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Multiphasic and tissue-specific roles of sonic hedgehog in cloacal septation and external genitalia development

Ashley W. Seifert[1], Cortney M. Bouldin[2], Kyung-Suk Choi[2], Brian D. Harfe[2] and Martin J. Cohn[1,2,3,*]

Malformations of the external genitalia are among the most common congenital anomalies in humans. The urogenital and anorectal sinuses develop from the embryonic cloaca, and the penis and clitoris develop from the genital tubercle. Within the genital tubercle, the endodermally derived urethral epithelium functions as an organizer and expresses sonic hedgehog (*Shh*). *Shh* knockout mice lack external genitalia and have a persistent cloaca. This identified an early requirement for Shh, but precluded analysis of its later role in the genital tubercle. We conducted temporally controlled deletions of *Shh* and report that *Shh* is required continuously through the onset of sexual differentiation. Shh function is divisible into two temporal phases; an anogenital phase, during which Shh regulates outgrowth and patterning of the genital tubercle and septation of the cloaca, and a later external genital phase, during which Shh regulates urethral tube closure. Disruption of Shh function during the anogenital phase causes coordinated anorectal and genitourinary malformations, whereas inactivation during the external genital phase causes hypospadias. Shh directs cloacal septation by promoting cell proliferation in adjacent urorectal septum mesenchyme. Additionally, conditional inactivation of smoothened in the genital ectoderm and cloacal/urethral endoderm shows that the ectoderm is a direct target of Shh and is required for urethral tube closure, highlighting a novel role for genital ectoderm in urethragenesis. Identification of the stages during which disruption of Shh results in either isolated or coordinated malformations of anorectal and external genital organs provides a new tool for investigating the etiology of anogenital malformations in humans.

KEY WORDS: Anorectal malformation (ARM), Cloaca, External genitalia, Urethra, Hypospadias, Sonic hedgehog

INTRODUCTION

Mammals develop separate anorectal and urogenital canals by septation of a single posterior sinus known as the cloaca. Anorectal malformations (ARM) frequently occur in association with urogenital anomalies (Saha et al., 2005), suggesting the presence of shared developmental mechanisms. Development of the external genitalia begins at approximately embryonic day (E) 10.5 in the mouse, when a pair of genital swellings emerges anterior to the cloacal membrane (Perriton et al., 2002). Coordinated outgrowth of the genital swellings results in formation of the genital tubercle, the anlagen of the penis and clitoris. The genital tubercle is composed of mesoderm, surface ectoderm and cloacal endoderm that extends into the tubercle to form the urethral plate epithelium. The urethral plate epithelium gives rise to the entire penile urethra (Seifert et al., 2008). Development of the genital tubercle is indistinguishable in male and female embryos until approximately E16, when the urethral plate begins to be masculinized to form the penile urethra, marking the beginning of the hormonally controlled period of sexual differentiation (Seifert et al., 2008).

Cloacal septation is initiated at the same stage as external genital development, when mesoderm dorsal to the urachus and ventral to the gut (known as the urorectal septum mesoderm, URSM) begins to accumulate at the anterior end of the cloaca. Mesenchyme of the urorectal septum is flanked by cloacal endoderm on its dorsal, ventral and posterior edges, and expansion of the URSM results in dorsoventral partitioning of the cloaca (Nievelstein et al., 1998; van der Putte, 2005). Disruption of this process can result in congenital anomalies including anogenital fistula and, in more severe cases, persistent cloaca, in which the urethra and/or bladder, reproductive tract and colon converge in a common exterior opening. Persistent cloaca is often associated with hypospadias, a partial or complete failure of urethral tube closure on the ventral side of the phallus.

The molecular mechanisms of cloacal septation and genital tubercle formation are not well understood, and even less is known about the basis of associated malformations of these systems. Two members of the Hedgehog (Hh) gene family, Sonic hedgehog (*Shh*) and Indian hedgehog (*Ihh*), are expressed early during anogenital development, and previous work has shown that hedgehog signaling is required for patterning of gastrointestinal organs (Haraguchi et al., 2001; Lipinski et al., 2006; Perriton et al., 2002; Ramalho-Santos et al., 2000; Roberts et al., 1995). Work by a number of labs has shown that Shh is required for outgrowth and patterning of the genital tubercle and for division of the cloaca into separate urogenital and anorectal sinuses. *Shh*$^{-/-}$ mice have complete agenesis of the external genitalia and persistence of the cloaca (Cheng et al., 2008; Haraguchi et al., 2001; Kim et al., 2001; Kimmel et al., 2000; Mo et al., 2001; Perriton et al., 2002). In humans, mutations affecting Hedgehog signaling and its downstream targets have been linked to syndromes involving ARM, such as the VACTERL complex, Pallister-Hall and Townes-Brocks syndromes (Kang et al., 1997; Kohlhase et al., 1998; Mo et al., 2001).

The early and severe genital and cloacal phenotypes of *Shh* knockout mice has precluded analysis of Shh function at later stages, as anogenital development fails to progress beyond the initiation of genital budding and early cloacal septation. To circumvent this problem, we have undertaken a temporal dissection of Shh function using an inducible cre allele. We show that following an early Shh-independent phase of initiation, there are two Shh-dependent phases, one in which anorectal and external genital development are

[1]Department of Biology, [2]Department Molecular Genetics and Microbiology, [3]Howard Hughes Medical Institute, University of Florida, Cancer/Genetics Research Complex, PO Box 103610, Gainesville, FL 32610-3610, USA.

*Author for correspondence (mjcohn@ufl.edu)

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coupled, and a later phase when Shh is required for development of the external genitalia but not the cloaca. In addition, using tissue-specific ablation of smoothened, we identify a novel role for Hedgehog-responsive genital ectoderm in urethral tube closure. The results identify specific developmental stages during which disruption of the Hedgehog (and perhaps other) signaling pathways can result in either isolated or coordinated malformations of anorectal and external genital organs.

MATERIALS AND METHODS

Animals

The *Shh*^{creERT2}, *Shh*^{GFPcre} (Harfe et al., 2004), *Shh*^{C} (Dassule et al., 2000), *R26R* (Soriano, 1999), *Msx2cre* (Sun et al., 2000) and *Smo*^{C} (Long et al., 2001) alleles have been previously described. For cre activation in *Shh*^{creERT2} embryos, tamoxifen (Sigma) was prepared fresh in corn oil (20 mg/ml) and pregnant females were given intraperitoneal injections of 4–7 mg, according to weight. *lacZ* staining was performed as previously described (Seifert et al., 2008).

