Clinical Trial/Experimental Study

Medicine®

OPEN

Enhancement of in vitro proliferation and bioactivity of human anterior cruciate ligament fibroblasts using an in situ tissue isolation method and basic fibroblast growth factor culture conditions

A pilot analysis

Ziming Liu, MD, PhD[a], Yuwan Li, MD, PhD[a], Youliang Ren, MD, PhD[b], Ying Jin, MD[c], Jibin Yang, MD[c], Shengmin Wang, MD[c], Xizhong Zhu, MD[c], Huazhang Xiong, MD, PhD[b], Gang Zou, MD[c], Yi Liu, MD, PhD[c], Wei Huang, MD, PhD[a,*]

Abstract

Background: Previous studies have reported poor proliferation and bioactivity of human anterior cruciate ligament fibroblasts (hACLFs) after injury. As hACLFs are one of the most significant and indispensable source of seed cells in constructing tissue-engineered ligament, enhancing hACLF proliferation would offer favorable cellular-biological ability and induce the extracellular matrix secretion of hACLFs after loading on multiple types of scaffolds. Enhancing the bioactivity of hACLFs would improve tissue repair and functional recovery after tissue-engineered ligament transplantation. This study compared cells prepared by collagenase digestion and the in situ culture of tissue pieces and investigated the effect of basic fibroblast growth factor (bFGF) on hACLFs.

Methods: Six adult patients participated in this study. Of these patients, tissues from three were compared after culture establishment through collagenase digestion or in situ tissue isolation. hACLF phenotypic characteristics were assessed, and the effect of bFGF on hACLF cultures was observed. hACLFs cultured with and without bFGF served as the experimental and control groups, respectively. Cell Counting Kit-8 was used to detect proliferation. The expression of ligament-related genes and proteins was evaluated by immunofluorescence staining, real-time polymerase chain reaction (PCR) assays, and Western blot assays.

Results: The morphology of hACLFs isolated using the two methods differed after the 2nd passage. The proliferation of cells obtained by in situ culture was higher than that of cells obtained by collagenase digestion. hACLFs cultured with bFGF after the 3rd passage exhibited a higher proliferation rate than the controls. Immunofluorescence staining, real-time PCR, and Western blot analysis showed a significant increase in ligament-related gene and protein expression in the hACLFs cultured with bFGF.

Conclusions: The in situ isolation of tissue pieces enhanced hACLF proliferation in vitro, and the hACLFs exhibited phenotypic characteristics of fibroblasts. hACLFs cultured with bFGF exhibited increased hACLF bioactivity.

Abbreviations: ANOVA = one-way analysis of variance, BCA = bicinchoninic acid, bFGF = basic fibroblast growth factor, BMSCs = bone marrow stem cells, ECM = extracellular matrix, FBS = fetal bovine serum, FCM = flow cytometry, FITC = fluorescein isothiocyanate, hACL = human anterior cruciate ligament, hACLFs = human anterior cruciate ligament fibroblasts, hAMSCs = human amnion mesenchymal stem cells, IGF = insulin-like growth factor, LG-DMEM/F12 = low-glucose Dulbecco's Modified Eagle's Medium and F12 Nutrient Mixture, LOX-1 = lysyl oxidase-1, MMP-2 = matrix metalloproteinase-2, MRI = magnetic resonance imaging, MSCs = mesenchymal stem cells, PRP = platelet-rich plasma, RT-PCR = reverse transcription quantitative real-time polymerase chain reaction, TBST = Tris-buffered saline with Tween, TGFβ-1 = transforming growth factor β-1, Triton X-100 = polyethylene glycol octylphenol ether, VEGF = vascular endothelial growth factor.

Editor: Ilke Coskun Benlidayi.

This study was funded by the National Natural Science Foundation of China (No. 81572142 to Wei Huang), Science & Technology Program of Guizhou Province (No. SY [2010]3091 to Yi Liu), the Science & Technology Program of Guizhou Province (No. LH [2017]7105 to Gang Zou), and the Science and Technology Program of Guizhou Province (No. LH [2016]7477 to Huazhang Xiong).

The authors declare that they have no conflicts of interest to disclose.

[a] Department of Orthopaedic Surgery, the First Affiliated Hospital of Chongqing Medical University, [b] Department of Orthopaedic Surgery, the Second Affiliated Hospital of Chongqing Medical University, Chongqing, [c] Department of Orthopaedic Surgery, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, P.R. China.

[*] Correspondence: Wei Huang, Department of Orthopaedic Surgery, the First Affiliated Hospital of Chongqing Medical University, No. 1 Youyi Road, Yuzhong District, Chongqing, 400016, P.R. China (e-mail: weih68@hotmail.com).

Medicine (2019) 98:22(e15907)

Received: 26 September 2018 / Received in final form: 9 May 2019 / Accepted: 10 May 2019

http://dx.doi.org/10.1097/MD.0000000000015907

Keywords: basic fibroblast growth factor, cell isolation and cultivation, human anterior cruciate ligament fibroblasts, ligament injury, tissue-engineered ligament

1. Introduction

The human anterior cruciate ligament (hACL) is a stiff connective tissue that allows the knee to rotate internally and externally and limits anterior tibial displacement to prevent excessive knee extension. The ability of the human ACL to self-repair is weaker than other soft tissues due to the poor tissue-blood supply.[1] Three lakhs (300,000) patients have newly acquired ACL injuries, and the overwhelming majority of these patients will require ACL reconstruction surgery.[2–4]

Tissue-engineered ligaments are continuously being developed.[5] From the point of view of cell biology, the poor healing ability of the ACL after injury can be overcome by enhancing the proliferative ability of hACL fibroblasts (hACLFs). Tissue-engineered ligament, aims to promote the regeneration and achieve the mechanical and biochemical characteristics similar to that of the original ACL. hACLFs are an important type of seed cell that has become a significant cell origin for constructing tissue-engineered ligaments.[6,7] Many emerging technologies have been used in vivo with promising results when combined with platelet-rich plasma (PRP),[8] growth factors,[9] transforming factors, different biomaterial scaffolds, and mechanical loading.[10] Choosing the appropriate seed cells is essential for constructing a tissue-engineered ligament. The seed cells must be able to proliferate, secrete extracellular matrix (ECM), be biocompatible with the scaffold, and augment ACL healing in vivo.[11–13]

