Androgen-regulated *MafB* drives cell migration via MMP11-dependent extracellular matrix remodeling in mice

**Highlights**

- Androgen-regulated *MafB* is required for cell migration in urethral masculinization.
- *MafB* upregulates *Mmp11* during urethral masculinization.
- MMP11 cleaves CollagenVI, resulting in fibrillar fibronectin deposition.
- *MafB*-expressing mesenchymal cells migrate on fibronectin, but not on CollagenVI.

Alcantara et al., iScience 25, 105609
December 22, 2022 © 2022
https://doi.org/10.1016/j.isci.2022.105609

Mellissa C. Alcantara, Kentaro Suzuki, Alvin R. Acebedo, ..., Kazuo Yamagata, Satoru Takahashi, Gen Yamada

k-suzuki@wakayama-med.ac.jp (K.S.)
genyama@yahoo.co.jp (G.Y.)
Article

Androgen-regulated MafB drives cell migration via MMP11-dependent extracellular matrix remodeling in mice

Mellissa C. Alcantara,1 Kentaro Suzuki,1,*, Alvin R. Acebedo,1 Daiki Kajioka,1 Satoshi Hirohata,2 Tsuneyasu Kaisho,3 Yu Hatano,4 Kazuo Yamagata,4 Satoru Takahashi,5 and Gen Yamada1,6,*

SUMMARY

While androgen is considered a pivotal regulator of sexually dimorphic development, it remains unclear how it orchestrates the differentiation of reproductive organs. Using external genitalia development as a model, we showed that androgen, through the transcription factor MafB, induced cell migration by remodeling the local extracellular matrix (ECM), leading to increased cell contractility and focal adhesion assembly. Furthermore, we identified the matrix metalloproteinase Mmp11 as a MafB target gene under androgen signaling. MMP11 remodels the local ECM environment by degrading Collagen VI (ColVI). The reduction of ColVI led to the fibrillar deposition of fibronectin in the MafB-expressing bilateral mesenchyme both in vivo and ex vivo. The ECM remodeling and development of migratory cell characteristics were lost in the MafB loss-of-function mice. These results demonstrate the requirement of mesenchymal-derived androgen signaling on ECM-dependent cell migration, providing insights into the regulatory cellular mechanisms underlying androgen-driven sexual differentiation.

INTRODUCTION

Androgen is the key regulator for the sexually dimorphic development of the external genitalia.1 Arising from a common anlage, the male and female embryonic external genitalia (eExG) of mice begin to show sexual differences at embryonic day (E) 15.5. Under androgen regulation, the urethra of male mice canalizes around E16.5, a process termed urethral masculinization. In contrast, the ventral side of the urethra remains open in females. V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) is a transcription factor that is expressed in the mesenchyme lateral to the presumptive urethra, hereafter referred to as the bilateral mesenchyme (biMs). Previously, MafB has been identified to be a direct androgen target that is essential for urethral masculinization: male MafB mutant mice show an abnormal, open urethra.2,3 Prior to androgen induction, MafB is required for vascular differentiation during testicular organogenesis (Li et al., 2021). Although several androgen-regulated cellular processes during urethral masculinization have been described,4,5,6 how MafB contributes to this event has yet to be defined.

During organogenesis, androgens have been reported to regulate the cytoskeletal elements during urethral masculinization.7,8 In this study, we observed defects in cell migration in the MafB knockout mice; therefore, a unique pathway is likely downstream of androgen-MafB signaling. Cell migration is highly influenced by either the composition, stiffness, or concentration of the ECM.9,10 Matrix metalloproteinases (MMPs) are endopeptidases that can cleave matrix proteins and remodel the ECM environment. Their function is essential for both developmental and pathological processes, especially in cancer.11 MMP11, also known as stromelysin-3, is an MMP that was first isolated from breast cancer tissue.12,13 During embryogenesis, Mmp11 is expressed transiently in mesenchymal cells that are associated with tissue remodeling14–16; however, in healthy adult human organs, Mmp11 is seldom expressed.17

MMP11 has been reported to cleave the α3 subunit of ColVI,18 a ubiquitous ECM protein that interacts with other ECM proteins to form a structural network for cells.19 One such interaction is with the ECM protein fibronectin (FN), wherein ColVI expression regulates the deposition pattern of FN.20–22 FN participates in cell differentiation, growth factor signaling, and cell migration.23 In several biological systems,
Figure 1. Mesenchymal MafB regulates Mmp11 during urethral masculinization

[A–D] Mesenchymal, not epithelial, MafB is involved in urethral masculinization. See also Figure S1A. (A) The formation of a urethral tube (arrow) was observed in E16.5 control mice. (B) Mesenchymal-specific MafB knockout mice (Sall1cre+/MafBflox/flox) failed to form a urethral tube. (C) Endodermal epithelium-specific MafB knockout mice (Shhcre/+;MafBflox/flox) exhibited a masculinized urethral tube. (D) Ectodermal epithelium-specific MafB knockout mice (Wnt7acre/+;MafBGFPflox) exhibited a masculinized urethral tube. Scale bar: 50 μm

[E–N] Mmp11 is involved in urethral masculinization. See also Figures S1B and S1C.
perturbations in FN expression led to phenotypes associated with impaired cell migration,24–26 and the fibrillar deposition of FN has also been correlated with cell movement.27–29 In fact, the FN network continuously changes during embryonic development: In the Xenopus embryo, FN fibrils are constantly remodeled from gastrulation until neurulation,30 while spatiotemporal differences in the pattern of FN deposition accompany palate shelf elevation.31 Both neurulation and palatogenesis are widely accepted models for the investigation of tissue fusion, which both point to the importance of spatiotemporal regulation of FN during midline fusion in organogenesis.32,33 Since the male-type urethra also undergoes migration and fusion events, we investigated whether FN was involved in this process.

Here, we elucidate the mechanism of androgen-driven cell migration during male eExG development. We demonstrate MMP11-dependent ECM remodeling during sexual differentiation. MMP11-degradation of ColVI changes the ECM microenvironment and promotes focal adhesion formation and cell contractility. Furthermore, we propose that MafB regulates Mmp11 under androgen signaling and reveal the significance of this signaling cascade during urethral masculinization.

RESULTS

MafB regulates biMs cell migration into the midline

Androgen-driven biMs cell migration is one of the essential processes for the eExG development.4,5 To understand the role of MafB during urethral masculinization, we cultured tissue slices from the eExG of MafBKO knockout (MafB KO) and control mice for 48 h. Cells of the biMs in the control slices actively migrated toward the midline, forming the urethral tube (Video S1). In contrast, biMs cells of the MafB KO, while motile, remained in place (Video S2). These data suggest that MafB is necessary for cell migration during urethral masculinization.

Mesenchymal MafB function is required for urethral masculinization

Although MafB is predominantly expressed in the biMs, it is also expressed in the epithelia (Figure S1A). To confirm whether only mesenchymal MafB is crucial to urethral masculinization, we analyzed several conditional knockout mice which specifically targeted the mesenchymal, endodermal-epithelial, and ectodermal-epithelial MafB in the eExG (Figures 1A-1D). Sall1 has previously been reported to be expressed in the biMs, while Shh (Sonic hedgehog) is specific to the endoderm-derived urethral epithelium.5 Meanwhile, Wnt7a is expressed in the ectodermal epithelium of the eExG.34 At E16.5, only the mesenchymal Sall1+/cre/MafB121 (MafB cKO) mice showed the female-like open urethra, similar to the conventional knockout mice. The urethral tube was formed completely in the other two mutants, indicating that mesenchymal MafB is essential for androgen-driven urethral masculinization.

