Midline-derived Shh regulates mesonephric tubule formation through the paraxial mesoderm

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Introduction

The vertebrate kidney is derived from the intermediate mesoderm (IM), which is a narrow strip of tissue located between the paraxial and lateral plate mesoderm (Schoenwolf, 2000). Depending upon the species, up to three separate kidney structures form in an anterior–posterior sequence during embryonic development (Saxen, 1987). The first to form, and most anterior, is the pronephros, which is the functional embryonic kidney in most fish and amphibians, and a transient embryonic anlage in amniotes. The mesonephros, which is the functional embryonic kidney in most species, up to three separate kidney structures form in an anterior–posterior sequence during embryonic development (Saxen, 1987). The pronephros, which is the last to form and is the most posterior, is specific to amniotes and becomes the definitive adult kidney (Saxen, 1987). The cranial domains are where a majority of nephrons along the nephrogenic cord vary across the animal kingdom.

In mice, the mesonephros consists of approximately 11 pairs of mesonephric tubules (MTs) and pretubular mesenchymal condensations extending from the level of somite 10–17, and has distinct cranial and caudal domains (Sainio, 2003; Vetter and Gibley, 1966). Cranial MTs are connected to the Wolffian duct (WD) at 4–6 sites, whereas the caudal MTs, which are the bulk of the mesonephros, are primitive, unbranched tubules that do not connect to the WD. The WD differentiates into the male reproductive tract including the epididymis. MTs in the cranial domain become the efferent duct connecting the epididymis and testis, whereas MTs in the caudal domain regress. MTs first appear as condensations of the nephrogenic cord.

During organogenesis, Sonic hedgehog (Shh) possesses dual functions: Shh emanating from midline structures regulates the positioning of bilateral structures at early stages, whereas organ-specific Shh locally regulates organ morphogenesis at later stages. The mesonephros is a transient embryonic kidney in amniotes, whereas it becomes definitive adult kidney in some anamniotes. Thus, elucidating the regulation of mesonephros formation has important implications for our understanding of kidney development and evolution. In Shh knockout (KO) mutant mice, the mesonephros was displaced towards the midline and ectopic mesonephric tubules (MTs) were present in the caudal mesonephros. Mesonephros-specific ablation of Shh in Hoxb7-Cre;Shhfl/fl and Sall1CreERT2;Shhflox/flox mice embryos indicated that Shh expressed in the mesonephros was not required for either the development of the mesonephros or the differentiation of the male reproductive tract. Moreover, stage-specific ablation of Shh in ShhCreERT2;Shhflox/flox mice showed that notochord- and/or floor plate-derived Shh were essential for the regulation of the number and position of MTs. Lineage analysis of hedgehog (Hh)-responsive cells, and analysis of gene expression in Shh KO embryos suggested that Shh regulated nephrogenic gene expression indirectly, possibly through effects on the paraxial mesoderm. These data demonstrate the essential role of midline-derived Shh in local tissue morphogenesis and differentiation.

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Nephrogenic cells undergo a mesenchymal-to-epithelial transition, forming a renal vesicle before elongating into an S-shaped body (Smith and Mackay, 1991). Lim1 and Pax2 are well-characterized markers of IM that also play essential roles in nephrogenesis (Bouchard et al., 2002; Torres et al., 1995; Tsang et al., 2000). In avian embryos, an unidentified paracrine signal from the paraxial mesoderm regulates Lim1 and Pax2 expression in IM, as well as the fates of IM progenitors (James and Schultheiss, 2003). Although the anatomy and gene expression patterns in the mesonephros are well characterized, the mechanisms that regulate MT patterning, including the role of early mesodermal tissue interactions, are poorly understood.

