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Embryonic tenocytes express Olfml2b, a potential direct target of Scleraxis

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Abstract

We identified Olfml2b as a candidate downstream target of the tendon transcription factor Scleraxis (Scx). Olfml2b is expressed in mouse embryonic tenocytes, colocalizes with a tendon marker Mkx. Luciferase assay identified a Scx-responsive element approximately 5 kb upstream of Olfml2b transcription start site. To investigate its function, we generated an Olfml2b knockout mouse. Despite its tendon-specific expression, loss of Olfml2b did not affect embryonic tendon formation or the gross morphology of adult tendons. These findings indicate that Olfml2b is a possible Scx-regulated tendon gene but is dispensable for tendon development. Further study is required on its physiological role.

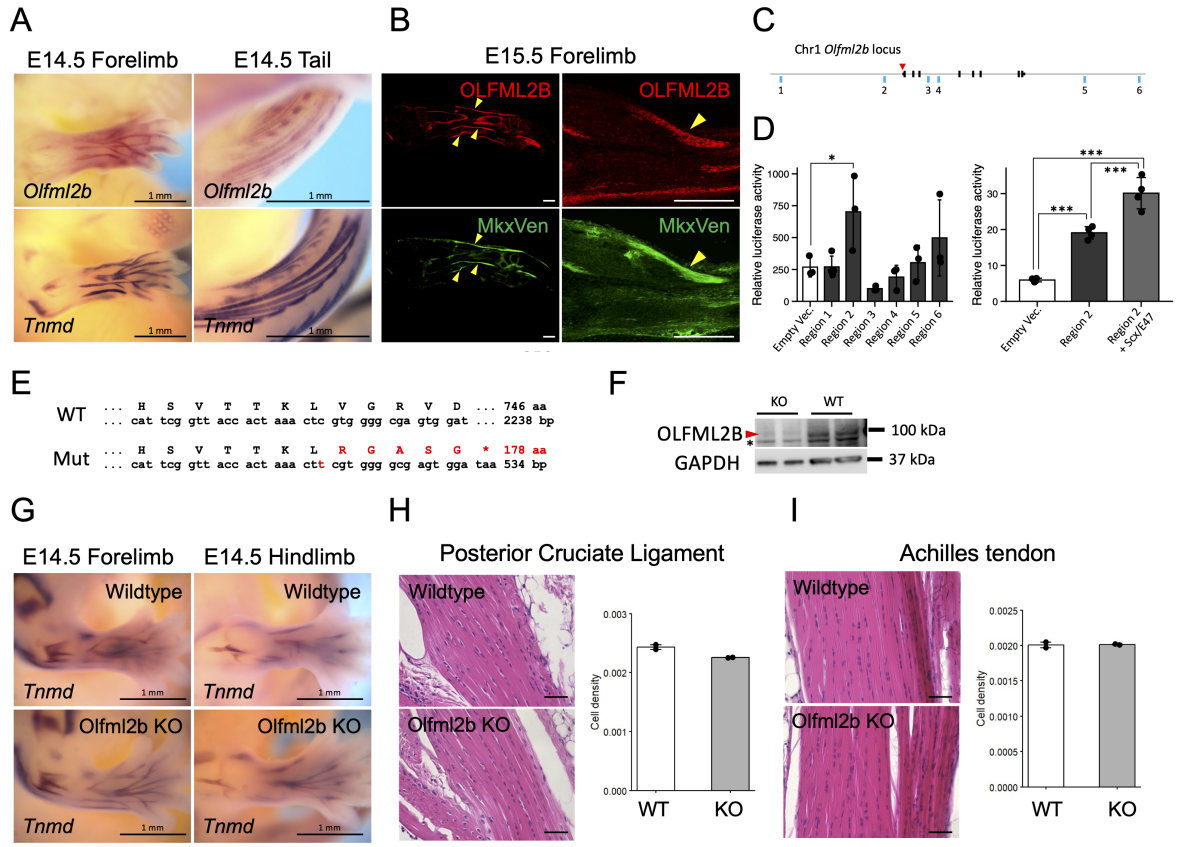


Figure 1. Olfml2b is expressed in embryonic tendon as possible direct target of Scx transcription factor:

(A) Whole mount in situ hybridization of *Olfml2b* mRNA (top row) and *Tnmd* mRNA (bottom row) in E14.5 embryo (n=3). Scale bar: 1 mm. (B) Immunofluorescence of OLFML2B (top row) and Venus (Mkx-Ven) (bottom row) in forelimb section of E15.5 Mkx-Ven embryo (n=3). Scalebar: 250 µm (C) Schematic drawing of *Olfml2b* gene and regulatory region candidates. Black boxes indicate *Olfml2b* exons. Blue boxes with numbers show the position of regulatory region candidates. Red arrowhead indicates the transcription start site of *Olfml2b* gene. (D) Left panel, a luciferase assay with pNL1.1-TK (Empty Vec) and pNL1.1-TK-candidate regions. Data is shown with mean + SD and a one-way ANOVA followed by Dunnett post hoc test was used for the statistical analysis. *p < 0.05. Right panel, a luciferase assay with pNL1.1-TK (Empty Vec), pNL1.1-TK-region2 vector with or without the overexpression of Scx and E47. Data is shown with mean + SD and a one-way ANOVA followed by Tukey's post hoc test was used for the statistical analysis. ***p <

0.001 (E) The deduced amino acid sequence (upper letters) from wildtype (WT) and mutant (MUT) DNA sequence (lower letters) around 1 base insertion (red letter) in *Olfml2b* mutant allele. Wildtype (WT) sequences are shown at the top and mutant (MUT) sequences are shown at the bottom. Red letters in DNA sequence and amino acid sequence indicate the inserted base and altered amino acids, respectively. (F) Western blotting of OLFML2B and GAPDH proteins in wildtype (WT) and Olfml2b KO (KO) embryonic limb (n=2). Red arrowhead and asterisk indicate OLFML2B protein and non-specific band, respectively. (G) Whole mount in situ hybridization of *Tnmd* mRNA in Wildtype (top row) and Olfml2b KO (bottom row) in E14.5 embryo (n=3). Scale bar: 1 mm. (H-I) H&E staining of posterior cruciate ligament (H) and Achilles tendon (I) of 11-weeks-old wildtype mice (top panels) and Olfml2b KO mice (bottom panels) cell density (cells/ µm2) within tendon tissue are shown at right (n=2). Scale bar 50 µm.

Description

Tendon is a dense connective tissue that transmits the muscle contractile force to the bones. Scleraxis (Scx) is one of the essential transcription factors for tendon formation and maintenance (Brent et al., 2003; Huang et al., 2015; Liu et al., 2021; Schweitzer et al., 2001; Shukunami et al., 2018; Yoshimoto et al., 2017), however its direct target gene as well as their roles in tendon biology is insufficiently understood (Liu et al., 2021). Previously, we have identified genes that are downregulated in the embryo where Scx-lineage cells were ablated, and thus are possible Scx downstream targets (Ono et al., 2023). Among them, we found that *Olfactomedin Like 2B* (*Olfml2b*) gene is expressed in embryonic limb and tail at E14.5, in domains similar to those of *Tnmd*, a well-known Scx direct target gene (Fig. 1A). Immunofluorescence analysis in embryonic fore limb sections confirmed that OLFML2B protein colocalized with Mkx-Venus (MkxVen) tendon reporter (Ito et al., 2010) (Fig. 1B). Specifically, OLFML2B signals were detected in the flexor and extensor tendons that are positive for MkxVen (Fig. 1B arrowheads). These results indicate that Olfml2b is expressed in embryonic tenocytes. Because *Olfml2b* encodes a secreted extracellular matrix (ECM) associated protein (Furutani et al., 2005), we hypothesized that it could contribute to the development and function of tendon tissue which is rich in ECMs.

