Fstl1 Antagonizes BMP Signaling and Regulates Ureter Development

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Abstract

Bone morphogenetic protein (BMP) signaling pathway plays important roles in urinary tract development although the detailed regulation of its activity in this process remains unclear. Here we report that follistatin-like 1 (Fstl1), encoding a secreted extracellular glycoprotein, is expressed in developing ureter and antagonizes BMP signaling activity. Mouse embryos carrying disrupted Fstl1 gene displayed prominent hydroureter arising from proximal segment and ureterovesical junction defects. These defects were associated with significant reduction in ureteric epithelial cell proliferation at E15.5 and E16.5 as well as absence of subepithelial ureteral mesenchymal cells in the urinary tract at E16.5 and E18.5. At the molecular level, increased BMP signaling was found in Fstl1 deficient ureters, indicated by elevated pSmad1/5/8 activity. In vitro study also indicated that Fstl1 can directly bind to ALK6 which is specifically expressed in ureteric epithelial cells in developing ureter. Furthermore, Sonic hedgehog (SHH) signaling, which is crucial for differentiation of ureteral subepithelial cell proliferation, was also impaired in Fstl1+/− ureter. Altogether, our data suggest that Fstl1 is essential in maintaining normal ureter development by antagonizing BMP signaling.

Introduction

Congenital malformations of the kidney and urinary tract are the primary causes of renal failure in children and young adults [1] and frequently affect human infants. Many of these hereditary diseases display hydroureter and/or hydronephrosis with dilatation of the ureter and/or the renal pelvis, caused by failure to conduct urine from the renal pelvis to the bladder [2,3]. The underlying causes of these congenital malformations are still largely unknown.

Marine urinary tract development is a model that is broadly used to understand the underlying mechanism of human urinary tract malformations. On gestational day 10.5 (E10.5), ureteric bud, an epithelial outgrowth from Wolffian duct (WD), appears at the level of the future hind limbs. Then the ureteric bud invades an epithelial outgrowth from Wolffian duct (WD), appears at the level of the future hind limbs. Then the ureteric bud invades

CND absorption process are important to the final position of ureterovesical junction and distal ureter maturation.

During ureter development, the epithelial cells differentiate into the urothelium, while a layer of smooth muscle cells are differentiated from the condensed mesenchymal cells around the ureteric epithelium, and mediate peristalsis, conducting urine from the renal pelvis to bladder. In later stage, another kind of mesenchymal cells is differentiated between smooth muscle layer and epithelium in ureter, called subepithelial ureteral mesenchymal cells. Recent report revealed that Shh from ureteric epithelium is required for differentiation of subepithelial ureteral mesenchymal cells. Deletion of Shh in urothelium causes absent of subepithelial ureteral mesenchymal cells. The mutant mice display congenital renal hypoplasia, hydronephrosis and hydroureter phenotype at birth [8].

BMP signaling pathway is essential for many development processes. During ureter development, Bmp4 and Bmp7 are expressed in ureteral mesenchymal cells, while Rmp7 is expressed in ureteric epithelium [9]. Gene targeting approaches have uncovered some of their important roles during ureter development. Bmp7 deficient mice display a dysplastic kidney and hydroureter phenotype [10,11]. Mice heterozygous for a null mutation of Bmp4 display abnormalities that mimic human congenital anomalies of the kidney and urinary tract (CAKUT), suggesting that Bmp4 has important functions in the early...
development of urinary tract by inhibiting ectopic budding from WD or the ureter stalk [12]. At later stage, Bmp4 is reported to have multiple biological functions in urinary system development. For instance Bmp4 can act on the metanephric mesenchyme, prevents cell death and promotes expansion and migration of mesenchymal cells [13]. Fstl1 encodes a secreted extracellular glycoprotein that belongs to the BM/SPARC/osteonectin family, which contains an extracellular calcium-binding (EC) domain and a follistatin (FS)-like domain [14,15]. In zebrafish, morpholino knockdown of Fstl2 (the mouse Fstl1 homolog) results in a ventralized body axis [16]. Recently, Fstl1 is indicated to act as a BMP4 signaling antagonist in lung development [17]. Nevertheless, how Fstl1 affects BMP signaling or the function of Fstl1 during ureter development remains unclear.

Clues about Fstl1 function in the developing urinary system is suggested by its dynamic expression in the nephric duct and the nascent nephron epithilia of the developing kidney [18]. In this study, we report that Fstl1 knockout mice (Fstl1−/−) display a prominent hydroureter beginning at E16.0. Our histological and biochemical results suggest that Fstl1 has important roles in regulating ureteric epithelial cell proliferation, subepithelial ureteral mesenchymal cells differentiation and distal ureter maturation, mainly via regulating BMP and SHH signaling pathways.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Animal Care and Use Committee of Model Animal Research Center, Nanjing University, in strict accordance with the Guide for the Care and Use of Laboratory Animals, China. The relevant approved animal protocol (MARC-AP#: XG28) is entitled “Fstl1 antagonizes BMP signaling by regulating epithelial-mesenchymal interaction during ureter development”.

Mouse Strains

Fstl1 floxed mice (Fstl1flox/+ ) were generated by inserting two loxP sites into intron 1 and intron 2 respectively, followed by a neomycin cassette and a third loxP site in same orientation [19]. Heterozygous Fstl1 knockout mice (Fstl1+/−) were generated by crossing Fstl1flox/+ mice with EIIa-Cre mice (B6.FVB-Tg(EIIa-cre);C5379Lmgd/J, 003724), in which Cre recombinase expression occurs prior to implantation in the ureter wall [20]. Intercross of Fstl1−/− mice produced null mutant mice (Fstl1−/−) with exon 2 deletion. Pit1lacZ−/− mice (STOCK Ptkh1m1Jdg/J, 003081) were obtained from The Jackson Laboratory (Bar Harbor, ME). Fstl1 knockout mice (Fstl1−/−) as well as Fstl1+/−:Pit1lacZ−/− mice were kept at 129;B6 mixed background. All mice were maintained in an AAALAC accredited SPF facility in Model Animal Research Center of Nanjing University.