The growth factor Sonic hedgehog (Shh) plays crucial roles in embryogenesis. Although Shh knockout (KO) mice show kidney hypoplasia, deciphering the basis of this defect is complicated by the fusion of the paired kidney primordia at an early stage (Chiang et al., 1996; Hu et al., 2006), when Shh is expressed in the notochord, floor plate, and endodermal epithelium. Ablation of the notochord and floor plate using diphtheria toxin in mice displays no effect on nephrogenesis but it results in kidney fusion (Tripathi et al., 2010). In contrast, Shh expressed in the ureteric epithelium promotes ureteric mesenchymal cell proliferation and regulates the timing of differentiation of smooth muscle progenitors (Yu et al., 2002). Based on these reports, Shh appears to possess dual functions in kidney development: midline-derived Shh regulates the position of the bilateral kidney primordia, whereas Shh in the WD regulates kidney morphogenesis and growth. Although Shh is also expressed in the tubular epithelia of the WD and MTs (Little et al., 2007), and the Shh receptor, Ptpn1, and the intracellular signaling transducer, Gli1, are expressed in the mesonephric mesenchyme (Barsoum and Yao, 2011; Yao et al., 2002), the role of Shh in MT development has not been characterized. In this study, we examined the roles of Shh in the mesonephros as well as the notochord and floor plate using mouse genetic models. Mesonephros-specific KO of Shh provided no significant effects on the mesonephros or male reproductive tract development. In contrast, temporal analysis of Shh function using tamoxifen (TM)-inducible gene recombination system strongly indicated that midline-derived Shh was essential for the regulation of MT number. We also found that hedgehog (Hh)-responsive cells contributed to the paraxial mesodermal derivatives. Foxc1 and Foxc2 expression in the paraxial mesoderm were downregulated and the expression patterns of some nephrogenic gene in IM were disorganized. These data indicate an indirect role for midline-derived Shh in the patterning of nephrogenic tissue, possibly through the regulation of paraxial mesodermal genes.

Materials and methods

Mice

The mouse strains used were Shh (Chiang et al., 1996), Shh-creERT2 (Dassule et al., 2000), Shh-CreERT2 (Harfe et al., 2004), Gaxb-cre (Yu et al., 2002), Tef1-CreERT2 (Inoue et al., 2010), Gli1-CreERT2 (Ahn and Joyner, 2004), R26-LacZ (Soriano, 1999), and ICR (CLEA, Tokyo, Japan). Shh-creERT2, Shh-CreERT2 and Hoxb-cre mice were obtained from Jackson Laboratory. All experimental procedures and protocols were approved by the Animal Research Committee of the Wakayama Medical University and Kumamoto University. Embryos were collected from at least three pregnant females. Noon on the day when a vaginal plug was detected was designated as E0.5. The TM-inducible Cre recombinase system removes the floxed sequence in the target genome (Danielian et al., 1998; Feil et al., 1996, 1997). TM (Sigma, St. Louis, MO) was dissolved in sesame oil (Kanto Chemical, Tokyo, Japan) at a final concentration of 10 mg/ml and administered to pregnant female mice by intraperitoneal (ip) injection (2 mg per 40 g body weight). Under these conditions, there were no overt teratologic effects or disorders of the reproductive organs in control mice (Haraguchi et al., 2007; Miyagawa et al., 2009).

Whole-mount immunofluorescence imaging

Whole-mount immunofluorescence imaging was performed using anti-Pax2 antibody (Zymed, 1:200) according to standard procedures (Thompson et al., 2010). Embryos were washed thrice in PBSTx (PBS + 1% Triton X-100), blocked with 10% FBS/PBSTx for 90 min, followed by incubation with the primary antibody diluted in the blocking solution for 3 days. Embryos were washed thrice in PBSTx, incubated for 3 days in Alexa-488 secondary antibody (Molecular Probes; 1:200), washed in PBSTx, mounted in PBS containing 90% glycerol, and visualized using a fluorescence microscope (Biorreme, Keyence, Japan).

Histology and immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin, and histological analysis was performed as previously described (Haraguchi et al., 2000). Anti-Pax2 (Zymed, 1:200) and anti-Wt1 (Dako, 1:50) antibodies were used for immunostaining as previously described (Murashima et al., 2011).

Whole-mount and section in situ hybridization for gene expression

Whole-mount and section in situ hybridization was performed using digoxigenin-labeled probes as previously described (Haraguchi et al., 2007). Probes for the following genes were used: Shh (Choi et al., 2012), Gli1 (kindly provided by Dr. J. Motoyama), Hoxd4, Hoxd11 (kindly provided by Dr. D. Duboule), Fgf8 (kindly provided by Dr. B. L. Hogan), Gdnf, Ret (Nishinakamura et al., 2001), Lim1 (kindly provided by Dr. K. Furushima), Bmp4 (Jones et al., 1991), Noggin (McMahon et al., 1998), Foxc1 (Sasaki and Hogan, 1993), Foxc2 (Genepaint, RNA probe 2548) and Pax2 (Dressler et al., 1990).

