Mesenchymal actomyosin contractility is required for androgen-driven urethral masculinization in mice

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The morphogenesis of mammalian embryonic external genitalia (eExG) shows dynamic differences between males and females. In genotypic males, eExG are masculinized in response to androgen signaling. Disruption of this process can give rise to multiple male reproductive organ defects. Currently, mechanisms of androgen-driven sexually dimorphic organogenesis are still unclear. We show here that mesenchymal-derived actomyosin contractility, by MYH10, is essential for the masculinization of mouse eExG. MYH10 is expressed prominently in the bilateral mesenchyme of male eExG. Androgen induces MYH10 protein expression and actomyosin contractility in the bilateral mesenchyme. Inhibition of actomyosin contractility through blebbistatin treatment and mesenchymal genetic deletion induced defective urethral masculinization with reduced mesenchymal condensation. We also suggest that actomyosin contractility regulates androgen-dependent mesenchymal directional cell migration to form the condensation in the bilateral mesenchyme leading to changes in urethral plate shape to accomplish urethral masculinization. Thus, mesenchymal-derived actomyosin contractility is indispensable for androgen-driven urethral masculinization.
Androgens are steroid hormones that are essential for the masculinization of the male reproductive tract such as the external genitalia, epididymis, and other organs. 

Defects of androgen signaling lead to various developmental defects in male-type sexually characteristic organogenesis. However, the molecular mechanisms underlying androgen signaling and how these regulate sexually dimorphic organogenesis are still unclear.

The development of mammalian external genitalia gives rise to sexually dimorphic structures, the male and female external genitalia. Embryonic external genitalia (eExG, also known as genital tubercle) develop as an outgrowing organ. During eExG outgrowth, the urethral plate epithelium (UPE) forms continuously from the proximal to the distal region of the eExG within the midline region. During development of the eExG in response to androgen signaling, the UPE forms a male-specific tubular urethra (hereafter designated as urethral masculinization). Disruption in androgen signaling gives rise to congenital anomalies including defects of urethral formation or hypospadias. Hypospadias encompasses phenotypes involving ventral ectopic urethral openings. The etiology of defects of urethral formation is likely to be multifaceted, involving multiple genetic anomalies (Supplementary Table 1). Thus, urethral masculinization in the mouse embryonic external genitalia provides a unique model to study the mechanisms of androgen-induced sexually dimorphic organ development.

Previously, we identified that androgen signaling in the mesenchyme adjacent to the UPE (hereafter designated as bilateral mesenchyme) is required for urethral masculinization. Several sexually dimorphic genes such as Mafb (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B), β-catenin, and Srd5α2 (5α-reductase type 2 mRNA) are expressed in male bilateral mesenchyme under androgen signaling. Furthermore, knockout mice for Mafb and β-catenin in the mesenchyme show urethral developmental defects. Additionally, it was reported that mesenchymal F-actin shows sexually dimorphic expression pattern in the eExG bilateral mesenchyme.

Actomyosin is a cytoskeletal system composed of an F-actin network bound to the motor protein nonmuscle myosin II. Nonmuscle myosin heavy chains, which are essential components of nonmuscle myosin II, exist in three isoforms (NMHCIIA, NMHCIB, and NMHCIC) with each isoform encoded by the Myh9, Myh10, and Myh14 genes respectively. Mechanical force is generated through the ATP-dependent contraction of nonmuscle myosin II which is then transmitted to the F-actin network and through cell–cell and cell-ECM interactions. Actomyosin contractility plays significant roles in various cellular processes such as cell adhesion and cell migration. Regulation of these cell processes by actomyosin is a driving mechanism in tissue morphogenesis. Actomyosin contractility also regulates changes in epithelial cell shape, which facilitates organogenesis. In the development of the neural tube, epithelia-derived actomyosin contractility induces apical constrictions, which results in the folding of the neural plate epithelium. Furthermore, epithelial actomyosin contractility by Myh9 and Myh10 elicits tissue fusion during organogenesis. Thus, the involvement of actomyosin contractility in shaping organogenesis has long been reported but is usually highlighted in the epithelia rather than mesenchyme. Currently, the contribution of actomyosin contractility in mesenchyme during organogenesis is not well understood.

