Timing of androgen receptor disruption and estrogen exposure underlies a spectrum of congenital penile anomalies

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Congenital penile anomalies (CPAs) are among the most common human birth defects. Reports of CPAs, which include hypospadias, chordee, micropenis, and ambiguous genitalia, have risen sharply in recent decades, but the causes of these malformations are rarely identified. Both genetic anomalies and environmental factors, such as antiandrogenic and estrogenic endocrine disrupting chemicals (EDCs), are suspected to cause CPAs; however, little is known about the temporal window(s) of sensitivity to EDCs, or the tissue-specific roles and downstream targets of the androgen receptor (AR) in external genitalia. Here, we show that the full spectrum of CPAs can be produced by disrupting AR at different developmental stages and in specific cell types in the mouse genital tubercle. Inactivation of AR during a narrow window of prenatal development results in hypospadias and chordee, whereas earlier disruptions cause ambiguous genitalia and later disruptions cause micropenis. The neonatal phase of penile development is controlled by the balance of AR to estrogen receptor α (ERα) activity; either inhibition of androgen or augmentation of estrogen signaling can induce micropenis. AR and ERα have opposite effects on cell division, apoptosis, and regulation of Hedgehog, fibroblast growth factor, bone morphogenetic protein, and Wnt signaling in the genital tubercle. We identify *Indian hedgehog* (*Ihh*) as a novel downstream target of AR in external genitalia and show that conditional deletion of *Ihh* inhibits penile masculinization. These studies reveal previously unidentified cellular and molecular mechanisms by which antiandrogenic and estrogenic signals induce penile malformations and demonstrate that the timing of endocrine disruption can determine the type of CPA.

sexual differentiation | external genitalia | congenital malformation | androgen | hypospadias

Congenital penile anomalies (CPAs) encompass a spectrum of malformations of the penis. Analysis of the Nationwide Inpatient Sample, the largest inpatient database in the United States, identified CPA in 7.8/1,000 newborns and showed that the frequency of CPA has increased over the past 40 y (1–3). The most common CPA is hypospadias (68.3%), followed by chordee (8.6%) and hypospadias plus chordee (5%), and 14% are reported as unspecified penile anomalies (2). The range of structural defects included in the CPA classification suggests that a single developmental mechanism is unlikely to account for the full spectrum of malformations. Furthermore, the rate at which reports of CPAs have increased in recent decades cannot be explained by genetics alone. There is increasing evidence that environmental factors, particularly exposures to environmental endocrine disrupting chemicals (EDCs), may play a causal role in these developmental defects (4); however, little is known about the interactions between EDCs and the gene networks that control external genital development, the temporal windows of sensitivity to EDC exposure, the endogenous role(s) of estrogen in penile development (5–7), or the relationship between androgen and estrogen signaling in normal genital development. At present, there are no mouse models for human-like CPAs, such as midshaft hypospadias (with or without chordee) or micropenis.

Hypospadias is a urethral tube defect in which the urethra opens ectopically on the ventral side of the penis, between the glans and the perineum. The severity of hypospadias can range from a slightly offset urethral meatus to complete failure of urethral tube formation, which can result in ambiguous genitalia. Epispadias is a less common urethral tube defect, in which the urethra opens on the dorsal side of the penis. Both malformations can be associated with chordee, an abnormal bending of the penis, which may involve soft-tissue tethering. Hypospadias and epispadias can occur in both sexes although detection in females is challenging (8, 9). Micropenis refers to an abnormally small but normally structured penis with a stretched penile length of >2.5 SDs below the mean human penis size for the same age individual (10, 11). Micropenis is often associated with both functional (related to sex and voiding) and psychological problems, and patients with micropenis can suffer from penile dysmorphic disorders (12). Analyses of mouse mutants have implicated a number of developmental control genes in hypospadias (reviewed in ref. 13), and, although association studies of affected patients have identified promising candidate mutations and copy number variants (14–16), the causes of hypospadias in humans remain largely unknown (17).

Androgens and estrogen are steroid hormones that play critical roles in sexually dimorphic genital development (18). Masculinization of male external genitalia is determined mainly by

Significance

Birth defects of external genitalia occur at a striking frequency, affecting ∼1:250 live births. Congenital penile anomalies (CPAs) encompass a range of malformations, including failure of urethral tube closure (hypospadias), penile curvature (chordee), micropenis, and feminization of male genitalia. Both genetic anomalies and exposures to endocrine disrupting chemicals (EDCs) are suspected to be involved; however, little is known about the underlying causes or the developmental window(s) of sensitivity to antiandrogenic or estrogenic signals. This study shows that disruption of androgen signaling at different stages of genital development can induce different types of CPA. We identify a cell type in which the androgen receptor (AR) is essential for genital masculinization and uncover previously unknown mechanisms through which antiandrogenic and estrogenic signals induce penile malformations.

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androgen signaling. Mice with mutations in the androgen receptor (AR) or 5α-reductase, which converts testosterone to dihydrotestosterone, develop feminized external genitalia (19, 20). In humans, mutations in these genes underlie androgen insensitivity syndrome, in which genetic males fail to respond to androgen signaling and consequently develop feminized genitalia and secondary sex characters (21). Prenatal exposure to antiandrogenic chemicals can feminize the external genitalia and induce hypospadias in male rodents (22–25).

It has long been thought that female external genitalia develop due to the low levels of androgen signaling activity; however, recent studies of mouse estrogen receptor α (ERα) mutants revealed virilization of the clitoris, demonstrating that estrogen signaling via ERα is required for normal development of female external genitalia (26). Deletion of ERα in mice affects only female external genitalia development (26) although mutations in ERα have been identified in human males with genital and reproductive abnormalities (27). Furthermore, exposure of neonatal rats to estrogen results in reduced penis size and weight, an effect mediated by ERα (26, 28, 29). Deletion of ERα is sufficient to rescue diethylstilbestrol (DES)-induced feminization of the penis (29), suggesting that ERα plays a crucial role in mediating estrogen-driven penile anomalies.

We combined mouse genetics with pharmacology to dissect the temporal and tissue-specific roles of androgen and estrogen signaling in penile development, and we show that disruption of AR and/or activation of ERα at discrete time periods can produce the complete spectrum of congenital penile anomalies found in humans. Here, we show that normal development of male external genitalia in mice involves two phases of AR and ERα activity; an early prenatal phase regulates formation of a closed urethral tube and development of the surrounding stromal tissue and prepuce, and a later neonatal phase controls proliferation of the glans penis mesenchyme. Our results demonstrate that modulation of androgen and estrogen signaling at discrete developmental time points can account for the entire spectrum of CPAs.

