Development of the mammalian urethra is controlled by Fgfr2-IIIb

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Summary

Development of external genitalia in mammalian embryos requires tight coordination of a complex series of morphogenetic events involving outgrowth, proximodistal and dorsoventral patterning, and epithelial tubulogenesis. Hypospadias is a congenital defect of the external genitalia that results from failure of urethral tube closure. Although this is the second most common birth defect in humans, affecting one in every 250 children, the molecular mechanisms that regulate morphogenesis of the mammalian urethra are poorly understood. We report that mice lacking the IIIb isoform of fibroblast growth factor receptor 2 (Fgfr2) exhibit severe hypospadias. Urethral signaling regions, as indicated by Shh and Fgf8 expression, are established in Fgfr2-IIIb null mice; however, cell proliferation arrests prematurely and maturation of the urethral epithelium is disrupted. Fgfr2-IIIb−/− mutants fail to maintain the progenitor cell population required for uroepithelial renewal during tubular morphogenesis. In addition, we show that antagonism of the androgen receptor (AR) leads to loss of Fgfr2-IIIb and Fgf10 expression in the urethra, and an associated hypospadias phenotype, suggesting that these genes are downstream targets of AR during external genital development. Genitourinary defects resulting from disruption of AR activity, by either genetic or environmental factors, may therefore involve negative regulation of the Fgfr2 pathway. This represents the first example of how the developing genitourinary system integrates cues from systemically circulating steroid hormones with a locally expressed growth factor pathway.

Key words: Fgfr2-IIIb, Tubular morphogenesis, Hypospadias, Urethral plate, Genital tubercle

Introduction

Development of mammalian external genitalia requires tight coordination of proximodistal outgrowth, three-dimensional patterning and tubular morphogenesis. Malformations of the external genital system occur at a frequency second only to cardiac defects, currently affecting approximately one in every 250 live births (Centers for Disease Control, 2002; Gatti et al., 2001; Paulozzi et al., 1997). The most common of these anomalies is hypospadias, a failure of urethral tube closure that is often accompanied by agenesis of the ventral aspect of the penis. Frequently, these children have multiple or oversized urethral openings, and males with severe hypospadias are born with ambiguous genitalia. Although generally considered to be a malformation of male genitalia, hypospadias can also occur in females and is characterized by clefting of the clitoris and mislocalization of vaginal and/or urethral openings (Miyagawa et al., 2002; Stadler, 2003). In the USA, the incidence of hypospadias approximately doubled, without explanation, between 1968 and 1993, and similar increases have been reported throughout the industrialized world (Paulozzi et al., 1997; Pierik et al., 2002; Toppuri et al., 2002). Paradoxically, the molecular mechanisms that regulate early development of the external genitalia remain largely unknown.

Vertebrate external genital development can be divided into two distinct phases: the first involves initial outgrowth and patterning of the genital tubercle, which occurs in both male and female embryos (George and Wilson, 1994); the second, hormonally controlled phase, involves either the continued growth and differentiation of the penis, or the arrest of outgrowth and differentiation of the clitoris. During the first phase, there are no discernable morphological differences between male and female external genitalia. In the mouse, genital tubercle development is initiated at embryonic day (E) 10.25, when paired buds of lateral plate mesoderm emerge beneath ventral body wall ectoderm on either side of the cloaca (Perriton et al., 2002). An epithelial extension of cloacal endoderm extends between these swellings to form the urethral plate, and the left and right swellings merge medially to form a single genital tubercle (Kurzrock et al., 1999a; Perriton et al., 2002). The urethral plate epithelium extends to the distal tip of the genital tubercle where, in the clitoris, it persists as an epithelial cord or, in the penis, it canalizes to form a urethral tube.