Recent ChIP-seq study identified several Scx binding peaks near Olfml2b gene (Liu et al., 2021). We have examined the transcriptional regulatory activity of sequences around these Scx binding peaks by luciferase assay using primary rat tenocyte (Fig. 1C), and found that 800 bp sequence at 5 kbp upstream of Olfml2b transcription start site (region 2) has positive transcriptional activity (Fig. 1D). Moreover, overexpression of Scx together with E47 increased the luciferase activity, indicating that this sequence could respond to Scx (Fig. 1D).

As Olfml2b is expressed in tenocyte as possible target of Scx transcription factor, we hypothesize that this gene could play some role in tendon formation and homeostasis. In line with this idea, *OLFML2B* mutation was identified in a family with twin sibling males diagnosed as an anterior cruciate ligament non-contact rupture (Caso et al., 2016). To elucidate the role of Olfml2b in tendon tissue, we have generated Olfml2b knockout (KO) mouse with genome editing technique. Established Olfml2b knockout mouse line has 1 bp insertion mutation in 3rd exon of Olfml2b gene that caused frameshift mutation and premature termination at 178th amino acid to OLFML2B protein (Fig. 1E). Western blotting analysis confirmed that OLFML2B protein is absent in embryonic limb extract of Olfml2b KO mouse (Fig. 1F). *Tnmd* expression of Olfml2b KO embryo at E14.5 was similar to that of wildtype embryo, indicating that Olfml2b is not essential for the embryonic tendon formation (Fig. 1G). Histological examination of the Achilles tendon and cruciate ligaments in 11-weeks-old Olfml2b KO mice revealed that the gross morphology undistinguishable from those of wildtype mice (Fig. 1H, I). Cell density of cruciate ligaments, but not of Achilles tendon, was slightly lower in Olfml2b KO mice (Fig. 1H, I). Altogether, our results indicated that Olfml2b is expressed in embryonic tendon cell but is not essential for the tendon/ligament tissue formation. We have identified possible regulatory sequence at ~5 kb upstream of Olfml2b gene. Further evaluation such as the narrowing down the crucial sequence and electrophoresis mobility shift assay (EMSA), would elucidate whether it contains direct regulatory element by Scx transcription factor (Shukunami et al., 2018). The role of Olfml2b in tendon tissue remains elusive. Considering the ECM associating nature of Olfml2b, it could be involved in collagen fiber organization in tendon tissue. Given the trend toward reduced cell density in Olfml2b KO cruciate ligament, further analyses with increased sample number and extending to other tendon/ligament tissues may help elucidate the function of Olfml2b. Other members of Olfml gene family could have redundant role that needs to be evaluated in future studies.

Methods

Animals

C57BL/6 mice, ICR mice, and Wistar rats were purchased from Sankyo lab-service (Tokyo, Japan). MkxVen mouse (Ito et al., 2010) is kindly provided by Dr. Asahara. All animal experiments were approved by the animal experiment committees of Meiji University (approval No. MUIACUC2022-04).

Generation of Olfml2b knockout mouse

Olfml2b KO mouse was generated by genome editing (Hashimoto and Takemoto, 2015). Briefly, a crRNA targeting exon 3 of the Olfml2b gene (5'-TCGGTTACCACTAAACTCGT-3'), tracrRNA, and S.p.Cas9 protein were purchased from IDT (Coralville, Iowa, USA). The fertilized eggs of C57BL/6 mice purchased from Ark-Resource (Kumamoto, Japan) were mixed with crRNA/tracrRNA/Cas9 protein and electroporation was performed using CUY21EDITⅡ electroporator (BEX, Tokyo, Japan). The electroporation conditions were 30 V (3 msec ON + 97 msec OFF) x 7 times. The surviving 2-cell-stage embryos were transferred to the oviducts of pseudo-pregnant ICR female mice. Genome sequencing of one F1 offspring revealed a 1-bp insertion in exon 3, predicted to cause a frameshift mutation.

Primary tenocyte culture

Primary tenocytes were isolated from 3-weeks-old male Wistar rat and cultured as described previously (Tsuchiya et al., 2025).

Luciferase assay

Genomic sequences from 6 regions neighboring Olfml2b gene were PCR amplified and cloned into pNL1.1 vector (Promega, Wisconsin, USA) together with thymidine kinase (TK) promoter. Primer sequences used to the cloning are listed as below.

