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Fkbp52 Regulates Androgen Receptor Transactivation Activity and Male Urethra Morphogenesis*

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Hypospadias is a common birth defect in humans, yet its etiology and pattern of onset are largely unknown. Recent studies have shown that male mice with targeted ablation of FK506-binding protein-52 (Fkbp52) develop hypospadias, most likely due to actions of Fkbp52 as a molecular co-chaperone of the androgen receptor (AR). Here, we further dissect the developmental and molecular mechanisms that underlie hypospadias in Fkbp52-deficient mice. Scanning electron microscopy revealed a defect in the elevation of prepucial swelling that led to the onset of the ventral penile cleft. Interestingly, expression of Fkbp52 was highest in the ventral aspect of the developing penis that undergoes fusion of the urethral epithelium. Although *in situ* hybridization and immunohistochemical analyses suggested that Fkbp52 mutants had a normal urethral epithelium signaling center and epithelial differentiation, a reduced apoptotic cell index at ventral epithelial cells at the site of fusion and a defect of genital mesenchymal cell migration were observed. Supplementation of gestating females with excess testosterone partially rescued the hypospadic phenotype in Fkbp52 mutant males, showing that loss of Fkbp52 desensitizes AR to hormonal activation. Direct measurement of AR activity was performed in mouse embryonic fibroblast cells treated with dihydrotestosterone or synthetic agonist R1881. Reduced AR activity at genes controlling sexual dimorphism and cell growth was found in Fkbp52-deficient mouse embryonic fibroblast cells. However, chromatin immunoprecipitation analysis revealed normal occupancy of AR at gene promoters, suggesting that Fkbp52 exerts downstream effects on the transactivation function of AR. Taken together, our data show Fkbp52 to be an important molecular regulator in the androgen-mediated pathway of urethra morphogenesis.

Male external genital development in mammals is composed of distinct hormone-independent and hormone-dependent stages (1–3). The first phase (androgen-independent) is characterized by initial patterning and outgrowth of genitourinary structures in both male and female early embryos (embryonic day (E)10.5–E13.5 in the mouse). In males, the second phase (androgen-dependent) involves further outgrowth and differentiation of the glans penis, closure of the urethral fold (urethral epithelium remodeling) and scrotal development. Insufficient or altered hormonal signaling during development leads to many birth defects in the reproductive system, including hypospadias (4, 5). Hypospadias is a common birth defect in humans, in which altered urethral epithelial fusion causes ectopic openings at the ventral surface of the male external tubercle. Hypospadias is thought to result from androgen insufficiency or insensitivity. However, most cases of hypospadias have no clear link to defects in androgen production or androgen receptor (AR)[3] mutations (5).

Fkbp52 and Fkbp51 are the best known of several tetratricopeptide repeat proteins that co-chaperone steroid receptors, including AR, progesterone receptor, and glucocorticoid receptor (6–8). Prior work has shown that hypospadias is one of the major physiological defects found in Fkbp52-deficient male mice, but not in Fkbp51-deficient males (9, 10). Initial characterization also demonstrated reduced AR-mediated activity when heterologous reporter constructs were transfected into Fkbp52-deficient MEF cells (9). However, the ability of Fkbp52 to regulate endogenous AR-induced genes, the mechanism for this control, and the precise morphogenic events of male urethral development controlled by Fkbp52 are still unknown.

In the current study, we show that Fkbp52 expression is enriched in the ventral aspect of the developing urethral epithelium, a region responsible for final urethra formation via closure of the enveloping epithelial layers. Histological and scanning electron microscopic analysis showed that E18.5 was the precise time at which urethral clefts were observed in Fkbp52 mutants due to retarded elevation of prepucial swelling and urethral seam formation, suggesting a lack of growth and migration by the enveloping epithelial cell layers. As a test of the migration hypothesis, an *in vitro* genital mesenchyme cell migration assay was developed, and it showed a dramatic defect

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[3] The abbreviations used are: AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; E, embryonic day; Fkbp, FK506-binding protein; MEF, mouse embryonic fibroblast; SEM, scanning electron microscopy; UE, urethral epithelium.

of cellular migration and outgrowth by mutant penile cells. To assess the contribution of AR, gestating females were dosed with high level testosterone, resulting in partial rescue of the hypospadias phenotype in developing mutant males. Consistent with this, reduced AR activity at genes responsible for sexual dimorphism was observed in Fkbp52-deficient cells. Our findings not only explain the pathogenesis of hypospadias in Fkbp52-mutant males, but also highlight the importance of functional regulators of the AR in contributing to this most common birth defect.

EXPERIMENTAL PROCEDURES

Fkbp52-deficient Mice—Fkbp52-deficient mice were generated as described previously (9). They were maintained in a C57BL/6J/129SvEv mixed background. All animal experiments were carried out using a protocol approved by the Indiana University School of Medicine Institutional Animal Care and Research Advisory Committee.

Histology and Scanning Electron Microscopy (SEM)—Tissue samples were rapidly excised from mice at the indicated ages and washed in PBS. The isolated tissues were fixed in 4% paraformaldehyde at 4 °C for 24 h and embedded in paraffin. Sections were stained with hematoxylin and eosin for general morphological and histological analysis as previously described (9). For SEM analysis, embryonic tissues (E15.5 and E18.5) were fixed in Karnovsky's fixative (pH 7.5), rinsed in 0.1 M phosphate buffer (pH 7.3), postfixed in 1% phosphate-buffered osmium tetroxide, dehydrated and dried to critical point with carbon dioxide, coated with gold-palladium, and examined with an Amray 1000A scanning electron microscope.

In Situ Hybridization—Both whole mount and section *in situ* hybridization were performed as previously described (11, 12). Complementary RNA probes of Shh, FgfR2-IIIb, Fgf10, and Fkbp52 were labeled with digoxigenin-UTP using a Roche DIG RNA labeling system according to the manufacturer's guidelines. These probes were hybridized with paraformaldehyde-fixed embryonic samples or isolated penile tissues for whole mount *in situ* staining or with either frozen or deparaffined sectioned samples for *in situ* staining.

