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Sonic hedgehog Signaling from the Urethral Epithelium Controls External Genital Development

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External genital development begins with formation of paired genital swellings, which develop into the genital tubercle. Proximodistal outgrowth and axial patterning of the genital tubercle are coordinated to give rise to the penis or clitoris. The genital tubercle consists of lateral plate mesoderm, surface ectoderm, and endodermal urethral epithelium derived from the urogenital sinus. We have investigated the molecular control of external genital development in the mouse embryo. Previous work has shown that the genital tubercle has polarizing activity, but the precise location of this activity within the tubercle is unknown. We reasoned that if the tubercle itself is patterned by a specialized signaling region, then polarizing activity may be restricted to a subset of cells. Transplantation of urethral epithelium, but not genital mesenchyme, to chick limbs results in mirror-image duplication of the digits. Moreover, when grafted to chick limbs, the urethral plate orchestrates morphogenetic movements normally associated with external genital development. Signaling activity is therefore restricted to urethral plate cells. Before and during normal genital tubercle outgrowth, urethral plate epithelium expresses *Sonic hedgehog* (*Shh*). In mice with a targeted deletion of *Shh*, external genitalia are absent. Genital swellings are initiated, but outgrowth is not maintained. In the absence of Shh signaling, *Fgf8*, *Bmp2*, *Bmp4*, *Fgf10*, and *Wnt5a* are downregulated, and apoptosis is enhanced in the genitalia. These results identify the urethral epithelium as a signaling center of the genital tubercle, and demonstrate that Shh from the urethral epithelium is required for outgrowth, patterning, and cell survival in the developing external genitalia.

Key Words: **external genitalia; urethra; polarizing activity; mouse embryo; Sonic hedgehog; Hox gene; apoptosis.**

INTRODUCTION

External genital defects are among the most common congenital anomalies in humans, with hypospadias, an ectopic ventral opening of the urethra, affecting one in every 125 male births (Kurzrock *et al.*, 1999a). Paradoxically, the molecular mechanisms controlling early stages of external genital development have not been intensively investigated, and even the embryology of this system is not well understood. The penis and clitoris develop from the genital tubercle, a medial outgrowth that emerges ventral to the cloaca during embryogenesis. Initially, the genital tubercles of males and females are morphologically indistinct. The early tubercle in both sexes has the potential to differentiate into either male or female genitalia, and exposure to androgens masculinizes the genitalia. In most mammals, males and females have a penile or clitoral skeleton, known as the os penis (or baculum) and os clitoris, respectively. Development of the os penis, which consists of a proximal segment of hyaline cartilage and membrane bone and a distal segment of fibrocartilage, is also dependent on androgen function (Murakami, 1987). Disruption of androgen signaling can result in feminization of the genitalia, which frequently includes hypospadias (Anderson and Clark, 1990; Baskin *et al.*, 1997; Kurzrock *et al.*, 2000; Brinkmann, 2001). Although androgens play key roles in external genital development, outgrowth and patterning of the genitalia are well underway before the onset of androgen production. For example, human genital development begins almost 2 weeks before the onset of testosterone synthesis by the testes (Kalloo *et al.*, 1993). Early patterning of the genital tubercle, therefore, must be controlled independently of androgens. Whereas the effects of androgens on mammalian genital development have been well studied

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(Glucksmann *et al.*, 1976; Murakami, 1987; Williams-Ashman and Reddi, 1991; Baskin *et al.*, 1997; Kurzrock *et al.*, 2000), the genetic mechanisms that regulate early development of the genital tubercle are not well understood.

Development of the genital tubercle has many similarities with the development of the vertebrate limb bud. Early development of both structures involves the initiation of budding from lateral plate mesoderm. Sustained distal outgrowth of the bud is controlled by the overlying epithelium. In a seminal paper, Murakami and Mizuno (1986) highlighted a striking parallel between the genital tubercle and the limb by demonstrating that removal of the epithelium from cultured rat genital tubercles results in stage-dependent loss of distal structures, with earlier removals resulting in more severe truncations. In the limb bud, the apical ectodermal ridge (AER) controls proximodistal outgrowth, and removal of the ridge results in loss of distal limb structures in a stage-dependent manner (Saunders, 1948; Summerbell, 1974). Interestingly, Murakami and Mizuno's experiments involved culturing genital mesenchyme after removal of surface epithelium and urethral epithelium. Surface epithelium is ectodermal in origin, whereas urethral epithelium is derived from endoderm (Kurzrock *et al.*, 1999a). Because both tissues were removed in those experiments, it is not clear whether the outgrowth signal was derived from ectoderm, endoderm, or both. Nonetheless, interactions between the epithelial and mesenchymal tissues in the tubercle are required for normal genital development.

Although the control of proximodistal outgrowth is beginning to be understood, little is known about the mechanisms that pattern the genital tubercle along its dorsoventral and proximodistal axes, or how patterning is coordinated with outgrowth. One possibility is that the tubercle contains a signaling center analogous to the polarizing region (or zone of polarizing activity; ZPA) of the limb bud. During limb development, the polarizing region, a specialized mesenchymal signaling center located at the posterior margin of the limb bud, controls anteroposterior patterning of the limb bud (Saunders and Gasseling, 1968; Tickle *et al.*, 1975). Transplantation of an additional polarizing region to the anterior margin of the chick limb bud causes anterior cells to be posteriorized, resulting in mirror-image duplication of the digits (Saunders and Gasseling, 1968; Tickle *et al.*, 1975). The extent of digit duplication is dose-dependent, with a weak polarizing signal (or fewer ZPA cells) resulting in duplication of the anteriormost digit (digit 2), and progressively stronger signals (or more ZPA cells) causing duplication of more posterior digits (digit 3 followed by digit 4) (Tickle, 1981). There has been no direct evidence for such a specialized signaling region in the genital tubercle; however, transplantation of the distal third of the genital tubercle from E12.5 mouse embryos (after removal of the ectoderm) to the anterior margin of the chick wing results in duplication of the most anterior digit (digit 2) (Dollé *et al.*, 1991; Izpisúa-Belmonte *et al.*, 1992). This discovery of weak polarizing activity in the genital tubercle provided further evidence that patterning mechanisms may be conserved in limbs and genitalia (Dollé *et al.*, 1991). The precise localization of polarizing activity within the tubercle is unknown; it may be a general property of the genital tubercle or restricted to a subset of cells within tubercle mesenchyme or urethral endoderm.

Similarities between external genital and limb development also extend to the molecular level. The posterior *Hox* genes are involved in patterning structures at the terminus of the main body axis (i.e., genitalia, anus, and posterior vertebrae) and at the terminus of the limbs (i.e., digits). Extensive studies of *Hoxd13* and *Hoxa13* have demonstrated their essential role in development of the genital tubercle and digits (Dollé *et al.*, 1993; Kondo *et al.*, 1997; Mortlock and Innis, 1997; Warot *et al.*, 1997; Zákány *et al.*, 1997). Interestingly, *Hoxd13* expression in the genitalia and limbs is controlled by the same regulatory element, perhaps reflecting evolutionary conservation of a common molecular mechanism of development (van der Hoeven *et al.*, 1996b). $Hoxd13^{-/-}$;$Hoxa13^{-/-}$ double mutants exhibit agenesis of the genital tubercle and digits, and heterozygosity for *Hoxa13* or *Hoxd13* causes patterning defects of the phallus and limbs (Dollé *et al.*, 1993; Warot *et al.*, 1997; Zákány *et al.*, 1997). In humans, mutations in the *HOXA13* gene are responsible for the range of phenotypes observed in Hand–Foot–Genital Syndrome, which affects development of the distal limbs and urogenital system (Goodman *et al.*, 2000). More recently, conserved functions of signaling molecules have been identified in limb and genital development. *Wnt5a* is expressed in a number of embryonic outgrowths, where it acts as a positive regulator of cell proliferation (Yamaguchi *et al.*, 1999). Expression of *Wnt5a* is graded from distal to proximal in both the limb bud and genital tubercle, and loss of Wnt5a function impairs distal outgrowth of these structures (Yamaguchi *et al.*, 1999). Recent work has added a molecular dimension to the pioneering experiments of Murakami and Mizuno, by demonstrating that the distal urethral epithelium of the genital tubercle can be replaced by fibroblast growth factors (FGFs) (Haraguchi *et al.*, 2000). *Fgf8* is normally expressed in these cells, and although removal of distal epithelium results in truncation of tubercle development, Haraguchi *et al.* (2000) demonstrated that outgrowth can be rescued by application of beads soaked in FGF8. This bears a striking resemblance to the role of FGF signaling in limb development. Several Fgf genes, including *Fgf4*, *Fgf8*, *Fgf9*, and *Fgf17*, are expressed in the apical ridge of the limb (Sun *et al.*, 2000), and application of FGF-soaked beads can rescue limb development after apical ridge removal (Niswander *et al.*, 1993; Crossley *et al.*, 1996). Thus, Fgf, Wnt, and Hox genes have important roles in genital development, and their functions in the genital tubercle appear to mirror their functions in the limb bud.

The function of Shh in external genital development is not yet understood. In the limb bud, *Shh* is expressed in the polarizing region, where it mediates the signaling activity of these cells (Riddle *et al.*, 1993). Shh acts in a dose-dependent manner to specify anteroposterior pattern in the

