



Fos Promotes Early Stage Teno-Lineage Differentiation of Tendon Stem/Progenitor Cells in Tendon

JIALIN CHEN,^{a,b,c*} ERCHEN ZHANG,^{a,b*} WEI ZHANG,^{a,b} ZEYU LIU,^{a,b} PING LU,^{a,b} TING ZHU,^{a,b,d} ZI YIN,^{a,b} LUDVIG J. BACKMAN,^c HUANHUA LIU,^{a,b} XIAO CHEN,^{a,b} HONGWEI OUYANG ^{a,b,e}

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^aCenter for Stem Cell and Tissue Engineering, School of Medicine, ^dDepartment of Orthopedics, Second Affiliated Hospital, ^eDepartment of Sports Medicine, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, People's Republic of China; ^bZhejiang Provincial Key Lab for Tissue Engineering and Regenerative Medicine, Hangzhou, Zhejiang, People's Republic of China; ^cDepartment of Integrative Medical Biology, Anatomy, Umeå University, Umeå, Sweden

*Contributed equally.

Correspondence: Hong Wei Ouyang, M.D., Ph.D., Mailbox #39, Center for Stem Cell and Tissue Engineering, School of Medicine, Zhejiang University, 866 Yu Hang Tang Road, Hangzhou, Zhejiang 310058, People's Republic of China. Telephone: 86-571-88208262; e-mail: hwoy@zju.edu.cn; or Xiao Chen, Ph.D., Mailbox #39, Center for Stem Cell and Tissue Engineering, School of Medicine, Zhejiang University, 866 Yu Hang Tang Road, Hangzhou, Zhejiang 310058, People's Republic of China. Telephone: 86-571-88982909; e-mail: chenxiao-610@zju.edu.cn

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ABSTRACT

Stem cells have been widely used in tendon tissue engineering. The lack of refined and controlled differentiation strategy hampers the tendon repair and regeneration. This study aimed to find new effective differentiation factors for stepwise tenogenic differentiation. By microarray screening, the transcript factor *Fos* was found to be expressed in significantly higher amounts in postnatal Achilles tendon tissue derived from 1 day as compared with 7-days-old rats. It was further confirmed that expression of *Fos* decreased with time in postnatal rat Achilles tendon, which was accompanied with the decreased expression of multiply tendon markers. The expression of *Fos* also declined during regular in vitro cell culture, which corresponded to the loss of tendon phenotype. In a cell-sheet and a three-dimensional cell culture model, the expression of *Fos* was upregulated as compared with in regular cell culture, together with the recovery of tendon phenotype. In addition, significant higher expression of tendon markers was found in *Fos*-overexpressed tendon stem/progenitor cells (TSPCs), and *Fos* knock-down gave opposite results. In situ rat tendon repair experiments found more normal tendon-like tissue formed and higher tendon markers expression at 4 weeks postimplantation of *Fos*-overexpressed TSPCs derived nonscaffold engineering tendon (cell-sheet), as compared with the control group. This study identifies *Fos* as a new marker and functional driver in the early stage teno-lineage differentiation of tendon, which paves the way for effective stepwise tendon differentiation and future tendon regeneration. *STEM CELLS TRANSLATIONAL MEDICINE* 2017;6:2009–2019

SIGNIFICANCE STATEMENT

This study identifies a new factor *Fos* for tendon early-stage differentiation. It paves the way for the stepwise differentiation from stem cells to mature tenocytes, which is beneficial for stem cells-based tendon regeneration.

INTRODUCTION

Tendon tissue engineering is promising for tendon repair and regeneration, which combines stem cells, scaffolds, and growth factors. However, current models are still far from ideal when it comes to tendon regeneration. A repaired tendon after injury is usually comprised of smaller-sized collagen fibrils, which accounts for the poor mechanical strength [1].

Stem cells have been widely used in tendon tissue engineering, including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), tendon stem/progenitor cells (TSPCs), and induced pluripotent stem cells (iPSCs). The properties that stem cells harbor make them potentially ideal for tendon regeneration. However, controlled teno-lineage differentiation is crucial for successful tendon regeneration and since stem cells have multi-differentiation ability, this renders an uncertainty of cell fate. We stand by our firm belief that stem

cells cannot fully differentiate into tenocytes, which causes the unsatisfactory repair effect in current tendon tissue engineering [2–4]. Thus, new effective differentiation factors need to be found.

The normal in vivo tendon development process is the ultimate environment to find new important differentiation factors. The cell types during tendon development transit from ESCs to MSCs to TSPCs and eventually to mature tenocytes. The cell fate is gradually defined toward teno-lineage during development, and this indicates that currently used stem cells may require different stimulation at different stages in order to achieve an effective and successful tendon differentiation. Actually, many known important genes have been found by studying the development process of tendons, such as *Scleraxis* (*Scx*) [5, 6], *GDF-5* [7], and *GDF-6* [8], most of which were discovered from the embryonic development stage. There are also some recent studies

that have tried to elucidate the postnatal tendon development process [9–12]. They evaluated the structural alteration and the roles of some previously known genes during development. However, inherent molecular basis of tendon development and differentiation has not been clearly elucidated. Therefore, new genes crucial for tendon differentiation need to be discovered for successful tendon regeneration.

We hypothesize that there are new markers for the direction of early teno-lineage differentiation to be found. Therefore, the aim of this study was to screen for potential candidate genes by microarray, and to confirm their role by doing gene overexpression and gene silence experiments. By comparing the tendon gene expression and *in vivo* function, we provide the first evidence that identifies the *Fos* gene as a tendon early stage differentiation factor.

MATERIALS AND METHODS

Microarray Analyses

Achilles tendons at different development stages (postnatal 1 day and 7 days, $n = 3$, each sample contains at least two individuals) were harvested for microarray analyses. Total RNA was extracted using Trizol reagent and was further purified using Qiagen RNeasy Mini Kit according to the manufactures' instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis. An aliquot of 200 ng of total RNA was used to synthesize double-stranded cDNA, and then produce biotin-tagged aRNA using MessageAmp Premier RNA Amplification Kit (Life Technologies, Grand Island, NY, <http://www.thermofisher.com/>). The resulting biotagged aRNA was fragmented to strands of 35–200 bases in length according to the protocols from Affymetrix. The fragmented aRNA was hybridized to Rat Genome 230 2.0 Array (Affymetrix, <http://www.thermofisher.com/>) containing 30,000 transcripts. Hybridization was performed at 45°C with rotation for 16 hours at hybridization oven 640. The GeneChip arrays were washed and then stained automatically on an Affymetrix Fluidics Station 450 followed by scanning on an Affymetrix GeneChip Scanner 3000 7G.

The scanned images were first assessed by visual inspection then analyzed to generate raw data files saved as CEL files using the default setting of Affymetrix GeneChip Command Console 3.2 (AGCC) Software. Then, the raw data were normalized and summarized with the Affymetrix Microarray Suite 5.0 (MAS5) and with the Robust Multi-array Average (RMA) algorithm [13]. In a comparison analysis, we applied a two-class unpaired method in the Significant Analysis of Microarray software (SAM, version 3.02) to identify significantly differentially expressed genes between two groups. Genes were determined to be significantly differentially expressed using fold change of 2.0 and q value < 5% as cutoffs in the SAM output result. Hierarchical clustering with the average linkage method was performed with Cluster3.0 software, and the cluster result was visualized through with the Treeview program. The array has been submitted to the GEO repository with accession number GSE70459.