Immunohistochemistry and TUNEL Assay—To analyze keratin-14 (PRB-155P; COVANCE) and Fkbp52 (N-17; Santa Cruz Biotechnology) expression and tissue distribution, an immunohistochemical staining system (Vector) was used according to the manufacturer's instructions. To detect apoptotic cells, an *in situ* labeling kit (ApopTag kit, S7110; Chemicon) was used, as previously described (12).

Organ-explant Culture and Genital Mesenchyme Cell Migration Assay—Embryonic genital tubercles were dissected in ice-cold medium (DMEM with L-glutamine, supplemented with 10% FCS, and penicillin/streptomycin) from E15.5 and E16.5 Fkbp52-deficient and littermate control embryos obtained from Fkbp52 heterozygous mating and transferred onto a 10-mm culture plate coated with gelatin (0.1%). Organ grafts were cultured with ventral side toward the slide. Culture medium (50% DMEM and 50% BJGB, supplemented with 10% FCS and L-glutamine and antibiotics) covered two-thirds of each graft. Typically, within 24 h of culture in 37 °C (5% CO_2), genital mesenchymal cells migrate out of cultured penile tissues via the scission opening and attach to the culture plate as a single cell layer. Experiments were performed double-blinded by proceeding with culture prior to genotyping. Cells migrating out of cultured tissue were scored and matched to their corresponding genotypes.

Assessment of Hormonal Response—To assess the effect of testosterone on urethral closure in Fkbp52-deficient males, pregnant heterozygous females mated with heterozygous males were subcutaneously implanted with testosterone pellets (5 mg/pellet, 21-day release, Innovative Research of America, A-151) at E12.5. This treatment yields testosterone serum levels of ~4 ng/ml during the course of treatment (a 100-fold increase over normal basal levels in female mice). Embryos were harvested at E18.5 via cesarean section, assayed for Fkbp52 genotype and gender, as previously described (9), and processed for histological analysis. Serial paraffin sections were performed, which allowed us to measure the distances between the sites of fusion to the distal end of various penile tissues by counting the number of sections (7 μm/section).

Androgen Receptor Transcriptional Activity Assays—The human Fkbp51 promoter containing the AR-binding site was amplified from human genomic DNA with the following primers: h_FKBP51_pr F, 5′-TAATGGTACCGCCTTGAATGCCACATGGAAGAA-3′; h_FKBP51_pr R, 5′-ATCAGATCTAAACATTATCCACCCCAGCCCC3-′. The amplified fragment was then cloned into the pGL3-luciferase vector (Promega) between the KpnI and BglII sites. After transfection for 24 h, cells were treated with 1 nM DHT or ethyl alcohol. Luciferase activity was analyzed 48 h after transfection, and transfection efficiency was normalized with a *Renilla* expression vector.

Real-time PCR analysis was performed as follows. Total RNA was isolated from MEF cells using 5 Prime PerfectPure RNA Cell kit (Fisher Scientific Company, LLC) according to the manufacturer's instructions. Total RNA concentration and purity were determined by measuring absorbance at 260/280 nm and confirmed on an RNA denaturing formaldehyde gel. Purified RNA (1 μg) was used to produce complementary strands of DNA (cDNA) using a first strand synthesis kit (Roche Applied Science). cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad). PCR amplification of the cDNA was performed by quantitative real-time PCR using a qPCR Core kit for SYBR Green I (Applied Biosystems). The thermocycling protocol consisted of 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 61 °C, and 20 s at 72 °C and finished with a melting curve ranging from 60 to 95 °C to allow distinction of specific products. Primers for Nkx3.1, eNOS, iNOS, and TNFα were designed using Primer Express 3.0 software (Applied Biosystems). All primer sequences were uploaded to the PrimerFinder database (Toledo, OH).

Chromatin Immunoprecipitation (ChIP)—WT and Fkbp52-deficient MEF cells stably transfected with AR (9) were seeded at ~50% confluence and grown overnight, then treated with 1 nM DHT or ethyl alcohol for 24 h prior to experiments. ChIP assays were performed using a ChIP assay kit (Millipore). The lysate was immunoprecipitated with either rabbit IgG or an anti-AR antibody (C-19; Santa Cruz Biotechnology). Primers used in PCR are shown in Fig. 7*A*.

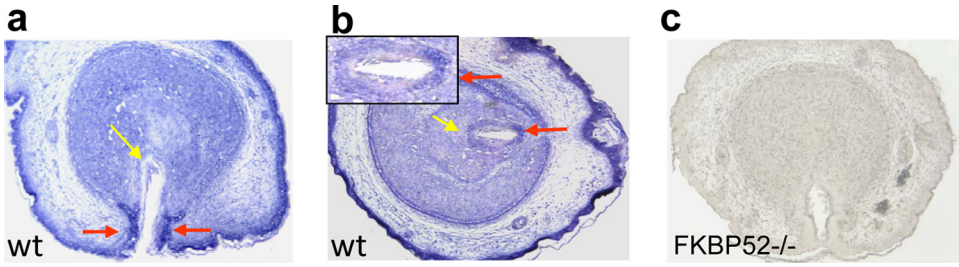

FIGURE 1. **Analysis of Fkbp52 expression in the developing penis.** *In situ* hybridization was performed on E18.5 penile tissues, as described under "Experimental Procedures." Although Fkbp52 expression is found in prepucial epithelium, genital mesenchyme, and urethral epithelium, expression was highest in the ventral aspect of urethral epithelium (*red arrows*) compared with dorsal aspect (*yellow arrows*). *a*, in unfused urethra, there is a clear border that separates Fkbp52 high expressing cells at the ventral side from Fkbp52 low expressing cells at the dorsal side. *b*, in fused urethra, Fkbp52 expression is also highest in the ventral aspect (*red arrows*). *c*, negative control using Fkbp52-deficient penile tissue is shown. *wt*, wild type.

