset of urine production in the kidney.

Our analysis did not uncover regionalization of cellular and/or molecular changes that may account for the proximal restriction of hydroureter formation in *Sox9*-deficient embryos. Although such changes may have remained undetected, it is also possible that harsher hydrodynamic conditions at the UPJ (e.g. higher hydrodynamic pressure) result in preferential proximal dilatation when the peristaltic and/or contractile urinary machinery is perturbed rather than completely absent. Evidence may also be derived from other mouse models [e.g. *Calcineurin* (6), *Shh* (7), *Dlgh1* (25), *Tshz3* (9) and *Bmp4*-deficient mice (10,26)] that to some—albeit clearly lower—degree feature enhanced proximal hydroureter formation. In all of these mutants, defects of SMC differentiation and organization have been observed, again pinpointing to the crucial importance of the SMC layer for ureter integrity.

Human urinary tract abnormalities are highly variable ranging from a weak unilateral proximal hydroureter to bilateral megaureters. Variability may reflect physiological parameters (e.g. in urine production) that differently interact with changes of tissue architecture and function (e.g. SMC integrity and peristaltic activity), and/or may reflect the strong dose-dependency of some gene products involved in ureter differentiation. Examples for the latter are provided by haploinsufficiency of *Bmp4* (26,27) and as shown in this study of *Sox9* in the development of the ureteric mesenchyme in both mouse and man. In any case, the *Sox9*-deficient mice...
presented in this study represent an excellent model for functional ureter obstruction in humans.

Expression and genetic analyses suggest additional functions of Sox9 in the urinary system. Sox9 is expressed in the tips of the collecting duct system as well as in the urethra (data not shown). Whereas the functional significance of the epithelial ureteric tip expression has not yet been explored, we detected a severe dilation of the bladder upon Sox9 inactivation in the entire posterior trunk mesenchyme. Since heterozygous mutants did not show this phenotype, urinary tract abnormalities of CMDP patients may primarily reflect the process of Sox9 in the ureter. Alternatively, different Sox9 gene dosage requirements in different tissues of mouse and human may exist.

The extracellular matrix may be critically involved in smooth muscle cell differentiation and function of the ureter

Differentiation of SMCs is accompanied by the transcriptional activation of muscle-specific genes that confer the unique contractile and physiological properties of this cell type. Most of the SM genes are controlled by serum response factor (SRF), a widely expressed transcription factor. Myocardin and myocardin-related transcription factors interact with SRF and stimulate SRF-dependent transcription, and myocardin has been shown to be sufficient and necessary for SMC differentiation (28). Although a functional requirement for myocardin or SRF in SMC differentiation in the ureter has not yet been demonstrated, activation of Myocd expression shortly before onset of ureteric SMC differentiation and absence of Myocd expression in Tshz3-mutant mice that show perturbed SM differentiation in the ureter strongly suggest a functional implication of Myocd in ureter SMC differentiation (9).

Bmp4 signaling in the undifferentiated ureteric mesenchyme is required for SM differentiation as well as for Tshz3 expression (29), suggesting that Bmp4 signaling triggers SM differentiation by activating Myocd. Furthermore, Bmp2/Smad signaling has been shown to activate Myocd protein levels and increase expression of Myocd target genes in cardiomyocytes (30), and Bmp4 signaling has been shown to recruit Myocd to regulate transcription of CArG box containing genes, such as Acta2, Tagln and Myh11 in SMCs (31). In Sox9-deficient and Sox9-overexpressing ureters, Bmp4, Tshz3 and Bmp signaling were not changed, arguing against the possibility that Sox9 acts as an upstream activator of this pathway. However, we cannot exclude the possibility that Sox9 acts as a necessary cofactor of P-Smad1,5,8 or Tshz3 protein in transcriptional activation of Myocd. Alternatively, Sox9 may be required indirectly by providing a permissive environment for Myocd activation.