Quantitative Polymerase Chain Reaction

RNA isolation, reverse transcription, and quantitative polymerase chain reaction (qPCR) were carried out as previously described [14]. All primers (Invitrogen, <http://www.thermofisher.com/>) were designed using primer 5.0 software. Representative results are

displayed as target genes expression normalized to house-keeping gene.

Lentiviral Production and Infection

A third-generation self-inactivating lentivirus vector containing a CMV promoter upstream of the multiple cloning sites (MCS) was used. The Coding DNA Sequence sequences of rat *Fos* gene and *lgfbp2* gene were inserted into MCS. Additionally, green fluorescence protein (GFP) was used as the control to discount any change in gene expression profile that may result from the delivery method. The constructed lentiviral vector and another three package vectors were cotransfected into 293FT cells (Invitrogen) with lipofectamine (Invitrogen) according to the manufacturer's instructions. The medium was replaced 16 hours after transfection. Forty-eight hours later, the virus-containing medium was pooled and passed through a 0.45 μ m filter to remove cell debris and was immediately used to infect cells in the presence of 10 ng/ml polybrene (Sigma, St. Louis, MO, <https://www.sigmaaldrich.com/>). The infected cells before passage 4 were used for further study.

Immunocytochemistry

Monolayer cultures of cells were fixed in 4% (vol/vol) paraformaldehyde and subjected to immunostaining with the primary antibody, rabbit anti-human MKX monoclonal antibody (LifeSpan BioSciences, Seattle, WA, <https://www.lsbio.com/>), followed with a goat anti-rabbit secondary antibody (Invitrogen). 4',6-diamidino-2-phenylindole (DAPI) staining was used to reveal the nuclei of the cells.

Gene Expression of Fos in Postnatal Rat Achilles Tendon

Postnatal rat Achilles tendons were collected at 1, 3, 7, 14, 28, and 56 days ($n = 3$ for each time point). RNA isolation and reverse transcription were carried out to obtain cDNA. Gene expression of *Fos* and multiply tendon markers were compared by qPCR between different developmental time points.

Cell-Sheet Model

As described previously [4], upon reaching confluence, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum and 50 μ g/ml ascorbic acid (Sigma). A multi-layered cell-sheet detached from the substratum and formed dense sphere-like structure automatically within 2 weeks in culture. To evaluate the extracellular matrix (ECM) deposition speed, the percentage of samples forming sphere-like structures was calculated. The cell aggregates were also harvested for transmission electron microscopy, RNA isolation, and animal experiments.

Three-Dimensional Cell Culture Model

Human TSPCs were cultured in nonadherent culture dishes, with DMEM supplemented with 10% (vol/vol) fetal bovine serum. Cells aggregated into sphere-like structures by themselves. RNA was isolated to evaluate the tendon-related gene expression.

siRNA Transfection

siRNA transfection in TSPCs to knock-down *Fos* was performed according to the manufacturer's protocol (RiboBio, Guangzhou, China, <http://www.ribobio.com>). Three *Fos* siRNA duplexes were used (siRfo1, siRfo2, and siRfo3). A scrambled siRNA was used as a control (siRNTC). All siRNA oligos were designed and synthesized

by RiboBio Company. Forty-eight hours post-transfection, cells were harvested to assess the gene expression.

In Situ Rat Tendon Repair Model

Eight mature female Sprague Dawley rats weighing 200–220 g were used for this experiment. The Zhejiang University Institutional Animal Care and Use Committee approved the study protocol (ethical No. ZJU20170413). Under general anesthesia, a gap wound was created and the Patella tendon was removed to create a defect of 4 mm in length. Nonscaffold engineering tendons (cell sheet of *Fos* overexpressed TSPCs or control group transfected with *GFP*) were sutured to the remaining Patella tendon using a nonresorbable suture (6-0 nylon). The wound was then irrigated and the skin was closed. The animals were allowed free cage activity after surgery. At 4 weeks postimplantation, eight samples from each group were harvested for the evaluation of histology and gene expression.

Histological Examination

Histological specimens treatment was performed as previously reported [15]. The collected specimens were fixed in 10% (vol/vol) neutral buffered formalin, dehydrated through an alcohol gradient, cleared, and embedded in paraffin blocks. Histological sections (7 μ m) were prepared by using a microtome and were stained with hematoxylin and eosin. A blinded semiquantitative scoring system was used to evaluate the repair of tendon tissue [16], which was based on six parameters (fiber structure, fiber arrangement, nuclear roundness, vascularity, inflammation, number of cells). For the evaluation of each parameter, 0 was allotted to normal tendon and 3 was allotted to maximally abnormal tissue.

Statistical Analysis

Student's *t* test (except for the microarray analysis) was performed to assess statistically significant differences in the results of different groups. Values of $p < .05$ were considered to be significantly different.

RESULTS

Screen of Tendon Early Stage Marker Genes

Postnatal Achilles tendon tissues at 1 day (1d) and 7 days (7d) were harvested for microarray experiment. A total of 571 transcripts out of 30,000 transcripts were found to be more than two-fold different when comparing 1d with 7d tendons (Supporting Information Table S1), in which 370 transcripts were upregulated (65%), and 201 transcripts were downregulated (35%) in tendon tissues at 1d as compared with tendon tissues at 7d. These transcripts were hierarchically clustered (Fig. 1A). The expression of six randomly chosen genes (*Adipoq*, *Comp*, *Cpxm2*, *Cpz*, *Mpz*, and *Fos*) were confirmed by qPCR (Fig. 1B). Gene Ontology (GO) analysis showed the top ten GO terms to be related to biological process (Fig. 1C) and pathway (Fig. 1D) between 1d and 7d tendon tissues. In biological process, the most significant GO term was "cell adhesion," followed by "spermine biosynthesis" and "proteolysis." The most significant GO term in pathway was "PPAR signaling pathway." Other GO terms were related to cell adhesion molecules and graft-versus-host disease.

To find out the early stage tendon marker genes for development and differentiation, the top 25 genes were ordered, which were enriched in postnatal 1d tendon tissue as compared with 7d

tendon tissue (Supporting Information Table S2). These genes were upregulated in tendon tissues at 1d, as compared with 7d, with at least 3.7-fold. Transcript factors usually play crucial roles in cell fate determination. Transcript factor *Igfbp2* is the most abundant binding protein in tendon, and sensitively responsive to tendon injury [17]. Transcript factor *Fos* plays an important role in mesoderm derived tissue differentiation and maintenance [18–20]. Thus, *Igfbp2* and *Fos* were chosen as candidates in the current study.

