



Contents lists available at ScienceDirect

Journal of Orthopaedic Translation

journal homepage: www.journals.elsevier.com/journal-of-orthopaedic-translation

Original Article

Fibrochondrogenic differentiation potential of tendon-derived stem/progenitor cells from human patellar tendon



Shengnan Qin^a, Wen Wang^{a,*}, Zhihe Liu^a, Xing Hua^b, SaiChuen Fu^c, Fei Dong^a, Aiguo Li^a, Zhen Liu^b, Pengzhen Wang^a, Libing Dai^a, Peihong Liang^a, Jinli Zhang^a, Wenjuan Cao^a, Xifeng Xiong^a, Honghui Chen^a, Jiake Xu^{d,**}

^a Guangzhou Institute of Traumatic Surgery, Department of Orthopaedics, Guangzhou Red Cross Hospital, Medical College, Jinan University, Guangzhou, China

^b Department of Pathology, Guangzhou Red Cross Hospital, Medical College, Jinan University, Guangzhou, China

^c Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong

^d School of Biomedical Sciences, The University of Western Australia, Perth, Australia

ARTICLE INFO

Keywords:

Bone–tendon junction

Fibrochondrogenic differentiation

Tendon-derived stem/progenitor cells

ABSTRACT

Background: Bone–tendon junction (BTJ) is a unique structure connecting tendon and bone through a fibrocartilage zone. Owing to its unique structure, the regeneration of BTJ remains a challenge. Here, we study the fibrochondrogenic differentiation of human tendon-derived stem/progenitor cells (TSPCs) both *in vitro* and *in vivo*. **Methods:** TSPCs were isolated from human patellar tendon tissues and investigated for their multidifferentiation potential. TSPCs were cultured in chondrogenic medium with transforming growth factor beta 3 (TGF-β3) and BMP-2 *in vitro* and examined for the expression of fibrochondrogenic marker genes by quantitative real-time reverse transcription polymerase chain reaction, enzyme-linked immunosorbent assay, and immunofluorescence. TSPCs pretreated were also seeded in collagen II sponge and then transplanted in immunocompromised nude mice to examine if the fibrochondrogenic characteristics were conserved *in vivo*.

Results: We found that TSPCs were differentiated towards fibrochondrogenic lineage, accompanied by the expression of collagen I, collagen II, SRY-box transcription factor 9 (Sox 9), and tenascin C. Furthermore, after TSPCs were seeded in collagen II sponge and transplanted in immunocompromised nude mice, they expressed fibrochondrogenic genes, including proteoglycan, collagen I, and collagen II.

Conclusion: Taken together, this study showed that TSPCs are capable of differentiating towards fibrocartilage-like cells, and the fibrochondrogenic characteristics were conserved even *in vivo*, and thus might have the potential application for fibrocartilage regeneration in BTJ repair.

The translational potential of this article: TSPCs are able to differentiate into fibrocartilage-like cells and thus might well be one potential cell source for fibrocartilage regeneration in a damaged BTJ repair.

Introduction

Bone–tendon junction (BTJ) is a unique structure linking tendon and bone. It consists of three district tissues: bone, tendon, and a special tissue, known as fibrocartilage that plays a major role in minimizing stress and strain from bone to tendon [1,2]. BTJ injuries are common in sports, such as tears or ruptures of anterior cruciate ligament (ACL) or rotator cuff and injuries of patellar–patellar tendon, but the healing or reconstruction of BTJ is very difficult to achieve clinically because of the

formation of fibrous scar tissue after BTJ injuries [3,4]. For instance, it was reported that the failure rates range 20%–94% in rotator cuff repair [5,6] and 10%–25% in ACL reconstruction [7].

The mechanism of fibrocartilage regeneration is not well known. Like chondrocytes, the fibrocartilage-like cells are generally round or oval in shape, with typical features of chondrocytes such as the expression of collagen II/X. In addition, they also exhibit certain fibroblastic phenotypes, such as the expression of collagen I [1], which was also evident in TSPCs but not in bone mesenchymal stem cells (BMSCs) [8]. Perez Castro

* Corresponding author. Department of pathology, Guangzhou Red Cross Hospital, Medical College, Jinan University, NO.396, Rd. Tongfu, Guangzhou, 510220, China.

** Corresponding author. School of Biomedical Sciences, The University of Western Australia, 35 Stirling Highway Perth WA 6009, Australia.

E-mail addresses: wangwarren88@hotmail.com (W. Wang), jiake.xu@uwa.edu.au (J. Xu).

* Co-first author.

<https://doi.org/10.1016/j.jot.2019.08.006>

Received 19 June 2019; Received in revised form 16 August 2019; Accepted 18 August 2019

Available online 1 November 2019

2214-031X/© 2019 Published by Elsevier (Singapore) Pte Ltd on behalf of Chinese Speaking Orthopaedic Society. This is an open access article under the CC BY-NC-

ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

et al. [9] found that at least 25% of the cells in fibrocartilage express collagen I and II. The difference between fibrocartilage and cartilage cells are likely attributed to the differential expression of collagen subtypes [10].

The role of BMSCs in fibrocartilage formation at BTJ has been reported [11]. BMSCs was able to differentiate to fibrocartilage-like cells *in vivo* and *in vitro* [12,13]. Interestingly, it is also evident that bone–tendon junction healing was facilitated by TSPCs transplanted in nude mice [14]. Consistently, fibrocartilage-like tissue was detected in TSPCs-transplanted nude rats [15]. Lui et al. [16] also reported that tendon stem cells sheet could enhance bone–tendon junction healing with more Sharpey's fibres, which have characteristics of fibrocartilage at BTJ.

It has been documented that TSPCs contain a population of mesenchymal progenitors that have multidifferentiation potential [14,15,17]. TSPCs derived from rat, mouse, rabbit, and human tendon tissue could differentiate towards osteoblasts, adipocytes, and chondrocytes *in vitro* [14,15,17]. TSPCs have some unique features which are different from BMSCs, including the expression of fibroblastic marker collagen I [8] and tenogenic markers, tenascin C, and tenomodulin [14]. The effect of TSPCs on tendon repair were previously documented [18,19]; however, their potential role in fibrochondrogenic differentiation has not been well studied.

In this study, we examined the potential of TSPCs to differentiate to fibrocartilage-like cells under differentiating conditions both *in vitro* and *in vivo*. Our data indicate that TSPCs are able to differentiate to fibrochondrocyte-like cells, and the fibrochondrogenic characteristics were conserved even *in vivo* and therefore might have potential application for fibrocartilage regeneration in the repair of BTJ.