In this study, we investigated the role of actomyosin during urethral masculinization. Intriguingly, Myh10 was expressed prominently and actomyosin contractility was increased in the condensed mesenchyme of the male eExG in an androgen-dependent manner during urethral masculinization. To analyze mesenchymal-derived actomyosin contractility, we established a slice culture system to recapitulate mouse embryonic urethral formation in vitro. Both mouse genetic study and chemical inhibitor experiments using the slice culture system suggest that actomyosin contractility is required for androgen-driven urethral masculinization. We thus report here a unique role for mesenchymal-derived actomyosin contractility for sexually dimorphic organogenesis.

Results

Dynamic urethral masculinization processes of mouse eExG.

During urethral masculinization of male eExG at E16.5, the UPE showed morphological changes characteristic of tissue fusion (Fig. 1), such as removal of midline epithelial structures and mesenchymal confluence. Formation of a male-specific tubular urethra (urethral masculinization) occurs proximally to distally during eExG development (Fig. 1a–d). The UPE curved medially and became thinner at the fusion site (Fig. 1b; blue arrow), eventually disappearing from the midline of the eExG (Fig. 1a). Disappearance of the UPE at the fusion site led to the confluence of the bilateral mesenchyme at the mid-ventral region of the eExG. Remaining UPE subsequently developed into the tubular urethra (Fig. 1a; yellow arrow). Confluent bilateral mesenchyme ventral to the tubular urethra differentiated simultaneously into a mesenchymal seam (Fig. 1a; red arrow). In female eExG, the UPE did not curve medially throughout its development (Fig. 1e–h).

SALL1 was found to be broadly expressed in both male and female mesenchyme adjacent to the urethral plate (Fig. 1i, n). On the other hand, MAFB, which is an essential sexualization gene for urethra, was strongly expressed in the male bilateral mesenchyme but not in the female mesenchyme (Fig. 1j, o). Expression of SALL1 overlapped with MAFB in the bilateral mesenchyme (Fig. 1k, l). Thus, we defined SALL1 as a marker indicating the bilateral mesenchyme in both male and female eExG. Intriguingly, the SALL1-expressing bilateral mesenchyme in male eExG condensed prominently at the fusion site during urethral masculinization (Fig. 1b; square, c, l). On the other hand, female eExG retained the uncondensed bilateral mesenchyme (Fig. 1f; square, g, q). We subsequently investigated the expression of the cell–cell adhesion marker, N-CADHERIN. N-CADHERIN has been previously reported to be necessary for cell condensations. N-CADHERIN was expressed prominently in male mesenchymal condensed region but not in female mesenchyme (Fig. 1m, r). Thus, the bilateral mesenchyme condenses close to the fusing UPE, which suggests its role for urethral masculinization.

Sexually dimorphic MYH10 expression in eExG mesenchyme.

Previously, we reported that androgen regulates sexually dimorphic F-actin assemblies in the genital organogenesis. Thus, we investigated further on actomyosin during urethral masculinization. One of the components of actomyosin, MYH10, was expressed prominently in the male SALL1-expressing bilateral mesenchyme at E16.5 while female eExG showed lower expression (Fig. 2a–d). In contrast, MYH9 was expressed mainly in the UPE and ectodermal epithelia without showing sexual dimorphism in E16.5 eExG. MYH9 also showed weak expression in the bilateral mesenchyme (Supplementary Fig. 1). Phosphorylation of the myosin light chain (p-MLC) is essential for the increased contractility of actomyosin indicating its role in force generation. Male eExG showed higher p-MLC expression in the bilateral mesenchyme and urethral epithelia near the fusion site (Fig. 2i–l) compared to female eExG at E16.5 (Fig. 2m–p). We further investigated the onset of MYH10 sexually dimorphic expression during urethral masculinization. The MYH10
expression pattern appeared similar between male and female 
eExG mesenchyme at E14.5 (Fig. 2g, h). MYH10 expression 
became higher in male eExG mesenchyme starting at E15.5 
when urethral masculinization can be first observed (Fig. 2e, f). 
These results suggest a correlation between the spatiotemporal 
expression of MYH10 and the occurrence of sexually dimorphic 
development of the eExG.