Cluster analysis indicated *Igfbp2* and *Fos* to be upregulated in postnatal tendon tissues at 1d as compared with 7d (Fig. 2A). qPCR results were consistent with microarray data. It was also found by qPCR that the expressions of *Igfbp2* and *Fos* in postnatal 1d tissues were higher than 56d tissues (Fig. 2B). Overexpression vectors of these two candidate genes were constructed. The transfection efficiency came to nearly 100% (Fig. 2C, 2D). qPCR results showed that the expression of *Igfbp2* and *Fos* in human ESC derived MSCs (hESC-MSCs) were upregulated by 400-fold and 30,000-fold, respectively, (Fig. 2E).

The expression of tendon related transcript factors and ECM genes were evaluated after overexpression of *Igfbp2* or *Fos* in human ESC-MSCs (Fig. 2F). There were no obvious upregulations in the group in which *Igfbp2* was overexpressed. However, the expression of *MKX* and *COL3* increased by overexpression of *Fos* (3.37-fold and 3.84-fold, respectively). Immunofluorescence detected the increased expression of *MKX* protein in the *Fos* overexpressed group. *MKX* and *COL3* are early stage related genes in tendon, thus *Fos* may play a role in maintaining early stage tendon phenotype.

Relevance Between *Fos* and Tendon Phenotype

The expression of *Fos* in postnatal rat Achilles tendon was compared at 1d, 3d, 7d, 14d, 28d, and 56d (Fig. 3). Continuous decrease in *Fos* gene expression was found during the development, in which significance was found since postnatal 7d, as compared with 1d. Similar decreases during development were found in expression levels of tendon markers, including *Mkx*, *Egr1*, *Col14*, *Tnc*, *Gdf5*, and *Gdf6*. As for *Scx*, *Tnmd*, *Dcn*, and *Bgn*, there was a peak expression at around 7d or 14d, which came to a low level when the tendon tissue matured. No obvious change was found for other tested tendon-related genes, such as *Hoxa11*, *Six1*, *Fmod*, *Col1*, and *Col3*.

Cells gradually lose their tendon phenotype during regular cell culture in vitro. The expression of the tendon related genes or proteins were downregulated. It was noticed that TSPCs at passage 1 expressed more than 3.5-fold higher *Scx* as compared with normal tendon tissues (Fig. 4A). Nevertheless, the expression of *Scx* decreased to 24% of normal tendon tissues in TSPCs at passage 4 in cell culture. The expression of *Scx* in in vitro cultured tenocytes was 63% of normal tendon tissues. *Tnmd* could only be detected in tendon tissue, but not in in vitro cultured TSPCs or tenocytes (Fig. 4A). *Mkx* and *Col14*, early tendon stage related genes, also gradually lost their expression during in vitro cell culture (Fig. 4A). Green fluorescence was shown in normal tendon tissue of *Scx*-GFP mice. However, the fluorescence could not be detected in cells that were cultured in vitro (Fig. 4C–4E). Notably, the expression of *Fos* was downregulated in accordance with the loss of tendon phenotype in vitro (Fig. 4B).

When TSPCs were cultured in vitro using a cell-sheet or a three-dimensional (3D) cell culture model (Fig. 4F, 4I). The expression of tendon related genes was upregulated in the two different

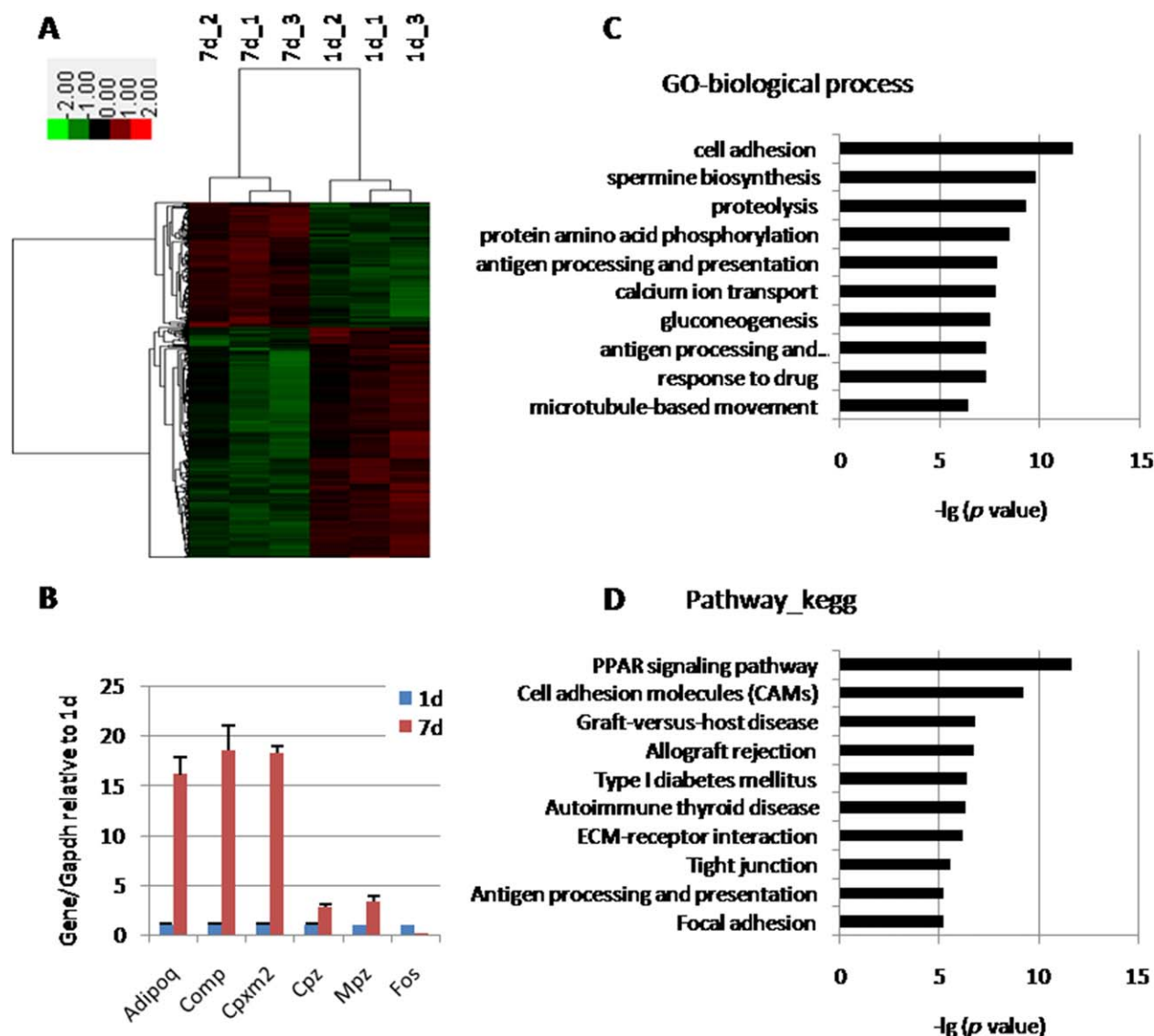


Figure 1. Microarray analysis of tendon tissues at postnatal 1d and 7d. **(A):** Gene cluster analysis of 571 transcripts, which showed more than twofold differences between 1d and 7d tendons. **(B):** qPCR results confirming six differently expressed genes from microarray. **(C):** GO related to biological process ordered according to $-\lg(p \text{ value})$. **(D):** GO related to pathway ordered according to $-\lg(p \text{ value})$. Abbreviation: GO, gene ontology.

models as compared with that in regular cell culture (Fig. 4G, 4J), accompanied by a great upregulation of *Fos* expression (Fig. 4H, 4K).