Materials and methods

Isolation of tendon-derived stem/progenitor cells (TSPCs) from patellar tendon

We obtained TSPCs from human patellar tendon samples of four patients ($n = 4$) who underwent ACL reconstruction using bone–patellar tendon–bone autografts with patients' consent. The age range of patients was from 22 to 32 years. TSPCs were isolated from the patellar tendon tissues [17]. First, 0.25% of trypsin was used to predigest the tendon for 15 min, and these tissues were cut into small pieces. Second, 3 mg/ml of collagenase I (Sigma-Aldrich, St. Louis, MO) in plain low glucose Dulbecco's modified Eagle's medium (LG-DMEM) (Gibco, Invitrogen, Carlsbad, CA) was used to digest these small pieces for at least 2 h at 37 °C, and then this digestion solution was passed through a cell strainer (70 μm) (Becton Dickinson, Franklin Lakes, NJ) to obtain a uniform single-cell suspension. After centrifugation and washing, the cells were resuspended in LG-DMEM supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). The cells were plated at three different cell density (50, 100, and 200 cells/ cm^2) and cultured in LG-DMEM containing with 10% FBS at 5% CO_2 , 37 °C for 12 days. Cell colonies formed from the isolated tendon cells were either subcultured for next experiments or stained with 0.5% crystal violet (Sigma-Aldrich) after being fixed with 70% ethanol. The number of colonies formed were counted. All the next experiments were performed with Passage 3–5 of human TSPCs.

Fluorescence-activated cell sorting (FACS) analysis of human TSPCs

10^5 TSPCs at Passage 3 were harvested to detect markers of stem cells, including cell surface markers (CD29 and CD105), monocytic and neutrophil markers (CD14), mesenchymal stem cell marker (CD44), leucocyte marker (CD45), and fibroblastic marker (CD90) using the flow cytometry analyses. TSPCs were incubated in $1 \times$ phosphate-buffered saline (PBS) with antibodies aforementioned so that cells could be immunolabeled with 1 μg of phycoerythrin (PE)- or fluorescein

isothiocyanate (FITC)-conjugated mouse antihuman monoclonal antibodies for 1 h at 4 °C, the proportion of positive cells can be analysed by an Epics-XL-MCL flow cytometer (Beckman Coulter). The results we obtained were calculated using the FACS and can be programmed (Becton Dickinson (BD) Biosciences).

Multidifferentiation of human TSPCs

The differentiation potential of human TSPCs towards osteocytes and adipocytes was made *in vitro* as reported previously [17]. TSPCs (Passage 5) were plated in 12-well plate and used for multidifferentiation experiments when reaching confluence. For osteogenic differentiation, medium was changed to osteogenic medium, and cells continued to be cultured for a further 14 days. Osteogenic induction medium was LG-DMEM containing 10% FBS and 1% penicillin-streptomycin-neomycin (PSN) as well as 1 nM dexamethasone, 20 mM β -glycerolphosphate, and 50 mM ascorbic acid. After 14 days, the cells were fixed and stained with crystal violet followed by staining with 0.5% (w/v) alizarin red S (pH 4.1, Sigma-Aldrich) for 30 min. For adipogenic differentiation, cells were cultured in adipogenic medium containing 10% FBS, 500 nM dexamethasone, 50 μM indomethacin, 0.5 mM isobutylmethylxanthine and 10 $\mu\text{g}/\text{ml}$ insulin (Sigma-Aldrich) or continued to be cultured in complete medium for another 14 days. The adipogenesis was measured by staining with 0.3% fresh oil red O (Sigma-Aldrich) so that red lipid droplets of adipocytes after staining can be seen. The cell plates, both osteogenic and adipogenic induction, were scanned and imaged by microscope.

Human TSPCs differentiation towards fibrocartilage cells

TSPCs at Passage 5 were plated at 1×10^4 cells/ cm^2 and cultured in complete medium until the cells reached confluence. Complete medium supplemented with 10 ng/ml of TGF- β 3 and 50 ng/ml of bone morphogenetic protein-2 (BMP-2) (R&D systems), 10^{-7} M dexamethasone, 40 $\mu\text{g}/\text{ml}$ proline, 100 $\mu\text{g}/\text{ml}$ pyruvate, 50 μg ascorbate-2-phosphate (all from Sigma-Aldrich), and 1:100 diluted insulin, transferrin, selenium (ITS) Universal Cell Culture Supplement Premix (Becton Dickinson, Franklin Lakes, NJ) was used as differentiation medium. When TSPCs started to be induced by differentiation medium, cells were photographed at Day 4, 7, and 14 to follow changes of morphology. The supernatant of TSPCs was collected at Day 4, 7, and 14 to measure the concentration of collagen I and II by enzyme-linked immunosorbent assay (ELISA) kits (R&D systems). Cells were also harvested at Day 0, 7, and 14 for detecting gene expression by quantitative real-time reverse transcription polymerase chain reaction (q-PCR) or fixed at Day 21 for immunofluorescence staining.

Heterotopic fibrochondrogenesis of TSPCs *in vivo*

To examine the fibrochondrogenesis differentiation effect of human TSPCs *in vivo*, we chose collagen II sponge prepared in our earlier study [20] as cell carriers, assuring that cells can be loaded and not leaked out under the dorsal surface of nude mice. TSPCs were labelled with PKH 67 (Sigma-Aldrich) to observe cell viability in collagen II sponge. Approximately 10^6 TSPCs were seeded in collagen II sponge and cultured in induction medium with TGF- β 3 and BMP-2 or complete medium for 14 days before being transplanted into the dorsal surface of 8- to 10-week-old female immunocompromised nude mice subcutaneously [8]. Collagen II sponge without cells was also transplanted as a control. Transplants were harvested after 4 weeks and then fixed by neural formalin, decalcified, and embedded with wax. The sections would be stained with Safranin O/Fast Green or immunohistochemistry staining (IHC).

Quantitative real-time reverse transcription PCR

q-PCR was performed in accordance with the manufacturer's protocols. In brief, TSPCs with or without treatment were harvested for total RNA extraction using a kind of RNA extraction mini kit (Invitrogen, Carlsbad, CA). Three hundred nanograms of mRNA was reverse transcribed to complementary DNA (cDNA) using first stand cDNA synthesis kit (Invitrogen). The mRNA expression levels were measured by real-time PCR by the SYBR green master mix and primers used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), alpha 1 type I collagen (Col1 α 1), Sox 9, alpha1 type II collagen (Col2 α 1), and tenascin C are shown in Table 1. PCR conditions were as follows: 95 °C for 10min and then 40 cycles of 95 °C for 10s, 60 °C for 30s, and 72 °C for 30s. The mRNA level of target genes was normalized to that of GAPDH gene. Relative mRNA expression was calculated with the $2^{-\Delta\Delta CT}$. At least three independent experiments were performed to calculate the standard deviation of $2^{-\Delta\Delta CT}$.