Seed cells used to construct tissue-engineered ligaments are mainly one of two types: fibroblasts arising from the ligaments or tendons from the ACL,[14] medial collateral ligament (MCL)[15] or patellar tendon[16]; and mesenchymal stem cells (MSCs)[17] from multiple tissue sources.[18] hACLFs are an important source of cells used to construct tissue-engineered ligaments due to their similar bioactivity to normal human ACL. hACLFs primarily contain collagen fibrils, which are mostly collagen type I and III fibrils, matrix proteins, and glycoproteins. hACLFs secrete multiple collagen and matrix proteins, and the cell-scaffold complex forms after cells are loaded into scaffolds that exert a biomechanical effect. The self-proliferation, expression of collagen, and cell matrix protein of hACLFs are superior to other fibroblasts originated from other tissues. Hence, the hACLFs attached importance in constructing tissue engineered ligament. The literature indicated that the fibroblasts were extracted from intact and impaired ACL and cultured on scaffold materials in vitro. The results showed that the cells appearance from both sources were nearly same. There was no difference in the structure of the new ligament and the synthesis of actin and integrin, suggesting that the impaired ACLFs could be used as the seed cell source. Furthermore, it was found that ACLFs could be used as seed cells for tissue engineering ligament because of its fast migration and stable phenotype in hydrogel.[6,7,19] Fibroblasts have been isolated from the MCL, the patellar tendon, the Achilles tendon, and uterine ligament.[20] However, effective in vitro amplification and proliferation of these isolated cells requires high biological activity. Basic fibroblast growth factor (bFGF) is a safe growth factor that has been applied to promote proliferation,[21] increase bioactivity and rapidly boost protein secretion.[22–24]

In this study, we compared hACLFs collected by collagenase digestion to cells prepared by in situ isolation from tissue pieces and investigated the effects of bFGF application to cultured hACLFs in vitro by assaying cellular morphology, proliferation rate, and specific gene and protein expression. We hypothesized following method: firstly, the two isolation methods (collagenase digestion and in situ isolation by culturing tissue pieces) would result in different morphologies and proliferation rates and secondly, bFGF use would improve early cell proliferation and relative gene expression and protein secretion in hACLFs.

2. Materials and methods

2.1. hACLF isolation

2.1.1. Collagenase digestion of hACLFs. This study was approved by the Ethics Committee of the Affiliated Hospital of Zunyi Medical University, and the written informed consent for participation of the present study was obtained from all the patients before surgical retrieval. The 12 ACL-rupture patients were diagnosed with inclusion criteria. The ACL-rupture samples were collected from a total of 6 patients according to our inclusion and exclusion criteria (Table 1). ACL tissues were isolated from six adult patients (5 males, 1 female) suffering from ACL ruptures who received arthroscopic ACL reconstruction (Fig. 1). The ACL ruptures were related to sports injuries. The ACL-rupture patients were diagnosed by clinical manifestation, physical examination, magnetic resonance imaging (MRI), and arthroscopy. The enrolled ACL-rupture patients received ACL reconstruction surgery under arthroscopy. ACL tissue of approximately 1 cm was obtained through resection of the ligament stump with a sharp scalpel under arthroscopy. No patient had concomitant cartilage lesions. Basic information regarding these patients is provided in Table 2. The

Table 1

Diagnostic criteria for ACL rupture.

Inclusion criteria	Exclusion criteria
I. Clinical manifestation	I. Patients with meniscus, collateral or PCL injuries and other ligament injuries need to be reconstructed at the same time
II. Physical examination	II. Patients with craniocerebral or pectoral and abdominal injuries
III. MRI	III. Patients who have contralateral knee joint surgery history
IV. Arthroscopy	IV. Patients under 18 years of age and over 30 years of age
V. Patients' age between the ages of 18 and 30	V. Patients who combine multiple basic diseases and are unable to conduct general activities
VI. Course less than 3 months	VI. Patients fail to complete the follow-up test
VII. Detection of simple rupture of ACL under knee arthroscopy	

ACL = anterior cruciate ligament, MRI = magnetic resonance imaging, PCL.

