Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFI in mice

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The sexual differentiation paradigm contends that the female pattern of the reproductive system is established by default because the male reproductive tracts (Wolffian ducts) in the female degenerate owing to a lack of androgen. Here, we discovered that female mouse embryos lacking Coup-tfII (chicken ovalbumin upstream promoter transcription factor II) in the Wolffian duct mesenchyme became intersex—possessing both female and male reproductive tracts. Retention of Wolffian ducts was not caused by ectopic androgen production or action. Instead, enhanced phosphorylated extracellular signal-regulated kinase signaling in Wolffian duct epithelium was responsible for the retention of male structures in an androgen-independent manner. We thus suggest that elimination of Wolffian ducts in female embryos is actively promoted by COUP-TFI, which suppresses a mesenchyme-epithelium cross-talk responsible for Wolffian duct maintenance.

Sexually dimorphic establishment of reproductive tracts epitomizes the anatomical difference between males and females. This dimorphic establishment depends on two concurrent events during embryogenesis: regression of one of the two primitive ducts (Müllerian and Wolffian ducts) and maintenance of the other. These two events ensure that the embryo retains only one reproductive tract that corresponds to its sex: Müllerian duct for the XX individual and Wolffian duct for the XY individual (1). In the 1950s, Alfred Jost provided the first evidence for what became the foundation of the sexual differentiation paradigm: XY embryos retain Wolffian ducts through the action of testis-derived androgen, whereas XX embryos lose Wolffian ducts as a result of a lack of androgens (2–5).

The action of androgen on the Wolffian duct is mediated through androgen receptors in the mesenchyme surrounding Wolffian ducts (6, 7). It is well established that mesenchyme-derived factors govern the fate and differentiation of ductal epithelium (8). The orphan nuclear receptor COUP-TFI (chicken ovalbumin upstream promoter transcription factor II, or NR2F2) is a mesenchyme-specific regulator in many developing organs, including the mesonephros, where Wolffian ducts develop (9). COUP-TFI expression in Wolffian duct mesenchyme overlapped with Wilms’ Tumor 1 (WT1) (fig. S1A), another mesenchyme-specific transcriptional factor (10).

To investigate the role of COUP-TFI in Wolffian duct regression, we used the tamoxifen-inducible Wt1CreERT2 mouse model that targeted Coup-tfII deletion in Wt1+ mesenchymal cells (fig. S1B). In the control (Wt1CreERT2; Coup-tfII+/−) female, COUP-TFI remained in the mesenchymal cells of mesonephros from embryonic day 12.5 (E12.5) to E16.5 (fig. S1, A to E) and completion (E16.5) of Wolffian duct regression in XX embryos (fig. S2A). In the knockout (Wt1CreERT2; Coup-tfII−/−) female, COUP-TFI ablation began 24 hours after the first tamoxifen injection (fig. S1F) and was completed by E14.5 (fig. S1, G and H). Ablation of Coup-tfII was further confirmed with reverse transcription polymerase chain reaction (RT-PCR) (fig. S2B). These results demonstrated an efficient ablation of Coup-tfII in WT1-positive Wolffian duct mesenchyme in XX embryos.

The impact of Coup-tfII ablation on XX mesonephros was first examined at E18.5, when dimorphic development of reproductive tracts is completed. The control XX embryos contained only Müllerian ducts that were visualized by immunostaining of the epithelial marker Pax2 (Fig. 1A). Other control genotypes that include Wt1CreERT2; Coup-tfII+/−, Coup-tfII+/−/−, and Coup-tfII−/− female embryos also developed normally, with only Müllerian ducts (fig. S3A). Knockout XX littersmates, however, had both Müllerian and Wolffian ducts in the mesonephros (Fig. 1D).