Lineage analysis of Hh-responsive cells

Cell lineage analyses were conducted by whole-mount X-gal staining (Haraguchi et al., 2000) to detect LacZ activity in Gli1CreERT2+/− R26-LacZ+ males (Ahn and Joyner, 2004). The Gli1-CreERT2 mice were crossed with R26-LacZ indicator mice (Soriano, 1999) to obtain Gli1CreERT2+/− R26LacZ/LacZ males, which were subsequently crossed with ICR females. Time-mated ICR females were administered TM as mentioned above.

Results

Shh KO mice embryos display ectopic MTs and mesonephric mesenchymal hypoplasia

To determine the effects of Shh on mesonephric development, we first examined Shh KO mice. Pax2 expression in the WD and mesonephric mesenchyme was similar in the control and Shh KO embryos at E9.5 (Supplemental Fig. S1). At E10.5 in control embryos, Pax2 expression was strong in the MTs forming within the cranial mesenchyme (Fig. 1A) but was weak and sporadic in the caudal mesenchyme along the WD (Fig. 1A, E and G). In contrast, strong and continuous Pax2 expression was observed along the cranio-caudal axis in the caudal mesenchyme of Shh KO embryos, indicating the formation of ectopic MTs in the caudal domain (Fig. 1B, F and H, arrows). In the caudal mesonephros, Pax2 and Wt1 were co-expressed in the condensed mesenchyme in control embryos, whereas in Shh KO embryos, Pax2 expression was...
Fig. 1. Shh KO embryos showing abnormalities in the mesonephros. Whole-mount immunofluorescence staining of Pax2 at E10.5 (A and B) and E13.5 (K and L). Histological appearances of the mesonephros at E10.5 (C–F), E13.5 (M–P) and E15.5 (Q and R). Ectopic MTs are visible in the Shh KO mesonephros (arrows). Reduction in the mesenchymal mass surrounding the WD is prominent in Shh KO embryos at E13.5 and E15.5 (N, P, and R). (G and H) Immunofluorescence of Pax2 (red) and Wt1 (green) in sections of caudal mesonephros at E10.5. Most of the MT cells in the control embryos are positive for Pax2 and Wt1 (G), whereas MTs in the Shh KO embryos are positive for Pax2 but not for Wt1 (H). Nuclei were stained with Hoechst 33342. (I) A scheme indicating the axial levels of the cranial and caudal embryos at E10.5. Scale bars: A, B, K, L = 0.5 mm; C–H, M–P = 50 μm; Q and R = 100 μm. Arrows, ectopic MTs; arrowheads, WDs; g, gonad.
upregulated in the condensed mesenchyme and Wt1 expression was absent in the ectopic MTs (Fig. 1G and H). The upregulation of Pax2 expression and the downregulation of Wt1 expression resembled the gene expression pattern normally observed in cranial MTs (Fig. 1C and D, data not shown). This suggests that tubule maturation in ectopic MTs in the caudal mesonephros of Shh KO embryos may be similar to cranial MTs.

Pax2 expression in whole-mount Shh KO embryos at E13.5 revealed further development of ectopic MTs in the caudal mesonephros (Fig. 1I). In Shh KO embryos, the gonad and WD located more medial than those in control embryos (Fig. 1K and L), as previously described (Chiang et al., 1996). It is noteworthy that the WDS in Shh KO embryos were formed medial to the gonad, and that the MTs extended toward the midline, on the opposite side of the gonad (Fig. 1L). Formation of the ectopic MTs was also confirmed by staining histological sections (Fig. 1M–P). The ectopic MTs in Shh KO embryos underwent partial involution at E15.5, and there was less mesenchyme surrounding the WD (Fig. 1Q and K). The mesonephros in Shh KO embryos almost regressed at E18.5 and the male reproductive tract did not develop (data not shown).

These results indicate that Shh regulates the number of MTs in the caudal mesonephros, and maintains the mesonephros to develop into the male reproductive tract.