Therefore, there is potential relevance between *Fos* and tendon phenotype.

Overexpression of *Fos* in Postnatal 7d Derived TSPCs

Fos was overexpressed in 7d derived TSPCs (TSPCs-7d). The expression of *Fos* increased with about 7-fold (Fig. 5A). The *Fos* overexpressed TSPCs had significant higher expression of tendon markers as compared with the control group (Fig. 5B, 5C), including *Scx* (2.70-fold), *Mkx* (2.17-fold), *Tnmd* (2.16-fold), *Egr1* (1.92-fold), *Col1* (3.17-fold), *Col3* (7.56-fold), *Dcn* (5.31-fold), *Bgn* (2.38-fold), *Gdf5* (3.57-fold), and *Gdf6* (4.75-fold). When cells were cultured in a cell-sheet model, qPCR results showed that *Fos* overexpression reversed the gene expression of *Mmp3*, *Col14*, and *Col3* from being 7d postnatal to be more like 1d postnatal (Supporting Information Fig. S1).

Besides, the *Fos* overexpressed TSPCs had advantages in colony-forming ability (Supporting Information Fig. 2), cell proliferation ability, and ECM deposition ability (Supporting Information Fig. S3), as compared with the control group. There was no significant difference of osteo-lineage and adipo-lineage differentiation potential between *Fos* overexpressed TSPCs and control group (Supporting Information Fig. S2).

siRNA Knock-Down of *Fos* in TSPCs

The expression of *Fos* in TSPCs was knocked down by siRNA. qPCR experiments confirmed the knock-down effect of *Fos* expression by the use of three siRNA sequences (69, 31, and 39%, Fig. 6A). The siRNA sequence siRfo1 showed highest knock-down efficiency as compared with the other two siRNA sequence and was therefore used to knock down the expression of *Fos* in the following experiments. The *Fos* knock-down TSPCs had significant lower expression of tendon markers as compared with the control group

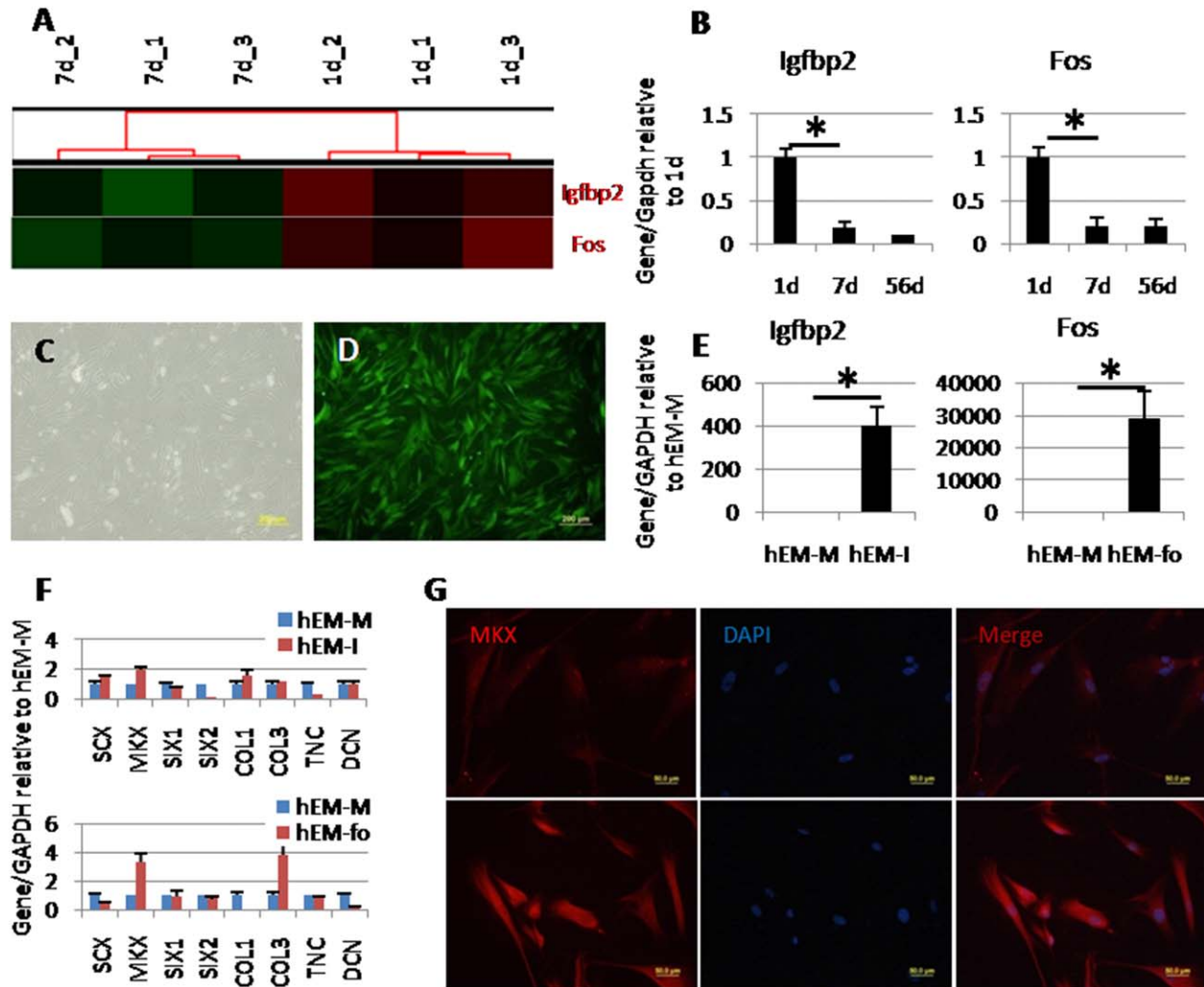


Figure 2. Screen of early stage differentiation factors. (A): Gene cluster analysis of *Igfbp2* and *Fos*. (B): qPCR results showing higher expression of *Igfbp2* and *Fos* in 1d tendon tissue compared with 7d and 56d. (C, D): Overexpression in human embryonic stem cells derived mesenchymal stem cells (hESC-MSCs). Green fluorescence indicating the transfection efficiency of green fluorescence protein (*GFP*) (control) came to nearly 100%. (E): Gene expression of *Igfbp2* or *Fos* in overexpressed hESC-MSCs. (F): Expression of tendon related genes in *Igfbp2* or *Fos* overexpressed hESC-MSCs, compared with *GFP* overexpressed hESC-MSCs. (G): MKX immunofluorescence in control group (upper) and *Fos* overexpressed hESC-MSCs (lower). Scale bars = 200 μ m (C, D) and 50 μ m (G). *Significant difference between two groups at $p < .05$. Abbreviations: hEM-fo, *Fos* overexpressed in hESC-MSCs; hEM-M, control group with *GFP* overexpressed; hEM-I, *Igfbp2* overexpressed in hESC-MSCs.