Like the limb, the genital tubercle undergoes proximal to distal outgrowth, during which cells within the tubercle are patterned along three axes. In the genitalia, however, these processes must be coordinated with epithelial tubulogenesis to generate a urethral canal. Epithelial-mesenchymal interactions
play an important role in outgrowth of the genital tubercle. Surgical removal of the distal epithelium leads to an arrest of outgrowth and truncation of the phallus (Kurzrock et al., 1999b; Murakami and Mizuno, 1986). This is analogous to the outgrowth of the early limb bud, which is controlled by the apical ectodermal ridge, a specialized population of distal epithelial cells. Indeed, both of these structures are sites of fibroblast growth factor (Fgf) expression, and signaling activity of these tissues can be replaced with beads soaked in Fgf8 protein (Crossley et al., 1996; Haraguchi et al., 2000). We recently identified a new signaling region, the endodermally derived urethral plate epithelium, which is situated along the ventral side of genital tubercle and is a site of sonic hedgehog (Shh) expression (Perriton et al., 2002). Shh is required for outgrowth and patterning of the genital tubercle, and mice with a targeted deletion of Shh have penile and clitoral agenesis (Haraguchi et al., 2001; Perriton et al., 2002). Members of the Hox paralogy group 13, Hoxa13 and Hoxa13, also play an essential role in external genital and limb development (Kondo et al., 1997; Morgan et al., 2003; Stadler, 2003). Loss of function of both genes results in agenesis of the genital tubercle, and heterozygosity for either causes patterning defects of the phallus. A recent study reports that Hoxa13-null mice exhibit hypospadias (Morgan et al., 2003), and mutations in the human HOXA13 gene are responsible for the range of phenotypes seen in Hand-Foot-Genital Syndrome (Goodman et al., 2000; Mortlock and Innis, 1997).

Whereas early genital development is controlled by a genetic program that operates prior to production of steroid hormones, the second phase of penis development requires exposure to an androgen, either testosterone or dihydrotestosterone (DHT) (George and Wilson, 1994). Androgenic steroids, synthesized by the Leydig cells of the testes, are first seen just prior to the onset of androgen-induced genital differentiation (Abney, 1999). In the developing external genitalia of mammals, androgen receptors (ARs) are abundant in the urethral epithelium, with lower concentrations found in the underlying stromal tissue (Kim et al., 2002). Similarly, 5α-reductase Type 2, an enzyme that converts testosterone to 5α-dihydrotestosterone (DHT), is highly expressed in the mesenchymal stroma surrounding the urethra (Kim et al., 2002; Tian and Russell, 1997). Loss of a functional AR or inability to synthesize DHT causes reduction in penis growth, hypospadias and, in severe cases, complete loss of phalic development (George and Wilson, 1994). By contrast, exposure of developing females to androgens leads to masculinization of the external genitalia (George and Wilson, 1994).

Normal genital development must involve integration of local and systemic cues; however, neither the interactions between signals that operate in the first and second phases of genital development nor the genetic control of urethral tube closure is well understood. We report that a loss of function mutation in mouse Fgfr2-IIib causes urethral tube development to arrest, resulting in severe hypospadias. Although Fgfr2-IIib−/− mice develop a urethral plate that expresses Shh and Fgf8, which mediate its signaling activity, the urothelial progenitor cell population becomes depleted and urethral plate maturation fails prior to the onset of tubulogenesis. We show that transcription of Fgfr2-IIib and Fgf10 requires AR signaling during the second phase of external genital development, and that the Fgfr2 promoter contains a stereotypic androgen response element (ARE) sequence. Together, these results demonstrate that Fgfr2-IIib-mediated signaling is essential for development of the urethral tube and suggest that the Fgfr2 gene is a transcriptional target of AR.

Materials and Methods

Mouse strains and tissue collection

Fgfr2-IIib−/− and Fgf10−/− mice have been previously described (Min et al., 1998; Revest et al., 2001). Fgf10−/− mice were kindly provided by D. Ornitz (Washington University Medical School, St Louis, MO). Embryos were harvested from pregnant mice obtained by timed matings at the desired stages of development, and genotyped by PCR using genomic DNA isolated from yolk sacs. Whole embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated and embedded in wax for cutting 5 μm sections. Embryos were sexed by PCR for the Sry gene or by morphological analysis of gonads. Wild-type embryos used for organ culture experiments were obtained from time-mated females of the CD1 strain (Charles River).

Histology, in situ hybridization, and immunohistochemistry

Histology and in situ hybridization were carried out using standard procedures and as previously described (Perriton et al., 2002; Revest et al., 2001). Probes used were for Fgfr2-IIib, Shh and Fgf8 (Mailleux et al., 2002; Revest et al., 2001). Wax sections transverse to the genitalia were dewaxed in xylene, rehydrated through graded alcohols and used for detection of keratin 14 (K14) (BabCO, USA), AR (N-A20) (Santa Cruz Biotech, USA) or Ki67 (Novocastra, UK). Sections were boiled twice for 10 minutes in 0.01 M citrate buffer pH 6 then blocked with swine serum (1:25). All dilutions were in PBS. Anti-K14 (1:1000), anti-AR (1:100) or anti-Ki67 (1:200) was applied for 2 hours at room temperature. A biotinylated swine anti-rabbit secondary antibody (DAKO, UK) was applied (1:500) for 40 minutes followed by streptavidin-peroxidase for 40 minutes (1:500). A DAB substrate kit (Vector Labs, USA) was used for detection. Apoptotic cells were either detected by incorporation of terminal deoxynucleotidyldtransferase-mediated dUTP end labeling (TUNEL) or stained using a commercially available kit (ApoTag ISOL) according to the manufacturer’s protocol (Intergen, UK).