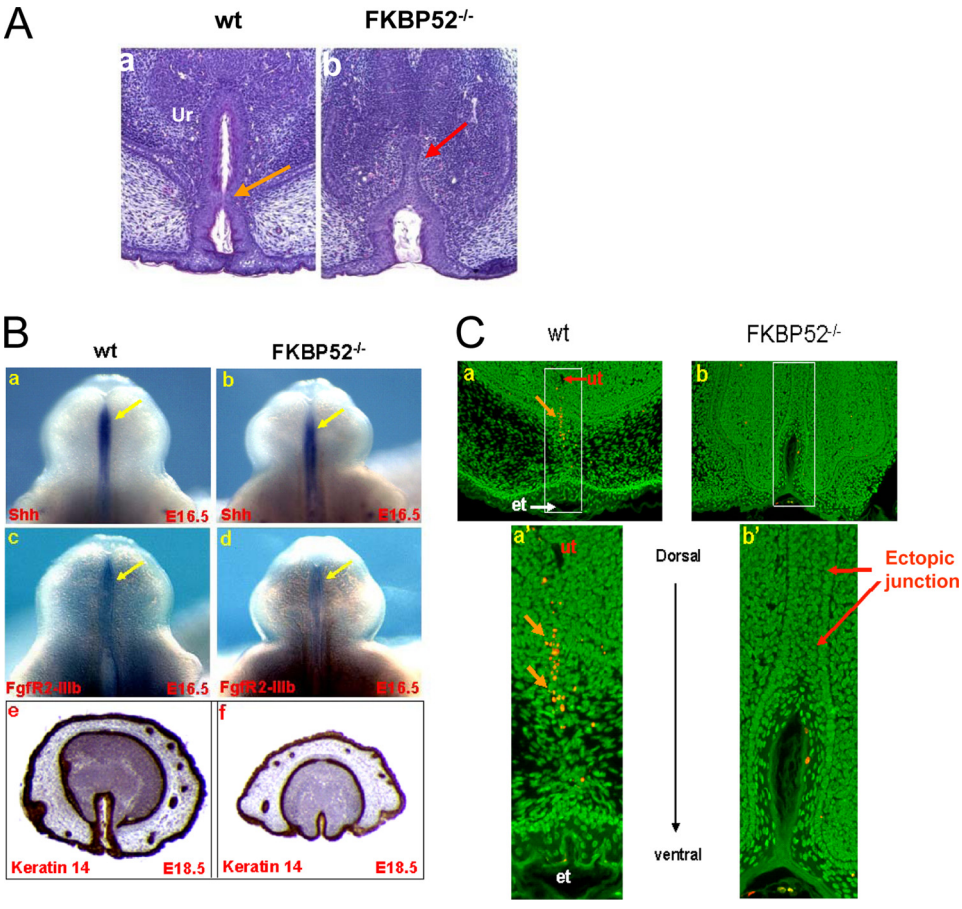

FIGURE 2. **Assessment of urethral epithelium development and urethral seam formation.** *A*, compared with normal urethral seam formation (*yellow arrow*), Fkbp52-deficient mutants have abnormal ectopic fusion at dorsal aspect of urethra (*red arrow*). *wt*, wild type. *B*, formation of the urethral epithelial signaling center and differentiation of epithelium are shown. *In situ* hybridization analysis of Shh and FgfR2-IIIb expression in wild-type is shown in *a* and *c*, and Fkbp52-deficient embryonic penile tissues (E16.5) are shown in *b* and *d*. The *blue* signals are positive staining (*yellow arrows*). Fkbp52-deficient mutants have normal expression, suggesting that UE signaling center is normally formed in Fkbp52 mutants. Immunohistochemistry analysis of epithelium marker keratin 14 was performed in wild-type (*e*) and Fkbp52 mutant embryonic penis (*f*). Keratin 14 is normally expressed in Fkbp52 mutant genital epithelium, suggesting that hypospadias in Fkbp52 mutants is not due to abnormal epithelial cellular differentiation. *C*, urethral cellular apoptosis in wild type and Fkbp52 mutants at E18.5 is shown. There are detectable amounts of apoptotic cells at ventral side of the developing urethra (*a* and *a'*, *arrows*), but a dramatic reduction in cellular apoptosis in Fkbp52 mutant urethral epithelium (*b* and *b'*). *Red arrows* point to the ectopic epithelial fusion in Fkbp52 mutant penis.

RESULTS

Fkbp52 Expression Pattern in Developing Urethral Epithelium—As previously reported, morphological examination of adult Fkbp52-deficient male external genitalia revealed severe hypospadias with 100% penetrance (9). In general, Fkbp52 transcripts are ubiquitously expressed (13), yet protein levels can vary widely across tissues (14), suggesting that protein turnover could be an active mechanism for regulation of Fkbp52-mediated responses. This may also explain why ablation of Fkbp52 appears to affect only select AR-regulated tissues, such as penis and prostate (9). In humans, a unique pattern of AR expression in the developing urethral epithelium has been observed (15), with AR expression higher along the ventral, rather than dorsal, portion of the urethra. However, this pattern has not been observed in the mouse because AR is uniformly present in the dorsal-ventral axis of the urethra (11). To test whether Fkbp52 expression is spatially regulated in the mouse developing penis, *in situ* hybridization with an antisense Fkbp52 probe was performed. Interestingly, although Fkbp52 is present in almost every cell type, expression at the ventral aspect of urethral epithelium was higher than that in the dorsal aspect of same cell layer (Fig. 1). This unique pattern along the dorsal-ventral axis suggests that in mice Fkbp52, rather than AR, has a novel spatial regulatory function during urethral closure.

Disrupted Urethral Seam Formation in Fkbp52-deficient Male Embryos—In the normal mouse developing urethra, the ventral urethral epithelial surfaces touch and form a closed urethral groove. Within this closed urethral groove two epithelial surfaces fuse to form the urethral seam, which results in a true urethra and a temporal tubular structure called the "epithelial tail" at the ventral side (3). Histological analysis on E18.5 Fkbp52-deficient embryonic genital tubercles revealed that there was a clear disruption in the spatial pattern of urethral seam formation. Instead of forming the epithelial tail at the ventral aspect, Fkbp52-deficient urethral epithelial surfaces either touch ectopically at the dorsal aspect or fail to touch at all (Fig. 2*A*).

