Title
Unique functions of Sonic hedgehog signaling during external genitalia development

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INTRODUCTION

The molecular mechanism that underlies the morphogenesis of external genitalia in mammals is poorly known. The genital tubercle (GT) constitutes the anlage of external genitalia for penis and clitoris (Dolle et al., 1991; Kondo et al., 1997; Murakami and Mizuno, 1984; Murakami and Mizuno, 1986). Coordinated growth and differentiation of the GT generates a proximodistally elongated structure that is suitable for copulation and efficient fertilization. The GT also establishes an upper-lower (dorsoventrally) differentiated structure that enables several essential functions, such as erection and urine/sperm transport. Formation of the urethral tube, which is first generated as a ventral urethral plate, is of the essential morphological differentiations necessary for uresis or ejaculation. During GT morphogenesis, other characteristic tissue differentiations must be accomplished to generate corpus cavernous glandis, penile bones (os penis or os clitoris) and the urethral plate/tube. Another feature of external genitalia development is sexual dimorphism (Cunha, 1975). During the perinatal/postnatal period, the development of external genitalia is influenced by steroid hormone (Anderson and Clark, 1990; Korach, 1994; Murakami, 1987). Impaired synthesis or signaling of steroid hormone has been implicated in human external genitalia malformations, such as hypospadias.

Several growth factors, including fibroblast growth factor (Fgf) and Wnt, have been implicated in the control of external genitalia development in mice (Haraguchi et al., 2000; Yamaguchi et al., 1999). We have recently shown that Fgf signaling plays a key regulatory component in orchestrating growth and differentiation of the GT (Haraguchi et al., 2000).
We have also found that several genes involved in the Shh signaling pathway are dynamically expressed during the embryonic process of external genitalia outgrowth and its concomitant differentiation. During murine external genitalia morphogenesis, Shh is initially expressed in the urogenital sinus and in the distal tip of the urethral epithelium (Haraguchi et al., 2000). Shh expression in the urogenital sinus epithelium has recently been shown to be important for prostate development (Podlasek et al., 1999).

The external genitalia constitute terminal appendage organs with endodermally derived tubular structures, i.e. initially a urethral plate and later a urethral tube. The developing GT is composed of a urethral plate/tube together with mesenchyme and outer ectoderm.

Gut and external genitalia might be considered to possess some shared aspects of organogenesis for generating endodermal tubular structures, despite performing distinct functions, such as transporting nutrients versus urine or sperms. Epithelial-mesenchymal interactions have been implicated in the development of the gastrointestinal tract, which is composed of esophagus, stomach, small intestine and colon. Embryonic gut development involves both regionalization along the anteroposterior (AP) axis, as well as radial patterning of the gut tube to achieve development of proper epithelium, connective tissue, muscle layers and glands. Shh signaling has been shown to be critical for the latter process (Ramalho-Santos et al., 2000; Roberts et al., 1995). Cross-regulation between Shh and other regulatory molecules has been reported such that misexpression of Shh leads to ectopic expression of Hoxd13 in the chick hindgut (Roberts et al., 1998). Shh signaling has also been shown to be required for foregut and lung development (Litingtung et al., 1998; Motoyama et al., 1998; Pepicelli et al., 1998). Shh overexpression in lung epithelium increased both epithelial and mesenchymal proliferation (Bellusci et al., 1997a). Taken together, Shh has been suggested to function in several endodermal sites of epithelial-mesenchymal interactions. Such interactions have been reported in developing urogenital systems, albeit not for external genitalia formation. Consistent with this notion, Hoxd13, a putative Shh downstream target, is expressed in the GT and the urogenital sinus (Dolle et al., 1991; Mortlock and Innis, 1997; Õfelelein et al., 1996; Podlasek et al., 1999).

In this report, the roles of Shh signaling in murine external genitalia formation were studied by in vitro organ culture of GT and mutant analysis. Our results suggest that Shh signaling is required during the initiation of GT outgrowth and the morphological differentiation of the GT, particularly during the formation of the urethral tube. Furthermore, we observed a dynamic pattern of apoptosis during external genitalia formation and our studies suggested Shh signaling might play a regulatory role in this process.