Shh expression in WD and MTs is dispensable for mesonephros or reproductive tract development

Shh is expressed in the epithelia of WD and MT throughout the development of mesonephros (Little et al., 2007). To determine the source of Shh required for normal MT patterning, we first eliminated Shh in WD using Hoxb7-Cre mice, which show the target gene recombination in the WD epithelia at E9.5 (Yu et al., 2002). Significant reduction of functional Shh expression in WD was confirmed by quantitative RT-PCR at E13.5 (data not shown). The mesonephros structure and male reproductive tract were unaffected throughout all fetal stages in these WD-specific Shh KO mice (Fig. 2A–D), and mesenchymal cell proliferation and smooth muscle cell differentiation were similar to the control (Fig. 2E and F, data not shown). Next, we introduced mutation in Shh in MT epithelia using TM-inducible Sall1-CreERT2 embryos. After administration of TM to Sall1-CreERT2 KO mice (Fig. 2).

Fig. 2. Phenotypic consequence of tissue specific Shh mutation. (A and B) The structure of WD and MTs are marked with Pax2 expression in control and WD-specific Shh KO embryos at E13.5. (C and D) Transverse sections of epididymis in control and WD-specific Shh KO embryos at E18.5. (E and F) Immunofluorescence staining to detect smooth muscle actin (SMA; green) in the epididymis of control and WD-specific Shh KO embryos. Nuclei were stained with Hoechst 33342. (G and H) Targeted recombination driven by Sall1-CreERT2 driver mice was assessed with the R26-LacZ reporter. TM was administrated at E10.5, and LacZ activity was assessed at E14.5 and E15.5. Sections were counterstained with eosin. (I and J) The structure of WD and MTs marked with Pax2 expression in control and MT-specific Shh KO embryos. (K and L) Histological analysis of MT-specific Shh KO embryos at E13.5, showing minor abnormalities in WD and MTs. Scale bar in A, B, G, I and J = 0.2 mm; C–F, K, L = 100 μm; ep, epididymis; ed, efferent duct; white and black arrows, WDs; red arrows, Mullerian ducts; arrowheads, MTs; k, kidney; g, gonad.
Fig. 3. Temporal regulation of MT development by Shh. (A–C) Gross anatomical features of the urogenital tract in the control and Shh\textsuperscript{CreERT2/lox} embryos. TM administration at E8.5 results in medial displacement of the gonad and mesonephros in the mutant embryos (B) compared with control embryos (A) and TM-induced embryos at E9.5 (C). (D and E) Whole-mount in situ hybridization to detect Pax2 expression in the reproductive tract of control and mutant embryos at E13.5, following TM administration at E8.5. Hyperplastic MTs are visible in the mutant mesonephros (E, arrowheads). (F–H) Histological analysis of the mesonephros in control and Shh\textsuperscript{CreERT2/lox} embryos. Ectopic MTs were visible in the caudal mesonephros of mutant embryos treated with TM at E8.5 (G) but not in control embryos (F), or mutant embryos treated with TM at E9.5 (H). (I and J) Gross abnormalities of the male reproductive tract in Shh\textsuperscript{CreERT2/lox} embryos at E18.5. Note the presence of disorganized and ectopic efferent ducts in the mutant treated with TM at E8.5 (J, arrowheads) compared with the controls (I). Arrowheads, ectopic MTs; arrows, WDs; t, testis; g, gonad; k, kidney. Scale bars: A–E, I and J = 0.5 mm; F–H = 100 μm.
R26LacZ/+ mice at E10.5, LacZ activity was observed in MT epithelia and, to a lesser extent, in the WD at E14.5 (Fig. 2G and H). In Sall1CreERT2/+;Shhfl/fl embryos, TM-induced recombination at E10.5 displayed no significant effects on mesonephric or male reproductive tract development (Fig. 2I–L). Significant reduction of functional Shh expression in MT was confirmed by quantitative RT-PCR at E13.5 (data not shown). These results indicate that Shh in mesonephric tissue was not required for mesonephros or male reproductive tract development.

The time window of Shh mutation for inducing ectopic MT development

Since tissue-specific ablation of Shh in the WD or MTs did not recapitulate the MT phenotypes observed in Shh KO embryos, we used ShhCreERT2/flox mice to determine the temporal requirement for the effect of Shh on ectopic MT development. In this genetic background, gene recombination is detected from 6–12 h after TM injection and lasts for 36 h (Joyner and Zervas, 2006). TM injection at E9.5, when WD reaches the urogenital sinus and MTs are present in IM, did not induce any major abnormalities in the gonad or mesonephros at E13.5 (Fig. 3A, C, F and H). In contrast, TM injection at E8.5, when the WD begins to form, led to the formation of ectopic MTs in the caudal mesonephros at E11.5 and E13.5, as is observed in the Shh KO embryos (Fig. 3D–G, arrowheads, Supplemental Fig. S2). Abnormal positioning of the mesonephros and gonads closer to the midline was observed at E13.5 of the mutant (Fig. 3B). Moreover, efferent duct of the mutant was hyperplastic at E18.5 (Fig. 3I and J, arrowheads). However, there were no obvious effects on mesenchymal mass or maintenance of the male reproductive tract (Fig. 3G and J). These results indicate that Shh expression before the onset of MT formation is required for the development of proper MT number at later stages.

