Tissue-specific roles of Fgfr2 in development of the external genitalia

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ABSTRACT

Congenital anomalies frequently occur in organs that undergo tubulogenesis. Hypospadias is a urethral tube defect defined by mislocalized, oversized, or multiple openings of the penile urethra. Deletion of Fgfr2 or its ligand Fgf10 results in severe hypospadias in mice, in which the entire urethral plate is open along the ventral side of the penis. In the genital tubercle, the embryonic precursor of the penis and clitoris, Fgfr2 is expressed in two epithelial populations: the endodermally derived urethral epithelium and the ectodermally derived surface epithelium. Here, we investigate the tissue-specific roles of Fgfr2 in external genital development by generating conditional deletions of Fgfr2 in each of these cell types. Conditional deletion of Fgfr2 results in two distinct phenotypes: endodermal Fgfr2 deletion causes mild hypospadias and inhibits maturation of a complex urethral epithelium, whereas loss of ectodermal Fgfr2 results in severe hypospadias and absence of the ventral prepucce. Although these cell type-specific mutants exhibit distinctive genital anomalies, cellular analysis reveals that Fgfr2 regulates epithelial maturation and cell cycle progression in the urethral endoderm and in the surface ectoderm. The unexpected finding that ectodermal deletion of Fgfr2 results in the most severe hypospadias highlights a major role for Fgfr2 in the developing genital surface epithelium, where epithelial maturation is required for maintenance of a closed urethral tube. These results demonstrate that urethral tubulogenesis, prepuce morphogenesis, and sexually dimorphic patterning of the lower urethra are controlled by discrete regions of Fgfr2 activity.

KEY WORDS: Fgf, Genitalia, Mouse, Sexual differentiation, Tubulogenesis, Urethra

INTRODUCTION

Tubular morphogenesis (tubulogenesis) is essential for normal embryonic development, and organs that undergo tubulogenesis are frequently affected by congenital anomalies (Ray and Niswander, 2012). A variety of mechanisms can drive tube formation, such as the wrapping mechanism of neurevolution, budding of the salivary and mammary glands, and the delamination/migration process of bile duct development (Andrew and Ewald, 2010; Caviglia and Luschning, 2014; Luschning and Uv, 2014; Zegers, 2014). Hypospadias, a urethral tube defect defined by a mislocalized urethral opening (meatus), is among the most common birth defects in humans, affecting ~1 in 250 live births (Paulozzi et al., 1997; Baskin et al., 2001).

External genital development involves a series of budding and fusion events. Paired genital swellings arise lateral to the cloacal membrane, around embryonic day (E) 10.5 in the mouse, and these swellings then merge to form a single genital tubercle by E11.5 (Perriton et al., 2002). By E13.5, paired preputial swellings have emerged from the lateral margins at the base of the genital tubercle and eventually give rise to the prepuce (foreskin and clitoral hood; Perriton et al., 2002). At E14.5, the labioscrotal swellings protrude caudally and later will either fuse to form the male scrotum or will remain unfused to form the female labia majora (Perriton et al., 2002). After the monomorphic phase of external genital paterning, which lasts until ~E15, sexually dimorphic patterning is regulated by sex hormones synthesized by the testes and ovaries.

The embryonic urethra develops via the inclusion of cloacal endoderm in the nascent genital tubercle (Perriton et al., 2002; Seifert et al., 2008), where it persists as a bilaminar epithelial plate through E14, when it begins to cavitate from proximal to distal to form the lumen of the urethral tube (Perriton et al., 2002; Cohn, 2011). In males, invasion of the urorectal septum mesenchyme into the genital tubercle displaces the urethral tube to a central position within the penis, whereas in females, the endoderm remains tethered to the ventral side of the glans (Glenister, 1954; Seifert et al., 2008). Thus, urethral tubulogenesis involves multiple morphogenetic processes, including evagination of the cloacal wall to form the bilaminar plate, epithelial stratification and maturation, and remodeling of the plate to form the lumen of the urethra.

A number of studies have reported that the incidence of hypospadias and other defects of the external genitalia (such as cryptorchidism and choreoed) has increased over the past several decades (Paulozzi et al., 1997; Oppari et al., 2010; Nordenvall et al., 2014). Although most instances of hypospadias are idiopathic (Van der Zanden et al., 2012), molecular genetic analyses of affected individuals have revealed nucleotide variants (Tannoury-Louet et al., 2010, 2014; Geller et al., 2014), epigenetic anomalies (Vottero et al., 2011), protein modifications (Qiao et al., 2011), and mutations in known developmental genes (Beleza-Meireles et al., 2007; Carmichael et al., 2013). The results of genetic, toxicological, and epidemiological studies have led to the proposal that urethral tube defects are caused by the combinatorial effects of aberrant hormone activity, such as that caused by exposure to environmental endocrine-disrupting compounds (Michalakis et al., 2014; Winston et al., 2014), and underlying genetic susceptibility (Wang and Baskin, 2008; Kalfa et al., 2009, 2011; Toppari et al., 2010; Yiee and Baskin, 2010; Van der Zanden et al., 2012).