Results

AR and ERα Show Dynamic Patterns of Expression During Sexual Differentiation of the External Genitalia. To determine the roles of AR and ER during sexual differentiation of mouse external genitalia, first we compared the distribution of AR and ERα during male and female genital development. *Ar* and *Esr1* mRNA were detected as early as embryonic day (E) 11.5 in the genital tubercle (GT) (Fig. S1); however, male and female genital tubercles show no differences in expression at this early stage. At E13.5, male and female genitalia show similar patterns of AR protein localization, with strong cytoplasmic staining occurring in the urethral epithelium and in the mesenchyme in the center of the genital tubercle (Fig. 1 *A–D*). By E15.5, AR shows marked sexual dimorphism in the genitalia; AR protein is almost undetectable in females but is abundant in males, where it is almost entirely nuclear, consistent with the localization pattern that occurs when signaling is activated after ligand binding (compare Fig. 1 *E* and *F* with Fig. 1 *G* and *H*). At postnatal day 1 (P1), AR-positive cells were detected in the mesenchyme of the penile prepuce and glans, with the strongest staining in the corpus cavernosum glandis, corpus cavernosum urethrae, and male urogenital mating protuberance (MUMP) ridge (Fig. 1*I*). In the few AR-positive cells that were found in the glans clitoris at P1, the protein was localized mostly to the cytoplasm, where AR resides before ligand binding and activation of genomic signaling (Fig.1 *K* and Fig. S2 *A* and *B*).

To test whether the sexually dimorphic pattern of AR at postnatal stages is caused by a differential response of male and female external genital cells to androgen or estrogen, we treated mice with either estradiol benzoate (EB) (300 μg/kg) or testosterone (T) (2 mg/kg methyltestosterone) at P0 and examined AR

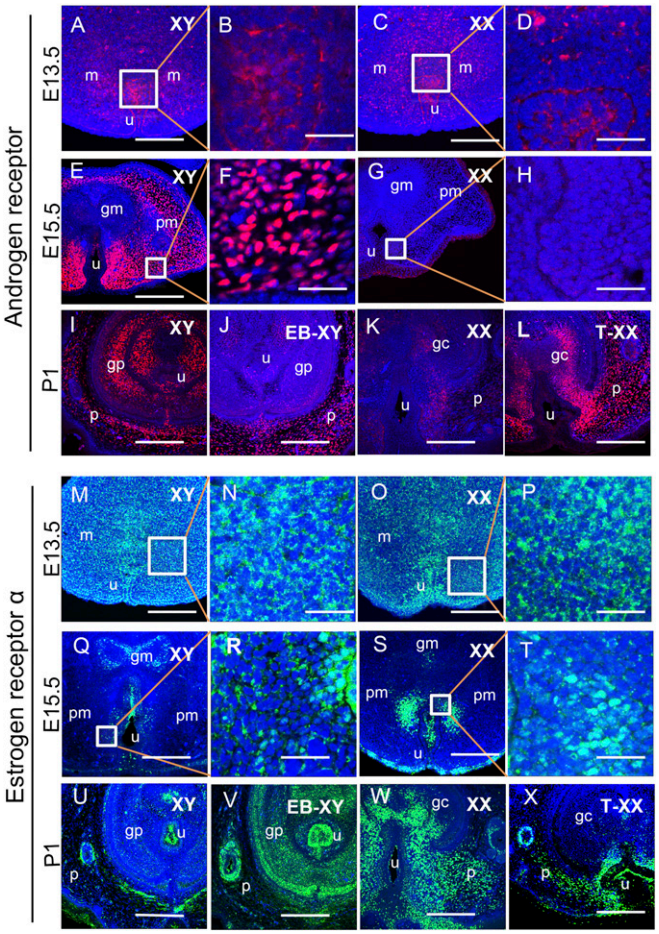

Fig. 1. AR and ERα distribution in developing genital tubercle. Immunolocalization of AR (red, *A–L*) and ERα (green, *M–X*) in mouse genital tubercle at E13.5 (*A–D*, *M–P*), E15.5 (*E–H*, *Q–T*), and P1 (*I–L*, *U–X*). Blue signal is DAPI. Stage is indicated at left, and sex is indicated by chromosome symbols XY (male) and XX (female). White boxes in *A*, *C*, *E*, *G*, *M*, *O*, *Q*, and *S* are shown at high magnification in *B*, *D*, *F*, *H*, *N*, *P*, *R*, and *T*, respectively. Note that males show more activated (nuclear) AR at E15.5 (*E* and *F*) and P1 (*I*) than females at the same stages (*G*, *H*, and *K*). Testosterone-treated female GTs (*L*) show more AR-positive cells than control females (*K*). EB-treated male GTs (*J*) show marked reduction of AR-positive cells compared with controls (*I*). ERα staining is higher in females than in males at E15.5 and P1 (*Q–U*, *W*). Testosterone-treated female GTs (*X*) show fewer ERα-positive cells than control female GTs (*W*) whereas EB-treated male GTs (*V*) show increased numbers of ERα-positive cells compared with controls (*U*). (Scale bars: *A*, *C*, *M* and *O*, 15 μm; *B*, *D*, *F*, *H*, *N*, *P*, *R*, and *T*, 5 μm; *E*, *G*, *I*, *J*, *K*, *L*, *Q*, *S*, *U*, *V*, *W*, and *X*, 30 μm.) gc, glans clitoris; gm, glans mesenchyme; gp, glans penis; m, mesenchyme; p, prepuce; pm, prepuce mesenchyme; u, urethra.

distribution in embryos 24 h later. EB treatment dramatically reduced the number of AR-positive cells in the glans penis of male pups but had no obvious affect on AR in the preputial mesenchyme (Fig. 1*J*). Testosterone treatment induced high levels of nuclear AR in female external genitalia, with the strongest effect seen in the clitoral glans and prepuce of P1 pups (Fig.1 *L* and Fig. S2*D*). We also compared *Ar* mRNA levels in prenatal (E15.5) and neonatal (P1) stages. At both stages, *Ar* mRNA levels in males were approximately threefold higher than in females. Testosterone treatment significantly increased female *Ar* mRNA to levels that approached those of control males, whereas EB treatment decreased *Ar* expression in males to levels similar to those of control females (Fig. S1 *E* and *F*).

To determine the spatial and temporal responsiveness of the external genitalia to estrogen signaling, we first investigated ERα

localization in males and females before and during sexual differentiation. Like AR, ERα showed cytoplasmic localization at E13.5, and no differences were found between male and females (Fig. 1 *M–P*). At E15.5, ERα had undergone a marked reduction in male and female genital tubercles (Fig. 1 *Q–T*). In genital tubercles of males ERα expression is weaker than AR at E15.5 although female genital tubercles showed stronger ERα staining than males and most of the ERα protein was localized to the nucleus (Fig. 1 *Q–T*). At P1, the penis showed relatively few ERα-positive cells in the urethra and preputial mesenchyme (Fig. 1*U*), and the protein localized predominantly to cytoplasm (Fig. S2*F*). Female external genitalia at this stage showed extensive nuclear localization of ERα, particularly in the glans and the mesenchyme adjacent to the urethral epithelium (Fig. 1*W*).

