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Research Article

Prenatal low-dose methyltestosterone, but not dihydrotestosterone, treatment induces penile formation in female mice and guinea pigs†

Shanshan Wang, John Lawless and Zhengui Zheng*

Department of Physiology, School of Medicine, Southern Illinois University Carbondale, Carbondale, IL, USA

***Correspondence:** Department of Physiology, School of Medicine, Southern Illinois University Carbondale, 1135 Lincoln Drive, Life Sci III, Carbondale, IL 62901, USA. Tel: +16184538430; E-mail: zzheng57@siumed.edu

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Abstract

Genital tubercle has bisexual potential before sex differentiation. Females exposed to androgen during sex differentiation show masculinized external genitalia, but the effects of different androgens on tubular urethral and penile formation in females are mostly unknown. In this study, we compared the masculinization effects of commonly used androgens methyltestosterone, dihydrotestosterone, and testosterone on the induction of penile formation in females. Our results suggested that prenatal treatment with low doses of methyltestosterone, but not same doses of dihydrotestosterone or testosterone, could induce penile formation in female mice. The minimum dose of dihydrotestosterone and testosterone for inducing tubular urethral formation in female mice was, respectively, 50 and 20 times higher than that of methyltestosterone. In vivo methyltestosterone treatment induced more nuclear translocation of androgen receptors in genital tubercles of female mice, affected Wnt signaling gene expressions, and then led to similar patterns of cell proliferation and death in developing genital tubercles to those of control males. We further revealed that low-dose methyltestosterone, but not same dose of dihydrotestosterone or testosterone, treatment induced penile formation in female guinea pigs. Exposure of female mouse genital tubercle organ culture to methyltestosterone, dihydrotestosterone, or testosterone could induce nuclear translocation of androgen receptors, suggesting that the differential effect of the three androgens in vivo might be due to the hormonal profile in mother or fetus, rather than the local genital tissue. To understand the differential role of these androgens in masculinization process involved is fundamental to androgen replacement therapy for diseases related to external genital masculinization.

Summary Sentence

Low-dose methyltestosterone treatment is sufficient to induce tubular urethral formation in female mice and guinea pigs through cellular processes similar to those of males, while same dose of dihydrotestosterone leads to female hypospadias in mice.

Key words: methyltestosterone, dihydrotestosterone, tubular urethral formation, penile formation, male reproductive tract, sex differentiation.

Introduction

External genitalia (EG) are sexually dimorphic among animals with internal fertilization. During development, the precursor structures of male and female EG are identical at early stages. The developing EG first becomes visible as two small mesenchymal swellings in mice [embryonic day (E) 10.75] [1] and guinea pigs (E20) [2]; the genital swellings then fuse to form a single genital tubercle (GT), which is the precursor of penis and clitoris. After this early bipotential phase, the development of the EG becomes sexually dimorphic. Androgen signaling is required to masculinize both internal and external genital organs [1–3].

Previous studies suggest that Wolffian duct stabilization and epididymal cell differentiation are mainly controlled by testosterone (T) [4], and dihydrotestosterone (DHT) is the main form of androgen to masculinize EG [5]. The interstitial cells in the testis differentiate into Leydig cells between E12.5 and E13.5 in mice and start to synthesize T [6]. 5α-Reductase is expressed in GT mesenchyme, and in males, it converts T into DHT [7]. Both T and DHT can bind to androgen receptor (AR) and activate AR signaling [8]. AR is differentially expressed in the developing male and female EG, and AR signaling leads to a series of morphological changes to form penises in males [9, 10].

Data of AR mutations have shown that genetic males fail to respond to androgen signaling and consequently develop feminized EG. A nonfunction or low levels of AR cause complete or partial androgen insensitivity in humans [11] and testicular feminization or ambiguous genitalia in animals [10, 12]. Mutations in the gene encoding 5α-reductase are another common cause of male pseudohermaphroditism. Such individuals are deficient in DHT, which is required for full masculinization of the EG and development of the prostate, but not for normal differentiation of the Wolffian ducts, epididymides, vasa deferentia, and seminal vesicles [13]. Mutations of 5α-reductase type 2 (*SRD5A2*) gene result in a wide spectrum of demasculinized EG phenotypes in humans [14]. These data suggest that DHT is required to fully masculinize EG. Both AR and 5α-reductase are also expressed in the female EG [15], but typically do not lead to masculinization owing to the lack of androgen. However, in female patients with congenital adrenal hyperplasia, the adrenal glands produce abnormally high levels of androgens, which causes varying degrees of virilization of the EG [16]. It has been known that in utero exposure to androgen can masculinize female EG [10, 17], but using tubular urethral formation and penile induction in females as a model to study external genital masculinization has never been reported.

As GT has bisexual potential before sex differentiation and castration in embryos is difficult, using androgen-induced female masculinization animal model is a methodological approach to detect the masculinizing effects of steroids, and it has been successfully used in brain masculinization and behavior studies [18, 19].

We have compared the difference among the effects of methyltestosterone (MT), DHT, and T on female external genital masculinization in both mice and guinea pigs, as well as the downstream genes expression. Here, we show that prenatal MT, DHT, and T induce tubular urethral and penile formation in female mice in a dose-dependent manner. We found that prenatal low doses of MT treatment are sufficient to induce penile formation in female mice, same doses of DHT treatment cause urethral malformation and female hypospadias, and the minimum dose of DHT and T for inducing female tubular urethral formation in mice is about 50 (DHT) and 20 (T) times higher than that of MT. Our results demonstrate that MT treatment activates genomic AR signaling, promotes differential cell proliferation and cell death, and induces tubular urethral formation in female mice, and the processes are similar to those of control males. Furthermore, as we have confirmed the similar effects of MT, DHT, and T on genomic AR activation in GT during sexual differentiation using an organ culture method, we believe that the in vivo differential effects of MT and DHT may be caused by the different hormonal profiles in mothers and fetuses.

Materials and methods

Animals and treatments

Shh^{gfpCre} and Rosa26-LacZ mice were purchased from The Jackson Laboratory (Bar Harbor, MN). ICR mice were purchased from Envigo (Indianapolis, IN). Hartley guinea pigs were purchased from Elm Hill Labs (Chelmsford, MA). Animals 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 Southern Illinois University Carbondale Animal Care and Use Committee-approved protocols 17-021 (mice) and 17-009 (guinea pigs). About 0.1–10 mg/kg of MT, DHT, or T were administered to pregnant female and/or neonatal mice through subcutaneous injection, 150 mg/kg flutamide was given to pregnant female mice by oral gavage, and the pups were collected at birth or postnatal day 21. Dosing schedules were provided in figure legends. Detailed preparation and administration procedures of these chemicals were the same as previously described [10, 20]. Mouse tail DNA was collected for genotyping of sex using SMCX/SMCY primers [21]. Shh^{gfpCre} and Rosa26-LacZ mice were genotyped using Jax lab protocols. Guinea pig breeding and embryo collection were performed as previously described [2]. MT and DHT administration in guinea pigs were similar to that in mice; dosing schedules and sample sizes were provided in figure legends.

Mouse GT organ culture and in vitro androgen administration

Embryonic mouse GT culture setup followed the methods described in publications with modifications [22, 23]. A 0.1% gelatin-coated 70 μm cell strainer (Falcon, 352350) was placed in the center of a Petri dish (6 cm diameter) as support. Mouse GTs were dissected from E14.5 embryos of ICR strain, and sex was determined by microscopic observation of gonadal tissues and then genotyping using SMCX/SMCY primers later. GTs were cultured on the strainer with the ventral side upwards, immediately below the air-medium interface, but with the top surface of GTs exposed to the air, at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures were sustained with DMEM/F12 (3:1) medium supplemented with 10% fetal bovine serum, nonessential amino acids, nucleotides, L-glutamine, and penicillin/streptomycin (The formula was a simplified modification of mouse embryonic stem cell growth medium [24]) with or without androgen. MT, DHT, and T were dissolved in ethanol (0.05 M stock, filter sterilized) and then diluted into the medium, each at 5×10^{-6} M, 5×10^{-7} M, 5×10^{-8} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, respectively. The GTs were cultured for 48 h and processed for immunohistochemical analysis.