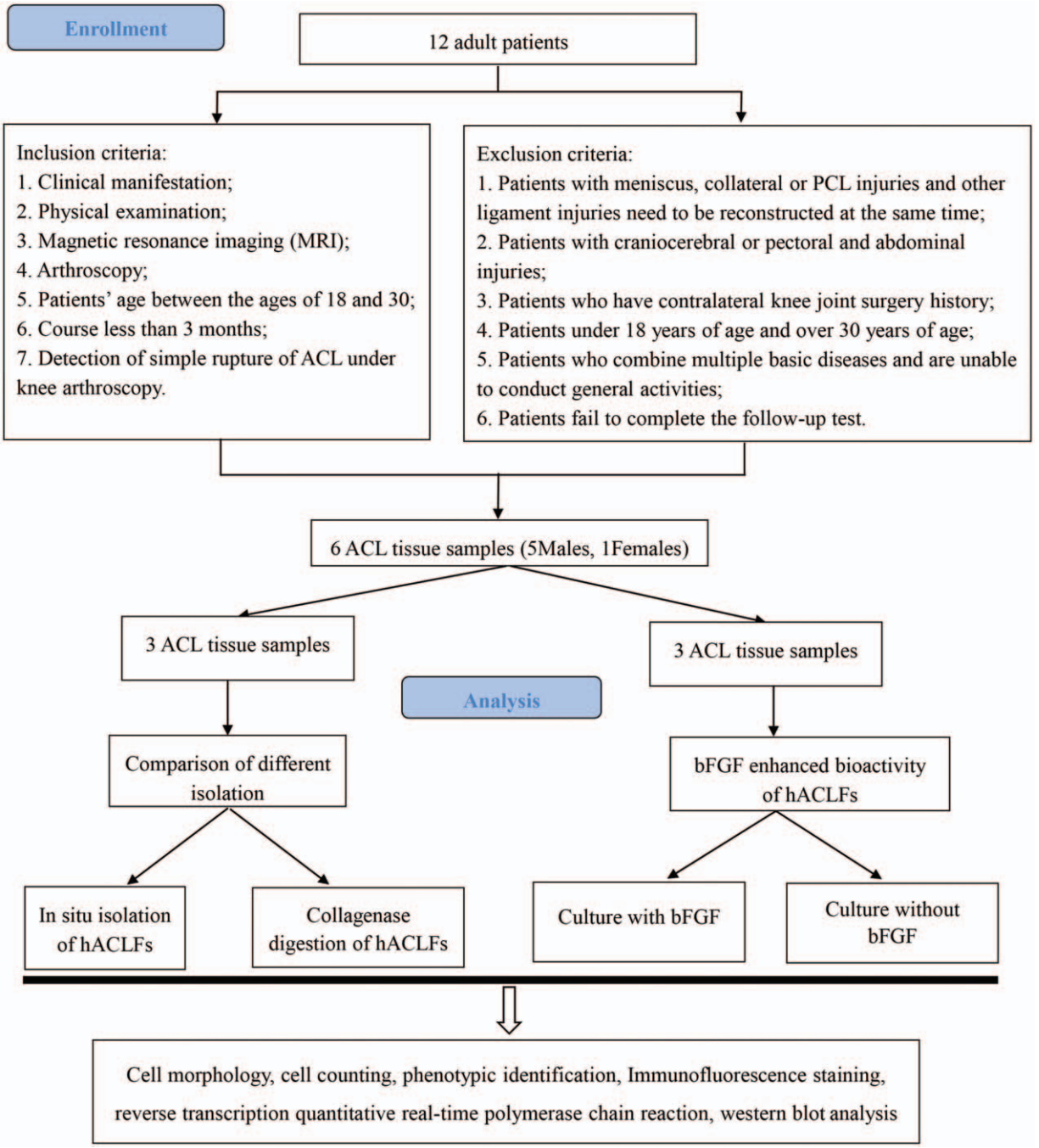

Figure 1. Flowchart of the present study. ACL tissues samples were collected from six adult patients (5 males, 1 female) suffering from ACL ruptures who received arthroscopic ACL reconstruction according to our inclusion and exclusion criteria.

Table 2

Clinical history of the patients with ACL rupture who provided the research materials.

	Isolation methods	Growth factors (bFGF)	*P* value	Significantly different
Patients number	3 samples (No. 1, 2, 3)	3 samples (No. 4, 5, 6)	/	/
Sex	3 males	2 males, 1 female	/	/
Age (year)	23.0 ± 1.0	23.67 ± 0.5774	.3739	No
Time from injury (days)	2.667 ± 0.5774	2.0 ± 1.732	.5614	No
Time from surgery (days)	4.667 ± 1.528	4.333 ± 0.5774	.7415	No

(Mean ± Std. Dev.).
bFGF = basic fibroblast growth factor.

ACL sample was stored in normal saline at 4°C and delivered to the laboratory.

After the ACL tissue was transferred to a Petri dish, the hematoma and other soft tissues were removed. The tissue was minced into approximately 1-mm^3 pieces that were digested with 0.2% collagenase type I in low-glucose Dulbecco's Modified Eagle's Medium and F12 Nutrient Mixture (LG-DMEM/F12) (Hyclone Company, Logan, UT)with 10% fetal bovine serum (FBS) at 37°C for 4 hours. The digested pieces were filtered with a 74 μm mesh filter to separate hACLFs from digested ACL pieces. The cells were washed three times with PBS, centrifuged at $1200 \times g$ for 5 minutes, resuspended in LG-DMEM-F12 medium with 10% FBS, 1% penicillin and streptomycin, 1% L-glutamine and nonessential amino-acids, and plated in T-25 flasks. The cells were incubated at 37°C with 5% humidified CO_2 and washed twice with PBS, and the medium was replaced every 4 to 5 days.

When the cells reached 80%–90% confluence, they were passaged by incubation with 0.125% trypsin for 5 minutes, and the cells were subcultured at a 1:2 ratio. Cells from P3 were used in subsequent experiments.

2.1.2. In situ isolation of hACLFs. ACL tissue was excised and obtained as described above. The ACL tissue was minced into 1-mm^3 pieces with sterile scissors and washed with PBS, and the pieces were placed in a T-25 flask with culture medium. The isolated cells were incubated at 37°C in a 5% humidified CO_2 atmosphere. The culture medium was replaced every 4 to 5 days after the cells were washed with PBS. The subculture protocol was the same as the protocol described above. hACLFs isolated using either collagenase digestion or the in situ culture of tissue pieces were observed with a phase-contrast microscope from P0 to P2.

2.2. Phenotypic identification

Cell markers for hACLFs at P3 were detected by flow cytometry (FCM). The cell density was adjusted to 2×10^6 cells/ml, and 100 μl of cell suspension was transferred to the FCM tube. Cells were incubated with purified fluorescein isothiocyanate (FITC)-conjugated primary antibodies against CD44, CD29 CD146, CD31, CD34, and CD45 for 30 minutes. After washing, flow buffer was added to the cells. The cells were centrifuged at $1000 \times g$ for 5 minutes, the supernatant was discarded, and the sample was resuspended in 250 μl of flow buffer. The number of hACLFs that were positive for surface markers was analyzed by FCM using Coulter Epics XL software, SYSTEM II Software, Version 3.0 (Beckman, Brea, CA).

2.3. Analysis of hACLFs cultured with bFGF

After subculture, the same number of hACLF cells on P3 was transferred to T-25 flasks. The experimental group was cultured in LG-DMEM-F12 medium (as described above) with 10 ng/ml bFGF, and the control group was cultured in LG-DMEM-F12 medium only. The cells were incubated at 37°C in 5% humidified CO_2. The morphology of hACLFs at P3 cultured in LG-DMEM-F12 medium was viewed with a phase-contrast microscope on days 7, 10, and 14.

2.4. Proliferation and cell count analysis

hACLFs isolated using collagenase digestion or the in situ culture of tissue pieces were seeded into 96-well plates with 100 μl of LG-DMEM-F12 medium. When the isolated cells reached 85% confluence, the cell suspensions were adjusted to a density of 4×10^3 cells/ml. The cells were cultured for an additional 7 days, and the viable cell density in every well was assessed with CCK-8 (DOJINDO, Kumamoto, Japan). After the plates were incubated at 37°C in 5% humidified CO_2 for 2 hours, the absorbance value at 450 nm was measured using a microplate reader (A5082-TECAN, Redwood City, CA). The cell number was counted with a cell analyzer (Beckman Vi-Cell XR). A proliferative curve was computed based on the data. The hACLF proliferation and cell count at P3 after culture with or without bFGF were determined using the same procedure.