MMP11 is a MafB-regulated, sexually dimorphic metalloproteinase in the embryonic external genitalia

We hypothesized that MafB drives urethral masculinization through the regulation of cell migration. Modifications in the composition or arrangement of the ECM are known to regulate cell migration,9 and one of the known mechanisms by which the ECM is remodeled is through MMPs. We performed RNA-seq analysis of the biMs to investigate their transcriptomic expression profile and observed that Mmp2, Mmp11, and Mmp14 were the most highly expressed MMPs in the biMs of the eExG at E16.5 (GEO: GSE185966). However, RT-qPCR analysis determined that only Mmp11 was a sexually dimorphic gene (Figures 1E-1G). Indeed, both Mmp11 mRNA and protein were strongly expressed in the biMs of the male eExG from E15.5 (Figure 1H) compared with that of the female (Figure 1I), concurrent with the onset of androgen-dependent urethral masculinization.7 This dimorphic expression extends to E16.5 (Figures 1J and 1K; Figure S1B), at which the urethral tube begins to form prominently. To explore the possibility that Mmp11 is...
regulated by androgen, we treated wild-type female mice with testosterone propionate (TP) during the masculinization window (E14.5-E15.5). Mmp11 was induced in the TP-treated female (Figures 1L and 1M), thus indicating that Mmp11 is an androgen-inducible metalloproteinase that is highly expressed during urethral masculinization.

We next determined whether Mmp11 is a downstream target of the androgen-dependent masculinization factor MafB. Conditional mesenchymal deletion of MafB resulted in the downregulation of the expression level of Mmp11 in the bMs of the eExG (Figures 1N and 1O). To further confirm the necessity of MafB in Mmp11 expression, we administered TP to MafB KO female mice and observed that its expression was not induced in the female mutant embryos even in the presence of androgen (Figure S1C). Moreover, the promoter region of Mmp11 was highly conserved among mice, humans, and chimpanzees (Figure S1D), and we identified a histone 3 lysine 27 acetylation (H3K27ac) positive site in this region (GEO: GSE158279) (Figure 1P). The Maf recognition element (MARE) (Figure 1Q) was identified within this H3K27ac positive element by using the JASPAR database, and ChIP-PCR analysis confirmed that MAFB was bound to this MARE site (Figure 1R). Taken together, these data suggest that, under androgen signaling, MafB regulates ECM remodeling through Mmp11 during urethral masculinization.

Requirement of Mmp11 activity for extracellular matrix remodeling

One of the known targets of MMP11 is the α3 chain of ColVI. Thus, we analyzed its expression and observed that ColVI was downregulated in the bMs of both E15.5 and E16.5 male eExG (Figure 2A, Figure S2A). This reduction was not observed in the female (Figures 2B and S2A), suggesting that the changes in mesenchymal ColVI are required for the process of urethral masculinization. Loss of ColVI expression has been reported to be necessary for the proper deposition and organization of FN. We next analyzed the expression of FN in the eExG and observed different FN deposition patterns between the male and the female bMs. Although a subtle difference between the sexes could be observed at E15.5 (Figure S2B), the sexually dimorphic FN deposition pattern became more prominent at E16.5. This difference was observed despite similar transcript expression levels between sexes (Figure S2C). FN fibrils were formed in the bMs at E16.5 (Figures 2C and 2E), coinciding with reduced ColVI expression; while a ring-like FN deposition was observed in the female bMs E16.5 (Figures 2D and 2F). These results suggest that mesenchymal ECM remodeling is required for androgen-driven urethral masculinization.

It has been reported that FN deposits in Col6a1 null fibroblasts appear as streaked fibrils as opposed to the ring-like FN deposited in control cells. To investigate whether the pattern of FN deposition is dependent on ColVI in the ECM, we analyzed the effect of exogenous ColVI on the pattern of FN deposition using an established eExG slice culture system. eExG slices were cultured on gels supplemented with 10% ColVI (ColVI [+]) or without ColVI (control) for 24 h (Figure 2G). Similar to the female phenotype, slices cultured on ColVI (+) exhibited a ring-like deposition of FN (Figure 2H); whereas the streak-like pattern was observed in the control group (Figure 2I). The pattern of FN deposition in the bMs, therefore, is affected by the ColVI within the ECM environment.

To investigate whether MMP11 degrades ColVI in the bMs, we analyzed its expression in the bMs after culturing in MMP11-containing conditioned media. As MMP11 is secreted in its active form, we transfected a Mmp11 expression vector into HEK293 cells and confirmed the presence of the MMP11 protein by immunostaining and Western blotting (Figures S3A and S3B). Female eExG slices were cultured in either the MMP11 conditioned medium (Mmp11[+]) or control media for 24 h (Figure 2J). The deposition of ColVI was reduced in Mmp11[+] slices (Figure 2K) compared to the control (Figure 2L), suggesting that MMP11 in the bMs likely degrades ColVI during androgen-driven urethral masculinization. On the other hand, Collagen I, a major collagen in the eExG, was not affected by this treatment (Figure S2C), suggesting the substrate specificity of MMP11 to ColVI. The addition of MMP11, however, induced the fibrillar deposition of FN fibrils formed in the gel only set-up (H) and a ring-like pattern in the presence of ColVI (I). Scale bar: 10 μm. (J) Schematic diagram of Mmp11 overexpression set-up. (K and L) ColVI expression was reduced in eExG slices cultured in Mmp11 conditioned medium (K) versus the control (L). Scale bar: 10 μm.
Figure 3. ECM remodeling is necessary for migration in the male biMcs

(A and B) BiMcs cells migrate more efficiently on FN rather than on ColVI. See also Videos S3 and S4. (A) Schematic diagram of primary cell migration assay. (B) Still images from live imaging video of biMcs on either FN or ColVI. Images taken at 0,
deposition of FN in the female biMs (Figure S2D). Altogether these data suggest that MMP11 remodels the ECM in the biMs by degrading ColVI which, in turn, alters the deposition pattern of FN during urethral masculinization.

MMP11-dependent extracellular matrix remodeling leads to cell migration in the male embryonic external genitalia

Since cell migration is necessary for urethral masculinization, we next investigated whether ECM remodeling is critical for this process. In the absence of ColVI, both epithelial cells and neural crest cells have been reported to become more migratory on FN substrate. To investigate whether MMP11-regulated ECM remodeling is required for cell migration in the eExG, we developed a migration assay using primary biMs cells. Following the recent methods of Hagiwara et al. (2021), polydimethylsiloxane (PDMS) rings were coated with either ColVI or FN, and cells were cultured within an inner well (Figure 3A). After the cells have attached, we removed the PDMS sheets and observed the cells for 48 h. Live imaging analysis revealed that biMs cells migrated into the FN-coated region (Figure 3B; Video S3) with in the first hour of culture. The cells proceeded to migrate efficiently into the coated region throughout the 48 h duration. In contrast, cells that were within the ColVI-coated region remained stationary (Figure 3B; Video S4).