Histology

Embryos were processed into either paraffin wax or OCT for histological analysis. Samples were cut at 12 μm thickness and counterstained with Biebrich Scarlet. For cryosectioning, embryos were taken through a graded series of sucrose/PBS and embedded in 100% OCT.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as previously described (Ormerod et al., 2004) using rat anti-bromodeoxyuridine (1:500; Accurate, Westbury, NJ, USA) and donkey anti-rat Cy3 antibodies (1:500 Jackson ImmunoResearch, West Grove, PA, USA). Lysotracker Red (1:5000; Molecular Probes) was used to detect cell death. In situ hybridization was performed as previously described (Perriton et al., 2002).

Proliferation index

Pregnant dams were injected with bromodeoxyuridine (BrdU) 4 h before harvest. Proliferation indices were calculated at posterior, dorsal and ventral positions within the URSM (see Results for details). Counting frames containing ~50 cells were placed at these positions and the number of BrdU-(S-phase) and DAPI-labeled cells were counted in each frame. Cells were counted in each frame on every other section spanning the width of the cloaca, averaged for each frame per specimen and converted to the proliferative index (BrdU cells/DAPI cells). For the proliferative index in the cloacal endoderm, transverse sections were cut from the bladder to the tail and endodermal cells were counted as either BrdU-positive or DAPI-positive on every other section.

Statistics

A MANOVA was used to explore differences in URSM proliferation following removal of Shh signaling with position (i.e. anterior, urogenital sinus, hindgut) and proliferative index as the dependent variables. All group differences in the dependent variables were revealed using Newman-Keuls post-hoc comparisons. Alpha levels were set at 0.05.

RESULTS

Continuous Shh signaling is required for patterning and outgrowth of the external genitalia

To circumvent the early and severe genital phenotype of *Shh*$^{-/-}$ mice, we conducted temporally controlled deletions of *Shh* in *Shh*$^{creERT2/C}$ embryos (Fig. 1). Expression of *Ptch1*, a direct target of the Shh signal transduction pathway, was monitored to determine the time required for Shh signaling to be terminated. Shh signaling activity was diminished within 12 hours of tamoxifen injection, with complete and irreversible inactivation of the pathway occurring between 18 and 24 hours after injection (see Fig. S1A,B in the supplementary material; further details of the kinetics of Shh inactivation will be described elsewhere).

In order to determine the role of Shh at each day of genital development between E10.5 and 17.5, a single tamoxifen injection was administered to pregnant females between E9.5 and 16.5 (allowing 24 hours after injection for complete inactivation of Shh signaling), and genital phenotypes were examined at E18.5 (Fig. 1). Inactivation of Shh activity at E10.5, before genital budding, phenocopied *Shh* knockout mice, in which two small swellings develop at the anterior margin of the cloaca but external genitalia are absent (Fig. 1A). In addition, septation of the cloaca into urogenital and anorectal tracts failed, and this resulted in the persistence of a common cloacal outlet (Fig. 1A). Removal of Shh at E11.5, 24 hours after initiation of genital outgrowth, resulted in formation of a severely reduced phallus, including a hypoplastic glans, and a persistent cloaca (Fig. 1B). When Shh signaling was inactivated at E12.5, a genital tubercle developed with ventral abnormalities, resulting in complete failure of urethral tube closure and absence of a ventral prepuce (Fig. 1C). Inactivation of Shh at these stages also blocked formation of separate anorectal and urogenital sinuses. Thus, disruption of Shh signaling between E10.5 and 12.5 results in severe underdevelopment of the external genitalia, hypospadias and persistence of the embryonic cloaca.

Removal of Shh from E13.5 onward revealed that Shh activity continues to be required for development of the ventral aspect of the phallus and for urethral tubulogenesis, but not for cloacal septation. Loss of Shh activity at E13.5 resulted in severe ventral hypospadias along with hypoplasia of the ventral prepuce (Fig. 1D). Inactivation of Shh at E14.5 also resulted in hypospadias, although a less severe form than that observed when Shh was disrupted 1 day earlier (compare Fig. 1D with 1E). In some cases, loss of Shh at E15.5 allowed normal closure of the proximal urethral opening, although there was a more distal hypospadias (Fig. 1F). Removal of Shh activity at E16.5 had little to no affect on external genital development, and the subtle variability at this stage probably depended on the precise time at which the Shh pathway was inactivated (Fig. 1G). Interestingly, inactivation of Shh signaling between E10.5 and E16.5 produced similar phenotypes in males and females (Fig. 1H,I).

Thus, Shh function in anogenital development can be divided operationally into two developmental windows after the initiation of budding (which is independent of Shh). The first Shh-dependent phase occurs from ~E10.5 to 13, when Shh is required for genital tubercle outgrowth and cloacal septation. The second Shh-dependent phase occurs from E13.5 to 16.5, when Shh is required for sustained growth and patterning of the ventral phallus and for closure of the urethral tube. During the early temporal window, the genital tubercle and cloaca are developmentally coupled, whereas after E13.5, external genital development can be perturbed without severe disruption to the separation of the anorectum from the urogenital sinus.

Early disruption of Shh: loss of Shh signaling before E13.5 results in a persistent cloaca

In order to address how inactivation of Shh signaling during the early developmental window leads to a persistent cloaca, we fate-mapped the cloacal endoderm using the *Gt(ROSA)26Sor* reporter (*R26R*) after deletion of *Shh* at different stages of development (Fig. 2). Inactivation of Shh signaling in *Shh*$^{creERT2/C}$*;R26R* mice at E10.5, 11.5 or 12.5 (by tamoxifen injection 24 hours earlier) inhibited outgrowth of the genital tubercle, and endodermally derived cells that normally form the perineum failed to reach the caudal surface (Fig. 2A-C,A′-C′). Although the cloacal membrane ruptures in mutant embryos, failure of septation leaves a common cloacal outlet, and *lacZ*-labeled endoderm (normally fated to form the perineum)