To test whether sexually dimorphic expression of ERα in external genitalia of neonates is regulated by systemic estrogen and/or androgen, P0 mice were treated with either EB or T, and then ERα was examined after 24 h. EB treatment induced widespread ERα in the penis at P1 (Fig. 1*V*), and the protein was detected both in the nucleus and cytoplasm (Fig. S2*H*), indicating that EB treatment results in up-regulation and activation of ERα. Treatment of females with testosterone had the opposite effect on ERα expression in the clitoris, leading to a marked decrease 24 h after treatment (Fig. 1*X*). *Esr1* mRNA levels in males at E15.5 and P1 were significantly lower than in control females (Fig. S1 *E* and *F*). *Esr1* mRNA levels were approximately threefold lower in control males and in testosterone-treated females compared with control females at P1, and EB-treated males had similar *Esr1* mRNA levels as control females. Our observations that androgen-treated females show increased AR and decreased ERα, whereas estrogen-treated males show decreased AR and increased ERα, suggest that sex hormone-induced masculinization and feminization of the external genitalia reflect the relative levels of AR to ERα signaling in both sexes, as has been reported for sexually dimorphic digit proportions (30).

We also monitored expression of *Atf3*, a gene associated with hypospadias in humans and previously reported to have sexually dimorphic expression (31, 32). *Atf3* expression was not significantly different between males and females at prenatal stage E15.5, nor was it affected by testosterone or EB treatment at this stage (Fig. S1*E*). At P2, however, *Atf3* mRNA levels were approximately threefold lower both in control males and in testosterone-treated females compared with control females, and *Atf3* expression was increased in EB-treated males to levels significantly higher than in control females (Fig. S1*F*). These data suggest that *Atf3* becomes estrogen-responsive in neonatal external genitalia.

The Timing of AR Disruption Determines the Class of Congenital Penile Anomaly: Uncovering Temporal Windows of Sensitivity. The finding that sexually dimorphic patterns of AR and ERα during external genitalia development are influenced by the balance of androgen to estrogen at prenatal and postnatal stages led us to investigate whether there are specific temporal windows of sensitivity to antiandrogenic or estrogenic cues. To determine the relationship between the prenatal stage of anti-androgen exposure and the class of penile anomaly, we treated pregnant mice with flutamide (120 mg/kg), an AR antagonist, at specific time periods between E12.5 and birth and then examined the phenotypic outcomes after sexual maturation of the genitalia (P60). Treatment over any two consecutive days between E12.5 and E16.5 (i.e., E12.5–E13.5, E13.5–E14.5, E14.5–E15.5, or E15.5–E16.5) resulted in varying degrees of hypospadias with chordee, in which urethral tube closure was incomplete and the ventral side of the penis was curved and tethered to the skin by the urethral epithelium (Table S1 and compare Fig. 2 *A–D* with Fig. 2 *E* and *F*). The most severe hypospadias with chordee occurred after treatment at E14.5–E15.5 (Fig. 2 *E* and *F*). Treatment at E17.5 and E18.5 had little

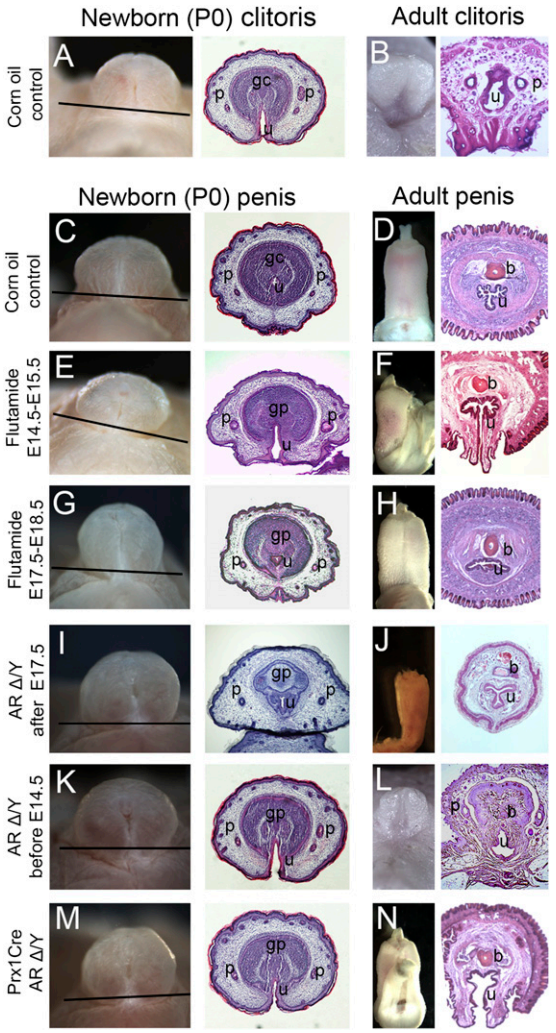

Fig. 2. Modulation of androgen signaling at different stages can produce a spectrum of congenital penile anomalies. (*A–D*) Control female (*A* and *B*; $n = 8$ for adults and $n = 10$ for newborns) and male (*C* and *D*; $n = 9$ for adults and $n = 12$ for newborns) external genitalia in newborn (*A* and *C*) and adult (*B* and *D*) mice. (*E* and *F*) Penises of mice treated with flutamide from E14.5 to E15.5 showing hypospadias with chordee ($n = 7$ for adults and $n = 9$ for newborns). (*G and H*) Penises of mice treated with flutamide from E17.5 to E18.5 ($n = 8$ for adults and $n = 9$ for newborns). (*I and J*) Micropenis in mice with conditional deletion of AR after E17.5 using *RosaCreErt2* ($n = 6$ for adults and $n = 8$ for newborns). (*K and L*) Male-to-female sex reversal of external genitalia in mice with deletion of AR before E14.5 ($n = 5$ for adults and $n = 6$ for newborns). (*M and N*) Hypospadias and chordee with a frenulum in penises of mice with deletion of AR using *Prx1Cre* ($n = 4$ for adults and $n = 6$ for newborns). Adult penises (*D, F, H, J, I,* and *N*) were dissected out from the prepuce; control female (*B*) and sex-reversed male (*L*) show clitoris together with prepuce. *F* and *J* are lateral views. Lines indicate plane of section. b, baculum; c, clitoris. Other labels are as described in Fig. 1.

affect (Fig. 2 *G* and *H*), indicating that this period is beyond the window of sensitivity for anti-androgen induction of hypospadias or other major penile anomalies.

Having identified the prenatal period during which the external genitalia are responsive to AR disruption, we then took a genetic approach to refine the temporal roles of AR. A floxed allele of AR was inactivated at specific time points using the tamoxifen-inducible *RosaCreErt2* allele (*Methods*). Genetic deletion of AR by tamoxifen administration at E17.5 resulted in micropenis but did not affect urethral tube closure (Fig. 2 *I* and *J*), consistent with the results of flutamide treatment after E16.5. When AR was

deleted by tamoxifen administration 4 d earlier, at E13.5, external genitalia were sex-reversed, showing a phenotype similar to that found in *tfm* mice (Fig. 2 *K* and *L*) (33) and human complete androgen insensitivity syndrome (34). Mutant genitalia showed an open urethral plate and a rudimentary phallus that was indistinguishable from a control female clitoris (compare Fig. 2 *K* and *L* with Fig. 2 *A* and *B*). Taken together, these results show that the stage of disruption of AR activity determines the class of penile malformation.