2.5. Immunofluorescence staining

hACLFs at P3 from the experimental and control groups were cultured on cover slips for 7, 10, and 14 days before the cell density was adjusted to 10^4 cells/ml. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% polyethylene glycol octylphenol ether (Triton X-100) for 10 minutes. The cells were then blocked with Lowenthal serum for 30 minutes. The following primary antibodies were incubated with the cells overnight at 4°C: anti-collagen I, collagen III, fibronectin, and tenascin-C (Abcam, Eugene). Secondary FITC- or CY3-labeled antibodies were then incubated with the samples for 1 hour. Cell nuclei were counterstained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine at room temperature for 5 minutes. Cover slips were slowly mounted onto slides with 90% glycerol and observed with an inverted fluorescence and phase-contrast microscope.

2.6. Reverse transcription quantitative real-time polymerase chain reaction

Total RNA from P3 hACLFs in the experimental and control groups was extracted on days 7, 14, and 21, and cDNA libraries were constructed from total RNA using a reverse transcription protocol. Collagen I and III, fibronectin, tenascin-C, matrix metalloproteinase-2 (MMP-2), and lysyl oxidase-1 (LOX-1) mRNA expression was analyzed using reverse transcription quantitative real-time polymerase chain reaction (RT-PCR). The relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta}$CT method. The PCR primers used in this study are provided in Table 3.

2.7. Western blot analysis of collagen type I and III proteins

Cells in the experimental and control groups on days 7, 10, and 14 were collected, and total protein was extracted using RIPA lysis buffer (Beyotime, Nanjing, China). The protein concen-

Table 3

Primer sequence of the target genes.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
Collagen type I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Collagen type III	GCCAAATATGTGTCTGTGACTA	GGGCGAGTAGGAGCAGTTG
Fibronectin	GAGAATAAGCTGTACCATCGCA	CGACCACATAGGAAGTCCCAG
Tenascin-C	TCCCAGTGTTCGGTGGATCT	TTGATGCGATGTGTGAAGACA
MMP-2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC
LOX-1	TTGCCTGGGATTAGTAGTGACC	GCTTGCTCTTGTGTTAGGAGGT
GAPDH	CCATGTTCGTCATGGGTGT	CCAGGGGTGCTAAGCAGTT

GAPDH, LOX-1 = lysyl oxidase-1, MMP-2 = matrix metalloproteinase-2.

trations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo, Waltham, MA), and equal amounts of protein were applied to SDS-PAGE gels and transferred onto PVDF membranes. After blocking with 5% evaporated milk for 2 hours at 37°C, the blots were incubated with anti-collagen I, collagen III, fibronectin, and tenascin-C (Abcam, Eugene) overnight at 4°C. After washing with Tris-buffered saline with Tween (TBST), the membranes were probed with a fluorescently labeled secondary antibody (Odyssey, San Gabriel, CA). Human GAPDH served as a control for equal protein loading. The relative density was measured using Image J software.

2.8. Statistical analysis

The data are expressed as the mean ± standard errors. The Student–Newman–Keuls test and one-way analysis of variance (ANOVA) were used to compare two or more groups. The statistical software Statistical Product and Service Solutions (SPSS, Chicago, IL) version 14.0 was used to calculate and analyze the results. Differences were considered significant at a P value $< .05$.

3. Results

3.1. Morphology and proliferation of hACLFs collected with different isolation methods

Patients were diagnosed with ACL injury by clinical exams that were confirmed by MRI and arthroscopy (Fig. 2A1 and A2). Nucleated cells were isolated from ACL tissue (Fig. 2A3). The morphology of hACLFs isolated using different methods was observed from P0 to P2. The hACLFs at P0 had polygonal, triangular, and irregular shapes. At P1, cells obtained using the in situ culture method exhibited a spindle-shaped, adherent, fibroblast-like morphology, and more hACLFs were obtained