TM treatment of embryos carrying the ShhCreERT2/flox allele at E9.5 and E8.5 resulted in the ablation of Shh expression in both the notochord and floor plate (Choi and Harfe, 2011; Choi et al., 2012). We confirmed gene recombination in this allele crossing R26LacZ/LacZ female to ShhCreERT2/+ males. After administration of TM at E8.5, embryos were harvested at E11.5 and examined for LacZ expression. The gene recombination was predominantly observed in the notochord and floor plate, but was rare in the epithelia of WD and MT (Supplemental Fig. S3A). At E10.5, Shh expression was detected in the notochord and floor plate of control embryos but was barely detectable in the ShhCreERT2/flox embryos. (Supplemental Fig. S3B and C). These results suggest that Shh from midline structures between E8.5 and 10.0 is essential for the development of the proper number of MT.

Hh-responsive cells are observed in the neural tube, splanchnic mesoderm and paraxial mesoderm, but are absent from the IM

Shh signaling plays crucial roles during patterning of the neural tube and mediolateral patterning of the somite in the early embryo

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**Fig. 4.** Hh-responsive cells and their lineage analyses. (A and B) Section in situ hybridization of Gli1 in E9.5 embryos at the craniocaudal levels indicated in (C). Gli1 expression is reduced in the sclerotome of Shh KO embryos. (D and E) X-gal staining patterns in Gli1CreERT2/+;R26LacZ/+ embryos treated with TM at E8.5, to detect the descendants of Hh-responsive cells at E12.5 (D) and E14.5 (E). Scale bars: A–B = 50 μm; IM; circled line, WD; pxm, paraxial mesoderm; spl, splanchnic mesoderm; da, dorsal aorta; nc, notochord; nt, neural tube; g, gonad; arrowheads, MTs; arrow, WD.
Genetic analysis using the TM-inducible Cre system indicates that Shh is essential for patterning during early stages, when it is expressed in the notochord, floor plate of the neural tube and endodermal epithelia (Choi et al., 2012). Gli1 expression is a readout of transcriptional activity in response to Hh signaling (Ahn and Joyner, 2004). We assayed Gli1 expression to identify candidate

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**Fig. 5.** Anterior–posterior patterning of IM in Shh KO embryos. Whole-mount in situ hybridization to detect Hoxd4 (A and B), Hoxd11 (C and D), Gdnf (E and F), Ret (G and H), and Fgf8 (I and J) in control (A, C, E, G, and I) and Shh KO (B, D, F, H, and J) embryos at E10.5. Arrows indicate the metanephric field. Arrowheads indicate ectopic expression in the mesonephric field. Scale bar = 0.5 mm.
target tissues of midline-derived Shh involved in regulating MT number. In control embryos at E9.5, Gli1 was expressed throughout the ventral neural tube, paraxial mesoderm and splanchnic mesoderm, but was absent in IM (Fig. 4A). In Shh KO embryos at E9.5, Gli1 expression in the neural tube and paraxial mesoderm was dramatically reduced but was still detected in the splanchnic mesoderm,

**Fig. 6.** Foxc1/2 and early IM marker expression in Shh KO embryos. (A and B) Whole-mount in situ hybridization of Foxc2 expression in control and Shh KO embryos at E9.5. The expression in the anterior trunk mesoderm is downregulated in Shh KO embryos relative to control embryos. (C–L) The expression of Foxc2 (C and D), Foxc1 (E and F), Bmp4 (G and H), Noggin (I and J), Lim1 (K and L) in the sections of control (C, E, G, I, and K) and Shh KO (D, F, H, J, and L) embryos at the craniocaudal levels indicated in (A) and (B). Scale bars: A–B=0.5 mm; C–L=50 μm. Arrow, notochord; arrowhead, WD.
indicating that Shh was not required for Hh signaling in the splanchnic mesoderm (Fig. 4B). We also performed lineage analysis of Hh-responsive cells using \( \text{Gli}^{\text{CreERT2};/;\text{R26loxGFP}} \) mice. After TM injection at E8.5, LacZ-expressing cells were detected in the sclerotome, but were rare in the mesonephric tissues at E12.5 and E14.5 (Fig. 4D and E). These results suggest that the effect of midline-derived Shh on IM is indirect, consistent with previous reports, indicating that the neural tube and notochord indirectly regulate IM induction through their effects on the paraxial mesoderm (Groebstein, 1955; Mauch et al., 2000).