Histology and Immunohistochemistry

For histological staining, urinary system was fixed in 4% paraformaldehyde, paraffin-embedded, sectioned (6μm), and stained with hematoxylin-eosin. Immunohistochemistry was performed on paraffin sections following a standard protocol with antibodies listed below [21].

For X-gal staining of urinary systems, the whole urinary tract was fixed for 30min in fixative buffer (0.05 M NaPO4 buffer, PH 7.3, 1.8% formaldehyde, 0.02% NP-40, 2 mM MgCl2, 0.01% deoxycholate) on ice. After fixation, the urinary systems were washed 5 min in rinse buffer (0.05 M NaPO4 buffer, PH 7.3, 0.02% NP-40, 2 mM MgCl2) for three times and stained by immersion in X-gal staining solution (0.05 M NaPO4 buffer, PH 7.3, 2 mM MgCl2, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% X-gal) overnight at 37°C in the dark. The X-gal-stained urinary systems were rinsed with PBS, and post-fixed with 4% paraformaldehyde in PBS at 4°C. Then the samples were paraffin embedded and sectioned (10 μm). The sections were counterstained with nuclear fast red.

Antibodies

Antibodies and reagents used are as follows: goat anti-Fstl1 (R&D system, AF1738), goat anti-Smad1 (R&D system, AF2039), rabbit anti-phospho-Smad1/5/8 (Cell Signaling Technology, 9151), rabbit anti-Pax2 (Zymed, 71-6000), DAPI (Sigma-Aldrich, D9542), rabbit anti-pan-Cytokeratin (Santa Cruz Biotechnology, SC-15367), mouse anti-α-SMA (Neomarkers, MS-113-P1), goat anti-SM22 α (Abcam, ab10135), mouse anti-smMHC (Abcam, ab53219), mouse anti-phospho-AKT (Ser 473) (Cell Signaling Technology, 4051), rabbit anti-AKT (Cell Signaling Technology, 9272), mouse anti-β-actin (Sigma, A5441), mouse anti-GAPDH (Santa Cruz Biotechnology, SC-32233), mouse anti-c-Myc (Santa Cruz Biotechnology, SC-40), mouse anti-HA (Sigma-Aldrich, H3663), FITC-conjugated rabbit (Santa Cruz Biotechnology, F3262) and Cy5-conjugated rabbit (BioMea Corp., SJ29004) secondary antibodies and DAB (Maixin, KIT-9710), Goat anti-mouse IgG (Fc) (Pierce, 31439), Rabbit anti-Goat IgG (Sigma-Aldrich, A5420), and Goat anti-Rabbit IgG (Sigma-Aldrich, A9169).

Dye Injection

To visualize the urinary tract lumen, Bromophenol blue was injected into the pelvic region of the kidneys using a three dimensional manipulator (MN-153, NARISHIGE).

Analysis of Peristalsis

E18.5 kidneys and associated ureters were dissected from wild-type and Fstl1−/− embryos and were incubated in an in vitro culture chamber attached to an Olympus X71 inverted microscope. Ureter movements were observed and captured by digital camera of the microscope apparatus.

Proliferation and Apoptosis Analysis

To determine proliferative activity of the developing ureter, Timed-mated pregnant females were intraperitoneal injected with 10μl/g body weight BrdU (5mg/ml) (Sigma-Aldrich). E15.5 urinary systems were harvested 1 hour after injection, and E16.5 urinary systems were collected 2 hours after injection. For E15.5 embryos, a total of over 20 ureter sections from three embryos of each genotype were collected for quantification. For E16.5 embryos, a total of 20 ureter sections from the distal segment of ureter of each genotype were collected for quantification. Proliferative activity was examined after treatment with Fstl1 in culture: Wild-type E15.0 urinary system (kidney, ureter and bladder) was dissected and positioned on top of a culture plate insert (0.4 μm pore size, Millipore Corporation, Bedford, MA01730, USA) within an individual well of a 24-well tissue culture plate and cultured in mock or Fstl1-containing conditioned media for 16 hours, with 10 μM BrdU added during the last 4 hours of treatment [22]. A total of 7 sections from 3 pairs of cultured ureters were used for quantification. The BrdU-labeling index was defined as the number of BrdU-positive nuclei relative to total number of nuclei, which were counterstained with hematoxylin.

Apoptotic cells were detected using TUNEL assay that performed on 6μm paraffin sections using DeadEnd™ Fluoro-
metric TUNEL System (Promega, G3250). For both E15.5 and E16.5 embryos, a total of 6 ureter sections from two embryos of each genotype were analyzed. For E12.5 embryos, a total of 6 sections containing CND region from two embryos of each genotype were analyzed.

In Situ Hybridization

In situ hybridization was performed as previously described [23]. A 619 bp 3’ UTR fragment of Fstl1 cDNA was subcloned into the pBluescript II KS (-) (Stratagene) plasmid, which was used to generate an in situ hybridization probe (PCR primers are listed in Table S1). Pax3 probe was kindly provided by Dr. Yingzi Yang from NIH as a gift. Pax2 probe was provided by Dr. Yeguang Cheng (Tsinghua University, Beijing, China) as a gift. 3 embryos of each stage were used for Fstl1 expression examination. 4 embryos of each genotype were used for whole mount in situ hybridization using Pax2 and Pax3 probes.