We previously reported that deletion of either Fgfs10 or its receptor Fgfr2 (the epithelial isoform of Fgfr2) results in severe hypospadias (Petiot et al., 2005). Fgfs10 is expressed in the genital mesenchyme, and Fgfr2 is transcribed in the adjacent urethral epithelium and in the surface ectoderm (Haraguchi et al., 2000; Satoh et al., 2004; Petiot et al., 2005; Ching et al., 2014). Fgfr2iiib−/− mice develop hypospadias associated with decreased proliferation

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and stratification of urethral cells that results from failure to maintain the progenitor cell layer of the urethral epithelium (Petiot et al., 2005). Importantly, Fgfr2 transcription in the genital tubercle can be downregulated by exposure to the androgen receptor (AR) antagonist flutamide in a dose-dependent manner (Petiot et al., 2005), suggesting that Fgfr2 acts as a link between hormonal and genetic regulation of external genital development.

The complex expression pattern of Fgfr2iiib and the compound phenotype of Fgfr2iiib−/− mice precluded a determination of the tissue-specific roles of Fgfr2. To determine which domain(s) of Fgfr2 expression regulate(s) urethral tubulogenesis, we generated conditional mutants in which Fgfr2 is deleted from either the urethral endoderm or the surface ectoderm. Conditional deletion of Fgfr2 results in two distinct phenotypes: endodermal Fgfr2 ablation causes mild hypospadias and hypotrophy of a complex urethral epithelium, whereas loss of ectodermal Fgfr2 induces severe hypospadias and failure of ventral prepuce closure. Although these compartment-specific mutants exhibit distinct genital anomalies, we find that at the cellular level, Fgfr2 plays similar roles in the urethral tube and surface ectoderm. These results demonstrate that urethral tubulogenesis, prepuce morphogenesis, and sexually dimorphic patterning of the urethra are three crucial processes in external genital development that are controlled by independent regions of Fgfr2 activity.

RESULTS

Distinct regions of Fgfr2 signaling mediate development of the prepuce and urethra

To dissect the roles of the urethral endodermal and the surface ectodermal domains of Fgfr2 in external genital development, we performed conditional deletions of Fgfr2loxlox using the ShhCre and Msx2Cre alleles (Sun et al., 2000; Yu et al., 2003; Harfe et al., 2004). Fgfr2 was ablated in urethral endoderm in ShhCre+/−; Fgfr2loxlox mice, which will be referred to as Fgfr2Endo mutants. The ectodermal domain of Fgfr2 was deleted in Msx2Cre; Fgfr2loxlox mice, which will be referred to as Fgfr2Ecto mutants. Removal of Fgfr2 from target tissues was verified by whole-mount in situ hybridization using a riboprobe that detects the floxed region of Fgfr2 (supplementary material Fig. S1). To examine whether Fgfr2 deletion in one epithelial population affects Fgfr2 signaling in another region, we used immunofluorescence for Fgfr2. We found no changes in the localization of ectodermal FGR2 in Fgfr2Endo mutants, of urethral FGR2 in Fgfr2Ecto mutants or of mesenchymal FGR2 in either mutant (supplementary material Fig. S1), indicating that Fgfr2 conditional deletion from the urethra does not affect ectodermal Fgfr2 signaling and vice versa.

To facilitate identification of Fgfr2 knockout cells, we used the Rosa26lacZ reporter (R26R; Soriano, 1999) and fate-mapped Cre-expressing lineages with X-Gal staining. In control (ShhCre+/−; R26R) male genitalia at postnatal day (P) 0, lacZ+ cells are present in three locations: the urethral epithelium, the perineal raphe, and the preputial glands (Fig. 1A; Seifert et al., 2008). External genitalia of male Fgfr2Endo mutants at P0 appeared generally normal (the prepuce enveloped the glans and was fused at the ventral midline); however, lineage tracing revealed that ectopic endodermal cells were exposed on the ventral surface of the penis and a distal urethral meatus was absent (Fig. 1B). The stripe of endodermally derived cells along the perineum was thinner mediolaterally, shorter anteroposteriorly and had an irregular border relative to controls (Fig. 1B). The intermingling of urethral endodermal and surface ectodermal cells suggests a relaxation of the boundary between these cell populations (Fig. 1B). These results indicate that in males, deletion of Fgfr2 in the endoderm results in incomplete internalization of urethral epithelial cells.

Fgfr2Ecto mutants developed hypospadias with a range of severity. In male mutants with the most extensive hypospadias, preputial ectodermal cells failed to fuse along the ventral margin of the penis, resulting in complete exposure of the (lacZ+) urethral plate (Fig. 1D), whereas mutants with localized ectopic urethral openings were characterized by partial fusion of the prepuce around the urethral plate (supplementary material Fig. S2). Mosaicism in Msx2loxlox activity was visible by discontinuous distribution of lacZ+ in both mutant and control embryos (Fig. 1C,D; supplementary material Fig. S2). These data show that deletion of ectodermal Fgfr2 results in penile hypospadias, indicating that Fgfr2 activity in the surface epithelium is required for morphogenesis of the ventral prepuce and enclosure of the urethral tube.

