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Cell lineage analysis demonstrates an endodermal origin of the distal urethra and perineum

Ashley W. Seifert [a], Brian D. Harfe [b], Martin J. Cohn [a,c,*]

[a] Department of Zoology, University of Florida, Cancer/Genetics Research Complex, PO Box 103610, Gainesville, FL 32610-3610, USA
[b] Department of Molecular Genetics and Microbiology, University of Florida, Cancer/Genetics Research Complex, PO Box 103610, Gainesville, FL 32610-3610, USA
[c] Department of Anatomy and Cell Biology, University of Florida, Cancer/Genetics Research Complex, PO Box 103610, Gainesville, FL 32610-3610, USA

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ABSTRACT

Congenital malformations of anorectal and genitourinary (collectively, anogenital) organs occur at a high frequency in humans, however the lineage of cells that gives rise to anogenital organs remains poorly understood. The penile urethra has been reported to develop from two cell populations, with the proximal urethra developing from endoderm and the distal urethra forming from an apical ectodermal invagination, however this has never been tested by direct analysis of cell lineage. During gut development, endodermal cells express *Sonic hedgehog* (*Shh*), which is required for normal patterning of digestive and genitourinary organs. We have taken advantage of the properties of *Shh* expression to genetically label and follow the fate of posterior gut endoderm during anogenital development. We report that the entire urethra, including the distal (glandar) region, is derived from endoderm. Cloacal endoderm also gives rise to the epithelial linings of the bladder, rectum and anterior region of the anus. Surprisingly, the lineage map also revealed an endodermal origin of the perineum, which is the first demonstration that endoderm differentiates into skin. In addition, we fate mapped genital tubercle ectoderm and show that it makes no detectable contribution to the urethra. In males, formation of the urethral tube involves septation of the urethral plate by continued growth of the urorectal septum. Analysis of cell lineage following disruption of androgen signaling revealed that the urethral plate of flutamide-treated males does not undergo this septation event. Instead, urethral plate cells persist to the ventral margin of the tubercle, mimicking the pattern seen in females. Based on these spatial and temporal fate maps, we present a new model for anogenital development and suggest that disruptions at specific developmental time points can account for the association between anorectal and genitourinary defects.

Introduction

Despite the high incidence of congenital malformations of the anorectal and urogenital systems in humans, the mechanisms that govern normal anogenital development are poorly understood. The most common of these malformations is hypospadias, an ectopic opening (or multiple openings) of the urethra on the ventral aspect of the phallus. Frequently, defects of anorectal and genitourinary organ systems occur together, which raises the possibility that they are linked mechanistically during early development. For example, failure of the embryonic cloaca to subdivide into separate anorectal and urogenital sinuses (clinically referred to as persistent cloaca) is often associated with ambiguous genitalia, and numerous other syndromes involve associated defects of the bladder, anorectum, and external genitalia (Mo et al., 2001). Insight into how development of the external genitalia, urethra, bladder, rectum and perineum is coordinated at both cellular and molecular levels is necessary for understanding the basis of their association in congenital anomalies.

In mammals, the embryonic cloaca undergoes septation to form separate genitourinary and anorectal sinuses, whereas in birds, reptiles and most anamniotes, the cloaca persists as a common outlet for the digestive, urinary and reproductive tracts. Surface ectoderm and endoderm are in direct contact at only two positions during vertebrate development; posteriorly at the cloacal membrane and anteriorly at the oropharyngeal membrane. Cloacal endoderm lines the posteriormost portion of the gut tube and contacts the overlying ectoderm at the cloacal membrane. The morphogenetic mechanisms that drive division of the cloaca into separate urogenital and anorectal tracts are unclear, although a variety of processes have been proposed, including descent of a urorectal septum (known as the Tourneaux fold), extension of the Rathke folds from the lateral walls of the cloaca, differential growth of the cloacal mesoderm, and reorganization of the cloacal epithelium (Hynes and Fraher, 2004c; Kluth et al., 1995; Nievelstein et al., 1998; van der Putte, 2005). After division of the cloaca, the anal and the genitourinary outlets are separated by the perineum on the

* Corresponding author. Department of Zoology, University of Florida, Cancer/Genetics Research Complex, PO Box 103610, Gainesville, FL 32610-3610, USA. Fax: +1 352 273 8284.
E-mail address: cohn@zoo.ufl.edu (M.J. Cohn).

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posterior surface of the embryo. Since the middle of the 19th century, it has been reported that the perineum forms by medial growth and fusion of the cloacal folds, in a manner similar to fusion of the palatal shelves (Larson, 2001; Nievelstein et al., 1998). Recent work in humans and in mice has challenged this interpretation, suggesting instead that the perineum is derived from the urorectal septum (Dravis et al., 2004; Hynes and Fraher, 2004c; Sasaki et al., 2004; van der Putte, 2005). Identifying the developmental origin of the perineum should clarify how the posterior-most part of the embryonic gut gives rise to anorectal and genitourinary organs.

In both male and female mammalian embryos, development of the external genitalia begins with the emergence of the paired genital swellings immediately above the cloaca (see Perriton et al., 2002 for a detailed description of normal external genitalia development in mouse). These swellings fuse medially and give rise to the bipotential genital tubercle, which can be masculinized to form the penis or feminized to form the clitoris. As the genital tubercle grows out, the ventral side of the cloacal endoderm forms a bilaminar urethral plate that extends into the genital tubercle, and this structure later cavitates in a proximal to distal direction to form the urethral tube (Hynes and Fraher, 2004a; Perriton et al., 2002). Classical accounts of external genital development reported that the urethra has a dual embryonic origin – with the distal (glandar) portion arising from an ectodermal invagination from the distal tip of the genital tubercle and the proximal portion coming from the endodermal urethral plate – a description that remains in contemporary embryology textbooks (Glenister, 1954; Larson, 2001; Moore, 2007; Sadler, 2006). An alternative model proposes that the entire urethra forms from endoderm, which undergoes differentiation in the glandar portion to form squamous epithelium (Felix, 1912; Kurzrock et al., 1999; Penington and Hutson, 2002a,b; Perriton et al., 2002), however neither model has been tested by direct analysis of cell lineage.

During sexual differentiation of the external genitalia in mice, which occurs under the control of androgens, the bilaminar urethral plate is transformed into a central urethral tube along the length of the penis (Baskin et al., 2001; Hynes and Fraher, 2004b; Mahendroo et al., 2001; Yamada et al., 2003). Prior to masculinization, the urethral plate extends from the center of the genital tubercle to its ventral edge, where it contacts the surface ectoderm at the cloacal membrane. The dorsal aspect of the urethral plate is thought to become the definitive urethral tube, while the ventral portion is remodeled such that the urethral tube becomes surrounded by stromal mesenchyme. It is unknown whether removal of the ventral aspect of the urethral plate is accomplished by apoptosis, an epithelial-to-mesenchymal transition or through morphogenetic movement of the urethral epithelium. By contrast, female genital development involves little remodeling of the urethral plate, resulting in a more proximal and ventral urethral opening. While apoptosis has been reported to occur in this region, the possibility of an epithelial-to-mesenchymal transition has not been excluded. Resolution of the cellular origin of the urethra and the fate of ventral urethral plate cells is important for identifying the cell population(s) affected in hypospadias and for investigations of gene function during urethragenesis.

