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5α-Dihydrotestosterone negatively regulates cell proliferation of the periurethral ventral mesenchyme during urethral tube formation in the murine male genital tubercle

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SUMMARY

Androgen is an essential factor involved in masculinization of external genitalia. Failure of the exposure to 5α-dihydrotestosterone (DHT) causes a hypoplastic penile size and urethral abnormality. The main pathology of hypospadias is defective urethral closure on the ventral side of the penis. Hormone-dependent genes are suggested as the causative factors. However, the detailed mechanisms of DHT functions on urethral tube formation remain unknown. Androgen is both a positive and negative regulator of cell proliferation. The roles of locally converted DHT in cell proliferation at the periurethral mesenchyme have not been elucidated. We revealed the expression pattern of 5α-reductase type 2 mRNA (*Srd5a2*) and local DHT distribution by direct measurement in this study. We also analyzed periurethral mesenchymal cell proliferation status using systematic three-dimensional (3D) reconstruction analyses. A prominent *Srd5a2* expression and localized DHT distribution on the ventral side of the genital tubercle were detected. Cell proliferation was reduced in this mesenchymal region during urethral formation. The current results suggest the presence of the possible negative regulation of cell proliferation by DHT. Moreover, cell proliferation related to urethral tube formation was revealed to be DHT dose dependent. These data are expected to contribute to the understanding of the mode of regulation of cell proliferation related to urethral tube formation by DHT. These findings may also offer insight into the understanding of human hypospadias and related hormone-dependent factors.

INTRODUCTION

External genitalia are sexually differentiated organs in mammals. The morphologies of the anlage of external genitalia in early staged embryos are identical between males and females. It has been shown that such embryonic stages are hormone-independent (Georgas *et al.*, 2015). The primordial morphology of external genitalia dramatically changes in a hormone-dependent manner in late embryogenesis (Yamada *et al.*, 2003). Androgen is an essential factor involved in masculinization, i.e. prostate, epididymis, gubernaculum, and for testis development (Tanaka & Baba, 2005; Hinton *et al.*, 2011; Kaftanovskaya *et al.*, 2012). Penile development is one of the masculinization processes, which includes urethral formation.

Hypospadias is human birth defect characterized by abnormal penile and urethral formation. The incidence of hypospadias has been on the rise in the past three decades (Paulozzi *et al.*, 1997; Nissen *et al.*, 2015). The main pathology of hypospadias is defective urethral formation on the ventral side in the penis with its ectopic meatus located between the perineum and the glans of the penis. The causative factors of the hypospadias are reported as multiple factors, including genetic and environmental factors. Some of the causative factors are hormone-dependent genes, i.e. androgen receptor (*AR*), steroid 5 alpha-reductase 2 *(SRD5A2)*, *HSD17B3,* and *MAMLD1* (Kojima *et al.*, 2010). The exposure to environmental chemical disruptors is also suggested as one of the causes of congenital anomalies of the penis (Beleza-Meireles *et al.*, 2008; van der Zanden *et al.*, 2012). In fact, failure of the exposure to 5α-dihydrotestosterone (DHT) causes a hypoplastic penile size and urethral defects (Wilson *et al.*, 1993; Maimoun *et al.*, 2011; Zheng *et al.*, 2015). Testosterone (T) is locally converted into DHT by 5α-reductase type 2 encoded by the *SRD5A2* gene (Russell & Wilson, 1994). Penile development is dependent on the activities of DHT (Imperato-McGinley & Zhu, 2002). When rodent models were treated with a 5α-reductase

type 2 inhibitor, the affected pups displayed some types of urethral abnormalities (Anderson & Clark, 1990; Clark *et al.*, 1990). Thus, elucidating the role of DHT in urethral formation can contribute to the understanding of the etiology of hypospadias. However, the DHT functions, especially local actions, on urethral formation are not completely understood.