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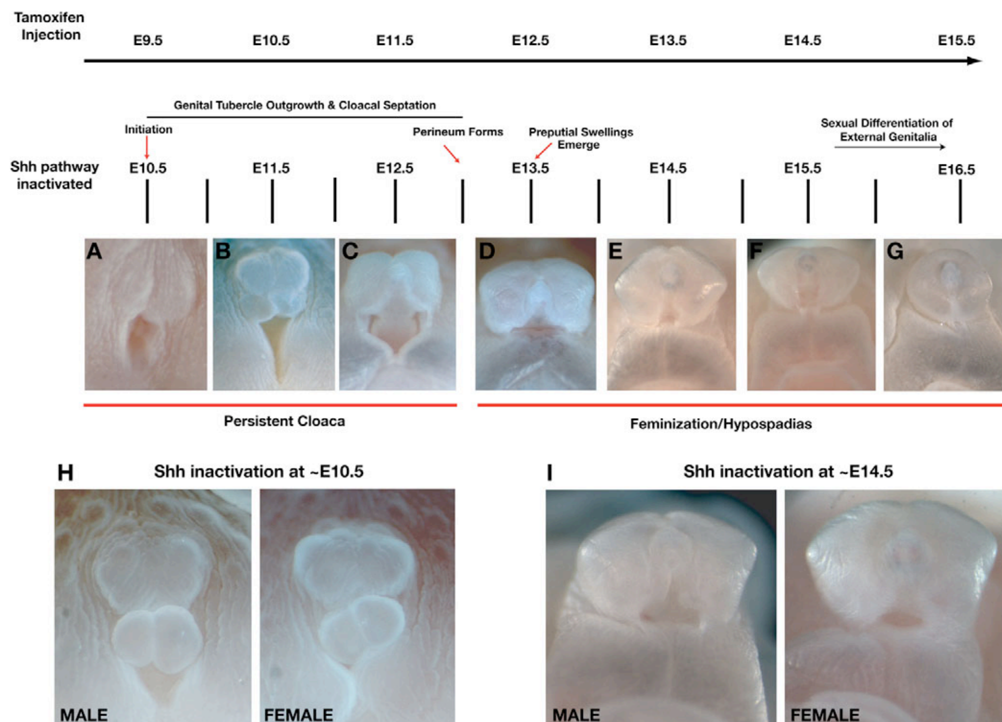

Fig. 1. Temporal dissection of Shh function in anogenital development. *Shh* was inactivated in $Shh^{creERT2/C}$ mutants by administration of tamoxifen to pregnant females at the embryonic days shown along the top line. Hedgehog signaling was inactive 24 hours after tamoxifen treatment (confirmed by absence of *Ptch1*; data not shown). (**A-C**) Shh is required both for genital tubercle outgrowth and for cloacal septation from E10.5 to 12.5 (the anogenital phase). Loss of Shh signaling during this time window results in a persistent cloaca, truncated outgrowth and ventral hypoplasia of the genital tubercle. Earlier removal of Shh results in more severe outgrowth defects. (**D-G**) Shh signaling continues to be required during a second period, from E13.5 to 16.5 (the external genital phase), for normal urethragenesis and genital patterning. This phase follows perineum formation at E13.0, and mutants have external genital defects but separate anorectal and urogenital sinuses. (**H**,**I**) Loss of Shh signaling at early (H) and late (I) stages has similar effects on males and females.

was observed deep within the cloaca (Fig. 2A′-C′). Consequently, mutants exhibited a severe truncation of the phallus, persistence of the cloaca and absence of a perineum.

By contrast, loss of Shh signaling at E13.5 did not disrupt cloacal septation or perineal development (Fig. 2D,D′). Although inactivation of the Shh pathway at E13.5 resulted in hypospadias, the endoderm at the leading edge of the urorectal septum reached the terminus of the cloaca and contributed to the perineum (Fig. 2D,D′). Together, these results indicate that Shh is required until ~E13 for outgrowth and patterning of the genital tubercle and for development of separate urogenital and anorectal sinuses, and that the duration of Shh signaling determines the degree of genital outgrowth and cloacal septation.

Duration of Shh signaling determines the extent of cloacal septation

The extent of urorectal septum development correlated with the duration of Shh signaling; earlier disruptions resulted in more severe truncations of the septum (compare Fig. 2A′, 2B′ and 2C′). In order to determine how disruption of Shh during the early phase results in persistence of a cloaca, we inactivated Shh signaling at E9.5, 10.5 and 11.5 and examined embryos at E14.5, 1 day after normal embryos complete septation of the urogenital and anorectal sinuses. We first mapped the distribution of *Shh*-expressing cells and their descendants in phenotypically normal $Shh^{GFPcre/+}$*;R26R* embryos at E14.5. Using optical projection tomography (OPT), we confirmed that these cells contribute to the epithelium of the small intestine, colon, rectum, bladder, urethra and urethral plate at E14.5 (Fig. 3D). By contrast, $Shh^{creERT2/C}$*;R26R* mutants that had short exposures to Shh (inactivation at E9.5) underwent only minimal septation of the hindgut, resulting in a short intestinal segment and an elongated cloaca with a rudimentary bladder (Fig. 3A). Longer exposures to Shh (inactivation at E10.5 or 11.5) allowed further septation of the cloaca and elongation of the hindgut, and Shh was required at least through E11.5 for formation of a recognizable intestinal loop and a longer bladder, urethra and urethral plate (Fig. 3B,C).

The anteroposterior position at which the gut joined the cloaca varied according to the duration of Shh signaling (Fig. 3A-D). High magnification analysis of E14.5 embryos revealed that septation progressed further posteriorly as the duration of Shh signaling increased, resulting in a posterior shift of the junction between the gut and cloaca (compare arrowheads in Fig. 3A′-C′). When Shh signaling was inactivated at E9.5, a short intestinal segment was situated at the anterior margin of the cloaca (Fig. 3A′), whereas inactivation at E10.5 led to a longer intestinal tract that joined to the mid-cloaca (Fig. 3B′). Inactivation at E11.5 allowed further intestinal elongation and a junction at the posterior aspect of the cloaca (Fig. 3C′). Thus, the duration of Shh signaling determines the anteroposterior extent of cloacal septation, with complete division of the cloaca into anorectal and urogenital sinuses requiring Shh activity through E12.5.

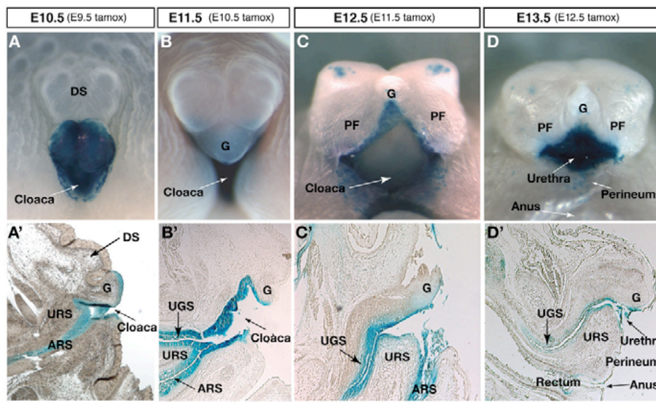

Fig. 2. Deletion of *Shh* during the anogenital phase results in a persistent cloaca and external genital malformation. Lineage mapping of *Shh* descendant cells in male *Shh*$^{creERT2/C}$*;R26R* embryos harvested at E18.5 following tamoxifen injections at E9.5-12.5. (**A-C**) Early (E10.5-12.5) loss of Shh signaling results in persistent cloaca and truncated genital outgrowth. (**D**) Deletion of Shh at E13.5 leads to incomplete closure of the preputial folds and hypospadias. (**A′-C′**) Sections of A-C. Septation of the cloaca is incomplete and the perineum is absent because Shh descendant cells fail to reach the cloacal membrane. (**D′**) Section through D. Note the severe proximal hypospadias and normal perineum. ARS, anorectal sinus; DS, dorsal swellings; G, glans; PER, perineum; PF, preputial folds; UGS, urogenital sinus; URS, urorectal septum.

