

Human muscle stem/progenitor cells are bi-potential adult stem cells

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Abstract

Tendon injury occurs at high frequency and is difficult to repair. Identification human stem cells being able to regenerate tendon will greatly facilitate the development of regenerative medicine for tendon injury. We identified human muscle stem/progenitor cells having tendon differentiation potential both in vitro and in vivo. Interestingly, the tendon differentiation potential is not present in mouse muscle stem cells. These findings reveal that human muscle stem cells are bi-potential adult stem cells and can serve as a new source for tendon regeneration.

Introduction

Tendons is a tissue transmitting forces from skeletal muscle to bone to generate active movement. Tendonopathy affects more than 10% of the population under 45 and compromises the tendon functions^{1,2}. Tendon injury healing is slow and incomplete due to the low number of cells in tendon and the hypovascular and anaerobic environment^{3,4}. Tendon stem/progenitor cells (TDSCs) are a cell population derived from tendon and considered to be a subgroup of mesenchymal stem cells that have abilities to improve tendon injury healing^{5,6}. However, the number of TDSCs in tendon is low and retrieving TDSCs is invasive and exacerbates tendon injury. Morphology and proliferation ability loss in the culture system also hampers the efforts to obtain sufficient amounts of active TDSCs. To find more cell types supporting tendon regeneration will facilitate the development of regenerative medicine to treat tendon injury.

Skeletal muscles directly connect to tendon and has strong regeneration ability due to the existence of unipotency muscle stem cells⁷⁻¹¹. Functional muscle stem cells can be expanded in vitro to provide large amount of muscle stem cells for transplantation¹²⁻¹⁴. These features empower muscle stem cells to be a promising cell source for injury reparation.

Due to the different motion patterns, rodent tendon have different features from human¹⁵. Though rodent models have been widely used to study tendon regeneration, species related differences on regeneration have not been fully characterized. Here we found that human muscle stem/progenitor cells can be robustly differentiated to tendon cells both in vitro and in vivo. Transplantation of human muscle stem/progenitor cells to injured tendon improved the tendon regeneration, suggesting that human muscle stem/progenitor cells are bipotential adult stem cells. Interestingly, the tendon differentiation potential in mouse muscle stem cells is minimal and the differences on the cell intrinsic signaling network attributes to the species difference.

Results

It is a common clinical practice to harvest the whole peroneus longus tendon for the reconstruction of anterior cruciate ligament. The surgery completely removed the peroneus longus tendon. Surprisingly, the peroneus longus tendon regenerated well based on MRI images after 24 months (Fig. 1a)¹⁶. Another interesting clinical observation is that the removal of peroneus longus tendon is always accompanied by muscle injury (SFig. 1a). It seems that more severe muscle injury correlated with better tendon regeneration. Since muscle injury activates muscle stem cells, we therefore hypothesis that muscle stem cells which are activated in muscle injury caused by tendon removal could contribute to the tendon regeneration observed in clinics.

We first analyzed the differentiation potential of human muscle stem/progenitor cells using the single cell sequencing data obtained from human muscle samples. The trajectory analysis revealed that Pax7 + muscle stem/progenitor cells displayed differentiation potentials towards two lineages. One is towards differentiated muscle cells, the other is towards tendon differentiation (Fig. 1b and c). Scx, a key transcription factor determining tendon lineage, was expressed in muscle stem/progenitor cells as indicated by single cell sequencing data (Fig. 1c) and confirmed by RT-qPCR and RNA FISH (Fig. 1d and e).

To further test this hypothesis, we next isolated human muscle stem/progenitor cells from human muscle biopsies by FACS sorting to isolate CD45- CD31- CD29 + CD56 + CXCR4 + cells as reported previously¹⁷. The majority of the FACS sorted cells showed high Pax7 expression that is the marker of muscle stem/progenitor cells (Fig. 1f and SFig. 1b). As expected, the isolated human muscle stem/progenitor cells were differentiated to myotubes robustly (Fig. 1g). Consistently, the expression levels of genes related to muscle stem/progenitor cells like Pax7, Myf5, and MyoD were down-regulated after differentiation; while the myogenic differentiation related genes such as MyoG, Myh1, and Myh3 were up-regulated after differentiation (Fig. 1h and i).

The isolated primary human muscle stem/progenitor cells were then induced to differentiate to tenocytes by GDF7 and ascorbic acid. After 12 days of tendon differentiation, the expression of tendon marker genes such as TNC and Scx was increased. In contrast, the expression of genes related to muscular differentiation was minimal and no enrichment was observed (Fig. 2a, b, c, and SFig. 2). Other genes expressed in more mature tendons, such as Col I, Mxk, Thbs4, and Comp, were also enriched after tendon differentiation induction (Fig. 2d). These results combined suggest that human muscle stem/progenitor cells are capable of tendon differentiation in vitro.

RNA sequencing was then performed to further determine the lineage of the differentiated cells. Myotubes and tendon differentiated from human muscle stem/progenitor cells displayed distinct expression patterns (Fig. 3a). The differentiated tendon cells share high similarity to fresh isolated tenocytes from human tendons (Fig. 3a), suggesting that human muscle stem/progenitor cells are capable of tendon differentiation. Consistently, two distinct sets of genes were up-regulated in muscle stem/progenitor cells after myogenic induction and tenogenic induction, respectively (Fig. 3b and c). Further GO analysis also displayed the activation of two distinct sets of cell features. Upon the skeletal muscle differentiation induction condition, terms related to skeletal muscle functions such as skeletal muscle thin filament assembly, skeletal muscle contraction, muscle organ development, and sarcomere organization were enriched (Fig. 3d), suggesting the muscular identity of the differentiated cells. In sharp contrast, terms related to tendon formation, tendon development, and tendon cell differentiation were enriched (Fig. 3e), suggesting that tendon identity is achieved after tenogenic induction of human muscle stem/progenitor cells. Together, these results suggest that human muscle stem/progenitor cells are capable of both myogenic and tenogenic differentiation in vitro.