Androgen is both a positive and negative regulator of cell proliferation (Hinton *et al.*, 2011; Castoria *et al.*, 2014). Local androgens in prostate cancer have been suggested to regulate tumor growth (Rahman *et al.*, 2004; Montgomery *et al.*, 2008). However, the proliferation of AR-transfected PC-3 cells is blocked by DHT (Heisler *et al.*, 1997). Epithelial AR signaling may also negatively regulate prostate cancer cell proliferation (Niu *et al.*, 2010). In androgen-dependent embryonic stages in mouse, morphological change including urethral tubularization is visible at the ventral region of the male genital tubercle (GT; primordium of external genitalia) (Seifert *et al.*, 2008). It has been suggested that AR promotes internalization of the urethra on the ventral side of GT using conditional mouse models (Zheng *et al.*, 2015). However, the extent of mesenchymal cell proliferation and its correlation with urethral tube formation have not been elucidated. Moreover, whether locally converted DHT can regulate mesenchymal cell proliferation remains unknown. Hence, we measured directly local DHT distribution during urethral tube formation. We also analyzed the proliferation status of periurethral mesenchymal cells, the mesenchyme adjacent to the urethral plate, and the expression pattern of 5α-reductase type 2 mRNA (*Srd5a2*) by analyzing systematic three-dimensional (3D) reconstruction data. Mesenchymal cell proliferation was reduced during male GT urethral formation with a prominent *Srd5a2* expression. These results may give insight into the regulation of urethral development by locally produced DHT.

MATERIALS AND METHODS

Mouse

Time-mated C57BL/6J and ICR mice (CLEA, Tokyo, Japan) were used. Embryonic day 0.5 (E 0.5) was defined as noon on the day when a vaginal plug was detected. All procedures and protocols were approved by the Committee on Animal Research at Wakayama Medical University, Wakayama, Japan.

Direct androgen measurement by liquid chromatography–tandem mass spectrometry

For measurement of androgen levels in whole male genital tubercles (GTs), more than five GTs were collected and subsequently processed for one analysis. For measurement of the regional androgen levels, we subdivided GT into three parts, the dorsal-proximal, ventral (lower)-proximal, and distal parts. The corresponding 25 parts of GTs were collected and pooled for measurement. The samples were immediately frozen in liquid nitrogen after dissection. Testosterone and DHT levels were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The LC-MS/MS measurement protocol was performed as previously described (Arai *et al.*, 2011).

In situ hybridization for the gene expression analysis

Section in situ hybridizations for the gene expression analysis were performed as previously described (Haraguchi *et al.*, 2007). The antisense riboprobe templates for *Srd5a2* were obtained by standard reverse transcription polymerase chain reaction (RT-PCR) procedures utilizing primers F: 5′-GCCAGTTACGGGAAA CACAG-3′, R: 5′-GGAACAGACCAAGTGGCCAAAG-3′. Digoxigenin-labeled probes were prepared according to the manufacturer's instructions (Roche, Basel, Switzerland).

Cell proliferation analyses

Pregnant females were injected with 25 mg 5-ethynyl-2′-deoxyuridine (EdU) (A10044; Invitrogen, Carlsbad, CA, USA) per kg of body weight. One hour after injection, the embryos were collected. For organ culture, an EdU working solution (C100338; Invitrogen) was added to the medium for 2 h. The EdU concentration was 10 μM in the medium. EdU-positive cells were detected using an EdU labeling kit (C10337, C100338; Invitrogen). All procedures were conducted according to the manufacturer's instructions. The GT samples were embedded in paraffin and prepared in 6 μm serial sections to detect EdU. EdU-positive cells and the total cell number in the area defined around the urethral plate were counted by microscopic images from the GT histological sections (Fig. 3I). Six sections per analysis were used for statistical analyses.

Three-dimensional reconstruction for imaging analyses

Mouse embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline and dehydrated through methanol. The samples were embedded in paraffin and sectioned at 10-μm thickness. 3D reconstruction analyses were performed using a digital imaging software program (Amira5; FEI Visualization Sciences Group, Hillsboro, OR, USA) according to the manufacturer's instructions. The co-stained images of the *Srd5a2* expression and EdU staining were used for these analyses. The 3D reconstruction of these sections allowed a more comprehensive understanding of cell proliferation compared with ordinary section analyses. To clarify the distribution of *Srd5a2* mRNA and proliferating cells, six sections were used for coronal view analyses (Fig. S1).