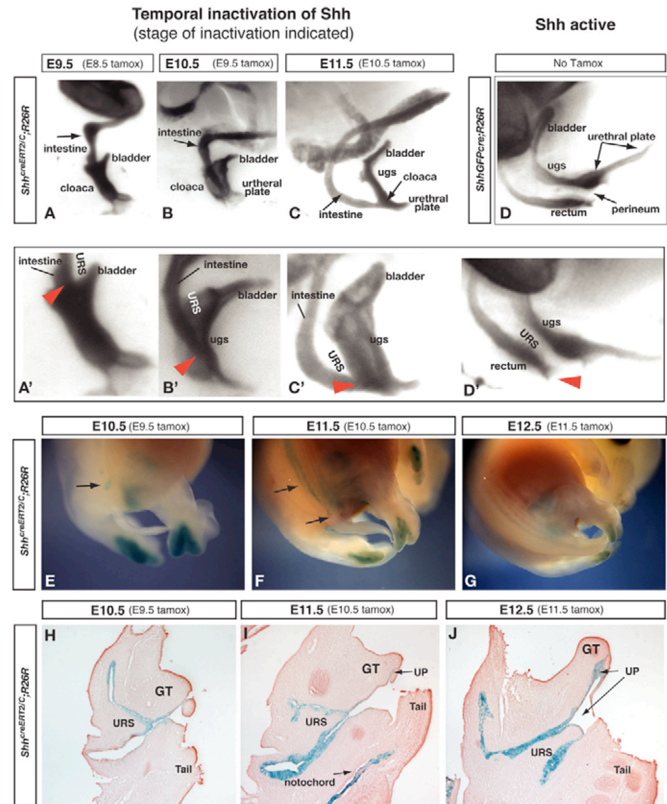

Fig. 3. Shh directs cloacal septation and genital tubercle outgrowth in a time-dependent manner. Analysis of anogenital and tail development in E14.5 embryos using OPT (A-D′), whole-mount X-gal staining (E-G) and histology (H-J). (**A-D**) Loss of Shh signaling in *Shh*$^{creERT2/C}$*;R26R* mice leads to a stage-specific shortening of the hindgut and urethral plate (A-C) compared with wild-type (*Shh*$^{GFPcre/+}$*;R26R*) mice (D). (**A′-D′**) High magnification images of specimens in A-D. (**E-G**) Loss of Shh signaling at E10.5 results in caudal truncation, loss of notochord and floorplate descendant cells, and loss of hindlimb digits (E), whereas longer exposure to Shh allows further development of caudal axis structures (F,G). Arrows (E,F) mark the posterior limit of midline Shh descendant cells. (**H-J**) Sagittal sections of the anogenital system shows that posterior expansion of urorectal septum mesoderm and growth of the urethral plate correlate with length of exposure to Shh. BL, bladder; GT, genital tubercle; HG, hindgut; UP, urethral plate; URS, urorectal septum.

Genital and cloacal defects in *Shh*$^{creERT2/C}$ mice are associated with malformations of the caudal axis

Malformations of the external genitalia often occur in association with other posterior structural anomalies. We therefore asked whether the duration of Shh signaling plays a role in coordinating development of the posterior body axis. Early termination of Shh signaling at E10.5 and 11.5 resulted in underdevelopment of the caudal end of the embryo, including truncation of the tail, loss of caudal vertebrae and intestinal hypoplasia (Fig. 3E-J), in addition to the anogenital defects described above (Fig. 3A-D). When the Shh pathway was inactivated at E10.5, the notochord atrophied posteriorly and Shh descendant cells were found scattered throughout the caudal mesoderm (Fig. 3E). Shh descendant cells were detected in the floorplate anterior to the hindlimbs, but they did not extend into the sacral or caudal regions (Fig. 3E,H). Inactivation of Shh at E11.5 resulted in less severe caudal truncations, further development of the hindlimbs (although with only four digits), and more posterior extension of the notochord and floorplate, although neither extended all the way to the tail (Fig. 3F,I). When Shh was inactivated at E12.5, development of posterior structures was nearly complete, although these mutants still exhibited truncation of the tail and the genitalia and incomplete septation of the cloaca (Fig. 3G,J). Analysis of the distribution of Shh descendant cells in the genital tubercle revealed that the duration of Shh activity also correlated with the length of the urethral plate and the size of the genital tubercle, with shorter periods of Shh activity resulting in decreased proximodistal outgrowth (Fig. 3H-J). These sections also show a decreased distance between the leading edge of the urorectal septum mesoderm and the distal tip of the glans, indicating that Shh is required for the posteroventral expansion of the urorectal septum mesoderm (Fig. 3H-J).

Shh regulates cloacal septation by maintaining cell proliferation

We next investigated whether Shh controls septation of the cloaca by regulating proliferation of mesenchymal cells of the urorectal septum (Fig. 4A,B, white outlines). In order to determine whether sustained Shh activity is required for URSM proliferation, Shh was inactivated at E11.5 (by tamoxifen injection at E10.5), BrdU was injected 20 hours later, and embryos were harvested at E12.5, 4 hours after BrdU administration (Fig. 4A). The ratio of BrdU-labeled to unlabeled cells was calculated in mesenchyme of *Shh*$^{creERT2/C}$ and control embryos at three positions: (1) the posterior/leading edge of the URSM; (2) the ventral URSM (adjacent to the urogenital sinus); and (3) the dorsal URSM (adjacent to the hindgut; see Fig. 4A-D and further details in Materials and methods). We quantified the effects of removing Shh on the proliferative index using a MANOVA test with the position of the