Establishment of a eExG slice culture system. To analyze the 
mechanism of urethral masculinization efficiently, we established 
a new eExG slice culture system (Fig. 3a, see Methods for details). 
eExG slices showed urethral tube-like structure in both male 
and female slices after 48 h with 10−8 M 5α-Dihydrotestosterone 
(DHT), which is a major androgen for the masculinization of 
external genitalia (Fig. 3i, j, s, t; yellow arrow). Further histological 
alyses clearly showed the urethral tube structure (Fig. 3k, u; yellow arrow) and mesenchymal seam (Fig. 3k, u; red arrow) in 
DHT-treated slices. In contrast, no tube-like structures were 
observed in both male and female eExG slices cultured without 
DHT (Fig. 3d–f, n–p).

Each specimen was scored by five stages (Score 0 to Score 4) 
according to histological observations by hematoxylin and eosin 
(H&E) staining and whole mount images of eExG slices (see 
Methods for details, Supplementary Fig. 2). Such quantification of 
fusion revealed that 43.6% (a total of 39 eExG slices) of DHT-
treated male eExG slices developed a tubular urethra and a 
mesenchymal seam (Score 3 and 4). On the other hand, DMSO-
treated male eExG slices showed no visible tubularization.

Similar to male eExG slices, 33.3% (a total of 30 eExG slices) of 
DHT-treated female eExG slices developed a tubular urethra 
and mesenchymal seam in contrast to DMSO-treated female 
eExG slices (Table 1, Supplementary Data, Fig. 3, Supplementary 
Fig. 2). A significant association between the presence of DHT 
during culture and urethral masculinization was observed for 
both male (n = 54, p = 0.002) and female eExG slices (n = 62, 
p = 0.0019). Confocal live imaging analyses further confirmed 
that male eExG slices cultured with DHT underwent masculini-
ization with the formation of tubular urethra from the UPE 
(Supplementary Movie 1). All DHT-treated eExG slices showed 
increased condensation of the bilateral mesenchyme compared to 
DMSO-treated eExG slices. Additionally, DHT-treated eExG 
slices showed induced expression of the androgen-responsive 
genes, AR, MAFB and β-CATENIN (Supplementary Fig. 3). E-
CADHERIN was expressed in eExG urethral and ectodermal 
epithelia suggesting the retention of the epithelial structural 
integrity (Supplementary Fig. 4). These results indicate that the 
current eExG slice culture system recapitulates the androgen-
driven embryonic urethral masculinization. Thus, we used this 
system to further analyze the contribution of the eExG 
mesenchyme to urethral masculinization.

Blebbistatin inhibits androgen-driven urethral masculiniza-
tion. We further analyzed the functional relevance of actomyosin 
contractility in androgen-driven urethral masculinization using 
males eExG slices treated with the nonmuscle myosin II inhibitor, 
blebbistatin (Fig. 4; Table 2, Supplementary Data). In control
masculinization.

Mesenchymal Myh9/10 is required for urethral masculinization. We examined the role of mesenchymal actomyosin contractility by utilizing the Sall1CreERT2/+ driver mice.
(Sall1 CreERT2) to delete MYH10 expression in the eExG bilateral mesenchyme (Supplementary Fig. 6a). Sall1CreERT2/+; Myh10lox/lox knockout mice (hereafter designated as Sall1 CreERT2 Myh10 DKO) did not show severe defects in urethral masculinization indicating functional compensation by MYH9 (Supplementary Fig. 7). These results prompted us to investigate Sall1CreERT2/+; Myh9lox/lox; Myh10lox/lox double knockout mice (hereafter designated as Sall1 CreERT2 Myh9/10 DKO). Sall1 CreERT2 Myh9/10 DKO mice showed defective urethral masculinization with unfused ventral cleft (Fig. 5b, e). Additionally, the UPE did not curve medially in the Sall1 CreERT2 Myh9/10 DKO mice (Fig. 5e, compared with Fig. 1b). In these mice, the mesenchyme also showed reduced condensation and N-CADHERIN expression, similar to the blebbistatin-treated eExG slices (Fig.5h, k, n). Since the Sall1 CreERT2 driver mice showed mosaic Cre recombinase expression in the urethral plate (Supplementary Fig. 6a), we investigated the phenotype of knockout mice using the ShhCreERT2 driver mice. In these mutant mice, Cre recombinase was expressed specifically in the UPE (Supplementary Fig. 6b). ShhCreERT2/+; Myh9lox/lox; Myh10lox/lox double knockout mice (hereafter designated as Shh CreERT2 Myh9/10 DKO) did not show obvious defects in urethral masculinization (Fig. 5c, f) and mesenchymal condensation (Fig. 5i, l). Moreover, their mesenchymal N-CADHERIN expression was unaffected by Myh9 and Myh10 deletion in the UPE (Fig. 5o). These results strongly suggest that mesenchymal-derived rather than epithelia-derived actomyosin contractility regulates urethral masculinization.