Intriguingly, our analysis detected reduced expression of several genes encoding ECM components, including Col2a1, Thbs1 and Vcan as the earliest molecular changes in Sox9-deficient ureters. Col2a1 is a direct target of Sox9 in chondrocytes, (32). It forms homotrimers that provide tensile strength to the tissue. In addition to being a structural protein, it also regulates matrix composition, acting as a scaffold onto which other fibrous collagens, such as type IX and type XI collagens bind (33). Thrombospondin 1 (Thbs1) is a matricellular protein that regulates inflammatory responses, developmental remodeling and wound repair. Thbs1 is considered to be a major activator of transforming growth factor (TGF) β-signaling in vivo (34) and can directly interact with versican to modify inflammatory responses (35). Versican is involved in cell adhesion, migration and proliferation in several organs. Versican is suggested to be central in mediating mesenchymal condensation by interacting with cell surface receptors via its chondroitin sulfate chains (36). However, despite reduced Vcan expression in Sox9-deficient ureters, mesenchymal condensation was not affected, suggesting that this process is not governed by Sox9-regulated gene expression. This is in stark contrast to the role of Sox9 in pre-cartilage condensation (16), and thus we confirmed that none of the possibly redundant Sox family members (Sox8, Sox10, Sox5 and Sox6) was expressed in the developing ureter during critical embryonic stages (data not shown).

Although urogenital defects have not been reported for mice individual mutant for Col2a1 (37), Thbs1 (38) or Vcan (39), combined loss of these as well as other ECM proteins may result in hydroureter formation. In fact, deficiencies in ECM deposition are associated with hydroureter in humans (40). However, the underlying pathogenetic mechanisms remain enigmatic. We hypothesize that defects in ECM composition may lead to hydroureter formation through at least two mechanisms. First, structural inadequacy due to defective ECM composition undermines the ability of the ureter to resist the pressure exerted by the urine and results in dilation of the UPJ—the region closest to the pelvis. Second, functional incompetence due to defective ECM composition may lead to general failure to induce SMC differentiation, resulting in a lack of the active machinery to propel urine to the bladder. This may result from inadequate accumulation or bioavailability of secreted factors by the ECM or perturbed ECM signaling. Intriguingly, integrin signaling is instrumental in activating Myocd expression and thereby the SMC differentiation program in vascular SMCs (41). Vascular inactivation of integrin β1 subunit, Itgb1 resulted in perturbed SMC differentiation, vascular aneurisms and local distension of blood vessels.

Requirement for Sox9 function in the ureteric mesenchyme is restricted to a narrow time window. In fact, prolonged expression of this transcription factor in SMCs perturbs their structural organization. Increased deposition of glycosaminoglycans supports the ECM as primary component to be controlled by this transcriptional regulator.

How or whether loss of Car3 contributes to hydroureter formation in Sox9-deficient ureters remains enigmatic. Car3 is considered to have a very low enzymatic activity as a carbonic anhydrase and loss of Car3 does not have a phenotype in mice (42). Clearly, further functional studies are required to determine whether and how the ECM and Car3 influence SMC differentiation during ureter development.

**Tbx18 and Sox9 genetically interact during ureteric smooth muscle cell differentiation**

In Tbx18+/− ureters, prospective ureteric mesenchymal coating fails to aggregate around the ureter stalk but disperses onto the kidney. The ureteric mesenchymal coating is diminished,
elongation and cytodifferentiation of the ureter are perturbed and hydrourereter develops (4). The finding that Sox9 expression in the prospective ureteric mesenchyme of the E11.5 metanephric field depends on Tbx18 suggested that loss of Sox9 accounts for some aspects of the Tbx18 phenotype. However, in Sox9-deficient ureters, cohesive aggregation of mesenchymal cells around the distal ureteric epithelium and subsequent proliferation occur normally, excluding a pivotal role for Sox9 in mediating Tbx18 function in these early stages of ureterogenesis. Therefore, the failure of SMC differentiation in Sox9-deficient ureters is likely to be caused by a mechanism other than that in Tbx18-mutant mice. Our earlier work detected down-regulation of Shh- and Bmp-signaling in the Tbx18−/− ureteric mesenchyme, whereas these signaling pathways were unperturbed in Sox9-deficient ureters. As discussed above, we favor the idea that abnormal ECM formation and/or signaling are the underlying mechanism other than that in Tbx18-deficient ureters. As discussed above, we favor the idea that abnormal ECM formation and/or signaling are the underlying mechanism other than that in Tbx18-deficient ureters. Intriguingly, we observed that loss of Tbx18 increased the phenotypic severity of SM defects in Sox9-deficient ureters. As discussed above, we favor the idea that abnormal ECM formation and/or signaling are the underlying cause(s) for the SMC differentiation defect in the Sox9-deficient ureter. Intriguingly, we observed that loss of Tbx18 increased the phenotypic severity of SM defects in Sox9-deficient mutant mice. Although we cannot exclude the possibility that the interaction occurs on the protein level, we favor the idea that Sox9 and Tbx18 act in independent molecular pathways that converge on the activation of Myocd, which results in SMC differentiation, or at least provides a permissive environment for this process.