Immunofluorescence staining

Immunofluorescence staining was performed as reported previously [21]. First, 70% ethanol was used to fix TSPCs for 10 min at room temperature and then the fixed cells were incubated by $1 \times$ PBS with 0.2% Triton-X100. Second, the cells were incubated in blocking solution with 1% bovine serum albumin and 5% goat serum, to block the unspecific binding of antibodies, and then incubated with primary antibody, also diluted in 5% goat serum/1% bovine serum albumin (BSA)-PBS, for overnight at 4 °C. The primary antibodies used were rabbit polyclonal anticollagen I (Abcam, 1:200), mouse monoclonal anticollagen II (Abcam, 1:200), rabbit polyclonal anti-Sox 9 (Santa Cruz, 1:50) and mouse monoclonal antitenascin C (Abcam, 1:200). The blocking solution without any antibody was chosen as a negative control. Third, the cells were incubated in corresponding secondary antibodies that were also diluted in 5% goat serum/1% BSA-PBS in the dark for at least 1 h at room temperature. The secondary antibodies used were Alexa Fluor® 594 goat antirabbit (1:500) (red), Alexa Fluor@ 488 goat antimouse (green), 2 μ g/ml (Invitrogen) (1:500). Finally, after several washes with $1 \times$ PBS, the cell nuclei were counterstained with DAPI (5 μ g/ml) (Invitrogen) for 1 min. The fluorescence was imaged using confocal microscopy (Leica Microsystems, Germany).

Immunohistochemistry staining

Transplants harvested from nude mice were fixed by 4% neutral buffered formalin for 48 h and embedded with paraffin wax. We prepared at least 10 sections in 5 μ m thickness using 1130/Biocut microtome (Reichert-Jung GmbH, Nussloch, Germany). These sections were stained with Safranin O/Fast Green [20]. The expression of collagen I and II was detected by immunohistochemistry staining. The primary antibodies used were as same as those used in immunofluorescence staining, whereas the secondary antibodies used were horseradish peroxidase (HRP) goat-anti-rabbit (1:1000) or HRP goat-anti-mouse (1:1000) (all

Table 1
Human primer sequences.

Human primers	Sequences
Col1 α 1	Forward 5'-CAGCCGCTTCACCTACAGC-3'
	Reverse 5'-TTTGTATTCAATCACTGTCTTGCC-3'
Col2 α 1	Forward 5'-GGCAATAGCAGGTTACAGTACA-3'
	Reverse 5'-CGATAACAGTCTTGCCCCACTT-3'
Tenascin C	Forward 5'-TCTCTGCACATAGTAAAAACAATACC-3'
	Reverse 5'-TCAAGGCAGTGGTGTCTGTGA-3'
Sox 9	Forward 5'-TACGACTGGACGCTGGTGCC-3'
	Reverse 5'-CCGTTCTCACCAGACTTCTCC-3'
GAPDH	Forward 5'-CGTAAAGACCTCTATGCCAACA-3'
	Reverse 5'-CGGACTCATCGTACTCTGCT-3'

from Santa Cruz company). Sections that were only incubated with secondary antibodies were used as negative controls. Subsequently, the immunoreactivity was detected by the mouse and rabbit-specific 3, 3'-diaminobenzidine (DAB) detection IHC kit (Abcam).

Data analysis

All data were presented as mean \pm standard deviation, and all reported representative data were from at least three independent experiments. All statistical analyses were performed with student's t-test. $P < 0.05$ was considered as statistically significant.

Results

Characterization and multidifferentiation potential of TSPCs isolated from human patellar tendon

To examine the colony-forming potential of TSPCs, human tendon cells were isolated and cultured at different cell densities and the colonies formed were observed by crystal violet staining (Figure 1A). It appears that isolated TSPCs were able to form colonies at different cell density with an increasing trend of colony formation in a higher cell density. In addition, the morphology of TSPCs after being subcultured was observed to have the difference between cells at Passage 0 and those at Passage 1. Star-shaped cells were seen at Passage 0, whereas fibroblast-like cells were observed at Passage 1 (Figure 1B).

Flow cytometric analysis was employed to examine if TSPCs express mesenchymal stem cell surface markers, CD29 and CD44. It was revealed that approximately 99.67% and 99.87% cells were positive for CD29 and CD44, respectively. They were mostly negative for CD45 (0.32%), a leucocyte marker, and only a small proportion of TSPCs was positive CD14 (12.12%), known as a monocytic and neutrophil marker (Figure 1C). In addition, CD 90 negative in BMSCs, was highly expressed in 99.11%.of cells. Interestingly, approximately, 84.75% of cells expressed CD105, a TGF-beta receptor accessory molecule (Figure 1C).

Isolated tendon cells were cultured in different condition media to examine their differentiation potential towards osteocytes and adipocytes. Red calcium nodules stained by alizarin red S were observed after 14-day osteogenic induction, which were not observed in the control group (Figure 1D). In addition, lipid droplets formation was detected in adipogenic induction medium after being induced for 14 days, which were not found in the control group (Figure 1E). These results indicated that TSPCs are stem cell-like cells with multidifferentiating potential.

Induction of fibrochondrogenic-like cells of TSPCs with TGF- β 3 and BMP-2 in vitro

Then, we examined the differentiation potential of TSPCs to become fibrochondrocyte-like cells. As shown in Figure 2A, after being cultured in an induction medium with TGF- β 3 and BMP-2 for 4 days, some TSPCs exhibited round shape, but those without induction showed fibroblastic cell morphology. At Day 7 after induction, some TSPCs in TGF- β 3 and BMP-2 treated group formed round or irregularly shaped with flat colonies or aggregates, and at Day 14, these colonies/aggregates became more spherical shaped. By comparison, TSPCs cultured in noninduced medium displayed fibroblastic morphology at all time points (Figure 2A).

To further determine the characteristics of TSPCs after induction, the level of collagen I and II protein secreted by TSPCs was examined by ELISA (Figure 2B). Our results showed that the expression levels of both collagen I and II in supernatant were higher in TGF- β 3 and BMP-2 group than that in noninduced group (Figure 2B). In addition, collagen I and II secretion of TSPCs increased with time in TGF- β 3 and BMP-2 group from Day 4 to Day 14, whereas no significant difference can be seen in noninduced group over the 14-day induction period (Figure 2B).