Mouse genital tubercle organ culture

The GT organ culture was prepared as previously described (Haraguchi *et al.*, 2000). The mouse GTs for organ cultures were isolated from female embryos of ICR mice at E14.5. Dissected GTs were oriented with the ventral surface up on Millicell Cell Culture Insert (EMD Millipore, Billerica, MA, USA) in tissue culture dishes. The GTs were cultured in 10% charcoal/dextran-treated fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA) in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc., Kyoto, Japan) with 10 or 100 nM DHT (Sigma-Aldrich, St. Louis, MO, USA) or dimethylsulfoxide (DMSO) (Sigma-Aldrich) for 9 h. EdU was added to the medium and incubated for 2 h. GTs were subsequently analyzed.

Finasteride administration

To investigate the effect of reduced androgen levels on cell proliferation, pregnant C57BL/6J mice were treated with 100 mg/kg/day of finasteride (Sigma-Aldrich) dissolved in sesame oil (Kanto Chemical, Tokyo, Japan) by gavage daily in the morning from E13.5 to E14.5 or from E13.5 to E17.5 (Fig. 6A,

B). Control embryos were treated with sesame oil by the same protocol. E15.5 embryos were collected to analyze cell proliferation. E18.5 embryos were collected for histological analyses. The sections were stained with hematoxylin and eosin.

Statistical analyses

Statistical analyses were performed using Student's *t*-test or Welch's *t*-test, followed by the *F*-test. Values of $p < 0.05$ were judged as significant.

RESULTS

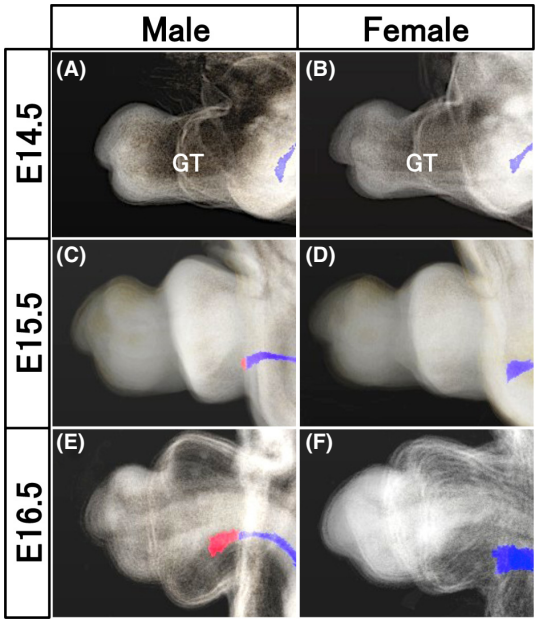
High concentration of DHT and *Srd5a2* expression at the ventral region of the male GT

In order to verify the distribution of androgens, the local androgen in the male genital tubercle (GT) was measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Testosterone (T) in the testis is detected from E14.5 (O'Shaughnessy *et al.*, 2006). Urethral tube formation starts at E15.5 from proximal to distal, and the proximal urethral closure completes until around E16.5 (Georgas *et al.*, 2015; Fig. 1A–F). T and 5α-dihydrotestosterone (DHT) were detected at E14.5 in the male GT (Fig. 2A; $n = 3$). The DHT concentration subsequently increased dramatically at E15.5, and such high concentration of DHT was maintained in the late embryonic stage. The level of T also increased from E15.5, albeit at a low level. DHT plays significant roles in urethral formation during embryonic GT masculinization (Suzuki *et al.*, 2015). We further investigated the regional androgen levels in three distinct GT regions. These regions corresponded to the dorsal-proximal, ventral (lower)-proximal, and distal parts of the male GT at E16.5 (Fig. 2B). Urethral tube formation occurs just at the ventral-proximal region of the male GT at such stage (Georgas *et al.*, 2015; Fig. 1E). The DHT concentration was higher in the ventral-proximal region compared with those of the other regions (Table 1). In contrast, the T level was lowest in the ventral-proximal region among the three regions. These results indicate that the distribution of DHT is not uniform, but restricted in specific regions of the male GT. Because the DHT concentration was high in the ventral-proximal region of the male GT, we next investigated the expression patterns of 5α-reductase type 2 mRNA (*Srd5a2*) in males and females. There were sexual differences in *Srd5a2* expression at E14.5 and E15.5, especially in the ventral region of the GT (Fig. 2C–J: black arrows, Fig. 3A–H: yellow cells). A prominent *Srd5a2* expression was detected in the ventral-proximal region of the male GT at E14.5 (Figs 2C,E & 3A,C). This expression spread toward the distal part at E15.5 compared with those at E14.5 in males (Figs 2C, G & 3A,E). On the other hand, a significantly reduced *Srd5a2* expression was detected in the ventral region of the female GT (Figs 2D,F,H,J & 3B,D,F,H).