Sonic hedgehog (Shh) is expressed throughout the endodermal epithelium of the gut, where it persists during division of the cloaca and formation of the urethral plate (Bitgood and McMahon, 1995; Echelard et al., 1993; Haraguchi et al., 2001; Perriton et al., 2002). *Shh*−/− mice fail to form a genital tubercle, indicating that Shh is required for development of the external genitalia (Haraguchi et al., 2001; Perriton et al., 2002). In addition, loss of Shh function results in a failure of cloacal septation, and mice are born with a persistent cloaca. The finding that *Shh*−/− mutants have severe defects of their genital and cloacal derivatives suggests that early Shh signaling from the hindgut endoderm may act to coordinate morphogenesis of the entire anogenital system.

Here we investigate the cellular origins of the distal urethra and the perineum, and test the hypothesis that the ventral aspect of the urethral plate is removed during urethragenesis by an epithelial-to-mesenchymal transition. We exploited the fact that endodermal cells of these organ systems express *Shh* during early development, in order to genetically label and fate map the cloacal endoderm during anorectal and genitourinary organogenesis. Our lineage analysis provides the first direct evidence that the entire urethra is derived from endoderm, and that the transformation of the solid urethral plate into the definitive male urethra occurs in the absence of an epithelial-to-mesenchymal transition. The epithelial linings of the bladder, rectum and anterior region of the anus also are derived from *Shh*-expressing endoderm. Moreover, we present the unexpected finding that cloacal endoderm gives rise to the perineum, which is the first demonstration that endoderm differentiates into skin. We also fate mapped the ectoderm of the genital tubercle and show that it does not contribute to the urethral tube. Finally, we followed *Shh* descendant cells after disruption of androgen signaling and show that feminization of the male genitalia results from persistence of the endodermal urethral plate along the ventral margin of the genital tubercle. Taken together, these results reveal the fate of the cloacal endoderm during anorectal and urogenital organogenesis, and highlight the importance of this cell population in the coordinated formation of the anogenital system.

Materials and methods

Transgenic mice and lineage analysis

The *ShhGFPcre*, *Msx2cre*, and *Rosa26* reporter (*R26R*) mice used in this study have been described previously (Harfe et al., 2004; Soriano, 1999; Sun et al., 2000). The *ShhGFPcre* allele was generated by knocking a *GFPcre* fusion cassette into the start site of the *Shh* locus, placing cre-recombinase under the control of the endogenous *Shh* promoter (Harfe et al., 2004). The *ShhGFPcre* allele is a null allele, however heterozygous animals are phenotypically normal and breed successfully (Harfe et al., 2004). In *Msx2cre* mice, cre recombinase is driven by the proximal *Msx2* promoter (Liu et al., 1994; Sun et al., 2000). To irreversibly label *ShhGFPcre*- or *Msx2cre*-expressing cells, we crossed heterozygous males to females carrying the *R26R* reporter allele. Females were inspected for vaginal plugs and the morning they were found was determined as embryonic day (E)0.5. Pregnant dams were sacrificed at specific time points to collect a staged series of embryos with either *ShhGFPcre;R26R* or *Msx2cre;R26R* genotypes. The genitalia and limbs were used to confirm age, and embryos were processed for X-gal staining and histological analysis.

Flutamide administration

Suspensions of flutamide (Sigma F9397) were prepared daily in corn oil. The corn oil was filtered and heated at ~55 °C to dissolve the flutamide. Flutamide was administered in 200 μl S.C. injections at 100 mg/kg/day. Injections began on E13.5 and continued until pups were born. Injection sites were altered each day between shoulder and haunch. Control females were injected daily with the vehicle alone in the same manner.

X-gal staining

β-Galactosidase activity was detected using X-gal. Embryos were harvested in PBS and fixed overnight in 0.2% PFA at 4 °C. Embryos were washed three times in LacZ rinse buffer (1 M sodium phosphate pH 7.4, 0.1% sodium deoxycholate, 1 M $MgCl_2$, 0.02% NP40), then stained with X-gal overnight rocking at room temperature. Embryos were then rinsed, post-fixed and stored in 4% PFA at 4 °C.

Histology

X-gal stained embryos were processed into either paraffin wax or OCT for histological analysis. For wax prepared specimens, samples were dehydrated in a graded ethanol series, taken through a xylene substitute (XS-3, Statlab) to preserve the β-galactosidase, and embedded in paraffin. Samples were cut at 10 μm thickness and counterstained with Biebrich Scarlet. For cryosectioning, embryos were taken through a graded series of 15% sucrose/PBS, 30% sucrose/PBS, and 30% sucrose/50% OCT before being embedded in 100% OCT and cut in 10 μm serial sections.

Results

Cloacal endoderm gives rise to the entire urethral epithelium

In order to resolve the origin of the glandar urethra, we first sought to determine the cellular origin of the entire urethral plate. It has been

shown previously that *Shh* is expressed in the developing gut endoderm and is excluded from the surrounding mesoderm and ectoderm during development of the urogenital system (Bitgood and McMahon, 1995; Haraguchi et al., 2001; Perriton et al., 2002). Therefore, in order to fate map the entire cloacal endoderm, we utilized the *ShhGFPcre* allele to irreversibly activate the *Rosa26* reporter (*R26R*) allele in all *Shh*-expressing cells and their descendants.

Prior to initiation of genital tubercle outgrowth at E10.5, all cloacal endoderm expresses *Shh*, and *R26R* activity confirmed that *ShhGFPcre* expression faithfully follows the endogenous *Shh* expression pattern (Fig. 1A). Histological sections through the cloacae of *ShhGFPcre;R26R* mice at E10.5 showed that labeled cloacal endoderm cells are in contact with surface ectoderm, and the junction of these two cell layers comprises the cloacal membrane (Fig. 1B). The caudal-most junction of these two cell layers, at the base of the tail, occurs at the proctodeum, the future site of the anal opening (Fig. 1C, white asterisk). Between E10.5 and E11.5, the lateral walls of the LacZ-labeled cloacal epithelium come into apposition at the distal tip of the tubercle to form the beginning of a bilaminar urethral plate (Fig. 1D). This apposition continues proximally as the tubercle grows out (Figs. 1D, F). The junction between urethral plate endoderm and surface ectoderm is maintained, and mesoderm lateral to the cloacal membrane does not invade this morphological boundary (Fig. 1F). As the genital tubercle grows out, expansion of the anterior mesodermal population on the dorsal aspect of the tubercle (visible as a dorsal swelling) results in ventral displacement of the urethral plate (Figs. 1C, E, G; see also Perriton et al., 2002). The urethral plate consisted of *ShhGFPcre* descendant cells at E13.5, and analysis of labeled cells during urethral tube formation indicated that this endodermal population gives rise to the entire urethral tube, including the glandar portion (Fig. 2).

Cloacal endoderm gives rise to the perineum

Activation of reporter gene expression in cloacal endoderm also allowed us to map morphogenesis of the urorectal septum, a population of mesoderm that divides the cloaca into the anorectal and urogenital sinuses. By E10.5, the leading edge of the urorectal septum extended into the labeled endoderm at the anterior side of the cloaca (Fig. 1A). Beginning at E11, we observed a caudal expansion of the peritoneal cavity into the urorectal septum mesoderm (Fig. 1C). The urorectal septum mesoderm continues partitioning the cloaca into the urogenital sinus and the hindgut between stages E11.0 and E13.5 (Figs. 1C, E, G).