To understand the mechanism leading to cell migration, we cultured biMs primary cells on either ColVI or FN (Figure 3C). After 24 h, a higher number of biMs cells was attached to the FN-coated well (Figures 3D and 3E). The presence of focal adhesions was marked using vinculin (VCL), and cell contractility was assessed by analyzing the expression of the phosphorylated myosin light chain (pMLC). Cells cultured on FN formed prominent lamellipodia that contained longer and more distinct focal adhesions (Figure 3F and 3F'), while those cultured on ColVI tended to form smaller adhesions (Figure 3G and 3G') (Figure S4A). Contractile stress fibers were also more prominently expressed by cells cultured in the presence of FN (Figure 3F' and 3F'') than on ColVI (Figure 3G' and 3G'') (Figure S4A). These results suggest

Figure 3. Continued
24, and 48 h. Yellow dotted line marks the border between the coated and non-coated regions. Red dotted line indicates the end of migrating cells.

(C–G) BiMs cells cultured on FN possess prominent focal adhesions and stress fibers. (C) Schematic diagram of primary cell culture on either FN or ColVI. (D and E) More biMs cells adhered to FN (D) than to ColVI (E). Scale bar: 50 μm. (F and G) On FN, VCL (F) was strongly expressed on the edges of the cell, while pMLC (F') was prominent throughout the cell body. On ColVI, VCL (G)-marked focal adhesions were smaller, and pMLC (G') was restricted to the sides of the cell. F' and G' show merged image. Scale bar: 10 μm.

Figure 4. Focal adhesions are assembled in the male biMs

(A and B) pFAK was expressed more prominently in the male biMs (A) compared to the female (B). Scale bar: 50 μm.

(C and D) VCL and ITGA5 expressions co-localized in the male biMs (C), marked with SALL1, but not in the female (D). (C' and D') VCL expression in the biMs marked by SALL1. (C'' and D'') VCL expression in the biMs marked by SALL1. Scale bar: 50 μm. See also Figure S4.
that biMs cells require a shift from ColVI to FN to develop focal adhesions and contractility, leading to cell migration.

These in vitro data prompted us to investigate the cytoskeletal dynamics in the tissue during androgen-driven urethral masculinization. pMLC is upregulated in the male E16.5 eExG, compared to the female5; Figure S4B). The sexually dimorphic expression occurred from E15.5 (Figure S4B), concurrent with the onset of morphologically dimorphic eExG development. Furthermore, focal adhesion markers - phosphorylated focal adhesion kinase (pFAK), VCL, and integrin α5 (ITGA5) - were also expressed prominently in the male biMs but not in females (Figures 4A–4D). The sexually dimorphic expression was observed from E15.5 (Figures S4C and S4D). Collectively, these findings suggest that to form the urethral tube during masculinization, MMP11-dependent ECM remodeling in the biMs is required for the MafB-positive cells to acquire a migratory state.

**MafB drives cell migration via MMP11-dependent extracellular matrix remodeling**

As mentioned, MafB regulates Mmp11 expression in the biMs. To reinforce the significance of upstream MafB signaling, we analyzed the expressions of several ECM proteins, cell-matrix adhesion markers, and
actomyosin activity in the MafB KO mice. A MafB-GFP knock-in mouse line (MafB<sup>GFP/+</sup>) was utilized to identify the MafB-expressing biMs cells (Figure S5). Expression of ColVI remained in the biMs of the KO mouse (Figures 5A and 5B) and FN was observed in a ring-like pattern (Figures 5C–5F), which was similar to the female ECM microenvironment. Moreover, pMLC, VCL, and ITGA5 were downregulated in the absence of MafB (Figures 5G–5J). These results indicate that MafB regulates cell migration through MMP11-dependent ECM remodeling in the biMs.

**DISCUSSION**

Androgens govern the development of the male reproductive organs. Epithelial androgen signaling regulates cell proliferation, differentiation, and survival during the development of the prostate, epididymis, and seminal vesicles. While mesenchymal androgen receptor (AR) signaling is necessary for epithelial cell proliferation and differentiation in the prostate gland, the role of mesenchymal androgen signaling during male external genitalia development has yet to be elucidated. Here, we demonstrate that local ECM remodeling, under mesenchymal androgen signaling, is essential during eExG development. Androgen-MafB drives cell migration through the regulation of MMP11-dependent ECM remodeling: MMP11-degradation of ColVI in the mesenchyme leads to the fibrillar deposition of FN, and this allows the biMs cells to form focal adhesions required for sexually dimorphic cell migration (Figure 6).

**MafB is a regulator of Mmp11 under androgen signaling**

Androgens regulate MMP expression in prostate and bladder cancers; however, its regulatory effect is context-, type-, and dose-dependent. While it is generally accepted that AR signaling can stimulate MMP activity, it has also been reported to downregulate the expression of MMPs through Ets transcription factors. AR induces the activity of target genes through transcription factors such as SRY, SP1, and the Activator Protein-1 (AP-1) superfamily. The AP-1 superfamily consists of the Jun, Fos, and Maf families. A highly conserved region, which includes a binding site for Jun/Fos dimers, is present in the cis-regulatory elements of the promoter regions of several MMPs. In chondrocytes, MafB has been reported to stimulate MMP3/13 expression in response to retinoic acid signaling, suggesting a possibility of MafB as a regulator not only of Mmp11, but of stromelysins. Here, we report that a MARE is present in the promoter region of Mmp11 and that MafB, another member of the AP-1 family of transcription factors, binds to this site during urethral masculinization. Since there are currently no known Mmp11 regulators during development, androgen-regulated MafB is the first suitable candidate as an upstream regulator of Mmp11.

**Androgen-driven cell migration via extracellular matrix remodeling during organogenesis**

In the prostate, androgens initiate ductal growth and cell differentiation. In the Wolffian duct, androgens are required for cell differentiation and proliferation. While androgen is known as the master regulator for sexually dimorphic reproductive organ formation, the mechanism through which it orchestrates organogenesis has yet to be defined. We previously reported that male-specific mesenchymal cell
dynamics is indispensable during androgen-dependent urethral masculinization. Here, we report that cell migration is defective in the MafB mutant mesenchyme, indicating that androgen-regulated MafB is required for mesenchymal cell migration during eExG development.