The identity of the Wolffian duct was confirmed by the presence of Wolffian duct epithelium marker transcription factor AP-2α (AP-2α) (Fig. 1, A and B) (9). Embryos in which Coup-tfII was knocked out died soon after birth. We therefore developed an organ culture system that allowed us to maintain E18.5 XX mesonephros with ovaries for 7 days to investigate whether Wolffian ducts remained present postnatally. At the end of culture, Wolffian ducts were still present in knockout tissues, along with the components of female reproductive tracts (Fig. 1, C and F), indicating that Wolffian duct maintenance in knockout XX persisted after birth and was not a transient event.

Retention of Wolffian ducts in the Coup-tfII knockout XX embryo points to a possible action of androgens based on the Jost paradigm (11). Wt1CreERT2 targets Coup-tfII deletion not only in mesonephros but also somatic cells of the ovary (12), raising the possibility that an ovary in which Coup-tfII has been knocked out could synthesize androgens ectopically. We compared the transcriptomes between control and knockout ovaries at E14.5 and E16.5, during which Wolffian duct regression occurs. The transcriptome of the knockout ovary was not different from the control ovary, with the exception of 10 differentially expressed genes (including Coup-tfII) (table S1). None of these genes were associated with androgen production. Furthermore, mRNA expression of two rate-limiting enzymes—hydroxy-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 1 (Hsd3b1) and cytochrome P450 17A1 (Cyp17a1)—for steroidogenesis was not different between control and knockout ovaries and nearly undetectable compared with the wild-type fetal testis (a positive control) (Fig. 2, A and B). A lack of androgen-producing capacity in the ovary was corroborated with unchanged anogenital distance (AGD), an androgen-sensitive parameter, between control and knockout XX at E18.5 (Fig. 2C). To exclude the possibility that androgens came from other resources in the knockout embryo, we removed the mesonephros from XX embryos and cultured them for 4 days. After culture, Wolffian ducts regressed in control XX mesonephros, as expected, whereas in knockout XX, either in the presence or absence of ovaries, Wolffian ducts were maintained (Fig. 2D and fig. S3B). These results indicate a lack of androgen production in the XX knockout ovaries and led us to speculate that Wolffian duct retention in XX embryos could be the result of ectopic activation of the androgen pathway in the absence of Coup-tfII. This possibility was excluded based on the finding that expression of androgen receptor (Ar) and two androgen-induced genes—folate hydrolase 1 (Folh1) and solute carrier family 26 member 3 (Slc26a3) (33, 14)—was not different between control and knockout XX mesonephros (Fig. 2, E to G). To rule out the involvement of androgens, we exposed the dam that carried control and knockout embryos to the androgen antagonist flutamide (Fig. 2H) (15). This regimen was sufficient to prevent Wolffian duct maintenance resulting from ectopic androgen action in XX embryos (16). Despite the verified action of flutamide (fig. S4), Wolffian ducts were still retained in knockout XX embryos (Fig. 2H). Thus, the maintenance of Wolffian ducts in the Coup-tfII knockout XX embryo is not due to ectopic production or action of androgens.

To identify the androgen-independent mechanism underlying Wolffian duct retention in the female in which Coup-tfII had been knocked out, we turned our attention to epidermal growth factor (EGF) and fibroblast growth factor (FGF) signaling pathways for their putative ability to promote Wolffian duct maintenance (17, 18). We first examined by means of RT-PCR the expression of Egf and its receptor Egfr. Their expression...
Fig. 1. Coup-tfII ablation leads to Wolffian duct retention in the XX embryo. Control and Coup-tfII knockout reproductive systems from E18.5 XX embryos were analyzed with (A and D) whole-mount immunofluorescence for the epithelial marker PAX2 or (B and E) AP-2α on frozen sections. (C and F) Control and knockout mesonephroi from E18.5 XX embryos were cultured for 7 days and analyzed with bright field microscopy or (insets) AP-2α immunofluorescence on frozen sections. Blue arrows indicate Wolffian ducts, magenta arrows indicate Müllerian ducts, and asterisks indicate ovary. ov, oviduct; ut, uterus; va, vagina. Scales bars, (A), (C), (D), (F), 0.5 mm; (B), (C) inset, (E), and (F) inset, 50 μm. n = 14 embryos in (A); n = 3 embryos per each genotype in (B), (C), (E), (F), and n = 23 embryos in (D).