Both male and female Fgfr2 null mutants were reported to develop hypospadias (Petiot et al., 2005). During normal development, newborn male and female external genitalia show clear dimorphism; females display a more proximal urethral meatus, a vaginal opening at the base of the glans, a broad domain of endodermally derived cells in the perineal raphe and a short agenital distance (Fig. 1E,G). Fgfr2Endo mutant females at P0 had a hypoplastic vaginal opening in which lacZ+ cells extended both laterally onto the surface of the perineum and distally onto the clitoral skin (Fig. 1F). This was reminiscent of the mislocated endodermal cells at the base of the glans in Fgfr2Endo mutant males. An additional similarity with Fgfr2Endo males was that the population of lacZ+ endodermally derived cells along the perineal raphe was thinner and shorter than in controls (Fig. 1F). The urethral meatus of control females was visible at the apex of the prepuce,

![Fig. 1. External genital morphologies and mutant cell fates in Fgfr2 conditional knockout mice. (A,B,E,F) Endoderm fate-mapping in ShhCre+/−; R26R mice demonstrates that Fgfr2Endo males have an irregular perineal raphe (triangles) and females have a dysmorphic vaginal opening (black arrows). Both sexes have ectopic endodermal cells in the preputial skin (inset) and lack a distal urethral meatus (white arrows). (C,D,G,H) Ectoderm fate-mapping in Msx2Cre; R26R mice reveals failed prepuce closure (white arrowheads) in Fgfr2Ecto males and severe hypospadias in Fgfr2Ecto males and females. Scale bars: 100 μm.](image-url)
where the lateral preputial folds abut the glans (Fig. 1E). This region was absent from Fgfr2EndoΔ clitorises, and ectopic punctate domains of lacZ+ endodermal cells were visible along the ventral midline (Fig. 1F, inset). Thus, the external genitalia of newborn male and female Fgfr2EndoΔ mutants have endodermally derived cells ectopically positioned in the surface epithelium of the prepuce, and an irregular boundary of endodermal and ectodermal cells along the perineal raphe.

Female Fgfr2EctoΔ mutants had hypospadias with a range of severities similar to those observed in males. In female Fgfr2EctoΔ mutants with severe hypospasias, the prepuce was completely unfused, resulting in a single urogenital opening (supplementary material Fig. S2). A subset of neonatal female Fgfr2EctoΔ mutants had a large, proximally displaced (hypospadic) urethral meatus and a partially fused prepuce that separated the tip of the urethral plate from the vaginal orifice (Fig. 1H; supplementary material Fig. S2). Thus, ectodermal Fgfr2 deletion causes severe hypospadias and perturbs prepuce morphogenesis in both sexes.

Fgfr2 conditional deletions disrupt urethral internalization and cause hypospadias

Histological analysis provided further insight into the external genital anomalies of Fgfr2 conditional mutants. In transverse sections through the distal penis of newborn control mice, the left and right sides of the prepuce fused ventrally, dividing the endodermal epithelium into a urethral tube dorsally and the penile raphe ventrally (Fig. 2A,C,E,G). In sections through this region of Fgfr2EndoΔ mutant males, the urethral epithelium was not septated into a dorsal tube and ventral seam, but instead persisted as a urethral plate that extended from the center of the tubercle to the ventral margin, similar to the morphology seen in the control female clitoris (compare mutant penis in Fig. 2J with control penis in 2I and control clitoris in 2U). The distal penis of Fgfr2EctoΔ males displayed an unfused prepuce and an open urethral sulcus (Fig. 2D,H). Within the penile shaft of controls, the urethral tube was positioned at the ventral margin of the glans, which was completely enveloped by the prepuce (Fig. 2L,K). By contrast, the urethral epithelium of both Fgfr2EndoΔ and Fgfr2EctoΔ mutants extended ventrally from the glans through the unfused prepuce and was contiguous with the ventral surface ectoderm, resulting in hypospasias (Fig. 2F). In control and Fgfr2EndoΔ females, the distal urethra formed a solid epithelial cord that lacked a lumen (Fig. 2Q,R), whereas the proximal urethra was an open tube that remained attached to the ventral ectoderm (Fig. 2U,V). In Fgfr2EndoΔ females, the ventral margin of the urethral plate developed an open, hypospadic phenotype along the proximodistal axis of the clitoris (Fig. 2, compare enclosed tube in S,W with open groove in T,X). These data indicate that, in males, both endodermal and ectodermal Fgfr2 deletions interrupt urethral internalization, endodermal Fgfr2 deletion disrupts urethral canalization, and ectodermal Fgfr2 deletion disrupts fusion of the ventral prepuce and urethral internalization. In females, only ectodermal Fgfr2 deletion results in hypospadias with an open ventral prepuce proximally.

Endodermal Fgfr2 regulates maturation of a complex urethral epithelium

To identify the cellular basis of hypospadias in mice lacking urethral Fgfr2, we examined Fgfr2EndoΔ and control genital tubercles at E14.5 by scanning electron microscopy (SEM), histology, fate mapping and immunohistochemistry. At E14.5, Fgfr2EndoΔ mutant external genitalia were morphologically similar to controls; each had developed a genital tubercle with a urethral seam along the ventral midline and preputial swellings on the lateral margins, labioscrotal swellings on the ventral body wall just below the preputial swellings, and a proximal urethral opening located at the base of the tubercle (Fig. 3A,B). At high magnification, the proximal urethral opening of control embryos was oval-shaped, in contrast to the diamond-shaped opening of Fgfr2EndoΔ mutants (Fig. 3C,D). Given that Fgfr2 was deleted only in the endoderm, we hypothesized that this malformation of the proximal urethra is a direct consequence of morphogenetic changes in the urethral epithelium. By histological analysis, three features of urethral