Whole organ culture of genital tubercles

Genital tubercles were dissected in ice-cold medium (MEM supplemented with 10% fetal calf serum, L-glutamine and antibiotic/antimycotic), and transferred onto Millipore nitrocellulose filters (0.8 μm) supported by wire mesh. Organs were cultured with the ventral side upwards, immediately below the media-gas interface, in a humidified incubator at 37°C and 5% CO2. Cultures were fed with BJGB medium supplemented with 10% fetal calf serum, L-glutamine and antibiotic/antimycotic. Glutamidine (Sigma) was dissolved in 100% ethanol and added to the cultures at concentrations of 10−5 M, 10−4 M, 5×10−4 M and 10−3 M. Control cultures were treated with an identical volume of ethanol. Dihydrotestosterone (Sigma) was dissolved in ethanol and used at a concentration of 5×10−6 M. Cultures were maintained for 48 hours before being fixed in 4% paraformaldehyde for in situ hybridization or 1% glutaraldehyde for scanning electron microscopy.

Results

Fgfr2-IIib is expressed in the developing urethra and prepuce of the genital tubercle

In order to identify genes involved in development of the mammalian urethral tube, we carried out an in situ
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hybridization screen of mouse genital tubercles between embryonic day (E) 9.5 and E16.5. Given that hypospadias results from abnormal development of the urethral epithelium, adjacent mesenchyme or the surrounding prepuce (the lateral outgrowths that surround the developing urethra), candidate genes were identified based on expression in one or more of these tissues. Fgfr2-IIIb expression was detected in the urethral epithelium prior to the initiation of genital tubercle outgrowth at E10.25, and expression persisted in the developing urethra through E16.5 (Fig. 1). During this period, Fgfr2-IIIb transcripts were localized throughout the urethral plate, but were excluded from the surrounding genital mesenchyme (Fig. 1A-C). Interestingly, Fgfr10, a major ligand of Fgfr2-IIIb, is expressed in the genital tubercle mesenchyme adjacent to Fgfr2-IIIb (Haraguchi et al., 2000; Perriton et al., 2002); however, we did not detect expression of Fgf7, another Fgfr2-IIIb ligand, at any stage of genital tubercle development (data not shown). At E13.5, Fgfr2-IIIb expression also appeared along the lateral edges of the genital tubercle, where the paired preputial swellings emerge (Fig. 1D). As the preputial swellings grew laterally and ventrally, they continued to express Fgfr2-IIIb (Fig. 1E-G). Thus, during genital tubercle development, Fgfr2-IIIb is expressed specifically in tissues that are involved in urethral tube closure.

Fgfr2-IIIb<sup>−/−</sup> mice exhibit severe hypospadias

The spatial and temporal expression pattern of Fgfr2-IIIb suggested a potential role for this receptor in urethraigenesis. To determine the function of Fgfr2-IIIb in external genital development, we examined mice with a targeted disruption of Fgfr2-IIIb (Haraguchi et al., 2000; Perriton et al., 2002); although the early stages of tubercle outgrowth were grossly normal in Fgfr2-IIIb<sup>−/−</sup> mutants revealed hypospadias, in which the ventral aspect of the phallus protruded from the skin (Fig. 2D). The ectopic ventral opening of the urethral plate continued to spread distally and, by E17.5, the interior walls of the urethra were exposed along the ventral surface of the penis (Fig. 2F). During normal development, preputial swellings grow dorsally and ventrally around the tubercle to further internalize the urethral tube, and by E17.5 the prepuce completely surrounds the glans and penile shaft (Fig. 2E). By contrast, preputial swellings failed to develop ventrally in Fgfr2-IIIb<sup>−/−</sup> mice (Fig. 2F). Histological analysis of Fgfr2-IIIb<sup>−/−</sup> mutant males confirmed the lack a tubular penile urethra and ventral prepuce (Fig. 2G,H). These results indicate that signaling through Fgfr2-IIIb is essential for normal closure of the urethral epithelium and for development of the ventral prepuce. We next examined genitalia of mice with a null mutation in Fgfr10, which codes for a ligand for Fgfr2-IIIb, and found that male and female Fgfr10<sup>−/−</sup> mutants also develop severe proximal hypospadias (Fig. 2I,J). The appearance of these anomalies in both sexes of Fgfr2-IIIb<sup>−/−</sup> and Fgfr10<sup>−/−</sup> null mutants as early as E13.5 indicates that, prior to the onset of sexual differentiation, Fgfr10-Fgfr2-IIIb signaling is essential for male and female urethral plate morphogenesis.