Ectopic MTs in Shh KO embryos may not reflect disrupted anterior–posterior patterning in IM

The effects of stage-specific Shh deletion indicated that MT development was dependent on Shh signaling at earlier stages of mesonephros formation. Therefore, we focused on the patterns of gene expression within the IM. Hoxd4 is a marker of IM and was expressed normally in Shh KO embryos (Fig. 5A and B). Hoxd11 expression, which is necessary for metanephros development and is sufficient to induce the metanephros specific tubules in the mesonephros, was restricted to the metanephric region in both control and Shh KO embryos (Fig. 5C and D). Hoxd11 was not expressed in the ectopic MTs of Shh KO embryos, indicating that the ectopic tubules were not metanephric identity.

Our analysis of Hoxd expression in IM suggested that the formation of ectopic MTs in Shh KO embryos was not associated with altered anterior–posterior patterning of IM. Therefore, we next analyzed the expression of nephrogenic genes in IM. Gdnf is essential for ureteric bud outgrowth (Sánchez et al., 1996). The expression of Gdnf and its receptor, Ret, was restricted mostly to the metanephros in control embryos, whereas their expression was anteriorly expanded up to the level of the mesonephros in Shh KO embryos (Fig. 5E–H). Fgf8 is expressed in and essential for the formation of cranial MTs (Kitagaki et al., 2011; Perantoni et al., 2005). Fgf8 was ectopically expressed throughout the IM in Shh KO embryos (Fig. 5I and J). These results indicate that loss of Shh promote ectopic ureteric bud outgrowth and/or the formation of supernumerary cranial-type MTs in the caudal mesonephros.

Downregulation of Foxc1 and Foxc2 expression in Shh KO embryos

Ectopic ureteric buds and Gdnf expression in the developmental mesonephros are associated with deficiencies in genes not directly involved in Hh signaling, such as β-catenin, Gata3, and Slit2/Robo2 (Grieshammer et al., 2004; Grote et al., 2008; Marose et al., 2008). The ectopic ducts in these mutants are accompanied by the swelling of the WD, indicating that almost all the ectopic ducts are due to ectopic budding from the WD. Similarly, Foxc1-deficient and Foxc1/2 double heterozygous embryos form ectopic ureteric buds. These Foxc mutants also develop medially located ectopic MTs that do not always connect to the WD in the caudal mesonephros (Kume et al., 2000; Mattiske et al., 2006). Moreover, Foxc1 and Foxc2 are required in the nephrogenic mesonephros for accurate patterning of the tubular tissue and the adjacent mesenchyme (Wilm et al., 2004). Based on the close similarities between the mesonephric phenotypes of Foxc1/2 mutants and Shh KO embryos, we examined Foxc1 and Foxc2 expression in Shh KO mice. In contrast to the expression of Foxc2 throughout the IM and the paraxial mesoderm in control embryos at E9.5 (Fig. 6A and C), Foxc2 was downregulated in the anterior mesoderm at the level of the presumptive mesonephros in Shh KO embryos, primarily in the paraxial mesoderm (Fig. 6B and D). Foxc1 expression was also downregulated in the paraxial mesoderm (Fig. 6E and F). These data indicate that the loss of Shh expression leads to the downregulation of Foxc1 and Foxc2 expression in the paraxial mesoderm at the level of the presumptive mesonephros. Since Foxc1 and Foxc2 expression is suppressed by BMP signaling during medio-lateral patterning of the mesoderm (James and Schultheiss, 2005), we analyzed the effects of Shh KO on the expression of Bmp4 and Noggin, a BMP antagonist. In control embryos, Bmp4 was expressed in the ventral mesoderm, lateral plate mesoderm, splanchnic mesoderm and weakly in the IM, whereas Noggin was expressed in the notochord and ventral side of the neural tube (Fig. 6G and I; Wijgnerde et al., 2005; Wilm et al., 2004). There were no significant changes in either Bmp4 or Noggin expression in Shh KO embryos (Fig. 6H and J). Furthermore, we examined Lim1 and Foxc2 expression to determine if the expansion of the mesonephros toward the midline correlated with the expansion of IM. No obvious expansion of gene expression was observed at E9.5 (Fig. 6K and L; data not shown). These results indicate that Shh positively regulates Foxc1 and Foxc2 expression in the paraxial mesoderm, which may be required to prevent ectopic MT formation in control embryos.