Quantitative Real-Time PCR

The ureters were dissected and separated from the rest of the kidney. Two pairs of ureter were pooled into one sample. Over 4 groups of each genotype were used for real-time PCR for each gene. Total RNA from E16.5 ureter samples was isolated using the Qiagen RNeasy Mini kit (Qiagen). First strand cDNA was synthesized using AMV reverse transcriptase (Takara). Primers targeting specific transcripts were designed for real-time RT-PCR (SYBR). β-actin was used as internal control in each reaction. Quantitative real-time PCR was performed using an ABI PRISM 7700 (ABI) with conditions recommended by the manufacturer. Each reaction was performed triplicate. The quantity of each experimental sample is first determined using a standard curve based on their Ct values and then expressed relative to the internal control. The primers used to amplify each gene are listed in Table S1.

Cell Culture and Transfection

HEK-293 (CRL-1573TM, ATCC) cells were routinely cultured in DMEM supplemented with 10% FBS. Fstl1 coding sequence was subcloned into pcDNA3.1 (Invitrogen) vectors to express Fstl1 with (pcDNA3.1- Myc-Fstl1) or without Myc-tag (pcDNA3.1-Fstl1). HA tagged ALK3, ALK5 and ALK6 plasmids were provided by Dr. Yeguang Cheng (Tsinghua University, Beijing, China) as gifts. 3 embryos of each stage were used for Fstl1 expression examination. 4 embryos of each genotype were used for whole mount in situ hybridization using Pax2 and Pax3 probes.

Immunoprecipitation and Western Blot

For immunoprecipitation assay, HEK293 and COS7 cells were co-transfected with pcDNA3.1-Myc-Fstl1 and Bmp receptors expression plasmids HA-ALK3, ALK6 or ALK5. After transfection, the cells were cultured for 48 hours. Whole cell lysate was incubated with anti-c-Myc antibody, and rotated at 4°C for 6 hours. Then Protein A/G Agarose beads (Biyuntian Bio) were added and incubated overnight at 4°C with rotation.

Protein extracts (50µg/lane) were separated on discontinuous 10% SDS-PAGE gel followed by transfer to PVDF membrane (Amersham Biosciences), 100V for 1 hr. The transferred membrane was blocked in 5% skim milk in TBS (0.1% Tween-20 in TBS) buffer for 1 hr at RT, then immunoblotted with primary antibody overnight at 4°C. After four washes with TBS for 10 min each, the membrane was incubated with peroxidase-coupled secondary antibody for 1 hr at RT. After three washes in TBS for 10 min each, the immuno-reactive bands were visualized with a chemiluminescent substrate for peroxidase (Super Signal West Pico substrate, Pierce). For ureter samples, ureters were dissected and separated from the rest of the kidney. 10 pairs of ureters from each genotype were pooled together as one sample. The experiments from both E15.5 and E16.5 ureters were duplicated. The results on E18.5 kidney samples were confirmed with more than 4 litters of embryos.

Statistics

All results were presented as mean ± SEM. All statistical analyses were done using GraphPad Prism5 software. Two-tailed Student’s t tests were used for comparisons between two groups. *, p<0.05; **, p<0.01; ***, p<0.001. p<0.05 was considered significant.

Results

Fstl1 is Expressed in Developing Ureter

We examined Fstl1 expression in the developing ureter by immunohistochemistry (IHC) at E11.5 (Figure. 1A), E13.5 (Figure. 1C), E15.5 (Figure. 1D), E16.5 (Figure. 1E), and E17.5 (Figure. 1F). IHC result of E11.5 Fstl1 null embryo was presented as negative control (Figure. 1B). In developing ureter, Fstl1 protein was detected in both epithelial cells of ureteric bud and the surrounding mesenchymal cells (Figure. 1A) as early as E11.5. In later stages, Fstl1 was detected in the mesenchyme (inner and outer layers) and epithelium of the developing ureter at E13.5 (Figure. 1C), E15.5 (Figure. 1D), E16.5 (Figure. 1E), and E17.5 (Figure. 1F).

Since Fstl1 is a secreted glycoprotein, Fstl1 mRNA expression was also examined by in situ hybridization. Fstl1 mRNA was mainly produced in ureteral mesenchymal cells at E13.5 (Figure. S1A, B) and E15.5 (Figure. S1C, D). The difference of expression pattern of Fstl1 protein and RNA in ureter indicated that the Fstl1 protein found in the ureteric epithelium might be derived from the ureteral mesenchymal cells by diffusion.

Fstl1−/− Embryos Developed Congenital Hydrouretere and Hydronephrosis

To study the function of Fstl1 in development and pathophysiology, Fstl1 conventional knockout allele was generated by crossing heterozygous conditional knockout mice (Fstl1Δ/Δ) [19] with EIIa-Cre mice (B6.FVB-Tg (EIIa-cre) C5379Lmgd/J, 003724) [20].

Fstl1−/− mice died shortly after birth due to severe lung developmental defects [17,24]. We found Fstl1 mutant embryos also displayed profound hydroureter and hydronephrosis at birth. In order to define the onset and progression of urinary tract malformations in Fstl1−/− embryos, we analyzed urinary systems of wild-type and Fstl1−/− embryos from E13.0 to E17.5 (Figure. 2A, B, C). Morphologically, obvious ureter dilation was observed from E16.0 (Figure. 2B) in Fstl1−/− ureters, but was not detected at as early as E15.0 (Figure. 2A). The dilatation was more severe in the proximal region than distal part of the ureter. At E17.5, mutant urinary tracts displayed prominent hydroureter phenotype. The
mutant ureters were dilated, convoluted, fluid-filled and increased in length (Figure 2C). All these abnormalities occurred bilaterally and were fully penetrant in both male and female null mutants.

At the histological level, HE staining did not show any obvious differences between wild-type and \( \text{Fstl1}^{-/-} \) ureters in the proximal segment at E15.0 (Figure 2E, F). At E16.0, the ureter became dilated from the proximal fragment (Figure 2G, H). At E16.5, the urothelium of wild-type embryo was multilayered and surrounded by multiple layers of mesenchymal cells (Figure 2I). In contrast, the \( \text{Fstl1}^{-/-} \) ureter was enlarged with thinner layers of urothelium and mesenchyme (Figure 2J).