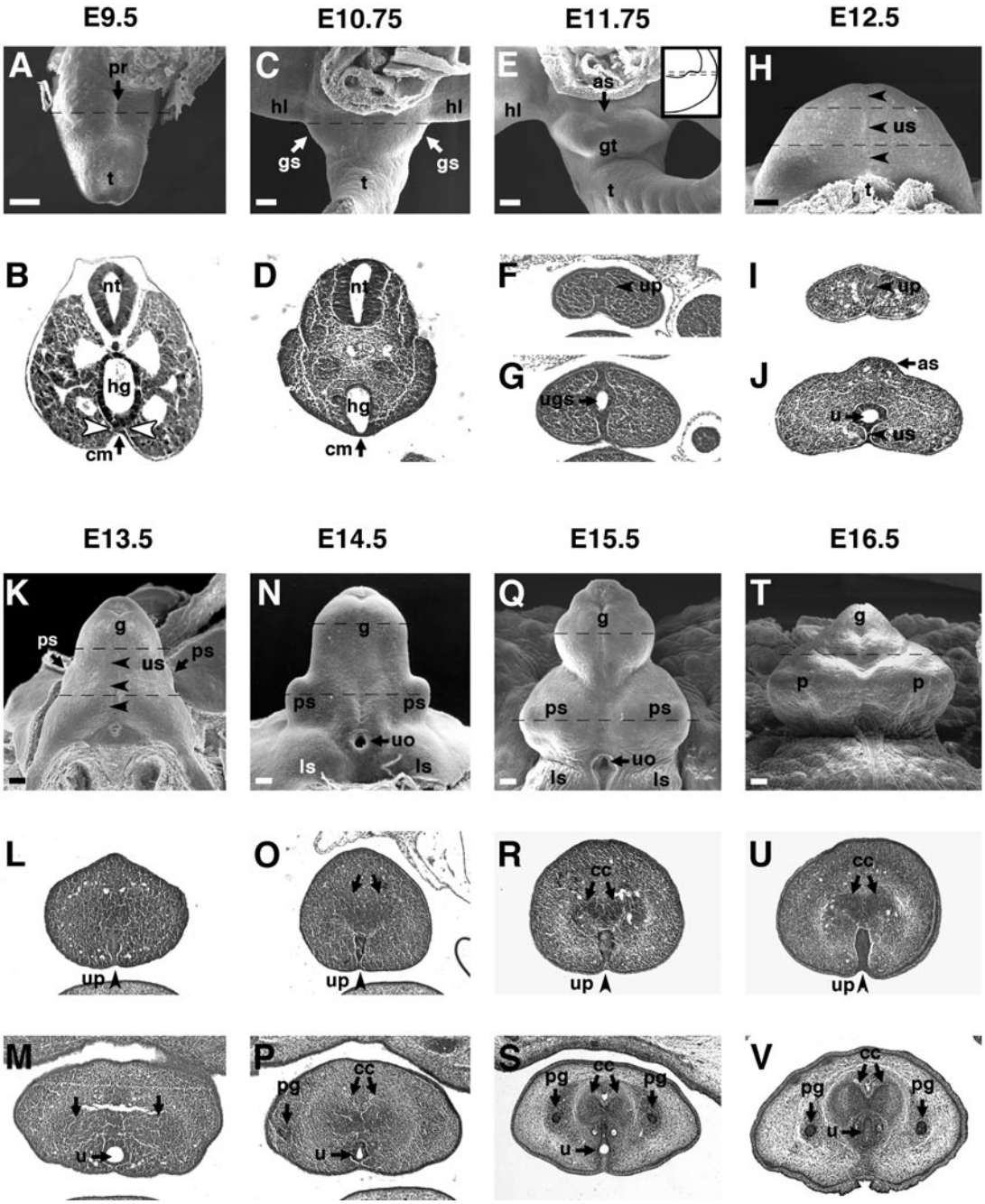

FIG. 1. Normal development of mouse external genitalia. SEMs and histological sections of mouse genitalia from E9.5 to E16.5. (A, C, E) Anterior is at the top. (H, K, N, Q, T) Distal is at the top. Dorsal is at the top in all sections. Sections are transverse to the genital tubercle, except for (B) and (D), which are transverse to the trunk. SEMs are ventral views of the genital tubercle, except for (A), (C), and (E), which are ventral views of the trunk. Broken lines on SEM images indicate the planes of section shown in images below. Scale bars, 100 μm. (A) E9.5 embryo showing region of proctodeum (pr). Budding of the external genitalia is not yet visible. (B) Transverse section through proctodeal region showing the junction of surface ectoderm and hindgut endoderm (white arrowheads) at the cloacal membrane (cm). (C) Early paired genital swellings (gs) are visible posterior to the hindlimbs (hl) at E10.75. (D) Section through genital swellings. Ectodermal–endodermal junction perisits between the swellings, at cloacal membrane. (E) Genital swellings are joined medially to form the singular genital tubercle (gt). An anterior swelling (as) is visible on the dorsal side of the tubercle. Inset shows planes of section below. (F, G) Sections transverse to the genital tubercle of E11.75 embryo. Sections 50 (F) and 100 μm (G) from the distal tip show extension of urogenital sinus (ugs) endoderm into the tubercle. (F) Lateral walls of the ugs are fused distally, forming the urethral plate (up). (G) Proximal region of the urogenital sinus remains patent. (H) Ventral view of the genital tubercle at E12.5 showing ventral urethral seam (us), indicating

limb, with high doses specifying posterior digits closest to the polarizing region, and lower doses specifying progressively more anterior digits further away (Yang *et al.*, 1997). Expression of *Shh* is maintained by Fgfs from the apical ridge, and Shh, in turn, feeds back to maintain ectodermal expression of these Fgfs (Sun *et al.*, 2000). Shh, like other members of the Hedgehog family, activates signal transduction by binding to the Patched (Ptc) transmembrane receptors (Goodrich *et al.*, 1996; Marigo *et al.*, 1996a,b; Pearse *et al.*, 2001). The binding of Hedgehog to Ptc alleviates repression of Smoothened (Smo), which, in turn, results in activation of signal transduction and subsequent expression of hedgehog target genes, including *Ptc* (Pearse and Tabin, 1998). Members of the bone morphogenetic protein (Bmp) family are also expressed in the limb bud, and it has been suggested that Bmps may be involved in relaying the hedgehog signal over long ranges (Yang *et al.*, 1997). More recent work, however, indicates that Shh itself can act over long- and short-range distances (Lewis *et al.*, 2001; Zeng *et al.*, 2001), and Bmp signaling may be involved in modulating *Ptc* expression (Pearse *et al.*, 2001). *Shh* expression has been detected in the urethral plate of the genital tubercle at E11.5 (Bitgood and McMahon, 1995; Haraguchi *et al.*, 2000), though the spatial and temporal dynamics of its expression pattern and its function in external genital development are unknown. Here, we report on experiments aimed at identifying signaling regions that operate within the genital tubercle. Since a staged account of external genital development is not available for the mouse, we first present a detailed embryological study of genital development. We also present evidence that localizes signaling activity within the genital tubercle to the urethral epithelium. Finally, we demonstrate that *Shh* plays a critical role in this signaling property and that it is required for normal external genital development.

MATERIALS AND METHODS

Transplantation of Genital Tubercle Cells to Chick Limb

CD-1 mice were time mated and embryos were staged according to Martin (1990) and Kaufman (1999). Genital tubercles were dissected from E11.5 and E12.5 embryos and incubated in either 2% trypsin or 1% dispase at 4°C for 20–40 min. After enzymatic treatment, tubercles were immediately transferred to ice-cold medium containing 10% fetal calf serum, and surface ectoderm was teased away and discarded [previous work (Dollé *et al.*, 1991) showed that ectoderm is not required for polarizing activity]. Urethral epithelium and genital mesenchyme were separated using tungsten needles, and both tissues were prepared for grafting. Limb buds of chick embryos at Hamburger–Hamilton stages 19–20 were prepared as hosts (Hamburger and Hamilton, 1951). Tungsten needles were used to tease away the anterior portion of the apical ectodermal ridge from the underlying limb mesenchyme to make a loop, and mouse tissue was grafted under the loop. Embryos were allowed to develop for 6 days after grafting and were then processed for cartilage staining and histology (see below).

Whole-Mount Cartilage Staining and Histology

Embryos were washed in 1× phosphate-buffered saline (PBS), fixed overnight in 5% trichloroacetic acid (TCA), and transferred to 0.1% alcian green in acid ethanol. Stained specimens were differentiated in acid ethanol, dehydrated in ethanol, and cleared in benzyl alcohol:benzyl benzoate for analysis of skeletal patterns. Strength of polarizing activity was quantified according to the method described by Tickle (1981), whereby duplication of digit 2 was scored as 25%, digit 3 as 50%, and digit 4 as 100%. Absence of extra digits was scored as 0. After photographing, limbs were given several changes of ethanol and 3 changes of toluene or xylene, and they were embedded in paraffin wax. Sections (10 μm) were cut and stained with either Mallory's triple stain or hematoxylin and eosin. Mice were fixed in 4% paraformaldehyde and processed for histology as above.

Scanning Electron Microscopy (SEM)

Embryos were washed briefly in PBS before fixation in 1% gluteraldehyde at 4°C. After postfixation in 1% osmium tetroxide, specimens were dehydrated in graded ethanols and placed in acetone. Specimens were then critical point dried, mounted on metal studs, sputter-coated with gold particles, and viewed under a Jeol JSM-T300 scanning electron microscope.

In Situ Hybridization

In situ hybridization was performed as described (Nieto *et al.*, 1996) with the following modifications: Embryos were bleached in 6% hydrogen peroxide in PBT for 1 h after rehydration, and

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the position of the urethral epithelium (arrowheads). (I, J) Growth of the anterior swelling displaces the urethra (u) ventrally in the proximal region of the tubercle (J). The urethral plate remains closed distally (I). (K) E13.5 genital tubercle shows early development of preputial swellings (ps). The glans (g) portion of the tubercle becomes distinct distally. Distal outgrowth of the dorsal side of the tubercle has advanced beyond the ventral side. (L, M) The urethral epithelium now lies along the ventral margin of the genital tubercle. (M) Localized mesenchymal condensations are seen dorsolateral to the proximal urethra (arrows). (N) E14.5 tubercle showing labioscrotal swellings (ls) and further outgrowth of preputial swellings. Proximal opening of urethra (uo) is indicated. (O) Faint mesenchymal condensations begin to appear distally (arrows). (P) Proximally, the paired, dense condensations of the corpus cavenosum (cc) are evident. Preputial glands (pg) are seen laterally, within the preputial swellings (section is slightly oblique so only one preputial gland is visible). (Q) E15.5 tubercle showing the increased size and ventral expansion of preputial swellings relative to the glans. The proximal urethral opening is still evident, but has begun to close. (R, S) Dense corporal bodies can now be seen distally (R) and proximally (S). (T) The proximal preputial swellings are fused ventrally to form the singular prepuce (p) that surrounds the glans. A proximal urethral opening can no longer be detected. (U) Section through the distal part of the glans, distal to prepuce. (V) The mature urethra is indicated centrally.

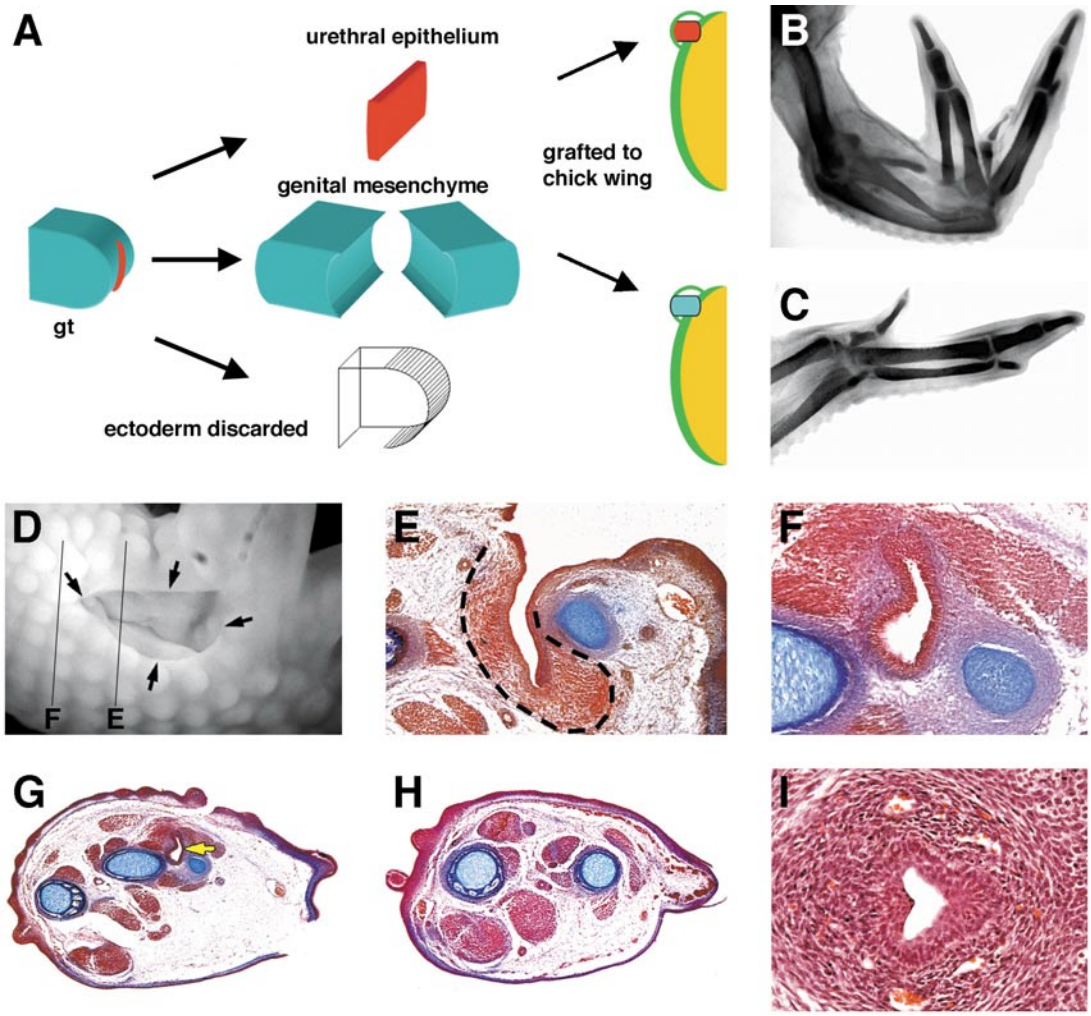


FIG. 2. Transplantation of urethral epithelium and genital mesenchyme to chick wing buds reveals signaling activity of the urethral epithelium. (A–H) Anterior is to the top. (I) Dorsal is to the right. (A) Schematic representation of transplantation experiments. (B, C) Chick wings stained with alcian green and cleared to show skeletal pattern following graft of urethral epithelium (B) and genital mesenchyme (C). (B) Wing shows mirror-image duplication of digits and an additional skeletal element in zeugopod following transplantation of urethral epithelium. (C) Normal wing skeleton that developed after genital mesenchyme graft. (D) Same wing as in (B), photographed prior to clearing shows furrow in skin (outlined by arrows). Parallel lines indicate plane of the sections shown in (E) and (F). (E) High magnification of section through the open, distal portion of the furrow reveals an epithelial invagination. Broken line indicates polarized muscle condensation around the invagination (F). High magnification of section proximal to the invagination showing a closed epithelial tube surrounded by a dense muscle condensation. (G) Low magnification of (F). Arrow indicates tube and associated muscle. (H) Low magnification of transverse section through a wing that received a graft of genital mesenchyme. Note absence of epithelial invagination. (I) Transverse section through a E16.5 penis showing urethra (u). Compare with (F).

incubated in glycine (2 mg/ml) for 5 min immediately after treatment with proteinase K (10 μg/ml) and prior to fixation. For the color reaction, 10% dimethyl formamide (DMF) was added to NBT/BCIP solution.