(Fig. 6B, 6C), such as *Mxk* (0.32-fold), *Tnmd* (0.20-fold), *Egr1* (0.56-fold), *Fmod* (0.60-fold), *Col14* (0.44-fold), *Tnc* (0.52-fold), *Gdf5* (0.24-fold), and *Gdf6* (0.34-fold). When cells were cultured in a cell-sheet model, the expressions of *Mmp3*, *Col14*, and *Col3* were the contrary when *Fos* was over-expressed (Supporting Information Fig. S4). Besides, the *Fos* knocked-down TSPCs had inferior ECM deposition ability as compared with the control group (Supporting Information Fig. S4).

Fos-Overexpressed TSPCs Improved the Healing of Injured Rat Tendon

Nonscaffold engineering tendons (cell-sheet) were constructed in vitro with *Fos* overexpressed TSPCs (FOS group) or control group transfected with *GFP* (Ctrl group). Implantation was performed in an in situ tendon repair model. After 4 weeks post-surgery, the cells in the repaired tissue of FOS group exhibited a more spindle-shaped morphology, as compared with the control group (Fig. 7A). More alignment of collagen fibers was also observed in the

FOS group (Fig. 7A). Histology score evaluation found that FOS group had significantly better score than the control group (5.94 ± 1.17 vs. 8.13 ± 1.08 , Fig. 7B). The mRNA transcript levels of multiply tendon markers were compared (Fig. 7C), in which significant upregulated expression was found in FOS group on *Scx* (5.7-fold), *Tnmd* (14.9-fold), *Fmod* (3.1-fold), *Col1* (3.6-fold), *Col3* (9.9-fold), and *Col14* (3.1-fold), as compared with the control group. Increased expression without significance was found in FOS group on other tendon-related genes.

DISCUSSION

This study identifies the *Fos* gene as a tendon early-stage differentiation marker. It was obvious that reduced expression of *Fos* corresponded to tendon phenotype loss during development and regular in vitro cell culture, and increased expression of *Fos* corresponded to upregulation of tendon related genes expression in cell-sheet and 3D cell culture model. Overexpression of *Fos* in

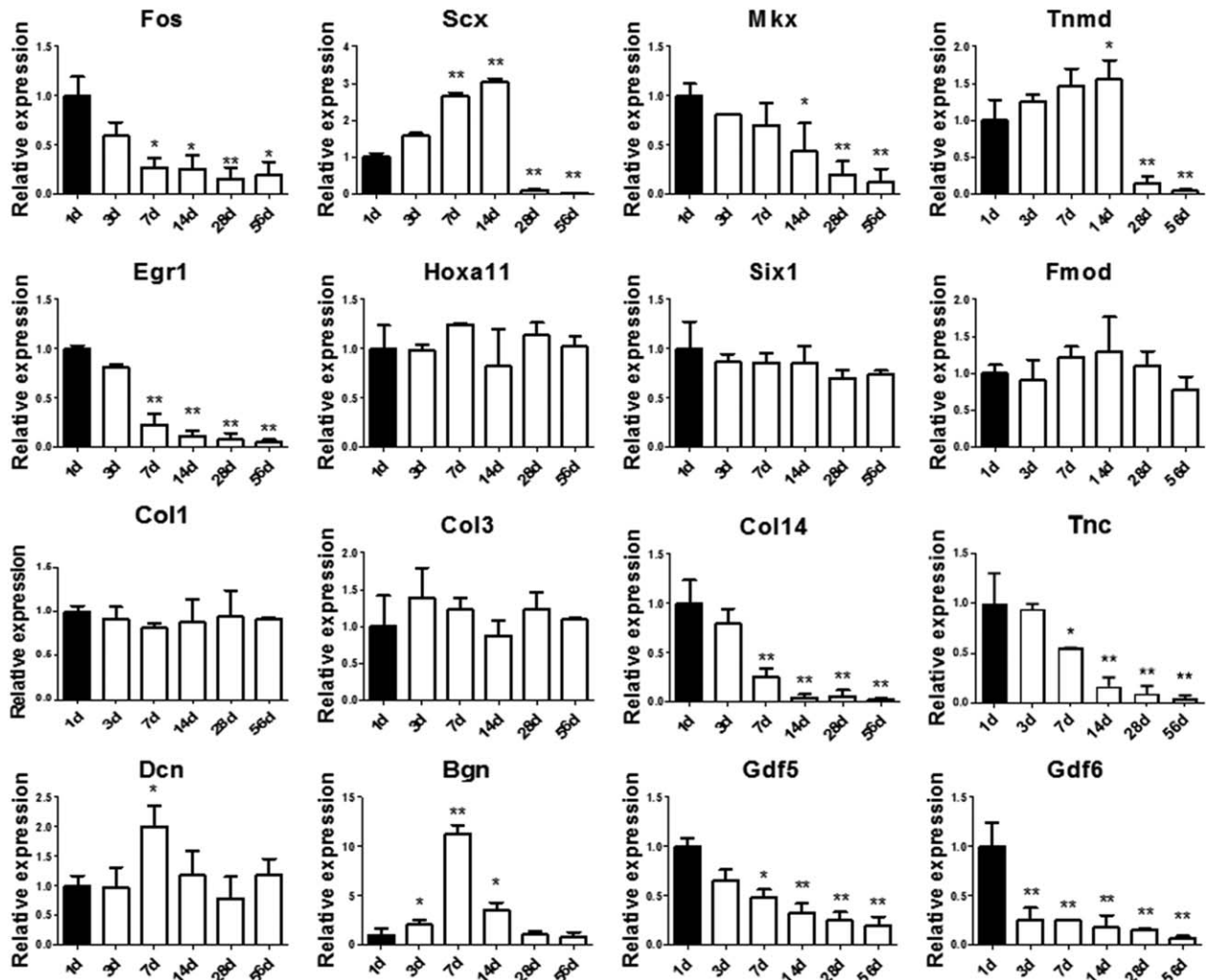


Figure 3. Expression of *Fos* and multiply tendon markers in postnatal rat Achilles tendon. The mRNA levels of *Fos* and multiply tendon markers in postnatal rat Achilles tendons were compared by qPCR at 1, 3, 7, 14, 28, and 56 days. The expression level of each gene at 1d was set as 1. *Significant difference between specific time point and 1d at $p < .05$. **Significant difference between specific time point and 1d at $p < .01$.

TSPCs caused upregulated gene expression of tendon markers, the opposite was seen in *Fos* knocked-down cells. Finally, accelerated healing of injured tendon was found by using *Fos* overexpressed TSPCs. The results of this study strongly suggest that *Fos* is a candidate marker for early teno-lineage differentiation and maintain.