We then investigated whether muscle stem cells have tendon differentiation potential and participate in tendon repair in vivo. Due to the difficulty for lineage tracing in human, we performed the lineage tracing experiments in mice. We first crossed Pax7CreERT2 to flox-Stop-flox-TdTomato mice to trace muscle stem cells and established a tendon injury mouse models mimicking the peroneus longus tendon removal surgery in human (SFig. 3). Mice were given 4 months to recover from the injury, respectively. Nevertheless, less than 0.5% tendon cells originated from mouse muscle stem cells were observed even after we induced muscle injury by injecting cardiotoxin (CTX) at the close proximate to the tendon injury site (SFig. 4a-c). These results suggest that murine muscle stem cells have poor tendon differentiation potentials. We thus isolated mouse muscle stem cells as reported previously to

test their tendon differentiation ability in vitro. In sharp contrast to human muscle stem/progenitor cells, murine muscle stem cells failed to be induced to tendon cells upon the same induction condition as that for human muscle stem/progenitor cells (SFig. 4d and f), even though they displayed efficient myogenic differentiation (SFig. 4e). These results further indicate that murine muscle stem cells have poor tendon differentiation potential.

Due to the difficulties in mouse lineage tracing, we set out to perform cell transplantation experiments to test whether ectopic muscle stem cells have tenogenic potential after being planted in tendon. We transplanted 50,000 Pax7 + human muscle stem/progenitor cells to the pre-injured tendon in SCID mice and harvested the tendon detecting the tendon cells originated from human cells (Fig. 4a). Two months after transplantation, the repaired tendon was harvested. The length of the tendon was obviously extended after transplantation of human stem/progenitor cells (Fig. 4b and c). The presence of tendon cells with human origin was detected by immunofluorescence staining with antibody specific for human laminA/C. At the PBS injection side, no human Lamin A/C signal was detected confirming the specificity of the antibody. In sharp contrast, human cells were detected after human muscle stem/progenitor cell transplantation (Fig. 4d and e). The majority of the human cells co-stained with tendon marker TNC, Scx, and Tnmd (Fig. 4e and f), suggesting that the transplanted human muscle stem/progenitor cells mainly differentiate to tendon cells in vivo when transplanted to the tendon. Only few transplanted human muscle stem/progenitor cells differentiated to MyHC + muscle cells (Fig. 4d and g). These results reveal that human muscle stem/progenitor cells have tendon differentiation potential in vivo. While when the same number of human muscle stem/progenitor cells were transplanted to pre-injured muscle, the same cell population differentiated predominantly to skeletal muscle (SFig. 5a-c), confirming their muscle stem/progenitor cell identities. Taken together, these results suggest that human muscle stem/progenitor cells are bi-potential adult stem cells and the differentiation potential is highly microenvironment dependent.

In sharp contrast to human muscle stem/progenitor cells, when 50,000 murine muscle stem cells constitutively expressing tdTomato were transplanted to pre-injured tendon in SCID mice, very low number of tdTomato + tendon cells were detected, though these cells can regenerate skeletal muscle efficiently (SFig. 6a-e). These results suggest that human muscle stem/progenitor cells have unique tenogenic differentiation potential both in vitro and in vivo.

We further examined the function of the regenerated tendons after human muscle stem/progenitor cell transplantation. The endurance time of the mice transplanted with human muscle stem/progenitor cells was longer than that of the mice with PBS injection while doing long distance run training (Fig. 4h). Consistently, the mice transplanted by muscle stem/progenitor cells can achieve higher maximum speed in running exercise (Fig. 4i) and longer fatigue time (Fig. 4j). All together, these results suggest that the transplanted human muscle stem/progenitor cells can improve the functions of injured tendons by differentiate to mature tendon cells.

The above results revealed the cell intrinsic species difference on the differentiation potentials of muscle stem/progenitor cells between human and mouse. We further compared the expression profiles of mouse and human muscle stem/progenitor cells. Interestingly, Notch signaling were significantly enriched in human muscle stem/progenitor cells (Fig. 4k), indicating that Notch signaling could be the key node for maintaining the tendon differentiation potential.

Muscle stem cells have long been considered to be the typical unipotent adult stem cells. Here we show that the differentiation potential of muscle stem cells is species dependent. Human muscle stem/progenitor cells are bipotent adult stem cell with both muscle and tendon differentiation potentials and could serve as new seed cell

type to treat tendon injury. Murine muscle stem cells barely have tendon differentiation potential. The species difference might be due to the higher Notch signaling level in human muscle stem/progenitor cells.

Notch signaling is critical for murine muscle stem cell quiescence maintenance by inhibiting differentiation¹⁸⁻²⁰. The elevated Notch signaling level in human muscle stem/progenitor cells may help maintaining a more naïve stage and enable the cells with more potentials to differentiate to multiple cell types. It will be interesting to explore the function of Notch signaling in human muscle stem/progenitor cells.

Studies using mouse models contributed to the majority of our knowledge about muscle stem cells and laid the foundation for our understanding of mammalian muscle stem cells. However, human is dramatically different from mouse in many aspects such as size, life span, and manners of motions. It is more demanding for humans to maintain the homeostasis of motion system with longer life span and bigger body size. Evolutionally, to evolve more differentiation potentials for a designated type of adult stem cells represents an economic aspects to maintain the longer life span and more complicated motion manner in human beings.

Methods

Human samples

Peroneal longus muscle and remanent tendon were obtained from the wastes of patients who underwent full-thickness peroneal longus tendon autograft for knee ligament reconstructive procedures. The study was approved by the ethical committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University, School of Medicine (Approval No. XHEC-D-2019-043) and written informed consents were obtained from all donors.

Animals

Animal care and use were in accordance with the guidelines of the animal facility hosted by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and the operations were approved by the ethical committee of Shanghai Institute of Biochemistry and Cell Biology. All mice were maintained in specific pathogen-free (SPF) animal facility in individually ventilated cages (IVC) with controlled temperature ($22\pm 1^{\circ}\text{C}$) and light (12h light/dark cycle). SCID mice were purchased from Animal Model Research Center of Nanjing University. Pax7CreERT2 and Rosa26-Flox-Stop-Flox-tdTomato mice were purchased from Jackson Laboratory.

Two years after peroneus longus tendon harvest, bilateral legs were scanned with a 3.0-Tesla MRI (Ingenia; Philips Healthcare, Eindhoven, Netherlands). The proton density-weighted and spectral attenuated inversion recovery (PDW-SPAIR) fat suppressed images were obtained in the axial planes. Imaging was performed from the distal end of fibula to the proximal cruris and 100 slices were obtained for each leg.