Detection of Apoptotic Cells

Acridine orange (AO) was used to localize apoptotic cells, as it is exclusively intercalated by cells undergoing apoptosis and not by those dying via necrosis (Abrams *et al.*, 1993). Embryos were rinsed briefly in PBS and incubated in AO (500 ng/ml) in PBS at 37°C for 30 min, then washed briefly in PBS. Samples were viewed and photographed immediately under epifluorescence, then photographed in the same plane of focus with bright-field illumination using DIC optics.

RESULTS

Embryology of the External Genitalia

Inconsistencies in the literature on mammalian external genital development have created confusion over several

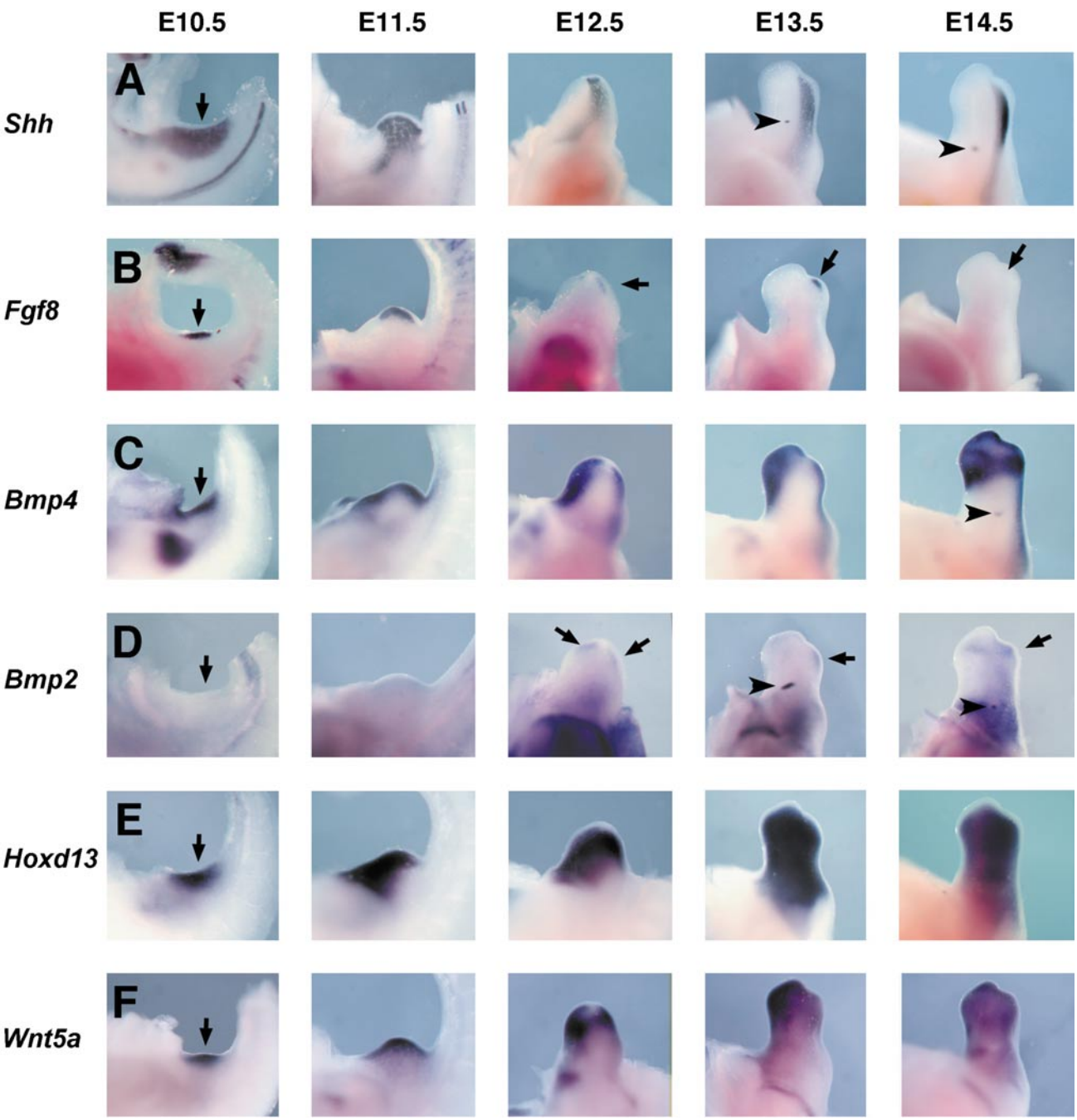


FIG. 3. Molecular analysis of the mouse genital tubercle. Images are lateral views, with the posterior end of the trunk (ventral side of the tubercle) to the right. The tail has been removed in all older and some younger embryos. Arrows in the E10.5 column indicate position of genital field. (A) *Shh* is expressed throughout the cloacal endoderm, prior to the onset of genital budding, and subsequently in the endoderm of the urogenital sinus and its derivatives, including urethral epithelium. Expression is also seen in the developing preputial glands from E13.5 (arrowheads). (B) *Fgf8* is expressed in the cloacal membrane at E10.5, and expression in the urethral plate at subsequent stages becomes progressively smaller and distally restricted (arrows). *Fgf8* expression is no longer detectable after E14.5. (C) *Bmp4* is expressed lateral to the cloacal plate at early stages, and becomes restricted to the dorsal and ventral surfaces of the genital tubercle by E12.5. Note strong expression seen on the dorsal surface at E12.5 becomes displaced distally by E14.5. *Bmp4* is also expressed in the preputial glands at E14.5 (arrowhead). (D) *Bmp2* is not detectable in the genital region until E12.5, when expression can be observed dorsally and ventrally (arrows). *Bmp2* is later expressed in the preputial swellings and preputial glands (arrowhead) and at the base of the tubercle. (E) *Hoxd13* is expressed in the cloacal and prospective genital regions at E10.5, and expression in the genital tubercle persists through E14.5. (F) *Wnt5a* is expressed in the prospective genital region at E10.5. As the tubercle grows out, expression becomes graded along the proximodistal axis, with the strongest expression at the distal tip.

aspects of the embryology of the genital tubercle, including the origin of the urethra and the morphogenetic movements involved in genital patterning (Wood Jones, 1910; Spaulding, 1921; Glenister, 1954; Kanagasuntheram and Anandaraja, 1960; Kluth *et al.*, 1988; Kurzrock *et al.*, 1999a). Some researchers, for example, have suggested that distal (glanular) regions of the human urethra form by invagination of apical ectoderm, whereas the proximal urethra develops from urogenital sinus endoderm (Glenister, 1954). This account is reproduced in several modern embryology texts (Moore, 1982; Larsen, 1997). Other descriptions of urethra development, from as early as 1912, indicate that the entire urethra is derived from endoderm (Felix, 1912; Kurzrock *et al.*, 1999a). Although aspects of external genital development have been studied in a number of mammals (Spaulding, 1921; Glenister, 1954, 1956; Kanagasuntheram and Anandaraja, 1960; Anderson and Clark, 1990; Kurzrock *et al.*, 1999a,b, 2000; Van der Werff *et al.*, 2000), a detailed, staged account of early genital development in the mouse has not been reported. Because an accurate and comprehensive picture of mouse external genital embryology is critical for understanding gene expression patterns and mutant phenotypes, we first carried out a detailed study of the embryology of the mouse external genitalia, beginning at embryonic day (E) 9.5, prior to the initiation of genital budding.

Scanning electron microscopy of the proctodeal region of mouse embryos at E9.5 indicates that genital outgrowth has not yet begun (Fig. 1A). Surface ectoderm abuts the endoderm of the hindgut at the cloacal membrane, and on either side of this junction, the surface ectoderm and gut endoderm are separated by lateral plate mesoderm (Fig. 1B). By E10, the proctodeal depression is less pronounced, but genital outgrowth is still not evident. At E10.5, a pair of lateral genital swellings can now be detected lateral to the cloacal membrane, at the level of the posterior edges of the hindlimb buds (data not shown). The swellings consist of mesenchyme covered by surface ectoderm, and a medial endodermally derived epithelium extends from the posterior hindgut to the cloacal plate. This region of the hindgut will form the roof of the urogenital sinus and, later, the urethra. Outgrowth of the paired swellings has increased by E10.75 (Fig. 1C), and the hindgut endoderm retains its contact with surface ectoderm of the cloacal membrane (Fig. 1D). By E11.75, the left and right swelling are contiguous and a single medial swelling has appeared anteriorly (Fig. 1E). These swellings form a single genital eminence (the genital tubercle) (Fig. 1E). As the genital tubercle emerges, the lateral walls of the urogenital sinus endoderm pinch together in the distal region of the tubercle, forming the urethral plate (Fig. 1F). In the proximal region of the tubercle, this endoderm remains as an open tube, which extends further proximally into the urogenital sinus (Fig. 1G). In the early genital tubercle, the urethral plate extends along the entire dorsoventral axis of the tubercle (Figs. 1F and 1G).

Between E11.5 and E12.5, the dorsal side of the tubercle grows at a faster rate than the ventral side, which displaces the urethral plate ventrally (Fig. 1J). At E12.5, the position of the urethral epithelium is marked by a seam along the ventral midline of the tubercle (Fig. 1H). The genital mesenchyme is still homogeneous and undifferentiated (Figs. 1I and 1J). By E13.5, faint mesenchymal condensations begin to form dorsal and lateral to the urethral epithelium, in the proximal region of the tubercle (Fig. 1M). By E14.5, these condensations have formed corporal bodies proximally (Fig. 1P) and condensations begin to appear distally (Fig. 1O), indicating that the pattern of mesenchymal differentiation proceeds from proximal to distal. Preputial swellings appear as secondary outgrowths on the lateral edges of the tubercle at E13.5 (Fig. 1K) and continue to grow laterally and ventrally to form the prepuce (Figs. 1N, 1Q, and 1T), within which the preputial glands are situated (Figs. 1P, 1S, and 1V). Rapid distal outgrowth of the dorsal side of the tubercle continues, which further displaces the urethra ventrally, and by E13.5, the dorsal side of the genital tubercle extends beyond the ventral side. Differential growth of the dorsal side results in formation of a pit at the distal boundary between the dorsal and ventral aspects of the tubercle, which marks the terminus of the urethral plate (Figs. 1K, 1N, 1Q, and 1T).

Labioscrotal swellings appear proximal to the preputial swellings from E14.5 (Fig. 1N). At the proximal limit of the urethral epithelium, between the labioscrotal and preputial swellings, the urogenital membrane ruptures at the position of contact with the urorectal septum (Fig. 1N). This hole is the only external opening of the urogenital system at this stage. The urethral plate persists in the glans at least through E16.5 (Figs. 1L, 1O, 1R, and 1U), although proximally it is an open tube, forming the proximal portion of the penile urethra (Figs. 1M, 1P, 1S, and 1V). By E16.5, the preputial swellings have fused to form a singular prepuce (Fig. 1T). Differentiation of the mesenchymal structures of the phallus appears to be centered around the urethral epithelium (Figs. 1O, 1P, 1R, 1S, 1U, and 1V). It is noteworthy that we did not detect, at any stage studied, an ectodermal ingrowth from the apex of the mouse genital tubercle. This contrasts with previous reports that the distal urethra forms by apical ectodermal invagination (Glenister, 1954; Moore, 1982; Larsen, 1997), but is consistent with observations in rat (Kluth *et al.*, 1988), dog (Kanagasuntheram and Anandaraja, 1960), and human (Kurzrock *et al.*, 1999a) embryos.