MATERIALS AND METHODS

Mouse genital tubercle organ culture

Procedures for filter supported organ cultures for murine GT were previously described (Haraguchi et al., 2000). To analyze the role of Shh signaling during GT morphogenesis, we developed a new rotating organ culture system. Dissected genital tubercles were cultured in chambers (2 ml/plastic cryogenic vial; Iwaki) with serum free BGJb medium (Gibco BRL). After incubation for 15 minutes at 37°C, vials were laid on rollers and rotated at 6 rpm during incubation (1-4 days) using a MiniPERM bioreactor system (Heraeus instruments). For antibody inoculation, anti-Shh (5E1) antibodies (DSHB, USA) or control Ig class-matched Ab (anti-CD90 antibody, Pharmingen) were added to the culture medium at a concentration of 1-10 µg/ml.

Mutant mice

Shh mutant mice with a targeted deletion of exon 2 of the gene were used (Chiang et al., 1996). Gli2 mutant mice with a targeted mutation of the DNA-binding zinc-finger motifs were used. The genotyping was performed as described elsewhere (Chiang et al., 1996; Mo et al., 1997).

In situ hybridization for gene expression

Whole-mount in situ hybridization was performed according to standard procedures. Probes for the following genes were used: Gli1 (Gli – Mouse Genome Informatics) Gli2, Gli3 (Hui et al., 1994), Pit1 (Pitch – Mouse Genome Informatics) (Goodrich et al., 1996), Bmp4 (Jones et al., 1991), Shh (kindly provided by C. Shukunami), Fgf8 (kindly provided by B. L. Hogan), Fgf10 (kindly provided by H. Ohuchi and N. Itoh) and Hoxd13 (Dolle et al., 1991).

Histological analysis

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 described (Haraguchi et al., 2000).

Preparation of Shh protein beads

Recombinant mouse Shh protein (R&D Systems) was used at a concentration of 1.0 mg/ml in phosphate-buffered saline (PBS). Heparin acrylic beads (Sigma) were washed with PBS and subsequently soaked in Shh protein for 1 hour at 37°C. Control beads were treated with PBS containing 0.1% bovine albumin (Sigma).

Analysis of cell proliferation and cell death in vivo and in vitro

Pregnant females were injected intraperitoneally with 100 µg per kg body weight of 5-bromodeoxyuridine (BrDU, Sigma) at 10.5 or 11.5 days post coitum (dpc). One hour after injection, embryos were collected, fixed in 70% ethanol, and washed several times with PBS. Cell proliferation was also analyzed by culturing tissues in the presence of BrDU. BrDU (1 µg/ml) was added in the BGJb culture medium for 30 minutes and tissues were fixed in 70% ethanol, and washed several times in PBS. Fixed samples were sectioned and stained as described (Bellusci et al., 1997a). Cells representing equal area of wild-type and Shh mutant GT were counted and average values were compared. TUNEL analysis for detection of apoptotic cells in the GT was performed using the in situ apoptosis detection kit (TAKARA, Japan). For Nile Blue staining, GT from 11.5-14.5 dpc embryos were dissected and stained with water-saturated Nile Blue (SERVA) diluted to 1:1000 in PBS. The tissues were kept in Nile Blue solution, washed with PBS several times and photographed.

RESULTS

Genes related to Shh signaling were expressed in the developing murine external genitalia

To obtain insight into the role of Shh signaling in murine external genitalia development, gene expression was examined for Shh and related signaling genes. Shh was expressed in the outermost part of the urogenital sinus epithelium at 10.5 dpc, before the onset of GT outgrowth (Fig. 1A,B). It was subsequently expressed in the distal tip of the urethral
epithelium (DUE) (Ogino et al., 2001) at 11.5 dpc although less evident than that of Fgf8 expression in the DUE (Fig. 1C,D). Later than 12.5 dpc, it was expressed in the urethral epithelium with its expression level moderately higher in the distal and lower in proximal region. The expression continued approximately until 15 dpc (Fig. 1E,F). Urethral expression of Shh was also previously reported (Bitgood and McMahon, 1995; Echelard et al., 1993; Haraguchi et al., 2000). Consistent with the expression pattern of Shh, Patched1 (Ptc1) encodes a transmembrane protein functioning as a receptor for Shh and its expression is transcriptionally activated by Shh signaling (Goodrich et al., 1996; Marigo et al., 1996; Milenkovic et al., 1999; Stone et al., 1996). Consistent with the expression pattern of Shh, Ptc1 expression was found in the mesenchyme with relatively higher level close to the urethral plate epithelium (Fig. 1G). Gli1, Gli2 and Gli3 encode zinc-finger transcription factors that are implicated in Shh signal transduction (Hui et al., 1994). All three Gli genes were expressed at the GT mesenchymal region (Fig. 1H-J). Bmp4, frequently reported as a cross-regulatory gene with Shh during organogenesis, was expressed in the GT mesenchyme (Fig. 1K). Hoxd13, a putative downstream target gene of Shh, was also expressed in the GT mesenchyme (Fig. 1L) (Dolle et al., 1991). The expression of these genes suggests a role for Shh signaling during murine external genitalia development.