Isolation of human muscle stem/progenitor cells

Human skeletal muscle from peroneal longus were dissected and digested as described previously²¹. Briefly, muscle tissues were cut into small pieces and digested by collagenase II (Worthington biochemical, 700-800U/ml, cat#LS004177) for 60min followed by 30min of digestion with the mixtures of collagenase II and dispase (Life Technologies, 11U/ml, cat#17105-041). Digested cells were passed 10 times through a 20-gauge needle. Cell

suspension was filtered through a 40µm cell strainer (BD Falcon, cat#352340). Erythrocytes were removed by red blood cell lysis buffer (Thermo Fisher Scientific, cat#00-433-57). The single cell suspension obtained from human muscle was stained with a cocktail containing PE-Cy5 anti-human CD45 (BD Pharmingen, cat#555484), Percp-Cy5.5 anti-human CD31 (BioLegend, cat#303132), AF-488 anti-human CD29 (BioLegend, cat#303016) and PE anti-human CD56 (BioLegend, cat#304606) for 45 min at 4°C. CD45- CD31- CD29+ CD56+ human muscle stem/progenitor cells were sorted by BD Influx sorter (BD Biosciences).

Isolation of human muscle stem/progenitor cells

Mouse muscle stem cells were FACS sorted as described previously^{22,23}. Briefly, dissected TA muscles were digested with 10ml muscle digestion buffer (DMEM containing 1% penicillin/streptomycin, 0.125mg/ml Dispase II (Roche, 04942078001), and 10mg/ml Collagenase D (Roche, 11088866001)) for 90 minutes at 37°C. The digestion was stopped by adding 2ml of FBS. The digested cells were filtered through 70µm strainers. Red blood cells were lysed by 7ml RBC lysis buffer (0.802% NH₄Cl, 0.084% NaHCO₃, 0.037% EDTA in ddH₂O, pH7.2-7.4) for 30s, then filter through 40µm strainers. After staining with antibody cocktails (AF700-anti-mouse Sca-1, PerCP/Cy5.5-anti-mouse CD11b, PerCP/Cy5.5-anti-mouse CD31, PerCP/Cy5.5-anti-mouse CD45, FITC anti-mouse CD34, APC-anti-mIntegrin a7+), the mononuclear cells were subjected for FACS analysis using Influx (BD Biosciences). The population of PI-CD45-CD11b-CD31-Sca1- CD34+ Integrin a7+ cells was collected.

Primary tenocytes isolation

Tendon tissues obtained from tendon autograft surgery were washed with PBS. Epi- and peri-tendon sheath were completely removed. Tenocytes were isolated as described previously⁵. Briefly, the tendons were minced to 1mm³ pieces and digested with 2 mg/ml collagenase II (Worthington biochemical, cat#LS004177) in DMEM (Gibco, cat#11965118) at 37°C for 3hrs with gentle agitation. The digested tissue was filtered through a 40µm cell strainer (BD Falcon, cat#352340) and the isolated cells were plated in for subsequent analysis.

Cell culture and differentiation

Primary human muscle stem/progenitor cells were plated in F10 basal medium (Gibco, cat#11550043) containing 20% FBS (Gibco, cat#10-013-CV), 2.5ng/ml bFGF (R&D, cat#233-FB-025) and 1% Penicillin-Streptomycin (Gibco, cat#15140-122) on collagen-coated dishes. Mouse muscle stem cells were plated in F10 basal medium (Gibco, cat#11550043) containing 20% FBS (Gibco, cat#10-013-CV), 2.5ng/ml bFGF (R&D, cat#233-FB-025) and IL-1α, IL-13, IFN-γ and TNF-α as described previously¹². DMEM (Gibco, cat#11965118) containing 0.4% Ultrosor G (Pall Corporation, cat#15950-17) and 1% Penicillin-Streptomycin were used to differentiate human muscle stem/progenitor cells as described previously²⁴. DMEM containing 2% horse serum (HyClone, cat#HYCLSH30074.03HI) and 1% Penicillin-Streptomycin were used to differentiate mouse muscle stem cells as described previously^{22,25}. DMEM containing 10% FBS, 100ng/ml GDF7 (R&D, cat#8386-G7-050), 0.2mM ascorbic acid (Sigma-Aldrich, cat#A4403) and 1% Penicillin-Streptomycin were used to induce tendon differentiation as described previously^{26,27}. Primary tenocytes were cultured in DMEM containing 20% FBS, 2.5ng/ml bFGF (R&D, cat#233-FB-025) and 1% Penicillin-Streptomycin.

Cell transplantation in TA muscle

A single dose of 18 Grey irradiation was administered to the hind legs of the recipient SCID mice. TA muscle was injured by injecting 15 μ l of 10 μ M CTX (Sigma), and 50,000 human muscle stem/progenitor cells or 50,000 mouse muscle stem cells suspended in 10 μ l PBS were injected intramuscularly to the injury site as described^{22,24}.

Tendon injury and cell transplantation

A 1.5mm long and 0.3mm width piece was removed from the medial gastrocnemius tendon beginning from myotendinous junction in recipient SCID mice. 50,000 human muscle stem/progenitor or mouse muscle stem cells resuspended in 20 μ l Matrigel (BD Biosciences, cat#354277) were injected to the injury site with 28-gauge needles. PBS mixed with 20 μ l Matrigel were injected as control.

Immunofluorescence staining

Immunofluorescence staining was performed as described^{12,25}. In brief, cryosections were fixed in 4% formaldehyde for 15min, permeabilized in 0.5% Triton X-100 for 15min, and stained with anti-Pax7 (Developmental Studies Hybridoma Bank), anti-MyoD (Santa Cruz, cat#sc-760), anti-Laminin (Abcam, cat#ab11575), anti-Lamin A/C (Abcam, cat#ab238303), anti-TNC (Abcam, cat#ab108930), anti-Scx (Abcam, cat#ab58655), anti-Tnmd (Abcam, cat#ab203676) or anti-MyHC (Millipore, cat#05-716) at 4°C overnight, and incubated with Alexa 488-, Alexa 594 or Alexa 647-labeled anti-mouse or anti-rabbit secondary antibodies (Invitrogen, 1:1000) at room temperature for 1hr. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, cat#H-1200). All images were acquired by Leica SP8 confocal microscope (Leica).

