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Stepwise direct reprogramming of adult human dermal fibroblasts into tendon-like cells via transcription factor optimization and 3D culture

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Abstract

Introduction

: Synthetic materials are widely used in pediatric surgery for congenital diaphragmatic hernia repair when primary closure is not feasible but remain associated with complications and lack growth potential. We investigated whether direct reprogramming could promote tenogenic differentiation of adult human dermal fibroblasts (aHDFs) toward patient-derived muscle-tendon constructs for pediatric reconstruction.

Methods

aHDFs were transduced into directly converted tenoblasts (dTBs) using retroviral transduction of tendon-related transcription factors and Yamanaka factors. Different factor combinations and culture conditions were evaluated. Tenogenic induction was assessed using real-time RT-PCR and immunostaining for tendon markers. *In vivo* potential was examined by subcutaneous transplantation into NOG/SCID mice.

Results

Six-factor transduction induced the expression of tendon-related markers tenomodulin (TNMD) and thrombospondin-4 (THBS4), and enabled short-term engraftment *in vivo*. Optimization identified SCX, MKX, OCT3/4, and MYCL as a sufficient combination for tenogenic conversion. OCT3/4 promoted TNMD expression, whereas SCX affected THBS4 expression. dTBs exhibited elongated tendon-like morphology and positive TNMD and THBS4 staining. Transfer from two-dimensional to three-dimensional culture further increased tenogenic marker expression.

Conclusions

Direct reprogramming with SCX, MKX, MYCL, and OCT3/4 promoted tenogenic differentiation of aHDFs and induced tendon-like characteristics. This simplified strategy may facilitate development of autologous muscle-tendon constructs for regenerative reconstruction in pediatric surgery.

Clinical trial registration

Not applicable. This study did not involve a clinical trial

1. Introduction

In pediatric surgery, synthetic materials such as expanded polytetrafluoroethylene (ePTFE; Gore-Tex) sheets are widely used to treat severe defects of the diaphragm in congenital diaphragmatic hernia (CDH) when primary closure is not feasible [1]. However, these artificial materials lack growth potential and are prone to infection, calcification, and recurrence [2]. These shortcomings limit long-term outcomes, particularly with respect to somatic growth in children [3], thus emphasizing the need to develop patient-derived, living tissue substitutes that can grow and adapt to the patient.

The diaphragm consists of both skeletal muscles and tendons, each of which is essential for durable repairs. Our long-term goal is to apply bioengineered sheets for the treatment of patients with diaphragmatic hernia; however, achieving this goal requires overcoming multiple technical challenges using a stepwise strategy. A previous study from our group demonstrated that adult human dermal fibroblasts (aHDFs) can be directly reprogrammed into myoblasts [4], and applied to biosheets, suggesting the feasibility of patient-derived, cell-based tissue engineering approaches [5]. Although direct reprogramming has been successfully applied to generate various cell types, such as cardiomyocytes and neurons [6, 7], its application in the tendon lineage remains limited. In particular, it remains unclear which transcription factors are essential for tenogenic induction and how their combinations can be optimized for this purpose. Furthermore, the role of microenvironmental cues, such as three-dimensional culture, in reinforcing tenogenic programs has not been systematically examined.

To address these issues, we focused on the tendon-specific transcription factors *SCX* and *MKX*, together with *MYCL* which has been shown to enhance reprogramming efficiency [8, 9], and combined them with selected Yamanaka factors *KLF4*, *OCT3/4*, and *SOX2* to promote cellular plasticity.

In this study, we aimed to promote tenogenic differentiation of aHDFs and induced tendon-like characteristics using direct reprogramming, and establish a stepwise strategy for tenogenic induction through transcription factor optimization and microenvironmental modulation.

2. Materials and Methods

2.1. Cells

aHDFs and PLAT-GP packaging cells were obtained from Toyobo (Osaka, Japan) and Cell Biolabs (San Diego, CA, USA), respectively. The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 mM non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. Primary human tenocytes (pHTCs) were purchased from ZenBio (Research Triangle Park, NC, USA) and cultured in tendon cell growth medium (Angio-Protémie, Bavaria, Germany).

2.2. Retroviral vectors

SCX and *MKX* coding sequences were synthesized using GenScript (Tokyo, Japan) and cloned into EcoRI-digested pMXs vectors (Cell Biolabs) using the GeneArt system (Invitrogen, Waltham, MA, USA).

pMXs vectors containing *KLF4*, *OCT3/4*, *SOX2*, *MYCL*, and *GFP* were kindly provided by S. Yamanaka (Kyoto University, Kyoto, Japan).

Briefly, PLAT-GP packaging cells were seeded at 5×10^6 cells per 100-mm gelatin-coated dish and cultured overnight. Cells were then co-transfected with 2.5 μg each of pCMV-VSV-G and a pMX plasmid containing the transcription factor genes described above using X-treme Gene 9 (Roche Applied Science, Penzberg, Germany) in 500 μL Opti-MEM. After 24 h, the medium was replaced with fresh, antibiotic-free DMEM. Viral supernatants were collected 24 h later, filtered through a 0.45-μm pore size filter, and used for infection of the cells.

2.3. Retroviral transduction of fibroblasts into tenoblasts

aHDFs were seeded at a density of 5×10^4 cells/well in 24-well plates (day 1). On day 2, retroviral supernatants encoding six transcription factors (*SCX*, *MKX*, and *MYCL* together with the Yamanaka factors, *KLF4*, *OCT3/4*, and *SOX2*) were prepared. To enhance transduction efficiency, the viral mixtures were supplemented with 5 μg/mL TransDux (System Biosciences, Palo Alto, CA, USA) and 100 μL MAX Enhancer (System Biosciences, Palo Alto, CA, USA) per 400 μL of medium and applied to the aHDFs after removal of the culture medium. After 24 h, the virus-containing medium was replaced with fresh tendon cell growth medium. Initially, aHDFs were transduced with six transcription factors to induce tendon lineage conversion in the cells. To identify the minimal factor combination required for induction, we tested multiple combinations by omitting individual factors from the initial six-factor cocktail. Based on these optimization experiments, the combination of SCX, MKX, OCT3/4, and MYCL was selected for subsequent analyses and designated as directly converted tenoblasts (dTBs). All cells were trypsinized and collected by centrifugation on day 14 unless otherwise indicated.