Organogenesis is generally associated with high levels of tissue remodeling and cell migration. During gut looping, the migration of the epithelial lateral plate mesodermal cells requires the loss of laminin through MMP activity, and the direction of looping is determined through asymmetric ECM deposition in the mesenchyme. Extensive remodeling of the ECM also occurs during the development of the lung, the tooth, and the palate. Our data demonstrate remodeling of the ECM alongside urethral tube development: MMP11 degrades ColVI, resulting in the fibrillar deposition of FN. It has been suggested that this is due to the competitive binding of ColVI for integrin β1 which prevents fibrillogenesis. We observed increased focal adhesion assembly and cell contractility in the male biMs and also in cells cultured with FN. FN has been reported to promote the formation of focal adhesions and cell migration. In the lung, FN polymerization is required for small airway epithelial cell migration. Myoblast cells have also been shown to migrate with persistent directionality on FN but not on gelatin. In addition, polymerization of soluble FN into fibrils has been reported to be required to generate cytoskeletal tension. It has further been suggested that a low ColVI/high FN environment leads to an increase in the phosphorylation of MLC through alterations in the calcium pathway. Lastly, in the exG, F-actin stress fibers and actomyosin contractility increase in response to androgens. Hormone-dependent ECM remodeling has also been previously reported during tadpole metamorphosis: MMP11 cleaves the basement membrane in response to thyroid hormone during intestinal development. We have shown that MMP11 is an androgen-dependent metalloproteinase in the exG. Intriguingly, androgen exposure is sufficient to induce cell migration in female exG slice cultures. These all support our finding that MMP11-regulated ECM remodeling is vital in androgen-driven mesenchymal cell migration during exG sexual differentiation.

Tissue fusion is classically studied through neural tube formation, palate morphogenesis, and heart development. Among these three, mesenchymal cell migration is most well-studied during heart development, focusing on neural crest cells as a model. Our study introduces the development of the external genitalia as an alternative model for investigating mesenchymal cellular processes that occur during tissue fusion. We report that this process is under androgen regulation. Several other organs suggest links between androgen signaling, MafB expression, and tissue remodeling. Organs of the cardiovascular system undergo androgen-dependent development, and MafB is a critical transcription factor for the development of the highly migratory cardiac neural crest cell. Similarly, the development of the CNS has also been reported to be sexually dimorphic. In this system, MafB is required for hindbrain segmentation and regional specification. It will be interesting to investigate whether androgen-MafB signaling can induce cell migration via MMP11-dependent ECM remodeling in other systems.

Limitations of the study

In this study, we elucidated the role of androgen-regulated MafB during cell migration using the masculinization of the mouse urethra as a model, and our in vitro assays on migration utilized mouse primary cells. Whether the same mechanism occurs in other mammals, including humans, remains to be explored. Furthermore, MafB is also expressed in other organs. Investigating downstream events to MafB in these systems would contribute to the generality of our proposed mechanism.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability statement
  - Data availability statement
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Histological analyses and imaging
  - Chromatin immunoprecipitation (ChIP) assay and ChIP-Seq analysis
Primary cell migration assay and culture
Mmp11 overexpression, eExG slice culture, and exogenous collagen 6 assays
RNA sequencing and qPCR analysis

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105609.

ACKNOWLEDGMENTS
We would like to thank Dr. Suneel Apte and Dr. Timothy Mead of the Lerner’s Research Institute; the Laboratory Animal Center, the Department of Immunology, and the Central Research Facility of the Wakayama Medical University; and Mr. Ryuya Taniguchi of Kindai University for their advice and technical support. We would also like to acknowledge Tomiko Iba, Yugi Rim, and the members of the Department of Developmental Genetics for their invaluable support. This work was supported by the Japan Society for the Promotion of Science KAKENHI (17H06432, 18K06837, 18K06938, 21K06822, and 21K19538) and the Mombukagakusho Scholarship from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

AUTHOR CONTRIBUTIONS
Conceptualization: K.S. and G.Y.; methodology: M.C.A., K.S., and A.R.A.; investigation: M.C.A., D.K., and Y.H.; writing – original draft, visualization: M.C.A.; writing – review and editing: K.S. and G.Y.; resources: S.H., T.K., K.Y., and S.T.; supervision: K.S. and G.Y.; funding acquisition: K.S. and G.Y.

DECLARATION OF INTERESTS
The authors have nothing to declare.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduct of research.

REFERENCES
1. Matsushita, S., Suzuki, K., Murashima, A., Kajioka, D., Acebedo, A.R., Miyagawa, S., Haraguchi, R., Ogino, Y., and Yamada, G. (2018). Regulation of masculinization: androgen signalling for external genitalia development. Nat. Rev. Urol. 15, 358–368. https://doi.org/10.1038/s41585-018-0008-y.
2. Matsushita, S., Suzuki, K., Ogino, Y., Hino, S., Sato, T., Suyama, M., Matsumoto, T., Omori, A., Iinoue, S., and Yamada, G. (2016). Androgen regulates mafb expression through its 3’UTR during mouse urethral masculinization. Endocrinology 157, 844–857. doi:https://doi.org/10.1210/en.2015-1596.
3. Suzuki, K., Numata, T., Suzuki, H., Raga, D.D., Iputan, L.A., Yokoyama, C., Matsushita, S., Hamada, M., Nakagata, N., Nishinakamura, R., et al. (2014). Sexually dimorphic expression of Mafb regulates masculinization of the embryonic urethral formation. Proc. Natl. Acad. Sci. USA 111, 16407–16412. https://doi.org/10.1073/pnas.1413273111.
4. Liu, L., Suzuki, K., Chun, E., Murashima, A., Sato, Y., Nakagata, N., Fujimori, T., Yonemura, S., He, W., and Yamada, G. (2017). Androgen regulates dimorphic F-actin assemblies in the genital organogenesis. Sex Dev. 11, 190–202. https://doi.org/10.1159/000477452.
5. Acebedo, A.R., Suzuki, K., Hino, S., Alcantara, M.C., Sato, Y., Haga, H., Matsumoto, K.I., Nakao, M., Shimamura, K., Takeo, T., et al. (2019). Mesenchymal actomyosin contractility is required for androgen-driven urethral masculinization in mice. Commun. Biol. 2, 95. https://doi.org/10.1038/s42003-019-0336-3.
6. Suzuki, H., Matsushita, S., Suzuki, K., and Yamada, G. (2017). Sex-Dihydrotestosterone negatively regulates cell proliferation of the periurethral ventral mesenchyme during urethral tube formation in the murine male genital tubercle. Andrology 5, 146–152. https://doi.org/10.1111/and.12241.
7. Zheng, Z., Armbfield, B.A., and Cohn, M.J. (2015). Timing of androgen receptor disruption and estrogen exposure underlies a spectrum of congenital penis anomalies. Proc. Natl. Acad. Sci. USA 112, E7194–E7203. https://doi.org/10.1073/pnas.1515981112.
8. Larkins, C.E., Enriquez, A.B., and Cohn, M.J. (2016). Spatiotemporal dynamics of androgen signaling underlie sexual differentiation and congenital malformations of the urethra and vagina. Proc. Natl. Acad. Sci. USA 113, E7510–E7517. https://doi.org/10.1073/pnas.1610471113.
9. Yamada, K.M., and Sixt, M. (2019). Mechanisms of 3D cell migration. Nat. Rev. Mol. Cell Biol. 20, 738–752. https://doi.org/10.1038/s41592-019-0172-9.
10. Hartman, C.D., Isenberg, B.C., Chua, S.G., and Wong, J.Y. (2017). Extracellular matrix type modulates cell migration on mechanical gradients. Exp. Cell Res. 359, 361–366. https://doi.org/10.1016/j.yexcr.2017.08.018.
11. Page-McCaw, A., Ewald, A.J., and Webb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. Nat. Rev.
Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C., and Chambon, P. (1990). A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 348, 699–704.