At the cloacal membrane, LacZ-labeled endoderm is in contact with surface ectoderm, and the strict boundary maintained between LacZ-positive and -negative cells suggests that these cell populations do not intermix (Figs. 1B, D, F). Endoderm and ectoderm abut one another along the ventral side of the genital tubercle and at the proctodeum until the cloacal membrane ruptures at ~E13.0, creating the anus and resulting in transient exposure of the urethra at the base of the tubercle (Figs. 1G, H). As the cloacal membrane ruptures, ectoderm degenerates between the anus and the base of the genital tubercle and is replaced by LacZ-labeled endodermal cells at leading edge of the urorectal septum (Fig. 1H). As a result, endodermal cells at the caudal end of the

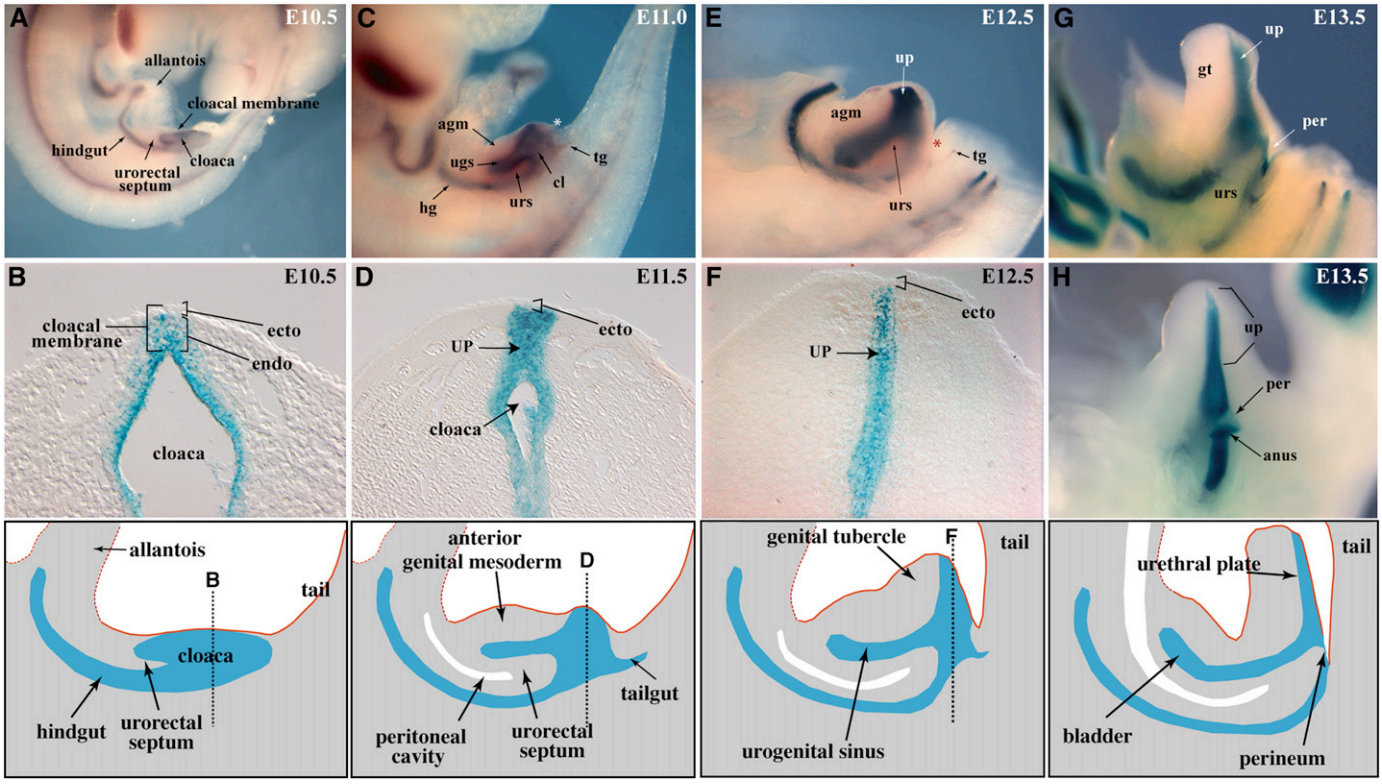

Fig. 1. *ShhGFPcre*-expressing endodermal cells give rise to the epithelium of the urethral plate, perineum, bladder and anorectum. *ShhGFPcre;R26R* mouse embryos stained with X-Gal to reveal LacZ-positive cells (labeled blue). Panels A, C, E and G are lateral views of genital region with the right hindlimb bud removed. Panels B, D and F are sections along the proximodistal axis of the genital tubercle, cut transverse to the trunk. (H) Ventral view of the genital tubercle. Stages shown in upper right corners. Schematic diagrams across the bottom refer to stages shown in panels A, C, E, and G; dotted lines indicate planes of section shown in panels B, D, and F (red, ectoderm; blue, endoderm, gray; mesoderm). (A) Urorectal septum mesoderm has begun to septate the cloaca. (B) Labeled cloacal endoderm lies in contact with the unlabeled surface ectoderm to form the cloacal membrane. (C) Distribution of labeled endodermal cells in urogenital sinus (ugs), hindgut (hg), cloaca (cl), tailgut (tg). Urorectal septum mesoderm (urs) has extended into the anterior region of the cloaca. Note position of anterior genital mesoderm (agm) relative to urogenital sinus. Asterisk marks the position of the proctodeum. (D) Labeled cloacal endoderm is beginning to form a bilaminar urethral plate (UP), which extends to distal tip of the genital tubercle where it abuts surface ectoderm (ecto). (E, F) Urethral plate (UP) spans the proximodistal length of the genital tubercle. The URS is approaching the proctodeum (asterisk). (G, H) Surface ectoderm at the base of the tail has ruptured and labeled endodermal cells have formed the central margin of the perineum (per).