**Induction of mesenchymal gene expression by Shh protein**

We have previously shown that the DUE, as well as several growth factors expressed in the DUE could augment the expression of several mesenchymal genes in GT explants (Haraguchi et al., 2000). These results indicate that epithelia-laden growth factors can affect mesenchymal gene expression in the developing GT and suggest a regulatory role of epithelial-mesenchymal interactions.

To investigate the roles of Shh signaling in the development of external genitalia, we first examined whether the expression of mesenchymal genes in the GT explants were affected by Shh protein administration. After 20-24 hours of culturing with Shh or control beads, GT explants treated with the Shh beads showed augmented expression of Ptc1, Bmp4, Hoxd13 and Fgf10 (Fig. 2A-D) (note the endogenous signals shown by arrows). Similar to Ptc1, the expression of Gli1, another universal transcriptional target of Shh signaling, was also augmented by Shh administration in the GT explants (data not shown). Some of the above upregulation, such as Bmp4, Hoxd13 and Fgf10, are speculated to incorporate several signaling cascades rather than reflect a direct gene upregulation because of the induction time. Such modes of gene regulatory cascades were reported for Fgf signaling during GT formation (Haraguchi et al., 2000). To further extend this observation, Ptc1 gene expression in the GT explant was examined upon the addition of anti-Shh antibody (5E1). The 5E1 antibody is a neutralizing antibody for Shh and has been frequently used in inhibition studies of Shh signaling in several systems, such as neural explant (Ericson et al., 1996). Consistent with previous observations in other systems, the 5E1 antibody also

**Fig. 1.** Expression of Shh, Ptc1, Gli1, Gli2, Gli3, Bmp4 and Hoxd13 during external genitalia development. (Top) Embryonic GT (genital tubercle) development in mice; red regions indicate the urogenital sinus (left) and the GT (middle and right). (A,B) Shh is initially expressed in the epithelium of the outermost part of urogenital sinus at 10.5 dpc. (C,D) At 11.5 dpc, Shh expression is mainly localized to the distal region of the urethral epithelium. (E,F) Shh is broadly expressed in the urethral plate epithelium in the GT from 12.5 to 14.5 dpc. (G) Gli1, Gli2, Gli3, Bmp4 and Hoxd13 (L) are expressed in the mesenchyme of developing GT at 13.5 dpc. (E,G-L) are ventral views, B,D are transverse sections and F is a coronal section. Scale bars: 250 μm in A,C; 62.5 μm in B,D,F; 312.5 μm in E,G-L.

**Fig. 2.** Effects of Shh protein and antibody administration on mesenchymal gene expression in the GT. GT explants were micro-dissected from 11.5 dpc embryos, implanted with Shh protein (s) and control BSA (b) beads, and cultured for 24 hours. (A-D) The expression of Ptc1 (A), Bmp4 (B), Hoxd13 (C) and Fgf10 (D) were augmented in regions adjacent to the Shh protein bead. Arrowheads and arrows indicate the augmented and endogenous expression, respectively. (E,F) Mesenchymal (endogenous) expression of Ptc1 in the GT explant was reduced after treatment with an anti-Shh neutralizing antibody for 24 hours. Scale bar: 250 μm in A-D; 312.5 μm in E,F.
blocked Shh signaling and inhibited endogenous *Ptchl* expression in the GT explants (Fig. 2E,F). Similar inhibition was also observed for endogenous *Fgf10* expression (see Fig. 5). These observations indicate a regulatory role of epithelial-derived Shh for mesenchymal gene expression in the GT.