The Urethral Epithelium, but Not Genital Mesenchyme, Has Polarizing Activity

Previous work showed that transplantation of the mouse genital tubercle to the anterior margin of the chick limb bud resulted in duplication of the most anterior digit (digit 2), indicating that the genital tubercle has mild polarizing activity (Dollé *et al.*, 1991; Izpisúa-Belmonte *et al.*, 1992). Absence of ectoderm from these grafts suggests that polarizing activity could be restricted to either the urethral endoderm or genital mesenchyme, or alternatively, both tissues may be required to generate the polarizing signal.

We hypothesized that the genital tubercle is patterned by a specialized signaling region, and that polarizing activity is therefore restricted to a subset of cells. In order to test this hypothesis, we separated the genital tubercles of E11.5 and E12.5 mice into constituent components of surface ectoderm, genital mesenchyme, and urethral epithelium (Fig. 2A). Urethral epithelium and genital mesenchyme were transplanted under the apical ectodermal ridge of stage 19–20 chick wing buds, and genital ectoderm was discarded (Fig. 2A). Grafts of urethral epithelium induced mirror-image digit duplications in all cases ($n = 5$; Fig. 2B). In contrast, grafts of genital mesenchyme did not alter the normal anteroposterior pattern of digits 2-3-4 (numbers indicate digit identity from anterior to posterior) ($n = 4$; Fig. 2C). Four of five embryos receiving urethral epithelium grafts developed duplications of digit 3, and one embryo developed an additional digit 2. The digit patterns observed were 3-3-2-3-4, X-3-3-4 (with X representing a thick, forked digit of indeterminate identity), 3-2-3-4, and 2-2-3-4. In two cases, a third long bone of indeterminate identity developed in the zeugopod, anterior to the normal radius (Fig. 2B). The strength of polarizing activity of the urethral plate was therefore calculated to be 45% (see Materials and Methods) and frequency of duplication was 100% (Tickle, 1981). These results indicate that polarizing activity of the genital tubercle resides exclusively in the urethral epithelium.

Surprisingly, limbs that received urethral epithelium grafts also developed a single deep furrow anteriorly (Fig. 2D). The dorsal and ventral margins of the furrow gaped open distally and narrowed to a point proximally. Histological analysis revealed that the furrow formed as a result of epithelial invagination into the subjacent mesenchyme (Fig. 2E). At the proximal end of the furrow, the superficial dorsal and ventral edges fused to form an internalized epithelial tube, which extended further proximally into the limb (Figs. 2F and 2G). This suggested that the margins zip together in a proximal to distal sequence to give rise to the epithelial tube (Figs. 2F and 2G). In limbs receiving grafts of genital mesenchyme, no such invaginations or tubes were observed (Fig. 2H). This property appears to be specific to the urethral epithelium, since ZPA grafts to the limb have not been reported to induce epithelial remodeling. Following urethral plate grafts, we also observed a dense condensation of muscle cells around the epithelial furrow and tube (Figs. 2E–2G). These structures bore a striking resemblance to the urethra and surrounding mesenchymal condensations of the phallus (compare Figs. 2E and 2F with 2I).

Spatial and Temporal Patterns of Gene Expression during External Genital Development

We next sought to identify the molecules that mediate the signaling activity of urethral plate cells, and therefore we carried out an *in situ* hybridization screen of developmentally important genes, focusing on expression in the mouse genital tubercle. Of over 30 limb development genes examined, 26 were expressed in the genital tubercle, and only 2 signaling molecules, *Shh* and *Fgf8*, were restricted to the urethral plate (Figs. 3 and 4; and data not shown). Below, we present expression patterns for what we believe to be the more pertinent molecules.

Previous work has reported the presence of *Shh* in the urogenital sinus and distal urethral plate at E11.5 (Bitgood and McMahon, 1995; Haraguchi *et al.*, 2000). Our screen revealed that *Shh* is expressed in hindgut endoderm at least as early as E10, before the initiation of the paired genital swellings (data not shown). As budding is initiated, *Shh* expression persists throughout the urethral endoderm (Fig. 3A). By E11.5, *Shh* expression is detected in endoderm of the urogenital sinus, including the urethral epithelium (Fig. 3A). The separation of the cloaca into the urogenital and anorectal sinuses has occurred by this stage. By E12.5, the domain of *Shh* expression is restricted to the ventral side of the genital tubercle, within the urethral epithelium, and the expression domain is contiguous between the distal urethral plate and the proximal urogenital sinus (Figs. 3A, 4A, and 4B). By E13.5, differential growth of the dorsal side of the tubercle has displaced the entire *Shh* domain to the ventral margin of the tubercle (Figs. 3A and 4I). Ventral views of the tubercle also indicate that the *Shh* expression domain narrows with the urethral plate of the glans region, but spreads out proximally as the plate opens to form a lumen (Fig. 4H). A similar pattern of expression is seen in the E14.5 embryo (Fig. 3A). At E13.5 and E14.5, *Shh* is also expressed focally on each of the lateral preputial swellings, within the developing preputial glands (Figs. 3A and 4H). *Ptc1* is expressed at high levels in a broad domain of the mesenchyme surrounding the *Shh*-expressing urethral epithelium at E12.5 (Fig. 4C), but *Ptc1* expression was not detected within the urethral epithelium (Fig. 4D).

Fgf8 expression is also detected in the urethral plate, but only in a subset of the *Shh* expression domain (Figs. 3B, 4E, 4F, 4J, and 4K). At E10.5 and E11.5, the *Fgf8* domain is restricted to the anterior region of the early urethral epithelium (Figs. 3B and 4G), and as the tubercle grows out, it remains localized to the distal tip of the urethral plate (Figs. 3B, 4E, and 4J). At E12.5, *Fgf8* is still expressed distally, but the size of the domain decreases from this stage until it is no longer detectable after E14.5 (Figs. 3B, 4E, and 4J). Transverse sections show that expression of *Fgf8* is restricted to the ventralmost part of the distal urethral plate at E12.5 (Fig. 4F) and E13.5 (Fig. 4K).

Bmp4 is expressed in the genital field on either side of the cloaca, and later in mesenchyme flanking the urethra (Figs. 3C, 4L, and 4M). At E11.5, the anterior/dorsal swelling also expresses *Bmp4*, and rapid growth of the dorsal side of the tubercle is associated with gradual movement of this domain toward the distal tip (Fig. 3C). The strong ventral expression flanking the urethral plate resembles expression of *Ptc1* in this region (compare Figs. 4D and 4M). By E14.5, *Bmp4* is expressed in the glans and in the developing preputial glands (Fig. 3C). In contrast to *Bmp4*, *Bmp2* was not detected in the genital region prior to E12.5. At E12.5, *Bmp2* was expressed laterally in the proximal region of the

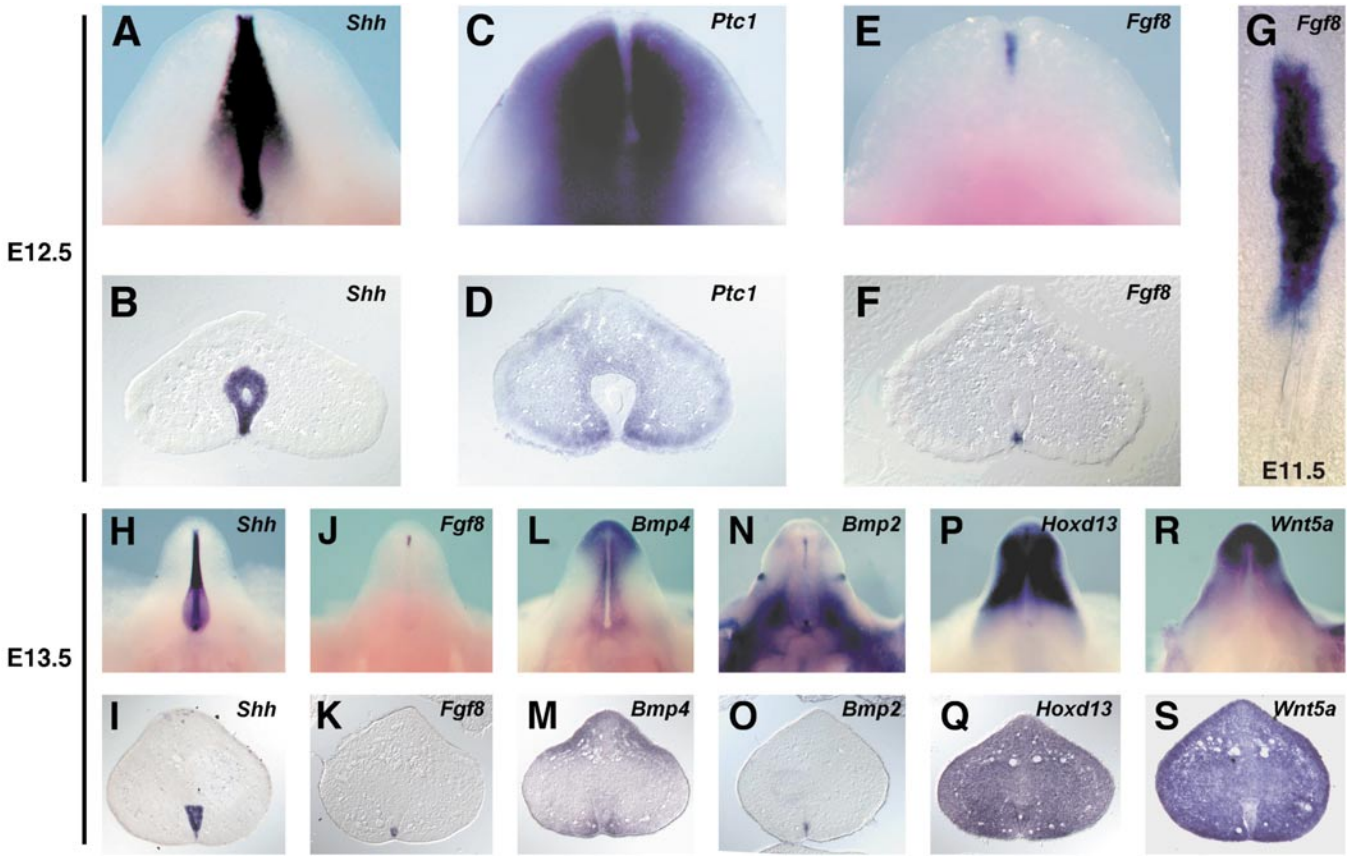


FIG. 4. Molecular analysis of the urethral epithelium. Genital tubercles are shown in ventral view, and sections are transverse to the tubercle. (A–F) E12.5. (G) E11.5. (H–S) E13.5. (A) *Shh* is expressed in the ventral midline of the genital tubercle. (B) Transverse section showing *Shh* expressed exclusively in urethral epithelium. (C) *Ptc1* is expressed in mediolateral gradient, with the strongest expression found medially, closest to the *Shh* domain. (D) Section shows *Ptc1* expression in the mesenchyme adjacent to the urethral epithelium, but not within the urethral epithelial cells. (E) *Fgf8* expression is detected at the distal tip of the urethral plate. (F) Section through distal tip of tubercle shows that *Fgf8* transcripts are restricted to cells at the ventral edge of the distal urethral plate. (G) High magnification of whole-mount genital eminence at E11.5 shows *Fgf8* expression localized to the anterior part of the urethral epithelium (anterior is at top). (H–S) Gene expression in genital tubercles at E13.5. (H, I) *Shh* expression is detected throughout the urethral epithelium, and laterally in the preputial glands (H). (J, K) *Fgf8* is detected in a small domain of the distal urethral plate (J), within the ventralmost cells (K). (L, M) *Bmp4* is detected in the mesenchyme flanking the *Shh* domain in the urethral plate, and in the glans. (M) *Bmp4* expression is also detected in dorsal mesenchyme subjacent to the dorsal ectoderm. (N, O) *Bmp2* is detected in the distal part of the urethral plate, in the developing preputial glands, and in the mesenchyme of the proximal tubercle. Faint expression is also detected in the distal mesenchyme of the glans, on either side of the urethral plate. (P, Q) *Hoxd13* is detected throughout the genital tubercle, although expression is weakest proximally. (R, S) *Wnt5a* is expressed throughout the distal mesenchyme of the glans, and expression weakens proximally. Laterally, strong expression is seen in the emerging preputial swellings (R).

tubercle and dorsally and ventrally in the distal region of the tubercle (Fig. 3D; and data not shown). By E13.5, a number of distinct *Bmp2* expression domains have appeared, including the preputial glands, the distal urethral plate, the proximal mesenchyme, and the ventral swellings of the glans (Figs. 3D, 4N, and 4O).