Discussion

Ectopic MT formation in mesonephros of Shh KO embryo

Characterization of mesonephros formation, especially the MT patterning, is still obscure. The mesonephros forms along the WD at E9.5 and the mesonephric mesenchyme begins to regresses at E10.5 extending in a caudal-to-cranial fashion (Hoshi et al., 2012). Delayed regression of mesonephric mesenchyme is suggested to cause the ectopic ureteric bud formation (Hoshi et al., 2012). There were no significant differences in Pax2 expression between the control and Shh KO embryos until E9.5 (Supplemental Fig. S1). This suggests the induction of Pax2 in condensed mesenchyme is normal and no obvious ectopic tubulogenesis have been induced in the Shh KO embryos until the mesonephric mesenchyme regression. However, at E10.5, the expression of Pax2 in the mesonephric mesenchyme adjacent to the WD was more prominent in the Shh KO compared with the control embryos. This may indicate the failure of mesonephric mesenchyme involution in Shh KO embryos. In contrast, tubulogenenic gene expression (Pax2, Fgf8 and Lim1) and epithelial structures were observed in the caudal part of the Shh KO mesonephros (Figs. 1 and 5, data not shown), suggesting the ectopic MTs were similar in character to cranial MTs. Taken together, these results may indicate that Shh regulates proper IM regression in addition to MT patterning through the regulation of tubulogenic gene expression in mesonephric mesenchyme.

Shh expression in the mesonephros is not required for the development of mesonephros and its derivatives

In the present study, we used genetic approaches to analyze the role of Shh in the formation of the mesonephros. Shh is expressed in the epithelia of WD and MTs throughout mesonephric development (Little et al., 2007). Ablation of Shh results in WD mesenchymal hypoplasia and regression of WD derivatives. To investigate the function of Shh expressed in mesonephros for its development, we analyzed Hoxb7-Cre;Shh\^{lox}–/– and \( \text{Sall1CreERT2};/;\text{Shh}^{\text{lox}–} \) mice which ablate Shh in WD and MTs, respectively. In contrast to Shh KO embryos, neither the mesenchymal mass nor its differentiation in the mesonephros were affected in either of these conditional mutants. Since the phenotype of Shh KO embryos showed that Shh was involved in WD and mesonephros development, the lack of phenotype in these mutant embryos may be due to functional redundancy among Hh signals, i.e., either between WD- and MT-derived Shh, or between Shh and other Hh ligands, such as Indian hedgehog (Ihh) and Desert hedgehog (Dhh).
Consistent with this, Shh and Ihh are functionally redundant in the gastrointestinal tract (Ramalho-Santos et al., 2000). Alternatively, the lack of phenotype may also be related to experimental variables, such as incomplete deletion of Shh in the target tissues. For these reasons, pathogenesis of WD mesenchymal hypoplasia or regression of WD derivatives in Shh KO embryos may not be fully explained by analysis of the current WD- or MT-specific Shh KO mutants. Hence, we also analyzed the temporal requirement for Shh using Shh\textsuperscript{CreERT2/\textsuperscript{lox}\textsuperscript{lox}} mutants. This analysis indicated that MT development was sensitive to Shh beginning around E8.5; however, WD mesenchymal mass and its derivatives, the male reproductive tract, developed normally following Shh ablation at either E8.5 or E9.5. Consistent with this result, Gli1 and Ptch1 were normally expressed in the mesonephros at E13.5 in Shh KO embryos (Supplemental Fig. S4, data not shown). Taken together, these data suggest that Shh expressed prior to E8.5 and/or other sources of Shh outside of the mesonephros are essential for its mesenchymal development and male reproductive tract differentiation. Indeed, Shh expression in the mesonephros was much weaker than that in the midline structures. Further investigation will reveal the specific roles of Hh signaling in mesonephric mesenchyme and male reproductive tract development.