2.4. Three-dimensional culture of tenoblasts

Cells cultured under three-dimensional (3D) conditions were embedded in a collagen gel matrix, whereas cells cultured under conventional two-dimensional (2D) conditions were maintained in standard culture dishes. For 3D culture, dTBs were resuspended at a density of 1.0×10^6 cells/mL in DMEM supplemented with 100 mM non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell suspension was then mixed with atelocollagen (3D Ready Atelocollagen; Koken, Tokyo, Japan) at 1:9 ratio. A total of 300 μL of the mixture was plated into 48-well plates and incubated at 37°C for 2 h to allow gelation. After gel formation, the appropriate culture medium for each cell type was added to the top of the gel, and the cells were maintained under 3D conditions.

Four experimental groups were established: (1) cells cultured under 2D conditions for 14 days; (2) cells transferred from 2D to 3D culture on day 1 after transduction and maintained until day 14; (3) cells transferred on day 7 and maintained until day 14; and (4) cells transferred on day 14 and maintained until day 21. To recover cells from the 3D cultures, collagen gels were completely digested using 0.2% collagenase solution, and the cells were subsequently collected.

2.5. Real-time RT-PCR

Total RNA was extracted from *in vitro*-generated dTBs and graft tissues following *in vivo* transplantation using ISOGEN II (Nippon Gene, Tokyo, Japan), and reverse-transcribed with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Real-time RT-PCR was performed using the Real-Time PCR Master Mix (Toyobo) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression levels were normalized to the expression of β-actin. All values are presented as the mean ± standard deviation, and each experiment was performed in triplicate (n = 3 independent biological replicates). Both *in vitro* and *in vivo* expression levels were calculated relative to those of unmodified HDFs (set at 1.0). The primer sequences are listed in Supplementary Table S1.

2.6. Immuno-staining

Cells were fixed with 4% paraformaldehyde at 4°C for 30 min. After rinsing with PBS, the cells were permeabilized with 0.1% Triton X (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature. The cells were incubated with anti-tenomodulin (TNMD) antibody (Thermo Fisher Scientific, Tokyo, Japan; Catalog No. PA5-112767; 1:50 dilution) and anti-thrombospondin 4 (THBS4) antibody (Thermo Fisher Scientific, Tokyo, Japan; Catalog No. PA5-149867; 1:50 dilution) at 4°C for overnight. After rinsing with PBS, the sections were incubated with Alexa Fluor 594–conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA; 1:2000 dilution) for 30 min at room temperature. Following another PBS wash, nuclei were stained with Hoechst® 33342. Immunostaining was performed on three cell types: aHDFs as negative controls, pHTCs as positive controls, and dTBs. Staining was observed using a fluorescence microscope, and the nuclei were imaged and quantified using a Keyence BZ-X710 microscope (Keyence, Osaka, Japan).

2.7. In vivo injection of reprogrammed fibroblasts

All animal experiments were approved by the Institutional Animal Care and Use Committee, and animal care was conducted following institutional guidelines. Male NOG/SCID mice (age, 8–10 weeks; CLEA Japan, Tokyo, Japan) were anesthetized using isoflurane. aHDFs transduced with all six transcription factors ($3–5 \times 10^5$ cells) were suspended in a 1:1 (v/v) mixture of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA). The cell–Matrigel mixture (total volume 100 µL) was subcutaneously injected into the flank of each mouse using a 27-gauge needle. Mice were monitored until recovery from anesthesia, sacrificed seven days after subcutaneous injection of the cells, and graft tissues were harvested for histological and immunohistochemical analyses.

2.8. Immunohistochemistry

The mice were sacrificed seven days after the cell inoculation. Graft tissues were excised, fixed in 4% paraformaldehyde at 4°C overnight, and embedded in paraffin. Sections (12 µm thick) were deparaffinized and rehydrated. After blocking with Blocking One Hist (Nacalai Tesque, Kyoto, Japan), the sections were incubated with an anti-TNMD antibody (Thermo Fisher Scientific, Tokyo, Japan; catalog no. PA5-112767; 1:50 dilution) at 4°C overnight, identical to the primary antibody used for immunocytochemistry. After rinsing with PBS, the sections were incubated with Alexa Fluor 594–conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA; 1:2000 dilution) for 60 min at room

temperature. The nuclei were counterstained with Hoechst® 33342. Fluorescence images were acquired using a Keyence BZ-X710 microscope (Keyence, Osaka, Japan). Hematoxylin and eosin (HE) staining was performed on sections from both aHDFs and dTBs grafts to evaluate the general histological morphology.

2.9. Data analysis

Data are presented as the mean ± standard deviation (SD). Differences between the two groups were assessed using Student's t-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Initial tenogenic induction using six transcription factors

As shown in Fig. 1, the positive control pHTCs exhibited the increased expression of tendon-related markers, including TNMD, THBS4, and SCX, confirming their tenogenic phenotype. In aHDFs transduced with the six transcription factors, TNMD and THBS4 were also significantly upregulated ($p < 0.05$). As expected, SCX and MKX expression levels were elevated, reflecting exogenous gene delivery rather than endogenous induction. In contrast, the expression levels of collagen type I alpha 1 (COL1A1), decorin (DCN), and major extracellular matrix components remained basal across all groups.

To assess the *in vivo* engraftment and persistence of induced cells, subcutaneous transplantation of aHDFs transduced with the six transcription factors was performed in NOG-SCID mice. Graft tissues were macroscopically identifiable at the injection sites without apparent necrosis or inflammation 7 days after transplantation. HE staining showed clusters of polygonal cells with prominent nuclei embedded within the graft matrix in aHDFs transduced with the six transcription factors, whereas such features were not observed in the aHDFs group (Fig. 2A, B). Immunohistochemistry revealed that TNMD-positive cells were observed only in the transduced group, whereas no TNMD-positive cells were detected in the aHDFs group (Fig. 2C, D). Real-time RT-PCR revealed a marked increase in the TNMD expression in the dTBs group relative to the aHDFs group (Supplementary Fig. S1). These findings support the feasibility of *in vivo* engraftment and short-term persistence of transduced cells, providing a basis for subsequent optimization studies.

3.2. Optimization of transcription factor combinations for tenogenic conversion

Figure 3A shows the effects of the five-factor combinations on the expression of TNMD. The TNMD expression in the group lacking OCT3/4 was not significantly different from that in the aHDFs, whereas all other combinations exhibited significantly higher TNMD expression levels. Similarly, in the four-factor

combinations (Fig. 3B), the TNMD expression was lower in the absence of OCT3/4. Among the combinations tested, cells transduced with SCX, MKX, OCT3/4, and MYCL showed no significant difference in TNMD expression levels relative to those transduced with all six transcription factors. These results suggest that OCT3/4 contributes to the induction of TNMD and that a four-factor combination may be sufficient. aHDFs transduced with SCX, MKX, OCT3/4, and MYCL (dTBs) were analyzed and used for subsequent experiments.