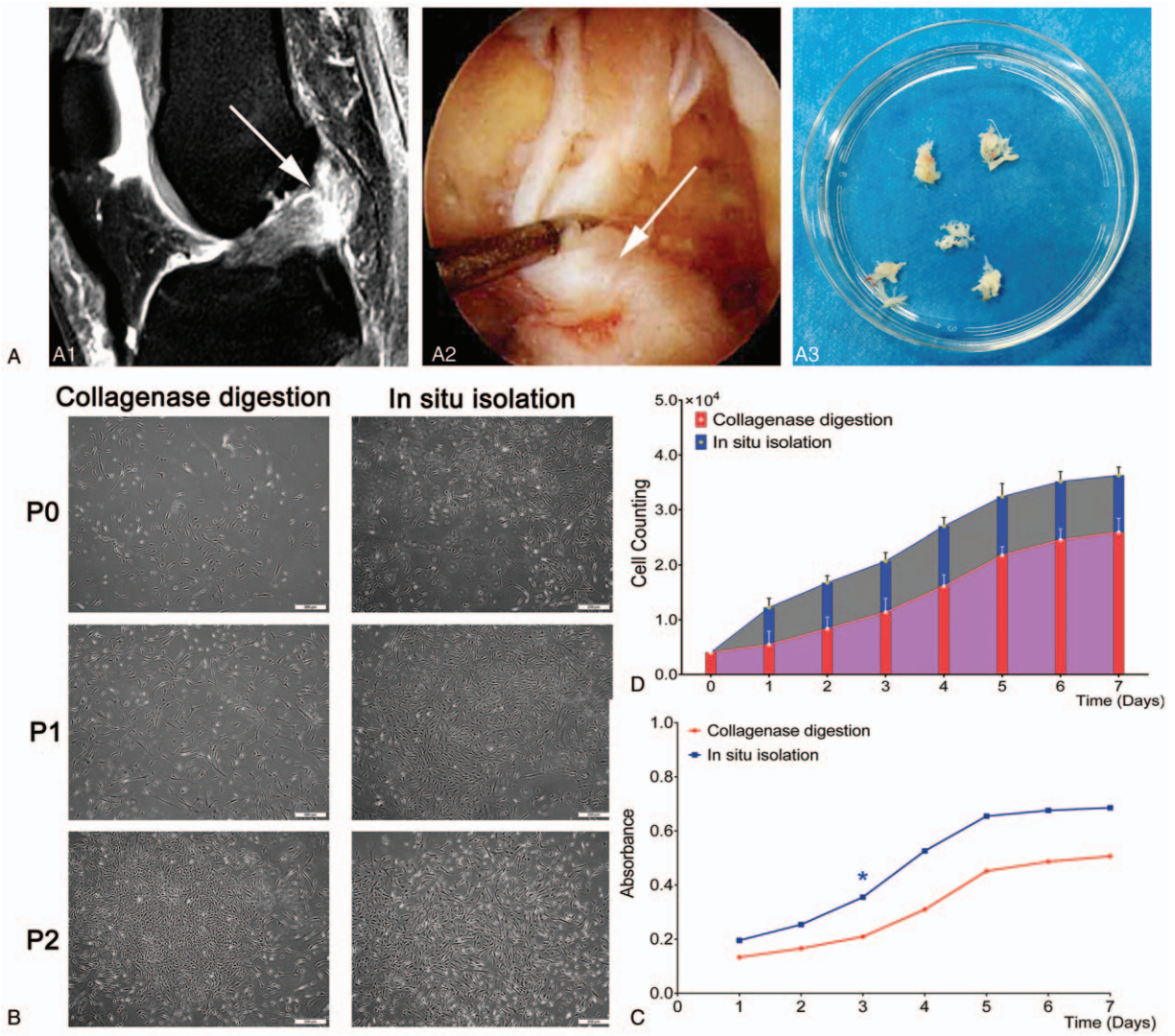

Figure 2. Morphology of isolated hACLFs and proliferative curves and cell counts of cells isolated using two methods. Magnetic resonance imaging (A1). Arthroscopic surgery showed emblematical ACL rupture (A2). ACL tissue was obtained during surgery (A3). Morphology of cultured hACLFs obtained by collagenase digestion or in situ culture of tissue pieces from P0 to P2 (B). (Magnification ×40, bars in Fig. 1B, 200 μm). Cell count (C) and proliferation curve (D) of primary hACLF cultures obtained from collagenase digestion or in situ culture of tissue pieces. Cell count and proliferative curves using the CCK-8 method showed that the proliferation and counts of hACLFs isolated through the in situ culture of tissue pieces were significantly higher than those of hACLFs obtained with the collagenase digestion method ($^{*}P < .05$).

with this method than with collagenase digestion. On P2, hACLFs obtained using the in situ culture method exhibited radial growth around the center of the adhesion, and a large number of cells migrated from the edge of the tissue block and connected to other cells. In comparison, fewer collagenase-digested hACLFs were observed, and the cells exhibited a primordial morphology with few fibroblast cells (Fig. 2B). The cell count and proliferation curve for hACLFs isolated using the two different methods showed an "S" pattern. The cells obtained with both isolation methods reached the logarithmic growth phase after 3 days of culture. The growth curve and cell count (Fig. 2C) of the cells obtained using the in situ culture method were significantly higher than those obtained for the collagenase-digested cells on day 3 ($P < .05$) (Fig. 2D).

3.2. Phenotypic characteristics of hACLFs

The phenotypic characteristics were analyzed by FCM, and the results showed that hACLFs on P3 expressed high levels of CD44 and CD29 and were negative for CD146, CD31, CD34, and CD45. Thus, these cells exhibited the phenotypic characteristics of ligament fibroblasts (Fig. 3A).