Immunohistochemistry, cell proliferation, and cell death assays

Immunohistochemistry was performed according to standard procedures using anti-BrdU (G3G4, DSHB, Cat# AB 2314035, RRID: AB_2618097), anti-AR (Santa Cruz Biotechnology, Cat# sc-816,

RRID: AB_1563391), anti-β-catenin (Abcam, Cat# ab32572, RRID: AB_725966), anti-frizzled-6 (Santa Cruz Biotechnology, Cat# sc-393791, RRID: AB_2736833), and anti-LEF1 (Santa Cruz Biotechnology, Cat# sc-374522, RRID: AB_10986008), and AR and LEF1 antibodies were detected using Tyramide SuperBoost Kits (Invitrogen, B40922) and ABC Kit (Vector Laboratories, PK-6100), respectively, according to the manufacturers' protocols. Cell proliferation and programmed cell death analyses were performed using BrdU and LysoTracker, respectively, following the previously published methods [10]. A Leica DM5500 Confocal Microscope (Leica microsystems Inc, Buffalo Grove, IL) was used for imaging. Sections of GTs (Sample size, $n = 5$) from three different litters were selected.

Quantitative RT-PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was performed according to a modification of previously described methods (30, 38). MT-treated and control female ICR mouse GTs were dissected at E16.5, two GTs from each litter were randomly selected for RNA extraction, and three litters of mice from each group were used. Total RNA was extracted using TRIzol method according to the operation manual (Invitrogen), and RNA quantity (>100 ng/μL) and purity (260/280 > 2.0, 260/230 > 1.65) were determined by using a Nanodrop. RNA integrity (28S/18S ratio > 1.5) was assessed by gel electrophoresis. A quantity of 500 ng of high-quality RNA for each sample ($n = 3$) was converted into cDNA by using the RT^2 First Strand cDNA Kit (Qiagen, Hilden, Germany). Pathway-specific gene expression was determined using the Wnt signaling PCR Array (PAMM-043Z, Qiagen, Hilden, Germany) and the CFX96 Real-time PCR system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The Web-Based PCR Array Data Analysis system (Qiagen, Hilden, Germany) was used to analyze PCR array results.

*Lac*Z reporter stain and histology

Whole mount *lacZ* staining was performed as previously described [25]. Leica M80 stereo microscope (Leica microsystems Inc, Buffalo Grove, IL) was used for imaging.

Statistics

We repeated the chemical treatment experiments four times. All the EG phenotypes after chemical treatment were observed and the malformation frequency was shown as percentage. To test the differences in cell proliferation and dorsal–ventral width of urethral plate between control females and other groups, the two-tailed Student *t*-test was used to calculate *P* values, the significance level (alpha) was set at 0.05, and data were mean ± SEM. The differences in anogenital distance (AGD) among different groups were determined using ANOVA, alpha was set at 0.05 and data were mean ± SEM.

Results

Prenatal treatment with low doses of MT, but not same doses of DHT or T, induced tubular urethral and penile formation in female mice

To reveal the effects of different androgens on masculinizing female EG during development, we treated the female mice with 1 mg/kg DHT or MT in utero (E12.5–16.5, once daily) and from newborn to postnatal day (P) 6 (P0–6, once every other day), then observed the phenotypes of EG on P21. The male mice showed no obvious difference among different androgen treatment groups (Figure 1A, B, E, F, I, and J). In DHT-treated female mice, an enlarged glans clitoris containing a bone with distal cartilage was observed at P21, but the treated mice failed to form a tubular urethra and failed to form penis (Figure 1C, D, G, and H). Moreover, all prenatally DHT-exposed females showed obvious hypospadias, similar to neonatal DES-induced malpositioning of vaginal and urethral openings with wide cleft clitoris [26]. When the female embryos were exposed to same dose of MT at the same stages, all of them formed penises with all characteristics a male penis typically has, including tubular urethra, os-penis with distal cartilage, corpus cavernosum, corpus spongiosum, and penis spines (Figure 1K and L). We also tested the penile induction ability of T injected following the same dosage schedule, and the results suggested that same dose of T had little effect on male mice, which was similar to DHT and MT, but induced enlarged glans clitoris with baculum in females, the urethra was outside of the clitoris epithelium (Supplemental Figure S1). As DHT has higher affinity to AR than T [27, 28] and MT [29, 30], we also examined dose responses of DHT on external genital masculinization in doses ranging from 0.5 to 4 mg/kg, and the results revealed similar effect among different dose groups; all DHT-treated females (100%) showed unclosed urethras and enlarged clitorises (Supplemental Figure S2A–D), but all the same doses of MT-treated females (100%) developed tubular urethras and penises (Supplemental Figure S2E–H).

AGD is another commonly used sexually dimorphic index. In our experiments, both 1 mg/kg MT- and DHT-treated females showed significantly increased AGD at both E16.5 and P21 compared with control females, but MT-treated females had significantly larger AGD than DHT-treated females (Supplemental Figure S3).

We next asked when is MT required to induce penile formation in females. In order to address this question, we performed MT treatment (1 mg/kg) either in utero (E12.5–16.5) or in the first week after birth, while we gave DHT treatment (1 mg/kg) at the other stage. The results showed that only the prenatally MT-treated females formed tubular urethra and penile structure (Figure 1M–P). The experiments revealed that only prenatal MT was required to induce urethral tube closure and lead to penile formation in females.

To discover the minimum effective dose of prenatal exposure of MT, DHT, and T to induce tubular urethral formation, we treated female mice with MT, DHT, or T once daily in utero (E12.5–16.5) using different doses ranging from 0.1 to 10 mg/kg (Table 1), collected the EG at the time of birth (P0), and observed the morphology and structure. We found no significant differences in litter size and male/female ratio among different groups, and all treated males showed no significant differences in AGD and penile structure (Table 1), and in 0.2 mg/kg MT-treated groups, more than 80% females formed a closed urethra surrounded by penile mesenchyme in the proximal region (Figure 2A–H and M–P; Table 1). When the dose of MT was increased to 1 mg/kg or higher, all treated female mice (100%) formed tubular urethra in the proximal region of EG, similar to control males (Table 1). In 0.1 mg/kg MT-treated group, all males group showed no difference from control males, but the AGD of females was significantly larger than that of control females ($P = 0.037$); however, only 10.7% treated females formed centralized tubular urethra in proximal region of EG (Table 1), and the urethra were unclosed in majority of treated females (Figure 2I–L). In DHT-treated groups, only 1 mg/kg or higher doses of DHT-treated females showed significantly increased AGD compared with control females, and no any female mice in 0.1, 0.2, or 1 mg/kg group presented centralized tubular urethra in proximal EG, only 14.3% females in 5 mg/kg DHT treatment group formed a tubular urethra (Table 1), and in most of 5 mg/kg DHT-treated females, the urethra remained