Figure 4 shows the effects of all possible combinations of the four transcription factors on the expression of TNMD and THBS4. The expression of TNMD was observed in cells transduced with OCT3/4 alone, whereas the highest expression levels were detected in cells transduced with all four factors. In contrast, the expression of TNMD was low in groups lacking OCT3/4 (Fig. 4A). The expression of THBS4 (Fig. 4B) was higher in cells transduced with SCX alone than in those transduced with other single factors, and no significant difference was observed between the SCX alone, SCX + MYCL, and the group transduced with all four factors. These results suggest that OCT3/4 predominantly contributes to the expression of TNMD, whereas SCX plays a predominant role in the expression of THBS4.

3.3. Phenotypic characterization of directly converted tenoblasts

Figure 5 shows the phenotypic characteristics of the dTBs. Phase-contrast microscopy revealed that aHDFs exhibited a typical spindle-shaped morphology with a relatively uniform size. In contrast, both dTBs and pHTCs were more elongated and had a fiber-like appearance, with enhanced alignment and intercellular connections. Although the dTBs exhibited an elongated morphology similar to that of the pHTCs, their overall size was smaller.

Immunocytochemistry showed that unmodified aHDFs were negative for TNMD and THBS4, whereas both pHTCs and dTBs were positive for these markers. The staining pattern of dTBs appeared fibrillar, resembling certain features of pHTCs. The fluorescence intensity in the dTBs was slightly lower than that in the pHTCs, but remained clearly above the background levels. These findings demonstrate the tendon-associated morphological and protein expression features of dTBs.

3.4. Enhancement of tenogenic properties by three-dimensional culture

To characterize the temporal changes in tenogenic marker expression following transduction, TNMD mRNA levels were evaluated over time. Kinetic analysis indicated that the TNMD mRNA expression in dTBs increased markedly from day 10 after transduction and reached a plateau by day 14 (Supplementary Fig. S2). Based on this time-course profile, subsequent three-dimensional culture experiments were conducted.

Figure 6 compares the dTBs according to the timing of transfer from 2D to 3D culture. Regarding the expression of TNMD mRNA, dTBs transferred to 3D cultures on day 14 showed significantly higher

expression levels relative to the other groups. In contrast, cells transferred to 3D culture at earlier time points did not show a significant difference from those transferred to the 2D until day 14.

Regarding THBS4, cells transferred to 3D culture at day 14 exhibited expression levels comparable to those of the 2D day 14 group, whereas no expression was observed in cells transferred to 3D culture at earlier time points. These results suggest that tenogenic induction is more effectively achieved in 2D culture, and that subsequent transfer to 3D culture after differentiation may enhance the expression of tenogenic markers, indicating the potential benefits of 3D culture.

4. Discussion

In this study, we demonstrated that direct reprogramming with SCX, MKX, MYCL, and OCT3/4 promoted tenogenic differentiation of aHDFs and induced tendon-like characteristics. The induced cells exhibited tendon-associated features, including upregulation of TNMD and THBS4, and acquisition of an elongated morphology. Notably, OCT3/4 and SCX were identified as key contributors to the induction of tendon-related markers, and a minimal combination of four transcription factors was found to be sufficient for tenogenic conversion. In addition, 3D culture further enhanced the expression of tenogenic markers in the induced cells.

This study parallels earlier direct reprogramming studies in other lineages. For example, fibroblasts have been converted into cardiomyocytes using GMT factors [6], neurons using BAM factors [7], skeletal myoblasts using MYOD1 [4, 10, 11], Schwann cells using SOX10 and Krox20 [12], osteoblasts using OCT4 and MYCL [13], brown adipocytes using C/EBP-β and C-MYC [14], and urothelial cells [15]. While these pioneering studies established the feasibility of lineage conversion, subsequent investigations are required to confirm functional maturation and stability. In line with this progression, we first verified the *in vivo* stability and persistence of the induced cells. To our knowledge, this study is the first to demonstrate direct transcription factor–mediated promotion of tenogenic differentiation in aHDFs through direct reprogramming. Notably, the induced cells exhibited tendon-like morphological features and upregulation of tendon-specific markers, including TNMD and THBS4 [16–19], providing proof-of-concept evidence that direct reprogramming can promote a tenogenic phenotype in fibroblasts and highlighting the novelty of our approach.

These results suggest that each transcription factor plays a distinct role in tenogenic reprogramming. SCX is a key regulator of tendon development, and tendon formation is severely impaired in Scx-deficient mice [20]. In our study, SCX was associated with THBS4 expression, suggesting its involvement in tendon-specific gene activation. OCT3/4 is a core pluripotency factor that regulates chromatin accessibility and maintains cellular plasticity during cell fate transitions [21, 22]. It has been reported to act as a pioneer factor that facilitates the activation of lineage-specific gene programs during reprogramming [23]. Thus, OCT3/4 may promote early cell plasticity and help induce TNMD by making cells more receptive to changes in cell type. MYCL likely improves reprogramming efficiency by promoting cell growth and suppressing fibroblast gene programs, as reported in other systems [4, 7, 24].

In this study, MYCL was preferentially expressed in groups with higher TNMD and THBS4 expression, consistent with its role in enhancing reprogramming efficiency. MKX is required for tendon maturation, as tendons do not fully develop in Mkx-deficient mice [25]. However, its role may be limited due to the immature tenoblast-like stage of the induced cells. Overall, these factors appear to act cooperatively, with SCX guiding tendon identity, OCT3/4 enhancing chromatin plasticity, MYCL improving efficiency, and MKX possibly supporting later-stage maturation.

3D culture has been reported to enhance tenogenic gene expression and promote tendon-like phenotypes, including upregulation of TNMD and activation of signaling pathways responsive to mechanical forces. Consistently, elongated morphologies and aligned 3D microenvironments are critical for maintaining the identity and function of tenocytes [26, 27]. In this study, dTBs cultured in collagen gel exhibited higher expression levels of tendon-related markers than those in 2D culture. The expression of TNMD was maintained even when cells were transferred early, whereas the expression of THBS4 was not. This likely reflects stage-dependent regulation, as TNMD is induced at earlier stages of tenogenic differentiation, whereas THBS4 is associated with a more mature matrix organization [28, 29]. Thus, early 3D transition may be insufficient to support the expression of THBS4, whereas prolonged 2D preconditioning may establish a permissive state that allows its maintenance in the 3D environment. Although previous studies have compared 2D and 3D cultures of tendon cells, it remains unclear which stage of induction or differentiation is advantageous for 2D or 3D culture conditions [30, 31]. These findings suggest that 3D culture reinforces, rather than initiates, tenogenic maturation in a stage-specific manner during direct reprogramming.