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Fig. 2. Urethral morphology in newborn males and females with conditional deletions of Fgfr2. Lineage tracing (A-D,M-P) and histological sections (E-L,Q-X) of the external genitalia in newborn male (A-L) and female (M-X) Fgfr2 conditional mutants. Urethral internalization is disrupted in Fgfr2EndoΔ (F,J) and Fgfr2EctoΔ (H,L) males. Fgfr2EndoΔ males (H,L) and females (T,X) develop severe hypospadias. Dashed lines in A-D and M-P indicate planes of section in E-L and Q-X, respectively. Black arrows mark the internal urethral tube, black triangles indicate the penile raphe, yellow arrows denote an endodermal epithelial cord, and red triangles mark hypospadic urethral openings. Histological images were converted to grayscale using Adobe Photoshop. Scale bars: 100 μm.
morpogenesis were evident in E14.5 control embryos: the proximal penile urethra contained an open lumen, the distal urethra was a bilaminar epithelial plate, and a complex, stratified epithelium was evident along the proximodistal axis of the urethra (Fig. 3E,G; Perriton et al., 2002). In controls and Fgfr2EndoΔ mutants, a urethral lumen was apparent in the proximal urethra while a closed urethral plate persisted distally; however, in Fgfr2EndoΔ mutants the urethral epithelium appeared thinner and less stratified (Fig. 3F,H). The finding that Fgfr2EndoΔ mutant urethrae show diminished stratification indicates that Fgfr2 is required for the endoderm to mature into a complex epithelium.

Previous studies of Fgfr2iiib knockout mice suggested that, between E13.5 and E15.5, the loss of the tubular urethra could be the result of an epithelial-mesenchymal transition (EMT) (Petiot et al., 2005). To test for the presence of endodermal cells in the genital mesenchyme, we sectioned β-galactosidase-stained Fgfr2EndoΔ and control genital tubercles and labeled epithelial cells with an antibody against cytokeratin 14 (K14), an intermediate filament produced in the genital epithelium but not in the mesenchyme (Kurzrock et al., 1999). In both mutants and controls, the margin of the K14+ urethral epithelium also marked the boundary of lacZ+ cells; blue cells were not observed in the mesenchyme (Fig. 3L,J), and this pattern persisted throughout the proximodistal length of control and mutant genital tubercles (supplementary material Fig. S3). Thus, deletion of Fgfr2 from the urethral endoderm disrupts maturation of the urethral epithelium but does not lead to an EMT.

During normal masculinization of the urethral plate, ingrowth of the urorectal septum into the proximal genital tubercle partitions the urethral plate into a definitive urethral tube dorsally and a penile raphe ventrally (Seifert et al., 2008). In light of our finding that urethral internalization is disrupted following endodermal Fgfr2 deletion, we investigated whether this process is disrupted in Fgfr2EndoΔ mutants. At E14.5, migration of the urorectal septum into the proximal genital tubercle was evident in control males (Fig. 3K,K′). Urorectal septum mesenchyme also was detectable at the base of the genital tubercle in Fgfr2EndoΔ mutants, although it did not extend into the genital tubercle as in controls (Fig. 3L,L′). These data are consistent with our finding that the glanular urethral tube is absent from male Fgfr2EndoΔ mutants at birth.

**Ectodermal Fgfr2 is required for preputial development and urethral morphogenesis**

We next investigated the possibility that the abnormal urethral opening and mislocalized preputial swellings of Fgfr2EctoΔ mutants might be associated with urethral tube defects internally. Analysis of transverse histological sections showed that the dorsal urethra of controls and Fgfr2EctoΔ mutants at E14.5 was a solid, bilaminar plate along the proximodistal axis of the genital tubercle, whereas the ventral urethra had opened into a lumen proximally and retained bilaminar morphology distally (Fig. 4E-H). However, in Fgfr2EctoΔ mutants, the ventral side of the urethral epithelium formed an open groove (sulcus) rather than a tube, exposing the underlying urethral plate such that it was visible on the surface of the genital tubercle (Fig. 4D,H). In coronal sections, the urethral tube of controls had a teardrop-shaped or cylindrical morphology that decreased in size from.
Nuclear Fast Red. Scale bars: 50 μm.

Coronal histological sections reveal a conical urethral lumen (asterisks) in ectodermal cells are stretched across the urethral lumen ventrally (arrowhead). Normal bilaminar plate arrangement in the dorsal urethra (arrow) but morphotype. Failure of ventral prepuce formation is associated with opening of the endodermal urethral plate (and, later, the tube) along the ventral midline, resulting in hypospadias.

**Fgfr2 regulates epithelial cell shape and stratification**

To better understand the nature of the epithelial defects that were observed in histological sections, we performed high-resolution transmission electron microscopy (TEM) analysis of Fgfr2\(^{Δ\text{Endo}}\) and Fgfr2\(^{Δ\text{Ecto}}\) genital tubercles at E14.5. To facilitate visualization of the epithelia, we pseudocolored cells based on their positions; those in contact with the urethral lumen (urethra) or the extraembryonic space (ectoderm) were tinted magenta, cells abutting the mesenchyme were tinted cyan and all remaining cells that contacted neither surface were tinted yellow. Control urethrae at E14.5 formed stratified epithelia of 3-6 cell layers (Fig. 5A), whereas Fgfr2\(^{Δ\text{Endo}}\) mutant urethral epithelia had 1-3 cell layers throughout the epithelial sheet (Fig. 5B). In the ectoderm of both Fgfr2\(^{Δ\text{Ecto}}\) mutants and controls, the epithelia were stratified 3-5 cell layers thick (Fig. 5C,D), suggesting that Fgfr2 mediates epithelial stratification in the urethra only.