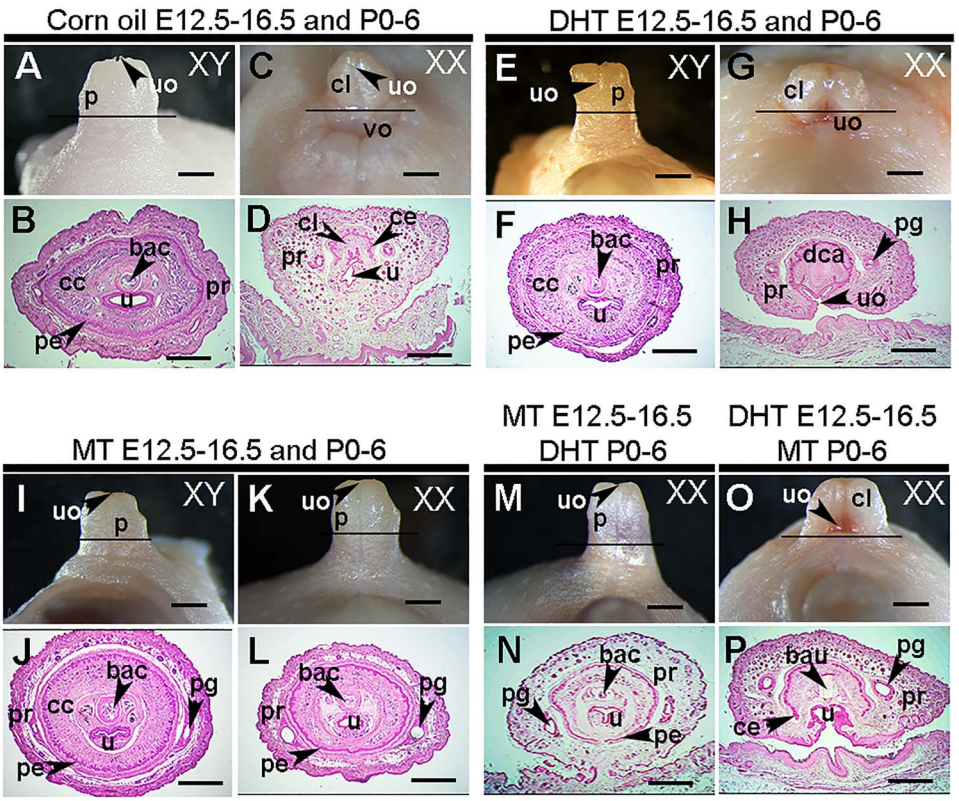

Figure 1. Low dose of MT, but not DHT, induced tubular urethral and penile formation in female mice. (A, C, E, G, I, K, M, and O) Ventral views of the P21 mouse EG with distal at the top; lines on images indicate the planes of sections shown in images below. All sections are transverse to the EG. Sexes are indicated by chromosome symbols XY (male) and XX (female). (A, B) Control male EG showing a closed urethra in the center of the penis. (C, D) Control female EG showing a urethra located outside of the clitoris. (E–H) DHT treatment had no obvious effect on male EG (E, F), but increased the glans clitoris size and developed a distal cartilage in females, with the urethral epithelium unclosed and female hypospadias formed (G, H). (I–L) MT treatment had little effect on male EG (I, J) but induced penis formation in females, no vaginal opening was observed, and the MT-induced female penis had the same structures as a typical male penis, including baculum, centralized urethral tube, corpus cavernosum, and penis spine (K, L). Prenatal MT and neonatal DHT treatment induced penis formation (M, N), but prenatal DHT and neonatal MT only increased glans clitoris size (O, P). The arrowheads in (A, C, E, I, K, M, and O) point to the urethral openings and in (B, D, F, H, J, L, N, and P) point at structures as indicated by adjacent labels. *bac*, baculum; *bau,* baubellum; *cc*, corpus cavernosum*; ce*, clitoris epithelium; *cl*, clitoris; *dca,* distal cartilage; *p*, penis; *pe*, penis epithelium; *pg*, preputial gland; *pr*, prepuce; *u*, urethra; *uo*, urethral opening; *vo*, vaginal opening. Scale bars in (A, C, E, G, I, K, M, and O) and histological sections (B, D, F, H, J, L, N, and P) are 1 mm and 500 μm, respectively.

open (Figure 2Q–T). When the dose of DHT was increased to 10 mg/kg, all the treated females had increased AGD and more than 80% of them developed a tubular urethra in proximal region (Figure 2U–X; Table 1), while all the males showed no noticeable difference from control males. The effect of T treatment was between those of MT and DHT (at the same doses); 1 mg/kg or higher dose of T treatment significantly increased the AGD in female mice and 2 and 5 mg/kg T succeeded to induce a closed penile urethra in proximal region in 19 and 90.5% of the females, respectively (Table 1; Supplemental Figure S4A–P).

To determine whether MT induced tubular urethral formation in female mice through AR genomic action, we performed MT plus AR antagonist flutamide (1 mg/kg MT plus 150 mg/kg flutamide) or flutamide only (150 mg/kg) treatment at the same stage (E12.5 to E 16.5 once daily) and pups were collected at birth. Both 1 mg/kg MT plus 150 mg/kg flutamide and 150 mg/kg flutamide treatment had no effect on litter size and male/female ratio. Nevertheless, 150 mg/kg flutamide treatment induced unclosed urethra (similar to that of control females at the same stage) in 90% of male mice with shorter AGD compared with that of control males at the same stage but had no effect on females; In MT plus flutamide-treated group, no females formed tubular urethra, and 84% of the males had unclosed urethra (Table 1).

More nuclear-localized ARs were found in MT- than DHT-treated female GT around urethral closing region

Nuclear translocation of AR is a key step in genomic AR action [31]. To determine how MT causes the masculinization of female EG, we first performed immunohistochemistry on E16.5 mouse GTs to visualize AR localization. Majority of ARs in mesenchymal cells adjacent to the urethral closing region were found in nuclei in control males and MT-treated (1 mg/kg) females (Figure 3A, B, E, and F). In control females and same dose of DHT-treated females, as no urethral closure happened, we observed the serial transverse sections of the GTs and found that majority of ARs in mesenchymal cells around the urethra were localized in cytoplasm and few nuclear-localized ARs could be detected in all sections examined; the representative sections were shown in (Figure 3C, D, G, and H).

Differential expression of Wnt pathway genes and proteins in MT- and DHT-treated female GTs

The finding that prenatal MT and DHT can induce differential nuclear translocation of AR during EG development led us to investigate the activation of AR downstream targets. Wnt/β-catenin pathway has been proved to be AR responsive genes during external genital masculinization [32]. In order to reveal

Table 1. The effect of prenatal androgen treatment on litter size, sex ratio, anogenital distance, and tubular urethra formation in male and female newborn mice[#].

		Litter size	Male/female sex ratio	Anogenital distance (mm)		Tubular urethra formation	
				Male	Female	Male	Female
Corn oil control		13.5 ± 1.22	0.89 ± 0.21	2.15 ± 0.32	0.96 ± 0.14	25/25 (100%)	0/28 (0%)
MT (mg/kg)	0.1	14.0 ± 1.10	1.02 ± 0.16	2.16 ± 0.16	1.32 ± 0.25*	28/28 (100%)	3/28 (10.7%)
	0.2	13.7 ± 1.03	0.90 ± 0.11	2.21 ± 0.18	1.61 ± 0.26**	19/19 (100%)	17/21 (81%)
	1.0	13.8 ± 1.05	0.96 ± 0.09	2.31 ± 0.12	1.95 ± 0.15**	27/27 (100%)	28/28 (100%)
	5.0	13.7 ± 1.08	0.95 ± 0.12	2.25 ± 0.15	2.08 ± 0.18**	20/20 (100%)	21/21 (100%)
	10[#]	13.5 ± 1.33	0.91 ± 0.14	2.11 ± 0.21	2.18 ± 0.11	21/21 (100%)	23/23 (100%)
T (mg/kg)	0.1	13.7 ± 1.12	1.04 ± 0.11	2.14 ± 0.23	0.97 ± 0.18	24/24 (100%)	0/23 (0%)
	0.2	13.3 ± 1.31	1.01 ± 0.08	2.14 ± 0.28	0.95 ± 0.12	20/20 (100%)	0/20 (0%)
	1.0	12.7 ± 1.25	1.03 ± 0.13	2.12 ± 0.19	1.31 ± 0.21*	19/19 (100%)	0/19 (0%)
	2.0	13.7 ± 1.03	0.95 ± 0.15	2.06 ± 0.27	1.42 ± 0.25*	20/20 (100%)	4/21 (19%)
	5.0	14.7 ± 1.01	1.06 ± 0.27	2.03 ± 0.22	1.72 ± 0.19**	23/23 (100%)	19/21 (90.5%)
	10[#]	13.9 ± 0.95	0.91 ± 0.16	2.05 ± 0.21	1.77 ± 0.22**	20/20 (100%)	22/22 (100%)
DHT (mg/kg)	0.1	13.7 ± 1.06	1.05 ± 0.12	1.98 ± 0.25	0.99 ± 0.17	22/22 (100%)	0/21 (0%)
	0.2	13.0 ± 1.05	1.05 ± 0.11	2.12 ± 0.15	0.98 ± 0.17	20/20 (100%)	0/19 (0%)
	1.0	13.3 ± 1.06	0.95 ± 0.10	2.11 ± 0.17	1.26 ± 0.16*	20/20 (100%)	0/21 (0%)
	5.0	13.7 ± 1.01	0.90 ± 0.09	2.04 ± 0.23	1.44 ± 0.18*	19/19 (100%)	3/21 (14.3%)
	10	12.2 ± 1.12	0.95 ± 0.13	1.96 ± 0.26	1.63 ± 0.31*	18/18 (100%)	16/19 (84.2%)
Flutamide (mg/kg)	150	13.7 ± 1.02	0.95 ± 0.17	1.24 ± 0.23**	0.92 ± 0.15	3/20 (15%)	0/21 (0%)
Flutamide (150 mg/kg) + MT (1 mg/kg)		13.3 ± 1.01	0.90 ± 0.13	1.36 ± 0.27**	1.17 ± 0.24	4/19 (21%)	0/21 (0%)