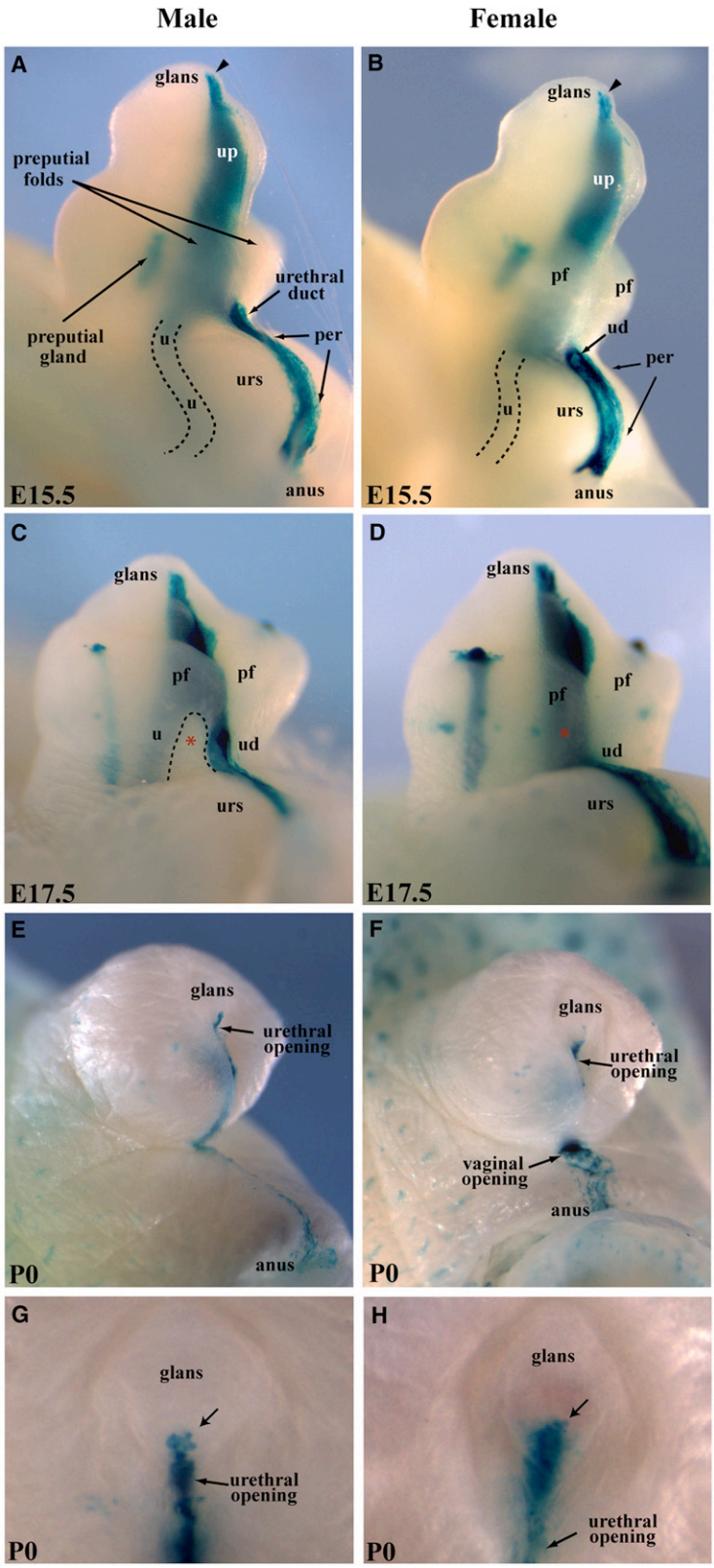

Fig. 2. Sexual differentiation of the urethral plate. Urethral tube development in male (A, C, E, G) and female (B, D, F, H) *ShhGFPcre;R26R* mice between E15.5 and birth (P0). *ShhGFPcre*-expressing cells and their descendants are stained blue. Ventral/Posterior surface of the genital tubercle is to the right in panels A–F and to the bottom in panels G and H. The tail is removed. (A, B) LacZ-labeled cells extend to the distal-most tip of the urethral plate (up; arrowheads) in the glans. The urethral duct (ud) is open in both males and females. Labeled cells are visible along the surface of the perineum (per; arrows). Dashed lines mark the position of the proximal urethra (u). Preputial folds (pf) and preputial glands are visible in panels A–D. (C) Male at E17.5, in which mesoderm of the urorectal septum (urs) and prepuce is seen invading the proximal end of the urethral plate (dashed line and asterisk). (D) Female at E17.5 showing the unseptated urethral plate (asterisk). (E) In neonatal males, the urethral duct has closed (compare with panels A and F) and endodermal cells along the surface of the perineum are contiguous with the ventral urethral seam. (F) In neonatal females, the urethral duct, which contains LacZ-positive cells, remains open and forms the posterior portion of the vagina. (G, H) The distal urethra in both males and females is derived from *ShhGFPcre*-expressing cells. The female urethral opening lies more proximal and ventral than the male urethra, which is positioned just beneath the apex of the glans.

urorectal septum come to lie on the surface of the embryo, where they form the central margin of the perineum (Figs. 1H, 2A, B).

Sexual differentiation of the urethra

We next investigated the mechanism by which the male forms a tubular urethra within the glans penis, whereas in females, an epithelial cord persists ventrally in the clitoris. At E15.5, the male and female genital tubercles are still morphologically similar, although the anogenital distance (the perineal area between the urogenital duct and the anus) is shorter in females (Figs. 2A, B). At E15.5, LacZ-labeled endodermal cells that previously covered the urorectal septum are visible along the central seam of the perineum (Figs. 2A, B). Beneath the perineum, mesoderm of the scrotal swellings in the male and labial swellings in the female is continuous with the mesoderm of the urorectal septum and with the proximal portion of the emerging preputial swellings (Figs. 2A, B). Thus, three distinct outgrowths (labioscrotal swellings, preputial swellings and urorectal septum) arise from a continuous population of cloacal mesoderm. The prepuce then envelops the glans from proximal to distal (Figs. 2A–F). At E15.5, the urethra extends to the distal tip of the genital tubercle and is composed entirely of cells descended from *ShhGFPcre*-expressing endoderm (Figs. 2A, B and 3E, F).

The proximal portion of the urethral plate has cavitated by E15.5 to form the proximal urethral tube in both males and females (Figs. 3A, B). The urethral duct is open at the proximal end of the phallus in both sexes. Within the distal portion of the glans, which is not yet surrounded by the prepuce, the urethral plate remains in contact with the overlying ectoderm (Figs. 3E, F). Preputial glands begin to develop in both sexes at E13.5, when focal spots of *ShhGFPcre*-expressing ectoderm begin to invaginate into preputial mesenchyme (Figs. 2A, B).

Beginning at E15.5, the distribution of *ShhGFPcre* descendants reveals the onset of sexual differentiation of the urethra (Figs. 2C, D and 3A–F). In males, the urethral plate is septated from proximal to distal to create the definitive urethral tube within the glans, whereas in females