Lefebvre, O., Wolf, C., Limacher, J.M., Wendling, C., LeMeur, M., Basset, P., and Rio, M.C. (1992). The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. J. Cell Biol. 119, 997–1002.

Lefebvre, O., Régnier, C., Chenard, M.P., Wendling, C., Chambon, P., Basset, P., and Rio, M.C. (1995). Developmental expression of mouse stromelysin-3 mRNA. Development 121, 947–956.

Ishizuya-Oka, A., Li, Q., Amano, T., Damjanovski, S., Ueda, S., and Shi, Y.B. (2000). Requirement for matrix metalloproteinase stromelysin-3 in cell migration and apoptosis during tissue remodeling in Xenopus laevis. J. Cell Biol. 150, 1177–1188.

Peruzzi, D., Mori, F., Conforti, A., La Monica, N., Capanni, C., Bergamin, N., Braghetta, P., and De Rinaldis, E. (2000). Live imaging of cell protrusive activity, and extracellular matrix controls cell migration during amphibian gastrulation. Int. J. Dev. Biol. 44, 139–147.

Rozario, T., Dzamba, B., Weber, G.F., Davidson, L.A., and DeSimone, D.W. (2009). The physical state of fibronectin matrix differentially regulates morphogenetic movements in vivo. Dev. Biol. 327, 386–398.

Davidson, L.A., Dzamba, B.D., Weber, G.F., and Desimone, D.W. (2008). Live imaging of cell protrusive activity and extracellular matrix assembly and remodeling during morphogenesis in the frog, Xenopus laevis. Dev. Dyn. 237, 2684–2692.

Davidson, L.A., Keller, R., and DeSimone, D.W. (2004). Assembly and remodeling of the fibrillar fibronectin extracellular matrix during gastrulation and neurulation in Xenopus laevis. Dev. Dyn. 237, 885–895.

Davidson, L.A., Keller, R., and DeSimone, D.W. (2011). Fibronecins, their fibrillogenesis, and in vivo functions. Cold Spring Harb. Perspect. Biol. 3, a005041. https://doi.org/10.1101/cshperspect.a005041

Loganathan, R., Potetz, B.R., Rongish, B.J., and Little, C.D. (2012). Spatial anisotropies and temporal fluctuations in extracellular matrix network texture during early embryogenesis. PLoS One 7, e36266. https://doi.org/10.1371/journal.pone.0036266

Miyagawa, S., Satoh, Y., Haraguchi, R., Suzuki, K., Iuchi, T., Taketo, M.M., Navagata, N., Matsumoto, T., Takeyama, K., Ito, S., and Yamada, G. (2009). Genetic interactions of the androgen and Wnt/beta-catenin pathways for the masculinization of external genitalia. Mol. Endocrinol. 23, 871–880.

Fornes, O., Castro-Mondragon, J.A., Khan, A., van der Lee, R., Zhang, X., Richmond, P.A., Mod, B.P., Correard, S., Gheorghe, M., Baranasic, D., et al. (2020). Jaspar 2020: update of the open-access database of transcription factor binding profiles. Nucleic Acids Res. 48, D87–D92. https://doi.org/10.1093/nar/gkz1001.

Pei, D., and Weiss, S.J. (1995). Furin-dependent intracellular activation of the humanstromelysin-3zymogen. Nature 375, 244–247. https://doi.org/10.1038/375244a0.

Hagihara, M., Maruyama, H., Akizawa, M., Koh, I., and Ara, F. (2021). Weakening of resistance force by cell-ECM interactions regulate cell migration directionality and pattern formation. Commun. Biol. 4, 808. https://doi.org/10.1038/s42003-021-02550-4.

Murashima, A., Kishigami, S., Thomson, A., and Yamada, G. (2015). Androgens and mammalian male reproductive tract development. Biochim. Biophys. Acta 1849, 163–170. https://doi.org/10.1016/j.bjbgmr.2014.05.020.

Cunha, G.R., and Chung, L.W. (1981). Stromal-epithelial interactions—1 Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. J. Steroid Biochem. 14, 1317–1324. https://doi.org/10.1016/0022-4731(81)90338-1.

Liao, X., Thrasher, J.B., Pelling, J., Holzebeierlein, J., Sang, Q.X.A., and Li, B. (2003). Androgen-stimulated extracellular matrix metalloproteinase-2 expression in human prostate cancer. Endocrinology 144, 1656–1663. https://doi.org/10.1210/en.2002-0157.

Lin, C., Lin, W., Yeh, S., Li, L., and Chang, C. (2015). Infiltrating neutrophils increase bladder cancer cell invasion via modulation of androgen receptor (AR)/MMP13 signals. Oncotarget 6, 43081–43089. https://doi.org/10.18632/oncotarget.56328.

Mountain, D.J.H., Freeman, B.M., Kirkpatrick, S.S., Beddies, J.W., Arnold, J.D., Freeman, M.B., Goldman, M.H., Stevens, S.L., Klein, F.A., and Grandas, O.H. (2013). Androgens regulate MMPs and the cellular processes of intimal hyperplasia. J. Surg. Res. 184, 619–627. https://doi.org/10.1016/j.jss.2013.05.070.

Hara, T., Miyazaki, H., Lee, A., Tran, C.P., and Reiter, R.E. (2008). Androgen receptor and invasion in prostate cancer. Cancer Res. 68, 1128–1135. https://doi.org/10.1158/0008-5472-CAN-07-1929.

Pang, S.T., Flores-Morales, A., Skoog, L., Chuan, Y.C., Nordstedt, G., and Pousset, A.
iScience Article

(2004). Regulation of matrix metalloproteinase 13 expression by androgen in prostate cancer. Oncol. Rep. 11, 1187–1192.

45. Schneker, J., Peterziel, H., Defossez, P.A., Klocker, H., de Launoit, Y., and Cato, A.C. (1996). Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-regulation of matrix metalloproteinase expression. J. Biol. Chem. 271, 23907–23913. https://doi.org/10.1074/jbc.271.39.23907.

46. Kajioka, D., Suzuki, K., Matsushita, S., Hino, S., Sato, T., Takada, S., Isono, K., Takeo, T., Kajimoto, M., Nakagata, N., et al. (2021). Sex-specific roles of SRY in the development of the female RVW system: evidence of a Y-chromosome-dependent cell migration. Am. J. Physiol. Cell. Physiol. 321, C85–C95. https://doi.org/10.1152/ajpcell.00079.2021.

47. Yuan, X., Lu, M.L., Li, T., and Balk, S.P. (2001). SRY interacts with and negatively regulates androgen receptor transcriptional activity. J. Biol. Chem. 276, 46647–46654. https://doi.org/10.1074/jbc.M108404200.

48. Takai, H., Nakayama, Y., Kim, D.S., Arai, M., Araki, S., Mezawa, M., Nakajima, Y., Kato, N., Masunaga, H., and Ogata, Y. (2007). Roles of the AP-1 site and MMP gene regulation: the AP-1 site and MMP gene regulation: the AP-1 site and MMP gene regulation: the AP-1 site and MMP gene regulation. J. Biol. Chem. 282, 2715–2722. https://doi.org/10.1074/jbc.M108196200.