Gene expression analysis

Total RNA was isolated using TRIzol Reagent (Invitrogen, cat#15596-018) according to the manufacturer's instruction. GAPDH served as internal control. The primers for RT-qPCR are listed as below:

Human GAPDH-F	5'-CAAGGCTGAGAACGGGAAGC-3'
Human GAPDH-R	5'-AGGGGGCAGAGATGATGACC-3'
Human Scx-F	5'-AGCGATTCGCAGTTAGGAGG-3'
Human Scx-R	5'-GTCTGTACGTCCGTCTGTCC-3'
Human Col I-F	5'-GGCTCCTGCTCCTCTTAGCG-3'
Human Col I-R	5'-CATGGTACCTGAGGCCGTTCC-3'
Human Col III-F	5'-CAGCGGTTCTCCAGGCAAGG-3'
Human Col III-R	5'-CTCCAGTGATCCCAGCAATCC-3'
Human Tnc-F	5'-GGTGGATGGATTGTGTTCCCTGAGA-3'
Human Tnc-R	5'-CTGTGTCCTTGTCAAAGGTGGAGA-3'
Human Thbs4-F	5'-TGCTGCCAGTCCTGACAGA-3'
Human Thbs4-R	5'-GTTTAAGCGTCCCATCACAGTA-3'
Human Mx-F	5'-TTCAAGGCAATGCTGAACGG-3'
Human Mx-R	5'-CTCCCGCTTTGATGACCGAA-3'
Human Dcn-F	5'-ATGAAGGCCACTATCATCCTCC-3'
Human Dcn-R	5'-GTCGCGGTCATCAGGAACTT-3'
Human Comp-F	5'-GATCACGTTCCCTGAAAAACACG-3'
Human COMP-R	5'-GCTCTCCGTCTGGATGCAG-3'
Human Pax7-F	5'-ACCCCTGCCTAACCACATC-3'
Human Pax7-R	5'-GCGGCAAAGAATCTTGGAGAC-3'
Human Myf5-F	5'-AATTTGGGGACGAGTTTGTG-3'
Human Myf5-R	5'-CATGGTGGTGGACTTCCTCT-3'
Human MyoD-F	5'-GACGGCATGATGGACTACAG-3'
Human MyoD-R	5'-AGGCAGTCTAGGCTCGACAC-3'
Human MyoG-F	5'-GCTCAGCTCCCTCAACCA-3'
Human MyoG-R	5'-GCTGTGAGAGCTGCATTCG-3'
Human MyH1-F	5'-GGGAGACCTAAAATTGGCTCAA-3'
Human MyH1-R	5'-TTGCAGACCGCTCATTTCAA-3'
Human MyH3-F	5'-ATTGCTTCGTGGTGGACTCAA-3'
Human MyH3-R	5'-GGCCATGTCTTCGATCCTGTC-3'
Human Desmin-F	5'-TCGGCTCTAAGGGCTCCTC-3'

Human Desmin-R	5'-CGTGGTCAGAACTCCTGGTT-3'
Human MYL1-F	5'-GTTGAGGGTCTGCGTGTCTTT-3'
Human MYL1-R	5'-ACCCAGGGTGGCTAGAACA-3'
Human MYMK-F	5'-CTTCATGCGTCACGACATCCT-3'
Human MYMK-R	5'-CCTCTTGGGTTCGTGGAAGT-3'
Human MYMX-F	5'-CAGTTCTGCCCCGCACTGA-3'
Human MYMX-R	5'-AGCAAAGCCTCTCGCATGTC-3'
Mouse GAPDH-F	5'-ACCCAGAAGACTGTGGATGG-3'
Mouse GAPDH-R	5'-ACACATTGGGGGTAGGAACA-3'
Mouse COL I-F	5'-CCAGCGAAGAAGTCTACAGC-3'
Mouse COL I-R	5'-GGACACCCCTTCTACGTTGT-3'
Mouse COL III-F	5'-CAACCAGTGCAAGTGACCAA-3'
Mouse COL III-R	5'-GCACCATTGAGACATTTTGAAG-3'
Mouse SCX-F	5'-GAGAACACCCAGCCCAAAC-3'
Mouse SCX-R	5'-TCACCCGCCTGTCCATC-3'
Mouse TNC-F	5'-GAGCCCCTTTGCCTCAACAA-3'
Mouse TNC-R	5'-CTTCGCCCCTGAAACCTTCTT-3'
Mouse MKX-F	5'-TGGTTTCCTGGACAATCCACA-3'
Mouse MKX-R	5'-CGCTTATGCCTTACCTTCCCTC-3'
Mouse THBS4-F	5'-GCCACAAGCACAGGAGACTTT-3'
Mouse THBS4-R	5'-TGACCTGCTGCCTCAGAAGA-3'
Mouse COMP-F	5'-ACTGCCTGCGTTCTAGTGC-3'
Mouse COMP-R	5'-CGCCGCATTAGTCTCCTGAA-3'
Mouse DCN-F	5'-TCTTGGGCTGGACCATTTGAA-3'
Mouse DCN-R	5'-CATCGGTAGGGGCACATAGA-3'

RNA-sequencing

Total RNA was isolated using TRIzol Reagent (Invitrogen, cat#15596-018) according to the manufacturer's instruction. mRNA was enriched with magnetic oligo (dT) beads (New England Biolabs, cat#S1419S). The cDNA library was constructed with mean inserts of 200bp with non-stranded library preparation using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, cat#E7530L). Sequencing was performed by a paired-end 125 cycles rapid run on the Illumina HiSeq2500. Sequencing data were filtered by SolexaAQ (Q > 20 and length \geq 25 bp)²⁸. The adapter sequences and low quality segments (Phred Quality Score < 20) were trimmed

using Cutadapt²⁹. Paired-end clean reads were then mapped to the reference genome GRCh38.98 using HISAT2³⁰. Htseq-count was used to quantify the gene expression value³¹. Read count of each gene was normalized using FPKM (Fragments Per Kilo bases per Million fragments). Differential expression (DE) analysis were performed using DESeq and significant DE genes were defined as those with absolute log2FoldChange>1 and p< 0.05. Heatmap and volcano plot of DE genes was generated by R package Pheatmap (<https://cran.r-project.org/web/packages/pheatmap/index.html>) and EnhancedVolcano (<https://bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html>), respectively. Gene enrichment analysis was conducted by R package topGO (Aibar et al., 2015) with p < 0.05 as the cut-off.