Hoxd13 expression in the genitalia has been described previously (Dollé *et al.*, 1991). We examined the dynamics of *Hoxd13* expression with respect to the other genes studied here. Prior to the initiation of outgrowth, *Hoxd13* is expressed throughout the cloaca and genital field at E10.5 (Fig. 3E). Strong expression is maintained in the genital tubercle at subsequent stages (Figs. 3E, 4P, and 4Q). Consistent with previous observations of histological sections analysed with ^{35}S-labeled riboprobes (Dollé *et al.*, 1991), we detected the most intense staining distally, in the glans, at E13.5 in whole mounts and section (Figs. 4P and 4Q).

Wnt5a is also expressed in the mesenchyme of the genital field at E10.5, during initiation of the genital swellings (Fig. 3F). From E11.5, expression in the genital tubercle is graded,

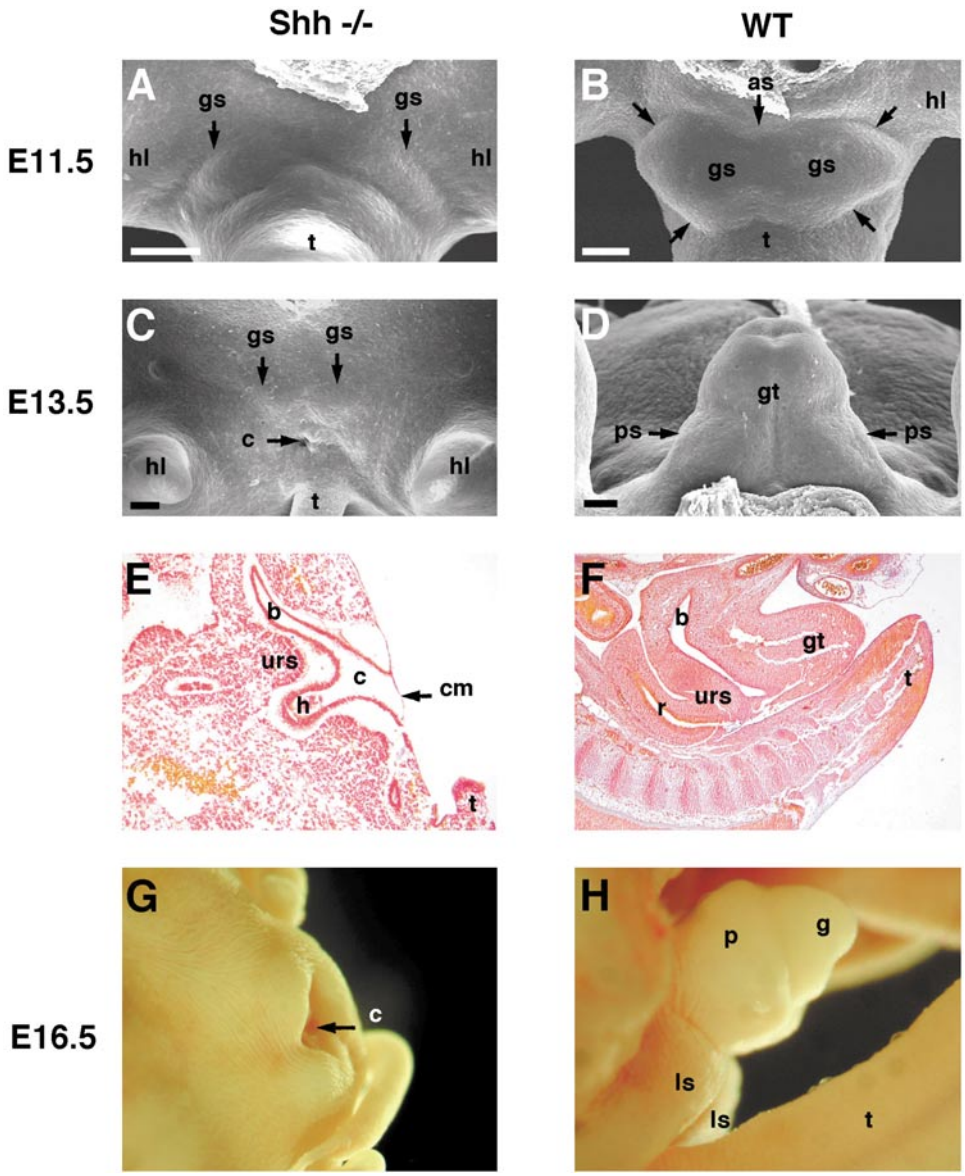


FIG. 5. Loss of Shh function results in agenesis of the external genitalia. Anterior is to the top, except in (D), where distal is at the top. (A–D) SEMs of genital region of *Shh*$^{-/-}$ (A, C) and wild type (B, D) mice at E11.5 and E13.5. Note that genital swellings (gs) are initiated but fail to form a tubercle in Shh null mutants (A, C). Bars, 100 μm. (A) Small genital swellings (gs) are visible at the base of the tail (t) between the hindlimbs (hl) of the mutant. (B) Wild type E11.5 embryo exhibits more advanced genital swellings, which have fused to the anterior swelling (as) to form a genital tubercle. (C) *Shh*$^{-/-}$ embryo at E13.5 with very slight genital swellings remaining anterior to the cloaca. (D) Wild type E13.5 embryo showing the ventral surface of the genital tubercle (gt) after the tail has been removed. The tubercle is well-developed and preputial swellings (ps) are beginning to emerge laterally. (E) Midsagittal section taken through an E13.5 *Shh*$^{-/-}$ embryo shows a persistent cloaca covered by a thin cloacal membrane (cm). The bladder (b) and hindgut (h) partially separated by the urorectal septum (urs). The proximal portion of the tail (t) can be seen at the bottom right. (F) Midsagittal section taken through wild type E13.5 embryo showing almost complete separation of the bladder and urogenital sinus from the rectum (r). (G) Light micrograph of E16.5 *Shh*$^{-/-}$ embryo. External genitalia are absent and a single cloacal opening is indicated (c). (H) Light micrograph of E16.5 wild type embryos showing the glans (g) portion of the genitalia surrounded proximally by the prepuce (p). Labioscrotal swellings (ls) are situated between the tail and the genital tubercle.

with the strongest expression at the distal tip (Fig. 3F) (Yamaguchi *et al.*, 1999). Ventral views of E13.5 genitalia show that *Wnt5a* expression in the glans and the preputial swellings is slightly stronger than other regions of the tubercle (Fig. 4R), consistent with previous work showing that *Wnt5a* is expressed in distal outgrowths (Yamaguchi *et al.*, 1999).

Taken together, these results reveal dynamic patterns of gene expression in the external genitalia. Additionally, they highlight a relationship between fine-scale morphogenesis of the tubercle and region- and tissue-specific patterns of gene expression.

Shh Is Essential for External Genital Development

Shh is known to mediate the signaling activity of several organizing and polarizing centers in vertebrate embryos, including the polarizing region of the limb bud (Riddle *et al.*, 1993), the floor plate of the neural tube (Echelard *et al.*, 1993), the node (Selleck *et al.*, 1996; Levin *et al.*, 1997), and the enamel knot of the tooth (Vaahtokari *et al.*, 1996). In light of our findings that the urethral epithelium has polarizing activity, can orchestrate tissue movements characteristic of genital morphogenesis, and expresses *Shh*, we hypothesized that Shh signaling from the urethral epithelium may regulate patterning and/or outgrowth of the external genitalia. To test this hypothesis, we examined whether a loss-of-function mutation in the mouse *Shh* gene (Chiang *et al.*, 1996) affects morphogenesis of the genitalia. We found that, in $Shh^{-/-}$ mice, genital development is severely disrupted, resulting in agenesis of the external genitalia (Figs. 5A, 5C, 5E, and 5G). Analysis of $Shh^{-/-}$ mutant mice by scanning electron microscopy showed that, at E11.5, a pair of faint genital swellings is present anterior to the cloacal membrane (Fig. 5A). In wild type mice at this stage, the swellings have undergone further outgrowth and are joined with the anterior swelling to form the genital tubercle (Fig. 5B). The swellings observed in $Shh^{-/-}$ mutants are reflective of the earliest stages of genital development (detailed in our description above), indicating that initiation of budding occurs in the absence of Shh, but outgrowth does not progress. At E13.5, $Shh^{-/-}$ mutant mice show no evidence of a genital tubercle, and the original paired swellings are still discernable anterior to the cloacal orifice (Fig. 5C). By contrast, in wild type mice at E13.5, the genital tubercle is well developed and the secondary preputial swellings are beginning to emerge from the lateral edges of the tubercle (Fig. 5D).

Histological analysis of $Shh^{-/-}$ mutant and wild type mice at this stage confirmed that external genitalia fail to develop in $Shh^{-/-}$ mutant mice, and revealed that mutants also have severe defects of the posterior hindgut and urinary tract (compare Figs. 5E and 5F). Sections of wild type mice at E13.5 show that the genital tubercle is well developed, and the bladder and urethra are separated from the rectum by the urorectal septum, which extends to the posterior end of the trunk (Fig. 5F). In contrast, $Shh^{-/-}$ mutant mice have a persistent cloaca, rather than a separate posterior urogenital sinus and rectum, and this is associated with incomplete development of the urorectal septum (Fig. 5E). Finally, to determine whether genital outgrowth had indeed arrested or, alternatively, may have been severely delayed, we examined $Shh^{-/-}$ mutant mice at E16.5 (Fig. 5G). Consistent with our observations at earlier stages, external genitalia were absent in $Shh^{-/-}$ mutants at E16.5 (compare Figs. 5G and 5H). Additionally, a single cloacal opening was present, indicating that the anorectal and urogenital sinuses remain undivided and have a common outlet from the cloaca (Fig. 5G). These results show that Shh is required for outgrowth and patterning of external genitalia, as well as posterior division of the urogenital sinus and rectum, but Shh is not required for the earliest initiation of paired genital swellings.

Shh Regulates Expression of Fgf8, Fgf10, Bmp2, Bmp4, and Wnt5a in the Genital Tubercle

Previous studies have shown that Hoxd13, Hoxa13, and Wnt5a are involved in outgrowth of the genital tubercle (Kondo *et al.*, 1997; Warot *et al.*, 1997; Yamaguchi *et al.*, 1999), and Fgf10 plays a role in ventral fusion of the prepuce in the glans penis and clitoris (Haraguchi *et al.*, 2000). FGF8-soaked beads can rescue outgrowth of genital tubercles following excision of the distal urethral plate epithelium (Haraguchi *et al.*, 2000), although its endogenous function in genital development is unclear. Proximity of these expression domains to the *Shh* domain in the urethral plate (see Figs. 3 and 4), together with their established genetic interactions in the limb (Cohn and Bright, 1999), raised the possibility that Shh may regulate transcription of these genes in the genital tubercle. We therefore examined gene expression in the genital region of $Shh^{-/-}$ mice. In wild type mice, *Fgf8* is expressed at high levels in the distal urethral plate until E14.5 (Figs. 3B, 6A, and 6C). In $Shh^{-/-}$ mutants at E11.5, we detected a small domain of *Fgf8* expression at the anterior margin of the cloacal epithelium (which normally gives rise to urethral plate) between the paired genital swellings (Fig. 6B). By E13.5, *Fgf8* could not be detected in this region (Fig. 6D), and analysis of E12.5 mutants indicated that *Fgf8* had already been downregulated (data not shown). *Bmp4* is normally expressed in genital mesenchyme flanking the urethral plate and, at later stages, throughout the glans (Figs. 6E and 6G). In $Shh^{-/-}$ mutants, weak expression of *Bmp4* was observed in two stripes lateral to the cloacal epithelium (Fig. 6F), but this was no longer detectable by E13.5 (Fig. 6H). Thus, transcription of both *Fgf8* and *Bmp4* is initiated in appropriate regions, but expression domains are reduced in size and intensity, and neither is maintained in the absence of Shh.