Urethral signaling activity is established but not maintained in Fgfr2-IIIb<sup>−/−</sup> genitalia

In light of our finding that Fgfr2-IIIb is required for morphogenesis of the urethral epithelium, we hypothesized that loss of Fgfr2-IIIb function would also result in a loss of signaling activity in urethral cells. We therefore examined expression of two key signaling molecules, Fgf8 and Shh, in mutant genital tubercles. At E13.5, both Fgf8 and Shh were expressed at appropriate positions in the mutant and wild-type genitalia; Fgf8 expression was detected at the distal tip of the urethral plate (Fig. 3A,B), and Shh was expressed throughout the urethral plate (Fig. 3C,D). In mutant embryos, the Shh expression domain appeared to bifurcate proximally as the ectopic opening formed at the base of the urethra (Fig. 3D). Although Shh expression normally persists in urethral cells through E15.5 (Fig. 3E,F), we were unable to detect Shh transcripts in the urethral region of Fgfr2-IIIb<sup>−/−</sup> mutants at this stage (Fig. 3G,H). Interestingly, downregulation of Shh expression in Fgfr2-IIIb<sup>−/−</sup> genitalia coincides with loss of urethral epithelial organization (described below). These findings indicate that, in Fgfr2-IIIb<sup>−/−</sup> mice, signaling regions are established normally during early genital development, but...
as urethral plate cells lose their epithelial character, Shh is downregulated prematurely.

**Fgfr2-IIIb is essential for urethral epithelial organization**

The effects of Fgfr2-IIIb deletion on morphogenesis of the urethral plate were further investigated histologically. Examination of Fgfr2-IIIb−/− embryos at E13.5 revealed disorganization of urethral plate epithelium (Fig. 4). In wild-type embryos, urethral development involves cell proliferation and cell shape reorganization to form a complex, stratified squamous epithelium (Fig. 4A). By contrast, the urethral plate of Fgfr2-IIIb−/− mice at E13.5 failed to develop beyond a thin sheet of cuboidal cells (Fig. 4B). By E15.5, the proximal urethral plate of wild-type embryos had separated medially to form a urethral tube (Fig. 4C), whereas the distal end remained a bilaminar epithelial plate. In Fgfr2-IIIb−/− mutants at the same stage, urethral plate epithelium could no longer be detected proximally, and only a thin, disorganized epithelium remained distally (Fig. 4D; data not shown). Beyond this stage, the only detectable remnant of the urethra in mutants was a shallow groove along the ventral surface of the phallus. Thus, in the absence of Fgfr2-IIIb, urethral cells fail to mature beyond a simple epithelium, which then disappears in a proximal to distal direction.

**Fgfr2-IIIb is required for proliferation of urethral progenitor cells**

The failure of Fgfr2-IIIb−/− mice to develop a structurally mature urethral epithelium suggested that Fgfr2-IIIb may be involved in maintenance of urothelial progenitor cells. To investigate the urethral epithelial defect in Fgfr2-IIIb−/− mutants, we examined expression of keratin 14 (K14), a marker of the basal, progenitor cell-containing layer of squamous epithelial populations (Byrne et al., 1994). In wild-type embryos at E16.5, K14 was detected in basal epithelial cells of the urethra, in the tunica albuginea surrounding the corpus cavernosum (Fig. 5A). By contrast, the urethral region of Fgfr2-IIIb−/− mutants had failed to express K14, although expression remained in the tunica and epidermis (Fig. 5B). Absence of urethral K14 expression in mutant embryos suggests that Fgfr2-IIIb is required for maintenance of the basal progenitor cell population of the urethral epithelium.