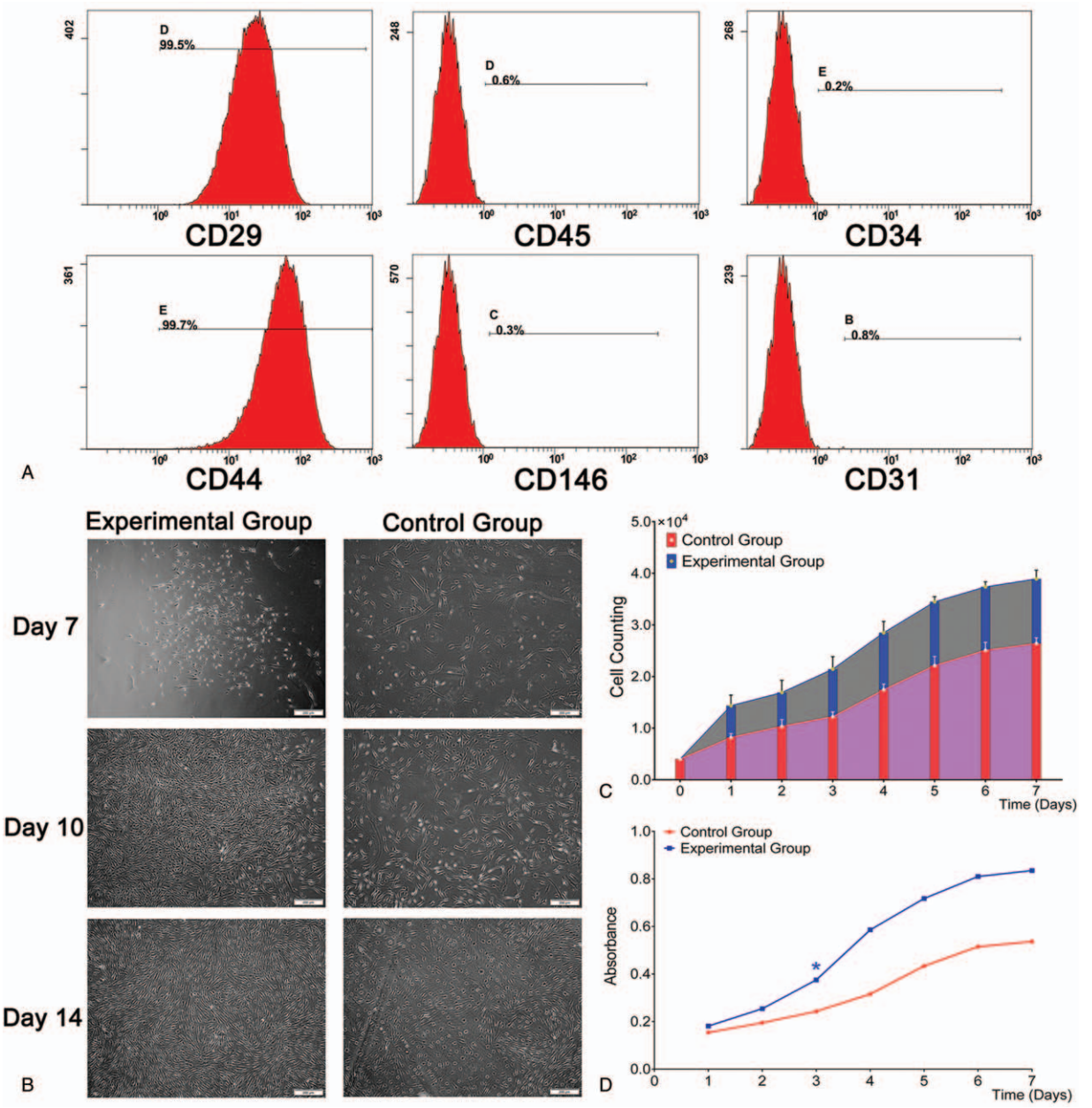

Figure 3. Phenotypic characteristics of hACLFs measured by FCM, the morphology of hACLFs in the experimental and control groups at P3, and the proliferative curve of the cells in each group determined by CCK-8. Based on flow cytometry, hACLFs in the 3rd passage were positive for the expression of CD29 and CD44 and negative for the expression of CD146, CD31, CD34, and CD45 (A). Morphology of cultured hACLFs in the experimental group and control group (B) (Magnification ×40, bars in Fig. 1B, 200 μm). Cell count (C) and proliferation rate (D) of hACLFs in the experimental and control groups. ($^{*}P < .05$). Cell count and proliferative curves were obtained using a cell count analyzer, and the CCK-8 results for cultured hACLFs in the 3rd passage showed that the cell count and proliferation of experimental group were significantly higher than those of the control group at day 3 ($^{*}P < .05$).

3.3. Appearance, growth, and cell count of hACLFs cultured with bFGF

The appearance of hACLFs in the experimental and control groups on days 7, 10, and 14 was observed. On day 7, the cells in the experimental group (Fig. 3B) exhibited a similar appearance (spindle-like, adherent morphology) and cell number compared with the control group. After the cells were cultured for 10 days, the cells in the experimental group exhibited a long spindle-shaped morphology and grew in a gyrate-like pattern, which was more similar to that of fibroblast differentiation than the control group, which demonstrated a basically unchanged morphology. On day 14, the hACLFs in the experimental group grew in a spiral pattern and exhibited a long spindle shape with over-tension. The cell number (Fig. 3C) and proliferation were significantly increased in the experimental group compared with the control group beginning on day 3 ($P < .05$) (Fig. 3D).

3.4. Immunofluorescence analysis of protein expression

Immunofluorescence was used to examine the distribution and excretion of collagen type I and type III, fibronectin and tenascin-C in hACLFs (Figs. 4–7). On day 10, the expression of collagen type I in the experimental group was significantly higher than that in the control group. On day 14, the intensity of immunofluorescence staining was markedly increased in the experimental group compared with the control group ($P < .05$). The expression and secretion of collagen type III was significantly increased in the experimental group. Web-like agglomerates were observed on day 10 in the experimental group, which indicates that large amounts of collagen III were secreted around cell nuclei, whereas the cells in the control group showed little change ($P < .05$). On day 14, a web-like shape was more apparent in the experimental group. Fibronectin expression was notably stronger in the experimental group on days 7, 10, and 14 than in the control group at the same time points ($P < .05$). Tenascin-C staining showed a mist-like expression pattern around the cell nuclei, and a greater density of fused staining emerged on days 7, 10, and 14 in the experimental group compared with the control group ($P < .05$).

3.5. Expression of ligament-related genes

Collagen type I and III, fibronectin, tenascin-C, MMP-2, and LOX-1 mRNA expression was upregulated to different extents over time after bFGF treatment (Fig. 8), and the expression of ligament-related mRNAs was significantly increased in the experimental group compared with the control group. Fibronectin gene expression was obviously increased in the experimental group on days 7, 10, and 14, whereas the control group exhibited a slight difference ($P < .05$). The tenascin-C mRNA level was slightly increased on days 7 and 10 in both groups ($P > .05$), but on day 14, tenascin-C expression was markedly increased in the experimental group. MMP-2 and LOX-1 mRNA expression was obviously upregulated in the experimental group on day 14 ($P < .05$).

3.6. Western blot analysis of collagen type I and III

Western blot analysis of collagen type I and III revealed an evident increase in the expression of these collagen proteins in the experimental group on days 10 and 14 compared with the control group, which exhibited a slight increase in collagen expression over time. However, the secretion of collagen I and III was only moderately increased in the experimental group between days 7 and 10 (Fig. 9).

4. Discussion

The tissue-engineered ligament is a type of anatomical reconstruction in which seed cells are expanded in vitro, carried on multiple bioactive scaffolds and then transplanted into the body

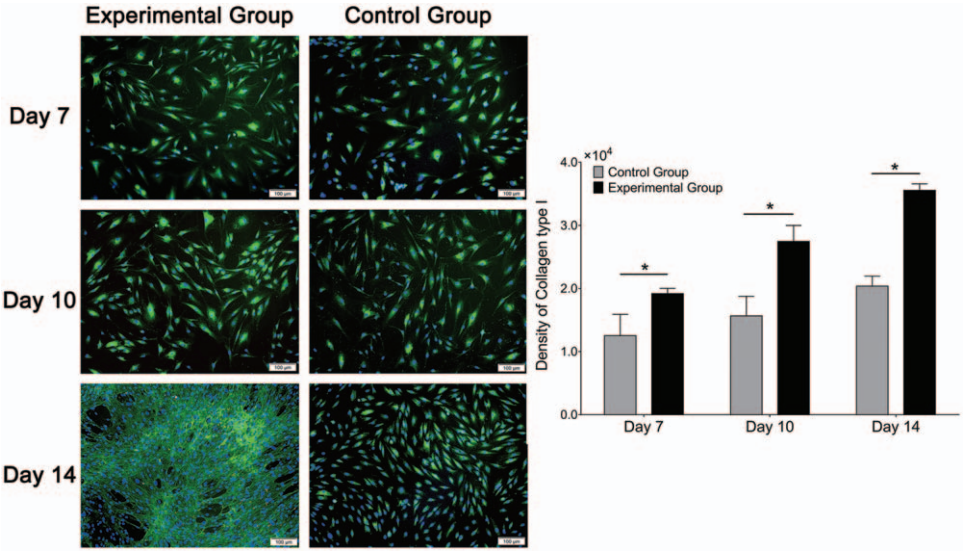

Figure 4. Protein expression of collagen type I in cultured hACLFs from the experimental and control groups at P3. The protein expression levels were quantified based on immunofluorescence. Immunofluorescence staining showed that collagen type I expression increased over time (from day 7 to 10 and 14) in hACLFs cultured at P3, and increased intracellular secretion was also detected. Collagen type I expression was greater in the experimental group than in the control group on day 7, and hACLFs showed the highest expression levels in the experimental group on day 14, during which fusion was observed. (Magnification ×100, bars, 100 μm, $^{*}P < .05$).