This study has several limitations. First, the functional properties of the induced tenocyte-like cells, including mechanical strength and force transmission, were not assessed. Second, *in vivo* analyses were limited to short-term engraftment, and the long-term survival, integration, and contribution to tendon remodeling remain unclear. Third, although tendon-associated molecular and morphological features were observed, comprehensive functional validation confirming full tenocyte identity was not performed. In addition, the mechanistic basis underlying transcription factor synergy and the effects of 3D culture are not fully understood at the level of intracellular signaling or gene regulation. These limitations highlight the need for further studies to determine whether the induced cells achieve functional tenocyte identity and to evaluate their biomechanical properties. In the future, co-culture with directly converted myoblasts may enable the evaluation of cell–cell interactions and functional properties, facilitating the development of muscle–tendon junction models and engineered composite sheets.

5. Conclusion

Direct reprogramming with a defined combination of *SCX*, *MKX*, *MYCL*, and OCT3/4 promoted tenogenic differentiation of aHDFs and induced tendon-like characteristics. The reprogrammed cells exhibited tendon-associated features, consistent with a tenogenic phenotype. Notably, this study identified a minimal set of transcription factors required for tenogenic conversion and indicated their potentially distinct roles in the reprogramming process. Although further functional validation is required, this

streamlined strategy provides a foundation for generating patient-derived tendon components for bioengineered muscle-tendon constructs and may contribute to future regenerative reconstruction for CDH and other pediatric surgical disorders.

Abbreviations

ePTFE
Expanded polytetrafluoroethylene
CDH
Congenital diaphragmatic hernia
aHDFs
Adult human dermal fibroblasts
pHTCs
Primary human tenocytes
dTBs
Directly converted tenoblasts
TNMD
Tenomodulin
THBS4
Thrombospondin-4
SCX
Scleraxis
MKX
Mohawk homeobox
COL1A1
Collagen type I alpha 1
DCN
Decorin
HE
Hematoxylin and eosin

Declarations

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Conflict of interest: The authors declare no conflicts of interest in association with the present study.

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Ethics approval

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of our institution (Approval No. M2022-558).

Consent to Participate

Not applicable. Commercially available human-derived cells were used in this study. According to the provider, all donor materials were obtained with informed consent and in compliance with ethical and legal requirements; therefore, no additional consent was required by the authors.

Consent for publication

Not applicable. This manuscript does not contain any individual person's data.

Conflict of interest

The authors declare no conflict of interest associated with this study.

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Authors' contributions

Kai Konishi Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft.

Tsunao Kishida: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Validation.

Masafumi Iguchi: Writing – review and editing, Supervision.

Yoshiaki Hirohata Data curation

Maho Inoue: Writing – review and editing.

Shohei Takayama: Writing – review and editing.

Kiyokazu Kim: Writing – review and editing.

Shigehisa Fumino: Writing – review and editing, Supervision.

Osam Mazda: Supervision, Project administration, Funding acquisition, Resources.

Shigeru Ono: Supervision, Project administration, Funding acquisition, Resources.

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Clinical trial registration

Not applicable. This study did not involve a clinical trial

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Figures

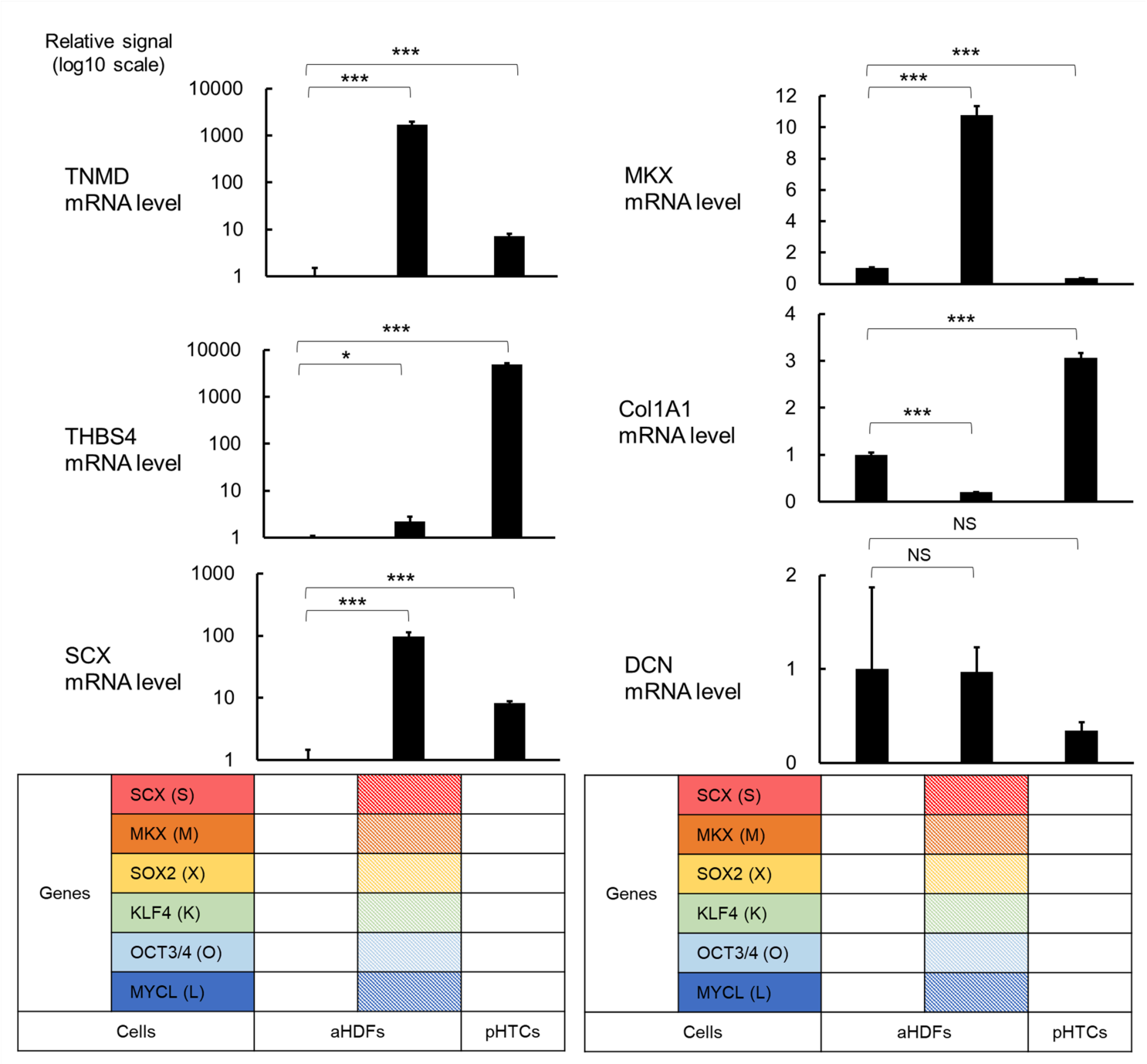


Figure 1

RT-PCR of the tendon-related gene expression in aHDFs transduced with six transcription factors