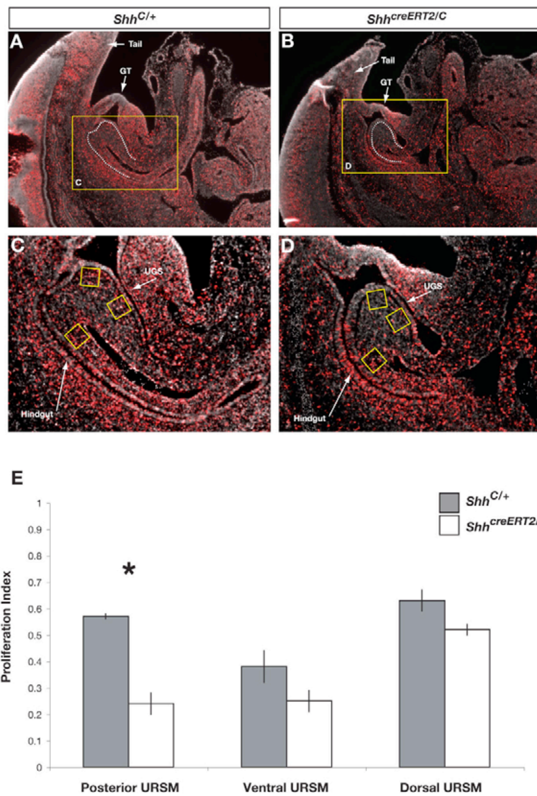

Fig. 4. Removal of Shh during the anogenital phase causes regionalized decreases in cell proliferation in urorectal septum mesenchyme. (**A-E**) BrdU labeling (A-D) and proliferative index (E) in anogenital region of control (*Shh*$^{+/C}$) and *Shh*$^{creERT2/C}$ embryos. Shh signaling was inactivated at E11.5 (tamoxifen injection at E10.5) and embryos were examined at E12.5. Posterior is towards the left. Dotted lines in A and B outline the URSM. Yellow boxes in C and D indicate counting frames at posterior, dorsal and ventral positions of the URSM. (E) Comparison of the proliferative index at all three positions in *Shh*$^{+/C}$ and *Shh*$^{creERT2/C}$ embryos shows that the proliferative index is significantly lower in the posterior URSM of *Shh*$^{creERT2/C}$ mutants (asterisk) but not significantly different at dorsal or ventral positions.

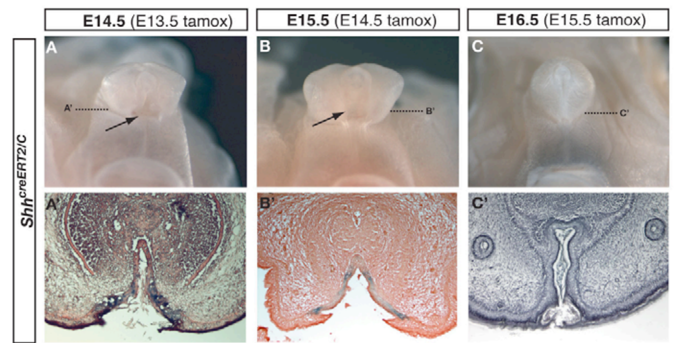

Fig. 5. Deletion of Shh during the external genital phase leads to hypospadias. (**A**,**B**) Inactivation of Shh at E14.5 and 15.5 causes an ectopic opening of the urethra in the proximal penis (arrows). (**A′**,**B′**) Histological analysis of *Shh*$^{creERT2/C}$ embryos shown in A,B shows a rupture of the ventral genital ectoderm. *lacZ*-positive urethral endoderm in a *Shh*$^{creERT2/C}$ embryo (B′) shows the boundary between endoderm and ectoderm. (**C**,**C′**) Loss of Shh signaling at E16.5 did not affect ectodermal integrity.

measurements as a covariate. The MANOVA revealed three major findings: (1) significant differences exist in the proliferative indices at the three positions regardless of treatment ($F_{(2,8)}$=19.93, P<0.001); (2) removing Shh caused a statistically significant decrease in proliferative index of the entire URSM ($F_{(1,4)}$=70.12, P<0.001); and (3) removal of Shh had a statistically significant effect on the proliferative index at the three positions ($F_{(2,8)}$=4.30, P<0.05).

In light of the statistical significance of the MANOVA results, we used Newman-Keuls post-hoc comparisons to examine each URSM position to further explore how removing Shh affected specific regions of the URSM. We found a significant decrease in the proliferative index at the posterior margin of the urorectal septum mesoderm ($t_{(4)}$=7.91, P=0.001), but not across the dorsoventral axis of the URSM (urogenital sinus; $t_{(4)}$=1.85, P=0.139 and hindgut; $t_{(4)}$=2.36, P=0.078 areas did not differ significantly; Fig. 4E). These findings indicate that Shh controls proliferation of the URSM and that loss of Shh leads to a lower proliferative index, especially at the leading edge. Thus, Shh regulates proliferation of the URSM in a position-dependent manner, and this can account for the defects in cloacal septation when Shh is inactivated.

Given the structural defects of the cloacal/urethral endoderm in *Shh*$^{creERT2/C}$ mutants, we also asked whether endodermal proliferation was affected by loss of Shh signaling. We measured the proliferative index in the cloacal endoderm extending from the position where the Mullerian/Wolffian ducts connect with the urogenital sinus to the end of the tail gut, and observed widespread proliferation in both *Shh*$^{creERT2/C}$ and control embryos. Comparison of the mean proliferative index between *Shh*$^{creERT2/C}$ (0.37±0.02, n=3) and control embryos (0.33±0.02, n=3) revealed no significant difference ($t_{(4)}$=1.17, P=0.153), suggesting that proliferation of epithelial cells in the anogenital sinuses is not dependent on Shh activity (Fig. 4C,D). Consistent with this result, the profile of p63, a marker of epithelial progenitors, was similar throughout the cloacal epithelium of *Shh*$^{creERT2/C}$ embryos and control littermates at E13.5, indicating that the progenitor cell population is maintained in the anogenital sinuses despite loss of Shh signaling (see Fig. S2 in the supplementary material). Together these results indicate that sustained Shh activity is required for maintenance of cell proliferation in the posterior urorectal septum mesenchyme, and that decreased cell proliferation in this population can account for the persistence of a cloaca in *Shh*$^{-/-}$ and *Shh*$^{creERT2/C}$ mutant mice.