In control urethral and ectodermal epithelia, basal cells were columnar and elongated perpendicular to the basement membrane, intermediate cells were smaller and rounded, and apical cells were rounded or, in the urethral epithelium, squamous (Fig. 5A,C). Fgfr2\(^{Δ\text{Endo}}\) urethral cell shape was more homogenous; along the apicobasal axis, all cells were cuboidal or rounded and had no obvious orientation within the epithelial sheet (Fig. 5B). Similarly, the basal cells in the surface epithelium of Fgfr2\(^{Δ\text{Ecto}}\) mutants failed to develop a columnar morphology; in each epithelial layer, cells were flat and elongated parallel, rather than perpendicular, to the basement membrane (Fig. 5D). These data indicate that endodermal Fgfr2 regulates epithelial stratification and basal cell morphogenesis within the urethra, and that Fgfr2 in the ectoderm controls basal cell shape, but not stratification, in the surface epithelium.

**Cytoskeletal and cell adhesion defects in Fgfr2 conditional mutants**

We next investigated the mechanisms responsible for the cellular defects that occur in the epithelia of Fgfr2 conditional mutants. Given that epithelial organization and polarity are perturbed in both conditional mutants, we asked how Fgfr2 deletion impacts actin dynamics and cell adhesion in Fgfr2\(^{Δ\text{Endo}}\) and Fgfr2\(^{Δ\text{Ecto}}\) epithelia. We used phalloidin staining to monitor filamentous actin and immunofluorescence for β-catenin (a required component in adherens junctions; also known as Ctnnb1 – Mouse Genome Informatics) to investigate cell adhesion. Our TEM results showed that normal basal urethral cells at E14.5 were columnar and elongated perpendicular to the basement membrane; phallolidin staining demonstrated that this organization was supported by a scaffold of actin filaments that localized to the cell cortices as long, fibrous bundles (Fig. 5E). At boundaries between adjacent basal cells, enriched F-actin staining was visible (Fig. 5E) and punctate regions of β-catenin were localized to basal lateral cell margins (Fig. 5K). In Fgfr2\(^{Δ\text{Endo}}\) basal urethral cells, as in controls, actin filaments were largely oriented perpendicular to the basement membrane; however, F-actin domains were short and hatched rather than long and fibrous (Fig. 5D), and basolateral β-catenin domains were not detected (Fig. 5L), suggesting that cell shape defects occur in conjunction with cytoskeletal disorganization and diminished cell adhesion.

The ventral seam of the genital tubercle showed regional differences in phallolidin staining along the proximodistal axis. In distal planes, where the urethral plate had not yet delaminated to form a tube, we found an enrichment of elongated actin filaments at...
the ventral seam (Fig. 5G). Increased F-actin was absent from the ventral genital ectoderm in proximal regions where the urethra had canalized to form an open lumen (Fig. 5I), although these regions displayed locally elevated β-catenin staining (Fig. 5O). In Fgfr2Ecto Δ mutants, local enrichment of filamentous actin bundles at the ventral midline was present along the entire proximodistal axis of the genital tubercle (Fig. 5H,J), and no increases in β-catenin staining were detected at the ventral ectodermal midline (Fig. 5N,P). These data suggest that urethral canalization involves dynamic changes in cytoskeletal rigidity and adhesion at the ectodermal-endodermal boundary, and that these processes are misregulated in Fgfr2Δ mutants.

**Fgfr2 controls proliferation in urethral and surface epithelia by regulating the G1/S transition**

To determine whether the epithelial defects in Fgfr2Endo Δ and Fgfr2Ecto Δ mutants include disrupted cell proliferation, we first quantified cell number in mutant versus control epithelia. Cell counting showed reduced numbers of urethral cells in quantified cell number in mutant versus control epithelia. Cell

![Fig. 5. Deletion of Fgfr2 disrupts epithelial cell shape, cytoskeletal organization, and cell adhesion.](image)

(A-D) Ultrastructural analysis by TEM reveals aberrant cell shapes in Fgfr2−/− genital tubercle epithelia; mutant basal cells fail to elongate perpendicular to the basement membrane in both Fgfr2Endo Δ and Fgfr2Ecto Δ mutant epithelia. Diminished stratification is evident in the Fgfr2Endo Δ urethra. Cells adjacent to basement membrane are pseudocolored cyan, those neighboring the lumen are magenta and intervening cells are yellow. Scale bars: 2 μm. (E-F) Fluorescent micrographs of control, Fgfr2Endo Δ and Fgfr2Ecto Δ genital tubercles stained with phalloidin (E-J) or immunolabeled with an anti-β-catenin antibody (K-P). Note the long cell cortices stained with F-actin in E (white arrows) and short, hatched phalloidin domains in F (arrowheads). Punctate, basolateral foci of β-catenin are evident in control basal urethral cells (yellow arrows) but were not detected in Fgfr2Endo Δ mutants. In controls, the ventral seam shows enriched actin stress fibers (white triangles) distally and increased β-catenin staining (yellow triangles) proximally. Elevated abundance of F-actin fibers is coincident with static levels of β-catenin intensity along the proximodistal ventral midline of Fgfr2Endo Δ mutants. Asterisks mark urethral lumen, dotted lines outline the urethral and surface epithelia. Scale bars: 20 μm.

To test the hypothesis that decreased cell numbers are caused by diminished cell proliferation, we used BrdU labeling to calculate the mitotic indices of urethral epithelial cells in Fgfr2Endo Δ mutants and of surface ectodermal cells in Fgfr2Ecto Δ mutants. We found a significantly lower mitotic index in Fgfr2Endo Δ urethral cells (17.7%) compared with control urethral cells (34.9%, P<0.001; Fig. 6B). We also found that the mitotic index of ectodermal cells in Fgfr2Ecto Δ mutants was significantly lower (17.9%) than control ectodermal cells (24.4%, P<0.005; Fig. 6B). As a control, we monitored the mitotic indices of Cre+ mesenchymal cells in Fgfr2Endo Δ mutants and controls and found no significant differences (33.4% and 34.5%, respectively; Fig. 6B). These data indicate that Fgfr2 regulates epithelial cell proliferation in the urethra and surface ectoderm of the genital tubercle.