Trajectory analysis

Single cell RNA-seq data were analyzed by Seurat R (Version 3.2.0) package³². Cells with less than 200 genes, more than 6,000 genes detected, and more than 10% mitochondrial genes were excluded. Cells expressing Pax7, Scx, Tnmd, Mxk, Myh1, Myh3, Myh5 or Myl1 were selected for cell clustering and trajectory analysis. Sequencing reads for each gene were normalized to total UMIs in each cell to obtain normalized UMI values by “NormalizeData” function. The “ScaleData” function was used to scale and center expression levels in the data set for dimensional reduction. Total cell clustering was performed by “FindClusters” function at a resolution of 0.1 and dimensionality reduction was performed with “RunUMAP” function. Trajectory analysis was done by R package Monocle2³³. The top 100 differentially expressed genes were identified to sort the cells in pseudo-time order, and cells were plotted along the inferred trajectory. Muscle stem cells were selected as the start of pseudotime and aligned via the “ordercells” function. ‘UMAP’ was applied to reduce dimensions and the visualization functions “plot_cell_trajectory” were used to plot each group along the same pseudotime trajectory.

Statistical analysis

The number of biological replicates and technical repeats in each experimental group are indicated in figure legends. Error bars indicated standard deviation. Two-tailed unpaired Student’s t-tests were used when variances were similar (tested with F-test) for comparison paired comparison. One-way ANOVA tests followed by Dunnett’s post-test or Tukey’s post-test were used for multiple comparisons. Shapiro-Wilk tests were performed to determine data normality. Statistical analysis was performed in GraphPad Prism 7 (GraphPad Software, San Diego, USA) or SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

The normality of continuous data was determined with the. Independent sample Student t test was used to compare parameters between experiment and control group. It was considered significant with P value less than 0.05. Data were presented as mean ± S.E.M. unless stated.

Accession numbers

The complete mRNA-seq data used to produce intensity maps have been uploaded on to Sequence Read Archive (SRA) database. The Accession number is PRJNA721361.

Declarations

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References

1. Gaida, J. E., Alfredson, H., Kiss, Z. S., Bass, S. L. & Cook, J. L. Asymptomatic Achilles tendon pathology is associated with a central fat distribution in men and a peripheral fat distribution in women: a cross sectional study of 298 individuals. *BMC Musculoskelet Disord* **11**, 41, doi:10.1186/1471-2474-11-41 (2010).
2. Voleti, P. B., Buckley, M. R. & Soslowsky, L. J. Tendon healing: repair and regeneration. *Annu Rev Biomed Eng* **14**, 47–71, doi:10.1146/annurev-bioeng-071811-150122 (2012).
3. Pennisi, E. Tending tender tendons. *Science* **295**, 1011, doi:10.1126/science.295.5557.1011 (2002).
4. Kannus, P. Structure of the tendon connective tissue. *Scand J Med Sci Sports* **10**, 312–320, doi:10.1034/j.1600-0838.2000.010006312.x (2000).
5. Bi, Y. *et al.* Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* **13**, 1219–1227, doi:10.1038/nm1630 (2007).
6. Kohler, J. *et al.* Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration. *Aging Cell* **12**, 988–999, doi:10.1111/accel.12124 (2013).
7. Charge, S. B. & Rudnicki, M. A. Cellular and molecular regulation of muscle regeneration. *Physiol Rev* **84**, 209–238, doi:10.1152/physrev.00019.2003 (2004).
8. Wosczyzna, M. N. & Rando, T. A. A Muscle Stem Cell Support Group: Coordinated Cellular Responses in Muscle Regeneration. *Dev Cell* **46**, 135–143, doi:10.1016/j.devcel.2018.06.018 (2018).
9. Baghdadi, M. B. & Tajbakhsh, S. Regulation and phylogeny of skeletal muscle regeneration. *Dev Biol* **433**, 200–209, doi:10.1016/j.ydbio.2017.07.026 (2018).
10. Relaix, F. *et al.* Perspectives on skeletal muscle stem cells. *Nat Commun* **12**, 692, doi:10.1038/s41467-020-20760-6 (2021).
11. Fu, X., Wang, H. & Hu, P. Stem cell activation in skeletal muscle regeneration. *Cell Mol Life Sci* **72**, 1663–1677, doi:10.1007/s00018-014-1819-5 (2015).
12. Fu, X. *et al.* Combination of inflammation-related cytokines promotes long-term muscle stem cell expansion. *Cell Res* **25**, 655–673, doi:10.1038/cr.2015.58 (2015).
13. Xu, C. *et al.* A zebrafish embryo culture system defines factors that promote vertebrate myogenesis across species. *Cell* **155**, 909–921, doi:10.1016/j.cell.2013.10.023 (2013).
14. Fang, J. *et al.* Skeletal muscle regeneration via the chemical induction and expansion of myogenic stem cells in situ or in vitro. *Nat Biomed Eng*, doi:10.1038/s41551-021-00696-y (2021).
15. Schneider, M., Angele, P., Jarvinen, T. A. H. & Docheva, D. Rescue plan for Achilles: Therapeutics steering the fate and functions of stem cells in tendon wound healing. *Adv Drug Deliv Rev* **129**, 352–375, doi:10.1016/j.addr.2017.12.016 (2018).
16. Shao, X. *et al.* Satisfactory functional and MRI outcomes at the foot and ankle following harvesting of full thickness peroneus longus tendon graft. *Bone Joint J* **102b**, 205–211, doi:10.1302/0301-620x.102b2.Bjj-2019-0949.R1 (2020).