49. Koochekpour, S. (2010). Androgen receptor signaling and mutations in prostate cancer. Asian J. Androl. 12, 639–657. https://doi.org/10.1038/ajandro.2010.89.

50. Eychene, A., Rocques, N., and Poupponnec, C. (2008). A new MAFa in cancer. Nat. Rev. Cancer 8, 683–693. https://doi.org/10.1038/nrc2460.

51. White, L.A., and Brinckerhoff, C.E. (1995). Two activator protein-1 elements in the matrix metalloproteinase-1 promoter have different effects on transcription and binding Jun D, c-Fos, and Fra-1. Matrix Biol. 14, 715–725. https://doi.org/10.1016/0945-053x(95)80014-9.

52. Chamberlain, S.H., Hemmer, R.M., and Brinckerhoff, C.E. (1993). Novel phorbol ester response element in the collagenase promoter binds Fos and Jun. J. Cell. Biochem. 52, 337–351. https://doi.org/10.1002/jcb.240520310.

53. Benziow, U., and Brinckerhoff, C.E. (1997). The AP-1 site and MMP gene regulation: what is all the fuss about? Matrix Biol. 15, 519–526. https://doi.org/10.1016/s0945-053x(97)90026-3.

54. Zhang, Y., and Ross, A.C. (2013). Retinoic acid and the transcription factor MaFb act together and differentially to regulate aggrecan and matrix metalloproteinase gene expression in chondrocytes. J. Cell. Biochem. 114, 471–479. https://doi.org/10.1002/jcb.24387.

55. Francis, J.C., and Swain, A. (2018). Prostate organogenesis. Cold Spring Harb. Perspect. Med. 8, a003053. https://doi.org/10.1101/cshperspect.a003053.

56. Lasnitzki, I., and Mizuno, T. (1977). Induction of the rat prostate gland by androgens in organ culture. J. Endocrinol. 74, 47–55. https://doi.org/10.1677/joe.0177400407.

57. Shaw, G., and Renfree, M.B. (2014). Wolffian duct development. Sex Dev. 8, 273–280. https://doi.org/10.1159/000363432.

58. Yamada, G., Suzuki, K., Haraguchi, R., Miyagawa, S., Sato, Y., Kamimura, M., Nakagata, N., Kato, H., Kuroiwa, A., and Chen, Y. (2006). Molecular genetic cascades for external genitalia formation: an emerging organogenesis program. Dev. Dyn. 235, 1738–1752. https://doi.org/10.1002/dvdy.20807.

59. Aman, A., and Piotrowski, T. (2010). Cell migration during morphogenesis. Dev. Biol. 341, 20–33. https://doi.org/10.1016/j.ydbio.2009.11.014.

60. Yin, C., Kikuchi, K., Hochgreb, T., Poss, K.D., and Stainier, D.Y.R. (2010). Hand2 regulates extracellular matrix remodeling for gut-looping morphogenesis in zebrafish. Dev. Cell 18, 973–984. https://doi.org/10.1016/j.devcel.2010.05.009.

61. Kurpios, N.A., Ibáñez, M., Davis, N.M., Lui, W., Katz, T., Martin, J.F., Ipirzia Belmonte, J.C., and Tabin, C.J. (2008). The direction of gut looping is established by changes in the extracellular matrix and in cell-cell adhesion. Proc. Natl. Acad. Sci. USA 105, 8499–8506. https://doi.org/10.1073/pnas.0803578105.

62. Zhou, Y., Horowitz, J.C., Nabi, A., Ambalavanan, N., Atabai, K., Bitterman, P.B., Corley, R.A., Ding, B.S., Engler, A.J., et al. (2018). Extracellular matrix in lung development, homeostasis and disease. Matrix Biol. 73, 77–104. https://doi.org/10.1016/j.matbio.2018.03.005.

63. Wang, X., Li, C., Zhu, Z., Yuan, L., Chan, W.Y., Li, M., Lim, C.T., and Ladoux, B. (2012). Emerging modes of collective cell migration? The case of the neural crest. Cell Adh. Migr. 5, 490–498. https://doi.org/10.4161/cam.5.6.18623.

64. Shi, W., Sheng, X., Dorr, K.M., Hutton, J.E., Emerson, J.I., Davies, H.A., Andrade, T.D., Wasson, L.K., Greco, T.M., Hashimoto, Y., et al. (2021). Cardiac proteomics reveals sex chromosome-dependent differences between males and females that arise prior to gonad formation. Dev. Cell 55, 3019–3034.e7. https://doi.org/10.1016/j.devcel.2021.09.022.

65. Tani-Matsunaga, S., Viecelli, F.M., Gandhi, S., Inoue, K., and Bronner, M.E. (2018). Transcriptome profiling of the cardiac neural crest reveals a critical role for MaFb. Dev. Biol. 444, S209–S218. https://doi.org/10.1016/j.ydbio.2018.09.015.

66. Sato, T., Matsutomo, T., Kawano, H., Watanabe, T., Uematsu, Y., Sekine, K., Fukuda, T., Aihara, K., Kust, A., Yamada, T., et al. (2004). Brain masculinization requires androgen receptor function. Proc. Natl. Acad. Sci. USA 101, 1673–1678. https://doi.org/10.1073/pnas.030530101.

67. Rosenfield, C.S. (2017). Brain sexual differentiation and requirement of SRY: why or why not? Front. Neurosci. 11, 632. https://doi.org/10.3389/fnins.2017.00632.

68. Giudicelli, F., Gilarde-Hebenstreit, P., Mechtza-Grigoriou, F., Poquet, C., and Charnay, P. (2003). Novel activities of MaFb underlie its dual role in hindbrain segmentation and regional specification. Dev. Biol. 253, 150–162. https://doi.org/10.1016/j.ydbio.2002.08.064.

69. Inoue, S., Inoue, M., Fujimura, S., and Nishinakamura, R. (2010). A mouse line expressing Sall1-driven inducible Cre recombinase in the kidney mesenchyme. Genesis 48, 207–212. https://doi.org/10.1002/dvg.20603.

70. Harfe, B.D., Scherz, P.J., Nissim, S., Tian, H., McMahon, A.P., and Tabin, C.J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell 118, 517–528. https://doi.org/10.1016/j.cell.2004.07.024.

71. Winuthayanon, W., Hewitt, S.C., Orvis, G.D., Behringer, R.R., and Korach, K.S. (2010). Uterine epithelial estrogen receptor α is dispensable for proliferation but essential for complete biological and biochemical responses. Proc. Natl. Acad. Sci. USA 107,
78. Moriguchi, T., Hamada, M., Morito, N., Terunuma, T., Hasegawa, K., Zhang, C., Yokomizo, T., Esaki, R., Kuroda, E., Yoh, K., et al. (2006). MafB is essential for renal development and F4/80 expression in macrophages. Mol. Cell Biol. 26, 5715–5727. https://doi.org/10.1128/MCB.00001-06.

79. Tran, M.T.N., Hamada, M., Nakamura, M., Jeon, H., Kamei, R., Tsunakawa, Y., Kulathunga, K., Lin, Y.Y., Fujisawa, K., Kudo, T., and Takahashi, S. (2016). MafB deficiency accelerates the development of obesity in mice. FEBS Open Bio. 6, 540–547. https://doi.org/10.1002/2211-5463.12058.

80. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

81. Komeya, M., Yamanaka, H., Sanjo, H., Yao, M., Nakamura, H., Kimura, H., Fujii, T., Sato, T., and Ogawa, T. (2019). In vitro spermatogenesis in two-dimensionally spread mouse testis tissues. Reprod. Med. Biol. 18, 362–369. https://doi.org/10.1002/rmb2.12291.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti Type VI Collagen (raised against cow) pAb (Rabbit, Antiserum) | Cosmo Bio Ltd. | Cat. No.: LSL-LB-1697, RRID:AB_10708895 |
| Anti-Fibronectin antibody produced in rabbit | Sigma | Cat. No.: F3648, RRID:AB_476976 |
| Mouse Anti-Vinculin Monoclonal Antibody, Unconjugated, Clone hVIN-1 | Abcam | Cat. No.: ab11194, RRID:AB_297835 |
| Anti-Integrin alpha 5 antibody [EPR7854] ab150361 | Abcam | Cat. No.: ab150361, RRID:AB_2631309 |
| Myosin light chain (phospho S20) antibody | Abcam | Cat. No.: ab2480, RRID:AB_303094 |
| Phospho-FAK (Tyr397) Recombinant Rabbit Monoclonal Antibody (31H5L17) | Thermo Fisher Scientific | Cat. No.: 700255, RRID:AB_2532307 |
| Anti human SALL1 mouse monoclonal antibody | Perseus Proteomics | Cat. No.: PP-K9814-00, RRID:AB_1964373 |
| Anti-mouse GFP | Roche | Cat. No.: 11814460001, RRID:AB_399013 |
| Alexa Fluor546 | Molecular Probes Oregon | Cat. No.: A-11010, RRID:AB_2534077 |
| Alexa Fluor488 | Molecular Probes Oregon | Cat. No.: A-21121, RRID:AB_2535764 |
| Alexa Fluor647 | Abcam | Cat. No.: ab150079, RRID:AB_2722623 |
| Rabbit Anti-Murine MafB Polyclonal, Unconjugated antibody | Novus | Cat. No.: NB 600-266, RRID:AB_525413 |
| Hoechst33342 | Sigma-Aldrich | Cat. No.: 875756-97-1 |
| MafB (P-20) antibody, Santa Cruz Biotechnology | Santa Cruz Biotechnology | Cat. No.: sc-10022, RRID:AB_648633 |
| Monoclonal ANTI-FLAG® M2 antibody | Sigma-Aldrich | Cat. No.: F1804, RRID:AB_262044 |
| MMP-11 antibody | Abcam | Cat. No.: 1881-1, RRID:AB_765032 |

### Chemicals, peptides, and recombinant proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Tamoxifen | Sigma-Aldrich | Cat. No.: T5648 |
| Testosterone prorionate | Sigma-Aldrich | Cat. No.: T1875 |
| Fibronectin | Sigma | Cat. No.: FC1141 |
| Collagen VI, Human | Corning | Cat. No.: 354261 |
| Signal Enhancer HIKARI for Western Blotting and ELISA | Nacalai Tesque | Cat. No.: 02267-41, 02270-81 |
| Chemi-Lumi One L | Nacalai Tesque | Cat. No.: 07880-70 |

### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Vector TrueVIEW Autofluorescence Quenching Kit | Vector Laboratories | Cat. No.: SP-8400 |
| Multi Tissue Dissociation Kit 1 | Miltenyl Biotec Inc. | Cat. No.: 130-110-201 |
| QIAquick PCR Purification kit | Qiagen | Cat. No.: 28104 |

### Deposited data

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| RNA sequencing data | Kajoka et al., 2021 | GEO: GSE158279 |
| RNA sequencing data | This paper | GEO: GSE185966 |

### Experimental models: Cell lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human: HEK293 cells | ATCC | CRL-1573, RRID:CVCL_0045 |

### Experimental models: Organisms/strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: Sall1<cre/+ | Inoue et al., 2010 | N/A |
| Mouse: Shh<cre/+ | Harfe et al., 2004 | N/A |
| Mouse: Wnt7a<cre/+ | Winuthayanon et al., 2010 | N/A |

(Continued on next page)
RESOURCES AND METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents and resources should be directed to and will be fulfilled by the lead contact, Gen Yamada (genyama77@yahoo.co.jp).

Materials availability statement

This study did not generate new unique reagents.

Data availability statement

RNA-seq data have been deposited at GEO (GEO: GSE185966) and are publicly available as of the date of publication.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All laboratory animals were maintained under standard conditions in accordance to the Animal Use and Care Guidelines of the Wakayama Medical University, Japan. The following mouse lines were used in this study: ShhCre/+ 76, Wnt7aCre/+ 77, Sall1Cre/+ 75, MafBff 79, and MafBGFP/+ 78. All lines were on a C57BL/6J genetic background. Mice were bred, and the presence of a vaginal plug was designated as E0.5. For conditional knock-out mice, the flox allele was deleted by oral administration of Tamoxifen (200 mg/kg body weight) on E11.5 for Sall1Cre/+MafBf/f and E9.5 for ShhCre/+MafBff. Heterozygous or wild-type littermates were used as control. To analyze the sexual dimorphism of mRNA and protein expressions in the eExG, ICR mice were utilized. TP was orally administered at E14.5 and E15.5 at 100 mg/kg body weight to induce masculinization in female mice prior to harvest.

METHOD DETAILS

Histological analyses and imaging

All tissue samples were fixed in 4% wt/vol paraformaldehyde in PBS (PFA/PBS) and serially dehydrated in methanol. The samples were paraffin-embedded and cut into 6 μm sections for immunofluorescence and hematoxylin/eosin (HE) staining, while a thickness of 10 μm was used for mRNA in situ hybridization. HE
staining was performed using standard protocol. For mRNA in situ hybridization, the samples were deparaffinized, rehydrated, and then incubated in 65°C overnight with the mRNA probe for either Mmp11 and FN (forward: 5’-GCATCACGGCAGTGTTCTAGA-3’; reverse: 5’-GGTTGGTGATGAAGGGGTGC-3’). The slides were then washed with 1X TBST and labeled with an anti-DIG probe (1:1000) prior to colorization with NBT/BCIP.

Proteins were detected using standard immunofluorescence protocol: the samples were deparaffinized and rehydrated before being subjected to antigen retrieval. HistoVOne (105°C, 15 min) was used as the antigen retrieval agent for all antibodies except for anti-fibronectin (FN; 5% w/v trypsin, 5 min). For detecting extracellular matrix proteins, rabbit monoclonal antibodies for Collagen VI (ColVI; 1:1000) and FN (1:200) were used in this study. For detecting focal adhesions and related proteins, we used mouse monoclonal antibody for vinculin (VCL; 1:800) and rabbit monoclonal antibodies for integrin α5 (ITGa5; 1:200), phosphorylated focal adhesion kinase (pFAK; 1:500), and phosphorylated myosin light chain (pMLC; 1:1000). We also used the following antibodies as markers for our regions of interest: rabbit monoclonal antibody for MAFB (1:1000), mouse monoclonal antibody for SALL1 (1:200), and GFP (1:200). To reduce autofluorescence by red blood cells, slides were incubated for 5 min in TrueVIEW Autofluorescence Quenching Kit. Immunostaining was visualized using Alexa Fluor 488, Alexa Fluor 546, and Alexa Flour 647 (1:200). Nuclei was marked using Hoechst33342 (1:1000).