Three to four litters of mice were used for each treatment, and each litter had 6–9 female pups. Subcutaneous injections once daily from E12.5 to E16.5 were used in all treatment groups. [#]10 mg/kg MT and 10 mg/kg T treatment groups had birth problems. As most of ICR mice give birth around E19, we collected E19.5 embryos instead of P0 pups for these groups. Numbers of tubular urethra formation were calculated by (number of animals with tubular urethra/number of total animals observed) based on observation of the urethral structure in proximal sections of each EG (see Figure 2), and percentages were also calculated. Data of litter size, sex ratio, and anogenital distance are means ± SDV. The comparison was performed between each treatment group and control group of the same sex. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

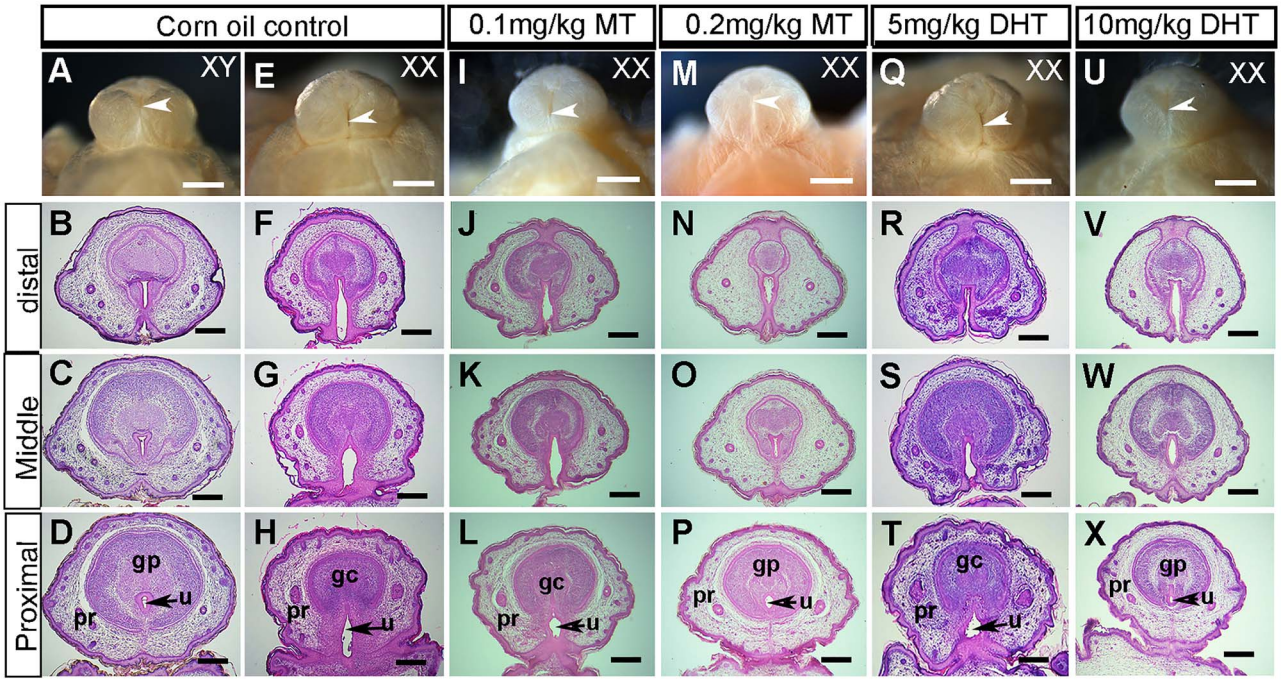

Figure 2. Dose-dependent effects of prenatal MT and DHT on inducing tubular urethral formation in female newborn mice. (A, E, I, M, Q, and U) Ventral views of the newborn mouse EG with distal at the top; white arrowheads indicate the urethral openings. (B–D, F–H, J–L, N–P, R–T and V–X) Transverse sections from distal to proximal through EG of (A, E, I, M, Q, and U), respectively; black arrows in (D, H, L, P, T, and X) point at the urethra. Sexes are indicated by chromosome symbols XY (male) and XX (female). All the MT or DHT treatment in experiments shown in this figure was administrated once daily at E12.5–16.5. *gc*, glans clitoris; *gp*, glans penis; *pr*, prepuce; *u*, urethra; Scale bars in (A, E, I, M, Q, and U) are 500 μm and in (B–D, F–H, J–L, N–P, R–T, and V–X) are 250 μm.

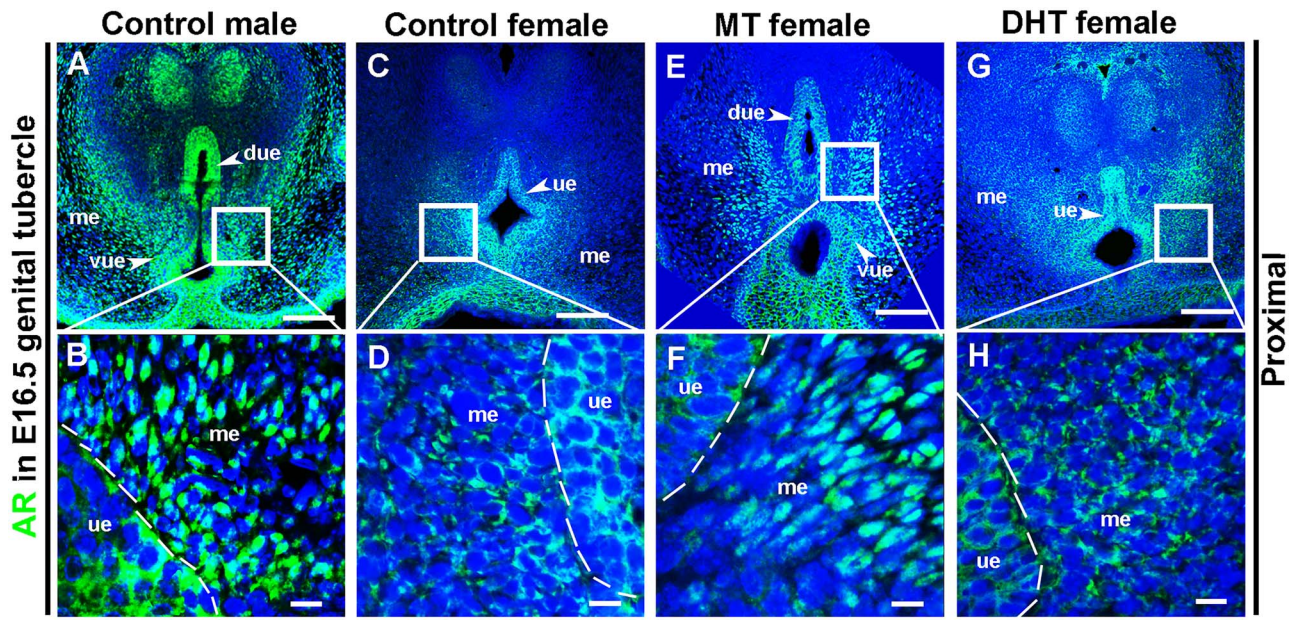

Figure 3. Distribution of ARs in E16.5 mouse GTs. All images (A–H) show transverse sections of mouse GTs with dorsal at the top. Different treatment group identifications are on top of the images. (B, D, F, and H) Higher magnification images of the areas in white boxes of (A, C, E, and G), respectively. White arrowheads in (A, C, E, and G) point at structures as indicated by adjacent labels. Dashed lines in (B, D, F, and H) indicate the boundary of urethral epithelium and mesenchyme. Green staining is AR and blue is DAPI. Note that E16.5 control male (A, B) and MT (1 mg/kg, E12.5–15.5, once daily)-treated female (E, F) GTs showed more nuclear localized ARs in mesenchyme than control female (C, D) and DHT (1 mg/kg, E12.5–15.5, once daily)-treated female (G, H) GTs of same stage. *due*, dorsal urethral epithelium; *me*, mesenchyme; *ue*, urethral epithelium; *vue*, ventral urethral epithelium. Scale bars in (A, C, E, and G) are 100 μm and in (B, D, F, and H) are 10 μm.

