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. © 2002 Elsevier Science (USA)

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 (Kaloo 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...
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). Hox13	extsuperscript{+/+};Hox13	extsuperscript{−/−} 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) (Hara-guchi 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 persists 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 μm (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 (Dolle 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 acetic acid. Stained specimens were differentiated in acetic acid, 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 (30 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 ethanol and placed in acetone. Specimens were then critical point dried, mounted on metal stubs, 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 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 (l). The urethral plate remains closed distally (l). (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 cavernosum (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.
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 urethral development, 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 genitourinary 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 2H).

**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 glans (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 glans (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
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 (Dolle 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 35S-labeled riboprobes (Dolle 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 tubercle, although expression is weakest proximally. Laterally, strong expression is seen in the emerging preputial swellings (R).
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 Shh⁰⁻¹ (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) Shh⁰⁻¹ 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 Shh⁰⁻¹ 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 Shh⁰⁻¹ 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 (Vahtokari 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 Tuberacle**

Previous studies have shown that Hoxd13, Hoxa13, and Wnt5a are involved in outgrowth of the genital tuberacle (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 tuberacle. 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 (Fig. 7E and 7F). Esenchymal 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, implicating 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
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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Shh Signaling Regulates External Genital Development

WT

Acridine Orange

Brightfield

E11.5

E13.5

Distal

Proximal

Shh-/-

Acridine Orange

Brightfield

Fgf8

Bmp2

Fgf10

Bmp4

Wnt5A

Hoxd13

Bmp4

/?

Outgrowth/Patterning

(Shh-dependent)

Initiation

(Shh-independent)

urethral

Shh mRNA

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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 (Kangasuntheram 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 (granular) 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 tube. Differentiation of mesenchymal structures in the tube 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 tube. The idea that the urethral epithelium functions in the genital tube 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.
The distal third of the genital tubercle to the limb (which than those previously reported following transplantation of
tation of urethral epithelium to the limb are more extensive
phallus.
the urethral epithelium (which is itself derived from hind-
gut endoderm) regulates development of muscle in the
region grafted directly to the anterior wing caused a com-
rior margin of the wing bud and a grafted polarizing region
reached the limb. This is analogous to the previous trans-
by the time it diffused through the barrier tissue and
intensity of the polarizing signal was weaker
Gritli-Linde et al., 1991). 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 mesen-chyme 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 ante-
rior 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 com-
plete 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 trans-
plantations 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 experi-
mental 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 possi-
bility that the basement membrane of the urethral epithel-
ium could play a role in the transfer of Shh protein to the
adjacent genital mesenchyme. Interestingly, several penile
tissues, including the corpora cavernosa, corpus spongio-
sum, 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 exter-
nal 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 up-
regulated at sites of Shh signaling (Mariño 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 expres-
sion. In contrast, Bmp2, Wnt5a, and Fgf10 seem to require
Shh for their initial expression (although we cannot ex-
clude the possibility that Bmp2, Wnt5a, and Fgf10 expres-
sion 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 expres-
sion persists throughout the genital field renders this un-
likely. 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 inde-
pendently, 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 tu-
bercle. 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, gener-
ally, 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

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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., 1995; Kraus et al., 2001). 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-Ezquerra 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 (Toftod 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 (Li廷g tung 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 chondrichthians 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
Gobiiformes 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 ventrified 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 the urogenital sinus appear to be generally conserved vertebrates (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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