Finally, we investigated whether mesenchymal actomyosin contractility is dependent on androgen signaling, we cultured eExG slices in the presence or absence of DHT. DHT-treated male eExG slices displayed increased expression of MYH10 (Fig. 6c, d) and p-MLC (Fig. 6l–n). In contrast, DMSO-treated
After 48 h of culture showing defects in mesenchymal condensation. Magnified view of bilateral mesenchyme. Scale bars in a = 300 µm. Scale bar in e = 20 µm

![Image 1](https://example.com/image1.png)

**Table 2** Incidence of urethral masculinization inhibition in response to blebbistatin treatment

| Blebbistatin | Score 4 | Score 3 | Score 2 | Score 1 | Score 0 | TOTAL |
|--------------|---------|---------|---------|---------|---------|-------|
| 0 µM (+DHT)  | 9       | 1       | 0       | 3       | 5       | 18    |
| 100 µM (+DHT)| 1       | 3       | 5       | 11      | 1       | 21    |

Fig. 4 Defective androgen-driven urethral masculinization by blebbistatin treatment. a–e Control male eExG slice (n = 18 eExG slices; Table 2) and f–j blebbistatin-treated male eExG slice (n = 21 slices; Table 2) before culture and b, c, g, h after 48 h culture with DHT. c, h Magnified view showing urethral morphology of control male eExG slice and h blebbistatin-treated male eExG slice at 48 h. d, e, i, j H&E staining and k, l, o, p MAFB immunostaining of d, e, k, l control male eExG slice (n = 4 eExG slices) and i, j, o, p blebbistatin-treated male eExG slice (n = 4 eExG slices) after 48 h culture showing defects in mesenchymal condensation. m, n, q, r N-CADHERIN expression in m, n control male eExG slice (n = 3 eExG slices) and q, r blebbistatin-treated male eExG slice (n = 3 eExG slices) after 48 h culture. Dashed lines; epithelial-mesenchymal border. Squares in d, i, k, o, m, q; magnified view of bilateral mesenchyme. Scale bars in a = 300 µm. Scale bar in e = 20 µm

Male eExG slices showed lower MYH10 (Fig. 6a, b) and p-MLC expression (Fig. 6i–k) after 48 h of culture. The expression of MYH10 (Fig. 6e, f) and p-MLC (Fig. 6o–q) was low in the female bilateral mesenchyme. These expressions were induced in the mesenchyme of female eExG slices after DHT treatment (Fig. 6g, h, r–t). These results suggest that mesenchymal-derived actomyosin contractility in the eExG is androgen-dependent.

**Discussion**

Actomyosin contractility is a highly conserved mechanism underlying cell adhesion, cell migration, cell shape and tissue morphogenesis. Regulation of cell adhesion and cell migration contributes to different processes of organogenesis. Previous reports on the role of actomyosin contractility during embryogenesis focused on the contribution of epithelial non-muscle myosin II. In palatal tissue fusion, epithelia-derived actomyosin contractility regulates the removal of medial epithelial structures, which leads to subsequent confluence of the palatal mesenchyme. In this study, we report a mechanism wherein mesenchymal-derived actomyosin contractility can also contribute to the removal of the midline epithelia during tissue fusion in the urethral masculinization of the eExG. Disruption in androgen signaling leads to congenital anomalies including hypospadias which encompasses phenotypes involving ventral ectopic urethral openings. Previously, we showed that mesenchymal androgen signaling is essential for urethral masculinization by using Sall1 CreERT2 driver mice to ablate androgen receptor function. In the Sall1 CreERT2 driver mouse model, Cre recombinase is expressed in the bilateral mesenchyme. MYH10 and p-MLC were expressed prominently in the male bilateral mesenchyme in an androgen dependent manner. Furthermore, Sall1 CreERT2 Myh9/10 DKO mice showed defects in urethral masculinization. These results indicate that androgen-induced, mesenchymal-derived actomyosin contractility is essential for urethral masculinization. Actomyosin contractility thus represents a process for the regulation of sexually dimorphic organogenesis.