Region 1 fw: 5'-CAAATAGGACCCGTGAGAGC-3', rev: 5'-TGGAGCTCGGATTACAGAGG-3'

Region 2 fw: 5'-TTTTCCCGGTCCCTAGTCTC-3', rev: 5'-AGCCTGTTGATTCCATGTCC-3',

Region 3 fw: 5'-GGATGTGAACCCTCAGGAAA-3', rev: 5'-GTGGTGGGTGCTCAGAAGTT-3'

Region 4 fw: 5'-CCGAGGGTCAGTGATGTTTT-3', rev: 5'-CAAATGTTGCTCCCAAGTCA-3'

Region 5 fw: 5'-GCAACCTCCTCCTGGTTGTA-3', rev: 5'-TGGTCTAGCAGTGCAGGATG-3'

Region 6 fw: 5'-CCGATTCCACAGATGATAAGG-3', rev: 5'-TCCAAGATTCCTTTGCGTTC-3'

For overexpression plasmid, mouse Scleraxis coding sequence was PCR amplified and cloned into pcDNA3.1. Expression vector for mE47 was purchased from Addgene (ID34585) (Lee et al., 2011). Tenocytes were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) and extracted with lysis buffer (50 mM HEPES pH 7.8, 200 mM NaCl, 5 mM EDTA, 1% NP40, 5% Glycerol, 1 mM DTT) after 48 hours. Luciferase activities were detected using Nano-Glo Dual-Luciferase Reporter Assay (Promega) as described previously (Saotome et al., 2020). pGL4.53 (Promega) was used as internal control.

Immunofluorescence

Immunofluorescence were performed essentially as described before (Ono et al., 2023; Yamasaki et al., 2026). Briefly, mouse embryos were fixed with 4% paraformaldehyde (PFA) overnight at 4 °C, embedded in optimal cutting temperature (OCT) compound (Sakurafinetek, Tokyo, Japan) and sectioned at 10 µm. Sections were blocked with 10% fetal bovine serum (FBS)/Phosphate buffer saline (PBS) for 1 hour at room temperature and incubated with primary antibodies overnight at 4 °C. The sections were washed with PBST (PBS supplemented with 0.1 % Tween 20), incubated with secondary antibodies for 1 hour at room temperature, washed with PBST and mounted with fluoroshield mounting medium (Immunochemistry Technologies, Davis, CA, USA). α-GFP (Abcam, Cambridge, UK, ab13970, 1:1000), α-Olfml2b (Merck HPA054136) were used as primary antibodies.

Whole-mount in situ hybridization (WISH)

WISH analysis was performed as described before (Sagasaki et al., 2026; Tanji et al., 2023). *Olfml2b* probe sequence were amplified from embryonic limb cDNA using primers below (fw 5'-cagtggagtggacttgctga-3', rev 5'-cataacgctgcttcaggtca-3') and cloned into pGEM T-easy vector (Promega). *Tnmd* probe was described previously (Shukunami et al., 2001).

Histology

Hindlimbs of 11-week-old mice were fixed with 4% PFA overnight at 4 °C, decalcified with 0.5 M EDTA solution, embedded with paraffin wax. Samples were sectioned at 6 µm and hematoxylin and Eosin (H&E) staining was performed as described before (Fukunaga et al., 2021). The cell density of the tendon/ligament tissue were calculated by counting hematoxylin positive nuclei within H&E image and normalized to the tendon area using Image J. Two individuals for each genotype were examined and both data were shown in the panel.

Western blotting

Western blotting was performed as described before (Inui et al., 2018; Saotome et al., 2020). α-Olfml2b (Merck, HPA054136, 1:500) and α-GAPDH (Millipore, MAB374, 1:2000) were used as primary antibodies and α-rabbit IgG-Peroxidase (SIGMA-Aldrich, A0545, 1:2000) and α-mouse IgG-Peroxidase (SIGMA-Aldrich, A2304, 1:2000) were used as secondary antibodies.

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