To determine whether the altered pattern of urethral seam formation is due to a defect in the urethral epithelium (UE)

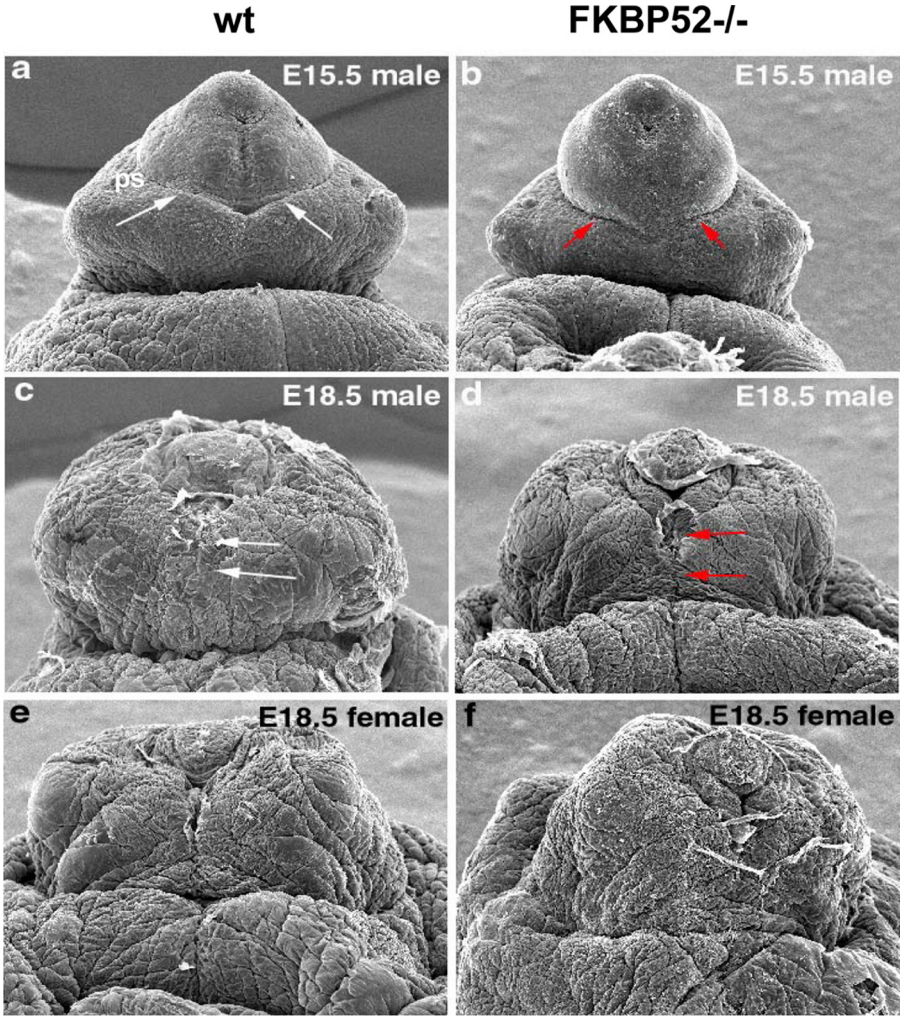

FIGURE 3. **Scanning electron micrographs of developing genital tubercle (ventral view).** *a* and *b*, comparison of male genital tubercle development at E15.5 between wild-type (*a*) and Fkbp52-deficient (*b*) embryos. There is no apparent defect in the formation of the urethral plate in the mutant, whereas the mutant glans is more exposed, suggesting that the encircling movement of prepucial swelling in Fkbp52 mutant is slower. *c* and *d*, comparison of male genital tubercle development at E18.5. There is a clear gap at the ventral side, and the interior epithelium is exposed in mutant penis (*red arrows* in *d*) compared with normal fused ventral side of the penis (*white arrows* in *c*). *e* and *f*, comparison of female genital development at E18.5. There is no difference between wild-type and Fkbp52-deficient mutant. A typical opening can be seen at ventral side of female genitalia. *wt*, wild type.

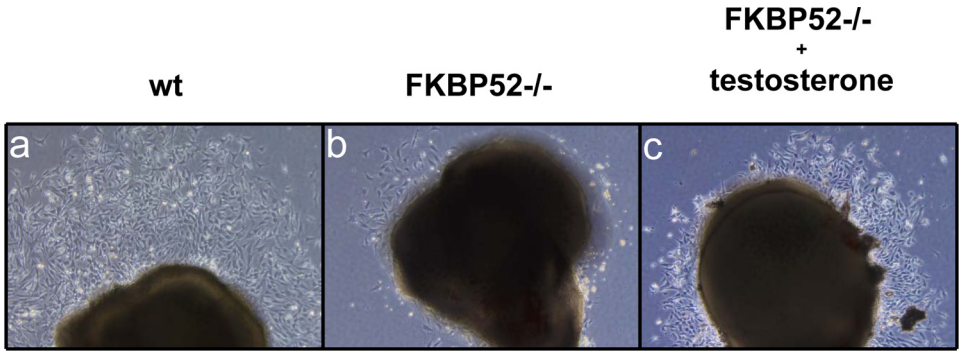

FIGURE 4. **Mesenchyme cell migration in embryonic genital organ culture.** *a*, mesenchymal cells migrate out from cultured wild-type (*wt*) penile tissues (E15.5) within 24 h. *b*, fewer cells migrate out from Fkbp52-deficient penile tissues. *c*, following treatment with testosterone in culture medium, more cells migrate out from Fkbp52 mutant penile tissues. However, the total cells migrating from cultured mutant penile tissues are fewer than wild-type untreated tissue (*a*).

signaling center controlling epithelial differentiation, growth, and maturation (1), we performed whole mount *in situ* hybridization with probes to Shh and FgfR2-IIIb in the Fkbp52-deficient developing penis at E16.5 (Fig. 2*B*). The results demonstrated that Shh and FgfR2-IIIb are normally present in Fkbp52-deficient UE. As an additional test of UE differentiation, immunohistochemical analysis of keratin 14 expression was performed. Strong expression of keratin 14 in Fkbp52-deficient urethral and prepucial epithelium at E18.5 was seen. These findings indicate that Fkbp52 ablation does not alter the establishment of UE signaling center and epithelium differentiation in the developing penis.