Fig. 2. Wolffian duct retention in Coup-tfII knockout XX embryo is independent of androgen production or action. (A and B) mRNA expression of two rate-limiting steroidogenic enzymes, Hsd3b1 and Cyp17a1, in E14.5 control testis (light blue), control ovary (light pink), and knockout ovary (dark pink). (C) AGD of the control and knockout embryos at E18.5. (D) PAX2 whole-mount immunofluorescence of ovaries and mesonephroi after 4-day culture. n = 7 embryos per genotype. (E to G) mRNA expression of androgen receptor Ar and androgen-responsive genes (Slc26a3 and Folh1) in control XY, control XX, and knockout XX mesonephroi. Results are shown as mean ± SEM. Asterisks in (A), (B), (C), (F), and (G) represent statistical significance of P < 0.05 compared with either control or knockout XX samples by means of one-way analysis of variance followed by Tukey’s test. [n = 8 embryos per each group in (A) and (B), n = 8 to 11 embryos in (C); and n = 8 embryos in (E) to (G)]. (H) Knockout XX embryos were exposed to either vehicle (oil) or androgen receptor antagonist flutamide in utero once daily from E12.5 to E17.5. Samples were collected at E18.5 and analyzed with PAX2 whole-mount immunofluorescence. n = 3 embryos per genotype. Blue arrows indicate Wolffian ducts, magenta arrows indicate Müllerian ducts, and white asterisk indicates ovary. Scale bar, (D) and (H), 0.5 mm.
was not different between control and knockout XX mesonephroi (Fig. 5A). Components of FGF signaling, in contrast, exhibited distinct changes in the absence of Coup-tfII. FGF2 is the major FGF receptor in the Wolffian duct epithelium (39), and its binding ligands include FGF1, -3, -7, -10, and -22 (20). mRNA expression of FGF receptors (Fgfr1, Fgfr2-b, and Fgfr2-c) and most ligands were unaltered (Fig. 5B and Fig. 5D) or undetectable (Fig. 5C and Fig. 5E) in knockout XX mesonephroi compared with the control (Fig. 3A). However, expression of Fgf7 and Fgf10 was increased significantly in knockout XX mesonephroi at E14.5 (Fig. 3A) and E16.5 (Fig. 3B). To investigate whether Fgf7 and Fgf10 were capable of reproducing the Wolffian duct maintenance phenotype in the wild-type female, we cultured E14.5 wild-type XX mesonephroi for 2 days in the presence of vehicle, Fgf7, Fgf10, or Fgf7+FGF10. In the vehicle-treated group, Wolffian ducts regressed after 2-day culture, similar to the in vivo situation. In contrast, presence of Fgf7, Fgf10, or Fgf7+FGF10 maintained the Wolffian duct in the wild-type XX mesonephroi (Fig. 3B).

FGF7 and FGF10 are expressed in the mesonephric mesenchyme, the same cellular compartment as Coup-tfII (21, 22). These FGFs bind FGR2 in Wolffian duct epithelium and activate two intracellular signaling components, phosphorylated protein kinase B (p-AKT) and phosphorylated extracellular signal-regulated kinase (p-ERK) (20). Loss of Coup-tfII did not change p-AKT activation in Wolffian ducts (fig. S5C). The presence of p-ERK, conversely, became detected in Wolffian duct epithelium of knockout XX compared with the control XX at E14.5 (Fig. 3C) and E16.5 (Fig. 3D). These results indicate that loss of mesenchymal Coup-tfII led to an enhanced activity of p-ERK signaling in Wolffian duct epithelium. We then tested whether elevated p-ERK signaling was the cause of Wolffian duct maintenance by culturing the Coup-tfII knockout mesonephroi with a p-ERK specific inhibitor PD0325901 (23). In the vehicle-treated group, Wolffian ducts in the knockout XX were maintained in culture. Conversely, the p-ERK inhibitor eliminated Wolffian ducts in the knockout XX mesonephroi (Fig. 3D), indicating that enhanced p-ERK signaling was involved in Wolffian duct retention in the Coup-