17. Barruet, E. *et al.* Functionally heterogeneous human satellite cells identified by single cell RNA sequencing. *Elife* **9**, doi:10.7554/eLife.51576 (2020).
18. Baghdadi, M. B. *et al.* Notch-Induced miR-708 Antagonizes Satellite Cell Migration and Maintains Quiescence. *Cell Stem Cell* **23**, 859–868 e855, doi:10.1016/j.stem.2018.09.017 (2018).
19. Verma, M. *et al.* Muscle Satellite Cell Cross-Talk with a Vascular Niche Maintains Quiescence via VEGF and Notch Signaling. *Cell Stem Cell* **23**, 530–543 e539, doi:10.1016/j.stem.2018.09.007 (2018).
20. Baghdadi, M. B. *et al.* Reciprocal signalling by Notch-Collagen V-CALCR retains muscle stem cells in their niche. *Nature* **557**, 714–718, doi:10.1038/s41586-018-0144-9 (2018).
21. Garcia, S. M., Tamaki, S., Xu, X. & Pomerantz, J. H. Human Satellite Cell Isolation and Xenotransplantation. *Methods Mol Biol* **1668**, 105–123, doi:10.1007/978-1-4939-7283-8_8 (2017).
22. Luo, Y. *et al.* Mangiferin attenuates contusive spinal cord injury in rats through the regulation of oxidative stress, inflammation and the Bcl2 and Bax pathway. *Mol Med Rep* **12**, 7132–7138, doi:10.3892/mmr.2015.4274 (2015).
23. Feng, X. *et al.* Dual function of VGLL4 in muscle regeneration. *EMBO J* **38**, e101051, doi:10.15252/emj.2018101051 (2019).
24. Ding, S. *et al.* Characterization and isolation of highly purified porcine satellite cells. *Cell Death Discov* **3**, 17003, doi:10.1038/cddiscovery.2017.3 (2017).
25. Yin, J. *et al.* Dkk3 dependent transcriptional regulation controls age related skeletal muscle atrophy. *Nat Commun* **9**, 1752, doi:10.1038/s41467-018-04038-6 (2018).
26. Otabe, K. *et al.* Transcription factor Mohawk controls tenogenic differentiation of bone marrow mesenchymal stem cells in vitro and in vivo. *J Orthop Res* **33**, 1–8, doi:10.1002/jor.22750 (2015).
27. Lou, J., Tu, Y., Burns, M., Silva, M. J. & Manske, P. BMP-12 gene transfer augmentation of lacerated tendon repair. *J Orthop Res* **19**, 1199–1202, doi:10.1016/S0736-0266(01)00042-0 (2001).
28. Cox, M. P., Peterson, D. A. & Biggs, P. J. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* **11**, 485, doi:10.1186/1471-2105-11-485 (2010).
29. Criscuolo, A. & Brisse, S. AlienTrimmer removes adapter oligonucleotides with high sensitivity in short-insert paired-end reads. Commentary on Turner (2014) Assessment of insert sizes and adapter content in FASTQ data from NexteraXT libraries. *Front Genet* **5**, 130, doi:10.3389/fgene.2014.00130 (2014).
30. Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* **37**, 907–915, doi:10.1038/s41587-019-0201-4 (2019).
31. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169, doi:10.1093/bioinformatics/btu638 (2015).
32. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411–420, doi:10.1038/nbt.4096 (2018).
33. Qiu, X. *et al.* Reversed graph embedding resolves complex single-cell trajectories. *Nat Methods* **14**, 979–982, doi:10.1038/nmeth.4402 (2017).

Figures

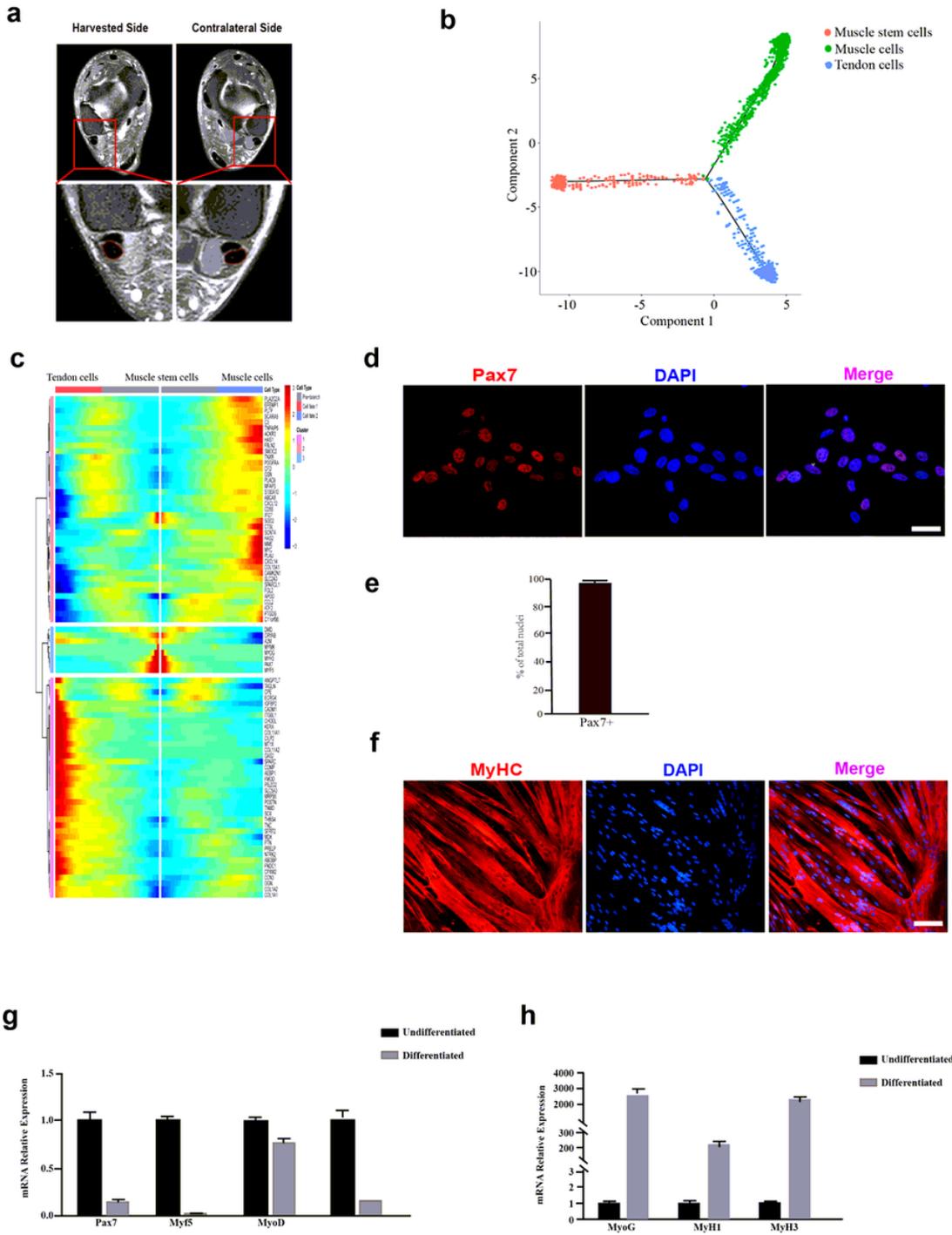


Figure 1

Human muscle stem/progenitor cells displayed potential tendon differentiation potential. a. MR imaging of distal leg 2 years after full-thickness peroneus longus tendon harvesting. The upper panels indicated the overall view of distal leg and the lower panel indicated the enlarged view of proneus longus tendon (PLT) location. The soft tissue with tendon like appearance and uniform signal similar to the tendon in the normal PLT tract was defined as evidence of tendon regeneration. The PLT was contoured by red line and the regenerated tendon could be clearly discriminated at the joint-line section of the harvested side in this case. Scale bars, 1 cm. b. Pseudotime trajectory inference using specific markers of muscle stem cells, tenocytes and muscle cells. Different cell types were annotated. c. Heatmap showing the different expression levels of specific markers during the trajectory. d.