In both *Shh*$^{-/-}$ and *Shh*$^{creERT2/C}$ embryos, there is some degree of cloacal septation anteriorly, which contrasts with the complete failure of genital tubercle formation (Fig. 4A,B; see also Fig. 3A′-C′). To determine whether the Hedgehog pathway had been completely inactivated around the cloaca in *Shh*$^{creERT2/C}$ embryos, we examined cloacal expression of *Ptch1* 24 hours after tamoxifen injection (see Fig. S1A,B in the supplementary material). Although *Ptch1* expression was absent from mesenchyme of the emerging genital swellings (described above), we observed some *Ptch1* expression around the ventral portion of the cloaca and around the urorectal septum (see Fig. S1A,B in the supplementary material). We therefore assayed for *Ihh* expression following *Shh* removal and found that *Ihh* was expressed in the anterior and ventral portions of the cloaca in *Shh*$^{creERT2/C}$ and in control embryos. *Ihh* expression persists in the cloaca until at least E13.5, but is absent from the genital tubercle (see Fig. S1C,D in the supplementary material; data not shown). This finding raises the possibility that the limited degree of cloacal division observed in both *Shh*$^{-/-}$ embryos and those with early deletions of *Shh* might be driven by Ihh activity in the urorectal septum. The data also

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indicate that whereas Ihh may compensate for Shh in the anterior region of the cloaca, Ihh is not sufficient to direct septation of the entire cloaca or for development of the external genitalia.

Late disruption of Shh: loss of Shh between E13.5 and 15.5 causes hypospadias due to degeneration of ventral genital ectoderm

Formation of the perineum at E13.0 marks the end of the early phase of Shh signaling, in which cloacal septation and genital tubercle outgrowth are coupled. The finding that hypospadias occurs when Shh signaling is disrupted between E13.5 and 15.5 indicated that Shh activity is required for urethral tube formation before and during sexual differentiation of the genitalia (Fig. 1D-G and Fig. 5A-C). Structural analysis of mutant genitalia following disruption of Shh at these stages revealed that hypospadias was associated with disintegration of the epithelium on the ventral surface of the genital tubercle, at the junction of the urethral plate and the surface ectoderm (Fig. 5A,A′,B,B′). Loss of epithelial integrity on the ventral surface of the genital tubercle resulted in exposure of the underlying urethra, which developed an ectopic proximal opening in a region that normally forms a closed tube (Fig. 5A′-C′). Thus, Shh signaling is required to maintain the structural integrity of the ectodermal seam along the ventral margin of the urethral plate.

Shh signals directly to genital ectoderm to maintain a closed urethral tube

Given that inactivation of *Shh* causes disintegration of the ventral genital ectoderm, we tested the hypothesis that ectodermal cells respond directly to Shh to maintain a closed urethral tube. Although previous studies reported expression of the Shh target gene *Ptch1* in the mesenchyme adjacent to the urethral plate, little attention was given to the surface ectoderm (Haraguchi et al., 2001; Perriton et al., 2002). Therefore, we re-examined *Ptch1* expression between E12.5 and 16.5. In situ hybridization showed *Ptch1* mRNA in the ventral genital ectoderm adjacent to the urethral plate epithelium and in the mesenchyme around the urethra, but not in the urethral endoderm itself (Fig. 6A,B). Activation of *Ptch1* in genital ectoderm suggests that ectodermal cells respond directly to Shh from the urethral plate. In order to test whether Shh signaling to the ventral ectoderm is required for normal development of external genitalia, we blocked the ability of the ectoderm to respond to Shh by conditionally inactivating smoothened (*Smo*) in these cells. *Smo* encodes a seven-pass transmembrane receptor that transduces Shh signaling. In the absence of Shh, Ptch1 normally represses Smo activity. Based on our previous finding that the *Msx2cre* transgene is expressed in the genital tubercle ectoderm (Seifert et al., 2008), we crossed *Msx2cre;Smo*$^{C/+}$ males to *Smo*$^{C/C}$ females to produce *Msx2cre;Smo*$^{C/C}$ embryos (Fig. 6C,D). In control littermates lacking the *Msx2cre* allele, the genitalia were morphologically normal (Fig. 6C). By contrast, *Msx2cre;Smo*$^{C/C}$ mutants developed hypospadias, and the severity of the urethral tube defects was similar to those that occur when Shh is inactivated between E13.5 and 15.5 in *Shh*$^{creERT2/C}$ mutants (compare Fig. 6C,D with Fig. 1D-F). In *Msx2cre;Smo*$^{C/C}$, the surface ectoderm did not cover the ventral midline and, consequently, the proximal portion of the urethra was open at the base (Fig. 6D).

We then investigated whether this phenotype was the result of cell death at the ectodermal/endodermal junction. Apoptosis was not increased in this region in *Shh*$^{creERT2/C}$ embryos, although there did appear to be more cell death in the glans at E16.5 (Fig. 6E,F). Thus, it appears that ectodermal cells require Shh not for their survival, but for maintenance of epithelial integrity. The results indicate that ventral genital ectodermal cells respond directly to Shh, and this is

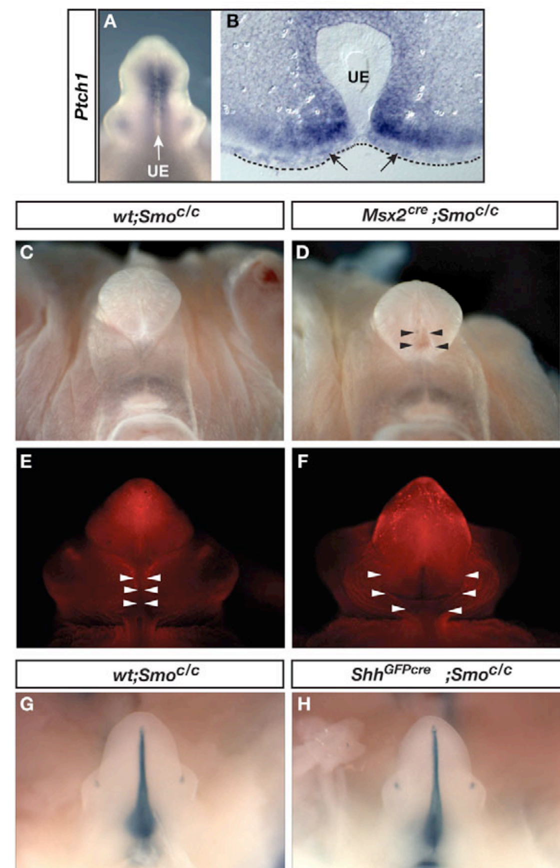

Fig. 6. Shh signals directly to genital ectoderm to maintain a closed urethral tube. Ventral views with distal towards the top in A,C-H; ventral is towards the bottom in B. (**A**,**B**) Genital tubercles showing *Ptch1* expression at E14.5 (A) and E12.5 (B). *Ptch1* transcripts are detectable in genital tubercle mesenchyme and in the ventral genital ectoderm (black arrows). The urethral epithelium is negative for *Ptch1*. (**C**,**D**) Control *Smo*$^{C/C}$ without cre (C) and *Msx2*^{cre}*;Smo*$^{C/C}$ (D) mouse genitalia at postnatal day 0 (P0). Arrowheads in D mark hypospadias. (**E**,**F**) Lysotracker Red staining at E16.5 shows that cell death is not increased along of the urethral seam (white arrowheads) of *Msx2*^{cre}*;Smo*$^{C/C}$ embryo. Note ectopic cell death in the glans (F). (**G**,**H**) Deletion of *Smo* in the endoderm of *Shh*^{GFPcre}*;Smo*$^{C/C}$ mutants (H) does not alter development of the perineum or urethral plate (compare H with control in G). UE, urethral epithelium.