MATERIALS AND METHODS

Generation of the HprtSox9 allele

A ‘knock-in’ strategy into the X-chromosomal Hypoxanthine guanine phosphoribosyl transferase (Hprt) gene locus was designed to replace major parts of the Hprt exon 1 (including the ATG) by a cassette suited for Cre-mediated (mis-)expression as described previously (43). Homologous recombination in a functional Hprt null allele, allowing direct selection of successfully targeted ES cells by 6-Thioguanine. The targeting vector contained a 2.2 kb 5′-homology region, followed by the ubiquitously expressed CMV early enhancer/chicken β-actin (CAG) promoter, the conditional expression cassette (43) and a 5.1 kb 3′-homology region (Supplementary Material, Fig. S8). The open reading frame (ORF) of mouse Sox9 was first subcloned in the vector pSL1180 (GE-Healthcare), 5′ of an IRES–EGFP sequence and then shuttled as 5′-NheI–ORF–IRES–EGFP–MluI-3′ fragment into the MluI and Nhel sites of the targeting vector. This results in a reverse orientation of the ORF, relative to the CAG promoter, avoiding ‘leaky’ expression. After Cre-mediated ‘flipping’ and excision events between pairs of LoxP and LoxM sequences, the ORF locates in sense direction, directly downstream of the CAG promoter. The targeting vector was verified by sequencing before linearization and electroporation in Hprt-positive SV129 ES cells (maintained beforehand in HAT medium). A two-step selection protocol was employed, starting 24 h after electroporation with the addition of 100 μg/ml G418, followed by the addition of 1.67 mg/ml 6-Thioguanine (Sigma) after five additional days. Surviving colonies were expanded and genotyped by PCR (conditions are available upon request). To test the functionality of the expression cassette in candidate ES cell clones, the GFP epifluorescence was analyzed 6 days after electroporation with a Cre-expression plasmid (pCAG::turbo-cre, kind gift from Achim Gossler). Southern blot-confirmed ES cell clones (data not shown) were microinjected into CD1 mouse blastocysts. Chimeric males were obtained and mated to NMRI females, to produce heterozygous F1 females.

Mouse strains and husbandry

Sox9flox (Sox9tm1Gor) (22) mice were crossed with the Pax3-Cre transgenic (20) or with the Tbx18Cre (Tbx18tm4(cre)akis) knock-in (21) mouse line. Resulting Cre+/;Sox9lox/lox offspring was backcrossed to Sox9flox/lox mice to obtain Cre+/;Sox9flox/lox mice. Tbx18lacZ heterozygous mice (44) were mated to generate Tbx18 null embryos. For conditional misexpression experiments, females homozygous for the HprtSox9 allele were crossed with Tbx18Cre+/+ males. Wild-type embryos for Sox9 expression analysis were obtained from matings of NMRI (Charles River) wild-type outbred mice. HprtSox9, Tbx18lacZ, Pax3-Cre, Tbx18Cre Sox9lox/lox and Rosa26lacZ (Gt(Rosa)26Sor) (45) mice were kept on an NMRI outbred wild-type background. Wild-type or heterozygous littersmates were used as controls for mutant embryos. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR. Details on PCR protocols are available on request. H. Hedrich, state head of the animal facility at Medizinische Hochschule Hannover, approved the care of animals and experiments.

Collection of embryos

For timed pregnancies, plugs were checked in the morning after mating. Noon was designated E0.5. Embryos were dissected in PBS. Kidneys and urogenital systems were isolated and fixed in 4% paraformaldehyde/PBS overnight, dehydrated in methanol and stored at −20°C.

Ink injection experiments

To visualize the ureteropelvic lumen, Royal Blue ink (Pelikan) solution was injected into the pelvic region of kidneys of isolated whole urogenital systems using a pulled-out Pasteur glass pipette. Hydrostatic pressure was then applied to push the ink though the ureter to the bladder.