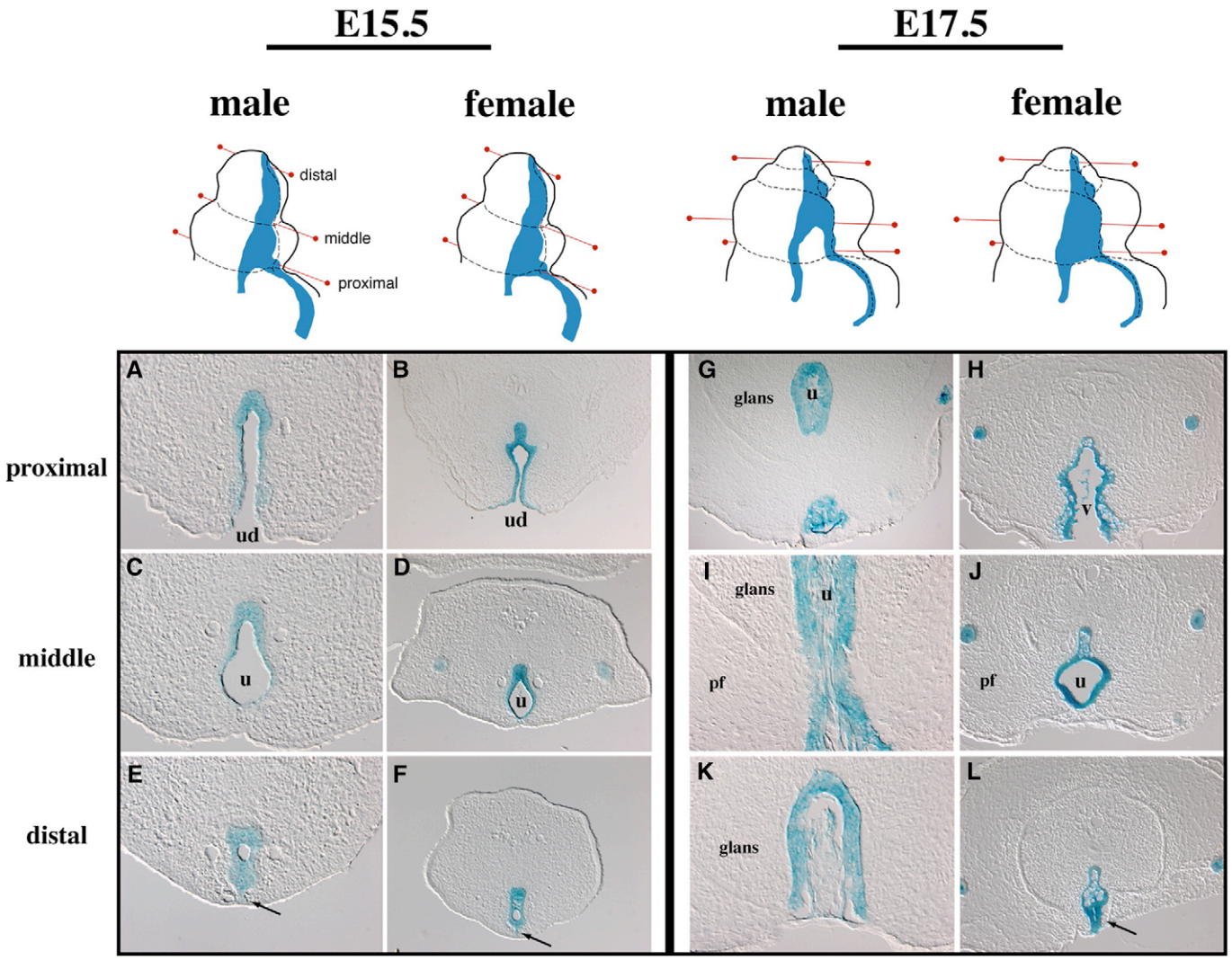

Fig. 3. Transformation of the urethral plate to a urethral tube. Comparison of urethral tube development in male (A, C, E, G, I, K) and female (B, D, F, H, J, L) *ShhGFPcre;R26R* mice at E15.5 and E17.5. Ventral is at the bottom. Cells derived from the *ShhGFPcre*-expressing population are stained blue. Sections are transverse to the genital tubercle at proximal, middle and distal levels (shown in schematic diagrams above each column). (A–F) Labeled cells are restricted to the urethral plate epithelium and are absent from the mesenchyme at E15.5. Cavitation of the urethral plate proceeds from proximal to distal and the urethral duct (ud) is open in both males and females (u, urethra). (G) Male urethral plate is septated by urorectal septum mesoderm at the proximal end. Note the remnant of the urethral plate at the ventral edge of the penis. (H) The female urethral plate remains unseptated and the proximal urethral duct remains open to form the posterior portion of the vagina (v). (I, K) Mesoderm has not yet invaded the middle (I) or distal (K) portions of the male urethral plate, which extends to the ventral edge of the penis. (J, L) Female urethral plate is tubular at the mid-shaft (J), but persists as a cord distally (L). Note the absence of mesoderm ventral to urethral plate in the female (arrow in panel L).

this septation fails to occur (Figs. 2C, D). By E17.5, the male urethral plate has been divided into a central urethral tube and a ventral seam (Fig. 3G), whereas the female urethral plate persists to the ventral edge of the clitoris (Fig. 3H). The distribution of *ShhGFPcre* descendant cells in males shows that septation occurs when mesoderm of the preputial swellings and urorectal septum converges ventral to the definitive urethra (Fig. 3G). During urethral septation, the preputial swellings continue to envelop the glans (Figs. 2C, E). As a result of these coordinated movements, the urethral plate is divided dorsoventrally, with the dorsal portion forming the definitive urethra and the ventral portion forming the urethral seam along the ventral edge of the penis (Fig. 3G). The absence of LacZ-labeled cells from the genital mesenchyme indicates that the urethral epithelium does not undergo a transition to mesenchyme during septation (Figs. 3G, I).

In females, proximal mesoderm fails to invade the genital tubercle and, consequently, the urethral plate is not septated (compare Fig. 2C with D, Fig. 3G with H, and I with J). As in males, the female preputial swellings continue to grow distally and both the cloacal and preputial folds expand medially to envelop the glans clitoris (Figs. 2D, F and 3H, J, L). At the same time, the proximal urethral plate is cavitated centrally to form the female urethra (Fig. 3J). This results in the female urethra remaining ventral to the glans, in contrast to the male condition, in which the urethral tube lies within the glans at E17.5 (Fig. 3 compare I and J). The urethral duct remains open and will form the posterior portion of the vagina, which we also found to be derived from *ShhGFPcre*-expressing endoderm (Fig. 3H). In the distal region of the clitoris, the labeled urethral plate cells form an epithelial cord, whereas in males the plate continues to cavitate distally to form the penile urethra (Figs. 3K, L). At birth, the female urethra lies distal to the vaginal opening, and is also derived entirely from endoderm (Figs. 2F, H).

In neonatal (P0) males, the glandar urethra is composed of *ShhGFPcre* descendants, indicating its endodermal origin (Figs. 2E, G). The distal glans is enveloped by the prepuce, and the penile urethra is positioned centrally within the glans (Fig. 2E). Endodermally derived cells are still visible at the apical opening of the urethra, along the ventral seam of the preputial folds, and on the exterior surface of the perineum (Figs. 2E, G). The proximal urethral duct in the male has closed, and labeled cells were restricted to the definitive urethral tube, including the distal meatus, and to the remnant of the ventral preputial seam (Figs. 2E, G).

There is no ectodermal contribution to the glandar urethra

The fate map of *ShhGFPcre*-expressing cells and their descendants showed an unequivocal contribution of endoderm to the glandar urethra, however these experiments could not exclude the possibility that some ectodermal cells are incorporated into the distal region. Therefore, we investigated whether the glandar urethra has an ectodermal component by using another *cre* allele to activate reporter gene expression in the genital ectoderm. We found previously that *Msx2* is expressed in the ectoderm overlying the developing genital tubercle at E11.5, and it remains restricted to the dorsal and distal ectoderm of the genital tubercle at later developmental stages (Fig. 4A inset). We first determined whether the *Msx2cre* allele (Sun et al., 2000) is expressed in distal genital tubercle ectoderm by crossing *Msx2Cre* males to females carrying *R26R*, and then examining reporter gene expression in embryos at E12.5. We found that *Msx2cre* activated *lacZ* in ectoderm of the developing genital tubercle in a domain that was broader than endogenous *Msx2* expression, and included the dorsal, distal and ventral medial ectoderm, but excluded the endoderm and mesoderm (Fig. 4A). Having determined this *cre* line to be an efficient marker of the genital tubercle ectoderm, we then examined the distribution of *Msx2cre* descendants in P0 males. *Msx2cre*-expressing cells and their descendants were distributed throughout the surface ectoderm of the penis, however labeled cells were notably absent from the urethral opening, the seam of preputial fold fusion, and the midline of the perineum (Fig. 4B). Histological analyses confirmed that blue cells were restricted to the ectoderm and did not invaginate into the glandar urethra (Fig. 4C). Transverse sections showed a sharp boundary between the ectodermally derived *Msx2cre*-expressing cells and the urethral tube (Fig. 4C). Taken together, these findings show that the glandar urethra is derived entirely from endoderm and that ectoderm makes no detectable contribution.