aHDFs were co-transduced with retroviral vectors encoding SCX (S), MKX (M), KLF4 (K), OCT3/4 (O), SOX2 (X), and MYCL (L). After 14 days of culture, total RNA was extracted, and the expression levels of TNMD, THBS4, SCX, MKX, COL1A1, and DCN were analyzed using real-time RT-PCR. The gene expression levels were normalized to those of non-transduced aHDFs (set as 1). Data are presented as the mean ± SD (n = 3). The expression of TNMD and THBS4 was significantly upregulated in transduced cells, whereas COL1A1, and DCN expression remained unchanged. The increased expression of SCX and MKX reflects exogenous gene delivery.

Asterisks above the individual bars indicate comparisons with non-transduced aHDFs, whereas asterisks above the brackets indicate comparisons between groups. *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant. The expression levels of TNMD, THBS4, and SCX are presented on a log10 scale to accommodate the wide dynamic range of expression.

Abbreviations: aHDFs, adult human dermal fibroblasts; TNMD, tenomodulin; THBS4, thrombospondin-4; SCX, Scleraxis; MKX, Mohawk homeobox; COL1A1, Collagen type I alpha 1; DCN, Decorin; pHTCs, primary human tenocytes

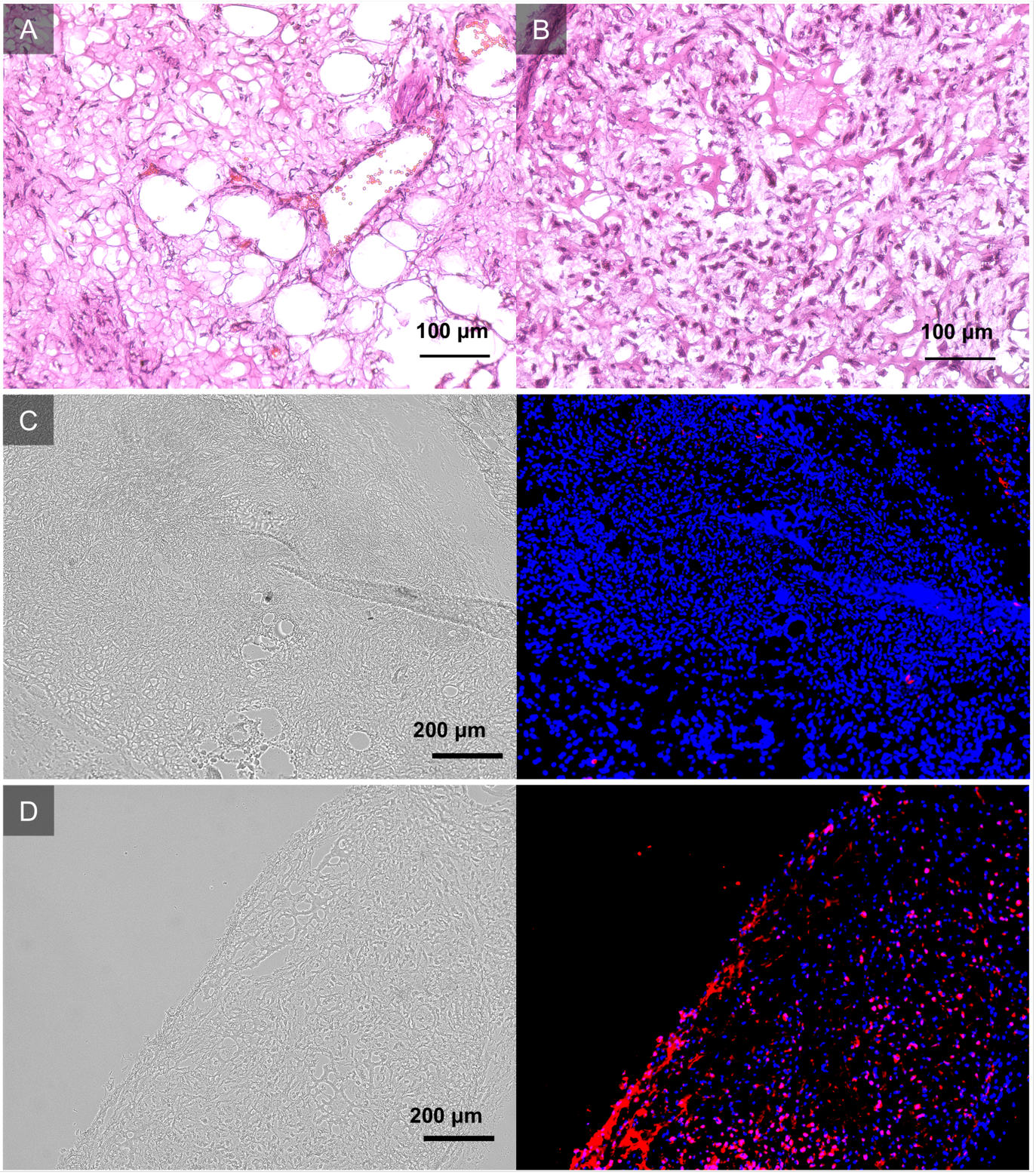


Figure 2

Histological and immunohistochemical evaluation of transplanted grafts

aHDFs transduced with the six transcription factors were subcutaneously transplanted into NOG-SCID mice. The graft tissues were harvested seven days after transplantation.

Hematoxylin and eosin (HE) staining of grafts derived from non-transduced aHDFs (A) and aHDFs transduced with six transcription factors (B). In the transduced group, morphologically distinct clusters of polygonal cells with prominent nuclei were observed within the graft matrix, whereas such features were not evident in the aHDFs group.

Fluorescent immunostaining of TNMD in non-transduced aHDFs (C) and aHDFs transduced with the six transcription factors (D). TNMD-positive cells were detected only in the transduced group, while no positive staining was observed in the aHDFs group.