**Agenesis of external genitalia in Shh knockout mice**

To investigate the role of Shh signaling in murine external genitalia development, we analyzed the phenotype of Shh knockout mice. A complete absence of external genitalia formation was observed at 12.5 dpc *Shh*−/− embryos (Fig. 3A,B). At perinatal stage, *Shh*−/− mice displayed small residual structures, which are probably derived from the truncated prepuce growth (data not shown). These observations provide genetic evidence that Shh signaling is essential for external genitalia development in mice and indicate an early function of Shh signaling in the initiation of GT outgrowth.

We next investigated whether the lack of external genitalia development in *Shh*−/− mice was correlated with altered expression of *Fgf8* gene. *Fgf8* is one of the Fgf genes expressed during the initiation of GT outgrowth. Previous analysis with GT explant cultures suggested its growth promoting role by gain-of-function assays (Haraguchi et al., 2000). Marked reduction of *Fgf8* expression was observed in the urogenital sinus at 10.5 dpc (Fig. 3D) and also at the distal urethral epithelium at 11.5 dpc (Fig. 3F). These results suggest that Shh might lie upstream of growth-promoting genes during the initiation of GT outgrowth.

**Roles of Shh in controlling cell proliferation and differentiation during external genitalia formation**

As *Shh*−/− mice show defects in the initiation of GT outgrowth, they are not useful for studying the function of Shh signaling at later stages of external genitalia development. To circumvent this limitation, we used the GT organ culture system (Haraguchi et al., 2000). Shh plays multiple roles in regulating cell proliferation and differentiation in several developmental systems, such as lung and neural tube. Consistent with the notion that Shh promotes cell proliferation, GT explants treated with Shh beads showed an increase of cell proliferation as tested by BrdU incorporation (data not shown). To examine the growth-promoting role of Shh in GT development, GT explants were incubated with the anti-Shh antibody (5E1). A rotating chamber device was used in these studies to allow the in vitro culturing of GT explants without the support of the membrane filter substrate. As shown in Fig. 4, the 5E1-treated specimen displayed retarded GT outgrowth along the proximodistal (PD) axis (Fig. 4C), when compared with the control specimen (Fig. 4B). Analysis of BrdU incorporation revealed a decrease of 50% and 20% in cell proliferation in the 11.5 dpc and 12.5 dpc GT explants, respectively (Fig. 4D).

In addition to the effects on cell proliferation, inhibition of Shh signaling by the 5E1 antibody also affected the differentiation of GT. In control explants, GT differentiated to form a ventral epithelium with mesenchyme locating bilateral to the midline urethral plate (Fig. 5A). By contrast, the 5E1-treated explants showed agensis of the midline urethral plate (the midline urethral plate was not formed in Fig. 5B; shown by an arrowhead in 5A). The 5E1-treated samples also showed marked reduction of *Fgf10* expression in the mesenchyme (Fig. 5C,D). By contrast, the 5E1-treated samples displayed similar expression of epithelial markers, such as keratin 14 in the ventral epithelium (data not shown).

These results may indicate a possible interaction between epithelial Shh and mesenchymal Fgf10 during the morphological differentiation of GT (see Discussion).

Inhibition of Shh signaling in vivo, using dihydrojervine, was also performed. Jervine and cyclopamine are structurally related alkaloids, that can block Shh signal transduction (Cooper et al., 1998; Gaffield et al., 1999; Incardona et al., 1998). Administration of jervine to pregnant female mice induces holoprosencephaly in embryos (Gaffield and Keeler, 1996). Similar to those observed in vitro, the inhibition of Shh signaling by administration of dihydrojervine to pregnant females at 9.5 dpc was found to induce abnormal GT development (data not shown).