For insight into the mechanism by which Fgfr2 regulates cell proliferation, we examined cell cycle kinetics of mutant cells in both Fgfr2Endo Δ and Fgfr2Ecto Δ embryos. We calculated the lengths of G1/G0, G2/M, and S phases, as well as total cell cycle duration for individual cell populations (Seifert et al., 2010). In the urethral epithelium of control embryos, the average length of G1 was 8.1 h and total cell cycle time was 12.1 h, but in Fgfr2Endo Δ urethral cells the average length of G1 was 25.5 h and total cell cycle time was 29.5 h (G1/S: P<0.001, total: P<0.001; Fig. 6C). We found no significant differences in the lengths of S phase and G2/M (Fig. 6C). Deletion of Fgfr2 from ectodermal cells has a similar effect on cell cycle kinetics; in control ectodermal cells, G1 length
was 18.1 h and the total cell cycle time was 23.2 h. By contrast, Fgfr2iiib/−− ectodermal cells have an average G1 length of 29.1 h and total cell cycle time of 34.1 h (G1/S: P<0.01, total: P=0.01; Fig. 6C). No significant differences in the times of S and G2/M phases were detected in Fgfr2iib/−− cells (Fig. 6C). As conditional deletion of Fgfr2 significantly lengthens G1, and, consequently, total cell cycle time is increased, we conclude that Fgfr2 regulates cell proliferation by promoting the G1/S transition both in the urethral epithelium and in the surface ectodermal epithelium.

**Transcriptional interactions among Fgfr2, Shh and β-catenin**

Global deletion of Fgfr2iiib results in a premature downregulation of Shh in the urethra (Petiot et al., 2005). Based on the Shh expression pattern and the phenotypes of tissue-specific Fgfr2 conditional knockouts, decreased Shh in Fgfr2iiib/−− mice could be a direct consequence of endodermal Fgfr2 deletion (meaning that Fgfr2 mediates Shh in a cell-autonomous manner), an indirect effect of fewer urethral cells (due to loss of endodermal Fgfr2), or an indirect effect of ectodermal Fgfr2 deletion (suggesting that Shh is regulated by an intermediate signal controlled by Fgfr2 in the ectoderm). To determine the tissue-specific roles of Fgfr2 in the regulation of Shh expression, we compared the levels of Shh mRNA in genital tubercles of Fgfr2iiib/−−, Fgfr2iib/−− and control mice by quantitative real-time PCR (qRT-PCR). We also examined levels of the Shh target gene Patched1 (Pch1) to monitor Shh signal transduction. Genital tubercles of Fgfr2iiib/−− mutants showed a 3.3-fold decrease in Shh expression (P=0.005) and a 2.3-fold decrease in Pch1 (P=0.006), whereas Fgfr2iib/−− genital tubercles showed no significant differences in Shh or Pch1 expression levels (Fig. 6D). Thus, hedgehog signaling is diminished as a result of endodermal Fgfr2 deletion but is unaffected by ectodermal Fgfr2 deletion.

In light of our finding that endodermal Fgfr2 deletion results in diminished Shh signaling, we tested whether Fgfr2 and Shh function in a feedback loop during development of the external genitalia. To test this possibility, we examined the expression levels of Fgfr2 in Shh mutant genital tubercles. As Shh−/− embryos fail to develop external genitalia beyond rudimentary paired genital swellings (Haraguchi et al., 2001; Perriton et al., 2002), we used inducible Cre [ShhCreERT2, Harle et al. (2004)] and floxed [Shh; Dassule et al. (2000)] alleles to conditionally delete Shh at E11.5, and we monitored the levels of Fgfr2 mRNA in E14.5 genital tubercles by qRT-PCR. ShhCreERT2/−− genital tubercles showed no change in Fgfr2 expression levels (Fig. 6D). Based on these data, we conclude that Fgfr2 is required for normal expression of Shh but that Shh does not feed back to regulate Fgfr2. This is consistent with our previous report that urethral epithelial cells do not express Shh (Seifert et al., 2009), and therefore do not respond directly to Shh.

Conditional deletion of β-catenin from the urethral endoderm causes transcriptional downregulation of Shh, Pch1 and Fgfr2 (Lin et al., 2008). Our immunohistochemical analyses demonstrated that β-catenin was mislocalized in mutant epithelia of Fgfr2iiib/−− and Fgfr2iib/−− conditional knockout mutants; to identify whether these defects were caused by changes in β-catenin transcription, and to determine whether Shh and β-catenin function in a feedback loop, we analyzed the levels of β-catenin expression in Fgfr2 and Shh conditional knockout genital tubercles by qRT-PCR. We found no changes in β-catenin transcription levels in Fgfr2iiib/−−, Fgfr2iib/−− or ShhCreERT2/−− genital tubercles as compared with control littermates (Fig. 6D). These data suggest that changes in β-catenin localization in Fgfr2iiib/−− epithelia occur at the level of the protein, and that β-catenin and Shh are not involved in a feedback loop in genital tubercle morphogenesis at E14.5.