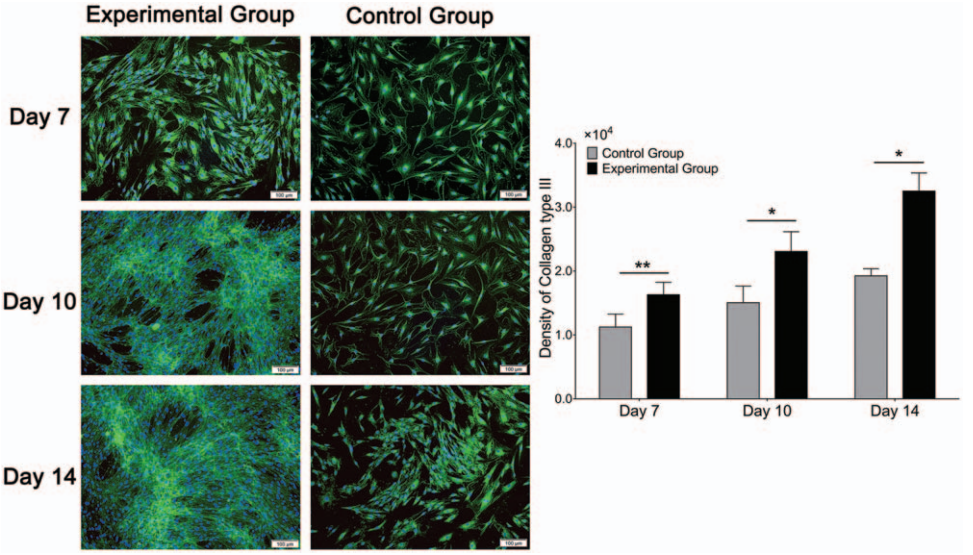

Figure 5. Protein expression of collagen type III in cultured hACLFs from the experimental and control groups at P3. The protein expression levels were quantified based on immunofluorescence staining. Immunofluorescence staining showed that the experimental group presented clearly increased expression and intracellular secretion of collagen type III over time compared with the control group, and a large fusion was observed starting on day 10. Quantification showed that the experimental group exhibited strong expression of collagen type III, with the highest expression on day 14. (Magnification ×100, bars, 100 μm, $^{*}P < .05$).

by ligamentous reconstruction surgery to achieve physiological reconstruction and biomechanical ability in vivo. Hence, accelerating the proliferation rate of hACLFs in vitro, enhancing their collagen secretion and promoting the synthesis of related matrix proteins are extremely important in the construction of tissue-engineered ligament. These processes are not only of great significance to the experimental study of cell biology but will also provide guidance for the treatment of ligamentous ligament injury with tissue-engineered ligament in the future.

The seed cell culture and induction methods determine cytoactivity, proliferation, and biocompatibility of hACLFs with scaffolds which are essential and necessary for the construction of tissue-engineered ligaments.[25–27] Subsequently, the seed cells must exhibit sufficient proliferation and bioactivity in vitro for use in the appropriate isolation and culture systems, which have received considerable attention in the tissue-engineering field.[28] In our study, we compared the proliferation and morphology of hACLFs isolated from the hACL using different methods and

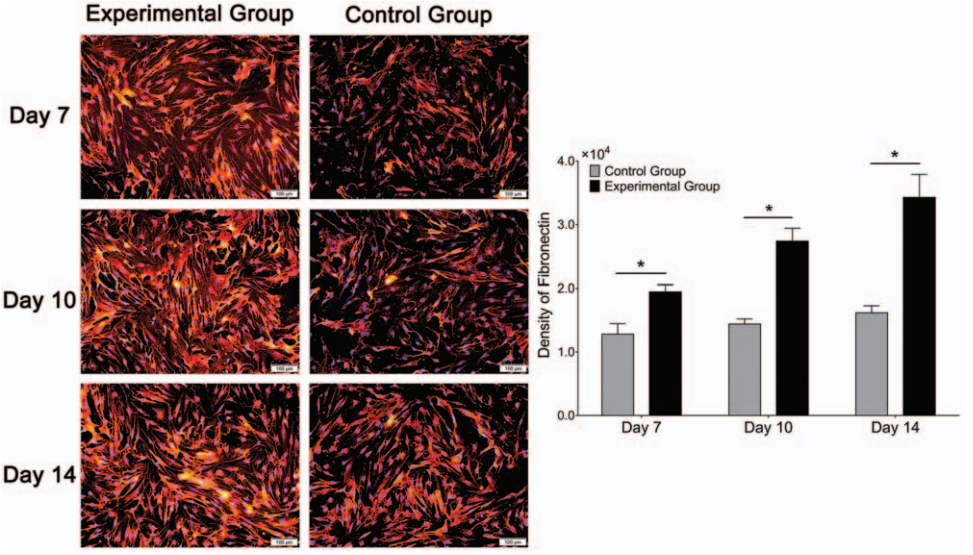

Figure 6. Protein expression of fibronectin in cultured hACLFs from the experimental and control groups at P3. The protein expression levels were quantified based on immunofluorescence staining. Immunofluorescence staining demonstrated that fibronectin expression increased over time. The fibronectin protein was secreted intracellularly in the control group and both intracellularly and extracellularly in the experimental group, and the experimental group showed the beginning of apparent fusion on day 7 compared with the control group. The experimental group exhibited a higher level of fibronectin expression and fusion than the control group, and fibronectin was most highly expressed in the experimental group on day 14. (Magnification ×100, bars, 100 μm, $^{*}P < .05$).