High level of the *Srd5a2* expression with decreased cell proliferation was detected in the ventral periurethral mesenchyme of the male GT

To verify the possibility that DHT regulates cell proliferation in GT, we investigated the *Srd5a2* expression and performed 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay using 3D analysis. The periurethral mesenchyme locates adjacent to the urethral plate which is the endodermal embryonic structure that subsequently forms the urethra. A decreased number of EdU-positive

Figure 1 Sexual differences in urethral tube formation in the genital tubercle (GT) by three-dimensional (3D) reconstruction images. (A, B) An anatomical difference between the male and female GTs is not prominent at E14.5. (C, D) The tubular urethra in the GT is formed at the base of the GT in males at E15.5. (E, F) The sexual difference in the tubular urethral length in the GT is prominent at E16.5. Blue regions, urethra in pelvic region. Red regions, tubular urethra in the GT.



cells were observed in the *Srd5a2*-expressing mesenchymal cells (Fig. 3C,G: Note the fewer green cells in yellow-marked cells). Fewer EdU-positive cells were detected in the ventral periurethral mesenchyme of the male GT compared with the female GT (Fig. 3C,D,G,H: green cells). The ratios of EdU-positive cells per total cell number in the ventral periurethral region are shown in Fig. 3I ($n = 4$). Significant differences between male and female GTs were detected at E15.5. The region of the mesenchyme containing such fewer numbers of proliferating cells also showed prominent androgen receptor (AR) expression in males (Fig. 4A: squares). These results indicate possibly negative regulation on cell proliferation by DHT in the periurethral mesenchyme.

DHT dose-dependent inhibition of cell proliferation in the periurethral mesenchyme

To verify the extent of DHT-dependent cell proliferation, we next analyzed the effect of DHT on cell proliferation using a GT organ culture system. DHT was added into the medium of the female GT culture at E14.5. The number of proliferating cells in the periurethral mesenchyme was reduced compared with the control (Fig. 5). A higher concentration of DHT (100 nM) prominently suppressed cell proliferation in the periurethral mesenchyme compared with a lower dose of DHT (10 nM; Fig. 5B, C). These results suggest that DHT may negatively regulate periurethral mesenchymal cell proliferation in a dose-dependent manner.

We recently established finasteride-induced hypospadias mouse model. Finasteride is a 5α-reductase type 2 inhibitor. The DHT concentration in GT is reduced by finasteride treatment (Suzuki *et al.*, 2015). Finasteride-treated male GTs showed a DHT dose-dependent reduction in the tubular urethral length between the glans of the penis and perineum. To further