the expression of Wnt pathway genes responsive to androgen treatment, we first quantified relative gene expression changes in E16.5 mouse GTs after MT treatment (1 mg/kg) using Wnt pathway-specific PCR array. Compared with control females, MT treatment induced significantly changes (over 1.8-fold) in the expression of 28 (out of 84) genes, and 22 of these 28 genes had more than twofold differences compared with controls (Figure 4Q). Among the 28 significantly changed genes, 22 were upregulated, including *Bcl9*, *Ccnd3*, *Csnk1a1*, *Ctnnb1*, *Dvl1*, *Dvl2*, *Foxn1*, *Fzd1*, *Fzd2*, *Fzd3*, *Fzd4*, *Fzd6*, *Jun*, *Lef1*, *Lrp5*, *Lrp6*, *Nlk*, *Rhou*, *Ppp2r5d*, *Tcf7l1*, *Wnt2*, and *Wnt5a*, while the other 6 (*Apc*, *Csnk1d*, *Dkk1*, *Fbxw2*, *Strp1* and *Wisp1*) were downregulated. The two- up to threefold change genes, *Ctnnb1* and *Fzd6*, which encode β-catenin and Frizzled-6 (FZD6), respectively, and LEF1, which is a nuclear effector in Wnt signaling pathway [33], were further studied at the protein level by performing immunohistochemical staining on MT- and DHT-treated E17 mouse GTs, and the results were shown in Figure 4A–P. β-Catenin protein was strongly expressed in urethral epithelium of control male and MT-treated female GTs (Figure 4A, B, E, and F), but reduced expression in DHT-treated females was obvious (Figure 4C, D, G, and H). FZD6 protein was expressed in urethral epithelium and mesenchyme adjacent to urethral closing region in control males and MT-treated females (Figure 4A, B, E, and F) but was detected in urethral epithelium only in control female and DHT-treated female GTs (Figure 4C, D, G, and H). As FZD6 regulates cell polarization [34], our results suggest that cell polarization may play a role in penile formation during sex differentiation of EG. LEF1 showed strong nuclear staining in mesenchyme of urethral closing region in control male and MT-treated female GTs (Figure 4I, J, M, and N). LEF1 protein was also detected in control female and DHT-treated female GTs, but the signal was obviously weaker than that in control male and MT-treated female GTs (Figure 4K, L, O, and P).

Low-dose MT, but not same dose of DHT, induced male patterns of cell proliferation and cell death in developing GTs in female mice

Androgen signaling masculinizes EG through regulating cell proliferation and programmed cell death [10]. Our findings that penile formation in female mice can be induced by transient exposure to MT, but not low doses of DHT and T, and that in vivo MT treatment, but not low dose of DHT, can induce majority nuclear translocation of AR in E16.5 female mouse GT suggest that MT and DHT treatment may have different effect on cell proliferation and cell death in E16.5 mouse GT. To test this hypothesis, we compared the urethral plate morphology, cell proliferation, and cell death patterns in E16.5 mouse GT among different treatment groups (both MT and DHT at 1 mg/kg, once daily from E12.5 to E15.5). Urethral plate was visualized using lacZ staining of $Shh^{gfpCre}R26R$ mouse GT. In control males and MT-treated females, mesenchyme of the urorectal septum and prepuce could be seen invading the proximal end of the urethral plate (Figure 5A and C); control females and DHT-treated females showed the unseparated urethral plate (Figure 5B and D). And compared with control females, the depth of urethral plate at the septation region was larger in both control males ($P = 0.0035$) and MT-treated females ($P = 0.0058$), but DHT-treated females had no significant difference ($P = 0.265$) from control females (Figure 5A–D and Q). MT treatment dramatically increased cell proliferation in urethral epithelium ($P = 0.001$) and adjacent mesenchyme ($P = 0.006$) compared with control females (Figure 5F, G, and R), and the pattern of cell proliferation

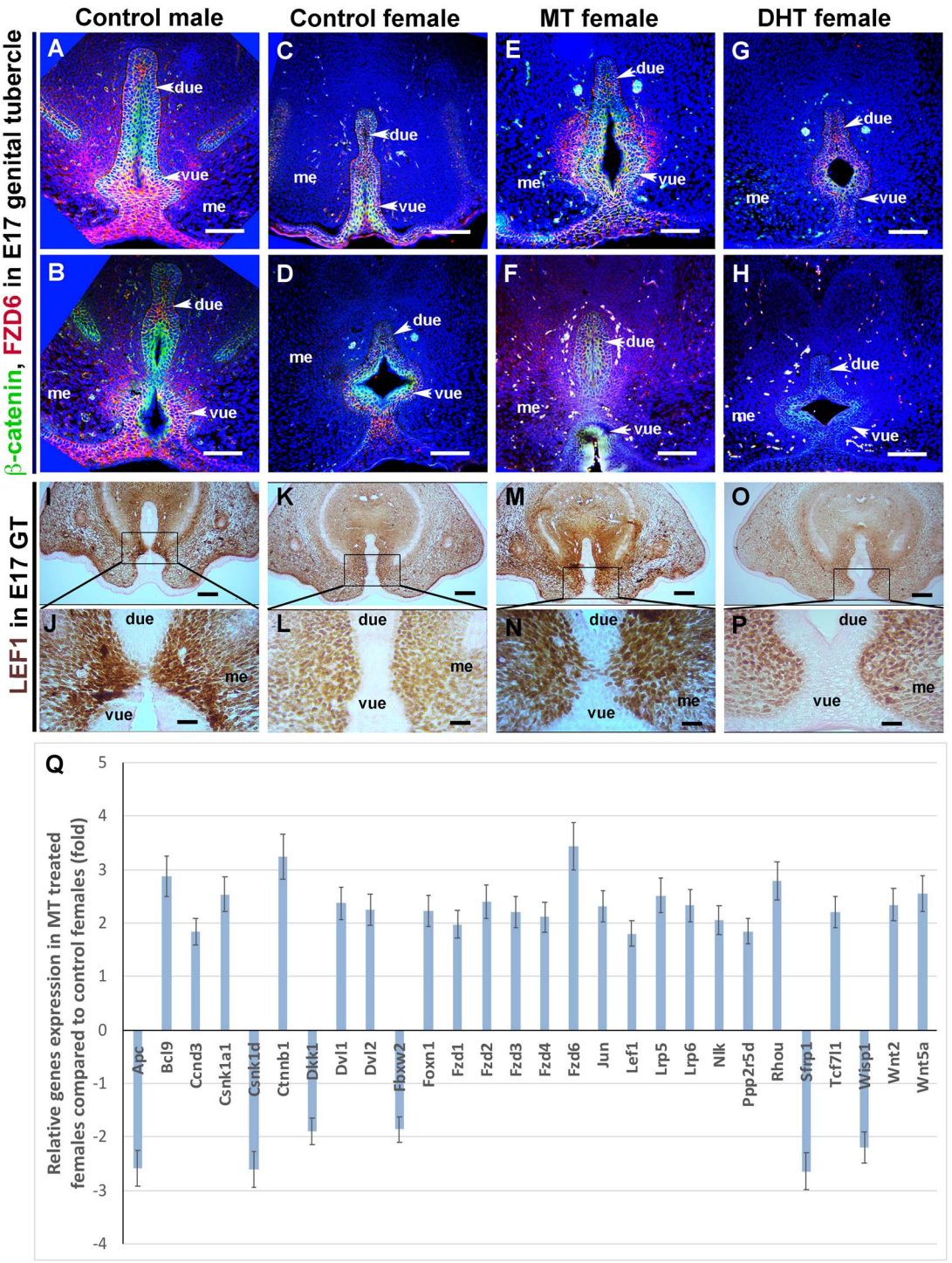

Figure 4. Expression of Wnt pathway genes and proteins in mouse GTs at sex differentiation. All images (A–P) show transverse sections of E17 mouse GTs with dorsal at the top. White arrowheads in (A–H) point at structures as indicated by adjacent labels. Different treatment group identifications are on top of the images. (A, C, E, and G) Distal sections and (B, D, F, and H) proximal sections. Green staining is β-catenin, red is Frizzled-6 (FZD6), and blue is DAPI. For images (I–P), (I, K, M, O) are proximal sections; brown shows LEF1 signal. (J, L, N and P) Higher magnification images of the areas in black boxes of (I, K, M, and O), respectively. (Q) Relative transcript levels (*y* axis shows fold change) of Wnt pathway genes in E16.5 GTs of MT-treated females compared with control females. Control was assigned a value of 0. The figure showed that the expression of 28 out of 84 genes had statistically significant ($P \leq 0.05$) changes of more than 1.8-fold, in which 22 genes were upregulated and 6 genes were downregulated. *due, me,* and *vue* are the same as Figure 3. Scale bars in (A–H, I, K, M, and O) are 100 μm and in (J, L, N, and P) are 25 μm.