Disruption of androgen signaling feminizes male genitalia without affecting urethral epithelial integrity

Disruption of androgen signaling during mammalian external genital development causes feminization of the male genitalia and can result in hypospadias. Having identified the cellular basis of masculinization and feminization of the urethra, we next investigated whether antagonism of androgen receptor (AR) activity, using the AR antagonist flutamide, altered the behavior of the male urethral cell lineage. Administration of flutamide (100 mg/kg/day) to pregnant dams from E13.5 resulted in complete feminization of the external genitalia and urethras of male pups (Figs. 5A–D). Analysis of *ShhGFPcre* descendant cells in flutamide-treated males revealed that the urethral plate did not undergo septation by the mesoderm, a process that occurred normally in control males treated with corn oil (Fig. 5, compare A, A′ with C, C′). Consequently, the urethral endoderm of flutamide-treated males failed to form a centralized urethral tube. The distribution of *ShhGFPcre* descendant cells in flutamide-treated mice showed that the urethral plate persisted from the midline of the phallus to the ventral margin, indicating that feminization of the male urethral plate by AR antagonism mimics the process that occurs in normal female development (i.e., there is no epithelial to mesenchymal transition and the urethral plate

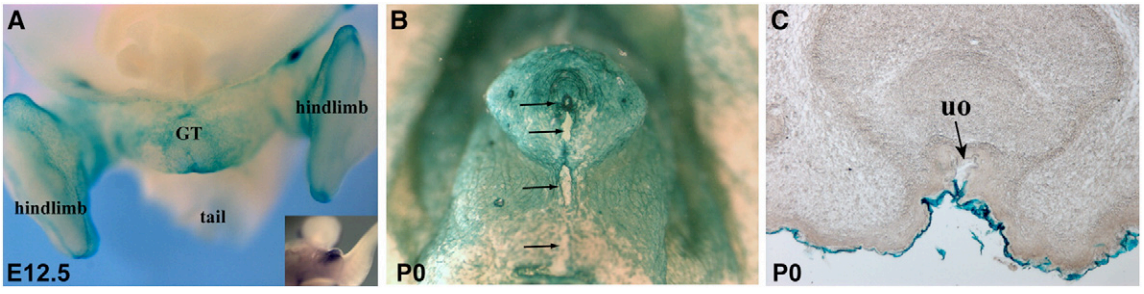

Fig. 4. Genital tubercle ectoderm does not contribute to the distal urethra. *Msx2cre;R26R* male mice stained with X-gal showing *lacZ* expression in ectoderm of the genital tubercle. (A, B) Ventral views of E12.5 (A) and P0 (B) mice showing genital tubercles (gt). Dorsal surface of genital tubercles is towards the top and tails have been removed. Inset of panel A shows *Msx2* mRNA expression in lateral view of E12.5 genital tubercle. (B) *Msx2cre* activates *lacZ* throughout the surface ectoderm of the penis. Arrows mark LacZ-negative domains along the central seam of the perineum and the ventral midline of the penis, two areas which contain cells derived from *ShhGFPcre*-expressing population (compare with Figs. 2E, F). (C) Distal, transverse section through penis shown in panel B reveals that labeled ectodermal cells contribute to skin but are excluded from the urethral tube (uo, urethral opening).

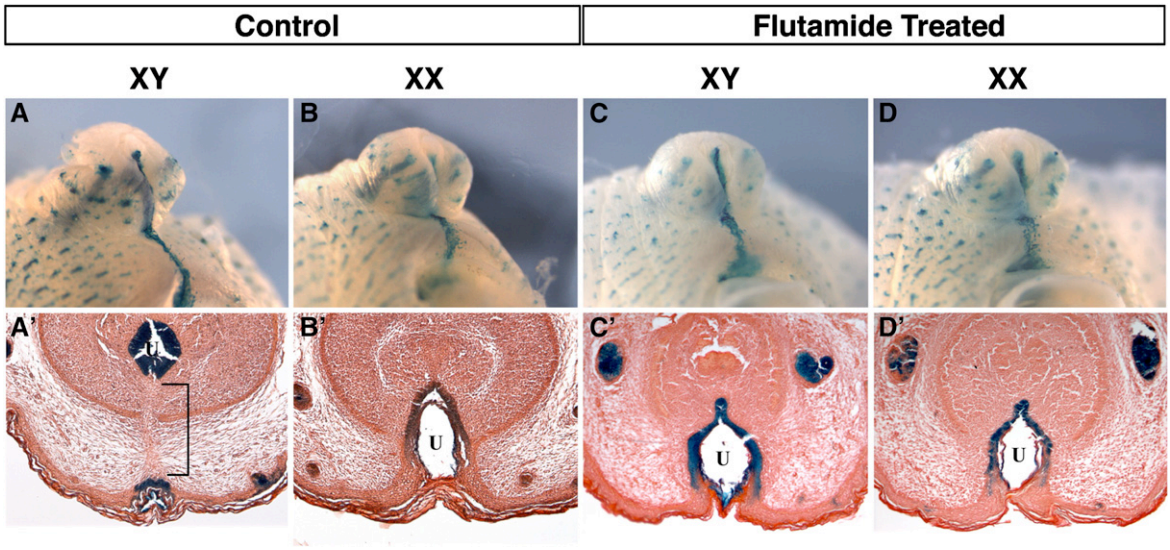

Fig. 5. Feminization of male genitalia by disruption of androgen receptor activity. Comparison of *ShhGFPcre;R26R* male (A, A′, C, C′) and female (B, B′, D, D′) mice at P0. (A, A′) Control male mice showing *ShhGFPcre* descendant cells of the definitive urethra (u) enveloped by the mature glans. Septation of the urethral plate has displaced some *ShhGFPcre* descendant cells to the ventral surface of the penis. Bracket marks mesodermal cells between urethral tube and ventral seam. (B, B′) Control females show that the *ShhGFPcre* descendant cells of the urethral plate persist in the midline between the preputial folds and glans. (C, C′) Flutamide-treated males show a feminization of the urethra, with *ShhGFPcre* descendant cells persisting from the center of the glans to its ventral surface. The urethral plate has failed to septate and mimics development of control and flutamide-treated females (compare C′ with B′ and D′). Restriction of *ShhGFPcre*-expressing cells to the urethral epithelium in treated males indicates that flutamide-induced feminization does not result in a transition of urethral plate epithelium to mesenchyme. (D, D′) Females treated with flutamide showing normal position of urethra.

remains in contact with the surface ectoderm on the ventral edge of the phallus; Figs. 5A′–D′).

Discussion

Endodermal origin of the distal urethra

Our fate map of the cloacal endoderm in mice provides the first direct evidence that the entire urethra is derived from endoderm. This finding challenges the longstanding view that the distal/glandar urethra arises from an invagination of distal ectoderm (Larson, 2001; Moore, 2007; Sadler, 2006). Histological studies and immunohistochemical analysis of cytokeratins had cast doubt on the hypothesis that the urethra has a dual origin (Kurzrock et al., 1999; Penington and Hutson, 2002a,b), however in the absence of a fate map, the origin of the urethra was unresolved. The cell lineage analysis presented above demonstrates that, in mice, the entire urethra (including the distalmost portion) originates from endodermal cells. This raises the possibility that a similar embryonic origin exists in human urethral development (Stadler, 2003).