Cell Type-Specific Functions of AR in the Genital Tubercle. After determining the stage-specific functions of AR, we next investigated the roles of AR in specific cell populations of the genital tubercle. A series of tissue-specific deletions of AR were performed using different *Cre* deleter alleles. Deletion of AR in urethral epithelial cells using *ShhGfpCre* did not affect penis development at either prenatal or postnatal stages (Fig. S3). Deletion of AR in male genital tubercle mesenchyme using *Dermo1Cre* led to ambiguous genitalia that were completely sex-reversed (Fig. S4*B*). When we used the *Prx1Cre* deleter allele to remove AR from genital tubercle mesenchyme, males developed hypospadias with chordee, mimicking the effect of flutamide treatment between E13.5 and E16.5 (Fig. 2 *M* and *N* and Fig. S4*D*).

We hypothesized that the different types of penile malformation induced by the two mesenchymal *Cre* deleter alleles (*Dermo1Cre* and *Prx1Cre*) reflected different levels of AR inactivation in the mutant genital tubercles. Therefore, we quantified AR-positive cells in the genitalia of controls and AR conditional mutants generated with each *Cre* allele. Deletion of AR using *Dermo1Cre* led to more than 80% reduction of AR-positive cells and resulted in complete sex reversal of the external genitalia (Fig. S4 *A* and *B*). Deletion of AR using *Prx1Cre* led to a 50% reduction of AR-positive cells (Fig. S4*C*) and resulted in hypospadias and chordee, with a frenulum tethering the penis to the body wall (Fig. 2 *M* and *N* and Fig. S4*D*). These results indicate that androgen signaling through AR regulates masculinization of the external genitalia in a dose-dependent manner.

Identification of an Estrogen-Responsive Phase of Penile Development. We next asked whether estrogen plays a role in development of the penis and, if so, whether the developing penis has a similar temporal window of sensitivity to estrogen signaling. Inhibition of estrogen receptor function by the ER antagonist fulvestrant (1 mg/kg) or augmentation of ER signaling by treatment with EB, between E14.5 and E16.5 (the window when antagonism of AR is known to cause hypospadias), had little affect on penis size of P21 or adult mice (Fig. 3*C* and Fig. S5 *A* and *B*). Although prenatal modulation of estrogen activity did not alter penis development, neonatal male mice treated with EB (every other day from P0 to P6) showed marked reduction of penis development and growth, which resulted in micropenis (Fig. 3*D*). Neonatal males treated with EB bore a striking similarity to males in which AR was conditionally deleted after E17.5 (compare Fig. 3*D* with Fig. 2*J*). By contrast, when EB or fulvestrant was given to males at puberty (P21–P30), there was little affect on adult penile morphology (Fig. S5 *C* and *D*). Finally, we investigated the effect of antagonizing AR during the prenatal window of sensitivity (E14.5–E16.5) and augmenting ER activity with EB during the neonatal period of sensitivity. Males undergoing this biphasic modulation of AR and ER developed ambiguous genitalia, often showing complete male-to-female sex reversal of the external genitalia (Fig. 3*E*), similar to the effects of AR deletion before E14.5 (Fig. 2*L*). When developing females with normal estrogen activity were treated with T during the prenatal (E14.5–E16.5) and neonatal (every other day from P0 to P6) windows of sensitivity, females developed a penis (Fig. 3*F*).

AR Masculinizes External Genitalia Through Stage-Specific Regulation of Apoptosis and Proliferation. Our findings that penile malformations can be induced by transient exposure to an anti-androgen at E14.5–E15.5 and that AR and ERα undergo translocation to the nucleus at E15.5 suggest that masculinization of the male GT is initiated around E15. Moreover, the observation that inhibition of AR during this period causes chordee with a persistent urethral epithelial frenulum raised the possibility that programmed cell death of the urethral epithelium may be required for normal masculinization of the penis. To test this hypothesis, we compared patterns of cell death in the genital tubercles of control males, control females, AR conditional mutant males (*Dermo1Cre;ARΔ*, which deletes AR in genital tubercle mesenchyme), and EB-treated males at E15.5 and E17.5. The apoptotic stain LysoTracker red revealed that control males have higher levels of apoptosis in the ventral ectoderm overlying the urethral plate compared with control females, which have a more localized domain of apoptotic cells within the proximal urethral plate (Fig. 4 *A* and *B*). Males lacking AR exhibit marked reduction of apoptosis in the ventral genital ectoderm (Fig. 4*C*); however, WT males treated with EB retain the WT male pattern of apoptosis (Fig. 4*D*). Thus, disruption of AR during the prenatal window of sensitivity induces a feminized pattern of cell death in the urethral plate and ventral ectoderm adjacent to urethral plate, but addition of EB during this period did not affect the normal domain of apoptosis in males. The male proximal urethral plate normally separates from the ventral ectoderm by apoptosis at the ectoderm–endoderm junction between E15.5 and E16.5, and the adjacent mesenchyme fills the space previously occupied by the ventral urethral plate (Fig. 4*A*). The reduction of cell death at the junction between the ventral ectoderm and urethral endoderm of *Dermo1Cre; ARΔ* mutants results in persistence of the ventral urethral seam

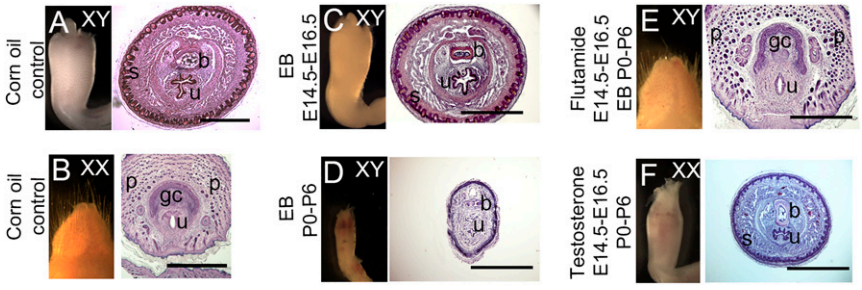

Fig. 3. Penile anomalies caused by modulation of estrogen signaling. For each panel, the whole mount is on the *Left* and the transverse section through the phallus is on the *Right*. (*A and B*) Shown are 21-d-old control male (*A*, $n = 7$) and female (*B*, $n = 6$) external genitalia. (*C*) Prenatal EB treatment had little affect on penis development ($n = 8$). (*D*) Neonatal EB treatment led to micropenis ($n = 6$). (*E*) Prenatal flutamide followed by neonatal EB treatment caused male-to-female sex reversal of external genitalia (compare *E* with control female in *B*; $n = 6$). (*F*) Testosterone treatment at both prenatal and neonatal stages caused female-to-male sex reversal of external genitalia ($n = 5$). (Scale bars: 400 μm.) s, spines. Other labels are as described in Figs.1 and 2.