in MT-treated female GTs was similar to that in control males (Figure 5E, G, and R). DHT-treated females had fewer proliferating cells than control females in both urethral epithelium (P = 0.026) and adjacent mesenchyme (P = 0.034, Figure 5F, H, and R). In addition, MT-treated females showed similar cell death pattern to control males in urethral plate and ventral portion of GT

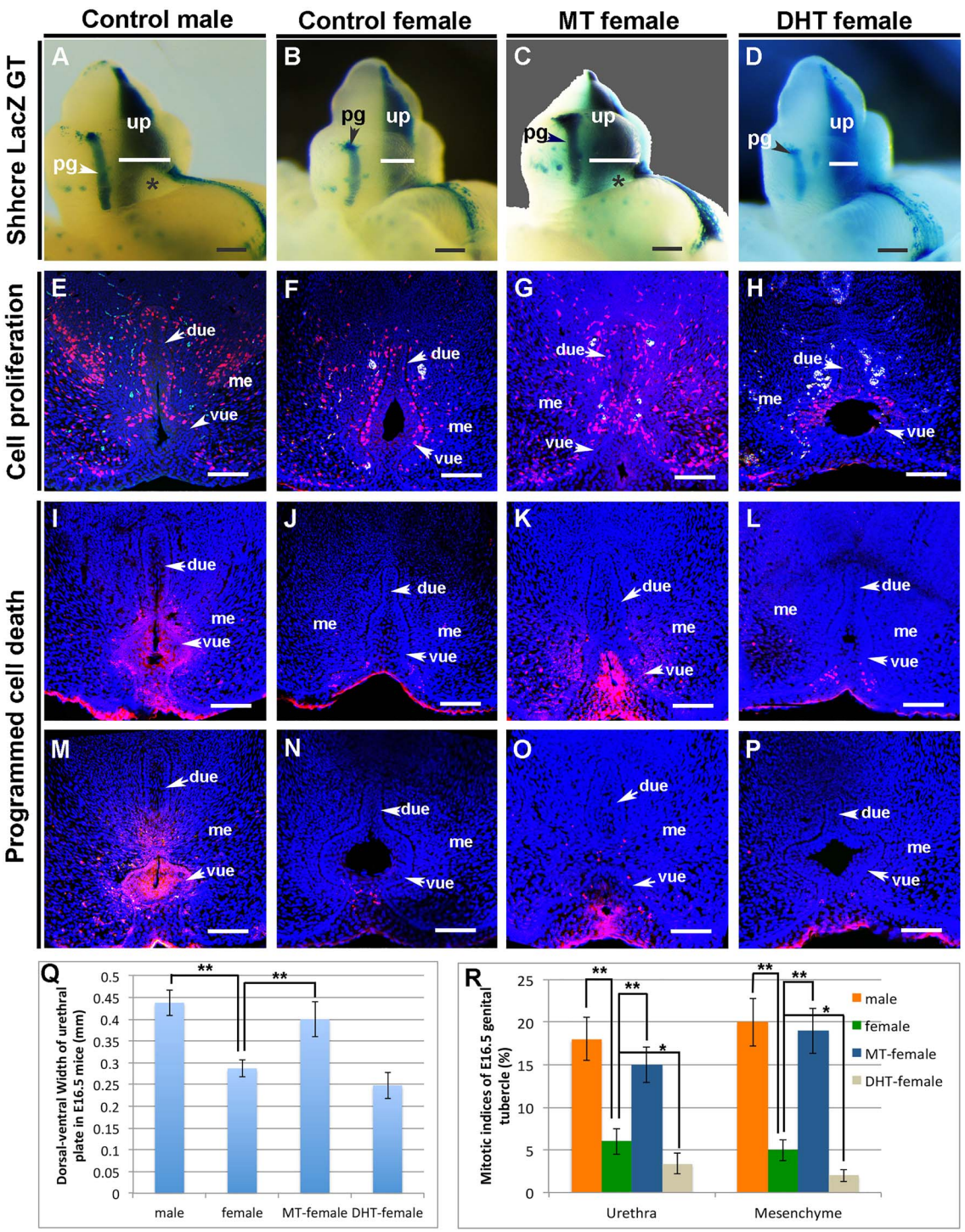

Figure 5. Differential cell proliferation and cell death in in vivo MT- and DHT-treated E16.5 GTs of female mice. (A–D) Side ventral views of E16.5 Shh^{gfpcre} LacZ mouse GTs in different treatment groups with distal at the top. Group identifications are labeled on top of the images. Blue X-gal staining shows the urethral plate and the length of the white bars indicates the dorsal–ventral width of urethral plate at these positions. "∗" marked areas in (A, C) show the mesenchyme of the urorectal septum and prepuce invading the proximal end of the urethral plate in control males and MT-treated females. All sections (E–P) are transverse to the GT with dorsal at the top. Arrowheads in (A–P) point at structures as indicated by adjacent labels. (E–H), Red BrdU labeling shows cell proliferation in E16.5 mouse GTs. (I–P), LysoTracker® Red labeling shows cell death in E16.5 GTs of control male, (I, M); control female, (J, N); MT-treated female, (K, O); and DHT-treated females, (L, P). (I, M) and (K, O) Serial transverse sections at urethral closing region of control male and MT-treated female GTs, respectively. (J, L) Distal and (N, P) proximal sections of control female and DHT-treated female GTs. (Q) Comparison of dorsal–ventral width of urethral plate among different groups. Sample size, 3 litters, and $n \geq 12$. (R) Mitotic indices in both urethral epithelium and mesenchyme among different groups. Sample size, 3 litters, and $n \geq 15$. *due, me, pg, ue,* and *vue* are the same as Figures 1, 3, and 4. *up*, urethral plate. Scale bars in (A–D) (black) are 250 μm and in all others (white) are 100 μm. In (Q, R), $*P \leq 0.05$, $**P \leq 0.01$.

(Figure 5I, K, M, and O), which caused the dorsal and ventral portions of urethra to separate, the ventral portion of urethra to disappear, and the dorsal portion of urethral epithelium to fuse to form tubular urethra. Control females and DHT-treated females showed much less programmed cell death in the ventral region of GT (Figure 5J, L, N, and P). The data suggested that in vivo low-dose MT could induce female external genital masculinization via increasing urethral epithelial and adjacent mesenchymal cell proliferation and cell death and switching to a male pattern of development, while in vivo same dose of DHT showed negative effect on urethral epithelial and mesenchymal cell proliferation.

All the three androgens (MT, DHT, and T) could induce nuclear-localized ARs in the urethral closing region of female GT in organ culture

To determine whether the androgen-induced differential nuclear translocation of AR in GT in female mice is caused by a local tissue reaction of GT or due to the metabolic effects of mother, placenta, or embryos, we established mouse GT organ culture and examined the AR nuclear translocation effect of the androgens on this in vitro model. Mouse GTs of ICR strain were dissected at E14.5 and put into culture using medium supplemented with androgen or androgen-free medium, and different concentrations of MT or DHT (5×10^{-6} M, 5×10^{-7} M, 5×10^{-8} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M) were administrated to the culture medium from the beginning in androgen treatment groups; the GTs were cultured for 2 days and then processed for AR immunostaining. Interestingly, AR nuclear translocation was found in the ventral mesenchyme adjacent to urethra in both in vitro MT- and DHT (at similar concentrations)-treated female GTs (Figure 6G–L; Supplemental Table S1), but not in control male and female GTs cultured without androgen (Figure 6A–F; Supplemental Table S1). Because we started culture at E14.5, the control males also showed very limited nuclear translocation of AR (Figure 6A–C; Supplemental Table S1). We have conducted the test in GT culture with the presence of similar concentrations of T as well, and the results showed that T could also induce AR nuclear translocation similar to MT and DHT (Supplemental Figure S5; Supplemental Table S1).