Neither *Wnt5a* nor *Fgf10* could be distinguished from low-level staining in the ventral body wall of $Shh^{-/-}$ mice at E11.5, indicating that if these genes are indeed expressed, then they are below levels of detection. In contrast to the genital region, strong expression of both genes was seen in

distal hindlimb buds (Figs. 6J and 6N). At E13.5, weak expression of both *Wnt5a* and *Fgf10* was detected in two small domains on the posterior, lateral edges of the cloaca (Figs. 6L and 6P). *Bmp2*, which first becomes apparent in the wild type tubercles at E12.5 (Fig. 3D) and localizes to the distal urethral plate and genital mesenchyme at E13.5 (Fig. 6U), is undetectable in the genital region of $Shh^{-/-}$ mutants (Fig. 6V). Like *Wnt5a* and *Fgf10*, *Bmp2* transcripts were detected posterior and lateral to the cloaca. It is noteworthy that, in Shh null mutants at this stage, the paired genital swellings are situated anterior to the cloaca (Fig. 5C), and this region lacks expression of *Wnt5a*, *Fgf10*, *Fgf8*, *Bmp2*, and *Bmp4* (Fig. 6).

Surprisingly, $Shh^{-/-}$ mutants exhibit strong expression of *Hoxd13* anterior to, and on either side of, the cloacal membrane at E11.5 (Fig. 6R). At E13.5, *Hoxd13* was expressed throughout the genital region anterior to the cloaca (Fig. 6T). In wild type embryos, *Hoxd13* is expressed throughout the genital mesenchyme before and during tubercle outgrowth (Fig. 3E). At E11.5, *Hoxd13* expression in $Shh^{-/-}$ mutants resembled the normal pattern of expression at E10.5 (Fig. 6R; and data not shown). Comparison of *Hoxd13* expression patterns in mutant and wild type mice at E13.5 also revealed a striking similarity in the position of the *Hoxd13* expression domains relative to their respective cloacal and urethral openings (compare Figs. 6S and 6T). Thus, *Hoxd13* does not require Shh in order to be expressed at high levels in the genital region. Taken together, these results, along with the observation that genital budding is initiated, suggest that the external genital field is specified, but the molecular machinery for outgrowth is not maintained in the absence of Shh.

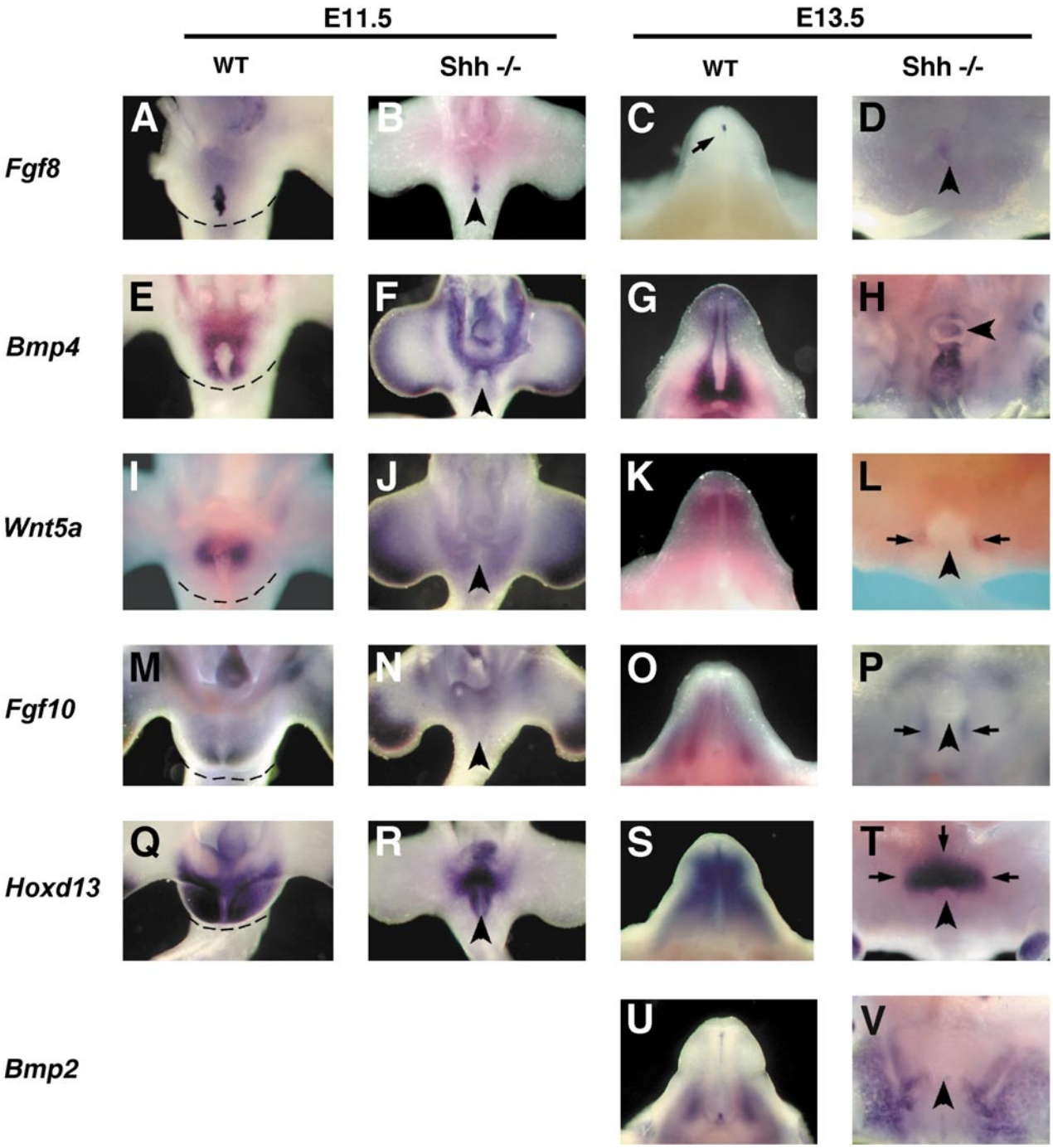
Shh Positively Regulates Cell Survival in External Genitalia

In addition to its role as a polarizing signal, Shh promotes cell survival in a number of cell types, including cranial neural crest cells (Ahlgren and Bronner-Fraser, 1999), myogenic and chondrogenic lineages of the somites (Teillet *et al.*, 1998), limb bud mesenchyme (Sanz-Ezquerro and Tickle, 2000), and specific neuronal populations (Miao *et al.*, 1997). To explore the possibility that *Shh* signaling by the urethral endoderm acts as a survival signal in the genital tubercle, we examined apoptosis in the genitalia of wild type and $Shh^{-/-}$ mutant mice using acridine orange (AO), which selectively labels apoptotic cells (Abrams *et al.*, 1993). In genital tubercles of E11.5 wild type mice, apoptosis was localized to the urethral plate (Fig. 7A). In the cloacal region of $Shh^{-/-}$ mice at E11.5, AO staining was more intense and covered a broader area than in wild type embryos, indicating that apoptosis is enhanced (Fig. 7B). In the wild type genital tubercle at E13.5, a dimple appears around the distal tip of the urethral plate, in the region of the future urethral meatus (Fig. 1K). AO staining revealed that this is a site of localized apoptosis (Fig. 7C). At the proximal end of the wild type genital tubercle, low levels of apoptosis were detected predominantly in the urethral epithelium (Fig. 7D), but apoptosis was not detected between this region and the distal tip (data not shown). By contrast, in $Shh^{-/-}$ mutants at E13.5, apoptotic cells are found at high density in genital mesenchyme and throughout the urethral epithelium (Figs. 7E and 7F). Mesenchymal cell death is particularly striking in these embryos, as the region of cell death appears to encompass the entire genital field anterior and lateral to the urethral plate (Fig. 7F). These results indicate that, in addition to regulating outgrowth signals, Shh is required for cell survival in the genital tubercle.

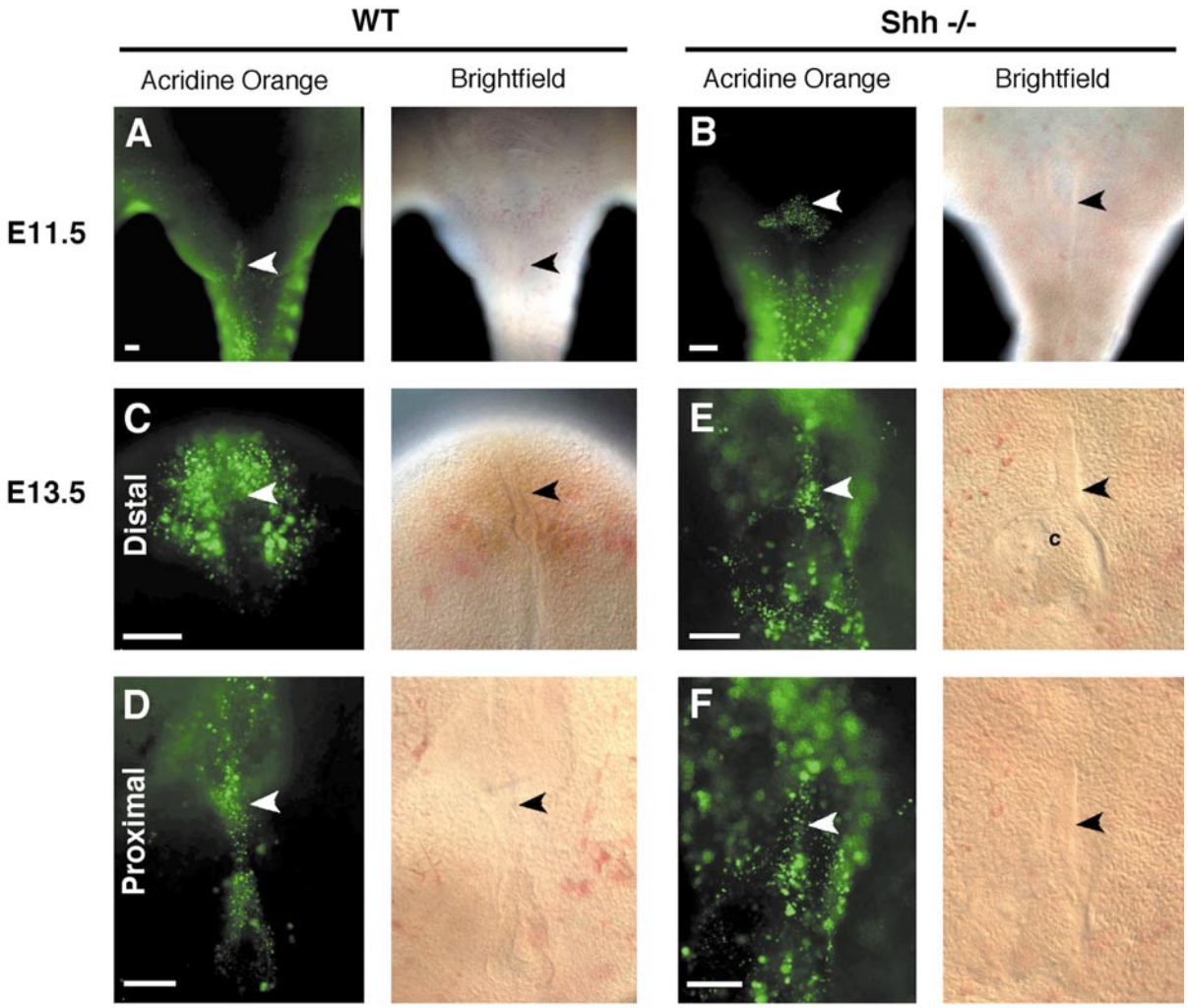
DISCUSSION

Our results identify the urethral epithelium as a signaling region in the genital tubercle, implicate Shh as the key urethral signal, and show that Shh is essential for external genital development. Although *Shh* expression in the cloacal endoderm can be detected prior to the initial budding of the genital swellings, it is not essential for establishment of the genital field or initial stages of outgrowth. We find that, in the absence of Shh, genital budding is initiated but outgrowth arrests before formation of the genital tubercle. With the exception of *Hoxd13*, none of the genes that we examined are maintained in the genital region of $Shh^{-/-}$ mice. We also find that Shh is required for cell survival in the genital tubercle. Similar conclusions regarding the role of Shh in genital development have been reached by Haraguchi *et al.* (2001) in an independent study. Finally, our