Later, we examined the mRNA expression of fibrocartilage-like cell marker genes. Total RNAs were extracted from TSPCs treated with TGF-

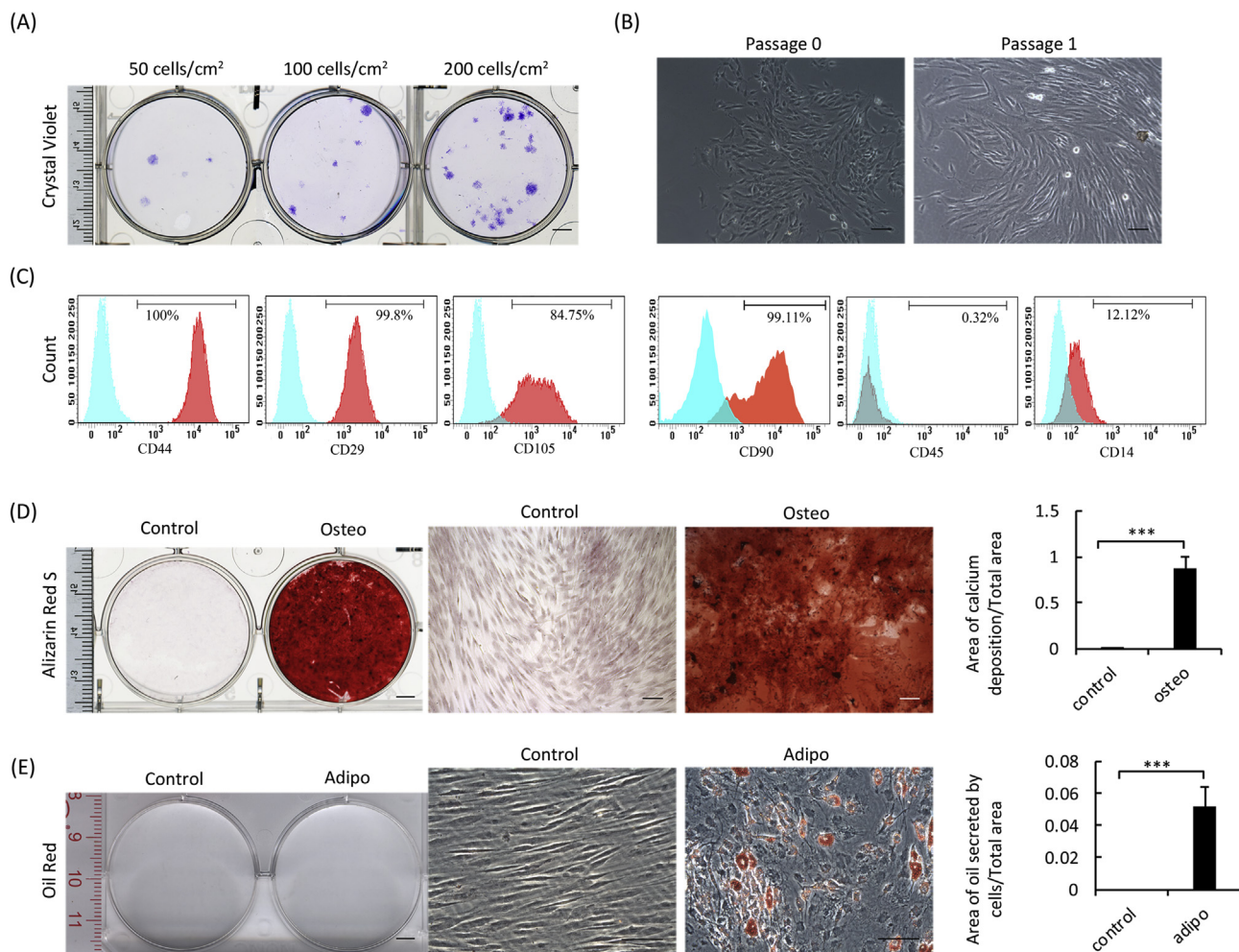


Figure 1. Isolation and identification of human tendon-derived stem/progenitor cells (TSPCs). (A) The colony-forming ability of TSPCs at different cell density, scale bar 0.5 cm (B) Morphology of tendon cells, star shaped cells were observed at Passage 0 (left) although fibroblast-like cells were observed at Passage 1 (right), scale bar 100 μm (C) Flow cytometry analysis of the expression of cell surface markers of TSPCs at Passage 3, including MSCs markers (CD29, CD44 and CD105), monocytic and neutrophil marker (CD14), leucocyte marker (CD45) and fibroblastic marker (CD90) (D) Osteogenic potential of TSPCs at Passage 5 *in vitro* as visualized by calcium nodules (red) with alizarin red staining, scale bar 0.5 cm (left), scale bar 100 μm (middle); and quantitative measurement of the area of calcium nodules (E) Adipogenic potential of TSPCs at Passage 5 *in vitro* as detected by oil drops (red) with oil red staining, scale bar 0.5 cm (left), scale bar 100 μm (middle); and quantitative measurement of the area of oil drops. ****p* < 0.001.

β3 and BMP-2 at Day 0, 7, and 14 and subjected to q-PCR analyses. It was revealed that the relative expressions of fibroblastic gene (Col1α1), chondrocytes-related genes (Col2α1 and Sox 9) and bone-tendon junction marker gene (tenascin C) were all upregulated in TSPCs treated with TGF-β3 and BMP-2 at different time point (Figure 2C). Notably, fibroblastic marker, Col1α1, increased sharply at Day 7 when compared with Day 0 but had no significant increase between Day 7 and Day 14. Chondrocytes-related genes (Col2α1 and Sox 9) slightly increased at Day 7, followed by a sharp rise at Day 14 in comparison to Day 0. Tenascin C gene was slightly upregulated at Day 7 and then strongly upregulated at Day 14 compared with Day 0. The expression pattern of the fibroblastic gene and chondrogenic marker genes suggested that TSPCs can undergo fibrogenesis at an early time point (Day 7) and subsequently chondrogenesis at a late time point (Day 14), in the presence of TGF-β3 and BMP-2.

The expressions of collagen I, collagen II, Sox 9, and tenascin C were further confirmed using immunofluorescence staining. As shown in Figure 3A–B, collagen I was detected in all groups, and further induced in the presence of TGF-β3 and BMP-2. Collagen II and Sox 9 were detected only in TGF-β3 and BMP-2 treated group, but not be found in the non-induced group. Tenascin C expressions were higher in TGF-β3 and BMP-2

treated group, when compared with the noninduced group. Collectively, these data suggested human TSPCs expressed fibrochondrogenic markers in the presence of TGF-β3 and BMP-2, indicating that TSPCs has been differentiated into fibrocartilage-like cells *in vitro*.