Abbreviations: aHDFs, adult human dermal fibroblasts; TNMD, tenomodulin

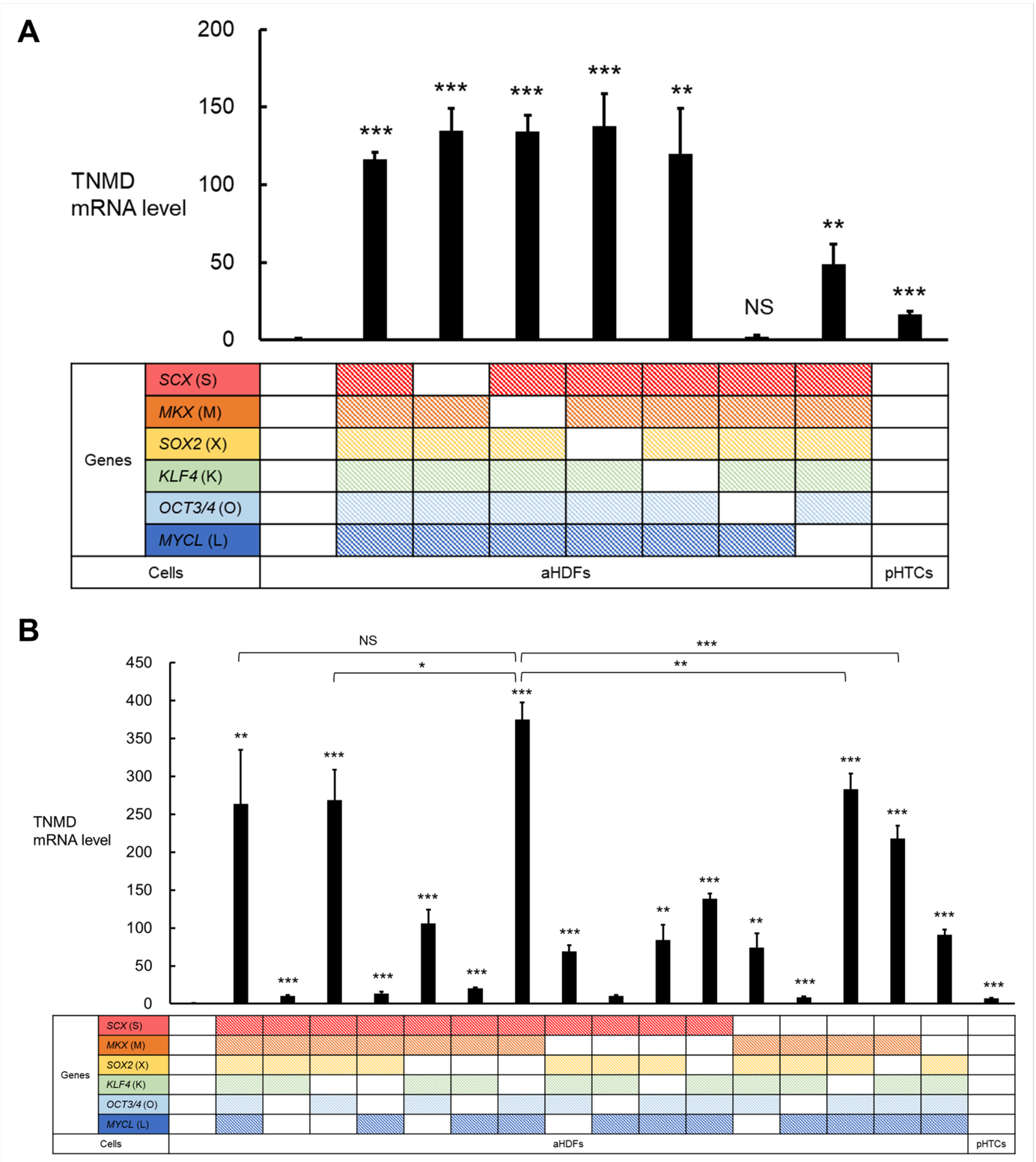


Figure 3

RT-PCR of transcription factor optimization identifies a minimal four-factor combination for TNMD induction

aHDFs were transduced with combinations of transcription factors, and the expression of TNMD was analyzed using real-time RT-PCR after 14 days of culture. The gene expression levels were normalized to

those of non-transduced aHDFs (set as 1). Data are presented as the mean ± SD (n = 3).

(A) Effects of five-factor combinations on the expression of TNMD. The combination lacking OCT3/4 did not show a significant difference relative to aHDFs, whereas all other combinations significantly increased the expression of TNMD.

(B) Effects of four-factor combinations on the expression of TNMD. The expression of TNMD was reduced in the absence of OCT3/4. aHDFs transduced with SCX, MKX, OCT3/4, and MYCL (dTBs) showed comparable TNMD expression levels to those transduced with all six transcription factors.

Asterisks above the individual bars indicate comparisons with non-transduced aHDFs, whereas asterisks above the brackets indicate comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; NS, not significant.

Abbreviations: aHDFs, adult human dermal fibroblasts; TNMD, tenomodulin; dTBs, directly converted tenoblasts