We identified that the urethral masculinization of the eExG was characterized by the androgen-driven condensation of the bilateral mesenchyme. Mesenchymal condensations have been observed during the development of a wide range of organs such as tooth, feather buds, gut villi, ear, and limb. Mechanical dynamics arising from actomyosin-regulated mesenchymal condensations have been implicated in changes in the architectures of adjacent epithelia leading to tissue folding. Different patterns of mesenchymal condensations can induce various types of tissue folding. Mathematical modeling for mesenchymal-epithelial interactions also reveals that mesenchymal-generated mechanical force is sufficient to induce
changes in epithelial shape. The mesenchymal condensation in
the male eExG may exert force on the UPE leading to initial
medial curvature and eventual removal of the UPE. In the current
study, loss of mesenchymal condensation due to actomyosin
inhibition resulted in reduced epithelial curvature leading to
defects of urethral masculinization. Proper regulation of cell
proliferation is generally required for organogenesis. Androgen
regulates negatively cell proliferation in bilateral mesenchyme
during urethral masculinization. This androgen-mediated
regulation of cell proliferation and other cell behaviors such
as migration and condensation are likely to play roles during
urethral masculinization. However, blebbistatin treatment did
not cause prominent differences in cell proliferation (Supple-
mentary Fig. 8). Our results strongly suggest that cell migration
and the formation of mesenchymal condensation by androgen-
induced actomyosin contractility is a sexually dimorphic process
to regulate morphogenesis of the urethral epithelia during
urethral masculinization. Previous studies have reported that
androgen can regulate myosin heavy chain expression in muscle
tissue such as cardiomyocytes. However, this study is the
first report, to our knowledge, to show the sexually dimorphic
expression pattern of MYH10 and p-MLC during organogenesis.
Currently, it is unclear how androgen regulates actomyosin
contractility. One possible mechanism is through the RhoA
(Ras homolog gene family, member A)-ROCK (Rho-associated
protein kinase) pathway which is a major regulatory mechanism
for actomyosin contractility. It has been reported that RhoA
is a mediator of androgen-regulated migration in prostate cancer