Loss of the urothelial progenitor cell population could be a consequence of decreased cell proliferation, increased apoptosis, or both. Fgf7 and Fgf10 signal via Fgfr2-IIIb to activate a mitogenic signaling pathway in epithelial cells of the prostate and bladder (Bagai et al., 2002; Lu et al., 1999; Thomson and Cunha, 1999). The epithelial cells lining the prostate, bladder and urethra have been characterized as having a ‘urothelial’ character (Baskin et al., 2001) and, if urothelial cell proliferation is regulated by a similar mechanism in all of
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these tissues, then one hypothesis is that reduction or arrest of cell division may underlie the loss of basal cells, and the resultant failure of urethral epithelial maturation, in Fgfr2-IIIb–/– mice. We therefore tested whether Fgfr2-IIIb is required for proliferation of urethral epithelial cells, using an antibody against the proliferation-associated nuclear antigen Ki67 (Gerdes et al., 1991). In both mutant and wild-type mice at E13.5, urethral epithelial cells were positive for Ki67 (Fig. 5C,D). In wild-type mice, proliferation continued in basal urethral cells through E15.5 (Fig. 5E); however, Ki67 was undetectable in the urethral region of E15.5 Fgfr2-IIIb–/– mutant (Fig. 5F). We also asked whether cessation of proliferation and loss of an obvious urethral plate were accompanied by increased apoptosis. Using both TUNEL and ApopTag methods to localize apoptotic cells, we detected no increase in apoptosis in mutant genitalia at E13.5, 15.5 or 16.5 (data not shown). Together, these results demonstrate that, in Fgfr2-IIIb mutants, progenitor cells of the urethra commit to an epithelial fate and begin to organize and proliferate, but between E13.5 and E15.5, proliferation of urethral epithelial progenitors arrests and, consequently, urethral maturation and tubulogenesis fails.

Transcription of Fgfr2-IIIb and Fgf10 requires AR activity

The hypospadias defect of Fgfr2-IIIb–/– mice resembles the phenotype that results from disruption of androgen signaling. In the prostate, Fgfr2 can mediate the effects of androgen on tumor progression, suggesting that Fgfr2 operates as an andromedin (Nakano et al., 1999). We therefore investigated the possibility that the Fgfr2 and the AR pathways interact in genital development. To determine whether AR is required for Fgfr2-IIIb expression in urethral cells, we cultured both male and female genital tubercles from E14.5-E15.5 wild-type embryos in the presence of flutamide, a pharmaceutical AR antagonist that induces hypospadias when administered to pregnant females (Imperato-McGinley, 1994). Because binding assays have shown that flutamide must be present at 500- to 1000-fold higher concentrations than circulating androgens, in order to compete for AR binding (Simard et al., 1986; Zuo et al., 2002), we first carried out a dose-response analysis to identify physiologically relevant concentrations required to produce a hypospadias phenotype in vitro. Genital

Fig. 3. Expression of Fgf8 and Shh in the urethral epithelium of wild-type and Fgfr2-IIIb+/− embryos. (A,B) Fgf8 expression in the distal urethral epithelium of male wild-type (A) and Fgfr2-IIIb−/− (B) genital tubercles at E13.5. (C,D) Shh expression in the urethral epithelium of male wild-type (C) and Fgfr2-IIIb−/− (D) genital tubercles. The Shh domain bifurcates proximally in the Fgfr2-IIIb−/− mutant (D), where the urethral plate has opened. (E,F) Transverse sections through the distal genital tubercle of E15.5 wild-type embryo, showing histological organization (E) and Shh expression (F) in urethral epithelium (boxed). (G,H) Transverse sections through the distal genital tubercle of E15.5 Fgfr2-IIIb−/− mutant embryo reveal the lack of a histologically distinct urethral epithelium (G) and the absence of Shh expression (H).