Kidney was also examined. Since \( \text{Fstl1} \) was highly expressed in collecting duct in kidney at E17.5 [18], Pax2 expression was examined as a collecting duct marker. From E15.5 to E17.5, Pax2 expression level in the collecting duct of mutant kidneys was normal (Figure S2). However, from these histological results, we found that \( \text{Fstl1} \) mutant kidney was reduced in size and displayed the dilatation of the pelvis and atrophy of papilla at E17.5, suggesting \( \text{Fstl1}^{-/-} \) kidneys exhibited hydronephrosis at this stage (Figure S2). Obvious enlargement of renal pelvis was not observed in \( \text{Fstl1} \) embryo at E15.5 (Figure S2A, B) and E16.5 (Figure S2C, D), therefore we speculate that hydronephrosis was possibly a secondary consequence of hydroureter.

Physical obstruction is a potential cause of ureter dilatation during urinary system development. To determine whether there was complete physical obstruction along the urinary tract in \( \text{Fstl1}^{-/-} \) mutant embryo, we injected dye directly into the pelvic region of E18.5 \( \text{Fstl1}^{-/-} \) kidneys. The purple dye could flow through the

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Figure 1. \text{Fstl1} protein expression in developing murine ureter. \text{Fstl1} immunohistochemistry analysis in sagittal sections of WT (A) and \( \text{Fstl1}^{-/-} \) ureteric bud (B, negative control for antibody) at E11.5 and transverse sections of ureters at E13.5 (C), E15.5 (D), E16.5 (E) and E17.5 (F). Note that \text{Fstl1} immunostaining was observed in the mesenchyme (um) as well as the ureteric epithelium (ue) from E15.5 to E17.5 (C-F). Scale bars: 40 \( \mu\)m. ub: ureter bud; m: metanephric mesenchyme; um: ureteral mesenchyme; ue: ureteric epithelium.
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Figure 2. Histological analysis of the \( \text{Fstl1}^{-/-} \) urinary system. (A-C) Kidneys and ureters from WT and \( \text{Fstl1}^{-/-} \) embryos at stages of E15.0 (A), E16.0 (B) and E17.5 (C). Arrows indicate mutant ureters, and arrowheads indicate wild-type ureters. (D) Dye injection experiments detected no complete physical obstruction of the \( \text{Fstl1}^{-/-} \) ureter at E18.5. (E-J) H&E staining on transverse sections of WT (E, G, I) and \( \text{Fstl1}^{-/-} \) (F, H, J) ureters at E15.0 (E, F), E16.0 (G, H) and E16.5 (I, J). (E, F) No obvious change was detected at E15.0 in \( \text{Fstl1}^{-/-} \) ureter. (G, H) \( \text{Fstl1}^{-/-} \) ureter became dilated from E16.0. (I, J) There are prominent changes in the histological structure of the \( \text{Fstl1}^{-/-} \) ureter compared to the WT ureter at E16.5. Scale bar: (E-J) 40 \( \mu\)m. k, kidney; p, pelvis; u, ureter; bl, bladder; um: ureteral mesenchyme; ue: ureteric epithelium.
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Defects of Distal Ureter and Ureterovesical Orifice in Fstl1<sup>−/−</sup> Embryos

Ureterovesical junction defects were observed in many hydrourteric mouse models [25,26] as well as human patients with CAKUT [1]. Previous report also revealed that Bmp4 heterozygote mutant embryos display ureterovesical junction defects with high penetrance [12]. Fstl1 is suggested to be a BMP signaling antagonist [17]. Therefore, we examined the ureterovesical orifice as well as the distal ureter of Fstl1<sup>−/−</sup> embryos. We found that the very distal segment of Fstl1<sup>−/−</sup> ureters was extremely narrow (Figure 3A, D, right, C, F) compared with wild-type (Figure 3A, D, left, B, E) ureter at E15.5 (Figure 3A, B, C) and E16.5 (Figure 3D, E, F). Histological analysis also revealed that distance between the two ureteral orifices is significantly shorter in Fstl1<sup>−/−</sup> embryos (Figure 2H, Figure 3B, D, I) than in wild-type (Figure 3G, Figure S3A, C, I) embryos at E15.5 (Figure 3G, H, Figure S3A, B, I) and E16.5 (Figure 3G, D, C) which was similar to the ureterovesical junction defects in Bmp4<sup>−/−</sup> embryo [12]. These results suggested that Fstl1 deficiency caused developmental defects in the distal part of ureter and ureterovesical junction.

During the development of urinary tract, ureteric bud first binds to the Wolffian duct, migrates to cloaca, and finally joins bladder. The abnormality of ureterovesical orifice could be caused by ectopic initial ureteric budding site or by defects of subsequent processes, such as the absorption of common nephric duct (CND) [7,12,25,27,28]. Therefore, we examined ureterovesical junction in earlier developmental stages. At E11.0, we investigated whether aberrant ureteral budding occurred. Wild-type (n = 4) and Fstl1<sup>−/−</sup> (n = 4) embryos were subjected to in situ hybridization using Pax2 and Pax3 antisense probes. Pax2 is expressed in the epithelial ureteric bud in the urinary system [29], while Pax3 is expressed in many tissues, including the somites [30]. Then we analyzed the embryos after in situ hybridization of both probes, and we found that both in wild-type (Figure 3I) and Fstl1<sup>−/−</sup> (Figure 3J) embryos, the position of the initial ureteric budding site aligned with the 28th somite. These observations demonstrated that position for initial ureteric bud outgrowth along the Wolffian duct was not affected by Fstl1 deficiency.