Immunofluorescence staining of Pax7 in primary human muscle stem/progenitor cells. Scale bars, 20 μ m. e. Statistical analysis of the percentage of Pax7+ cell. Error bars indicated standard error based on 5 independent experiments. f. Immunofluorescence staining of MyHC in myotubes differentiated from human muscle stem/progenitor cells. Primary human muscle stem/progenitor cells were isolated and differentiated to myotubes for 5 days followed by MyHC immunofluorescence staining. Scale bars, 100 μ m. g. Relative expression level of Pax7, Myf5, and MyoD in human muscle stem/progenitor cells and differentiated myotubes. RT-qPCR assays were performed with human muscle stem/progenitor cells. Error bars indicated standard error and based on 4 independent experiments. *** indicated $p < 0.005$; * indicated $p < 0.1$. h. Relative expression level of MyoG, MyH in human muscle stem/progenitor cells and differentiated myotubes. RT-qPCR assays were performed with human muscle stem/progenitor cells after myogenic differentiation. Error bars indicated standard error and based on 4 independent experiments. *** indicated $p < 0.005$.

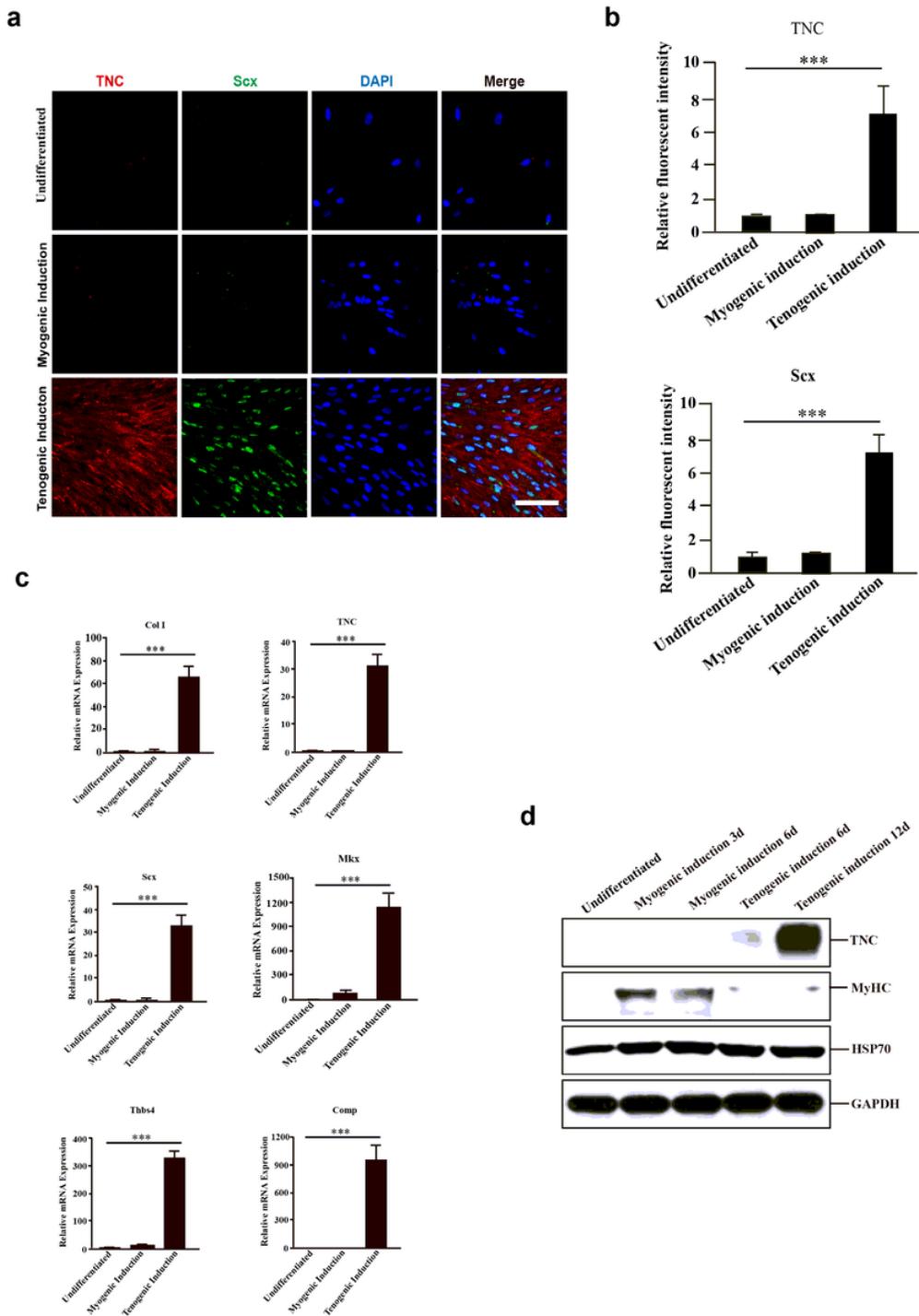


Figure 2

Human muscle stem/progenitor cells are capable of tendon differentiation. a. Immunofluorescence staining of tendon cell marker TNC and Scx in human muscle stem/progenitor cells undergone myogenic and tenogenic differentiation, respectively. Scale bars, 100 μ m. b. Quantification of TNC and Scx fluorescent intensity in human muscle stem/progenitor cells undergone myogenic and tenogenic differentiation, respectively. c. Relative gene expression levels of genes enriched in tendon cells. RT-qPCR assays were performed with human muscle stem/progenitor cells before and after myogenic and tenogenic differentiation. Error bars indicated standard error and based on 4 independent experiments. *** indicated $p < 0.005$. d. Protein level of tendon enriched protein TNC. Immunoblotting was performed with total proteins extracted from human muscle stem/progenitor cells before

Bubble chart of GO analysis of cellular process up-regulated in tenogenic differentiation of human muscle stem/progenitor cells.