Three Gli transcription factors possess distinct and overlapping functions in Shh signal transduction. Analysis of Gli mutant mice indicated that Gli2 functions as the major transcriptional activator in Shh signaling: Gli2−/− mice generally show a mutant phenotype similar to, but less severe than, those of *Shh*−/− mice (Motoyama et al., 1998). Although Gli1 and Gli2 possess overlapping functions, Gli1−/− mice are viable and fertile, and do not exhibit any obvious mutant phenotypes (Park et al., 2000). Both Gli1 and Gli3 mutant mice did not exhibit any abnormalities in GT development (data not shown). By contrast, Gli2−/− mice exhibited ventral malformations of the GT, which are strikingly similar to the 5E1-treated GT explants. Although the outgrowth of GT was initiated, Gli2−/− GTs displayed hypoplasia of the ventral mesenchyme (Fig. 5E,F). Later staged Gli2−/− mouse completely lacked urethral tube formation (data not shown). Taken together, the above results indicate that inhibition of Shh signaling induces ventral GT dysmorphogenesis with mesenchymal hypoplasia.

**Apoptosis during normal external genital morphogenesis**

While analyzing the histogenesis of GT, we have noticed the presence of apoptosis, which also offered a clue in analyzing Shh functions. Apoptosis is an essential regulatory process during various types of organogenesis (Coucouvanis and Martin, 1999; Ganan et al., 1996; Roberts et al., 1999) and is also involved in the control of the disappearance/maintenance of signaling centers during embryogenesis (Pizette and Niswander, 1999; Vaahktokari et al., 1996b). Apoptosis during the formation of the anterior urethra has been recently reported (Van der Werff et al., 2000), although its role in external genitalia formation is not well known. To determine the role of apoptosis during normal GT development, the presence of apoptosis was examined during normal GT development (Fig. 6A-C). Our results indicate a dynamic pattern of apoptosis in the developing murine GT. At 10.5 dpc, cell death was found in the outermost part of the urogenital sinus epithelium (Fig. 6A). Later at 11.5 dpc, apoptotic cells were observed at the epithelium and its adjacent mesenchyme (Fig. 6B). At 13.5 dpc, apoptosis was confined to the distal mesenchyme (Fig. 6C). These observations suggest that GT development involves precise temporal and spatial control of programmed cell death.
**Shh signaling is involved in both cell proliferation and death during GT development**

To determine the role of Shh signaling in the control of cell death versus cell proliferation, TUNEL and BrdU analysis were performed on 10.5 and 11.5 dpc Shh \(^{-/-}\) embryos. Apoptosis was drastically increased in Shh \(^{-/-}\) embryos at 10.5 dpc before the initiation of GT outgrowth, and at 11.5 dpc (summarized in Fig. 7G). Apoptotic cells are mostly localized to the urethral epithelium of the ‘truncated’ GT suggesting that massive apoptosis might be the major cause leading to the lack of GT outgrowth in Shh \(^{-/-}\) mice (Fig. 7B,D). Consistent with the in vitro observations (Fig. 4), BrdU analysis revealed a significant reduction of cell proliferation in the truncated GT of Shh \(^{-/-}\) embryos at both 10.5 dpc and 11.5 dpc (Fig. 7H). Similar to the GT explants, cell proliferation was reduced in both the urethral epithelium and surrounding mesenchyme. Thus, GT agenesis in Shh \(^{-/-}\) mice is accompanied by both an increase of apoptosis and a decrease of cell proliferation.

Bmp4 has been implicated in programmed cell death during organogenesis (Barlow and Francis-West, 1997; Chen and Zhao, 1998; Ganan et al., 1996). Significant alteration of Bmp4 gene expression was observed in the urogenital sinus of 10.5 dpc Shh \(^{-/-}\) embryos (Fig. 7E,F). Interestingly, Bmp4 gene expression in Shh \(^{-/-}\) embryos site was found to shift from mesenchyme to epithelium (Fig. 7F). These results strongly suggest a regulatory pathway involving both Shh and Bmp4 in the control of apoptosis during the initiation of the GT outgrowth (see Discussion).

**DISCUSSION**

The molecular mechanisms underlying the development of the external genitalia are poorly known. There have been numerous reports of the significance of Shh signaling during organogenesis (Bellusci et al., 1997a; Lewis et al., 2001; Litintung et al., 1998; Marti et al., 1995; Ogura et al., 1996; Riddle et al., 1993; Zuniga et al., 1999). Given the unique mode of Shh expression, initially in the urogenital sinus epithelium before GT outgrowth and subsequently in the proximodistally elongated urethral epithelium, the role of Shh signaling during external genitalia formation was investigated in this study.