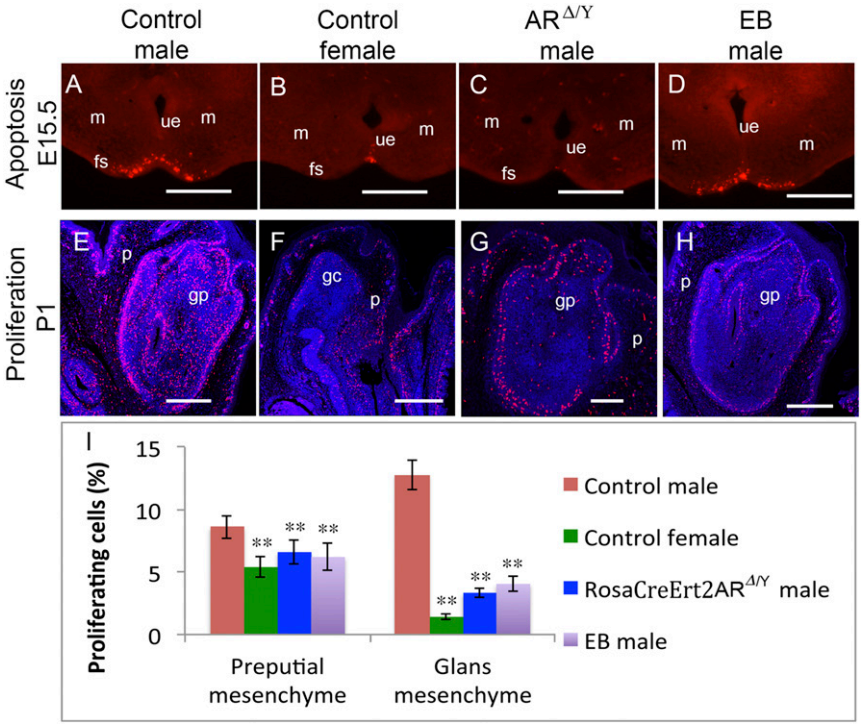

Fig. 4. Androgen and estrogen signaling controls cell death and proliferation in developing penis. (*A–D*) Transverse sections through E15.5 external genitalia showing cell death detected with LysoTracker Red. Strong apoptosis is detected in the ventral epithelium at the junction with the urethral plate in control males (*A*) and EB-treated males (*D*). Comparatively few apoptotic cells are detected in this region in control females (*B*) and *Dermo1Cre; AR*$^{\Delta/Y}$ males (*C*). Sample size: $n = 5$ in *Dermo1Cre*; AR$^{\Delta/Y}$ males, and $n = 6$ in others. (*E–I*) BrdU labeling shows cell proliferation in P1 mice. (*E*) Control male. (*F*) Control female. (*G*) *RosaCreErt2;* AR$^{\Delta/Y}$ male with tamoxifen injection at E17.5. (*H*) Estradiol benzoate-treated male; EB injection at P0. (*I*) Mitotic indices showing cell proliferation differences among male, female, *RosaCreErt2* AR$^{\Delta/Y}$ male, and EB-treated male groups. Sample size: $n = 4$ in *RosaCreErt2* AR$^{\Delta/Y}$ males, and $n = 6$ in others. For each sample, three central slides and a total of eight counting boxes (four in glans and four in prepuce) per slide were used to calculate mitotic indices. Error bars show SEM; $^{**}P < 0.01$. (Scale bars: *A, B, C,* and *D*, 30 μm; *E, F, G,* and *H*, 100 μm.) fs, foreskin; ue, urethral epithelium. Other labels are as described in previous figures.

(Fig. 4*C*), which may explain the presence of a frenulum-like tether and the failure of urethral internalization.

To test for sexually dimorphic patterns of cell proliferation in the genital tubercle, we calculated the mitotic indices of males and females using BrdU labeling. At E16.5, control male genital tubercles showed significantly higher levels of cell proliferation in proximal, middle, and distal regions compared with control females (Fig. S6). Our discovery that penis size could be markedly reduced by either neonatal addition of estrogen or by late prenatal (after E17.5) deletion of AR (Figs. 2 *I* and *J* and 3D) raised the possibility that differential control of cell proliferation by AR and ERα at perinatal stages may play a role in sexually dimorphic development of external genitalia. To test this hypothesis, we calculated the mitotic indices of the developing genitalia in normal males and females, in males with conditional deletion of AR, and in males exposed to EB after birth. Control females as well as males in which AR was inactivated in all cells at E17.5 by *RosaCreErt2* showed fewer BrdU-positive cells than did control males (Fig. 4 *E–H*). In preputial mesenchyme, females had 42% fewer proliferating cells than did males ($P = 0.004$) (Fig. 4 *E, F,* and *I*). In glans mesenchyme, females had 8.8 times less proliferation compared with males ($P < 0.001$) (Fig. 4 *E, F,* and *I*). After deletion of AR after E17.5, cell proliferation was significantly decreased in mutant males compared with control males ($P < 0.001$), resulting in a mitotic index similar to that of control females ($P = 0.085$) (Fig. 4 *G* and *I*). Neonatal exposure to estrogen significantly reduced proliferation in male glans mesenchyme ($P = 0.007$), similar to the effect of late prenatal deletion of AR (Fig. 4 *H* and *I*). Taken together, these results suggest that AR determines growth of the neonatal penis by controlling mesenchymal cell proliferation and promotes internalization of the urethra by regulating cell death. The finding that mesenchymal cell proliferation can be diminished by either exposure to estrogen or deletion of AR highlights the importance of the balance between androgen and estrogen signaling during penile development.

AR and ERα Regulate Sex-Specific Gene Expression in External Genitalia. Based on the discovery that androgen and estrogen control sexual differentiation of external genitalia by regulating urethral epithelial apoptosis in the embryo and mesenchymal cell proliferation in the neonate and the observation that male and female genitalia show differences in AR and ERα activity at these two stages, we tested the hypothesis that the gene network controlling early genital development may become differentially regulated in males and females during sexual differentiation. Given that the Hedgehog, bone morphogenetic protein (Bmp), Wnt, and fibroblast growth factor (Fgf) signaling pathways play key roles in genital development and that mutations of genes in these pathways result in malformations of external genitalia (13, 35–41), we tested whether AR or ERα signaling regulates the expression levels of genes in these pathways. Quantification of the transcript levels of 88 genes in males treated with flutamide and in control males at E15.5 identified 22 genes with significant responses to flutamide (Fig. 5*A*). Antagonism of AR with flutamide significantly decreased the expression levels of *Bmp5, Btrc, Crim1, Cep76, Ctnna1, Fgf9, Fgfr2, Fgfr3, Npc1, Npc1l1, Numb, Ihh, Gli1, Ptch1, Gsk3b, Ktcd11, Lrp2, Siah1a, Stk36*, and *Wnt2b* but increased expression of *Bmp2 and Wnt10a* (Fig. 5*A*).