Low-dose MT, but not same dose of DHT or T, induced tubular urethral and penile formation in female guinea pigs

The process of human tubular urethral formation was described as a distal opening and proximal closing zipper [3], which has not been observed in mice. The urethral closure process of a relatively longer gestation animal guinea pig has been shown to be similar to human double zipper, and antiandrogen-induced hypospadias in guinea pigs is morphologically more analogous to human hypospadias [2]. To reveal the differential effects of prenatal in vivo exposure of MT, DHT, and T on guinea pig external genital development, we treated the guinea pigs in utero with 1 mg/kg MT, DHT, or T at sex differentiation stages from E27 to E31 every other day and compared the external genital morphology at E40. The results showed that 1 mg/kg MT, DHT, or T had no obvious effect on EG development of males (Figure 7A, C, E, and G). Among all the treated females, only MT-treated ones formed tubular urethras and penises similar to control males [100% (5 out of all the 5 females from 3 litters)] (Figure 7A and D); DHT (1 mg/kg) exposure partially masculinized female EG but failed to induce penile tubular urethral formation in female guinea pigs [100% (8 out of all the 8 females from 4 litters)] (Figure 7B and F). The EG of female guinea pigs [100% (4 out of all the 4 females from 2 litters)] exposed to same dose of T showed no obvious difference from that of control females (Figure 7B and H).

Discussion

Our results showed that prenatal in vivo exposure to low doses of MT but not same doses of DHT or T succeeded in inducing penile formation in female mice and guinea pigs. The minimum dose of DHT and T required to induce tubular urethral formation in female mice is 50 (DHT) and 20 (T) times higher than that of MT. MT treatment could induce AR nuclear translocation, activate genomic AR signaling, regulate Wnt/FZD/β-catenin pathway, promote cell proliferation in both urethral epithelium and mesenchyme, and increase programmed cell death in ventral part of urethral epithelium. All these molecular and cellular processes worked together; it led to the dorsal part of urethral epithelium to form the tubular urethra and the GT to develop into a penis in females. Our findings suggest that only low doses of in vivo MT are sufficient to induce tubular urethral and penile formation, same doses of DHT and T in vivo treatment can partially masculinize female EG but is inefficient to induce centralized penile tubular urethral formation, low doses of DHT actually induced female hypospadias in mice in our experiments. MT-induced tubular urethral formation in female mice can be diminished by AR antagonist flutamide, suggesting that prenatal MT induces female penile formation mainly through AR genomic action.

Both DHT and T binding that can induce AR translocation from cytosol to nucleus has been established half a century ago [35]; it is surprising that 1 mg/kg DHT in vivo prenatally treated female GT showed much less nuclear-localized ARs than same dose of MT-treated female GT. As DHT and MT were given to pregnant mothers, not directly to developing embryos, to identifying causal mechanisms was therefore complicated. Similar amount of nuclear-localized ARs were found in the mesenchyme adjacent to the urethral epithelium in low concentrations of DHT-treated female GTs compared with in similar concentrations of MT-treated female GTs in organ culture, suggesting the main cause of the in vivo differential effects of MT and DHT on AR nuclear translocation may not be related to the local genital tissue response, but the hormone metabolism of mother, placenta, or even embryos. Multiple possibilities may be involved. One of them is that the metabolism of MT and DHT in mother or placenta may be different and another possibility is that the protection effect of pregnant mother against DHT and MT maybe different. We already know alpha fetal protein can bind to estradiol and protect the fetus [36], but what about DHT and MT? Our results showed that very high dose of prenatal in vivo DHT treatment could induce penile formation in females; this suggests that only limited protection effect against DHT may exist from the pregnant mother during embryonic development. On the other hand, the different roles of T and DHT interaction between AR and its co-factors had been reported in ovary [37]. It is very likely that MT and DHT has different effect on placental aromatase or some other enzymes' activity. In addition, DHT that is required in penile masculinization has been established through investigations of mutations in humans, but 5-alpha reductase type 1 and 2 double knockout in mice had little effect on penile formation [38]. It is possible that the function of DHT in external genital masculinization is different between humans and rodents, such as mice and guinea pigs. Clearly, the mechanism

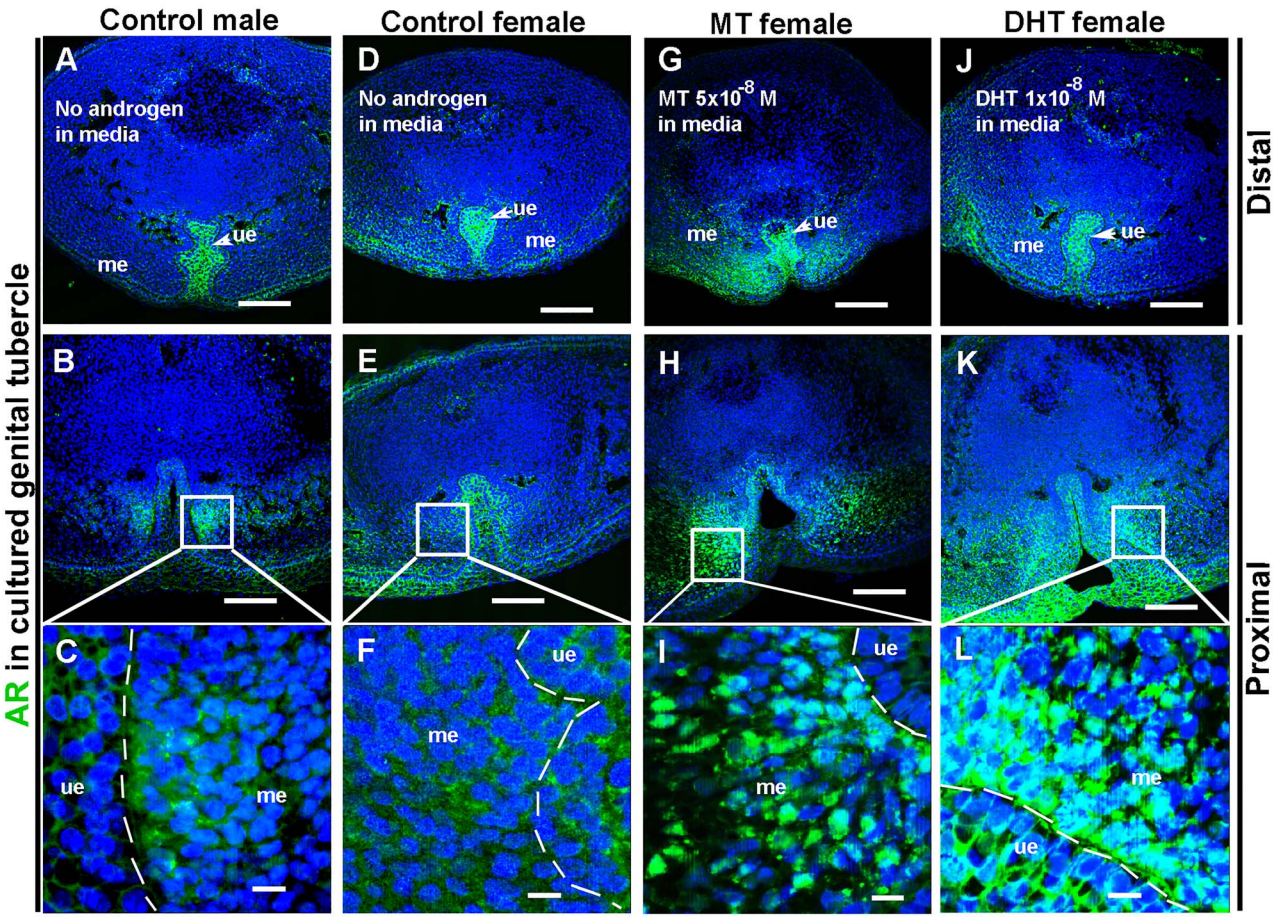

Figure 6. Distribution of ARs in cultured mouse GTs. E14.5 GTs of ICR mice were dissected and cultured with or without androgen for 2 days and then processed for AR immunostaining. Different treatment group identifications are on top of the images. All images (A–L) show transverse sections of mouse GTs with dorsal at the top. (C, F, I, and L) Higher magnification images of the areas in white boxes of (B, E, H, and K), respectively. Each column shows one same GT. (A, D, G, and J) Distal sections and (B, E, H, and K) proximal sections. White arrowheads in (A, D, G, and J) point at the urethral epithelium as indicated by adjacent labels. Dashed lines in (C, F, I, and L) indicate the boundary of urethral epithelium and mesenchyme. Green staining is AR and blue is DAPI. Note that both in vitro MT (5×10^{-8} mol/L) and DHT (1×10^{-8} mol/L)-treated GTs (G–L) showed more nuclear localized ARs in ventral mesenchyme adjacent to urethra than nonandrogen-treated control female GTs (D-F). As sexual differentiation of GTs was just initiated at the time when the culture was started, nonandrogen-treated control male GTs (A–C) also showed much less nuclear localized ARs compared with MT- and DHT-treated female GTs (G-L). *me*, mesenchyme; *ue*, urethral epithelium. Scale bars in (A, B, D, E, G, H, J, and K) are 100 μm and in (C, F, I, and L) are 10 μm.

of how transient in vivo exposure to MT, DHT, or T differentially regulates AR signaling in EG needs further study.