**DISCUSSION**

Complete loss of Fgfr2 from the external genital epithelia results in three major anomalies: hypospadia due to defective ventral growth of the prepuce, loss of progenitor cells in the urethral endoderm and concomitant decrease in proliferation and stratification (Petiot et al., 2005). Fgfr2 deletion from urethral endoderm or the surface ectoderm resulted in two distinct phenotypes: endodermal Fgfr2 ablation causes mild hypospadias with arrested development of the urethral epithelium, and loss of ectodermal Fgfr2 induces severe hypospadias and hypoplasia of the ventral prepuce. We conclude that Fgfr2 activity in the endoderm mediates urethral epithelial maturation, whereas ectodermal Fgfr2 is required for formation of the prepuce. In both the urethral and the surface epithelia of
the genital tubercle, Fgfr2 promotes proliferation by mediating the G1/S cell cycle transition and is required for adhesion and morphological maturation of basal epithelial cells. We found significant decreases in Shh and Ptch1 upon deletion of endodermal Fgfr2, but it is possible that this is an indirect result of fewer urethral cells rather than a direct consequence of Fgfr2 regulating hedgehog signaling; our finding that the mesenchymal cells of Fgfr2 conditional mutants show no changes in proliferation support this interpretation. Our findings highlight the interdependence of urethral tubulogenesis with formation of the ventral prepuce and dimorphic patterning of the distal urethra, and suggest that structural integrity of the ectodermal-endodermal boundary at the ventral midline integrates these morphogenetic events and is regulated by Fgf signaling. Despite the distinctive phenotypes resulting from Fgfr2 conditional deletions, our data suggest that Fgfr2 mediates the same cellular processes in both the endoderm and the ectoderm of the developing genital tubercle.

Both endodermal and ectodermal Fgfr2 maintain the structural integrity of the ventral seam that is required for tubulogenesis and prepuce formation

Cavitation of the bilaminar urethral plate epithelium progresses from proximal to distal, resulting in a cylindrical urethral tube (Van Der Werff et al., 2000; Hynes and Fraher, 2004; Cohn, 2011). Our finding that Fgfr2ΔEndo mutants fail to develop a penile urethral lumen suggests that cavitation requires endodermal Fgfr2 activity. We previously reported that a thin layer of ectoderm overlies the endoderm at the ventral margin of the developing urethra (Seifert et al., 2009). In Fgfr2ΔEndo mutants, this boundary is not strictly maintained; an irregular border between endoderm and ectoderm, identified by lineage tracing, indicates that Fgfr2ΔΔ endodermal cells intercalate with ectodermal cells, resulting in a relaxation of lineage-restricted compartmentalization. A loss of tissue integrity due to decreased cell proliferation and epithelial maturation following Fgfr2 deletion in the urethra disrupts lineage compartmentalization at the ventral ectodermal-endodermal boundary, and failure to form a discrete urethral epithelium covered by surface ectoderm results in exposure of the underlying urethral lumen and mislocalization of endodermal cells.

Mechanosensation of changes in environmental rigidity involve extension of actin filaments (Vogel and Sheeetz, 2006). We interpret the local upregulation of actin fibers at the ventral seam of the genital tubercle as evidence of cells sensing mechanical tension, and that response to this tension involves increased cell adhesion (evidenced by increased β-catenin). The finding that actin-rich stress fibers are produced in this region of Fgfr2ΔEndoΔ mutants suggests that the cells are able to sense increases in mechanical force; however, the absence of increased β-catenin suggests an inability of the ectodermal cells to respond to increased strain. Given the decreased proliferation in the surface epithelium of Fgfr2ΔEndoΔ mutants, we postulate that this results in a discrepancy between the rates at which the volume and surface area of the genital tubercle increase, causing rupture of the surface epithelium and an open (hypospadic) urethra. Signs of mechanical failure are evident in Fgfr2ΔEndoΔ external genitalia; we noted thin, damaged cellular remnants overlying the hypospadic urethra and infer that these projections are the result of a loss of epithelial contact at the endodermal-ectodermal boundary. Simultaneous loss of epithelial integrity along the ventral midline and misdirected lateral growth of the preputial swellings in Fgfr2ΔEndoΔ mutants suggests that normal ventrolateral preputial development requires strong epithelial integrity at the ectodermal-endodermal boundary. We propose that this axis along the ventral midline of the genital tubercle acts as a scaffold around which the preputial swellings eventually fuse. Therefore, disruption of this hinge point due to mechanical stress in Fgfr2ΔEndoΔ mutants causes lateral displacement of the preputial swellings and precludes formation of a complete prepuce.

Tissue-specific Fgfr2 deletions disrupt distinct aspects of organogenesis via a common cellular mechanism

Coordinated cell shape changes mediate tubulogenesis of many organs (Vogel and Sheeetz, 2006; Davidson, 2008; Davidson et al., 2010; Sawyer et al., 2010; Eom et al., 2011; Joshi and Davidson, 2012; Plosa et al., 2012; Cavigilia and Lusch nig, 2014; Girdler and Röper, 2014). Cytoskeletal reorganizations underlie cell shape changes, require cell adhesion and occur in a cell-cycle-dependent manner (Yonemura et al., 1995; Girdler and Röper, 2014). Fgfr receptor activation can induce cytoskeletal rearrangement in conjunction with stimulating the G1/S transition (Steele et al., 2006), and promotes the G1/S transition in other contexts of tubulogenesis (Yin et al., 2008). We found that Fgfr2 mediates columnar morphogenesis of basal cells and the G1/S cell cycle transition throughout the developing external genital epithelia. Our finding that Fgfr2ΔΔ basal urethral cells have decreased basolateral β-catenin and disorganized actin filaments implicates insufficient cell adhesion as a cause of aberrant epithelial morphogenesis. Together, these data raise the intriguing possibility that Fgfr2 in the genital tubercle epithelia drives epithelial maturation by coupling proliferation with cellular morphogenesis.