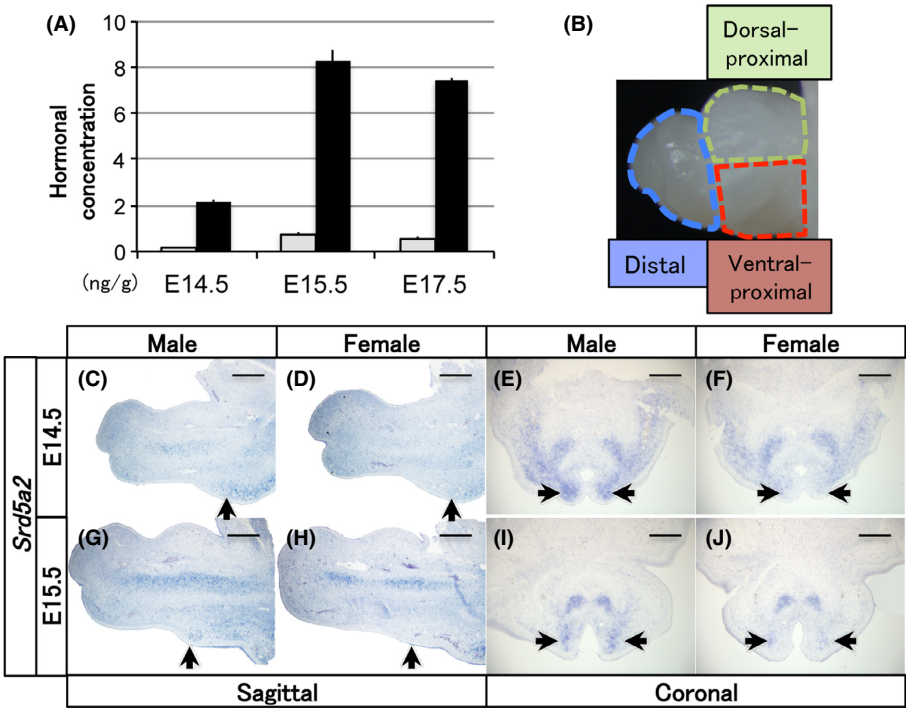


Figure 2 Measurement of local androgens and expression pattern of 5α-reductase type 2 mRNA (*Srd5a2*). (A) Concentration of T and 5α-dihydrotestosterone (DHT) in the GT measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Gray bars, T. Black bars, DHT. (B) Definition of three regions for measuring androgens: dorsal–proximal, ventral–proximal, and distal regions. (C–J) Expression of *Srd5a2* in male and female GTs at E14.5 (C–F) and E15.5 (G–J). Black arrows indicate male dominant regions of *Srd5a2* expression. Scale bars are 100 μm (C, D, G, H) and 50 μm (E, F, I, J).

Table 1 Concentration (ng/g) of T and DHT in the male GT subregions (E16.5)

	T	DHT
Distal	0.77	5.18
Dorsal–proximal	1.13	6.25
Ventral–proximal	0.49	10.83

T, testosterone; DHT, 5α-dihydrotestosterone; GT, genital tubercle.

investigate DHT-mediated regulation of cell proliferation, we analyzed periurethral mesenchymal cell proliferation during urethral formation in the finasteride-treated male GT. Although the tubular urethra is formed in the proximal region in the finasteride-treated group, its length was significantly decreased compared with the control group at E18.5 (Fig. 6C,D). The tubular urethra was not formed in female GT at this stage (Fig. 6E). A significant level of DHT has not been detected in the female mouse GT (Suzuki *et al.*, 2015). The number of EdU-positive cells was higher in the periurethral mesenchyme in the finasteride-treated group compared with the control group at E15.5 (Fig. 6F,G,I). Moreover, cell proliferation of female samples was increased compared with finasteride-treated male samples (Fig. 6G,H,I). Hence, normal GTs containing a longer urethral length at E18.5 displayed lower cell proliferation in the ventral periurethral mesenchyme at E15.5. These results indicate that the presence of DHT dose-dependent cell proliferation in the male GT is associated with urethral formation.

DISCUSSION

We observed that DHT negatively regulates periurethral mesenchymal cell proliferation. The regulation of cell proliferation is one of the major functions of androgens. It is well-known that the addition of androgen, especially DHT, stimulates prostate growth (Takeda *et al.*,1986; Andersson *et al.*, 1991). Androgen can function as both a negative and positive regulator in prostate cancer (Niu *et al.*, 2010; Sakamoto & Kyprianou, 2010). It has been suggested that androgen positively regulates cell proliferation of the androgen-dependent human prostate cancer cell line LNCaP at low concentrations, whereas cell proliferation is suppressed by administering a high level of androgen (Olea *et al.*, 1990; Soto *et al.*, 1995). Because of such positive and negative regulation, an investigation of the dynamics and local distribution of DHT in association with the extent of cell proliferation is essential. Nevertheless, the production and dynamic distribution of locally synthesized androgen, DHT, has been unclear in embryonic reproductive organ formation, including the GT. We revealed that a high level of DHT was locally produced, and its distribution was dynamically different spatiotemporally in the male GT. A prominent *Srd5a2* expression related to locally and highly concentrated DHT was revealed in the ventral region of the GT. These findings suggest that local DHT may suppress cell proliferation. This ventral region is in fact suggested to be responsible for urethral tube formation, the midline fusion of the urethra. Although further detailed analyses are required, current results indicated the presence of negative regulation of mesenchymal cell proliferation by DHT, which contributed to male embryonic tubular urethral formation.