At E12.5, when the ureter still binds the WD, the CND is actively absorbed into the cloaca by apoptosis [7]. We thus examined the length and apoptotic level of the CND in Fstl1<sup>−/−</sup> embryos. At this stage, the length of CND was similar in both wild-type (Figure 3E) and Fstl1<sup>−/−</sup> (Figure 3F) embryos. The apoptosis of CND was detected by TUNEL assay. TUNEL positive nuclei were highly localized in the epithelium of the CND (Figure 3G, H). No obvious difference of CND apoptosis was detected between wild-type (Figure 3G, J) and Fstl1<sup>−/−</sup> (Figure 3H, J) embryos. These results suggested that the defects of ureterovesical junction and distal ureter in Fstl1 mutant embryo are not caused by ectopic ureteric budding site or inappropriate regression of CND.

Inactivation of Fstl1 Results in Defective Ureteric Epithelial Cell Proliferation and Differentiation

The hydroureter is often associated with cell proliferation and/or apoptosis defects during ureter development [8,13,31,32,33,34]. Beside the dilation of ureter at proximal part, we also found the distal ureter of Fstl1 mutant embryo is significantly narrower than that of wild-type embryo. Therefore, we examined proliferation and apoptosis in Fstl1<sup>−/−</sup> embryonic ureters. Very few cells in wild-type and Fstl1<sup>−/−</sup> ureters underwent apoptosis at E15.5 as determined by the TUNEL assay. There was no significant difference in ureteric epithelium and ureteral mesenchyme between wild-type and mutant embryos (Figure S4A, B). Similar results were obtained in E16.5 ureters (Figure S4C, D).

We examined proliferation of ureteric epithelium and ureteral mesenchyme by BrdU incorporation in E15.5 wild-type and Fstl1<sup>−/−</sup> ureters (Figure 4A, B, E). There was no change in the rate of mesenchymal cell proliferation (Figure 4E). On the contrary, proliferation of Fstl1<sup>−/−</sup> ureteric epithelium was significantly reduced at E15.5 (Figure 4E). At E16.5, the proximal segment of Fstl1<sup>−/−</sup> ureter was already dramatically dilated. Therefore, we analyzed the proliferation rates of distal segment of ureter, which was narrower in Fstl1<sup>−/−</sup> embryo compare to wild-type. Similar with E15.5, BrdU incorporation indicated that the proliferation of ureteric epithelial cell was significantly decreased in distal segment of Fstl1<sup>−/−</sup> ureter (Figure 4C, D, E), and the ureteral mesenchymal cell proliferation was not affected by Fstl1 deficiency (Figure 4C, D, E).

To further confirm the effect of Fstl1 on ureteric epithelium proliferation, we cultured wild-type E15.0 ureters with conditioned media from cells transfected with pcDNA3.1 vector (Mock) (Figure 4F) or Fstl1 expression plasmid (Figure 4G). The presence of Fstl1 in conditioned media was confirmed by western blot (Figure 4H). Proliferation of epithelial cells was significantly increased in wild-type ureters treated with Fstl1-containing media compared to control media, as quantified by BrdU incorporation (Figure 4I). Our results indicate that Fstl1 is required for maintaining normal ureteric epithelial cell proliferation during development.

To determine whether urothelium differentiation was affected in Fstl1 deficient mice, we examined the expression of Upk3a, a urothelium differentiation marker [35,36], in E15.5 and E16.5 ureters. Expression of Upk3a was down-regulated in Fstl1<sup>−/−</sup> ureters compared to wild-type at E15.5 and E16.5 as determined by real-time PCR (Figure 5), suggesting that urothelium differentiation is also impaired in the Fstl1<sup>−/−</sup> ureter.

Subepithelial Ureteral Mesenchymal Cells were Absent in Fstl1<sup>−/−</sup> Ureter

Previous studies have suggested that the hydroureter may result from defects in ureteral mesenchymal cell differentiation [8,31]. So we examined the expression of mesenchymal cell markers in the Fstl1<sup>−/−</sup> ureter. At E15.5, the expression level of smooth muscle differentiation markers α-SMA (Figure S6A, B), SM22α (Figure S6C, D), and smMHC (Figure S6E, F) were indistinguishable between wild-type (Figure S6A, C, E) and Fstl1<sup>−/−</sup> ureters (Figure S6B, D, F). Western blot assay also confirmed that the expression level of these three markers was not changed in Fstl1<sup>−/−</sup> ureter at E16.5 (Figure S6G).

To further examine the contractile function of ureter smooth muscle, E18.5 (Movie S1, S2) kidneys and associated ureters were isolated and cultured. Ureter movements were observed. We found that even in late stages that the morphology of mutant ureters dramatically changed, contraction of the smooth muscle seemed to be normal in Fstl1<sup>−/−</sup> embryos (Movie S2) compared with wild-type embryos (Movie S1).

A layer of α-SMA-negative mesenchymal cells was differentiated between the ureteric epithelium and the multilayer α-SMA-positive mesenchymal cells in later developmental stages [8]. These cells, referred to as the subepithelial ureteral mesenchymal cells, are also absent in Shh and Dhh1 mutants displaying hydroureter phenotype [8,37]. We traced differentiation of these
cells in both wild-type and Fstl1−/− ureters from E15.5 to E18.5, by double staining of epithelial cell marker pan-cytokeratin and smooth muscle cell marker α-SMA. At E15.5 and E16.0, we could not detect that type of cells negative for both of these markers in either wild-type (Figure S7A, C and Figure 5A, G) or Fstl1 mutant ureter (Figure S7B, D and Figure 5B, H). But from E16.5 on, subepithelial ureteral mesenchymal cells developed in wild-type ureter (Figure 5C, I, arrows) and became a thicker cell layer at E18.5 (Figure 5E, K, arrows). However, this layer of cells was always missing in the Fstl1−/− ureter at E16.5 (Figure 5D, J) and E18.5 (Figure 5F, L). We didn’t detect any cell that is negative for both markers in dilated Fstl1−/− ureters.