**Roles of Shh in controlling cell proliferation and cell death in the developing mammalian GT**

Agensis of GT in Shh \(^{-/-}\) mice clearly illustrates an essential role of Shh signaling in GT development. Phenotypic analysis of Shh \(^{-/-}\) mice revealed that GT development is arrested at the stage of initial outgrowth. It was also shown that Shh induced cell proliferation by organ culture studies. These observations suggest that Shh is a critical signal for the initiation of GT outgrowth.

Significantly, we found that Fgf8 expression was lost in the urogenital sinus of Shh \(^{-/-}\) embryos. In explant studies, Fgf8 could substitute for the DUE in promoting GT outgrowth (Haraguchi et al., 2000). Together, these observations suggest that the lack of initial outgrowth in Shh \(^{-/-}\) GT is likely partly due to a downregulation of Fgf8 expression. However, it does not exclude the possibility of Shh per se functioning also as a regulator for cell proliferation. It has been shown that Fgf8 expression is activated, but not properly maintained, in the apical ectodermal ridge of Shh \(^{-/-}\) limbs (Chiang et al., 2001; Kraus et al., 2001). It remains to be determined whether Fgf8 is a direct target of Shh signaling during the initiation of GT outgrowth. Ablation of Shh might hamper sustaining the essential region, the urogenital sinus epithelium, resulting in the elimination of essential genes normally expressed there.

Involvement of other Fgf gene(s) might also occur because a redundant mode of Fgf gene function was suggested previously (Haraguchi et al., 2000; Sun et al., 2000). Wnt genes have been also suggested as one of the downstream target genes of Hedgehog (Tabata and Kornberg, 1994). Several Wnt genes and their antagonists are dynamically expressed during GT formation (K. Suzuki and G. Y., unpublished). Further studies on the interplay between signaling pathways are required.

In earlier studies, Shh is implicated as a growth-promoting factor (Dassule et al., 2000; Oro et al., 1997). Shh has been shown to suppress cell death in some areas of embryos, such as somites (Teillet et al., 1998). In the developing tooth, Shh may be involved in pattern formation as well as cell proliferation of the early dental epithelium (Dassule and McMahon, 1998; Hardcastle et al., 1998; Vaathokari et al., 1996a). We present evidence here that programmed cell death occurs in a temporal and spatial pattern during GT development and that Shh signaling may play a role in this process. In Shh \(^{-/-}\) embryos, massive apoptosis was detected in the urogenital sinus before GT outgrowth, which also showed high level of Bmp4 expression. This correlation of high Bmp4 expression and increased apoptosis is consistent with the role of Bmp4 in programmed cell death. In addition to a reduction of growth-promoting activities, such as Fgf8, massive apoptosis in the urogenital sinus and in the DUE is probably an important factor accounting for the lack of initial outgrowth of GT in Shh \(^{-/-}\) mice.

**Role of Shh signaling in GT morphogenesis; ventral midline defects caused by perturbing Shh signals**

As Shh \(^{-/-}\) mice completely lack GT development, we have performed several other studies to gain insights about the role of Shh signaling in later stages of GT development.