Fig. 4. Disorganization of the urethral plate in Fgfr2-IIIb−/− mutants. Transverse histological sections through the genital tubercle of wild-type (A,C) and Fgfr2-IIIb−/− mutant (B,D) genitalia. Urethral epithelium is shown within the boxes and at higher magnification on right sides of A and B. (A) At E13.5, cells in the wild-type urethral plate are stratified and have a complex squamous organization. (B) Urethral plate of Fgfr2-IIIb−/− mutant is a thin, unstratified plate of cuboidal cells. (C) Proximal transverse section through wild-type phallus at E15.5 showing separation of the bilaminar urethral plate to form a urethral tube. Boxed area indicates urethral epithelium. (D) Proximal transverse section through phallus of Fgfr2-IIIb−/− mutant showing that by E15.5, epithelial organization of the urethra has been lost.
tubercles from male and female embryos were cultured separately; however, we detected no differences in their responses to flutamide. Genital tubercles cultured for 48 hours with 10\(^{-5}\) M flutamide (n=19) developed similar to the controls (see Fig. S1A,B in the supplementary material). At 10\(^{-4}\) M flutamide, 38% of the tubercles exhibited hypoplasia of the prepuce and malformation of the glans (n=13; see Fig. S1C in the supplementary material). In cultures with 5 \times 10^{-4} M (n=4) and 10\(^{-3}\) M (n=20) flutamide, 100% of the genital tubercles had a hypoplastic phenotype, consisting of a prepuce that was either hypoplastic or completely absent, a superficial urethral plate and a poorly differentiated glans (see Fig. S1D,E in the supplementary material). Histological analysis of cultured tubercles revealed that urethral plate differentiation was perturbed by flutamide treatment. After 48 hours in the presence of 5 \times 10^{-4} M flutamide, the urethral plate still could be detected, but the tissue was severely disorganized and hypoplastic relative to the neatly stratified, complex urethral epithelium observed in control cultures (compare Fig. S1G with Fig S1F in the supplementary material). We next examined Fgfr2-IIIb expression in a series of genital tubercles cultured under the same conditions. Organ cultures of male and female genital tubercles treated with flutamide showed a dose-dependent decrease in Fgfr2-IIIb expression, which mirrored the dose-dependent induction of hypospadias. Treatment with 10\(^{-5}\) M flutamide resulted in no detectable change in Fgfr2-IIIb expression relative to controls (Fig. 6A,B); however, raising the flutamide dose to 10\(^{-4}\) M resulted in a marked decrease in Fgfr2-IIIb expression in the urethral plate (Fig. 6C). When the flutamide concentration was increased further to 5 \times 10^{-4} M or 10\(^{-3}\) M, Fgfr2-IIIb became undetectable (Fig. 6D,E). Given that the urethral plate is present, although disorganized, 48 hours after flutamide treatment (see Fig. S1G in the supplementary material), we conclude that absence of Fgfr2-IIIb transcripts after 48 hours in culture is due to downregulation of the gene, rather than to loss of Fgfr2-IIIb-expressing cells.

To confirm that the effects of flutamide treatment on Fgfr2-IIIb expression were specific to diminished AR activity, we attempted to rescue Fgfr2-IIIb expression in flutamide-treated genital tubercles by addition of dihydrotestosterone (DHT), the activated form of testosterone. DHT and flutamide compete for AR binding and, when bound by DHT, AR undergoes conformational changes that stabilize it and allow the recruitment of transcriptional coactivators (Furutani et al., 2002). Flutamide antagonizes AR activity by abrogating its stabilization, leading to degradation, and by preventing transactivation (Furutani et al., 2002). We found that addition of 5 \times 10^{-4} M DHT to cultures containing 5 \times 10^{-4} M flutamide successfully rescued Fgfr2-IIIb expression in the urethra, and the cultured genital tubercle developed normally (Fig. 6F). Thus, loss of Fgfr2-IIIb expression in flutamide-treated genitalia is a specific effect of diminished AR activity.

The ability of AR to modulate Fgfr2-IIIb transcription raised the possibility that the Fgfr2 gene may contain an ARE. Direct contact between AR protein and DNA requires a highly conserved AR recognition site, the hexanucleotide T-G-T-T-C-T, which functions as common DNA-binding element for androgen, glucocorticoid and progesterone receptors (De Vos et al., 1991; Scheidereit and Beato, 1984). We therefore performed an in silico analysis of the Fgfr2 promoter sequence (GenBank Accession Number, X66455) (Avivi et al., 1992) using TRANSFAC (Wingender et al., 2001), which revealed the presence of the T-G-T-T-C-T motif between nucleotides 1193-1198 (Fig. 6G). The presence of a putative ARE sequence within the Fgfr2 promoter, together with our finding that antagonism of AR results in loss of Fgfr2-IIIb transcripts in cultured genital tubercles, suggests that Fgfr2 could be a direct transcriptional target of the AR.

Fgf10 expression also can be regulated by androgen in the prostate (Donjacour et al., 2003; Lu et al., 1999), although whether this is direct or indirect is unclear (Thomson, 2001; Thomson and Cunha, 1999). To determine whether Fgf10 expression in the genital tubercle may require androgen signaling, we repeated the flutamide dose-response experiment described above and, after 48 hours in culture, genitalia were assayed for Fgf10 expression. Fgf10 transcripts persisted in control cultures and in the presence of 10\(^{-3}\) M flutamide; however, as with Fgfr2-IIIb, doses of flutamide between 10\(^{-4}\) M and 10\(^{-3}\) M resulted in progressive downregulation of Fgf10 in male and female genital tubercles (Fig. 6H-J; data not shown).