**Fig. 3.** Enhanced FGF signaling is involved in Wolffian duct retention in the absence of Coup-tfII. (A) mRNA expression of FGF receptors and ligands in the mesonephroi at E14.5. UD, undetected. Results are shown as mean ± SEM. Asterisks represent statistical significance of P < 0.05 compared with control females by using Student’s t test (n = 8 embryos for each genotype). (B) Whole-mount immunofluorescence of PAX2 of 2-day cultured E14.5 wild-type XX mesonephroi in the presence or absence of signaling, in contrast, exhibited distinct changes in the absence of Coup-tfII. FGF2 is the major FGF receptor in the Wolffian duct epithelium (39), and its binding ligands include FGF1, -3, -7, -10, and -22 (20). mRNA expression of FGF receptors (Fgfr1, Fgfr2-b, and Fgfr2-c) and most ligands were unaltered (Fig. 5B and Fig. 5D) or undetectable (Fig. 5C and Fig. 5E) in knockout XX mesonephroi compared with the control (Fig. 3A). However, expression of Fgf7 and Fgf10 was increased significantly in knockout XX mesonephroi at E14.5 (Fig. 3A) and E16.5 (Fig. 3B). To investigate whether Fgf7 and Fgf10 were capable of reproducing the Wolffian duct maintenance phenotype in the wild-type female, we cultured E14.5 wild-type XX mesonephroi for 2 days in the presence of vehicle, Fgf7, Fgf10, or Fgf7+FGF10. In the vehicle-treated group, Wolffian ducts regressed after 2-day culture, similar to the in vivo situation. In contrast, presence of Fgf7, Fgf10, or Fgf7+FGF10 maintained the Wolffian duct in the wild-type XX mesonephroi (Fig. 3B).

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We have shown that instead of a passive process occurring as a result of the absence of androgens, elimination of the male reproductive tract in the female embryo is actively promoted by Coup-tfII through its action in the Wolffian duct mesenchyme. Coup-tfII in the mesenchyme inhibits expression of FGFRs, which otherwise activate the p-ERK pathway in the Wolffian duct epithelium for its maintenance. The function of Coup-tfII in facilitating Wolffian duct elimination is not restricted to XX embryos; when the testis was removed from the Coup-tfII knockout XY mesonephroi, Wolffian ducts remained present despite a lack of androgens (fig. S6). These findings reveal unexpected mechanisms underlying the dimorphic development of the Wolffian ducts via Coup-tfII. In addition, maintenance of male reproductive tracts without androgens prompts a reassessment of the role of androgens in this process, which presumably is to antagonize the action of Coup-tfII.

**REFERENCES AND NOTES**

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SUPPLEMENTARY MATERIALS
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The makings of the reproductive tract
Every embryo, regardless of its sex, contains both male and female primitive reproductive tracts before sexual differentiation. To establish a sex-specific reproductive system, female embryos need to remove the components of male tracts. The general consensus contends that removal of the male tracts occurs by default, a passive outcome owing to a lack of testis-derived androgens. Working in mice, Zhao et al. discovered that this process instead was actively promoted by the transcription factor COUP-TFII (see the Perspective by Swain). Without the action of this factor, embryos retained male reproductive tracts, independently of androgen action. These findings unveil unexpected mechanisms underlying the sexually dimorphic establishment of reproductive tracts.

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