Next, we asked whether the same genes that respond to flutamide at prenatal stages also respond to estrogen treatment during the neonatal period. Quantification of the expression of the same 88 genes in EB-treated and control penises at P2 identified 12 genes with significant responses to estrogen exposure (Fig. 5*B*). Treatment of males with EB increased the relative expression of *Bmp8b, Dkk2, Hhip*, and *Stk3* but decreased the expression of *Frmd6, Grem1, Ihh, Ptch2, Stk36*, and several Wnt genes, including *Wnt2, Wnt4, and Wnt7b.* Thus, whereas many of the same pathways were targeted by antiandrogenic and estrogenic treatments, only two genes, *Ihh* and *Stk36*, showed similar responses to flutamide and EB (Fig. 5 *A* and *B*).

Given the central role of *Shh* in genital development, we were particularly interested in the discovery that EB treatment

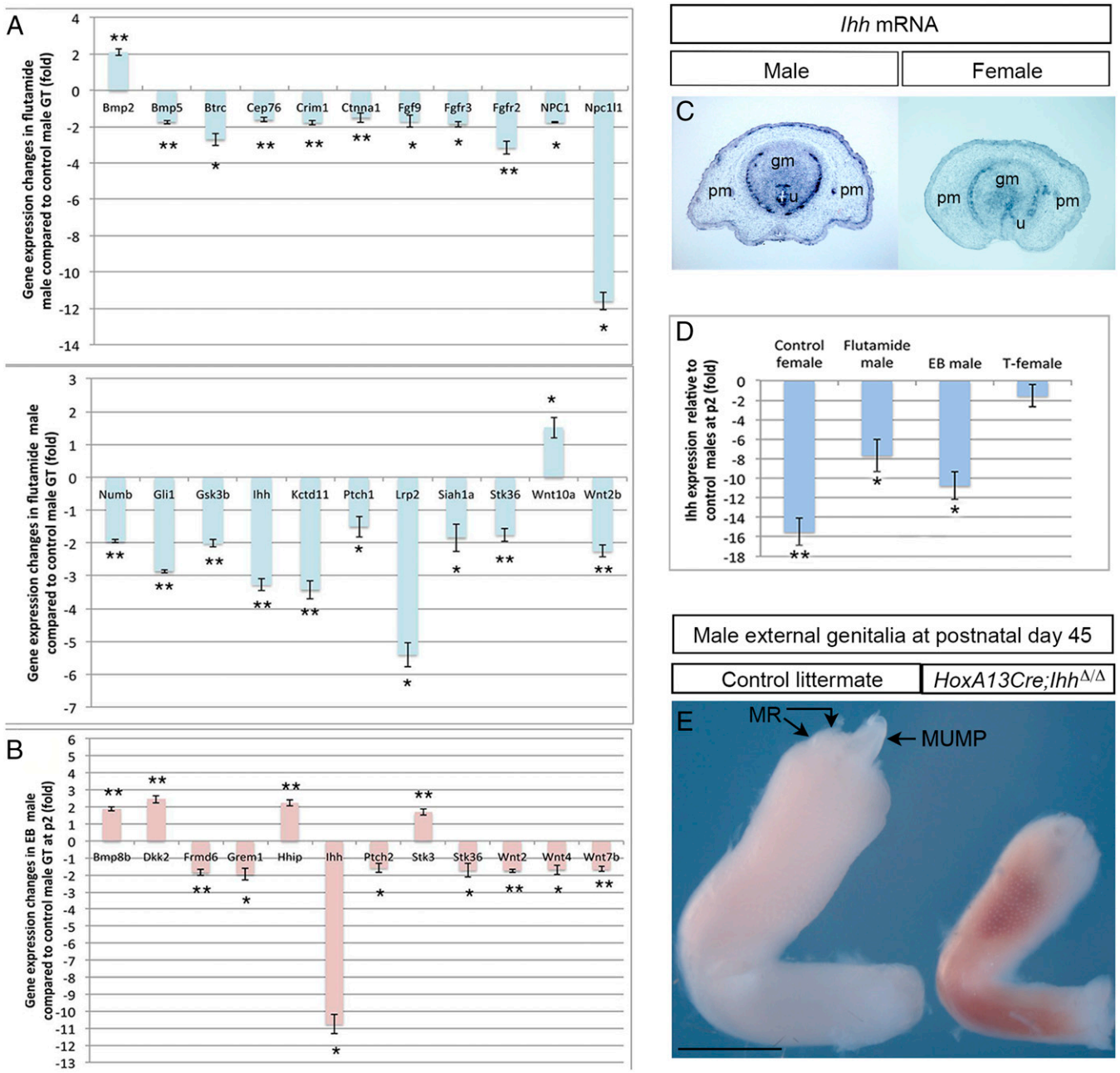

Fig. 5. Transcriptional targets of androgen and estrogen signaling and function of *Ihh* in the developing penis. (*A*) Relative transcript levels (*y* axis shows fold change) in E15.5 flutamide-treated male genital tubercles compared with control males. Control is assigned a value of 0. Twenty-two of 88 genes assayed show statistically significant changes of >1.5-fold. (*B*) Relative transcript levels (fold change) in P2 EB-treated male penises compared with control males. Twelve of 88 genes assayed show statistically significant differences of >1.5-fold ($P < 0.05$). Error bars show ±SEM, and asterisks denote significant differences (*$P \leq 0.05$, **$P \leq 0.01$). (*C*) Section in situ hybridizations showing *Ihh expression* in male (*Left*) and female (*Right*) external genitalia at E17. (*D*) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis shows that levels of *Ihh* are significantly decreased in control females, flutamide-treated males, and EB-treated males compared with control males at P2 (treatments occurred at P0 and P1, and RNA was collected at P2). Females treated with testosterone at P0 and P1 and assayed at P2 show levels of *Ihh* expression similar to P2 control males. The *y* axis shows fold changes relative to control males, which is set at zero. (*E*) Penises of *Hoxa13Cre;Ihh*$^{\Delta/\Delta}$ conditional mutant (right) and littermate control (left) males at stage P45. Note that deletion of *Ihh* causes demasculinization of the penis, characterized by reduced penis size and reduction of the MUMP and MUMP ridge at the tip of the glans. (Scale bar: 1 mm.) MR, MUMP ridge. Other labels are as described in previous figures.

decreased expression of *Ihh,* a *Shh* paralog, in P2 male genitalia by more than 10-fold (Fig. 5*B*). This finding led us to examine the spatial pattern of *Ihh* expression in male and female genital tubercles. *Ihh* was expressed in urethral, preputial, and glans epithelial cells of both male and female genitalia at E17.5 although staining seemed to be stronger in males (Fig. 5*C*). Quantitative analysis confirmed that *Ihh* mRNA levels were significantly lower (13.5-fold) in female relative to male genitalia at P2 (Fig. 5*D*). Furthermore, significant reductions in *Ihh* expression occurred in flutamide-treated males (7.68-fold) and EB-treated males (10.76-fold) at P2 (Fig. 5*D*). Reciprocally, in external genitalia of testosterone-treated females, *Ihh* expression increased to a level not significantly different from the mRNA level found in the penises of control males (Fig. 5*D*). These results suggested that *Ihh* is downstream (and could be a mediator) of androgen and estrogen signaling during sexual differentiation of the external genitalia. To test whether *Ihh* plays a role in masculinization of the penis, we knocked out *Ihh* in the mouse genital tubercle using *Hoxa13Cre*, which shows widespread expression in the external genitalia, including the urethral epithelium (42). Conditional deletion of *Ihh* by *Hoxa13Cre* led to underdevelopment of the penis and reduction of male-specific features, including the MUMP and the MUMP ridge, indicating an essential role for IHH in masculinization of mouse external genitalia (Fig. 5*E*).