The critical phase of androgen activity for GT masculinization has been suggested to function from E13.5 to E16.5 in previous studies (Miyagawa *et al.*, 2009; Zheng *et al.*, 2015). A hypospadias-like phenotype, which is characterized by defects of the tubular urethra in the male mouse GT, was suggested to be caused by the disruption of androgen signaling in this phase. It has been suggested that cell proliferation of the whole GT in male embryos significantly increases at the late embryonic stage compared with those of females (Zheng *et al.*, 2015). We have previously reported that the formation of sexual differences in the GT length is visible from the late stage (E18.5; Suzuki *et al.*, 2015). In contrast, such differences in urethral tube formation are visible from the earlier stage (E16.5). Moreover, the urethral

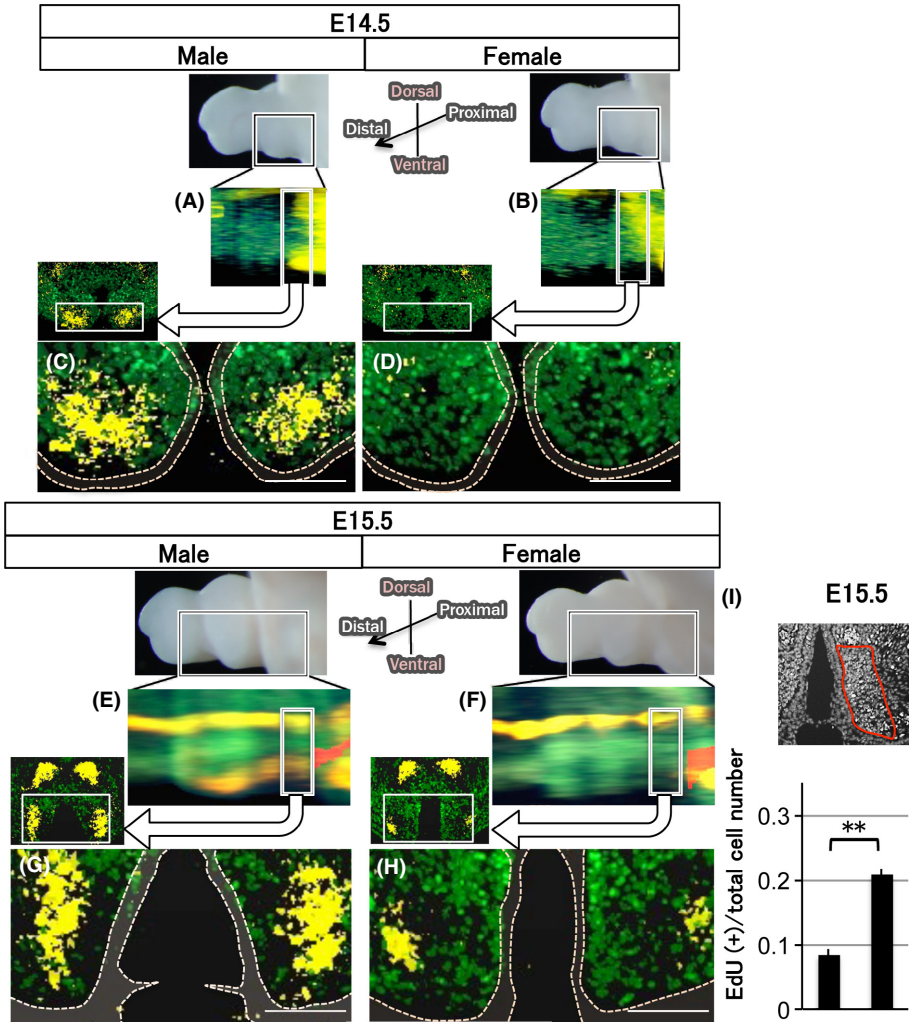


Figure 3 Sexual differences in cell proliferation and *Srd5a2* expression pattern on the ventral side of the genital tubercle (GT) at E14.5 and E15.5. (A, B, E, F) Sagittal 3D reconstruction images. (C, D, G, H) Coronal 3D reconstruction images on the ventral side of the GT. Yellow, *Srd5a2*-expressing cells. Green, EdU-positive cells. Red regions, urethra. Dotted lines, epithelium. Scale bars are 50 μm. (I) Ratio of EdU-positive cell numbers per total cell numbers inside the areas marked by red lines. Left bar, male. Right bar, female. Error bars represent SEM. $^{**}p < 0.001$.