Cloacal morphogenesis and development of the genital tubercle

Previously, Perriton et al. (2002) showed that an asymmetric dorsal swelling appears as the genital tubercle begins to emerge from the ventral body wall. Hynes and Fraher (2004b) further clarified the importance of this outgrowth by suggesting that it contributes to the glans of the genital tubercle. By using *ShhGFPcre* to distinguish endoderm from mesoderm during cloacal development, we have been able to visualize the dynamics of these two cell populations relative to one another. The data show that the glans forms from mesoderm situated anterior to the cloaca and ventral to the urogenital sinus, along with mesoderm of the initial genital swellings. Expansion of the dorsal swelling and the urorectal septum mesoderm, respectively, on the dorsal and ventral sides of the urogenital sinus is associated with a dorsoventral compression of the urogenital sinus. Thus, morphogenesis of the cloacal mesoderm may result in the distinct shape of the bladder and the ventral position of the urethral plate.

After formation of the coelomic cavity, lateral plate mesoderm in contact with the gut epithelium is defined as splanchnic, whereas that in contact with the surface ectoderm is defined as somatic. The mesoderm of the genital tubercle is unique, in that it is sandwiched between endoderm and ectoderm (i.e., it is not divided by the peritoneal cavity). This raises the possibility that genital tubercle mesoderm may differ from the splanchnic and somatic populations, both in the signals that it receives and in its responses to these signals. The expression patterns of a number of genes, including *Ptc1*, *Fgf10*, *Hoxd13* and *Hoxa13*, encircle the cloacal endoderm and are reminiscent of the response of gut mesoderm to endodermally derived *Shh* (Burns et al., 2004; Perriton et al., 2002; Petiot et al., 2005; Roberts et al., 1995). While previous work has compared emergence of the genital tubercle to early outgrowth of the limb bud (Haraguchi et al., 2001; Murakami and Mizuno, 1986; Perriton et al., 2002; Suzuki et al., 2003; Yamada et al., 2006), we propose that early development of external genitalia may be more similar to formation of the posterior gut tube, in which signaling occurs between endoderm and adjacent mesoderm.

Origin of the perineum

Our observation of *ShhGFPcre*-descendant cells along the central margin of the perineum reveals an unexpected endodermal origin of perineal skin in newborn mice. Perineal ectoderm does not express *Shh* (Perriton et al., 2002), confirming that LacZ-labeled cells of the perineal seam are derived from endoderm. As the urorectal septum extends towards the site of the future perineum, cloacal endoderm is driven towards the posterior surface of the embryo, where it ultimately comes to lie between the anus and the base of the genital tubercle. The discrete population of LacZ-labeled endodermal cells that we observed along the central margin of the perineum appears to result from caudal movement of the hindgut, and marks the terminal point at which the cloacal swellings meet the urorectal septum to form the definitive perineum. This result clarifies the longstanding confusion over how the embryonic cloaca is divided into separate urogenital and anorectal tracts (Hynes and Fraher, 2004c).

The classical view of anogenital septation is that the perineum forms from fusion of the cloacal shelves, in a manner similar to palatal

shelf fusion (Larson, 2001; Nievelstein et al., 1998). However, if movement of the mesoderm of the cloacal swellings and urorectal septum was lateral-to-medial in an *inward* direction, then the central seam of the perineum would be expected to move deep within the perineum as these shelves fused in the midline. We found no evidence for this in our lineage map. Rather, cloacal endodermal cells were found on the surface along the central margin of the perineum. This supports the hypothesis that the urorectal septum contributes to the perineum (Dravis et al., 2004; Hynes and Fraher, 2004c; Sasaki et al., 2004; van der Putte, 2005). Therefore, we propose that morphogenesis of the perineum involves posterior–lateral *eversion* of urorectal septum mesoderm, which results in cloacal endoderm being displaced to the posterior surface of the embryo.

The role of stromal mesoderm in urethral tube development

The results presented here show that proximal-to-distal invasion of the urethral plate by the urorectal septum and preputial mesoderm drives masculinization of the urethral plate. This occurs in association with preputial fold fusion along the ventral midline of the tubercle. Moreover, our fate map shows that *ShhGFPcre* descendants do not contribute to the mesenchyme of the external genitalia, which allows us to reject the hypothesis that the remodeling of a urethral plate into a centrally positioned urethral tube is due to an epithelial-to-mesenchymal transition. It is intriguing that apoptosis was not reported to occur in the epithelium of the urethral plate at E17.5 (when the urethral plate is undergoing septation), but was restricted to the mesenchyme between the urethra and the ventral ectoderm (Baskin et al., 2001). Taken together these two findings suggest that septation of the urethral plate results from morphogenetic reorganization of the epithelium, which may be a response to signals or mechanical influence from the adjacent mesenchyme, and this does not involve significant apoptosis or an epithelial-to-mesenchymal transition.

It has long been appreciated that disruption of androgen signaling (or treatment with estrogens) can lead to hypospadias in male genitalia (Agras et al., 2006; Gehring and Tomkins, 1974; Lyon and Hawkes, 1970). Despite extensive work on these pharmacological effects, the underlying developmental mechanisms responsible for hypospadias have been unclear. Recent work has shown that disruption of androgen signaling can modulate gene expression and alter epithelial organization within urethral plate cells (Dravis et al., 2004; Petiot et al., 2005). Our spatiotemporal lineage map of endodermal morphogenesis during urethral tube formation suggests that the timing of such disruptions may determine whether affected individuals have mild, moderate or severe hypospadias (see below). By identifying the cellular differences that occur during sexual differentiation of the genital tubercle, our results suggest that hypospadias can be interpreted as a morphogenetic feminization of the male external genitalia.