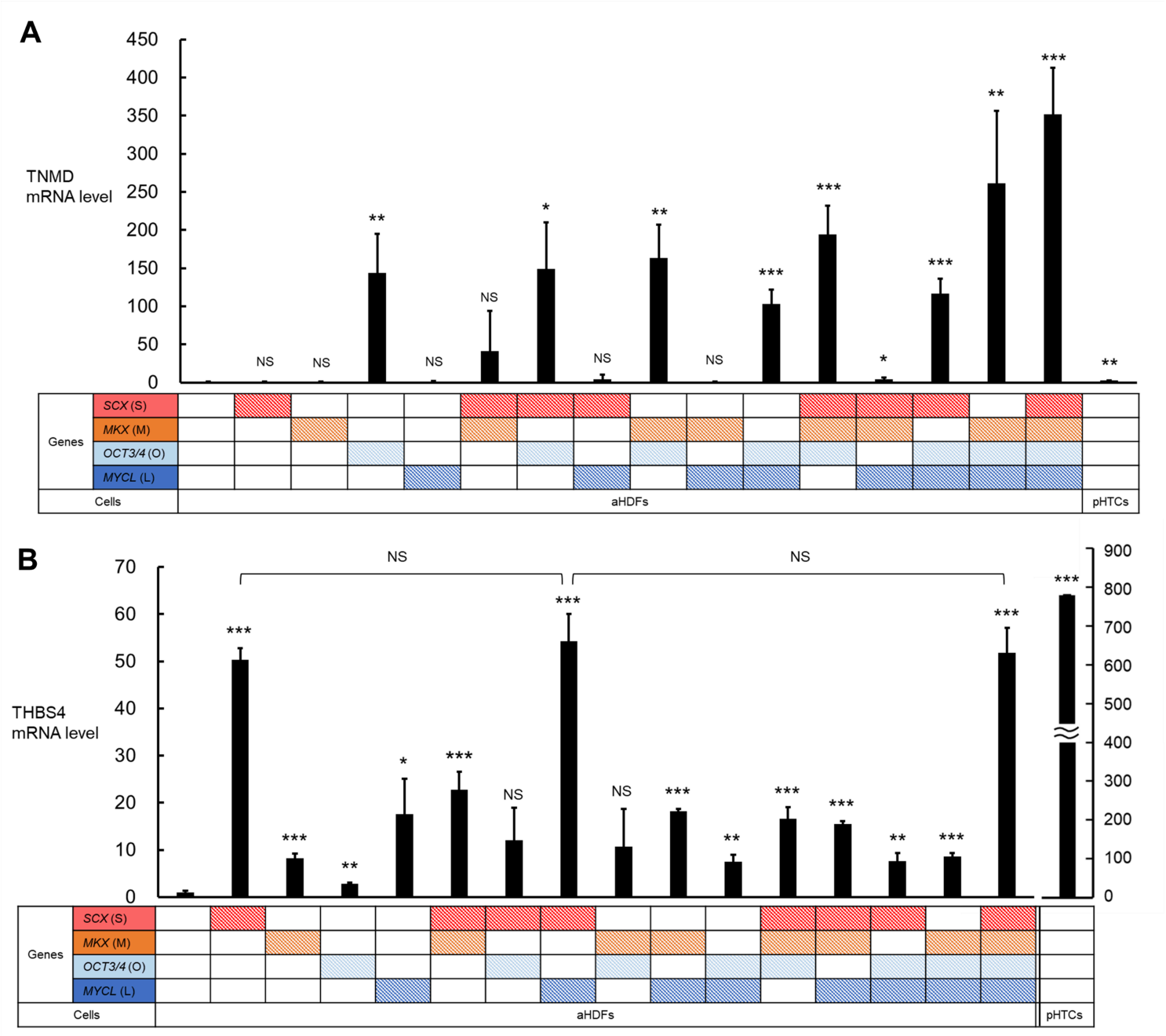


Figure 4

RT-PCR of the TNMD and THBS4 expression in all combinations of four transcription factors in dTBs

aHDFs were transduced with all possible combinations of SCX, MKX, OCT3/4, and MYCL genes. After 14 days of culture, TNMD (A) and THBS4 (B) expression levels were analyzed using real-time RT-PCR. Expression levels were normalized to those of non-transduced aHDFs (set as 1) and are presented as the mean ± SD (n = 3).

(A) The expression of TNMD was detected in cells transduced with OCT3/4 alone and was highest in cells transduced with all four factors (dTBs). In contrast, the expression of TNMD was reduced in the absence of OCT3/4.

(B) The expression of THBS4 was higher in cells transduced with SCX alone than in other single-factor conditions. No significant differences were observed between the SCX alone group, SCX + MYCL group, and four-factor combination. Due to the markedly higher THBS4 expression in pHTCs relative to the other groups, a secondary y-axis is shown.

Asterisks above the individual bars indicate comparisons with non-transduced aHDFs, whereas asterisks above the brackets indicate comparisons between groups. *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

Abbreviations: TNMD, tenomodulin; THBS4, thrombospondin-4; dTBs, directly converted tenoblasts; aHDFs, adult human dermal fibroblasts; pHTCs, primary human tenocytes

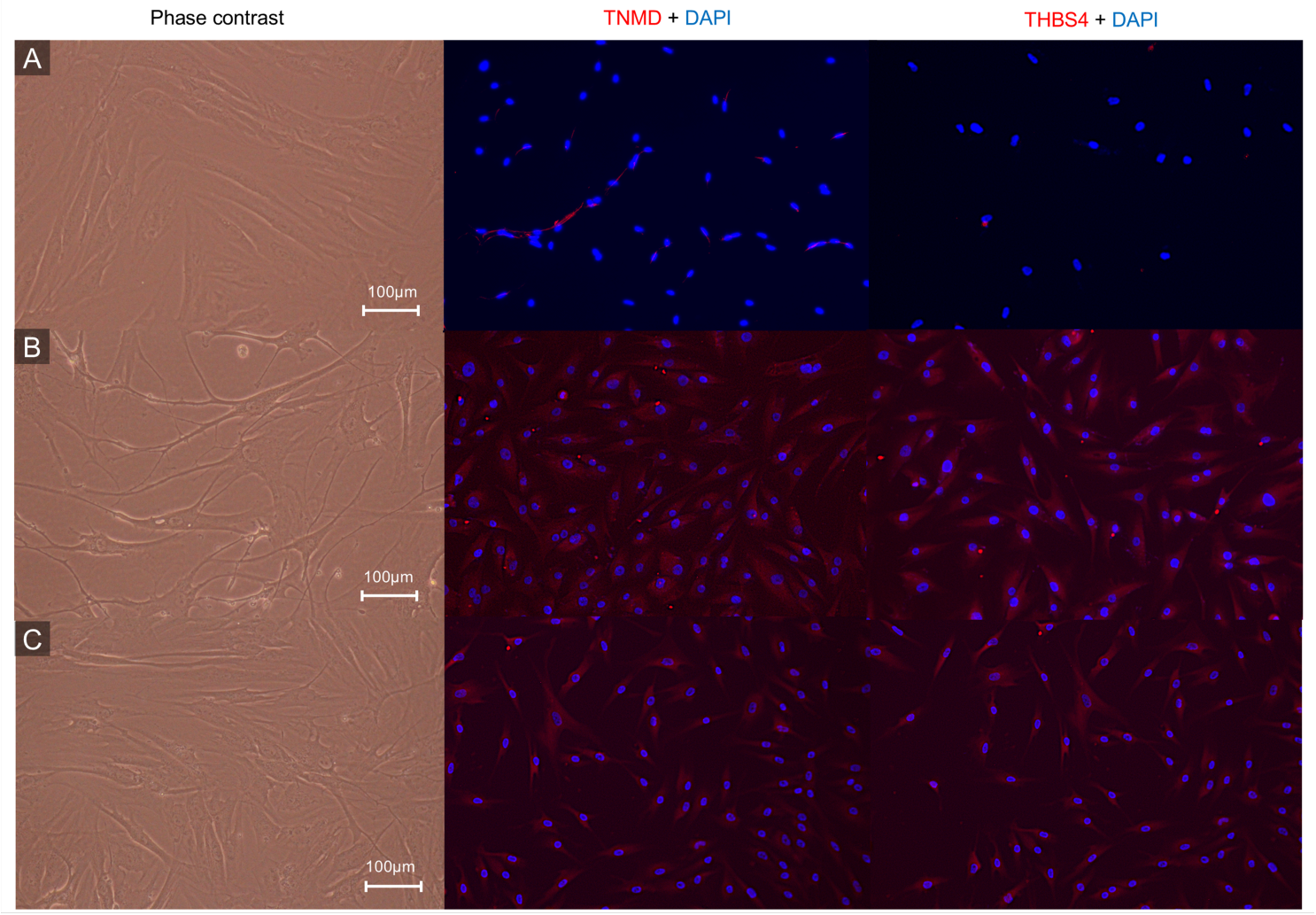


Figure 5

Morphological and immunocytochemical comparison among aHDFs, pHTCs, and dTBs

(A) A morphological evaluation of aHDFs was performed. Spindle-shaped cells are observed. Immunostaining was performed using anti-TNMD and anti-THBS4 antibody. aHDFs were not stained.