Discussion

Our results show that disruption of androgen and estrogen signaling at specific developmental time points and in specific cell populations can produce a broad spectrum of CPAs, including ambiguous genitalia, micropenis, hypospadias, and chordee. The experiments presented here identify two critical windows of sensitivity during normal penile development, a prenatal window that is controlled by AR and a neonatal window that is controlled by balance between androgen and estrogen signaling (Fig. 6*A*). Normal female external genital development depends on higher estrogen relative to androgen signaling during both the prenatal and postnatal phases (Fig. 6*B*). The critical prenatal phase of androgen activity occurs over E13.5–E16.5, and disruption of androgen signaling during this period causes hypospadias and chordee (Fig. 6*C*). Disruption of androgen signaling alone during the early phase (~E15.5) down-regulates genes in the Hh and Fgf pathways and perturbs normal apoptosis in the ventral ectoderm–endoderm connection region, which results in hypospadias and chordee (Fig. 6*C*).

The second (neonatal) phase of responsiveness occurs after birth in mice and involves both androgen and estrogen signaling. During this neonatal window of sensitivity, either disruption of androgen signaling or augmentation of estrogen signaling can result in micropenis (Fig. 6*D*). During this second phase, disruption of AR or activation of ERα signaling causes down-regulation of Ihh and Wnt signaling and reduction of cell proliferation (Fig. 6*D*). If sex steroid activity is modulated during both the prenatal and the neonatal phases, either by disruption of androgen signaling at both stages or by diminished AR activity prenatally and augmentation of ERα postnatally, then the external genitalia undergo sex reversal and males develop with ambiguous genitalia (Fig. 6*E*). Thus, tight spatial and temporal regulation of AR and ERα signaling is essential for normal development of male and female external genitalia, and this sensitivity is reflected by the ability of exogenous or genetic perturbations to cause different types of congenital defects at different stages of development.

Previous studies have shown that prenatal exposure to antiandrogens, such as flutamide, can cause hypospadias in male rodents and that AR mutant mice exhibit sex reversal (33). For example, Miyagawa et al. exposed mice to flutamide and reported that the critical window for masculinization of the GT by AR is from E15.5 to E16.5; however, phenotypes were not examined beyond P0 (25). Our analysis of the temporal roles and cellular specificity of AR and ERα signaling was broader in scope, combined pharmacologic and genetic disruptions, and used a finer grained phenotypic analysis than previous studies. This approach uncovered a series of temporal windows during which androgen and estrogen act on different aspects of sexual differentiation and revealed that a wide range of disorders of sexual differentiation of external genitalia can be induced simply by varying the stage of AR disruption or ER activation.

One of the first signs of sexual differentiation of the external genitalia occurs at E15.5, when a sexually dimorphic pattern of apoptosis in the ventral ectoderm–endoderm junction separates the urethral epithelium from the foreskin, allowing internalization of the urethra within the penis. Deletion of AR in genital tubercle mesenchyme caused male embryos to develop a feminized pattern of apoptosis, which likely contributed to development of hypospadias. By contrast, conditional deletion of AR in urethral epithelial cells had no affect on genital development or sexual differentiation, suggesting that cell death at the ventral ectoderm–endoderm junction may be mediated via the adjacent mesenchyme rather than by a cell autonomous response of urethral endodermal or ventral ectodermal cells to AR activity. Our hypothesis that AR acts primarily on genital mesenchyme and that a relay mechanism mediates its effect on adjacent epithelia is supported by evidence that androgen signaling regulates expression of genes in the Fgf, Bmp, and Hh pathways, which are known to control apoptosis during early development of the genital tubercle (35, 37, 39, 40, 43).

Hedgehog signaling is required for patterning and outgrowth of the genital tubercle at early stages and for sexual differentiation at later stages of development (36, 37, 43). Deletion of *Shh* at E10.5 results in agenesis of external genitalia, and deletion of *Shh* after E13.5 cause hypospadias (36). *Shh* is androgen-responsive in other developmental contexts, and, in the genital tubercle, *Shh* is restricted to the urethral epithelium; however, deletion of AR in urethral epithelial cells has no effect on genital patterning. The finding that AR is not required in the urethral endoderm was unexpected, but we found that a second hedgehog gene, *Ihh*, is expressed in the urethral epithelium and mesenchyme, is androgen-responsive, and is required for normal penile development. The discovery that *Ihh* expression is significantly reduced in male genitalia after either prenatal exposure to flutamide or neonatal exposure to estrogen suggests that *Ihh* is a downstream target of both AR and ERα. Conditional deletion of *Ihh* using *Hoxa13Cre* results in a marked reduction of penile size and of male-specific penile features, including the MUMP and MUMP ridge, demonstrating that *Ihh* is required for normal sexual differentiation of the penis. These findings highlight a previously unknown role for IHH in hormone-dependent sexual differentiation of male external genitalia.

Fgf signaling also is important in genital development and sexual differentiation (40, 44–46). Our experiments revealed that *Fgf9*, *Fgfr2*, and *Fgfr3* expression is diminished by prenatal exposure to flutamide, but *Fgf9* and *Fgfr2* show no response to neonatal exposure to estrogen. We found similar hormone-specific responses of Wnt and Bmp pathway genes. These findings suggest that, although antagonism of AR and agonism of the ER can have similar phenotypic effects on male genitalia, the target genes that respond to these receptors are only partially overlapping.