Reduced Epithelial Cellular Apoptosis in FKBP52 Mutant Genital Tubercles—A unique feature of ventral UE in the normal developing penis is that it meets and touches at midline and triggers epithelial apoptosis to complete a patterned fusion process (3, 16). To determine whether the ectopic epithelial junction observed in the Fkbp52-deficient penis triggers a similar apoptotic response, we performed TUNEL assay on an E18.5 mouse developing genital tubercle. Our data demonstrated that the ectopic epithelial touching event does not proceed to cellular apoptosis as occurs in normal littermates (Fig. 2*C*). Thus, no fusion event occurs in Fkbp52-deficient mutants, even in regions where the UE makes contact.

Reduced Prepucial Swelling in the Fkbp52-deficient Embryonic Penis—SEM was used to assess penile development in FKBP52-deficient males (Fig. 3). SEM analysis not only demonstrated a failure of ventral urethral tube closure at E18.5, but also revealed a defect of elevation in the prepucial swelling in Fkbp52 mutants. At an earlier stage (E15.5), mutant embryos showed normal development of the urethral plate contained within the developing glans and prepuce. Similar to wild type (WT), the distal meatus in mutants was able to form at this stage, and a transient proximal urethral meatus was present in the developing genital tubercle. The latter observations are in sharp contrast to more severe models of hypospadias, such as mice deficient in Hoxa13 (17), Shh (18), and FgfR2-IIIb(11), in which

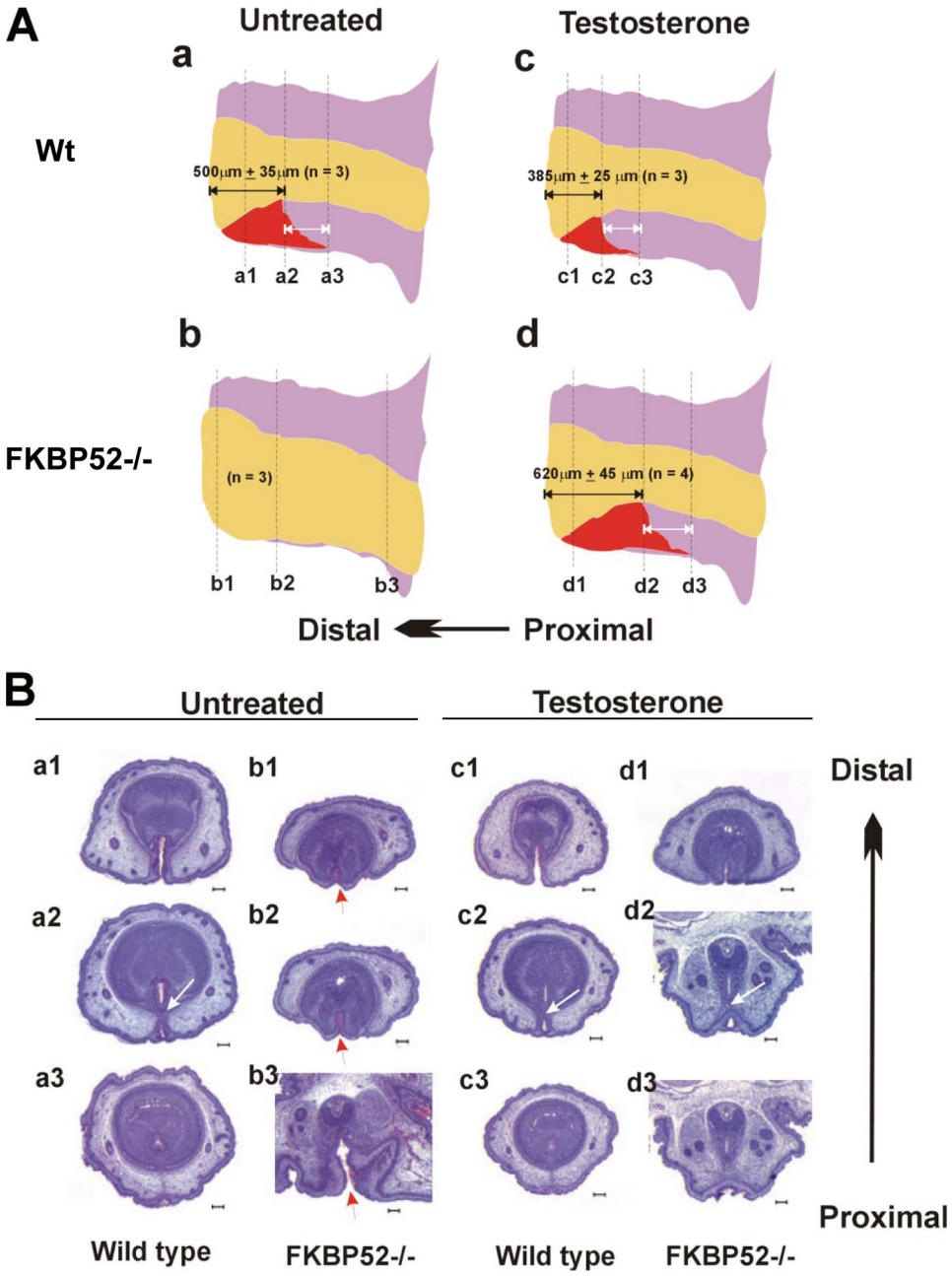

FIGURE 5. **Testosterone partially rescues hypospadias in Fkbp52-deficient embryos.** *A*, schematic summary (lateral view of penis) of the partial rescue of hypospadias by testosterone treatment of gestating females (E18.5). Urethras and epithelial tails are colored *yellow* and *red*, respectively. Without hormone treatment, compared with normal urethral fusion event (*a*, 500 ± 35 μm between distal end to the point of fusion, $n = 3$ tissues/3 different litters), Fkbp52-deficient mutant has no urethral fusion (*b*). With hormone treatment, wild-type male has a more advanced fusion event (*c*, 385 ± 25 μm between distal end to the point of fusion, $n = 3$ tissues/3 different litters), mutant appears to have a fusion event, but at a much more proximal region (*d*, 620 ± 45 μm between distal end to the point of fusion, $n = 4$ tissues/3 different litters). *B*, representative sections from serial histological sections. Section planes are indicated in *A*.

genital tubercles exhibit open urethral plates at E15.5. There was, however, a clear and consistent sign of slower encircling movement of the developing prepuce around the glans at E15.5 in mutant embryos. During normal development, prepucial swellings grow dorsally and ventrally around tubercles to internalize the urethral tube. By E18.5, the prepuce of WT animals completely surrounds the glans and penile shaft and fuses at ventral midline. In contrast, although the prepucial swellings were able to move ventrally, the prepucial swellings of Fkbp52-deficient males are misshaped, suggesting that the encircling process of prepucial swellings is altered. This observation is consistent with the histological findings (Fig. 2) that the prepucial swelling fails to completely surround the developing urethral tube in Fkbp52-deficient males, suggesting an additional defect in the interaction between genital mesenchymal and epithelial cells.