Induction of fibrochondrogenic-like cells of TSPCs *in vivo*

Finally, to examine the fibrochondrogenic potential of TSPCs *in vivo*, TSPCs were cultured in collagen II sponge. It is noted that collagen II sponge has appropriate pore structure suitable for cell growth, such as appropriate pore size (50–130 μm) with good porosity, as observed by bright field microscope (Figure 4A). The viability of TSPCs cultured in collagen II sponge for 14 days was evident by PKH67 dye, which was used to label TSPCs (Figure 4B). To determine the fibrochondrogenic potential of TSPCs *in vivo*, TSPCs were seeded in collagen II sponge and cultured in induction medium with TGF-β3 and BMP-2 or in complete medium for 2 weeks, which were then transplanted into the immunocompromised nude mice. Collagen II sponge alone without TSPCs was used as a control. After 4 weeks, proteoglycan (red), which was surrounded by undegraded collagen II (blue), was found to be higher in the TGF-β3- and BMP-2-treated TSPC group than in the noninduced TSPC

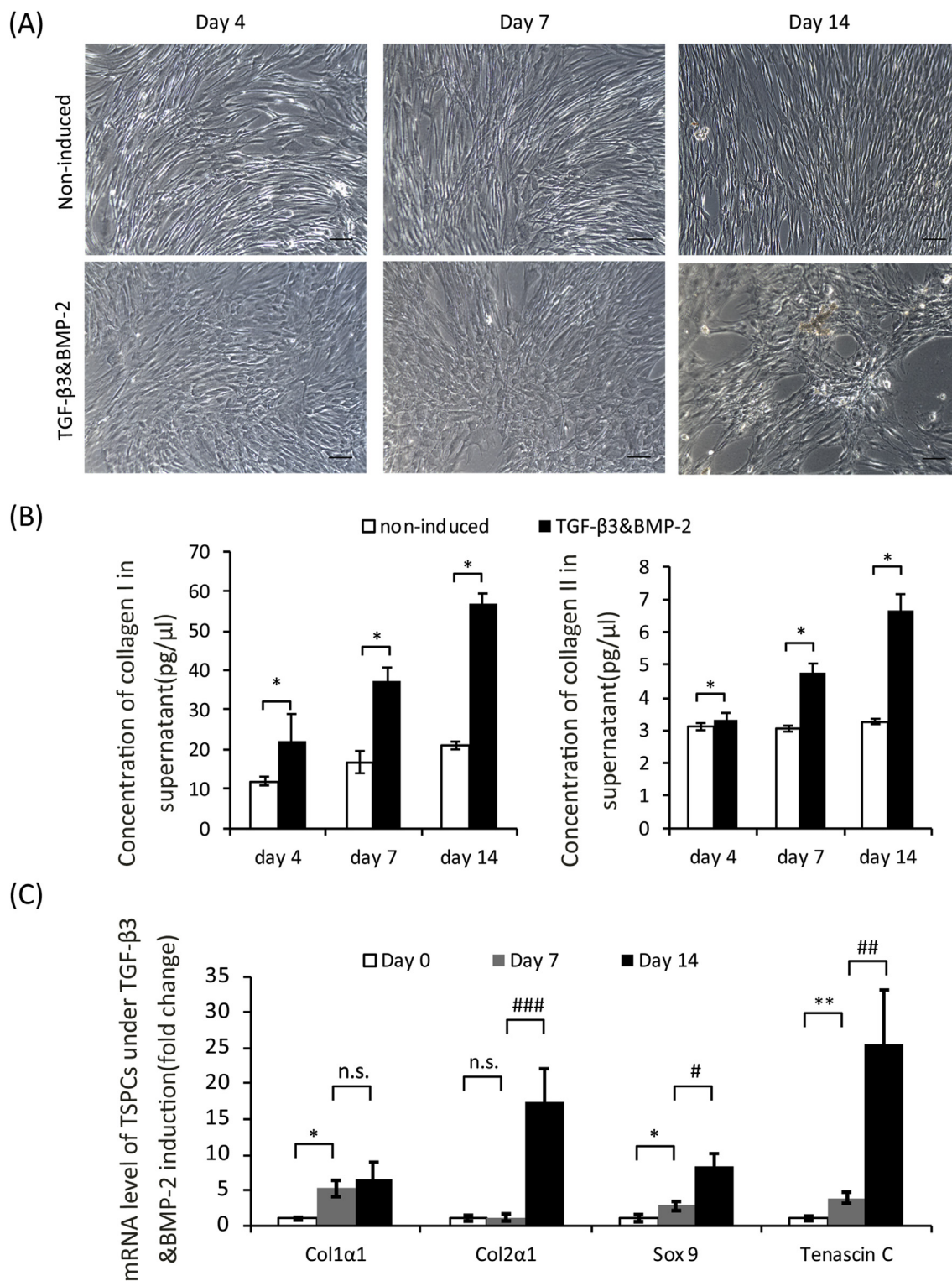


Figure 2. Fibrochondrogenesis of human TSPCs at different time points. (A) Cell morphology of TSPCs treated with or without TGF-β3 and BMP-2 was observed by microscopy at different time points, scale bar 100 μm (B) Expression levels of collagen I and II in the culture supernatant of TSPCs induced by TGF-β3 and BMP-2 at different time points were detected by ELISA kits, and culture supernatant of cells without TGF-β3 and BMP-2 was used as control group, * $p < 0.05$ (C) mRNA levels of fibrocartilage-like cells, including fibroblastic marker (Col1α1), chondrogenic markers (Col2α1 and Sox 9), and bone-tendon junction marker (tenascin C) were examined by qRT-PCR at different time points, and cells cultured in complete medium before induction were referred as Day 0, * $p < 0.05$. ** $p < 0.01$.

group, suggesting TSPCs have been induced towards chondrocytes lineage (Figure 4C). No cells were found in collagen II sponge group, which indicated that proteoglycan was produced from TSPCs seeded in the collagen II sponge. In addition, positive area of proteoglycan in the TGF-β3 and BMP-2 pretreated TSPCs group was greater than that in

noninduced TSPCs group. To confirm if these cells exhibit characteristics of fibrocartilage-like cells, the expressions of collagen I and II by IHC staining was performed (Figure 4D). There was significantly more collagen I than collagen II in the TGF-β3 and BMP-2 pretreated TSPCs group (Figure 4D).