Although Fgfr2-IIIb expression can be regulated by AR, we
found no evidence of a positive feedback loop between these two receptors. Immunohistochemical analysis of AR distribution in external genitalia of Fgfr2-IIIb–/– and wild-type embryos at E16.5 revealed no discernable differences (Fig. 7). AR staining was observed in genital mesenchyme and urethral epithelium, with particularly strong staining in the corporal bodies and basal layer of the urethra (Fig. 7A,B). Sections through the pelvic region of the trunk, proximal to the genital tubercle, showed that the proximal urethra remained intact and was positive for AR in Fgfr2-IIIb–/– mutants (Fig. 7A,B, insets). Thus, although AR is a positive regulator of Fgfr2-IIIb in the external genitalia, expression of AR appears to be independent of Fgfr2-IIIb.

**Discussion**

We have shown that the IIIb isoform of Fgfr2, which is expressed in the urethral epithelium and surrounding prepuce of the mouse genital tubercle, is required for normal development of the urethra and ventral phallus. Loss-of-function mutations in Fgfr2-IIIb or its ligand, Fgf10, result in severe proximal hypospadias. Although urethral signaling regions, as indicated by Shh and Fgf8 expression, are established in Fgfr2-IIIb-null mice, proliferation arrests prematurely and maturation of the urethral epithelium fails. The data suggest that hypospadias results from the inability of Fgfr2-IIIb–/– mutants to maintain the progenitor cell population required for renewal of the urethral epithelium during genital development. Finally, we have shown that Fgfr2-IIIb and Fgf10 transcription can be ablated by antagonism of the AR. These findings, taken together with the discovery of a putative ARE in the Fgfr2 promoter, raise the possibility that AR may be a direct transcriptional regulator of Fgfr2. Thus, genitourinary defects resulting from disruption of AR activity, by either genetic or environmental factors, may involve negative regulation of the Fgfr2 pathway.

**Fgf10-Fgfr2-IIIb signaling is required for maintenance of urethral progenitor cells**

Normal patterning of uroepithelium (urothelium) involves interaction between stromal (mesenchymal) cells and the adjacent epithelium (Kurzrock et al., 1999b). We have shown that, during external genital development, morphogenesis of the urethral tube is regulated by mesenchymally produced Fgf10 signaling through Fgfr2IIIb in the urethral epithelium. Although hypospadias is generally considered to result from defective
Development of the urethral tube, our findings show that hypospadias in Fgf2-IIIb-deficient mice results from a differentiation defect that begins to be manifested prior to the onset of tubulogenesis. Differentiation of stratified epithelia depends upon sustained regenerative proliferation, which requires a population of progenitor stem cells in the basal layer (Hong et al., 2004; Yang et al., 1999). Our results suggest that the mechanism by which Fgfr2-IIIb-mediated signaling regulates urethral tubulogenesis involves the sustained proliferation of basal cells in the urothelium, where the progenitor cell population resides. The cessation of cell division in this population causes an abrupt arrest of urethral differentiation, resulting in development of a thin, disorganized urethral plate that fails to undergo normal maturation and stratification. These findings are, to some degree, reminiscent of the bladder defects observed in Fgf7-null mutant mice, in which bladder urothelium is abnormally thin and fails to undergo normal stratification (Tash et al., 2001). Although the intermediate cell layer is absent, basal cells are retained in bladder urothelium of Fgf7+/− mice, suggesting that another factor may be able to at least partially compensate for Fgf7 in the bladder urothelium. Fgf10 is expressed in the lamina propria, adjacent to bladder urothelium and compensation by Fgf10 may be sufficient to maintain the basal progenitor cell layer in Fgf7−/− mice. The presence of two Fgfr2-IIIb ligands in the bladder mesenchyme contrasts with the situation in the genital tubercle, where we detected Fgf10 but not Fgf7. Thus, our observation that loss of Fgf10 alone results in a hypospadias phenotype of similar severity to that seen in Fgf2-IIIb−/− mice can be explained by a lack of redundancy in the expression of Fgfr2-IIIb ligands during external genital morphogenesis.