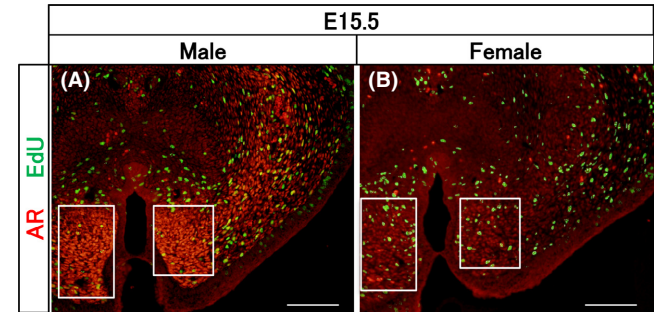


Figure 4 Double staining of androgen receptor (AR) and EdU in the male and female genital tubercles (GTs) at E15.5. (A, B) Coronal sections of the GT. Red, AR. Green, EdU-positive cells. Squares, periurethral mesenchyme. Scale bars are 100 μm.

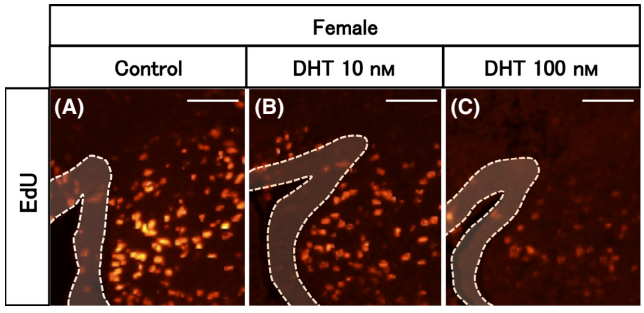


Figure 5 5α-Dihydrotestosterone (DHT) dose-dependent reduction in cell proliferation in female genital tubercles (GTs). (A–C) The EdU-positive cells in the periurethral mesenchyme in GTs cultured with 10 or 100 nM of DHT. Orange signals, EdU-positive cells. Dotted lines, epithelium. Scale bars are 50 μm.

masculinization process is sensitive to the actions of DHT. The inhibition of DHT production augmented mesenchymal cell proliferation at E15.5 with abnormal urethral formation in this study. These findings suggest that different sensitivities and/or downstream genes of DHT may be involved in GT length regulation and urethral tube formation. Moreover, the local expressions of AR and several androgen responsible genes, such as *Mafb*, β-*catenin*, and *Dkk2*, are detected in the periurethral region (Miyagawa *et al.*, 2009; Suzuki *et al.*, 2014). Thus, further experiments focusing on the regulatory mechanisms of cell proliferation by DHT may provide new information about androgen signaling. Further investigations into the cells possessing different sensitivities to androgen and analyses on mesenchymal cell proliferation in conditional knockout mouse models are necessary.

Abnormal urethral formation is the main pathological characteristic of human hypospadias. There are several degrees of urethral closure defects in human hypospadias, including glanular,