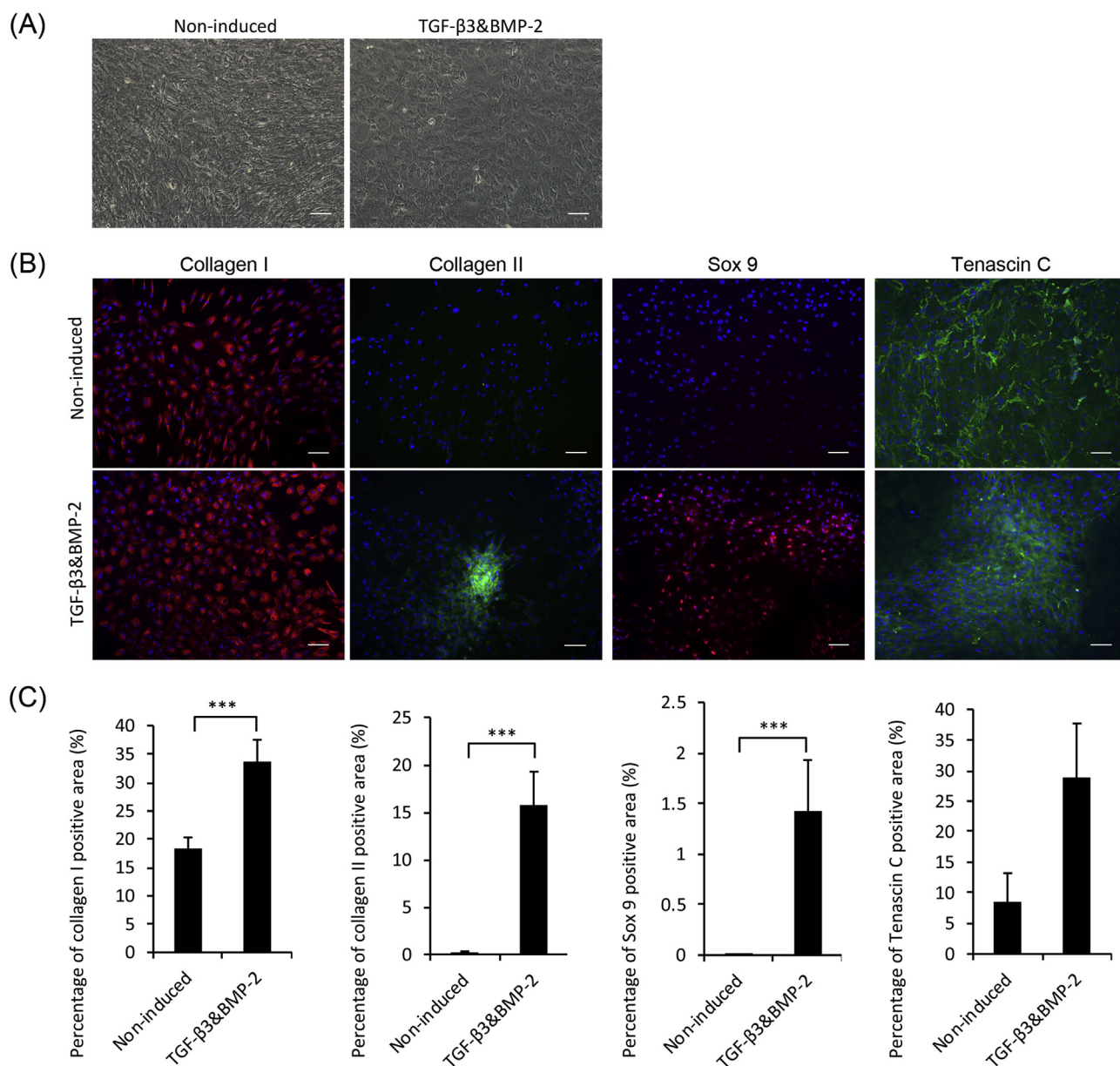


Figure 3. The protein expression of fibrocartilage-like cells by using immunofluorescence staining. (A) Morphology of TSPCs was photographed by phase-contrast microscope, scale bar 100 μ m (B) The protein expression of fibrocartilage-like cells was detected by immunofluorescence staining after TSPCs were cultured in chondrogenic medium with TGF- β 3 and BMP-2 for 21 days. TSPCs cultured in completed medium without induction for 21 days were used as a control. Fibroblastic marker (collagen I, red), chondrogenic markers (collagen II, green and Sox 9, red), and bone-tendon junction marker (tenascin C, green) were shown, and all nuclear area were stained by DAPI (blue), scale bar 100 μ m (C) Quantitative measurement of positive areas was analysed by Image ProPlus software, *** $p < 0.001$.

Discussion/conclusion

Previous studies have reported the chondrogenic differentiation from different sources of stem cells [8,17,22]. Little, however, was evident that tendon stem cells could be differentiated into fibrochondrocyte-like cells. In particular, owing to the unique location of fibrocartilage tissue at bone-tendon junction and the limitation of the small number of tendon stem cells, it remains elusive that tendon stem cells could be readily differentiated into fibrocartilage-like cells. In this study, we have successfully isolated and identified stem cells/progenitors from human patellar tendon. We showed that TSPCs could be induced toward fibroblastic and chondrogenic-like cells in the presence of TGF- β 3 and BMP-2. We provided evidence that TSPCs are capable of differentiating into fibrochondrocyte-like cells *in vitro* and *in vivo*.

The successful isolation of TSPCs is important because it provides a new cell resource to study its potential in fibrocartilage regeneration of

bone-tendon junctions. The coexpression of the fibroblast markers, such as collagen I and the chondrocytes markers, such as collagen II, have been used to identify fibrocartilage cells in bone-tendon junction [23]. Nevertheless, it is still difficult to define these cells, partly because a combination of different cell types are present in fibrocartilage at bone-tendon junction [1,24,25], and there is no specific markers that can be used to distinguish them.

There are many factors which would influence the chondrogenic differentiation of stem cells, such as cell density, growth factors, and culture conditions [26,27]. Rui et al. [29] found that TGF- β 3 could induce more proteoglycan and collagen II than TGF- β 1, and BMP-2 could enhance the chondrogenic effect of TGF- β 3 on synovium-derived stem cells *in vitro* [28]. BMP alone has been shown to inhibit tendon formation during development, whereas TGF- β alone would inhibit mRNA expression of Col1 α 1 in chondrogenic induction of BMSCs [30–32]. Our results showed that TSPCs express fibroblastic marker (collagen I) and