FIG. 6. Molecular analysis of $Shh^{-/-}$ embryos. Posterior is to the bottom, and tails have been removed from E13.5 wild type embryos to show the ventral surface of the genital tubercle. Broken lines outline the distal boundary of the genital tubercle in the E11.5 wild type embryos. Arrowheads indicate cloacal region of mutant embryos. (A–D) *Fgf8* is expressed in the urethral plate at E11.5 of wild type genitalia (A) and in the cloacal epithelium of the Shh null embryo (B). Note that the expression domain in the $Shh^{-/-}$ embryos is reduced in size and intensity. At E13.5, *Fgf8* expression is restricted to a small region distally in the wild type (C) (see arrow), and is not detectable in the genital region of the mutant embryo at E13.5 (D). (E–H) *Bmp4* is expressed in the mesenchyme surrounding the urethral plate in the wild type embryo at E11.5 (E), and is expressed in a similar pattern in the mutant, although expression is weaker (F). At E13.5, *Bmp4* is normally expressed immediately lateral to the urethral plate and throughout the entire distal region (G). (H) In $Shh^{-/-}$ mutants, no expression is detected in the genital region, although transcripts can be seen within, and posterior to, the cloaca. (I–L) *Wnt5a* is normally expressed immediately lateral to the urethral plate at E11.5 (I); however, in $Shh^{-/-}$ mutant mice, expression could not be detected above background



levels in this region (J). By E13.5, *Wnt5a* expression is seen throughout the genital tubercle of the wild type and is strongest distally (K). (L) In $Shh^{-/-}$ mutants, *Wnt5a* expression is seen only in two small domains at the posterior edges of the cloaca (see arrows). (M–P) *Fgf10* is expressed lateral to the urethral plate in the wild type embryo at E11.5 (M). (N) *Fgf10* could not be detected in the genital region of $Shh^{-/-}$ mutants, although expression is visible in distal hindlimbs (N). (O) At E13.5, *Fgf10* is expressed in the preputial swellings and in the mesenchyme flanking the urethral plate of the glans. (P) In $Shh^{-/-}$ mutants, *Fgf10* is expressed in two stripes posterior to the cloaca (see arrows), similar to the pattern observed for *Wnt5a* (compare with L). (Q–T) *Hoxd13* is expressed throughout the entire genital tubercle at E11.5 (Q), and strong expression is also detected in $Shh^{-/-}$ mutants, although in a smaller domain (R). At E13.5 *Hoxd13* is expressed throughout the genital tubercle of wild type mice (S), and throughout the genital region anterior to the cloacal opening in the mutant (T; see arrows). (U) *Bmp2* is expressed in the distal urethral plate and proximal tubercle mesenchyme of the wild type embryos at E13.5. (V) *Bmp2* expression is not detectable in the genital region (anterior to the cloaca) of $Shh^{-/-}$ mutants.



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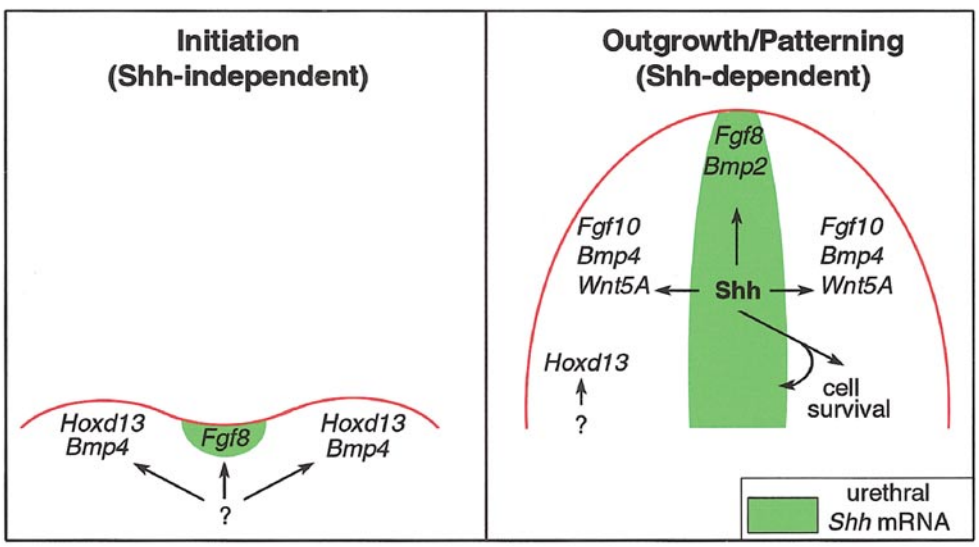

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analysis of external genital development highlights areas of genital embryology that differ significantly from descriptions in the classical literature and modern embryology texts.

Embryology of the External Genitalia

The literature concerning external genital development is controversial, owing largely to inconsistent descriptions of genital development (see Introduction and Kurzrock *et al.*, 1999a for review). Development of the penile urethra has been a particular area of controversy, with such fundamental issues as the embryonic origin of the distal urethral plate and morphogenesis of the tubular urethra remaining unclear. Although modern embryology texts describe development of the urethra as a two-step process in which the proximal urethra forms by fusion of the urogenital folds around the urethral plate, and the distal urethra forms by invagination of the apical ectoderm (Moore, 1982; Larsen, 1997), we find no evidence for an apical ectodermal invagination in the mouse, and other researchers have demonstrated that it does not occur in other mammals (Kanagasuntheram and Anandaraja, 1960; Kluth *et al.*, 1988; Kurzrock *et al.*, 1999a). The Y-shaped depression that appears at the tip of the tubercle results not from apical ectodermal involution, but rather from growth of the dorsal side of the tubercle beyond the distal limit of the ventral side of the tubercle. Indeed, the dynamic patterns of gene expression in the tubercle show a consistent displacement of gene expression domains from the dorsal to the ventral side of the glans. The urethral endoderm is present from the proximal limit to the distal tip of the genital tubercle from the earliest stages of tubercle outgrowth. Our finding that *Shh* expression is contiguous throughout the endodermal urogenital sinus and urethral epithelium suggests that the entire urethra develops from *Shh*-expressing endoderm. Collectively, these data indicate that the classical account of urethral development, in which two separate processes (ventral endodermal and distal ectodermal involutions) generate the urethra, does not apply to the mouse. As an alternative, we favor a model in which the entire urethra of the mouse is derived from endodermal urethral epithelium, with the distalmost (glanular) region of the urethral plate being the last to cavitate.

Signaling Activity of the Urethral Epithelium

The abilities of the urethral epithelium to polarize limb bud mesenchyme, orchestrate epithelial tubulogenesis, and generate mesenchymal condensations similar to those observed in the genitalia suggests that the urethral epithelium may function as an organizing region in the genital tubercle. Differentiation of mesenchymal structures in the tubercle is polarized around the urethral endoderm, which, taken together with the experimental results reported here, raises the possibility that the urethral epithelium controls dorsoventral polarity, in addition to outgrowth, of the tubercle. The idea that the urethral epithelium functions in the genital tubercle like the polarizing region (ZPA) functions in the limb bud is further supported by our observation that Shh signaling by the urethral plate maintains expression of *Fgf8* in the distal urethral epithelium and *Bmp2* and *Bmp4* in the mesenchyme, thereby mirroring the interaction between Shh in the polarizing region and Fgfs in the apical ridge of the limb (Zuniga *et al.*, 1999; Sun *et al.*, 2000; Chiang *et al.*, 2001). Previous findings that the distal tip of the urethral plate has an apical ridge-like function in maintaining tubercle outgrowth, possibly via Fgf8, and that *Hoxa13* and *Hoxd13* are required for development of the genital tubercle and autopod, extend the parallels between limb and genital patterning mechanisms (Murakami and Mizuno, 1986; Kondo *et al.*, 1997; Kurzrock *et al.*, 1999b; Haraguchi *et al.*, 2000). We think it unlikely, however, that Shh alone can account for all of the effects observed following transplantation of urethral epithelium, as direct application of Shh to chick limb buds leads to digit duplications, but not epithelial invagination. Interestingly, Shh signaling from gut endoderm regulates splanchnic muscle development (Litingtung *et al.*, 1998), and Shh regulates skeletal muscle development in the trunk and limbs

FIG. 7. Apoptosis in wild type and $Shh^{-/-}$ embryos. Embryos at E11.5 and E13.5 stained with AO to reveal apoptotic cells (which appear as fluorescent dots). Each fluorescence micrograph is paired with a light micrograph in the same plane of focus. Scale bar, 100 μm. Anterior is to the top. Arrowheads indicate lateral edge of urethral and cloacal epithelia. (A) E11.5 wild type embryo showing apoptotic cells in the urethral plate. (B) $Shh^{-/-}$ embryo of same stage showing a broad, dense domain of apoptosis in the cloacal epithelium. (C) E13.5 wild type showing cell death only in the most distal urethral plate and in the surrounding mesenchyme. (D) E13.5 wild type genital tubercle showing apoptosis predominantly in urethral epithelium. (E) E13.5 $Shh^{-/-}$ showing intense staining in the cloacal epithelium and mesenchyme (cloaca labeled "c"). (F) Same region as seen in (E), but at a more ventral plane of focus, showing high levels of apoptosis in genital mesenchyme.

FIG. 8. Two phases of external genital development. Initiation of paired external genital swellings (left) does not require Shh signaling, although *Shh* is expressed (green) prior to the onset of budding. We refer to this as the Shh-independent phase. Question mark on the left represents unknown signal or signals that initiate budding and gene expression in the genital swellings. During the subsequent Shh-dependent phase (right), Shh signaling by the urethral plate maintains outgrowth of the tubercle, expression of *Fgf8*, *Fgf10*, *Wnt5a*, *Bmp2*, and *Bmp4*, and cell survival in genital mesenchyme and urethral epithelium. Question mark on the right represents unknown signal responsible for maintenance of *Hoxd13* expression. See text for further details.

(Amthor *et al.*, 1998). It is therefore possible that Shh from the urethral epithelium (which is itself derived from hindgut endoderm) regulates development of muscle in the phallus.

The digit duplications we observed following transplantation of urethral epithelium to the limb are more extensive than those previously reported following transplantation of the distal third of the genital tubercle to the limb (which results in duplication of digit 2 only) (Dollé *et al.*, 1991; Izpisúa-Belmonte *et al.*, 1992). One explanation for this difference may be that our grafts consisted only of the urethral endoderm, whereas in the distal tubercle grafts, the urethral endoderm was contained within the surrounding genital mesenchyme (surface ectoderm had been removed). In the latter experiments, the urethral plate signal would have had to be transmitted through the genital mesenchyme in order to reach the limb. In a classic series of experiments, Honig (1981) showed that insertion of a 200-μm mesodermal barrier of leg tissue between the anterior margin of the wing bud and a grafted polarizing region resulted in duplication of wing digit 2, whereas a polarizing region grafted directly to the anterior wing caused a complete duplication of digits 4, 3, and 2. This demonstrated that the polarizing signal could be transmitted over a distance of approximately 200 μm, through a block of leg tissue, but the intensity of the polarizing signal was weaker by the time it diffused through the barrier tissue and reached the limb. This is analogous to the previous transplantations of distal genital tubercle, in which the graft was also 200 μm in size, and the genital mesenchyme would have been situated between the urethral plate (the signaling region) and the limb bud (Dollé *et al.*, 1991). Our finding that genital mesenchyme does not have polarizing activity further supports the idea that this tissue could have acted as a barrier through which the signal would have had to diffuse. Based on our finding that the polarizing signal of the urethral plate is Shh, we conclude that the different experimental outcomes can be explained in terms of Shh range, in which direct application of *Shh*-expressing cells (urethral endoderm) to the anterior limb results in specification of posterior digit identities, and indirect application of Shh (through the intact genital tubercle) results in specification of anterior digits. The medial-to-lateral gradient of *Ptc1* expression in the genital tubercle is also consistent with the idea that Shh activity decreases with distance from the urethral endoderm.