Reduced Genital Mesenchymal Cell Migration in Fkbp52-deficient Embryos—Epithelial-mesenchyme interaction plays an important role in supporting outgrowth of the developing genital tubercle. It has been shown that surgical removal of the distal epithelium leads to an arrest of outgrowth and truncation of the phallus (19, 20). The reduced prepucial swelling observed in Fkbp52-deficient males may therefore reflect a defect either in the proliferation or migration of genital mesenchymal cells that reside in the preputial swelling. To measure genital mesenchyme cellular proliferation in Fkbp52-deficient mutants, we performed a [^{3}H]thymidine labeling assay. The labeling index of genital mesenchyme and UE cells in Fkbp52-deficient penises was normal (data not shown), suggesting that cell cycle activity of genital mesenchyme in the Fkbp52-deficient penis remains unchanged.

To determine the migration activity of genital mesenchyme, an organ-explant culture assay was established. As an initial test, WT embryonic genital tubercles isolated from E14.5, E15.5, E16.5, and E18.5 embryos were used. The result demonstrated that explants from E15.5 and E16.5 yielded the maximal number of migrating cells (data not shown). By E18.5, virtually no migration activity could be observed under these conditions. This observation is consistent with the fact that the processes of encircling movement and urethral remodeling are complete by E18.5 (1, 3). When Fkbp52-deficient genital tubercles at E15.5 were tested, all explants (7/7) exhibited severe deficiency in mesenchyme cell migration, whereas all samples (23/23) from WT or Fkbp52 heterozygous animals (data not shown) exhibited normal migration (Fig. 4). This finding suggests a defect in genital mesenchyme cell movement in Fkbp52-deficient males,

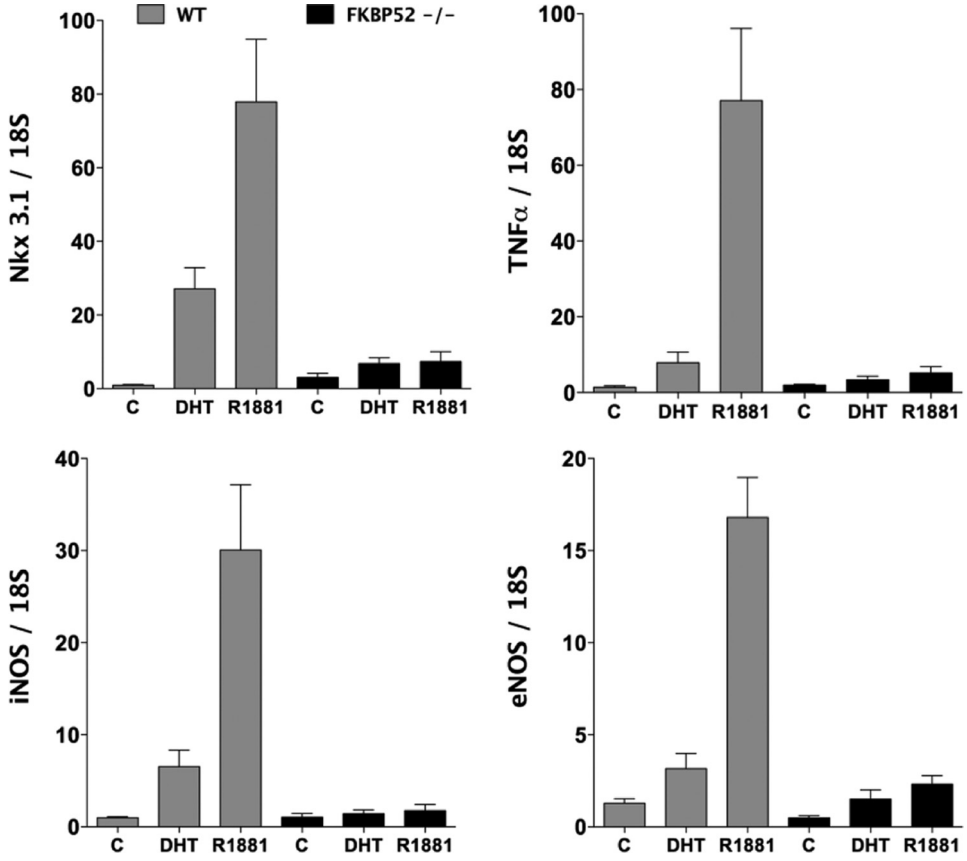

FIGURE 6. **Fkbp52 is required for AR activity at dimorphic and growth factor genes.** WT and Fkbp52-deficient MEF cells stably transfected with androgen receptor were treated with vehicle (*C*), 10 nM DHT, or 10 nM R1881 for 2 h, followed by real-time PCR analysis for the indicated genes. Values are SEM for two independent samples, assayed in triplicate. *Error bars* indicate standard error.

Fkbp52 Is Required for Optimal AR Activity at Gene Promoters—We have shown previously that the hormone-binding and nuclear translocation functions of AR are normal in Fkbp52-deficient cells, but that the receptor has reduced transcriptional activity when Fkbp52-deficient cells were transfected with heterologous reporter constructs (9). To assess whether Fkbp52 controls AR activity at endogenous genes, real-time PCR analysis was performed in WT and Fkbp52-deficient MEF cells exposed to DHT or synthetic agonist R1881. Genes were chosen with potential roles in AR-mediated sexual dimorphism and function (21, 22). They are Nkx3.1, iNOS, eNOS, and TNFα. The results show moderate induction by DHT and stronger induction by R1881 at all genes in WT MEF cells (Fig. 6). More importantly, a dramatic loss of hormone responsiveness was observed in the Fkbp52$^{-/-}$ cells (Fig. 6). These results suggest that Fkbp52 is essential to AR-mediated induction of genes involved in sexually dimorphic development of the penis (Nkx3.1), in erectile function of the mature organ (iNOS and eNOS), and in promotion of apoptosis during development (TNFα).

which likely contributes to the abnormal genital outgrowth and urethral remodeling observed during the later phases of external genital development. Interestingly, testosterone addition to the organ-explant cultures noticeably improved Fkbp52-mutant mesenchyme cell migration (Fig. 4*C*), suggesting that AR-mediated signaling is critical to this genital mesenchyme cell function.