**Regulation of urethral tube closure by androgen is mediated by Fgfr2-IIIb**

Disruption of androgen signaling during external genital development has long been known to result in hypospadias (Glucksmann et al., 1976). Here, we have shown that hypospadias can also result from disruption of the Fgfr2-IIIb pathway. Our discovery that AR activity is required for Fgfr2-IIIb expression in the urethra suggests that the androgen and Fgf10 pathways converge at the point of Fgfr2-IIIb to regulate morphogenesis of the urethral tube. Our observations that both male and female mutants develop hypospasias, and that the hypospadias phenotype begins to develop in Fgfr2-IIIb mutants prior to the stages at which androgens act on genital development suggest that Fgfr2-IIIb also has an early, androgen-independent role in development of the urethral plate. We favor a model in which pattern formation of external genitalia at sexually indifferent stages is controlled by locally regulated expression of Fgfr2-IIIb; however, as systemic endocrine signals are integrated at later stages, expression of Fgfr2-IIIb (and perhaps other genes) may be modulated by this new set of factors. Although the presence of an AR binding site in the Fgfr2 promoter suggests a possible direct interaction, the data do not rule out the possibility of intermediate steps. These findings represent, to our knowledge, the first example of how the developing genitourinary system integrates cues from systemically circulating steroid hormones (e.g., androgen) with signaling by locally expressed growth factors.

**Divergent functions of Fgf10-Fgfr2-IIIb signaling in limb and external genital development**

Investigation of the molecular genetic control of external genital development has only recently begun, a striking number of parallels with the mechanisms of limb development have emerged. Maintenance of limb bud and genital tubercle outgrowth is controlled by a specialized epithelial signaling region (the apical ectodermal ridge and the distal urethral epithelium, respectively), which is a source of Fgf8 (Crossley et al., 1996; Haraguchi et al., 2000; Murakami and Mizuno, 1986). Wnt5a, which is expressed in a distal to proximal gradient in both structures, is required for their distal outgrowth (Yamaguchi et al., 1999), as are Hoxd13 and Hoxa13 (Kondo et al., 1997; Morgan et al., 2003). A polarizing region that expresses Shh in an asymmetric pattern is also present in limb buds and genital tubercles, and loss of Shh results in loss of digits and agenesis of the phallus (Chiang et al., 1996; Perriton et al., 2002). Curiously, although both Fgf10 and Fgfr2-IIIb are essential for early outgrowth of the limb buds (De Moerlooze et al., 2000; Min et al., 1998; Revest et al., 2001; Sekine et al., 1999), our findings indicate that neither is required for outgrowth of the genital tubercle. In the limb, Fgfr2-IIIb acts upstream of Shh (Revest et al., 2001). In the genital tubercle, by contrast, Shh expression does not require Fgf10-Fgfr2-IIIb signaling. These results, taken together with our previous finding that Shh is an upstream regulator of Fgf10 expression in the genital tubercle (Perriton et al., 2002), indicate that the relative positions of Fgf10/Fgfr2-IIIb and Shh are inverted in the pathways that control limb and genital tubercle outgrowth. Although Shh is essential for maintenance of genital tubercle outgrowth, it is not required for early induction of the genital swellings (Perriton et al., 2002). Thus, the identity of the gene that initiates the process of genital tubercle budding remains to be determined.
Development and disease

Implications for the etiology of hypospadias in humans

Humans with Beare-Stevenson cutis gyrata syndrome, which is characterized in part by external genital defects such as hypospadias and bifid scrotum, have mis-sense mutations in FGFR2 (Akai et al., 2002; Przyjelka et al., 1996; Vargas et al., 2003; Wang et al., 2002). Our data suggest that a defect in urethral epithelial maturation may underlie this condition. Malformations of the urogenital tract also occur in Split Hand/Split Foot syndrome, limb-mammary syndrome, ectodactyly-ectodermal dysplasia-cleft lip/palate (EEC) and Hay-Wells syndrome, all of which are associated with mutations in p63, a homolog of the tumor suppressor gene p53. Interestingly, it has recently been shown that the p63 sterile-α motif regulates Fgfr2 splicing and is required for generation of the IIb isoform (Fomenkov et al., 2003). Although it remains unclear whether these malformations result from deficiencies in epithelial stem cell proliferation, one possible explanation for the associated genital malformations is an underlying Fgfr2-IIb deficiency. Finally, our findings highlight a mechanism that may have implications for understanding the global increase in hypospadias (Paulozzi et al., 1997). Fgfr2 and Fgf10 null mutations have widespread effects on multiple organ systems (De Moerloose et al., 2000; Min et al., 1998), making it highly unlikely that the large number of children presenting with hypospadias carry mutations in any of the genes examined here. However, our finding that antagonism of AR leads to downregulation of Fgfr2-IIb in mouse genitalia raises the possibility that exposure to anti-androgenic compounds during pregnancy may lead to diminished Fgfr2 activity. This represents a crucial step towards understanding how the genetic program for urethragenesis is affected by signals from the environment and the endocrine system during genitalourinary development.

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Supplementary material

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