necessary for the maintenance of structural integrity of the ectodermal epithelium and an adherent junction between the surface ectoderm and the urethral tube endoderm. Disruption of either Shh signaling from the endoderm or the ectodermal response to Shh results in separation of the ectoderm, loss of its junction with the urethral endoderm, and an ectopic opening of the urethral tube. This identifies a novel role of the genital ectoderm in urethral tube formation and provides the first demonstration that Shh mediates an epithelial-epithelial signaling interaction between the endodermal urethral plate and the surface ectoderm of the genital tubercle.

Shh-expressing endodermal cells of the cloaca and urethra are not Shh-responsive

Our finding that *Ptch1* is expressed in genital mesenchyme and ectoderm suggests that these two cell populations respond directly to Shh, which was confirmed by the temporal deletion of the Shh

signal and the removal of Smo from the ventral ectoderm. Although *Ptch1* was not detected in the cloacal or urethral endoderm, we observed striking defects in morphogenesis of the endodermal epithelium. This raised the question of whether Shh-expressing cells were signaling to themselves, or whether defective morphogenesis of the endodermal epithelium resulted from absence of a secondary signal feeding back to the endoderm from an adjacent cell population. To address the possibility that Shh is required by *Shh*-expressing endoderm, we blocked the ability of the cloacal and urethral endoderm to respond directly to Shh by conditionally removing *Smo* using a Shh^{GFPcre} allele. $Shh^{GFPcre};Smo^{c/c}$ embryos underwent normal septation of the cloaca and closure of the urethral tube, suggesting that endodermal cells do not respond directly to Shh (Fig. 6G,H). Thus, Shh functions in a paracrine manner in anorectal and external genital development, signaling to adjacent mesoderm and to ventral ectoderm. Taken together, these experiments suggest that Shh-responding cells, in turn, produce a secondary signal that feeds back to regulate endodermal morphogenesis.

DISCUSSION

Multiphasic role of Shh in anogenital development

Spatial and temporal dissection of Shh function shows that anogenital development can be divided into three distinct phases with respect to Shh activity. The earliest phase, which we term the initiation phase, is independent of Shh activity. This conclusion is supported by the finding that removal of Shh before the onset of genital budding does not disrupt initiation of cloacal septation or emergence of the paired genital swellings, which is consistent with our earlier analysis of genital development in $Shh^{-/-}$ mice (Perriton et al., 2002). During the anogenital phase, which follows the initiation of septation and genital budding, Shh facilitates continued septation of the cloaca and outgrowth of the genital tubercle. This phase is defined operationally by the coupling of cloacal and external genital defects, and it extends from E10.5 to 13.0. Without the early phase of Shh activity, genital development arrests before the formation of a genital tubercle and the embryonic cloaca is not partitioned into anorectal and urogenital sinuses. Shh drives cell proliferation in the mesoderm that divides the cloaca and gives rise to the genital tubercle. Thus, disruption of Shh activity during the anogenital phase leads to both agenesis of the external genitalia and persistence of the cloaca. Disruption of Shh function during the latter part of the anogenital phase results in less severe defects than those that occur when Shh is removed from the outset, but always affects both organ systems. The resultant structural defects include incomplete septation of the cloaca, failure of perineum formation, hypoplasia of the prepuce and incomplete development of the urethra. There is a complete failure of urethral tubulogenesis, although the malformation presents as persistent cloaca rather than a localized hypospadias. At the end of the anogenital phase, embryos have developed separate anorectal and urogenital sinuses, and the genital tubercle is separated from the anus by the perineum.

Disruption of Shh activity during the external genital phase, which begins around E13.5 and lasts through E15.5, results in localized defects of the external genitalia. All embryos in which *Shh* was removed during the external genital phase exhibited hypospadias. The longer that Shh was allowed to signal during the external genital phase, the more complete the closure of the urethral tube. Disruption of Shh during this phase also resulted in defective ventral growth of the prepuce, which normally envelops the glans and urethral plate, and results in a centrally positioned urethra. When Shh activity remained until E16.5, urethral tube formation was generally complete and the external genitalia developed normally, which suggests that the external genital phase ends around this time. Interestingly, disruption of Shh activity during either of these phases produces similar defects in male and female mice, indicating that Shh is required throughout the sexually indifferent period of anogenital development.

Early phase of Shh is required for cloacal septation

Previous studies of Hedgehog gene expression patterns in mouse embryos showed that both *Shh* and *Ihh* are expressed in the gut endoderm (Ramalho-Santos et al., 2000). Our observations that the cloaca undergoes a limited degree of septation and that *Ptch1* persists in the urorectal mesoderm even when Shh is inactivated before E10.5 suggests that Ihh may partially compensate for loss of Shh in these areas. Ihh is insufficient, however, to drive complete septation of the cloaca in the absence of Shh. In light of these findings, it is interesting that division of the rectum and the urogenital sinus appears more complete in $Shh^{-/-}$ mice than in $Gli2^{-/-};Gli3^{-/-}$ double mutants, which suggests that the limited degree of cloacal septation in $Shh^{-/-}$ mice does not occur when the hedgehog pathway is completely inactivated (Cheng et al., 2008). Recent examination of the zebrafish anogenital system, which undergoes septation, also found a requirement for Hedgehog signaling during cloacal development, with, the most severe anorectal defects occurring in *smu* (smoothened) mutants or in embryos treated with cyclopamine (Parkin et al., 2009). By contrast, embryos null for *shha* displayed similar defects to those observed in $Shh^{-/-}$ mice, with some *ptc1* expression persistent in the posterior hindgut. Taken together, data from zebrafish and mouse support the hypothesis that initial division of the primitive cloaca is Hedgehog-dependent.