SHH signaling pathway is crucial for subepithelial ureteral mesenchymal cells differentiation and ureter development [8]. Therefore, we analyzed Shh expression in the developing ureter at E16.5. We found that Shh was down-regulated in the Fstl1−/− ureter as determined by real-time PCR (Figure 5N). X-gal staining signal was weaker in the Fstl1−/−;Ptch-lacZ−/− ureters (Figure 5O) than in the Fstl1+/+;Ptch-lacZ−/− ureters (Figure 5N). In E15.5 embryos, X-gal staining signal in Fstl1+/+;Ptch-lacZ−/− ureters was strong in subepithelial ureteral mesenchymal cells (Figure 5P, arrow), whereas no signal was detected in Fstl1−/−;Ptch-lacZ−/− littermates (Figure 5Q, arrow). These results confirm that subepithelial ureteral mesenchymal cells are absent, and that SHH signaling is down-regulated in Fstl1−/− ureters.

Fstl1 Deficiency Led to up-regulation of BMP Signaling in Developing Ureter and Kidney

Previous reports have suggested a correlation between Fstl1 and BMP signaling [16,17,39], and that BMP signaling is important for urinary system development [2]. We examined the effect of Fstl1 deficiency on BMP signaling in the urinary system. Smad1/5/8 phosphorylation levels in the ureter and kidney were analyzed by western blot (Figure 6A, first, second, and third panels). There was an increase of Smad1/5/8 activities in Fstl1−/− ureter at E15.5 (Figure 6A, left panels), and E16.5 (Figure 6A, second panels) compared to wild-type ureter. The increased Smad1/5/8 phosphorylation level was also detected in Fstl1−/− kidneys.

Figure 3. Defects of UV orifice and distal ureter in Fstl1−/− embryo. (A-F) Fstl1+/− mice were crossed to Ptch-lacZ+/− mice. Fstl1+/−; Ptch-lacZ+/− and Fstl1−/−; Ptch-lacZ−/− ureters were stained for β-galactosidase (blue) at E15.5 (A) and E16.5 (D). The Fstl1−/− and wild-type ureters were paraffin sectioned and stained with hematoxylin-eosin at E15.5 (distal segment) (B, C), E16.5 (distal segment) (E, F). Note that the Fstl1−/− ureter (A, D, C, F) is narrower than wild-type ureter (A, D, B, E) at both E15.5 and E16.5. The arrows indicated that lacZ staining was reduced in distal part of ureters in Fstl1−/− embryos compared to wild-type embryos. (G, H) Histological analysis of the ureterovesicle orifice at E15.5. Note that the distance between the left and right orifices (asterisk) is shorter in the Fstl1−/− embryo (H), compared with the wild-type embryo (G) at E15.5. (I, J) At E11.0, Pax2 and Pax3 whole mount in situ hybridizations were performed for wild-type (n = 4) (I), Fstl1−/− (n = 4) (J). The somites, which were labeled by the Pax3 in situ probe, in the caudal region of the embryo were numbered. The initial site of ureteric bud, which was labeled by Pax2 and indicated by a triangle, in both the wild-type (I) and Fstl1−/− (J) embryos aligned with the 28th somite level. Scale bar: (B-F) 40μm, (G, H) 100μm.

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proximal segment as well as ureterovesical junction defects. Furthermore, Fstl1 deficiency also results in down-regulated SHH signal, which in turn may affect subepithelial ureteral mesenchymal cells differentiation through mesenchymal-epithelial interaction (Figure 7).

The function of FSTL1 on regulating BMP signaling may be mediated at both receptor and ligand level. For receptors, the key issue is that FSTL1 interacts with different targets depending on specific cell types. In our previous paper analyzing lung phenotype of Fstl1 gene targeting mice, the Hep3B cell line was used mostly because of the endoderm tissue origin of lung epithelium [17]. For Hep3B cell line, FSTL1 can pull down BMPRII, but not BMPR1B (ALK6). In this study, we used HEK293 cell line which is derived from human embryonic kidney cells because we focused on ureter development. We found that only ALK6 can be co-precipitated with FSTL1 in HEK293. When we used another kidney derived cell line, COS7, both ALK6 and ALK3 can be co-precipitated. Nevertheless, Alk6 is specifically expressed in the ureteric epithelial cells in the urinary tract [12]. Consistent with the previous studies, our results suggest that Alk6 may be potential Fstl1 binding target in vivo that mediates its antagonizing effect during ureter development. It will be interesting, however, to elucidate the detailed mechanism how FSTL1 functions in vivo in different tissues in the future.

In addition, structure prediction and previous work in zebrafish, Xenopus and chick indicates that FSTL1 functions as a BMP antagonist similar to follistatin [16,39,42]. Because FSTL1 also binds to BMPs [17,43], it is possible that the interaction between FSTL1 and other TGF-β superfamily members may also contribute to the role of FSTL1 in vivo. Therefore, Fstl1 may have a broad inhibitory effect on BMP signaling pathway by targeting both ligands and receptors.

Our results are consistent with the previous suggestion that BMP signaling regulates ureter development. Bmp4 heterozygous mutants and Bmp7 mutants caused defects in urinary tract development. Bmp7 deficiency caused renal dysplasia and hydroureter phenotype [10,11], while Bmp4 heterozygous mutants exhibit multiple defects in urinary system, which is similar with human congenital anomalies of the kidney and urinary tract CAKUT [12]. Indirectly, Gata2 mutant animals resemble human congenital anomalies of the kidney and urinary tract as a result of reduction in BMP4 abundance [27,44]. These data indicated reduced BMP signaling is deleterious for urinary tract development. On the other hand, genetic inactivation of the BMP antagonist gremlin 1 (Grem1) leads to disruption of metanephric development at the stage of ureteric bud outgrowth initiation [45], indicating BMP antagonists play an essential role in negatively modulating the activity of BMP signals during early kidney development [45,46]. Our data also add more evidence that precise BMP signaling regulation is important in ureter development after ureteric bud initiation.