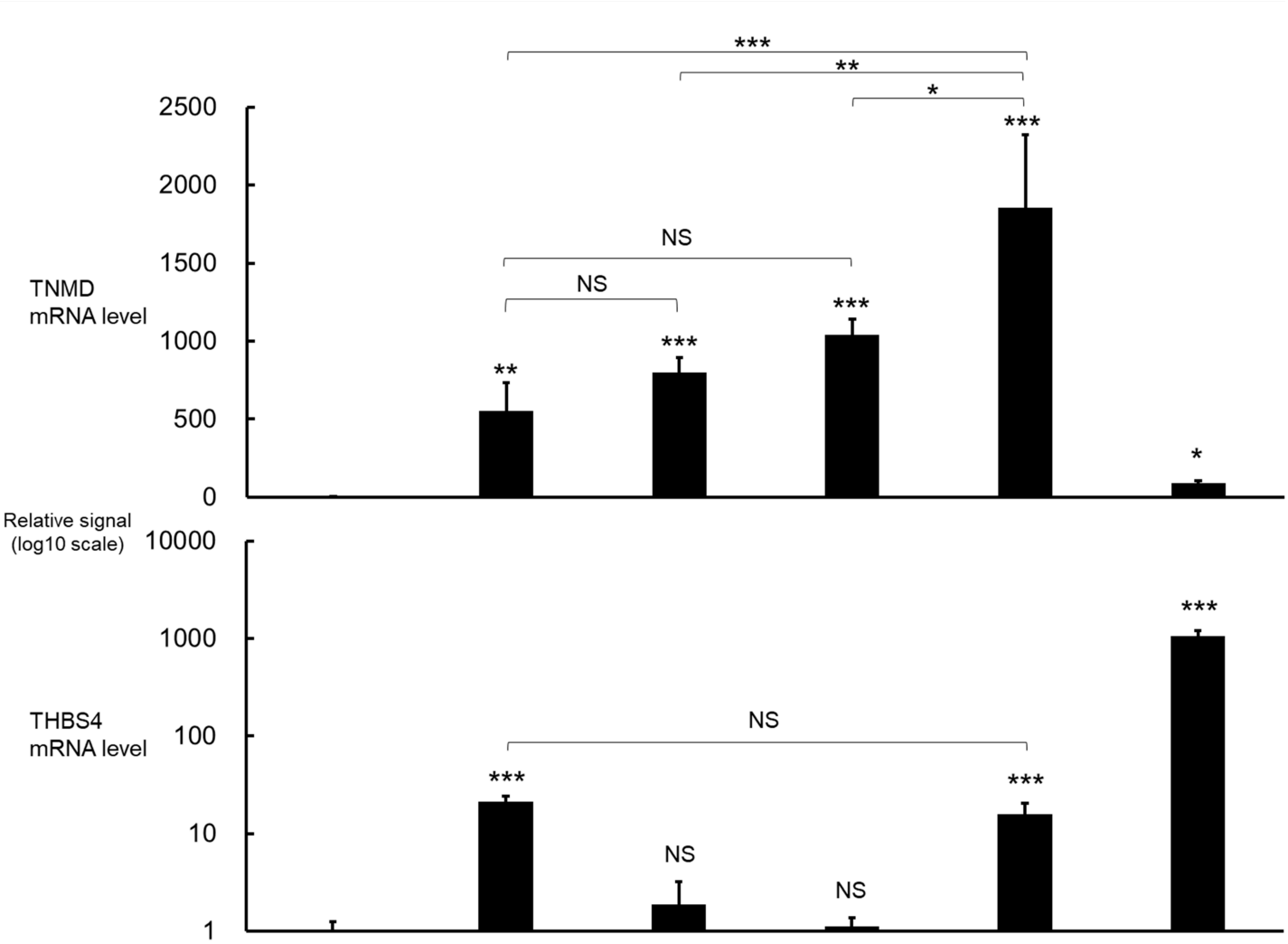
(B) A morphological evaluation of pHTCs was performed. In addition to spindle-shaped cells, abundant connections between the cells through extracellular matrix were observed.

pHTCs were stained with both anti-TNMD and anti-THBS4 antibodies.

(C) Morphological evaluation of the dTBs was performed. The dTBs acquired an elongated shape similar to that of the pHTCs, although their overall size was smaller than that of the pHTCs.

dTBs were stained with both anti-TNMD and anti-THBS4 antibodies to the same extent as pHTCs

Abbreviations: aHDFs, adult human dermal fibroblasts; pHTCs, primary human tenocytes; dTBs, directly converted tenoblasts; TNMD, tenomodulin; THBS4, thrombospondin-4



Cells		aHDFs					pHTCs
Days after transduction	2D	0 - 14	0 - 14	0 - 1	0 - 7	0 - 14	0 - 14
	3D	-	-	1 - 14	7 - 14	14 - 21	-

Figure 6

RT-PCR of the TNMD and THBS4 expression in dTBs following transfer from 2D to 3D culture at different time points

dTBs were transferred from 2D to 3D collagen gel culture at the indicated time points, and TNMD (A) and THBS4 (B) expression levels were analyzed using real-time RT-PCR. The expression levels were normalized to non-transduced aHDFs (set as 1) and are presented as the mean ± SD (n = 3).

(A) The expression of TNMD was significantly higher in cells transferred to 3D culture on day 14 than in the other groups. Cells transferred at earlier time points showed no significant difference relative to those maintained in 2D until day 14.

(B) The expression of THBS4 in cells transferred to 3D culture on day 14 was comparable to that of cells maintained in 2D culture until day 14, whereas no detectable expression was observed in cells transferred at earlier time points.

Asterisks above the individual bars indicate comparisons with non-transduced aHDFs, whereas asterisks above the brackets indicate comparisons between groups. *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

Abbreviations: TNMD, tenomodulin; THBS4, thrombospondin-4; dTBs, directly converted tenoblasts; aHDFs, adult human dermal fibroblasts

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx
- Submissionver.SupplementFigureS2.tif
- Submissionver.SupplementFigureS1.tif