Dimorphic urethral patterning requires Fgf signaling from multiple spatial domains

An important but poorly understood feature of sexually dimorphic external genital development is internalization of the male urethra (Glenister, 1954; Seifert et al., 2008). At initiation of external genital development, urethral endoderm contacts the ventral side of the genital tubercle (Glenister, 1954; Van der Meulen, 1964; Hynes and Fraher, 2004). In males, proximodistal invasion of urorectal septum mesenchyme into the genital tubercle contributes to displacement of the urethra to a central position within the glans (Glenister, 1954). It has been postulated that this remodeling is due to a reorganization of the urethral epithelium rather than apoptosis or EMT (Seifert et al., 2008). Our finding that male Fgfr2ΔEndoΔ mutants fail to separate the urethral tube from the ventral ectoderm supports this hypothesis and might result from deficient maturation of the urethral epithelium. This phenotype can be interpreted as a feminization, and our finding that the distal urethra of Fgfr2ΔEndoΔ mutants forms an epithelial cord similar to the clitoral endoderm supports this conclusion. Our observations that the distal urethra of Fgfr2ΔEndoΔ mutants fails to form a lumen and that this is associated with a disruption of urethral internalization suggest that urethral tubulogenesis and internalization are interdependent processes that each require Fgfr2.

Androgen-Fgf interactions and human hypospadias

Fgfr2 can be transcriptionally downregulated by treatment with the anti-androgen flutamide in a dose-dependent manner (Petiot et al., 2005). Flutamide treatment also results in hypospadias and persistence of urethral attachment to the ventral ectoderm (Seifert et al., 2008), phenotypes which closely resemble that of Fgfr2ΔEndoΔ mutants. Our data suggest that disruption of Fgfr2 signaling in the urethral endoderm could be one mechanism by which anti-androgen exposure causes hypospadias, failed urethral internalization, and hypoplasia of the ventral prepuce. Deviation of the penoscrotal
raphe from the ventral midline has been demonstrated to correlate with mild hypospadias in humans (Mohan et al., 2014). The observation that deletion of Fgfr2 from the urethral endoderm perturbs compartmentalization of the endodermal-ectodermal boundary along the ventral midline of the developing penis raises the possibility that mild hypospadias with penoscrotal raphe anomalies could result from aberrant urethral Fgfr2 signaling.

Taken together, these results provide new insights into the cellular mechanisms that govern morphogenesis of the prepucce and internalized urethra. The endodermal and ectodermal domains of Fgfr2 play distinct roles in urethral tubulogenesis; however, in both tissue compartments Fgfr2 controls epithelial cell proliferation, stratification and adhesion. Coordination of these cellular processes in the urethral epithelium and the overlying surface ectoderm is required both for synchronous development and for structural integrity of the developing urethral tube and prepuce.

**MATERIALS AND METHODS**

**Transgenic embryo generation and collection**

Mouse strains with the Mx2-Cre (Sun et al., 2000), ShhCre (Harfe et al., 2004), Fgfr2^floxtg (Yu et al., 2003), Rosa26mCAGlacZ (Soriano, 1999), ShhCreERT2 (Harfe et al., 2004) and ShhC (Dassule et al., 2000) transgenes have been previously described. Timed matings were generated by crossing male mice carrying Cre and one floxed allele to females homozygous for the floxed allele. Pregnant ShhCre females were dosed with 10 mg/kg tamoxifen by oral gavage at E10.5 to induce recombination. Pregnant dams were euthanized by cervical dislocation; neonatal mice were anesthetized by oral gavage at E10.5 to induce recombination. Pregnant dams were euthanized by cervical dislocation; neonatal mice were anesthetized by isoflurane inhalation and euthanized by decapitation. Protocol-specific euthanization of pregnant dams 2 h prior to embryo harvest and immunofluorescence was performed as above. Cell-cycle phase times were calculated according to published methods (Seifert et al., 2010) on 16 transverse sections/sample.

**Quantitative real-time PCR**

RNA was extracted from whole E14.5 genital tubercles (Qiagen RNaseasy Plus Micro Kit) and cDNA was reverse-transcribed from 500 ng of extracted RNA (Bio-Rad iScript RT cDNA Synthesis Kit). qRT-PCR reactions (18 µl volume) were synthesized using the Bio-Rad iQ SYBR Green Supermix and measured in three technical replicates. Primers are listed in supplementary material methods. At least three biological replicates were used for each primer-embryo pair. Relative expression values were calculated by the 2^(-AΔCT) method.

**Cell cycle analysis**

An intraperitoneal injection of BrdU at 50 mg/kg was given to pregnant dams 2 h prior to embryo harvest and immunofluorescence was performed as above. Cell-cycle phase times were calculated according to published methods (Seifert et al., 2010) on 16 transverse sections/sample.

**Histology, immunohistochemistry and immunofluorescence**

Preparation of tissue sections and histological stains are described in supplementary material Table S1. Tail biopsies were used to genotype for sex and the presence of Cre, conditional (Fgfr2 or Shh), and reporter alleles by standard PCR (supplementary material Table S4).

**Lineage tracing**

In mice carrying the R26R allele, β-galactosidase activity in the external genitalia of neonates and embryos was detected by X-Gal staining according to published methods (Hogan et al., 1994).

**Quantitative real-time PCR**

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