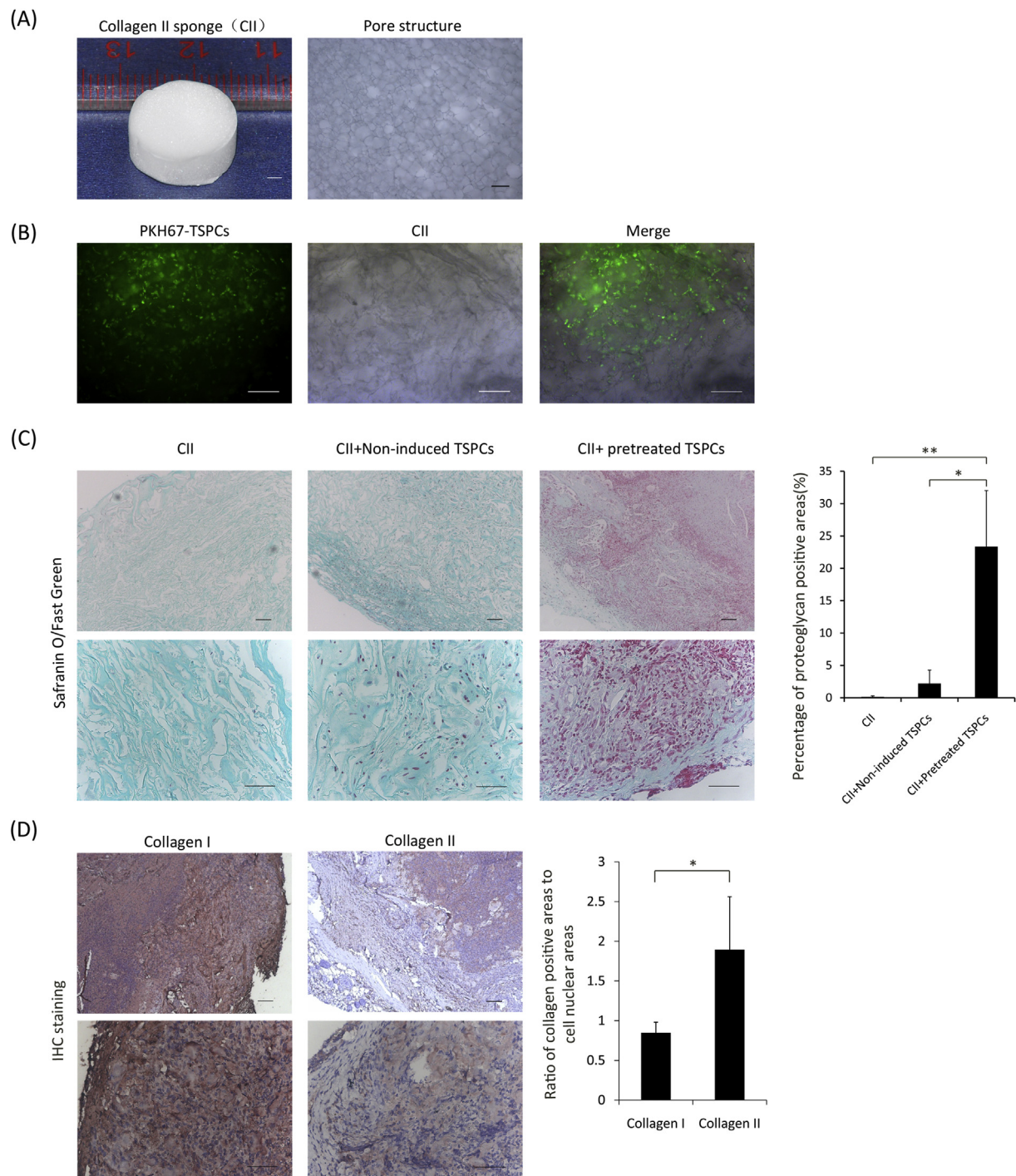


Figure 4. Induction of fibrochondrogenic-like cells of TSPCs *in vivo*. (A) Morphology of Collagen II sponge (left), scale bar 2 cm. Pore structure of collagen II sponge was photographed by bright field microscope (right), scale bar 100 μ m (B) PKH67 labelled TSPCs were viable in the collagen II sponge after being cultured for 14 days. TSPCs were labelled by PKH67 (green) (left), and collagen II sponge was imaged by phase-contrast microscope (middle). The merged image of TSPCs and collagen II sponge (right) was shown, scale bar 100 μ m (C) Proteoglycan-rich extracellular matrices were examined by Safranin O/Fast Green staining in all groups after TSPCs transplanted into nude mice for 4 weeks. Proteoglycan (red) was surrounded by collagen II sponge (blue), scale bar 100 μ m (top). Higher magnification of images showed that the expression of proteoglycan was mainly around cells, scale bar 50 μ m (bottom); and proteoglycan positive area was analysed by Image ProPlus software, * $p < 0.05$, ### $p < 0.001$ (D) IHC staining of TSPCs in collagen II sponge showed strong expression of collagen I and moderate expression of collagen II, scale bar 100 μ m (top) and 50 μ m (bottom). Collagen positive area was normalized to total cell nuclear area by Image ProPlus software to quantify the collagen expression levels, * $p < 0.05$.

chondrogenic markers (Sox 9 and collagen II) when cultured in the presence of TGF- β 3 and BMP-2 (Figure 2), indicating that TSPCs were induced to fibrocartilage cells which are able to express two kinds of markers simultaneously or at least a mixture of fibroblasts and chondrocytes. This is in line with a previous study showing that fibrocartilage

cells were likely to contain a mixture of fibroblasts and chondrocytes that express both collagen I and II [9]. However, whether or not these cells contain the tenogenic phenotype remained to be further investigated.

The repair of bone-tendon junctions after injury is a slow process. Growth factors or mechanical stimuli are often used to stimulate the

regeneration of fibrocartilage zone [23,33–35]. In this study, we found that abundant proteoglycan was detected in TSPCs seeded in collagen II sponge after being transplanted into mice, indicating that the presence of fibrocartilage-like cells. These cells also expressed collagen I and certain levels of collagen II. Taken together, in this study, TSPCs were isolated and are able to be differentiated into fibrocartilage-like cells, which might serve as a potential cell source for the treatment of TSPC-based bone–tendon junction repair.

Data availability statement

The data used to support the findings of this study are included within the article.

Ethical approval

This study was approved by the Chinese University of Hong Kong Territories East Cluster Clinical Research Ethics Committee (Approval NO.CRE-2010.248), and by Guangzhou Red Cross Hospital Ethics Committee (Approval NO.2014-107-01).

Funding

This work was supported by receiving grants from National Natural Science Foundation of China (No. 30973067, No.81228013), Science and Technology Project of Guangzhou City, Key Project (No.201704020145), General guidance project of Guangzhou (No.20151A010031, No.20181A010020), and Medical Research Foundation of Guangdong Province (No.A2015559).

Author contributions

W.W and SN.Q contributed equal to this study. SN.Q, JK.X and ZH.L draft the manuscript. SN.Q, W.W, X.H, Z.L, F.D, AG.L, PZ.W, LB.D, PH.L, JL.Z, WJ.C and XF.X performed the experiments. SN.Q analyzed the data. SC.F contributed the discussion part. HH.C, AG.L and SC.F contributed experiment instruments and analysis tools. W.W, SN.Q and HH.C conceived and designed the experiments. W.W, JK.X and HH.C co-supervised the study.

Conflict of interest

The authors have no conflicts of interest to disclose in relation to this article.

Acknowledgements

We thank Dr. Yunfeng Rui, Dr. Ting Zhang, Dr.Qi Tan and Dr. Lian-guang Xu for technical assistance on the project, Ms. YauChuk Cheuk for isolation of tendon stem/progenitor cells.