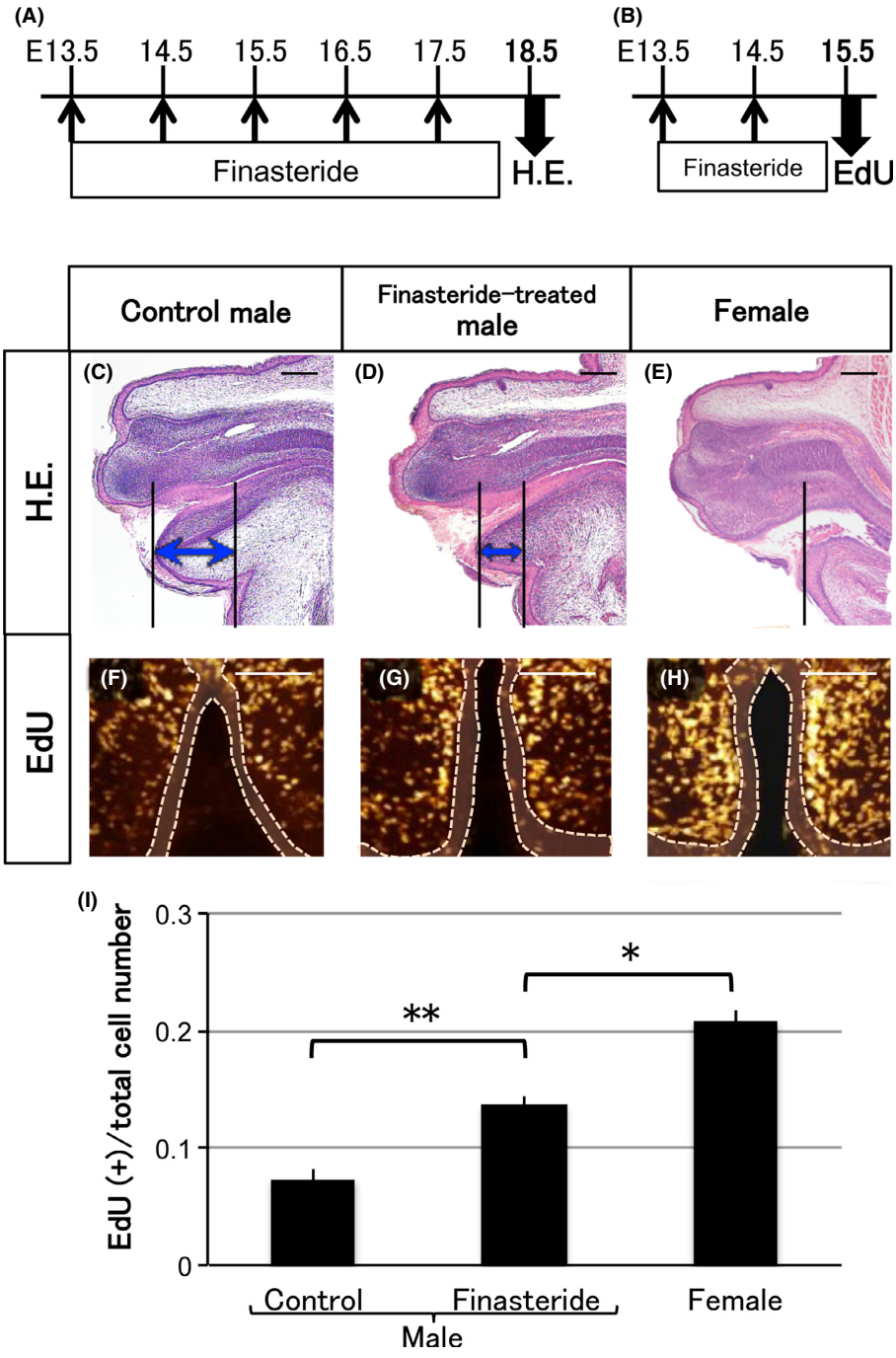


Figure 6 Augmented cell proliferation in the male genital tubercle (GT) by finasteride treatment. (A, B) Experimental schematic diagram of finasteride administration to pregnant mice. (C–E) Sagittal hematoxylin and eosin (H.E.) sections of the GT at E18.5. Blue arrows indicate the length of the tubular urethra in the GT. Scale bars are 200 μm. (F–H) Coronal 3D reconstruction images on the ventral side of the GT at E15.5. Yellow signals represent EdU-positive cells in the periurethral mesenchyme. Dotted lines, epithelium. Scale bars are 50 μm. (I) Ratio of EdU-positive cell number per total cell number. Error bars represent SEM. $*p < 0.005$. $**p < 0.001$.

penile, penoscrotal, scrotal, and perineal types (Kojima *et al.*, 2010; Cunha *et al.*, 2015). By utilizing finasteride-treated mice as a hypospadias model, we showed that mesenchymal cell proliferation was negatively regulated by androgen, and such inhibition was necessary to form the male urethra. The severity of the hypospadias-like phenotype was dependent on the local concentration of DHT in the ventral GT mesenchyme in this model (Suzuki *et al.*, 2015). Such defective regulation of cell proliferation may lead to various urethral closure abnormalities in human hypospadias. In previous researches of the hypospadias-like phenotype in mouse models, the presence of a urethral tube defect was the focus of discussion. To the best of our knowledge, this is the first study to present various degrees of such phenotypes with corresponding degrees of cell proliferation and local androgen. These findings may contribute to the understanding of different types of human hypospadias and the downstream causative hormonal factors involved in hypospadias.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Overview of the 3D reconstruction analysis.