Shh directs cloacal septation by regulating cell proliferation

The results presented here show that both division of the cloaca and outgrowth of the genital tubercle require Shh signaling from the cloacal endoderm. Disruption of Shh in the cloacal endoderm results in a lower proliferative index in mesenchyme at the anterior end of the urorectal septum. Decreased proliferation leads to reduced expansion of urorectal septum mesenchyme and, in turn, incomplete septation of the cloaca. This provides a cellular explanation for development of an anorectal fistula (incomplete separation of the anorectal and urogenital sinuses) in $Gli2^{-/-}$ mice

The group of cells commonly referred to as the 'urorectal septum' originates from mesoderm at the anterior and lateral margins of the cloaca. Expansion of these populations towards the cloacal membrane results in division of the cloaca into anorectal and urogenital sinuses. Our spatial analysis of BrdU incorporation in the urorectal septum mesenchyme shows that cell proliferation is highest at the posterior end of the urorectal septum, suggesting the existence of a posterior growth zone. Our experiments show that Shh from the adjacent endoderm plays a crucial role in stimulating proliferation of these mesenchymal cells.

Abnormal morphogenesis of hindgut epithelium is an indirect effect of Shh deletion

Although proliferation of the endoderm is not affected by Shh inactivation, loss of Shh signaling nonetheless disrupts epithelial morphogenesis in the terminal hindgut. OPT imaging revealed a relationship between the duration of Shh signaling and elongation

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of the intestine, with earlier truncations resulting in anterior displacement of the intestinal junction with the cloaca. Extension of the urethral plate was similarly affected by early termination of Shh signaling. The range of phenotypes observed when *Shh* was inactivated between E9.5 and 11.5 resembled those reported for *Gli2/3* double null mutants (Mo et al., 2001). *Gli2*$^{-/-}$ and *Gli3*$^{-/-}$ mutants display normal genital tubercle outgrowth, indicating redundancy in the genital tubercle, but *Gli2* is necessary for extension of the hindgut to the cloacal membrane (Cheng et al., 2008; Kimmel et al., 2000; Mo et al., 2001). Removal of one or both copies of *Gli3* in a *Gli2*$^{-/-}$ background does not increase the severity of the septation defect. This suggests that *Gli2* is required for normal development of the anorectum. If remodeling of the hindgut epithelium was mediated through an autocrine mechanism, in which *Shh*-expressing cells themselves responded to Shh, then removal of *Smo* in *Shh*-expressing cells would be expected to produce defects in cloacal septation and urethral tubulogenesis. However, Shh^{GFPcre};$Smo^{c/c}$ mutants have separate anorectal and urogenital sinuses and show no evidence of urethral tube defects, indicating that activation of the Hedgehog pathway in endodermal cells is not required for anogenital development. Our finding that *Ptch1* is expressed in the genital mesenchyme and the ventral genital ectoderm, but not the urethral or cloacal endoderm, supports the hypothesis that only the mesoderm and ectoderm respond directly to Shh. Thus, we propose that the defective morphogenesis of the endodermal epithelium in $Shh^{creERT2/C}$ mutants results from disruption of a secondary signal produced by adjacent cells in response to Shh signaling.

A novel role for surface ectoderm in hypospadias

The results presented here highlight a novel role of the ventral genital ectoderm in urethral tube closure. Interestingly, in both urethral tubulogenesis and neurulation, the surface ectoderm adjacent to the epithelial tube is required for development of a closed tube, although the mechanisms of tubulogenesis are distinct in these two structures. Neurulation involves bending and fusion of a flat sheet of neuroepithelium. Removal of ectoderm adjacent to the neural plate leads to a failure of neural tube closure (Hackett et al., 1997). Two explanations have been proposed for the role of the surface ectoderm in neurulation: (1) that it plays a mechanical role by generating forces required to drive bending of the neural folds (Hackett et al., 1997); and (2) that a very narrow strip of surface ectoderm attached to the neural plate serves as a signaling center to induce remodeling and flexure at the dorsolateral hinge points (Ybot-Gonzalez et al., 2002; Ybot-Gonzalez et al., 2007). In contrast to the neural tube, the distal urethra begins as a bilaminar epithelial plate, and tubulogenesis involves separation of the two layers of the plate along their basement membranes. In order to form a tube rather than an open sulcus, the urethral plate must remain joined at its dorsal and ventral margins. If the epithelial walls of the plate separate at their ventral margin, the tube opens and hypospadias results.

Deletion of Shh by tamoxifen injection at E13.5 resulted in hypospadias, and this was associated with loss of ventral ectodermal integrity. Our finding that the ventral ectoderm expresses *Ptch1* suggests that these cells are responsive to Shh. When either Smo is removed from the ectoderm or Shh is removed from the urethral endoderm after E13.5, the ventral ectoderm adjacent to the endodermal plate loses its structural integrity, the ventral margin of the urethral plate detaches and the tube opens, resulting in hypospadias. Thus, Shh signals directly to the ventral genital ectoderm, where it is required for the structural integrity of this tissue. Whether disruption of the ventral ectoderm leads to hypospadias due to mechanical forces or loss of reciprocal signaling requires further investigation.

The anogenital phase as a target period for ARM

The etiology of ARM is poorly understood. Our findings provide a temporal framework in which to examine how cloacal septation and genital outgrowth can be affected both by genetic and environmentally induced disruptions of Shh activity. Interestingly, many of the animal models of ARM have been linked to the Shh pathway. The anal atresia in pigs documented by Van der Putte and Neeteson (Van der Putte and Neeteson, 1984) has been traced to a heritable mutation on chromosome 15 that maps near the location of *Gli2* (Cassini et al., 2005). Both the *Shh* and *Gli2/3* mouse mutants have been suggested as models of the VACTERL complex (Arsic et al., 2002; Kim et al., 2001; Mo et al., 2001), and ARMs caused by treatment with either retinoic acid or etretinate (a vitamin A derivative) produce ARM that resemble the phenotypes presented here (Dawrant et al., 2008; Ioannides et al., 2003; Kubota et al., 2000; Kubota et al., 1998; Qi et al., 2002). A number of other mutations, such as ephrin B2 (*Efnb2* – Mouse Genome Informatics), *Trp63*, *Bmp7* and *Fgf10*, also result in anorectal and urogenital malformations, and in light of our findings it will be interesting to determine whether these pathways, as well as environmental contaminants, result in coordinated malformations of anorectal and urogenital organs because of a shared temporal window of sensitivity.

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Supplementary material
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