Role of midline-derived Shh in mesonephric tubulogenesis

Shh KO embryos show ectopic MTs and MT extensions directed toward the midline throughout IM, which normally extend toward the gonadal side in control embryos. Neither phenotype was observed in either WD- or MT-specific Shh KO embryos indicating that MT development depends upon Shh signaling derived from outside of the mesonephros. Temporal analysis of the requirement for Shh using the conditional mutant allele Shh\textsuperscript{CreERT2/\textsuperscript{lox}\textsuperscript{lox}} resulted in extensions from MTs toward the midline and ectopic tubule formation following Shh ablation at E8.5. These observations suggest that midline structures, i.e., the notochord and floor plate, were the sources of Shh for proper mesonephros development.

Midline-derived Shh is proposed to act as a "midline barrier" to prevent metanephrus fusion and ensure bilateral kidney development (Tripathi et al., 2010). Our results from TM-treated Shh\textsuperscript{CreERT2/\textsuperscript{lox}\textsuperscript{lox}} embryos indicate repulsive signals from midline cells may also be a component of the "midline barrier," in addition to the growth of midline tissues acting as a physical barrier. Furthermore, our results have uncovered novel functions of midline-derived Shh signaling in regulating MT number and direction. Although the mesonephros is transient in amniote embryos, it develops into the adult kidney in some anamniotes. In these species, it is more mesonephros is transient in amniote embryos, it develops into the adult kidney in some anamniotes. In these species, it is more

Possible downstream targets of Shh signaling during mesonephric tubulogenesis

Our results indicate that midline-derived Shh between E8.5 and E10.0 was essential for the correct positioning of mesonephros and regulates MT number. Caudal somitogenesis and IM differentiation occur during this period. IM marker genes, such as Lim1 and Pax2, are expressed between the paraxial and lateral plate mesoderm until E9.5 (James and Schultheiss, 2003; Tsang et al., 2000). Gli1 expression and the distributions of Hh-responsive cells in Gli1\textsuperscript{CreERT2/\textsuperscript{lox}\textsuperscript{lox}};R26\textsuperscript{foxC2/\textsuperscript{foxC2}} embryos (TM injection at E8.5) indicated Hh signaling activity in the paraxial mesoderm, predominantly in the sclerome. Thus, we suggest that midline-derived Shh may influence IM indirectly through effects on the paraxial mesoderm. In addition, the normal expression of Lim1 and Pax2 (Fig. 6L; data not shown) in Shh KO embryos at E9.5 indicates the cell fate decision between paraxial and IM is not dependent on Shh.

The expression and importance of Foxc transcription factors in the paraxial mesoderm and derivatives of the sclerome are well established. In mouse embryos lacking Foxc1 and Foxc2, IM marker gene expression expands into the paraxial mesoderm (Wilm et al., 2004). Moreover, alterations in Pax2 and Wt1 expression indicate disorganized MT cell differentiation in these mutants. Thus, Foxc1 and Foxc2 are required for mesonephric kidney development during IM formation, and for the subsequent differentiation of the nephrogenic region into the duct and tubule tissue, and adjacent mesenchyme (Kume et al., 2000; Mattiske et al., 2006; Wilm et al., 2004). We found that Foxc1 and Foxc2 expression in the anterior trunk mesoderm were downregulated in Shh KO embryos, especially in the paraxial mesoderm and its derivatives. Therefore, Foxc1 and Foxc2 may regulate the patterning of tubular tissue and mesenchyme in Shh KO embryos and may contribute to the repulsive effect of midline-derived Shh.

Although the cranial and caudal MTs and mesanephric kidney are different in size, structure and functional maturity, they are regulated by common inductive and developmental programs. In mice, all MTs are derived from the mesenchyme adjacent to the WD through a mesenchymal-to-epithelial transition (Mugford et al., 2008). The transcription factors Pax2, Wt1, and Foxc1/2 are involved in the formation of mesonephric nephrons, in addition to the metanephros (Bouchard et al., 2002; Kobayashi et al., 2007; Mugford et al., 2008; Sainio et al., 1997; Torres et al., 1995). The expression of these fundamental nephrogenic genes is regulated by the surrounding mesodermal tissues. Our data demonstrate that midline-derived Shh was essential for proper mesanephros formation and provides insights into a novel mechanism of tubulogenic cell determination and the diverse origins of the adult kidney in different animal phyla.

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Appendix A. Supplementary information

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References