Relatively little is known about the mesenchymal-epithelial interaction in ureter development, compared to detailed studies that have focused on the ureteric buds interaction with its surrounding metanephric mesenchymal cells during kidney induction [4,47]. Recently, it was discovered that Fstl1 was a diffusible mesenchymal factor that determined the epithelium fate during oviduct development [48]. Coincidentally, both Fstl1 and Bmp4 are expressed in the ureteral mesenchyme [12]. Our model provides genetic evidence of the existence of signals, such as

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**Figure 5. Subepithelial ureteral mesenchymal cells were absent in the Fstl1**

(A-L) Immunofluorescence staining of α-SMA (green), pan-Cytokeratin (red), and DAPI (blue) of transverse sections from WT ureters at E16.0 (A, G), E16.5 (C, I) and E18.5 (E, K), and Fstl1**

**mutant**

ureters at E16.0 (B, H), E16.5 (D, J) and E18.5 (F, L). (G-L) Enlarged views of the boxed area in (A-F). The arrows indicate subepithelial ureteral mesenchymal cells which are negative for both markers. (M) Quantitative real-time PCR of Shh (H), E16.5 (D, J) and E18.5 (F, L). (G-L) Enlarged views of the boxed area in (A-F). The arrows indicate subepithelial ureteral mesenchymal cells which are negative for both markers. (M) Quantitative real-time PCR of Shh (H), E16.5 (D, J) and E18.5 (F, L). (G-L) Enlarged views of the boxed area in (A-F). The arrows indicate subepithelial ureteral mesenchymal cells which are negative for both markers.
BMP, from the ureteral mesenchymal layer that affect ureteric epithelium function.

Hydroureter is often associated with cell proliferation and/or apoptosis defects during ureter development in several animal models [8,12,13,31,33]. In most of these published mouse models, decreased ureteral mesenchymal cell proliferation causes impaired smooth muscle differentiation, finally resulting in a functional obstruction due to defective smooth muscle movement. Although Fstl1 mRNA is mainly expressed in the ureteral mesenchyme, there were no significant anomalies in proliferation, apoptosis, or differentiation of Fstl1 null ureteral mesenchyme. On the contrary, the impaired ureteric epithelial proliferation was observed and considered as cause of very narrow distal segment ureters, which makes it difficult for urine to go through, and contributes to hydroureter/hydronephrosis phenotype.

There is increasing evidence that BMP signaling modulates cell proliferation during development. For instance, overexpressing BMP4 in lung epithelium and hair follicles result in a reduction of proliferation in lung epithelium and the outer root sheath cells in transgenic mouse models [49,50]. Studies using in vitro models suggest that exogenous BMP4 inhibits epithelial cell proliferation and ductal budding in cultured urogenital sinus tissues [51]. Genetic inactivation of Fstl1 leads to an increased BMP signaling, especially in ureteric epithelium. Consistent with BMP signaling
activation, ureteric epithelial cell proliferation was reduced in Fstl1 mutant ureters at E15.5 and E16.5. However, the detail mechanism still needs more studies.

Besides impaired epithelial cell proliferation, Fstl1-/- ureter also displays defects in subepithelial ureteral mesenchymal cell differentiation. The absence of subepithelial ureteral mesenchymal cells in the Fstl1-/- ureter is similar with the phenotype in Shh mutant ureter. Deletion of Shh in the urothelium results in congenital obstructive phenotypes and the subepithelial ureteral mesenchymal cells is missing in this mouse model, suggesting SHH signal plays crucial role in inducing subepithelial ureteral mesenchymal cells differentiation [8]. In Dlgh1 mutant ureter, the subepithelial ureteral mesenchymal cells which are referred as Raldh2 positive ureteric stromal cells, are also absent. The authors further speculate that this population of cells might provide flexibility during the contraction and relaxation phases of peristalsis, so the missing of these cells might contribute to the hydroureter phenotype [37]. In our study, the time point that subepithelial ureteral mesenchymal cells differentiated and the time point of hydroureter phenotype in Fstl1-/- to display are also concomitant. Consistent with previous reports suggesting that SHH signaling is crucial for subepithelial ureteral mesenchymal cells differentiation [8], we also observed a down-regulation of SHH signaling in the Fstl1-/- ureter. Our results indicate that Fstl1 is required to maintain or establish the subepithelial ureteral mesenchymal cells through at least in part of SHH signal. However, the origin of this cell population is still largely unknown. One possible model is this cell population is derived from the ureteral mesenchymal cells. However, more studies are needed to elucidate the origin and the function of this specific ureteral cell population.

Although we observed that SHH signal is down-regulated in Fstl1-/- ureter, the hydroureter phenotype of Fstl1 ureter is more severe than Shh conditional mutant in ureteric epithelium. Fstl1 deficiency caused a down-regulated SHH signal as well as up-regulated BMP signal in urinary tract. Both signals play important regulatory roles in ureter development. The interactions between BMP and SHH signaling are important regulatory mechanisms in multiple developmental processes, including the neural tube patterning, tooth morphogenesis, hair follicle growth induction, limb bud formation, gut development, and left-right determination [52,53,54,55,56,57,58]. Many BMP antagonists regulate the interaction between these two signals [52,54,57]. During ureter development, previous reports indicate that SHH signaling could induce Bmp4 activation and promote ureteral mesenchyme proliferation [8]. Our results provide hints that BMP signal could negatively regulate SHH signal, and Fstl1 plays important roles to maintain the balance between these two signaling pathways.
However, detailed studies are required to elucidate the exact way that Fstl1 regulates SHH signal. Anyway, Fstl1 mutant mice provide a good model to study the mechanisms of the interaction between SHH and BMP signaling pathways to regulate cell proliferation and differentiation during ureter development and in congenital malformations of the urinary tract.