References

- Benjamin M, Ralphs JR. Biology of fibrocartilage cells. *Int Rev Cytol* 2004;233:1–45 [eng].
- Lui P, Zhang P, Chan K, Qin L. Biology and augmentation of tendon-bone insertion repair. *J Orthop Surg Res* 2010;5:59 [eng].
- Thomopoulos S, Williams GR, Soslosky LJ. Tendon to bone healing: differences in biomechanical, structural, and compositional properties due to a range of activity levels. *J Biomech Eng* 2003;125(1):106–13 [eng].
- Wong MWN, Qin L, Tai JKO, Lee SKM, Leung KS, Chan KM. Engineered allogeneic chondrocyte pellet for reconstruction of fibrocartilage zone at bone-tendon junction? A preliminary histological observation. *J Biomed Mater Res* 2004;70B(2):362–7.
- Harryman 2nd DT, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen 3rd FA. Repairs of the rotator cuff. Correlation of functional results with integrity of the cuff. *J Bone Jt Surg Am* 1991;73(7):982–9 [eng].
- Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K. The outcome and repair integrity of completely arthroscopically repaired large and massive rotator cuff tears. *J Bone Jt Surg Am* 2004;86-A(2):219–24 [eng].
- Vergis A, Gillquist J. Graft failure in intra-articular anterior cruciate ligament reconstructions: a review of the literature. *Arthroscopy* 1995;11(3):312–21 [eng].
- Bi Y, Ehrlichou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007;13(10):1219–27.
- Perez-Castro AV, Vogel KG. In situ expression of collagen and proteoglycan genes during development of fibrocartilage in bovine deep flexor tendon. *J Orthop Res* 1999;17(1):139–48 [eng].
- Freemont AJ, Hoyland J. Lineage plasticity and cell biology of fibrocartilage and hyaline cartilage: its significance in cartilage repair and replacement. *Eur J Radiol* 2006;57(1):32–6 [eng].
- Connelly JT, Vanderploeg EJ, Mouw JK, Wilson CG, Levenston ME. Tensile loading modulates bone marrow stromal cell differentiation and the development of engineered fibrocartilage constructs. *Tissue Eng A* 2010;16(6):1913–23.
- Baker BM, Nathan AS, Gee AO, Mauck RL. The influence of an aligned nanofibrous topography on human mesenchymal stem cell fibrochondrogenesis. *Biomaterials* 2010;31(24):6190–200.
- Wang IE, Lu HH. Role of cell-cell interactions on the regeneration of soft tissue-to-bone interface. *Conf Proc IEEE Eng Med Biol Soc* 2006;1:783–6 [eng].
- Bi Y, Ehrlichou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007;13(10):1219–27.
- Zhang J, Wang J. Characterization of differential properties of rabbit tendon stem cells and tenocytes. *BMC Musculoskelet Disord* 2010;11(1):10.
- Lui PP, Wong OT, Lee YW. Application of tendon-derived stem cell sheet for the promotion of graft healing in anterior cruciate ligament reconstruction. *Am J Sports Med* 2014;42(3):681–9 [eng].
- Rui YF, Lui PP, Li G, Fu SC, Lee YW, Chan KM. Isolation and characterization of multipotent rat tendon-derived stem cells. *Tissue Eng A* 2010;16(5):1549–58.
- Adams Jr SB, Thorpe MA, Parks BG, Aghazarian G, Allen E, Schon LC. Stem cell-bearing suture improves Achilles tendon healing in a rat model. *Foot Ankle Int* 2014;35(3):293–9 [eng].
- Yang Z, Cao H, Gao S, Yang M, Lyu J, Tang K. Effect of tendon stem cells in chitosan/beta-glycerophosphate/collagen hydrogel on achilles tendon healing in a rat model. *Med Sci Monit* 2017;23:4633–43 [eng].
- Chen H, Yang X, Liao Y, Zeng X, Liang P, Kang N, et al. MRI and histologic analysis of collagen type II sponge on repairing the cartilage defects of rabbit knee joints. *J Biomed Mater Res B Appl Biomater* 2011;96(2):267–75 [eng].
- Zhang L, Tran N, Chen HQ, Wang X. Cyclic stretching promotes collagen synthesis and affects F-actin distribution in rat mesenchymal stem cells. *Bio Med Mater Eng* 2008;18(4–5):205–10 [eng].
- Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnol Bioeng* 2006;93(6):1152–63 [eng].
- Liu H, Yang L, Zhang E, Zhang R, Cai D, Zhu S, et al. Biomimetic tendon extracellular matrix composite gradient scaffold enhances ligament-to-bone junction reconstruction. *Acta Biomater* 2017;56:129–40 [eng].
- Thomopoulos S, Genin GM, Galatz LM. The development and morphogenesis of the tendon-to-bone insertion - what development can teach us about healing. *J Musculoskelet Neuronal Interact* 2010;10(1):35–45 [eng].
- Benjamin M, McGonagle D. Entheses: tendon and ligament attachment sites. *Scand J Med Sci Sport* 2009;19(4):520–7.
- Chen MJ, Whiteley JP, Please CP, Schwab A, Ehlicke F, Waters SL, et al. Inducing chondrogenesis in MSC/chondrocyte co-cultures using exogenous TGF-beta: a mathematical model. *J Theor Biol* 2018;439:1–13 [eng].
- Kim M, Erickson IE, Choudhury M, Pleshko N, Mauck RL. Transient exposure to TGF-beta3 improves the functional chondrogenesis of MSC-laden hyaluronic acid hydrogels. *J Mech Behav Biomed Mater* 2012;11:92–101 [eng].
- Rui YF, Du L, Wang Y, Lui PP, Tang TT, Chan KM, et al. Bone morphogenetic protein 2 promotes transforming growth factor beta3-induced chondrogenesis of human osteoarthritic synovium-derived stem cells. *Chin Med J (Engl)* 2010;123(21):3040–8 [eng].
- Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 2001;128(19):3855–66 [eng].
- Danisovic L, Varga I, Polak S. Growth factors and chondrogenic differentiation of mesenchymal stem cells. *Tissue Cell* 2012;44(2):69–73 [eng].
- Havlas V, Kos P, Jendelova P, Lesny P, Trc T, Sykova E. [Comparison of chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells with cultured chondrocytes and bone marrow mesenchymal stem cells]. *Acta Chir Orthop Traumatol Cech* 2011;78(2):138–44 [cze].
- Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. *Osteoarthr Cartil* 2007;15(6):597–604 [eng].
- Song F, Jiang D, Wang T, Wang Y, Chen F, Xu G, et al. Mechanical loading improves tendon-bone healing in a rabbit anterior cruciate ligament reconstruction model by promoting proliferation and matrix formation of mesenchymal stem cells and tendon cells. *Cell Physiol Biochem* 2017;41(3):875–89 [eng].
- Xu D, Zhang T, Qu J, Hu J, Lu H. Enhanced patella-patellar tendon healing using combined magnetic fields in a rabbit model. *Am J Sports Med* 2014;42(10):2495–501 [eng].
- Chen CH, Chang CH, Wang KC, Su CI, Liu HT, Yu CM, et al. Enhancement of rotator cuff tendon-bone healing with injectable periosteum progenitor cells-BMP-2 hydrogel in vivo. *Knee Surg Sport Traumatol Arthrosc* 2011;19(9):1597–607 [eng].