Mice are emerging as a powerful system for modeling human genitourinary defects, although there are notable differences in tissue morphogenesis and in signaling interactions that remain to be understood (47). For example, prenatal exposure to estrogen can feminize male external genitalia in humans (16, 48, 49) but has little effect in mice. Such differences likely reflect differences in gestational time because humans have a relatively long period of prenatal development compared with mice and, therefore, the neonatal phase of genital development in mice is comparable

Fig. 6. A spectrum of congenital penile anomalies can be generated by modulating AR and ER signaling during specific temporal windows of sensitivity. Active signaling pathways are indicated by black text; diminished signaling activity is indicated by gray text. Green boxes indicate increased activity, and red boxes indicate decreased activity. *A* and *C–E* are genetic males; *B* is genetic female. (*A*) In normal male mice, higher androgen (T)/AR relative to estrogen (E)/ERα signaling during the prenatal window of sensitivity up-regulates Hedgehog, Fgf, and Bmp signaling pathways and promotes cell death at the boundary of the urethral epithelium and ectodermal prepuce, producing a male genital tubercle (GT) with a tubular urethra. After birth, sustained T/AR signaling promotes cell proliferation through up-regulation of Hedgehog and Wnt pathway genes, resulting in growth of a normal penis. (*B*) In normal female mice, higher E/ERα relative to T/AR signaling during the prenatal window negatively regulates Hedgehog, Fgf, and Bmp pathway genes and reduces urethral cell death, producing a female genital tubercle. During the neonatal phase, sustained ERα activity down-regulates Hedgehog and Wnt signaling genes, inhibiting cell proliferation in the phallus and leading to formation of a normal clitoris. (*C*) Disruption of T/AR signaling at prenatal stages results in a feminized genital tubercle (GT), and, when followed by normal T/AR signaling at neonatal stages, males develop hypospadias, often with chordee. (*D*) Normal T/AR signaling at prenatal stages masculinizes the GT, but, when followed by either disrupted T/AR signaling or augmented E/ERα signaling at neonatal stages, males develop micropenis. (*E*) Disruption of T/AR signaling at prenatal stages feminizes the GT, and, when followed by sustained disruption of T/AR signaling or augmentation of E/ERα signaling at neonatal stages, males develop sex-reversed/ambiguous external genitalia.

with prenatal development in humans (47, 50). Although the penis and clitoris are well-differentiated in human neonates (51), penile and clitoral differentiation in the mouse occurs mainly during the postnatal period (52). Thus, the different effects of estrogen exposure on mice and humans could reflect species-specific differences in their developmental timing (i.e., mice are born at a relatively earlier stage of development) rather than species differences in the role of estrogen. In light of the developmental differences that exist at parturition, our finding that the relative activity of androgen and estrogen receptor signaling controls sexual differentiation during the neonatal phase of mice may be applicable to late prenatal stages of human development.

If the reported increase in the incidence of CPAs (such as hypospadias) over the past few decades is caused by a true increase in the numbers of affected children, rather than changes in reporting, then this observation would suggest involvement of factors other than mutations. Our findings identify molecular mechanisms by which CPAs can be induced by exogenous factors that modulate AR and ER activity. Taken together, the results presented here indicate that AR antagonism and ERα agonism, by either environmental or genetic mechanisms during specific developmental windows, can cause the complete range of congenital penile anomalies, including hypospadias, chordee, micropenis, and ambiguous genitalia, by modulating tissue-specific expression

of developmental control genes that regulate morphogenesis of male and female external genitalia. Environmental disruption of gene expression during these critical periods could explain the appearance of mutant phenotypes in individuals with normal genotypes.

Methods

Animals and Treatments. *AR^{flox}*, *Ihh^{flox}*, *Hoxa13Cre, and ShhGfpCre* mice were kindly provided by, respectively, Dr. Guido Verhoeven [Katholieke Universiteit Leuven, Leuven, Belgium (via Drs. Marvin Maestrich and Connie Wang, University of Texas M. D. Anderson Cancer Center, Houston)], Dr. Beate Lanske (Harvard School of Dental Medicine, Boston), Dr. Marie Kmita (Institut de Recherches Cliniques de Montréal, Montreal), and Dr. Brian Harfe (University of Florida, Gainesville). *RosaCreErt2*, *Prx1Cre*, and *Dermo1Cre* mice were purchased from The Jackson Laboratory. Mice were housed in a specific pathogen-free barrier facility on 12-h light/dark cycles with access to food and water ad libitum, and all experiments were conducted in accordance with University of Florida Institutional Animal Care and Use Committee protocol 201203399. Tamoxifen was administered by oral gavage (80 mg/kg in corn oil) to activate CreErt2-mediated recombination of floxed alleles, and control experiments confirmed that this dose of tamoxifen does not cause malformation of male genitalia (Fig. S7). Hormone and receptor agonist and antagonist treatments were performed as described previously (30). In brief, flutamide (120 mg/kg), fulvestrant (1 mg/kg), methyltestosterone (2 mg/kg), or estradiol benzoate (300 μg/kg) were dissolved in ethanol and diluted in corn oil. Treatments and corn oil vehicle controls [corn oil with 2% (vol/vol) ethanol] were administered by oral gavage (flutamide and methyltestosterone) or i.p. injection (estradiol benzoate) of pregnant females. For treatments of neonates, chemicals were further diluted 1:5 with corn oil and delivered by i.p. injection. Treatment days and sample sizes are provided in figure legends. The effect of treatment on embryos was validated by measurements of anogenital distance as described (30).

In Situ Hybridization, Cell Death, and Cell Proliferation Assays. RNA whole mount in situ hybridization and LysoTracker Red staining (Molecular Probes) were performed as described previously (38). Mouse *Ar* and *Esr1* probes were made from 500-bp PCR products amplified from 3′ cDNAs and cloned into pGEM-T vectors. LysoTracker Red-labeled genital tubercles were maintained in darkness and embedded in paraffin, and 10-μm sections were cut, analyzed, and photographed on a Leica DMRE compound fluorescence microscope. For cell proliferation analysis, BrdU (100 mg/kg) was injected i.p. into $AR^{\Delta/Y}$ mice or CD1 mice 24 h after administration of steroids or steroid receptor inhibitors, and external genitalia were collected 4 h later for immunohistochemistry.

Immunohistochemistry. Immunohistochemistry was performed using anti-AR (sc-816) and anti-ERα (sc-542) primary antibodies (Santa Cruz) diluted 1:200, and anti-BrdU (G3G4) diluted 1:10; sections were then incubated overnight at 4 °C. AR and ERα antibodies were detected using the TSA kit (Invitrogen) according to the manufacturer's protocol, BrdU antibody was detected using goat anti-mouse Alexa Fluor 488 (Invitrogen), and all slides were visualized on a Leica TSM Sp5 Confocal Microscope. BrdU-positive cells and total cells (visualized by DAPI) were counted in sections through each penis using five counting boxes per section and six sections from the midpoint of each sample ($n = 6$).

Quantitative RT-PCR. Quantitative RT-PCR was modified from previously described methods (30, 38), and CD1 mouse penises were dissected from E15.5 and P2 mice and were pooled by treatment. Pathway-specific gene expression was determined using the Hedgehog PCR Array (PAMM-078Z; Qiagen) and the 7900 HT Fast Real-time PCR system (Applied Biosystems) according to the manufacturer's protocol. Sequences of real-time PCR primers for *Ar, Esr1*, *Ihh,* and the control gene *Actb* were previously published (30, 38, 53, 54). *Atf3* primers (forward, GGCAGGAAGAGCCAAAGATAA; reverse, GTGCCATT AACATCCCACAATG) were designed from National Center for Biotechnology Information reference sequence NM_007498.3 using PrimerQuest software (Integrated DNA Technologies).

Statistical Analysis. For each treatment and control group, pairwise comparisons were carried out using two-tailed Student's *t* tests with α-level set at 0.05 to detect significant differences in dependent variables.

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