For immunocytochemical staining, cells were fixed in 4% PFA/PBS for 10 min, washed with PBS and permeabilized with 0.5% Triton X-. Slides were then incubated with the monoclonal antibodies targeting VCL (1:1000) and pMLC (1:1000). The cells were fluorescently labeled at a 1:300 dilution and counterstained with Hoechst 33342 and Hoechst 33342 (1:2000) prior to visualization.

All sections were viewed using an Olympus BX51 microscope and processed with Cell Sans Standard (v1.6, Olympus). Confocal fluorescence images were taken using the ZEISS LSM 900 with Airyscan 2 (Carl Zeiss). The images then were processed using the ZEN 2012 SP1 v8.1 software (black edition, Carl Zeiss). Live imaging videos were taken using ZEISS LSM 900 with Airyscan 2 equipped with an incubation chamber under 5% CO2 and 37°C.

Chromatin immunoprecipitation (ChIP) assay and ChIP-Seq analysis

The proximal and ventral portions of 30 eExG samples were dissected from E16.5 male ICR mice and homogenized in lysis buffer containing 10 mM HEPES-KOH (pH 7.3), 10 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. The chromatin was cross-linked for 30 min with 2 mM ethylene glycol bis(succinimidyl carbonate) (EGS) (Thermo Fisher Scientific, Inc) then with 1% formaldehyde for 5 min at RT. The resulting chromatin was digested using MNase (Takara) prior to being sonicated in SDS lysis buffer (50 mM Tris-HCl [pH8.1], 10 mM EDTA, 1%SDS). For the antibody reaction, 50 μg of DNA was immunoprecipitated with a specific antibody for MAFB (2.0 μg) at 4°C overnight. The immunoprecipitation buffer constituted of 16.7 mM Tris-HCl (pH8.1), 1.2 mM EDTA, 1.1% Triton X-, 0.01% SDS, 167 mM NaCl, 0.2 mM PMSF, and protease inhibitor cocktail. Dynabeads with Protein G (Life Technologies) were added to isolate protein-DNA complexes. Cross-linking was reversed at 65°C for 8 h. DNA fragments were purified by a QIAquick PCR Purification kit (QIAGEN). Polymerase chain reaction (PCR) was performed under the following conditions: 5 min at 95°C then 95°C for 10s, 55°C for 30s, 72°C for 1 min, and 72°C for 10 min for 40 cycles. The primer used for Mmp11 is listed in Table S2. Rabbit immunoglobulin (Dako) was used as a control.

Primary cell migration assay and culture

The biMs of E15.5 male ICR embryos were dissected, and the cells were separated using the gentleMACS Octo Dissociator (Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, the dissected tissues were placed inside gentleMACS C tubes with 1.1 mL of serum-free DMEM and the enzyme mix provided by the manufacturer. The tissues were processed at room temperature for 30 min. The resulting single-cell suspension was precipitated and resuspended in charcoal-filtered FBS- and DHT-supplemented media. For substrate migration assay, polydimethylsiloxane (PDMS) sheets were trimmed, submerged in 30 ng/cm² ColVI (Corning) or 30 ng/cm² FN (Sigma), and placed on a culture dish. The plates were incubated at 37°C for at least 20 min prior to use. Primary cells were cultured at a density of 50,000 cells and allowed to attach for 24 h before removing the PDMS sheets and imaging. For protein expression
analysis, the cells were plated at 100 cells/µL on an 8-well cell culture plate coated with either 30 ng/cm² CoVI (Corning) or 30 ng/cm² FN (Sigma) for 24 h before fixation and staining. (N ≥ 3)

**Mmp11 overexpression, eExG slice culture, and exogenous collagen 6 assays**

HEK293 cells were thawed and cultured until confluent in DMEM supplemented with FBS and 1% penicillin-streptomycin. The cells were harvested and a MPP11-myc-DDK-tagged plasmid vector (OriGene) was electroporated into the cells. A control setup was electroporated with a CMV6-Entry vector. The cells were allowed to recover for 24 h in serum-free DMEM, then cultured in 10% charcoal FBS-supplemented DMEM for an additional 48 h. The conditioned media was collected and used as culture medium for eExG slices (described below). After 24 h of culture, the tissue slices were collected, fixed, and analyzed. The media from both setups were collected to confirm MPP11 overexpression using Western blotting.

The proteins were separated using SDS-PAGE then blotted onto an Immobilon-P PVDF (polyvinylidene difluoride) membrane (Millipore). The membrane was blocked with 1% skim milk (BD Difco) in 1X TBST for 1 h, RT, prior to incubation with anti-FLAG antibody (1:1000) diluted in Signal Enhancer HIKARI for Western Blotting and ELISA Solution A (Nacalai Tesque) at 4°C, overnight. The membrane was then washed and incubated in the Signal Enhancer HIKARI Solution B (Nacalai Tesque) with HRP goat-conjugated anti-rabbit IgG (H + L) (Invitrogen) antibody. The signal was visualized using Chemi-Lumi One L (Nacalai Tesque) under the ChemiDoc XRS + system (BioRad Laboratories).

The eExG slice culture system was performed according to.5 The eExG of E15.5 mice were dissected and embedded in 4% low-melting point agarose in PBS. The tissues were sliced to a thickness of 140 µm using a 7000smz vibratome (Campden Instruments). The Z-deflection was adjusted to 0.03 mm or lower to reduce tissue damage. For Mmp11 overexpression assay, the slices from female eExG were placed on a Millicell Culture Insert (EMD Millipore) and cultured with either the conditioned or control media. For the exogenous CoVI assay, the slices were cultured on top of 1.5% agarose blocks that were supplemented with or without 10% CoVI (Corning), surrounded by DMEM supplemented with 10% charcoal-treated FBS, 1% penicillin-streptomycin, and 10⁻⁸ M DHT. The slices were kept for 24 h under 37°C and 5% CO₂ before fixation and analysis.

**RNA sequencing and qPCR analysis**

The biMs from the eExG of both male and female ICR mice (E13.5 and E16.5), along with the MafB KO and control (MafBGFP/+), was collected (n ≥ 3 per group). Total RNA was isolated using ISOGEN II (Nippon Gene Co., Ltd.) and reverse transcribed with PrimeScript RT Master Mix (Perfect Real-time, Takara Bio) following the manufacturer’s instructions. The preparation of the RNA libraries were entrusted to Novogene Japan K.K., and sequencing was performed using the Illumina HiSeq 4000. The data has been deposited in GEO under the accession number GSE185966. qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara Bio) in triplicate. At least three biological replicates were analyzed. GAPDH was used as internal control. Primer information is listed in Table S2.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

qPCR data is presented as mean relative expression ± SEM Statistical significance was assessed through t-test using Microsoft Excel. A p value of less than 0.05 was considered as statistically significant difference. All experiments were performed with at least three biological replicates.