The concept of stepwise differentiation is important for tissue engineering and tissue regeneration when using stem cells [2]. It has been confirmed in other tissues that stepwise differentiation of stem cells into defined cell types brings success and new hope for tissue repair, such as myoblasts [21–24], chondrocytes [25], liver [26], heart [27], insulin-secreting beta cells [28], neurons [29], and oligodendrocytes [30]. Goudenege and collaborators generated skeletal myoblasts from hESCs through mesodermal transition by two steps [24]. The mesenchymal differentiation was achieved by culturing hESCs in myogenic medium. After that, the myogenic factor MyoD was overexpressed in cells to cause the final conversion. This stepwise differentiation not only overcame the difficulties of differentiating ESCs into myoblasts, but also lowered the risks of teratomas formation. Oldershaw and

collaborators reported a refined protocol for differentiation of hESCs to chondrocytes using chemicals by three steps [25]. Different chemicals were combined in a specific order based on the knowledge from tissue development. There is not much reported about the stepwise differentiation in tendon, and is far away from elaborate and controllable, which hinders work on tendon repair and regeneration. To apply hESCs in tendon regeneration, our group differentiated hESCs into ESC-MSCs, which has shown good potential for tendon differentiation and regeneration when using scaffolds, mechanical stimulation, or overexpression of *SCX* in hESC-MSCs [1, 3, 4, 14]. It was found by Alberton and collaborators that overexpression of *SCX* in human BMSCs could convert the cells into tenogenic progenitor cells, which is an important step for fully differentiation of MSCs toward mature tenocytes [31]. Our recent work [32] showed that TSPCs isolated at different postnatal time-points possessed different self-renew, multi-potent differentiation potential, cell proliferation, and ECM deposition ability. The transition of TSPCs during development indicates the importance of stepwise differentiation from MSCs/TSPCs to mature tenocytes. Based on this, the current study analyzed the

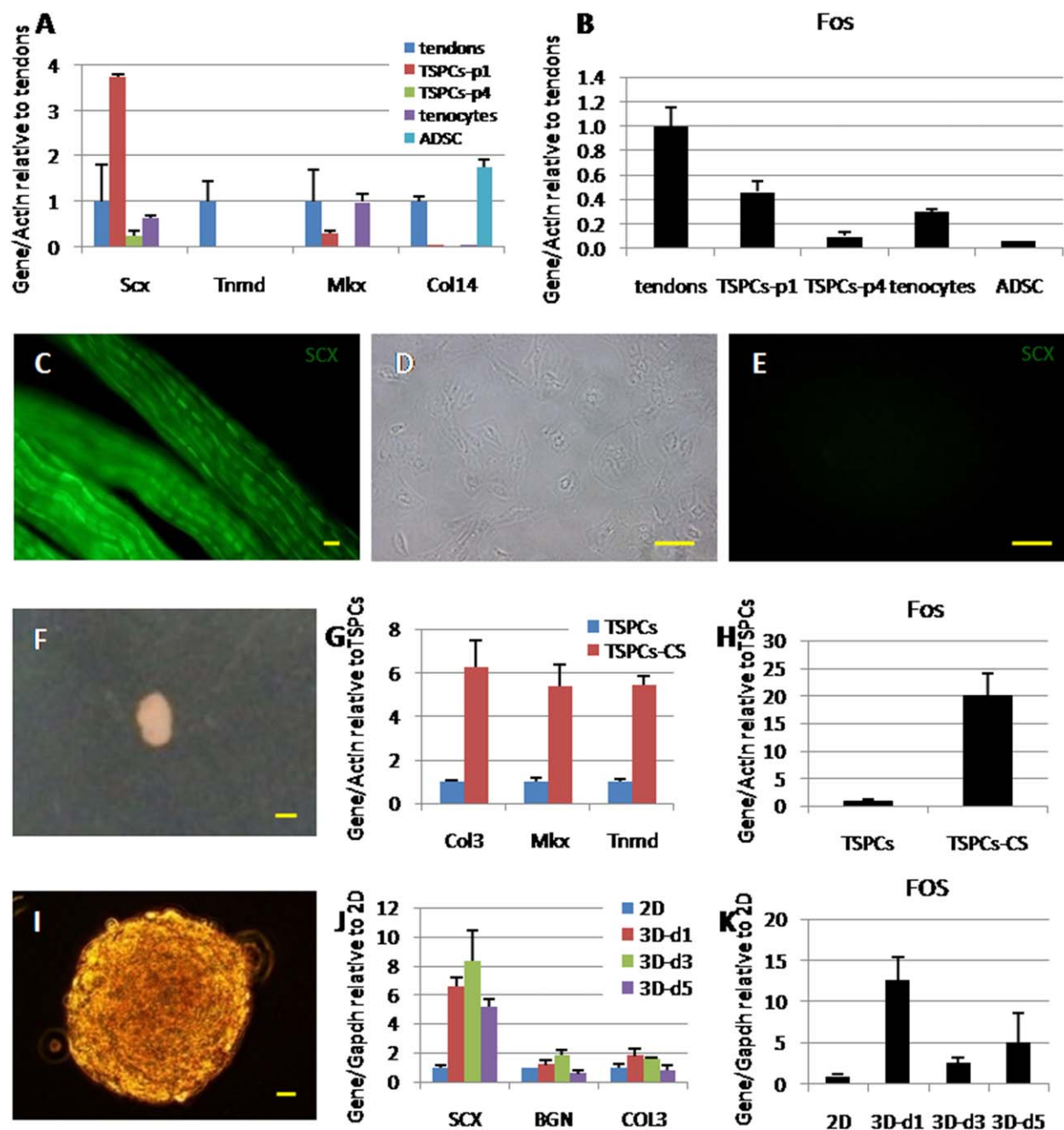


Figure 4. Relevance between *Fos* and tendon phenotype. (A): The expression of tendon related genes decreased during in vitro cell culture. (B): The expression of *Fos* in tendon tissues and cells cultured in vitro. (C): Green fluorescence could be observed in tendon tissue of Scx-GFP mice. (D, E): Cells isolated from tendons of Scx-GFP mice lost fluorescence when cultured in vitro. (F): Cell-sheet model: cells self-crimped into a sphere when cultured with ascorbic acid within 14 days. (G): Tendon related gene expressions compared between regular cell culture and the cell-sheet model. (H): The expression of *Fos* in the cell-sheet model. (I): Three-dimensional (3D) model: cells cultured with a 3D sphere structure. (J): Tendon related gene expressions compared between two-dimensional regular cell culture and 3D model at 1 day, 3 day, and 5 day. (K): The expression of *FOS* in 3D model. Scale bars = 50 μ m (C), 100 μ m (D, E), 1 mm (F), and 20 μ m (I). Abbreviations: ADSC, adipose derived stem cells; TSPCs, tendon stem/progenitor cells; TSPCs-CS, TSPCs formed cell-sheet; TSPCs-p1, TSPCs at passage 1.

highly expressed genes in postnatal 1d tendon tissue, by microarray, to screen for important differentiation factors for stepwise tenogenic differentiation.