Wnt signaling directs cell proliferation, cell polarity, and cell fate determination during embryonic development through polarizing asymmetric cell division and endo-mesoderm linage specification, anteroposterior axis patterning, and posterior growth [39–41]. The interaction between Wnt/β-catenin pathway genes and androgen signaling plays important roles in penile formation [32], and the interaction between β-catenin and AR can be enhanced by the presence of AR agonist [42]. Through comparison of MT-treated females with control females using Qiagen premade Wnt signal pathway PCR array, we discovered 28 Wnt pathway genes significantly responsive to MT. Some of these genes has been reported to play important roles in early stage of EG development before sex differentiation, such as *Wnt5a*, *Ctnnb1*, *Lef1*, and *Lrp6* [43–45], and some of them were found to be expressed in developing EG, but no function study available, including *Fzd1* [46] and *Dkk1* [47]. *Ctnnb1* and *Fzd6* were upregulated more than threefold, and immunohistochemistry showed that β-catenin protein was strongly expressed in urethral epithelium of control male and MT-treated female GTs, but reduced expression in DHT-treated females was obvious. Although β-catenin protein is mainly localized in cytoplasm in developing GT during sex differentiation, but the stronger expression of the Wnt signaling nuclear effector protein LEF1 in control male and MT-treated female GTs suggested that low-dose MT, but not DHT in low dose, can promote canonical Wnt signaling in developing GT in female mice. FZD6 protein was expressed in urethral epithelium and mesenchyme adjacent to urethral closing region in control males and MT-treated females but was detected in urethral epithelium only in control female and DHT-treated female GTs. Multiple Fzd receptors, Lrp co-receptors, β-catenin co-activator *Bcl9*, and *Lef1* were upregulated in MT-treated females compared with control females. In addition, APC, a core component of the β-catenin destruction complex; Wnt signaling pathway inhibitor *Dkk1*; and Wnt antagonist *Sfrp1* were downregulated by MT treatment. *Ctnnb1*(β-catenin) has been discovered to play important roles in penile masculinization and *Sfrip1* and *Dkk2* were found to be differentially expressed in male and female developing EG during sex differentiation [32]. *Dkk2* was

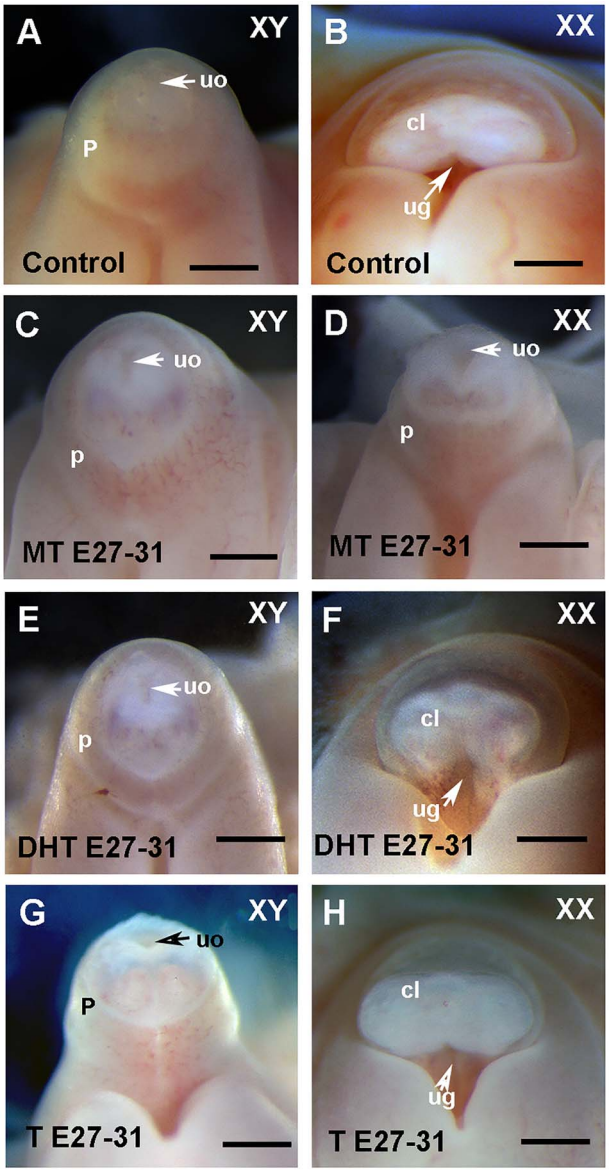

Figure 7. Low dose of MT, not DHT or T, in vivo treatment induced penile formation in female guinea pig embryos. All images are ventral views of E40 guinea pig GTs with distal (A, C, D, E, and G) or dorsal (B, F, and H) at the top. Arrows in (A–H) point to structures as indicated by adjacent labels. Sexes are indicated the same as Figure 1. (A, B) Corn oil (E27–E31, once every other day)-treated control male and female GTs. (C–H) MT (C and D), DHT (E and F), or T (G and H)-treated male and female GTs, respectively. All androgens were administrated at a dose of 1 mg/kg from E27–31, once every other day. *ug*, urethral groove; *cl, p,* and *uo* are the same as Figure 1. Scale bar: 1 mm.

not in our array list, but our data showed that *Dkk1* and *sfrip1* were downregulated and *Ctnnb1* was highly expressed in MT-treated female GTs, which was consistent with previous findings [32]. Most of differentially expressed genes we found have never been reported in EG development research; our results suggested that these genes may play roles in the patterning of external genital sexual differentiation, and our data revealed more androgen responsive candidate genes in masculinization of EG. It is interesting to note that many upregulated genes, such as Fzd class receptor genes (*Fzd2, Fzd3, Fzd4* and *Fzd6*), dishevelled segment polarity protein genes (*Dvl1* and *Dvl2*), and noncanonical Wnt signaling activator Wnt5a are known to play important roles in the planar cell polarity (PCP) pathway (noncanonical signaling pathway) during organogenesis [48]. *Fzd3, Fzd6, Dvl1,* and *Dvl2* are core PCP components; the PCP pathway governs cellular polarization and tissue movements and patterns and regulates the process of convergent extension in gastrulation, neural tube closure, and tubulogenesis in mammals [49, 50]. Our results indicate that Wnt/PCP signaling, as well as Wnt/β-catenin pathway, may play important roles in penile tubular urethral formation.

AR that promotes proliferation in mesenchymal cells of developing GT in males has been reported [10]. In this study, we discovered that in vivo MT-treated female and control male GTs showed increased cell proliferation, especially in dorsal portion of urethral epithelium before tubular urethra being closed, then the ventral part of the urethra disappeared gradually through programmed cell death, which revealed a new cellular mechanism of androgen-induced tubular urethral formation. In vivo low-dose DHT-treated females showed even less urethral epithelial and mesenchymal cell proliferation compared with control females. The results were consistent with a recent study in DHT-treated males [51]. Programmed cell death during tubular urethral formation was reported [52]; we had also found increased cell death at the junction of endoderm and ectoderm in male GT at E15.5 [10]. The programmed cell death in developing GTs of control males and MT-treated females was highly organized; the dying cells were only detected in ventral portion of urethral epithelium, but the dorsal part of urethral epithelium remained and formed the tubular urethra. How does steroid hormone signaling interact with local developmental genes to regulate this highly organized process needs further investigation.

Previous studies showed some differences in the tubular urethral formation process between human and mouse, and local 5-alpha reductase was considered unnecessary in mouse penile formation [3, 38]. We have shown that EG development in guinea pigs is similar to that of humans [2]; the results of in vivo MT, but not DHT, at certain doses that can induce tubular urethral and penile formation in both female mice and guinea pigs suggested that our discovery of the effects of androgens on inducing penile tubular urethral formation in mice maybe similar to that in guinea pigs, and these conclusions may also be applicable to humans.

Author contributions

Z.Z. and S.W. designed the project. S.W., J.L, and Z.Z performed experiments. S.W. and Z.Z. analyzed the results. S.W. and Z.Z. wrote the paper.

Conflict of interest

All authors declare that there is no conflict of interest.

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