Organ cultures

Embryonic ureters were dissected and placed on 1 μm pore size polycarbonate membrane filters (Corning Inc., Corning, NY, USA). Explants on the filters were cultured at the air-medium interface in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (HyClone Laboratories, San Angelo, TX, USA) at 37°C with 5% CO2. Culture medium was replaced every 48 h and explants were examined daily for viability, morphology and peristaltic activity.
Histological and histochemical analyses

Skeletal preparations of newborns were performed as previously described (46). Kidneys for histological stainings were fixed in 4% paraformaldehyde or in Bouin’s fixative, paraffin embedded and sectioned to 5 or 10 μm. Sections were stained with hematoxylin and eosin or alcan blue. Histochemistry for β-galactosidase activity was carried out as described previously (47). X-gal-stained urogenital systems were paraffin embedded and sectioned to 10 μm.

For the preparation of semithin sections, ureters from Sox9+/− and control E13.5 embryos were removed and fixed with 1% glutaraldehyde in 4% PFA, and then left in the fixative overnight. Ureters were post-fixed in 2% osmium tetroxide for 30 min, dehydrated in a graded series of ethanol solutions and embedded in Epon. Epon blocks were cut in semithin (0.5 μm) sections and stained with methylene blue.

For the preparation of antigens on 5 μm paraffin sections, the following primary antibodies and dilutions were used. Polyclonal rabbit antisera against Myh11 (SMMHC, SM myosin heavy chain, a kind gift from R. Adelstein, Bethesda, USA, 1:200), Sox9 [a kind gift from Michael Wegner, Erlangen, Germany; (48), 1:500], Tgln (SM22a, Abcam, ab14106-100, 1:200), pan-uroplakin [a kind gift of T.-T. Sun, New York, USA, (49), 1:2000] and monoclonal mouse antibody against Acta2 [alpha SM actin, aSMA, clone 1A4; FITC-labeled (Sigma), unlabeled (NatuTec), 1:200]. Fluorescent staining was performed using fluorophore-coupled secondary antibodies (Rhodamine-Red-X-conjugated donkey-anti-mouse/goat-anti-rabbit, Dianova, 1:200) or Biotin-conjugated secondary antibodies (goat-anti-mouse/goat-anti-rabbit, Dianova, 1:200) and either Dylight488-conjugated-Streptavidin (1:500) or the TSA Tetramethylrhodamine amplification kit (PerkinElmer). Non-fluorescent staining was performed using goat anti-rabbit IgG-HRP secondary antibodies (Santa Cruz Biotechnology Inc., 1:200, for pan-uroplakin) and/or kits from Vector Laboratories (Vectastain ABC peroxidase kit (Rabbit IgG), Mouse-on-Mouse Kit, DAB substrate kit). Incubation with primary antibodies was performed at 4°C overnight after antigen retrieval (Antigen Unmasking Solution, Vector Laboratories, 15 min, 100°C), blocking of endogenous peroxidases with 3% H2O2/PBS for 10 min (required for DAB and TSA) and incubation in 2.5% normal goat serum in PBST or blocking solutions provided from the kits.

Cell proliferation rates in tissues of E12.5 and E14.5 wild-type and Sox9+/− embryos (three each) were investigated by the detection of incorporated BrdU on 5 μm paraffin sections according to the published protocols (44). For each specimen, 10 adjacent sections of the proximal ureter were assessed. The BrdU-labeling index was defined as the number of BrdU-positive nuclei relative to the total number of nuclei as detected by DAPI counterstaining in histologically defined regions. To determine the total number of cells in E14.5 sections, DAPI positive cells were counted on the BrdU stained specimens. For both assays, statistical analysis was performed using the two-tailed Student’s t-test. Data were expressed as mean ± standard deviation. Differences were considered significant when the P-value was below 0.05. Apoptosis in tissues was assessed by TUNEL assay using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon) on 5 μm paraffin sections. All sections were counterstained with DAPI.

RNA in situ hybridization analysis

Whole-mount RNA in situ hybridization was performed following a standard procedure with digoxigenin-labeled anti-sense riboprobes (50). Stained specimens were transferred in 80% glycerol prior to documentation. RNA in situ hybridization on 10 μm paraffin sections was done essentially as described (51).

Documentation

Whole-mount specimens were photographed on Leica M420 with Fujix digital camera HC-300Z, sections on Leica DM5000 B with Leica digital camera DFC300 FX. All images were processed in Adobe Photoshop CS.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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