Fig. 5 Mesenchymal deletion of Myh9 and Myh10 induces defective urethral masculinization with unfused ventral cleft in the male eExG. Whole images of E18.5 male eExG. Control eExG (Myh9lox/lox; Myh10lox/lox), Sall1 CreERT2 Myh9/10 DKO eExG and Shh CreERT2 Myh9/10 DKO eExG. H&E staining of E16.5 male eExG. Control eExG, Sall1 CreERT2 Myh9/10 DKO eExG (n = 31 eExG) and Shh CreERT2 Myh9/10 DKO eExG (n = 15 eExG). MAFB immunostaining of E16.5 male eExG showing mesenchymal condensation in control eExG (n = 3 eExG), Sall1 CreERT2 Myh9/10 DKO (n = 3 eExG) and Shh CreERT2 Myh9/10 DKO eExG (n = 4 eExG) and N-CADHERIN expression in control eExG (n = 4 eExG) and Shh CreERT2 Myh9/10 DKO eExG (n = 3 eExG). Tamoxifen (200 mg/kg body weight) was administered to pregnant mice at E9.5. Dashed lines; epithelial-mesenchymal border. Squares in g–i; magnified view of bilateral mesenchyme. Scale bar in a = 500 µm, scale bar in d = 100 µm, scale bar in g = 50 µm, scale bars in d = 20 µm.
Fig. 6 Androgen responsiveness of actomyosin in the bilateral mesenchyme of male and female eExG slices. a–h MYH10 expression in SALL1-expressing bilateral mesenchyme of a, b DMSO-treated male eExG slice (n = 8 eExG slices), c, d DHT-treated male eExG slice (n = 8 eExG slices), e, f DMSO-treated female eExG slice (n = 10 eExG slices) and g, h DHT-treated female eExG slice (n = 7 eExG slices) after 48 h culture. Magnified view of representative parts of the SALL1-expressing bilateral mesenchyme showing increased MYH10 expression after DHT treatment in both d male and h female eExG slices. i–t Confocal images showing p-MLC expression in SALL1-expressing bilateral mesenchyme of i–k DMSO-treated male eExG slice (n = 3 eExG slices), l–n DHT-treated male eExG slice (n = 3 eExG slices), o–q DMSO-treated female eExG slice (n = 4 eExG slices) and r–t DHT-treated female eExG slice (n = 4 eExG slices) after 48 h culture. Magnified view of representative parts of the SALL1-expressing bilateral mesenchyme showing increased p-MLC expression after DHT treatment in both m, n male and s, t female eExG slices. Dashed lines; epithelial–mesenchymal border. Squares; representative areas of the SALL1-expressing bilateral mesenchyme. Asterisks in k, n, q; fluorescent signal from non-bilateral mesenchyme (vasculature). Scale bars in a, i = 50 µm, scale bars in b, j = 25 µm

Fig. 7 Androgen induces actomyosin contractility, which condenses eExG bilateral mesenchyme and regulates morphogenesis of the urethral epithelia structure during urethral masculinization.
N-CADHERIN expression in kidney metanephric mesenchyme deletion also impairs cell adhesions in vivo urethral masculinization while useful for the analysis of gene expression and epithelial organogenesis. However, previous organ culture systems have been established to study different organ culture systems have been established to study further analyze effects of androgens on urethral masculinization.

The condensation of mesenchyme is defined by cell–cell adhesions through increased N-CADHERIN expression. Both pharmacological inhibition of actomyosin contractility and genetic deletion of Myh9 and Myh10 resulted in reduced N-CADHERIN expression. In a previous study, it was reported that nonmuscle myosin II activity regulates the accumulation of cadherins at cell–cell adhesion sites. Furthermore, mouse embryos with ablated Myh9 develop defects in cell–cell adhesions resulting in disruption of tissue morphology. Similarly, Myh9 and Myh10 deletion also impairs cell–cell adhesions with reduced N-CADHERIN expression in kidney metanephric mesenchyme. Our results indicate the requirement of actomyosin contractility for cell–cell adhesion in male eExG bilateral mesenchyme during androgen-driven urethral masculinization. Mesenchymal condensations are also associated with directed cell migration, which results in increased cell density. Cell migration is regulated by actomyosin contractility which is transmitted throughout the cell by F-actin. In the eExG, male mesenchyme shows a unique pattern of F-actin consistent with the directional cell migration toward the UPE. This directional cell migration is responsive to androgen signaling and may be crucial for urethral morphogenesis during masculinization. In this study, we showed that androgen induced actomyosin contractility regulating the male-type directional cell migration. Inhibition of actomyosin contractility drastically reduced androgen-induced directional cell migration and prevented urethral masculinization. Thus, it is assumed actomyosin-regulated directed cell migration contributes to condensation of the bilateral mesenchyme and to urethral masculinization.

In this study, we established a new slice culture system to further analyze efficiently urethral masculinization. Several different organ culture systems have been established to study organogenesis. However, previous eExG organ cultures, while useful for the analysis of gene expression and eExG outgrowth, have been unable to properly show urethral masculinization. The current slice culture system recapitulates in vivo urethral masculinization including fusion of the UPE, male-specific mesenchymal cell migration and androgen-inducible expression of several eExG masculinizing genes. To our knowledge, this is the first report that a slice culture method was applied successfully to analyze androgen-driven sexually dimorphic organogenesis. Currently, slice cultures have extensively been used to study brain-related biological processes. It is possible that a similar slice culture method may be applied for the visualization of developmental processes of other organs.