By comparison of the gene expression profiles, 370 transcripts were upregulated in tendon tissues at 1d as compared with 7 days. The gene *Fos* was picked out due to the following reasons: (a) The *Fos* gene was more highly expressed in postnatal 1d

tendon tissue (more than fourfold) as compared with in 7d tendon, (b) *Fos* is a transcript factor, and it is well known that transcript factors are crucial for cell fate determination during development and differentiation. When *Fos* was overexpressed in hESC-MSCs, it promoted the expression of *MKX* and *COL3* by more than threefold as compared with the control group. *Mlx* is an important transcript factor for tendon differentiation [33–35].

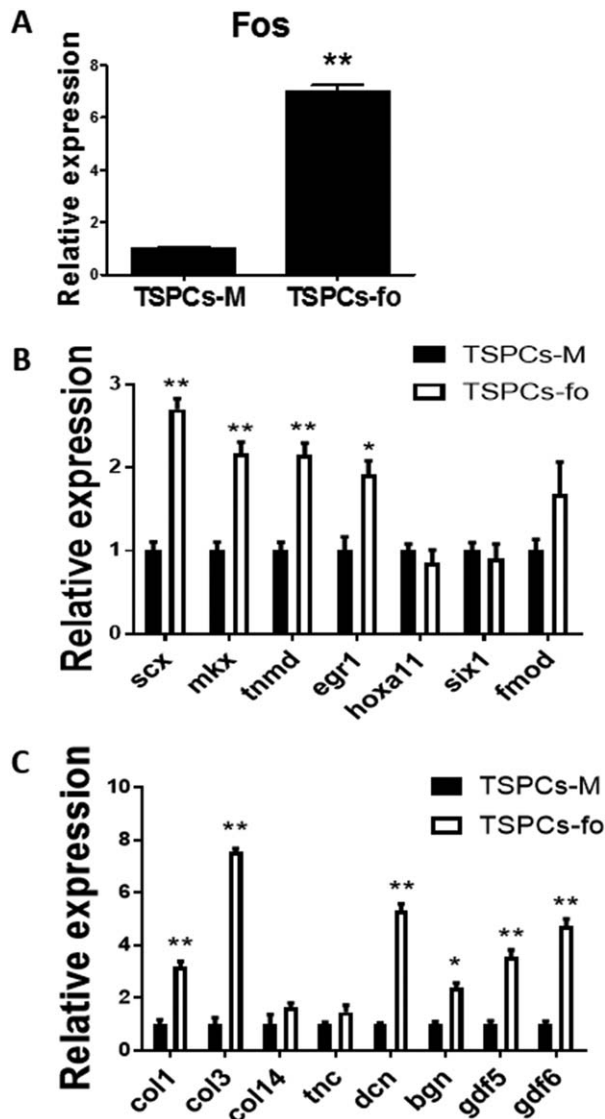


Figure 5. Overexpression of *Fos* in TSPCs caused upregulated gene expression of tendon markers. (A): Gene expression of *Fos* in overexpressed TSPCs. (B, C): Gene expression of multiply tendon markers in TSPCs-fo versus TSPCs-M. The expression level of each gene in TSPCs-M group was set as 1. *Significant difference between two groups at $p < .05$. **Significant difference between two groups at $p < .01$. Abbreviations: TSPCs, tendon stem/progenitor cells; TSPCs-fo, TSPCs transfected with *Fos*; TSPCs-M, control group which is transfected with *GFP*.

It was reported that dense *Mkx* mRNA could be detected in mice at E13.5 and E14.5 but decreased a lot at postnatal 0d and 14day [33]. *Col3* regulates the initial fibril assembly [36]. During development, the amount of *Col3* decreased with the increase of collagen fiber diameter [37–39]. Thus, the elevation of *MKX* and *COL3* expression after *Fos* overexpression indicates the conversion of cells into an early stage tendon. Besides, in regular in vitro cell culture or in vivo postnatal tendon development, the expression of tendon related genes or proteins were decreased. This is also seen in the expression of *Fos*. In vitro cell-sheet and 3D models maintained the tendon phenotype, causing an increase of the tendon related genes and *Fos*. Overexpression of *Fos* in TSPCs caused upregulated gene expression of tendon markers, while siRNA knock-down of *Fos* showed opposite results.

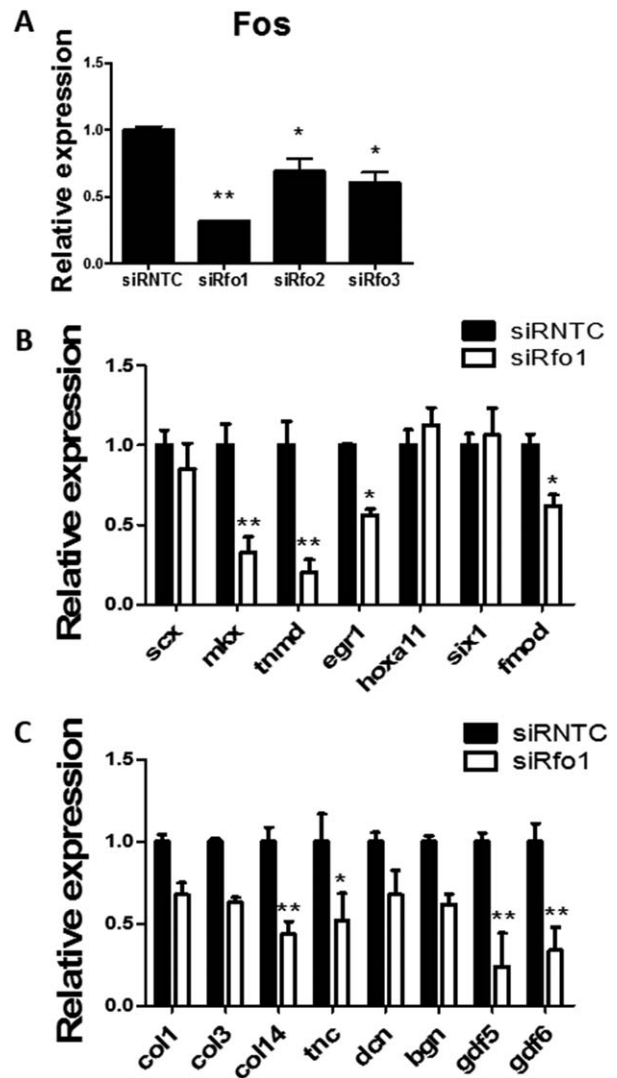


Figure 6. *Fos* knock-down by siRNA in tendon stem/progenitor cells caused downregulated gene expression of tendon markers. (A): qPCR showed the downregulation of *Fos* by three different siRNA sequences (siRfo1, siRfo2, and siRfo3), compared with siRNTC. (B, C): Gene expression of multiply tendon markers in siRfo1 versus siRNTC. The expression level of each gene in siRNTC group was set as 1. *Significant difference between specific group and siRNTC group at $p < .05$. **Significant difference between specific group and siRNTC group at $p < .01$. Abbreviation: siRNTC, control siRNA.