The use of neutralizing Shh antibody in GT explant culture indicates that inhibition of Shh signaling results in defects in growth and differentiation of GT; the final outcome is ventral GT dysmorphogenesis with the ventral (bilateral) mesenchyme hypoplasia. Similar but less severe defects could be obtained by jervine administration. The cause of this difference is likely attributed to a less efficient inhibition of Shh signaling in vivo. By explant culture experiments, Shh induced Bmp4 and Fgf10 expression in GT mesenchyme and inhibition of Shh signaling leads to downregulation of mesenchymal genes, such as Ptc1 and Fgf10. The reduction of mesenchymal Fgf10 gene expression may underlie such hypoplasia consistent with the previous data that Fgf10 gene mutation induces severe ventral GT dysmorphogenesis (Haraguchi et al., 2000). It has been stated that urethral plate/tube formation is attributed partly to endodermal morphogenesis although very little has been reported for the underlying molecular mechanisms (Hayward et al., 1998; Kurzrock et al., 1999a; Kurzrock et al., 1999b). Further studies will be required to incorporate these data to elucidate the mechanisms of human hypospadias because of the differences in GT histogenesis among several species (Kurzrock et al., 2000).
The morphogenetic processes of gut formation could be referred to as a comparison. Shh was recently identified as a crucial regulator emanating from the inner epithelium to the outer gut mesenchyme, and thus described as a radially acting regulatory molecule (Roberts et al., 1995; Roberts et al., 1998; Sukegawa et al., 2000). Shh signaling from urethral epithelium in the developing GT might be regarded as partly similar to the regulatory mode of Shh function during gut development as mesenchymal gene expression, e.g. Fgf10 expression could be controlled. However, one could also point to the differences in which GT development includes dynamic epithelial and mesenchymal tissue arrangements, e.g. urethral tube formation, which forms initially as a ventral groove, unlike radially arranging tissue layers.

Judged by comparisons to previous publications on histological observation, the current data may be related to the canalization process involved in the formation of the urethral plate/tube, because ablation of Shh signaling leads to an aberrant ventral GT histogenesis. Several explanations have been offered for such processes (Anderson and Clark, 1990; Kurzrock et al., 1999a; Van der Werff, 1999; Van der Werff et al., 2000). Glenister reported that the urogenital folds fuse to form the urethra in humans and claimed that the urethral plate is derived from the urogenital sinus (Glenister, 1954). Concerning the midline fusion, Van der Putte and Neeteson described that the penile urethra is not formed by a fusion of urogenital folds in pig embryos, but develops instead by a process in which the penile orifice moves ventrally by the growing perineum (Van der Putte and Neeteson, 1983). Recent detailed analysis suggested that the urethral plate is an extension of the endodermal urogenital sinus, as suggested by an observation that the glandar urethra is composed of stratified squamous epithelium with tissue recombination assay (Kurzrock et al., 1999a). Given such a dynamic mode of urethral tube formation, involvement of Shh for the epithelial-mesenchymal signaling in the GT indicates its important roles together with other factors.

**Fig. 3.** Agenesis of GT in Shh−/− mice. (A,B) 12.5 dpc Shh−/− embryos show severe defects in the initiation of GT outgrowth (A, wild type; B, Shh−/− mice). Sagittal sections (as illustrated in a) were obtained and stained with Hematoxylin and Eosin. (D,F) Drastic reduction of Fgf8 expression in the GT of Shh−/− mice compared with wild type (C,E). Note that Fgf8 expression was detected in the limb buds, but was very reduced in the GT, of Shh−/− mice (D). Scale bars: 62.5 µm in A,B; 250 µm in C-F.

**Fig. 4.** (A) The effect of inhibition of Shh activity by anti-Shh (5E1) antibody addition during GT formation. GTs from 12.5 dpc embryos (A) were cultured for 96 hours with 5E1 antibody (C) or with control antibody (B). The region above the line corresponds to GT (A). GT formation was retarded by 5E1 antibody application but not with control antibody. Reduced cell proliferation of anti-Shh antibody treated GTs is shown by a graph (D; control treated cell number is shown as 100%). Cultures of 11.5 dpc derived GTs showed more prominent reduction. Scale bar: 250 µm in A-C.

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The use of Gli2 mutant mice offers an alternative way to examine the role of Shh signaling in later stages of GT development. Gli2\(^{-/-}\) mice exhibited ventral malformations of the GT, which are similar to the 5E1-treated GT explants. Analysis of Gli mutant mice indicated that Gli2 functions as the major transcriptional activator in Shh signaling (Motoyama et al., 1998).

Gli genes have been involved in several organogenesis in the Shh pathway (Grindley et al., 1997; Hardcastle et al., 1998; Mo et al., 1997). Gli2\(^{-/-}\) mice possess bilateral unilobar lungs and also exhibit other foregut defects and malformation of the anus (Motoyama et al., 1998; Kim et al., 2001). Genetic studies have suggested Gli2 and Gli3 appear to have essential and overlapping functions during embryonic foregut development (Motoyama et al., 1998). Gli compound mutants manifested more severe phenotypes at the ventral GT midline region suggesting a genetic interaction between these genes (data not shown). Further studies are required for degree of genetic interaction in the Shh signaling. The point at which initiation of GT outgrowth is initiated, but not completed, in Gli2 mutant mice remains to be determined. These observations suggest that, besides the initial outgrowth process, Shh signaling is also required in the later stages for growth as well as the morphological differentiation.