In summary, actomyosin contractility plays an important role for androgen-driven urethral masculinization. Actomyosin contractility is necessary for the androgen-driven and sexually dimorphic condensation of the male mesenchyme. Additionally, actomyosin-regulated mesenchymal condensations contribute to urethral epithelium morphogenesis. These findings further highlight the importance of mesenchymal dynamics for epithelial morphogenesis as a mechanism for the regulation of sexually dimorphic organogenesis.

Methods

Animals. All animals were bred and maintained according to the regulations of the Animal Research Committee of Waseda University and Kumamoto University, Japan. The embryos of pregnant mice were staged according to the day when a vaginal plug was observed, which was designated as E0.5. Myh9fl/fl (43, Myh10fl/fl, ShhCreERT245, and R26R-EYFP47 mice were utilized in this study. These mice were maintained in a C57BL/6J genetic background. E14.5–E18.5 male and female mouse embryos were analyzed. Deletion of the floxed sequence was performed through administration of tamoxifen (Sigma-Aldrich). Tamoxifen was dissolved in sesame oil and treated to pregnant mice (200 mg/kg body weight) through oral gavage at E9.5. ICR mice were utilized for the slice culture and for analysis of sexually dimorphic expression of MYH10 and p-MLC.

eExG slice culture. Mouse eExG were dissected in PBS at E15.5 and embedded in 4% (wt/vol) low melting point agarose/1× PBS using Tissue-tek cryomolds. Gels were placed on slides and analyzed with NIS Elements BR 64 bit 3.22.00 software (Build 710, Laboratory Imaging, Nikon). Live imaging of urethral masculinization was performed using a Confocal Scanner Box, CellVoyagerTMCV1000 microscope and resulting images were analyzed with Cell Voyager® DH CV1000 Software (Yokogawa Electric Corporation). Image processing and analysis of individual cell movements were performed using Imaris software (Imaris Track, Imaris Measurement Pro, and Imaris XT modules, after ver. 7) (Bitplane).
E-CADHERIN (mouse, 1/100, 6010182, BD Transduction Laboratory), GFP (rabbit, 1/500, ab6556, Abcam), SALL1 (mouse, 1/200, PP-K9814-06, Perseus Proteomics). For immunofluorescence staining, primary antibodies were detected with Alexa Fluor 546-conjugated or 488-conjugated IgG (Molecular Probes, Oregon) and counterstained with Hoechst 33342 (Sigma-Aldrich). For primary antibodies raised in mouse, staining was performed using M.O.M. Immunodetection kit (Vector Laboratories) according to manufacturer’s suggested protocols with some modifications. For immunohistochemical staining, amplification using Vectastain ABC kit (Vector Laboratories) and TSA Indirect kit (Perkin Elmer) was performed. Signal was visualized through incubation with diaminobenzidine solution (Wako Pure Chemical Industries Ltd.). Images were visualized using an Olympus BX51 Fluorescence microscope and analyzed with Cell Sens Standard software (ver. 1.6, Olympus). Confocal images were visualized using a Carl Zeiss LSM 700 laser scanning confocal microscope and analyzed with Zen 2012 SP1 black edition 64-bit software (ver. 8.1, Carl Zeiss).

Data analyses. Associations between treatment condition and urethral masculinization scores were analyzed through cross-tabulation. Statistical significance was tested using Chi Square test (two-tailed values of p < 0.05 were considered to be significant) with Graphpad software (https://www.graphpad.com/quickcalc/contingency1/).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability All data generated or analyzed during this study are included in this published Article and its Supplementary files.

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Author contributions

G.Y. supervised the work. A.A., K. Suzuki, S.H., and G.Y. conceptualized and designed the study. All manuscript text and figures were prepared by A.A. and K. Suzuki. M.A. analyzed the slice culture data. Y.S. analyzed individual cell movements in the live imaging data. K. Shimamura supervised the collection and preparation of samples for live imaging. H. H., K. M., M. N., T. T., N. N., S. M., R. N. and R. A. contributed reagents/analytic tools. Mutant mice were supplied and previously analyzed by T. T., N. N., R. N. and R. A. All authors discussed and reviewed the results of the manuscript.

Additional information

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