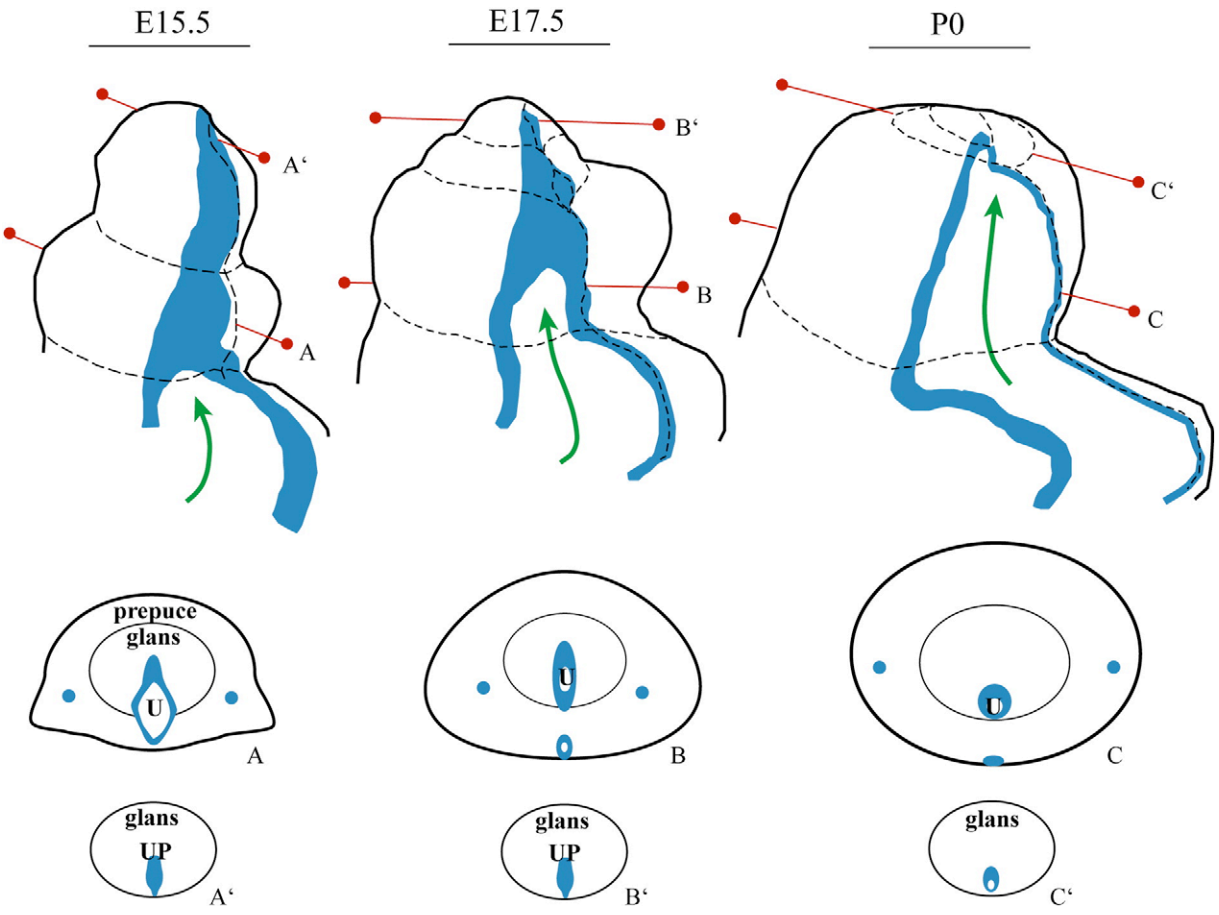

Fig. 6. Model for masculinization of the urethral plate in the mouse penis. Diagrams at the top show proximal-to-distal invasion of urorectal septum and preputial mesoderm (green arrows) into the male genital tubercle during masculinization, between E15.5 and P0. Red lines indicate planes of sections below (A–C and A′–C′), which show the spatial relationships of the urethral plate (up), urethra (u), prepuce and glans. The urethral plate is derived from endoderm (blue) and gives rise to the entire urethra. Beginning around E15.5, this process is mediated by androgen signaling. As the preputial mesoderm grows towards the distal glans, preputial cells move in a ventral direction towards the urethral plate. Simultaneously, urorectal septum mesoderm, which is continuous with proximal preputial mesoderm, grows into the genital tubercle, and together these two continuous populations septate the urethral plate. As this occurs, the urethral tube becomes internalized within the maturing glans. The remaining ventral portion of the urethral plate begins to disintegrate (B) and will form the ventral seam (raphe) of the penis (C). Absence of *ShhGFPcre* descendants in mesenchyme indicates that this division does not involve an epithelial-to-mesenchymal transition.

Urethral tubulogenesis and cloacal septation are linked by a common developmental mechanism

The data presented above suggest that the cellular processes underlying septation of the cloaca also underlie septation of the urethral plate to form the definitive male urethra. Historically, formation of the external genitalia and septation of the cloaca have been considered separate developmental processes. Our findings indicate that these two processes are coordinated along a spatiotemporal continuum, beginning with formation of the urorectal septum and ending with formation of the urethral meatus. As such, disruption of urorectal septum development during morphogenesis of the cloaca would be expected to result in malformations of both the urogenital and anorectal systems. Whole mount and histological data from both male and female mice show that the primary cellular difference that occurs during masculinization of the urethral epithelium is the division of the urethral plate by the mesenchyme of the urorectal septum and proximal preputial folds. Therefore, disruption of mesodermal septation of the urethral plate at earlier time points would be expected to result in more severe (i.e., proximal) hypospadias, with severity being classified by proximodistal position of the urethral opening. According to our model, described in detail below, an arrest of urethral plate septation at E15.5 would lead to a complete feminization of the male genitalia, whereas arrest at later time points would allow formation of a centralized urethra proximally but persistence of a ventrally open urethral plate distally.

A model for masculinization of the urethral plate

Based on the above results, we present a new model for morphogenesis and sexual differentiation of the urethra (Fig. 6). The model suggests that posterior urogenital and anorectal development is divisible operationally into three integrated phases. Firstly, during initiation of external genital outgrowth, paired genital swellings emerge ventro-lateral to the cloaca, which is coordinated temporally with convergence and extension of cloacal mesoderm at the urorectal septum anterior to the cloaca. Disruption of either event will lead to external genital reduction or agenesis and persistent cloaca, consistent with the phenotypes found in *Shh−/−*, *Gli2−/−*, *Gli2−/−;Gli3+/−*, *p63−/−* and *Hoxa13−/−;Hoxd13−/−* mutants, and in mice exhibiting caudal regression syndrome (Cheng et al., 2006; Haraguchi et al., 2001; Ince et al., 2002; Kimmel et al., 2000; Mo et al., 2001; Perriton et al., 2002; Warot et al., 1997). The second phase involves cloacal morphogenesis and outgrowth of the genital tubercle, which covers the period from the end of Phase I through septation of the cloaca into urogenital and anogenital sinuses, and includes formation of the urethral plate and perineum. Disruption in Phase II would lead to associated malformations of both the external genitalia and perineum (e.g., proximal hypospadias, micropenis, imperforate anus, persistent cloaca, etc). *Shh* has been suggested to act as an organizer during formation of the external genitalia, and our results suggest that its role as an organizing signal from the cloacal endoderm may act also to coordinate morphogenesis of the mesoderm surrounding the cloaca (Perriton et al., 2002). Consistent with our hypothesis, null mutations in the Gli family of proteins, which are key modulators of the *Shh* pathway, display malformations of this type (Kimmel et al., 2000; Mo et al., 2001). Lastly, the third phase of development includes the period from the completion of anorectal and urogenital septation (perineum formation) through sexual differentiation of the external genitalia. The behavior of cloacal endoderm in response to AR antagonism shows that, in contrast to the previous two phases, Phase III is androgen-dependent, and thus both genetic and hormonal disruption can affect normal morphogenesis during this time period. Phase III is defined by the invasion of urorectal septum and preputial mesoderm into the genital tubercle and a proximal-to-distal septation of the urethral plate to form a tubular urethra in the male. This is accompanied by growth and fusion of the prepuce along the ventral margin of the genital tubercle. These three phases provide a developmental framework for interpretation of congenital malformations and allow for the identification of the precise temporal windows during which morphogenesis has been disrupted in patients with urogenital and anorectal malformations.

Our finding that septation of the urethral plate involves sustained growth of the mesoderm surrounding the glans and urethra identifies a morphogenetic mechanism for the proximal-to-distal progression of urethral tubulogenesis. This suggests that disruption of morphogenesis during Phase III will result in hypospadias of varying severity, with earlier perturbations resulting in more proximal hypospadias. Most importantly, unlike Phase I and Phase II morphogenesis, development during Phase III is directed by both local and systemic signals. How systemically circulating endocrine signals interact with the gene networks that operate locally within the genital tubercle is only beginning to be understood (Dravis et al., 2004; Petiot et al., 2005), however this dual nature of developmental control during sexual differentiation potentially increases the number of perturbations that can affect urethral tube formation at later stages of development.

Acknowledgments

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