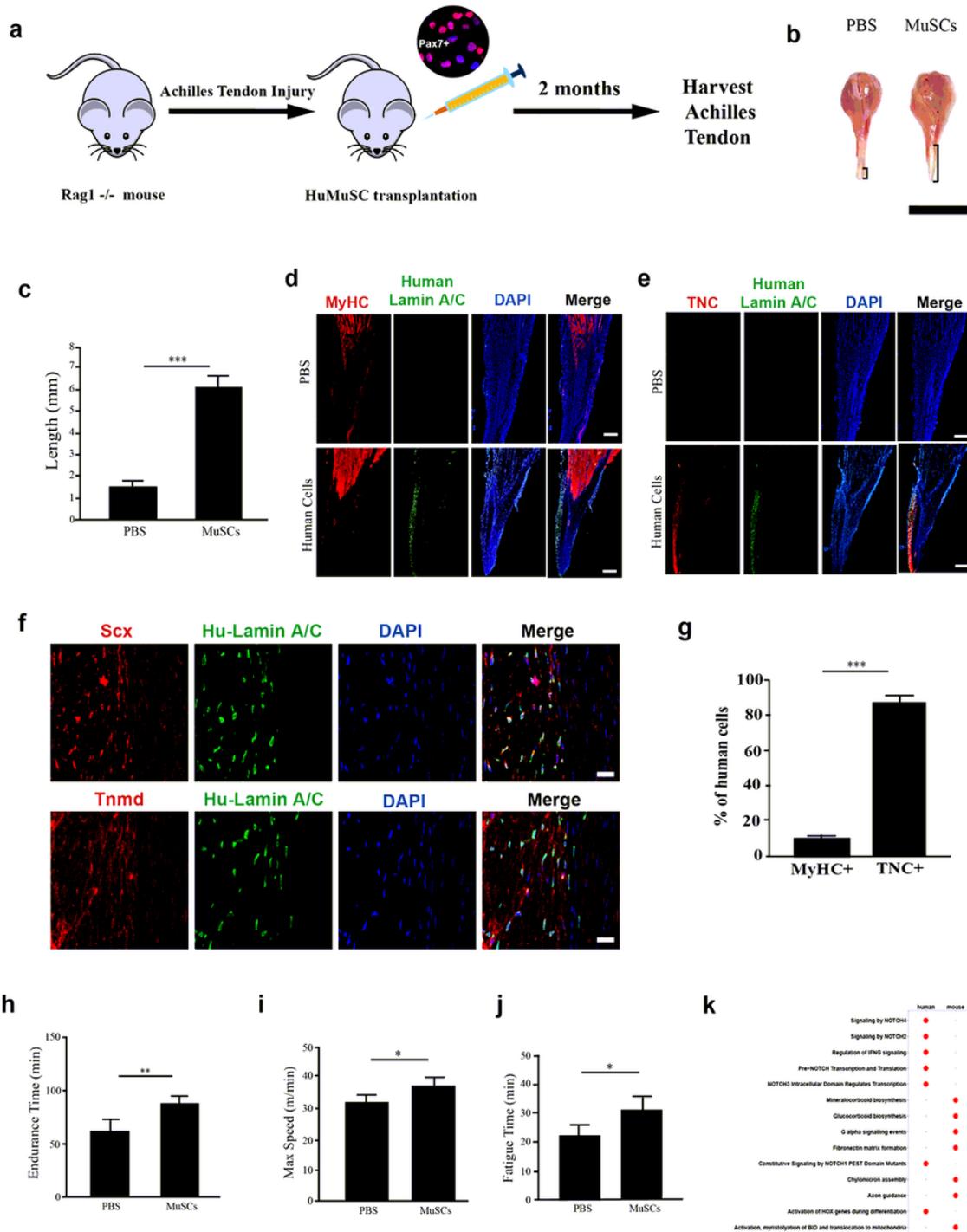


Figure 4

Human muscle stem/progenitor cells displayed tendon differentiation potential in vivo. a. Schem of human muscle stem/progenitor cell transplantation. b. Regenerated tendon after human muscle stem/progenitor cell transplantation. Tendon injury was induced in recipient SCID mice and 50,000 human muscle stem/progenitor cells were transplanted. Tendon was completely dissected out 2 months after human muscle stem/progenitor cell transplantation. Scale bars, 1cm. c. Statistic analysis of the length of the regenerated tendons after human muscle stem/progenitor cell transplantation. Error bars indicated standard error based on 10 independent

experiments. *** indicated $p < 0.005$. d. Immunofluorescence staining of MyHC and human Lamin A/C. The regenerated tendon and the connected skeletal muscle were dissected out and subjected for cryosection preparation. The cryosections were stained for MyHC which is specifically expressed in skeletal muscle and human Lamin A/C which is specifically expressed in human cells. Scale bars, $100\mu\text{m}$. e. Immunofluorescence staining of TNC and human Lamin A/C. The cryosections generated from the regenerated tendon and the connected skeletal muscle were stained for TNC which is enriched in tendon and human Lamin A/C which is specifically expressed in human cells. Scale bars, $100\mu\text{m}$. f. Immunofluorescence staining of Scx, Tnmd, and human Lamin A/C in cryosections derived from the regenerated tendon and the connected skeletal muscle 2 months after human muscle stem/progenitor cell transplantation. Scx and Tnmd were enriched in tendon cells. And human Lamin A/C was specifically expressed in cells originated from human. Scale bars, $20\mu\text{m}$. g. Statistic analysis of the percentage of human cells expressing tendon marker after being transplanted to the injured tendon sites. Error bars indicated standard errors and based on 10 independent transplantations. *** indicated $p < 0.005$. h. The endurance time of treadmill exercise. Mice were trained with the constant speed of $22\text{m}/\text{min}$ until exhaustion. Error bars indicated standard deviation based on 5 independent experiments. ** indicated $p < 0.01$. i. The maximum speed achieved during exhaustion test. Mice were trained on the treadmill with increased speed until exhaustion. Error bars indicated standard deviations based on 5 independent experiments. * indicated $p < 0.05$. j. The Fatigue time during exhaustion test. Mice were trained on the treadmill with increased speed until exhaustion. Error bars indicated standard deviations based on 5 independent experiments. * indicated $p < 0.05$. k. Bubble chart of differently activated biological pathways between human and mouse muscle stem cells.

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