Partial Rescue of Hypospadias by Testosterone—To test further of the involvement of AR, we asked whether high level dosing with androgen would rescue the hypospadias phenotype in Fkbp52-deficient males. To achieve this, testosterone tablets were implanted in gestating Fkbp52$^{+/-}$ females previously mated to Fkbp52$^{+/-}$ males. Tablets were implanted at E12.5, and embryos were harvested at E18.5 by cesarean section, followed by genotyping for Fkbp52 and gender. Testosterone-treated Fkbp52-deficient males were compared histologically with littermate wild-type and heterozygous males. By analyzing serial histological sections, measurements of the distances between the distal edge of glans to true urethral fusion and to the end of epithelial tail were obtained. Fig. 5 demonstrates that testosterone can partially rescue the hypospadias phenotype. In contrast to complete lack of fusion of the ventral UE in Fkbp52-deficient males, testosterone-treated mutant tubercles exhibited a fused UE, albeit at a more proximal position than wild-type treated and untreated genital tubercles.

Because of the dramatic loss of AR activity in the Fkbp52-deficient cells did not correlate with reduced hormone-binding or nuclear translocation (9), we asked whether loss of Fkbp52 prevented AR recruitment to gene promoters. This was tested by ChIP analysis in WT and Fkbp52-deficient MEF cells using the Fkbp51 promoter as site of AR occupancy (Fig. 7). Previously, we and others have shown that Fkbp51 is an androgen- and AR-regulated gene (23–25) whose expression is down-regulated in Fkbp52-deficient cells (9). AR binding sites have been found in the Fkbp51 promoter and enhancer regions (23). We used bioinformatic analysis to confirm the androgen response element (ARE) in a proximal region of the Fkbp51 promoter, and primers were designed that spanned the putative ARE (Fig. 7*A*). The results show no difference in AR binding to the Fkbp51 ARE in Fkbp52-deficient and WT cells (Fig. 7). To confirm reduced AR activity at this locus, the WT and Fkbp52-deficient cells were transfected with a luciferase reporter driven by the Fkbp51 promoter. Results show reduced AR activity in Fkbp52-deficient cells (Fig. 7*C*). These data demonstrate that Fkbp52 is critical to AR activity by controlling the intrinsic transactivation function of the promoter-occupied receptor, perhaps by affecting the extent and nature of co-activator recruitment. Further studies will be needed to investigate this new observation fully.