Recent work has shown that Shh signaling acts over graded long-range as well as short-range distances (Gritli-Linde *et al.*, 2001). Although initial comparisons of Shh mRNA and protein suggested a close spatial distribution (Marti *et al.*, 1995), it is now known that Shh protein extends well beyond *Shh*-expressing cells (Gritli-Linde *et al.*, 2001). Gritli-Linde *et al.* (2001) demonstrated that Shh protein associates with proteoglycans/glycosaminoglycans (PG/GAGs), and preservation of these proteins is essential for localization of Shh in target tissues. Moreover, they proposed that the distribution of PG/GAGs in the embryo may modulate movement of Shh. Shh protein can be detected over long distances in basement membranes of the neural tube and notochord, which are known to be rich in glycoproteins (Gritli-Linde *et al.*, 2001). These data, taken together with our finding that *Shh* mRNA localizes to the urethral epithelium of the genital tubercle, raise the possibility that the basement membrane of the urethral epithelium could play a role in the transfer of Shh protein to the adjacent genital mesenchyme. Interestingly, several penile tissues, including the corpora cavernosa, corpus spongiosum, and tunica albuginea, are rich in glycosaminoglycans, and different GAG proteins have tissue-specific patterns of expression in the penis (Goulas *et al.*, 2000). Widespread distribution and complex regulation of GAGs in the external genitalia may therefore be involved in the transmission of, and differential responses to, Shh protein. The broad, graded domain of *Ptc1* expression in the genital tubercle suggests that *Shh* signaling may indeed act over a long-range gradient. This is supported by the findings that Shh is essential for high level expression of *Ptc1* and *Ptc2* (Pearse *et al.*, 2001) and that expression of Ptc genes is strongly upregulated at sites of *Shh* signaling (Marigo and Tabin, 1996).

Genetic Interactions during External Genital Development

Our analysis of gene expression in $Shh^{-/-}$ mice also highlights roles for Shh in both induction and maintenance of gene expression in the genital tubercle. *Bmp4* and *Fgf8* require Shh for maintenance, but not induction, of expression. In contrast, *Bmp2*, *Wnt5a*, and *Fgf10* seem to require Shh for their initial expression (although we cannot exclude the possibility that *Bmp2, Wnt5a*, and *Fgf10* expression is initiated and immediately downregulated). Although it is formally possible that the observed changes in gene expression are caused by the increased apoptosis resulting in loss of expressing cells, the finding that *Hoxd13* expression persists throughout the genital field renders this unlikely. The expression pattern of *Hoxd13* observed in the genital region of $Shh^{-/-}$ mice bears striking similarity to the wild type pattern. These results suggest that *Hoxd13*, in contrast to other genes examined, may be regulated independently, or perhaps lie upstream, of *Shh*. $Shh^{-/-}$ mutants share several aspects of the urogenital phenotype with *Hoxa13* and *Hoxd13* compound mutants. In particular, $Hoxd13^{-/-}$;$Hoxa13^{-/-}$ double mutants lack a genital tubercle. A morphologically normal tubercle develops in mice with at least one copy of *Hoxa13* or *Hoxd13* (although skeletal malformations were detected in the proximal part of the baculum in $Hoxd13^{-/-}$ mice), indicating that, generally, *Hoxa13* and *Hoxd13* act in a functionally redundant manner during genital outgrowth (Dollé *et al.*, 1993; Kondo *et al.*, 1997; Warot *et al.*, 1997). Genes situated at the 5′ end of the HoxA and HoxD complexes play an essential role in regulating cell proliferation (Zákány and Duboule, 1999), and this may account for absence of external genitalia in $Hoxd13^{-/-}$;$Hoxa13^{-/-}$ mutants (Warot *et al.*, 1997). It is also

noteworthy that these *Hox* mutants, like *Shh*$^{-/-}$ mice, have hindgut and urogenital sinus defects, including persistence of a common cloaca in place of distinctive anorectal and urogenital sinuses (Warot *et al.*, 1997). It will be informative to examine *Shh* expression and urethral epithelial organization in *Hoxa13*/*Hoxd13* mutants.

Our finding that *Hoxd13* expression persists in the genital region of *Shh*$^{-/-}$ mice was nonetheless surprising, given the ability of Shh to regulate *Hoxd13* in the hindgut and limbs when ectopically expressed in chick embryos (Riddle *et al.*, 1993; Roberts *et al.*, 1998). Recent studies have shown that, in the absence of Shh, *Hoxd13* is expressed in the autopod at E12.5 (Chiang *et al.*, 2001; Kraus *et al.*, 2001). This Shh-independent *Hoxd13* expression correlates with expression of *Indian hedgehog* (*Ihh*), *Ptc1*, and *Gli1* expression in the distal limb of *Shh*$^{-/-}$ mutants, suggesting that Ihh may act in place of Shh to induce expression of *Hoxd13* and promote outgrowth of distal structures in the limb (Kraus *et al.*, 2001). It seems unlikely that a similar compensatory hedgehog signal regulates *Hoxd13* in the genital region of *Shh*$^{-/-}$ mice because none of the other genes that we examined are maintained. Although *Ihh* is not expressed in the urogenital sinus, it is coexpressed with *Shh* in the rectal endoderm (Bitgood and McMahon, 1995), and *Ihh* continues to be expressed in the gut endoderm of *Shh*$^{-/-}$ mutant mice (Zhang *et al.*, 2001). Persistent *Ihh* expression in *Shh*$^{-/-}$ mutants could therefore account for the bilateral patches of *Bmp2*, *Wnt5a*, and *Fgf10* expression that we observed at the posterior edge of the cloaca. The molecular basis for persistent expression of *Hoxd13* in the genital region of *Shh*$^{-/-}$ mice, however, is unclear.

Taken together, our results indicate that there are two phases of genital development with respect to Shh. Establishment of the genital field and initiation of paired genital swellings occurs independently of Shh, whereas subsequent outgrowth and patterning of the genital tubercle is Shh-dependent (Fig. 8). Although *Shh* is expressed prior to the formation of the paired genital buds, budding is initiated in its absence. However, outgrowth cannot proceed beyond the initiation of these faint swellings without Shh. This contrasts with the situation in the limb, in which considerable outgrowth, and development of the stylopod, zeugopod, and a single digit, can occur in *Shh*$^{-/-}$ mice (Chiang *et al.*, 2001; Kraus *et al.*, 2001). In the limb, FGFs are responsible for the initiation of limb budding (Cohn *et al.*, 1995; Crossley *et al.*, 1996; Ohuchi *et al.*, 1997; Min *et al.*, 1998; Sekine *et al.*, 1999), and *Fgf* expression in the lateral plate may be regulated by Wnt signaling (Kawakami *et al.*, 2001). Haraguchi *et al.* (2000) have demonstrated that the levels of expression of a number of genes can be augmented when FGF8-soaked beads are grafted into wild type genital tubercles, and application of FGF8-neutralizing antibody can reduce the intensity of *Bmp4* expression. It remains to be determined whether a targeted deletion of one or more Fgfs in the genital region has an effect on early genital tubercle outgrowth. Our results also indicate that *Fgf8* is expressed in the appropriate position at the time the genital swellings emerge (albeit after the onset of *Shh*), but the signal responsible for initiation of genital development remains to be identified.

Cell Survival in the Genital Tubercle

The dramatic increase in apoptosis observed in genitalia of *Shh*$^{-/-}$ mutants implicates Shh as a cell survival factor in the genital tubercle. Low-level apoptosis detected in the urethral epithelium during normal development is reminiscent of the buffering system in the polarizing region of the limb, in which the number of *Shh*-expressing cells is autoregulated by Shh-induced apoptosis (Sanz-Ezquerro and Tickle, 2000). The role of Shh as a cell survival signal is widespread in the embryo, and the enhanced apoptosis in the genital region of *Shh*$^{-/-}$ mutants is likely to be a consequence of Shh (or another survival factor situated downstream of Shh) being eliminated. *Shh* is expressed at high levels in the notochord and floor plate, and Shh is required for survival of many types of midline cells, including trunk neural crest progenitors, migratory cranial neural crest cells, ventral motor neurons, and sclerotome cells (Ahlgren and Bronner-Fraser, 1999; Borycki *et al.*, 1999; Britto *et al.*, 2000). In the limb, the anterior necrotic zone is expanded in *Shh*$^{-/-}$ mice, mirroring previous observations that surgical removal of the polarizing region results in increased anterior cell death (Todt and Fallon, 1987; Chiang *et al.*, 2001). Shh also regulates cell survival in gut derivatives, as *Shh*$^{-/-}$ mice show enhanced cell death in lung mesenchyme (Litingtung *et al.*, 1998). Endoderm of the lung bud is derived from foregut and urethral endoderm of the genital tubercle is derived from hindgut. The lung phenotype most closely approximates our observations in the genital tubercle. In both the lung and the genital tubercle, Shh signaling by the endoderm regulates cell survival in the surrounding mesenchyme. This highlights a broader parallel of budding systems, in which active inhibition of cell death (or promotion of cell survival), in addition to proliferation, is required for outgrowth.

Evolution of External Genitalia

Our results implicate Shh as a regulator of early outgrowth of the external genitalia. During vertebrate evolution, *Shh* expression in the cloacal endoderm may have played a role in outgrowth and/or patterning of the earliest genital appendage. The timing of the evolutionary origin of external genitalia remains unclear, but comparative anatomy of extant vertebrates suggests that a discrete genital outgrowth from the cloacal region is restricted to tetrapods, and perhaps to amniotes. Within chondrichthans and ray-finned fish, fin structures have been coopted for use as intromittent organs, such as the anal fin-derived gonopodium of guppies and the pelvic fin-derived claspers, or myxoptergia, of sharks. However, these structures are clearly modifications of the fins and, as such, are not true external genitalia. Internal fertilization is facilitated in

Gobiformes and *Blenniformes* by a cloacal papilla that lies dorsal to the cloaca, but its position and structure suggest that the papilla is not homologous to tetrapod external genitalia. If external genitalia coincided with the origin of tetrapods, then one might expect to find amphibians with external genitalia. In a key discovery, Duboule and colleagues found that *Hoxd13* expression in the digits and genital tubercle of mice is under the control of a shared transcriptional enhancer (van der Hoeven *et al.*, 1996b; Kondo *et al.*, 1997). They suggested that the origin of tetrapod digits and genitalia may have resulted from a genetic regulatory innovation, which gave rise to these two novel sites of *Hoxd* expression. One prediction of this model is that early tetrapod amphibians may have had external genitalia, and Kondo *et al.* (1997) noted that this possibility cannot be excluded by the fossil record (Kondo *et al.*, 1997).

In light of this model, it is interesting that a basal anuran, the tailed frog *Ascaphus truei*, possesses an elaborate tail-like structure, which contains erectile tissue and can be ventriflexed for delivery of sperm to the cloacal orifice of the female (Van Dijk, 1955). The relationship of the *Ascaphus* "tail" to the penis of amniotes has been a longstanding subject of debate (de Villiers, 1933). While it is clear that the *Ascaphus* "tail" is a complex organ that shares many structural and functional properties with the penis, its morphology, position, and developmental origin suggest that this structure may be an extension of the cloaca rather than a homolog of the penis. Further developmental studies are required, however, to resolve this question of homology. The absence of external genitalia in extant amphibians, and the equivocal status of the *Ascaphus* tail, lends a degree of support to the idea that the evolutionary origin of external genital organs occurred at the base of amniotes [though we concur with the argument of Kondo *et al.* (1997) that an origin at the base of tetrapods cannot be excluded]. The urethral epithelium is derived from urogenital sinus endoderm, and we find that, at early stages of mouse genital outgrowth, *Shh* is expressed in a contiguous domain from the hindgut into the prospective urethral endoderm. *Hoxd13* expression in the cloaca and *Hoxa13* expression in the urogenital sinus appear to be generally conserved vertebrate characters, having been detected in amniotes and zebrafish (Sordino *et al.*, 1996; van der Hoeven *et al.*, 1996a). Based on our findings, it could be speculated that expression of *Shh*, in addition to Hox13 genes, in the cloacal endoderm was required for evolution of external genital outgrowth. If cloacal or urogenital expression of *Shh*, *Hoxa13*, and *Hoxd13* is a primitive condition for vertebrates, then the molecular software for external genital development was already localized to the appropriate region of the embryo prior to the origin of an outgrowth initiation signal. Distal outgrowth from the ventral wall of the cloaca, under the initial control of such a signal, may have resulted in recruitment of *Shh*-expressing cloacal endoderm as a genital organizer.

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