**Involvement of Shh signaling in the formation of epithelia and mesenchymes during external genitalia development**

In this study, it was demonstrated that Shh is required not only for the regulation of GT outgrowth initiation but also for subsequent tissue differentiation, e.g. the ventral side of GT differentiation. Such a dual mode of Shh function in...
organogenesis may be regarded as a unique aspect of Shh signaling when compared with other types of organogenesis such as gut and limb formation. Characteristic issues of epithelial-mesenchymal interactions with Shh in several experimental systems has been described (Narita et al., 2000; Roberts et al., 1998; Sukegawa et al., 2000).

The interaction between Shh and Bmp has been characterized during vertebrate organogenesis. Bmp4 could be ectopically induced by Shh in the gut mesenchyme but not in the lung mesenchyme, where Shh and Bmp4 are co-expressed (Bellusci et al., 1997a). Roberts et al. showed that ectopic expression of Shh in the developing hindgut induced expression of Bmp4 and Hoxd13 (Roberts et al., 1995). Bmp4 was also suggested as a key factor for the developing prostate emanated from the urogenital sinus mesenchyme (Lamm et al., 2001).

The Bmp4 gene expression region was shifted from the mesenchyme to the epithelium before GT outgrowth by Shh mutation as shown in this study. It remains to be studied that such shifting reflects the alteration of the general nature of urogenital sinus epithelium or other adjacent epithelium.

Augmented apoptosis at the urogenital sinus epithelium associated with the shifted Bmp4 gene expression may be considered to be responsible for ablated GT growth initiation of Shh−/− mutants. It was previously shown that micro-surgical removal of the distal tip of the urethral epithelium ablated GT outgrowth consistent with this analysis (Haraguchi et al., 2000). Taken together, the developing GT would be an intriguing system to investigate gene cascade.

A relationship between Shh and Fgf8/10 was also suggested during GT formation (Fig. 8). The distal lung epithelium has several target genes in the mesenchyme controlled by Shh (Bellusci et al., 1997b). Treatment of embryonic lung mesenchymal cells with recombinant Shh inhibits Fgf10 (Lebeche et al., 1999). Bellusci et al. have proposed that a feedback interaction is established between the distal epithelium and Fgf10-expressing cells during lung development (Bellusci et al., 1997b). Fgf10 expression was downregulated in GT explant when Shh signaling is inhibited. The 'ventral' GT phenotypes of Fgf10 knockout mice also indicate that Shh-Fgf10 interaction may constitute one of the important components for GT development (Haraguchi et al., 2000).

The Fgf8 to Shh signaling was suggested during limb development (Vogel et al., 1996). The epithelial Fgf8 expression was lost in Shh−/− embryos during the initiation of GT outgrowth. Further analysis would be required for analyzing their functions in the GT epithelium.

Another area of interest that is related to endoderm formation involves evolutionary speculations. It has previously been stated that the expression and function of hedgehog in the gut endoderm might be an ancestral feature of chordates. Possibly, this ancestral role during gut development extends back even earlier, as hedgehog has been shown to be expressed in the ectodermally derived epithelium of the Drosophila gut (Pankratz and Hoch, 1995). It might be hypothesized that proper gut development among species is essential for all species, which has resulted in the conservation of developmental programs. The divergent structures of the copulatory system development may reflect evolution of the various mode of copulatory behaviors among vast number of species in water or on land. The highly divergent structures of penis/clitoris, in addition to the structures of other copulatory organs, might have implications for such intriguing questions. Molecular and evolutionary considerations would be further required related with the evolutionary divergence of appendages (Cohn and Tickle, 1999; Duboule, 1992; Duboule and Dolle, 1989; Holland, 1999). It may be worthwhile to examine to what extent the molecular developmental programs for external genitalia formation display divergence of genetic programs comprised of Shh, Fgfs and Bmps among different species (Sanchez et al., 2001).

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