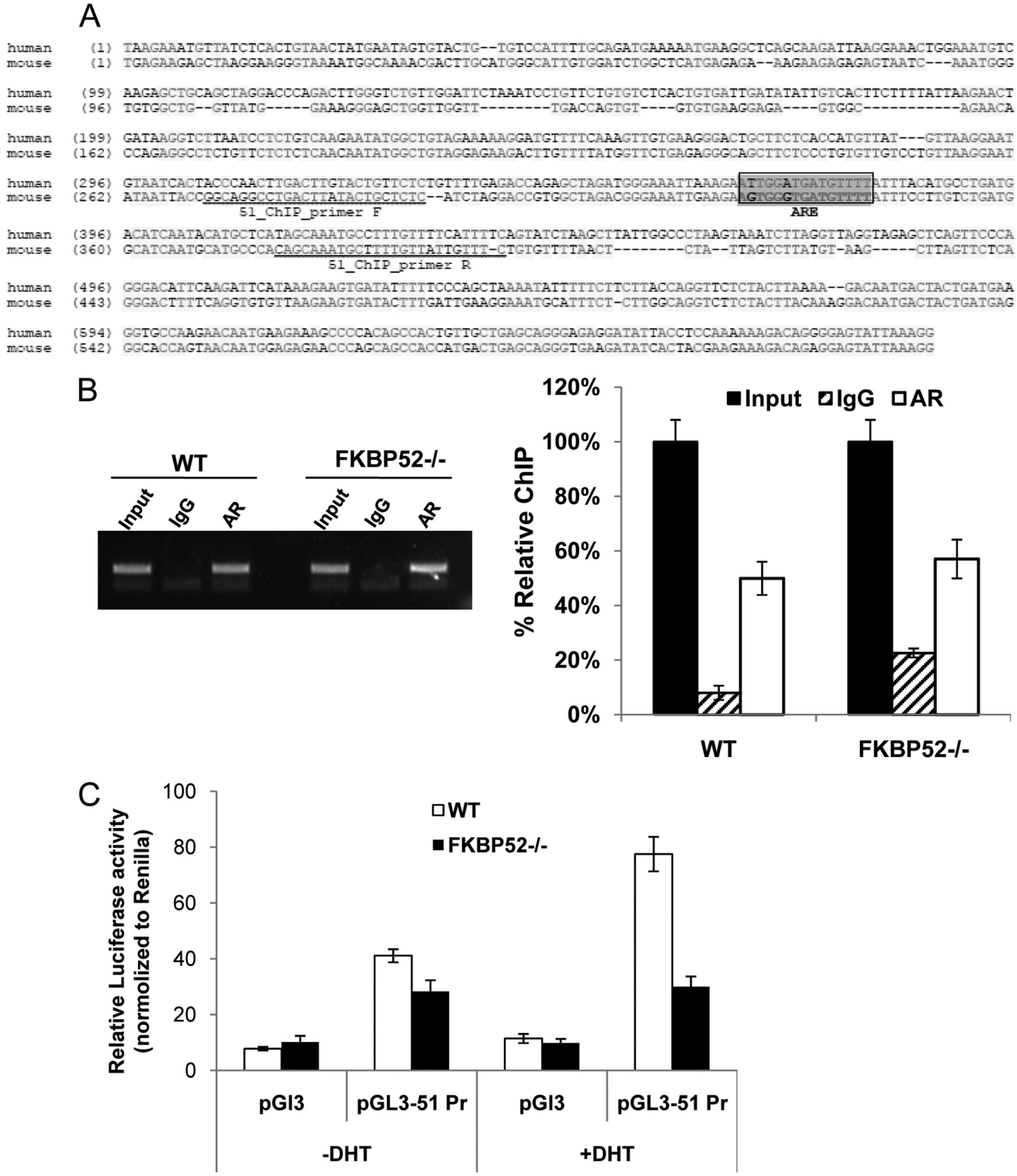

FIGURE 7. **Fkbp52 regulates AR activity at gene promoters.** *A*, locations of ARE and primer sites in the human and mouse Fkbp51 promoter are shown. *B*, AR binding to mouse Fkbp51 promoter was determined by ChIP assay. AR binding to the FKBP51 promoter in response to DHT treatment was the same in WT and Fkbp52-deficient MEF cells. The *right panel* shows the quantitative PCR analysis of ChIP DNA. The result was the average of two independent experiments, quantified using the standard curve method on Roche LightCycler 480 II thermocycler, and normalized to input (set at 100%). Primers used in PCR are shown in *A*. *C*, WT and FKBP52-deficient MEF cells stably expressing AR were transiently transfected with a Fkbp51-luciferase reporter (pGL3–51) or empty vector (pGL3), followed by treatment with vehicle or DHT (10 nM).

DISCUSSION

Development of mammalian external genitalia is a complex morphogenetic process requiring three-dimensional patterning, coordinated outgrowth, and tubulogenesis. It is composed of several developmental steps, including the formation of urethral groove and urethral seam, epithelial-mesenchymal interaction, and epithelial apoptosis, all of which have to coordinate in the genesis of urethra. At the androgen-independent phase, the external genitalia remain in the indifferent stage between male and female. The urogenital groove on the ventral surface of the genital swelling (phallus) is generated by the urethral fold. Establishment of the UE signaling center is the most important morphogenetic event at this stage (1), which provides instructive signals to genital-shelf mesenchyme and controls the distal outgrowth, cellular proliferation, epithelial-mesenchymal transformation or interaction in the developing external genitalia (1). Urethral epithelium is endoderm in origin. This UE signaling center relies on a series of morphogenetic signaling molecules, such as Shh (18, 26, 27), Hoxa13 and Hoxd13 (28), BMP7 (17), Fgfs (17, 29, 30), and receptor Fgfr2 (11). These molecules are expressed in the UE and play a critical role in various morphogenetic events. Ablation of these molecules in mice disrupts the formation of UE signaling center as visualized by dramatic reduction of the level of cytokeratin 14 (molecular marker for epithelium) and leads to severe genitourinary defects, including penile agenesis or hypospadias in mutant males and clitoral agenesis in mutant females.

Our data indicate that proper development of the UE center is not altered in Fkbp52 mutants, because expression of Shh, FgfR, and keratin 14 was all normal in this structure. Thus, Fkbp52 may not be vital to any role of AR in the UE center formation. However, we cannot exclude that other tetratricopeptide repeat co-chaperones, such as Fkbp51, Cyp40, or PP5 (31), may be more important to AR activity in the UE center. Because Fkbp52 ablation does not affect all AR-regulated tissues, even those (*e.g.* testes) where Fkbp52 is present (9), we currently favor a model in which AR signaling in Fkbp52 mutant embryos is sufficient for UE signaling center formation and function due to compensation by other co-chaperones. This interpretation is also consistent with the fact that androgen can partially rescue genital mesenchymal cell migration and hypospadias in mutant males.

An important event during external genital development is establishment of cellular sensitivity to androgen via the up-regulation of AR and/or molecules required for androgen-mediated signaling. For example, cross-talk between FGF and AR occurs during male genital development (1, 11). The importance of Fkbp52 to this morphogenetic process is reflected by its unique expression pattern. Along the dorsal-ventral axis in developing UE, Fkbp52 expression is more enriched in the ventral aspect, the region responsible for rapid encircling migration by mesenchyme cells. Interestingly, we have uncovered that the cellular migration function of genital mesenchyme cells is severely affected in Fkbp52 mutants and that androgen treatment improved this defect. We also found reduced cellular apoptosis on the ventral side of the developing urethra. In MEF cells, loss of Fkbp52 caused a reduction in androgen-induced expression of TNFα, which has been implicated in AR mediated growth and apoptotic processes (17). These findings suggest that Fkbp52 may be essential to controlled growth in this region by modulating AR-induced expression of growth factors.

An important question arising from our observation is how Fkbp52 coordinates the encircling movement of the underlying genital mesenchyme cells in relation to other developmental layers. Although the molecular mechanism for encircling movement is largely unknown, a possible interpretation is that Fkbp52 is involved in the establishment of a "spatial reference" to the migrating adjacent mesenchyme cells. Clearly, the loss of Fkbp52 disrupts this spatial reference, as evidenced by the appearance of ectopic epithelial junctions at the dorsal side of the urethra in the Fkbp52-deficient penis. At first, we reasoned that the UE signaling center might serve to set the Fkbp52 spatial reference. This seemed logical, as the interaction between urethral epithelium and genital mesenchymal cell is essential to the outgrowth of the developing penis (19, 20), and AR signaling had been shown to play a role in this process. For example, Baskin and colleagues showed that in humans expression of both AR and 5α-reductase type 2, the enzyme responsible for conversion of testosterone to DHT, is highest along the ventral portion of urethral epithelium (15). However, mice appear to have AR that is uniformly expressed throughout these tissues. Thus, our current model is that developmentally controlled expression of Fkbp52 is a key event in the coordination of growth between the UE center and ventral layers. These issues aside, we believe our data provide evidence of a novel and dynamic interactive model of urethral tubulogenesis, in which androgen, AR, and Fkbp52 play an important role.

In summary, our studies have uncovered a novel mechanism for Fkbp52 in male genital development and AR-mediated signaling, providing an important model for analyzing the pathogenic pathways leading to this common congenital birth defect. A current effort is to determine whether Fkbp52 is a genetic contributing factor for hypospadias in humans.

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