The expression of *Fos* has been detected in fetal liver, growing bone and developing central nervous system [40]. It is a quickly responsive gene to various stimulations and is of importance for signal transduction, cell proliferation and differentiation [41, 42]. *Fos* is the first transcription factor identified for the regulation of cell fate during development and maintenance of skeleton. It also plays an important role in the early osteogenic differentiation [19] and in the chondrocyte gene transcription [20]. Tendon, bone, and cartilage are all derived from mesenchyme, thus *Fos* may play a potential role in the regulation of tenogenic differentiation. A recent report by Eliasson and collaborators showed that *Fos* is one of the four genes significantly upregulated (almost sevenfold) in mechanically induced rat tendon repair, which was screened by microarray containing 27,342 genes [43]. However, to our

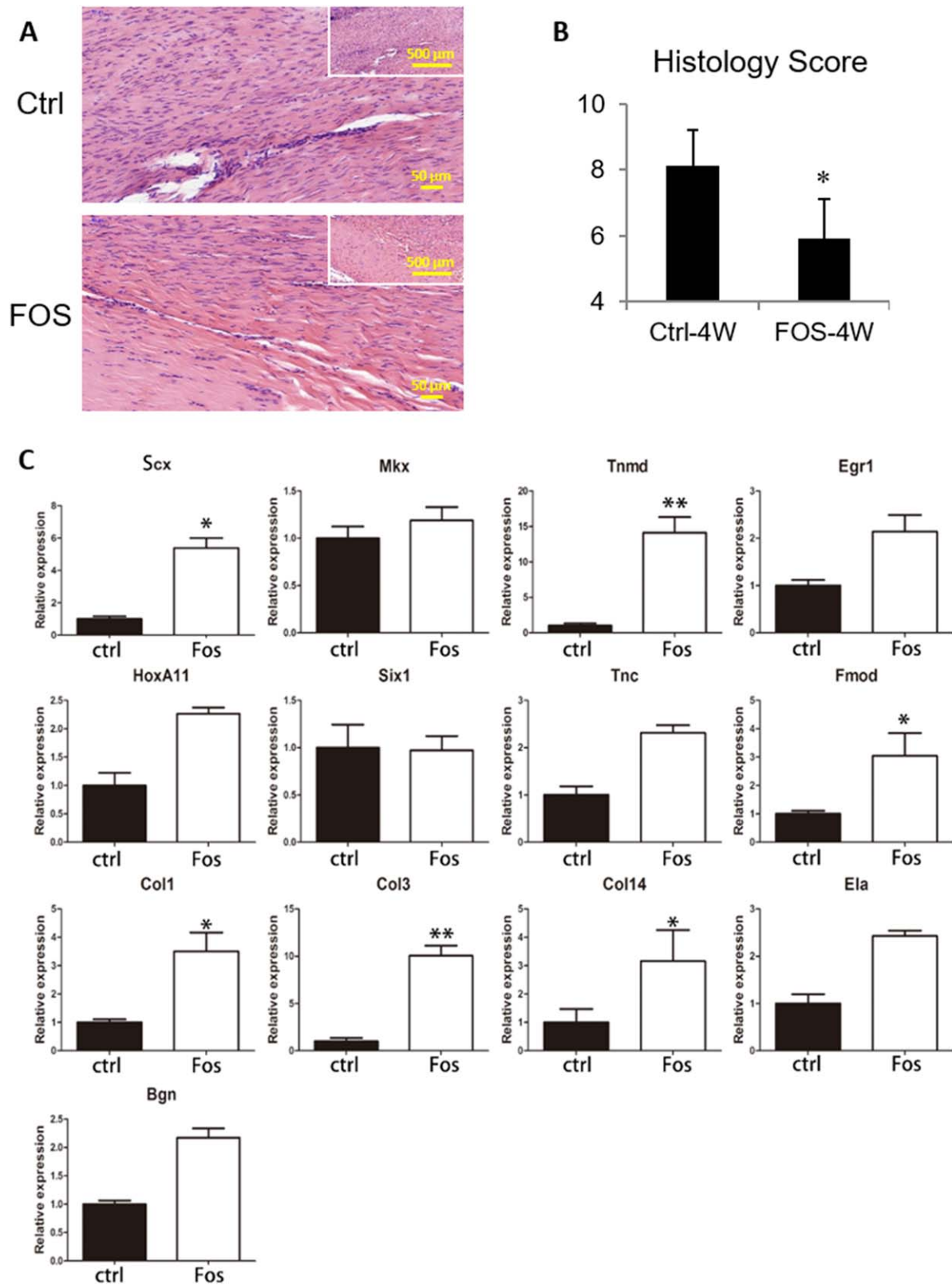


Figure 7. *Fos*-overexpressed tendon stem/progenitor cells (TSPCs) improved the healing of injured rat tendon. Cell-sheet constructed with *Fos* overexpressed TSPCs (FOS group) or control group transfected with *GFP* (Ctrl group) was compared in in situ rat Patella tendon repair model. **(A):** Hematoxylin and eosin staining after 4 weeks post-surgery. **(B):** Histology score evaluation was performed to compare between FOS group and Ctrl group. **(C):** Gene expression of multiply tendon markers after 4 weeks post-surgery. The expression level of each gene in Ctrl group was set as 1. *Significant difference between specific group and siRNTC group at $p < .05$. **Significant difference between specific group and siRNTC group at $p < .01$. Abbreviation: Ctrl, control.

knowledge, our research is the first that tries to find the relationship between *Fos* and tendon differentiation.

Previous studies have shown that *Fos* is sensitive to mechanical stimulation both in vitro and in vivo, and in many different cell types, such as tenocytes, ligament cells, osteoblasts, chondrocytes, and so forth [18, 20, 41, 42, 44, 45]. The natural exposure to mechanical stimulation at birth might be the reason for the high expression of *Fos* in postnatal 1d tendon tissue. Besides, to some extent, the cell-sheet and 3D models used in our study caused cell aggregation in vitro, and enhanced the mechanical strength during cell–cell interaction, which may induce *Fos* expression and tendon phenotype maintain. The promoter region of *Mkx* contains the binding site of *Fos*. *Mkx* plays important roles in tenogenesis and tendon regeneration [33–35] and thus may be the downstream effector of *Fos*. The inherent mechanism of *Fos*-regulating tenogenic differentiation, and whether mechanics regulate *Fos* during this process, should be studied in our future work.

CONCLUSION

This study is the first to identify the *Fos* gene as a tendon early-stage differentiation factor. The expression change of *Fos* is related to the loss and regain of tendon phenotype during in vivo tendon development, regular in vitro cell culture, and in cell-sheet and 3D cell culture model. Overexpression of *Fos* in TSPCs induced upregulated gene expression of tendon markers. In the case of *Fos* knock-down, the opposite results were seen. Cell-sheet derived from *Fos* overexpressed TSPCs promoted the healing of injured

tendon. This study paves the way for the stepwise differentiation from MSCs/TSPCs to mature tenocytes, which is beneficial for tendon regeneration.

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AUTHOR CONTRIBUTIONS

J.C. and E.Z.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; W.Z.: collection and assembly of data, data analysis and interpretation, manuscript writing; Z.L., P.L., T.Z., Z.Y., and H.L.: collection data; X.C.: conception and design, data analysis and interpretation, provision of study material, final approval of manuscript; L.J.B.: manuscript writing; H.O.: conception and design, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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