Supporting Information

Figure S1 Fstl1 mRNA expression in developing murine ureter. Fstl1 whole mount in situ hybridization of kidney and ureter at E13.5 (A, B) and E15.5 (C, D). In the cross sections of proximal segments of ureter (B, D), Fstl1 transcript was detected in ureteral mesenchymal cells (B, D, um), but not in ureteric epithelium at E15.5 (B, D, ue).

(TIF)

Figure S2 Fstl1-/- embryos developed congenital hydrourephrosis. (A-F) Immunohistochemistry of Pax2 in wild-type (A, C, E) and Fstl1-/- (B, D, F) kidneys at stages of E15.5 (A, B), E16.5 (C, D) and E17.5 (E, F). Note that the size and the collecting duct system of Fstl1-/- kidneys were not affected at E15.5 and E16.5 compared to those of the wild-types (A-D), whereas Fstl1-/- kidney at E17.5 showed hydrourephrosis and reduced size (F) compared to wild-type (E). Scale bar: (A-F) 400 μm.

(TIF)

Figure S3 Defects of UV orifice in Fstl1-/- embryo. (A-D) Fstl1-/− mice were crossed to Pth-lac−/− mice. Fstl1+/−; Pth-lac−/− and Fstl1−/−; Pth-lac−/− ureters were stained for β-galactosidase at E15.5 and E16.5. After sectioned and stained with hematoxylin, histological analysis of the ureterovesical orifice was performed at E15.5 (A, B) and E16.5 (C, D). Note that the distance between the left and right orifices (asterisk) is shorter in the Fstl1−/− embryo (B, D), compared with the wild-type embryo (A, C), (E-H) At E12.5, when the ureter still binds to WD, the length of the CND is similar in both wild-type (E) and Fstl1−/− (F) embryos. At E12.5, wild-type (G) and Fstl1−/− (H) CND showed no obvious differences in apoptosis detected by TUNEL assay. (I) Quantification of distance between two ureteral orifices at E15.5 (p<0.001, n=7). (J) Quantification of cell apoptosis in CND by TUNEL assay (p=0.29, n=7). Scale bar: (A-D) 100 μm, (E-H) 20 μm. CND: common nephric duct.

(TIF)

Figure S4 Apoptosis in E15.5 and E16.5 ureter. Wild-type (A, C) and Fstl1−/− (B, D) ureters at E15.5 (A, B) and E16.5 (C, D) showed no difference in apoptosis detected by TUNEL assay. Arrows point to representative cells positive for apoptosis. Scale bar: 20 μm.

(TIF)

Figure S5 Expression of Upk3a was down-regulated in Fstl1−/− ureter. Quantitative real-time PCR of Upk3a of E15.5 (n=6, p=0.04) and E16.5 (n=4, p=0.01) ureter.

(TIF)

Figure S6 Normal ureteral mesenchymal cell differentiation in Fstl1−/− ureter. (A-F) Expression of smooth muscle differentiation markers, α-SMA (A, B), α-SM22 (C, D) and smMHC (E, F) in transverse sections of Fstl1−/− ureters (B, D, F) shows no obvious difference compared with wild-type ureters (A, C, E) at E15.5. (G) Western blot analysis of smooth muscle differentiation markers. The expression of α-SMA, SM22a and smMHC were not altered in Fstl1−/− ureters at E16.5 compared to Wild-type control. Scale bar: (A-F) 20 μm.

(TIF)

Figure S7 Subepithelial mesenchymal cells are not detectable at E15.5. Co-Immunofluorescence staining of α-SMA (green), pan-Cytokeratin (red), and DAPI (blue) in transverse sections of WT ureters (A, C) and Fstl1−/− ureters at E15.5 (B, D), (C, D) Enlarged views of the boxed area in (A, B). Scale bar: (A, B) 50 μm, (C, D) 10 μm. um: ureteral mesenchyme; ue: ureteric epithelium.

Figure S8 Upregulation of phosphorylated Smad1/5/8 level in Fstl1−/− ureter. pSmad1/5/8 immunohistochemistry on transverse sections from WT (A, C, E) and Fstl1−/− (B, D, F) ureters at E15.5 (A, B), E16.5 (C, D) and E18.5 (E, F). Note that pSmad1/5/8 staining was stronger in the Fstl1−/− ureter (B, D, F). Scale bar: 40 μm.

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Figure S9 Normal TGF-β signal in Fstl1−/− kidney and ureter. Western blots of pSmad2 for E18.5 kidney protein (left panels), and E15.5 ureter protein (right panels).

(TIF)

Figure S10 Fstl1 can antagonize BMP4/BMP2-induced stimulation in vitro. (A) Western blots of pSmad1/5/8, Smad1, PAKT (Src170), AKT, GAPDH of HEK293 cells treated by adding BMP4 (20 ng/ml) and BMP2 (10 ng/ml) the conditional media transfected either by Fstl1 or pcDNA3.1 vector (Mock) for 30min. (B) Co-immunoprecipitation of Myc-Fstl1 and HA-tagged BMP type I receptors in COS7 cells. Myc-Fstl1 can be immunoprecipitated with the anti-c-Myc antibody. Note that both HA-ALK6 and HA-ALK3 were co-immunoprecipitated by the anti-c-Myc and detected by the anti-HA antibody (lane 4, 6).

(TIF)

Table S1 primers for in situ probe and real time PCR.

(DOC)

Movie S1 Ureteral peristalsis in a wild-type ureter.

(MP4)

Movie S2 Ureteral peristalsis in an Fstl1−/− ureter.

(MP4)

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Author Contributions

Conceived and designed the experiments: XG. Performed the experiments: JC. Analyzed the data: QJ. Contributed reagents/materials/analysis tools: QJ. Wrote the paper: QJ.

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