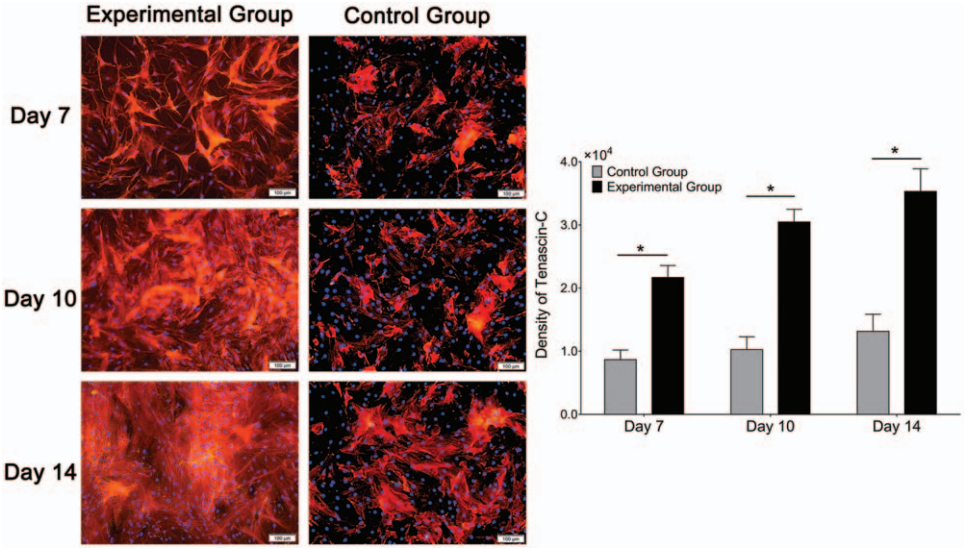

Figure 7. Protein expression of tenascin-C in cultured hACLFs from the experimental and control groups on P3. The protein expression level was quantified based on immunofluorescence staining. Immunofluorescence staining showed that tenascin-C protein was mostly secreted extracellularly and appeared as an indistinct mass, and the secretion of tenascin-C protein was not in the area around the nucleus. hACLFs in the experimental group showed a significant increase in tenascin-C expression over time. In the control group, the tenascin-C expression increased slightly at each time point, but this increase was not significant. The highest expression of tenascin-C was obtained in the experimental group on day 14 compared with the control group. (Magnification ×100, bars, 100 μm, $^{*}P < .05$).

analyzed the effect of bFGF on hACLF cultures. In our study, we demonstrated that the in situ culture of tissue pieces is a better method for isolating hACLFs than collagenase digestion and that bFGF addition has favorable effects. The cells rapidly proliferate in vitro when cultured with bFGF-supplemented media. Based on the results of our study, the in situ culture of tissue segments is an easy method that reduces the duration of the isolation process. In addition, the number of cells collected was higher than the number of cells obtained using the collagenase digestion method, which might enhance proliferation and bioactivity and cause less cellular damage. The isolated hACLFs possessed the phenotypic characteristics of fibroblasts.

Based on the morphological analysis performed in our study, the use of collagenase digestion to isolate hACLFs resulted in cells with short rod, polygonal, and irregular shapes, whereas the in situ culture of tissue pieces generated cells with a long spindle and bipolar shape. It is reported that potential source of hACLFs is the synovial fluid, and these cells are in a static state under healthy physiological conditions, but once the knee tissue ligament is injured or ruptured, these cells are mobilized and recruited to migrate to the injured ligament site to renovate and proliferate during the ACL tissue repair process.[29] Regardless of the origin of hACLFs (hACL or synovial fluid), the general function of hACLFs is to form the basic structure of hACL tissue, in addition, to repair and regenerate the injured ACL tissue, although the exact source of hACLFs requires further study.

The use of bFGF supplementation in the culture medium accelerated hACLF growth and upregulated mRNAs and proteins associated with ligament fibroblasts. According to previous studies, hACLFs exhibit substantial and favorable proliferation, self-renewal, and the secretion of collagen and matrix proteins.[10,30] hACLFs are mature somatic cells that have been widely utilized as classic seed cells to construct tissue-engineered ligaments.[31] Hence, the ability to rapidly expand hACLFs in vitro has become increasingly important. Based on accumulating evidence, bFGF is a mitogen with high affinity for heparin that exerts a strong angiogenic effect and might stimulate proliferation,[32] migration and collagen production in cells.[33,34]

In a recent study, cell proliferation significantly increased by more than 70% upon bFGF application.[35] As shown in a study, cells from a preloaded bFGF group showed reformative proliferation and increased cell numbers compared with the unloaded group.[36,37] Chen and Kohno colleagues reported that bFGF is one of most vital growth factors promoting collagen protein secretion and tendon cell development.[38,39] In their research, a comparison with the control group revealed that the cells in the experimental group showed increased vessel formation, resulting in enhanced healing of the tendon-bone-interface. In addition, bFGF increases the expression of other growth factors, such as TGF-β1, VEGF, and CTGF.[40]

In our study, the bFGF-treated hACLFs showed upregulated collagen type I, collagen type III, fibronectin, tenascin-C, MMP-2, and LOX-1 mRNA expression compared with the control group. Collagen type I, collagen type III, fibronectin, and tenascin-C are major connatural substances present in ligament fibroblasts and are used to evaluate the bioactivity and secretion of hACLFs after ACL reconstruction. MMP-2 regulates ECM proteins deposited by hACLFs. A previous study reported that bFGF regulates the expression of inflammation-, tumor-, and cytothesis-related factors through the nuclear factor (NF)-kappaβ-Jun N-terminal kinase (JNK) and phosphatidylinositol 3-kinase (PI3K)-Rac1-JNK signaling pathways.[41–43] hACLFs in the experimental group exhibited increased MMP-2 expression, which might indicate that bFGF induces inflammation and cytothesis in hACLFs. LOX-1 is known to be an important marker of collagen and elastin secretion, which increases the ECM content. As shown in a study by Xie, TGF-β1-induced LOX-1 expression increased in the MCL to a greater extent than in the ACL after knee ligament injury.[44] In addition, collagen type I is a crucial factor for detecting an increase in ligament fibroblast activity in

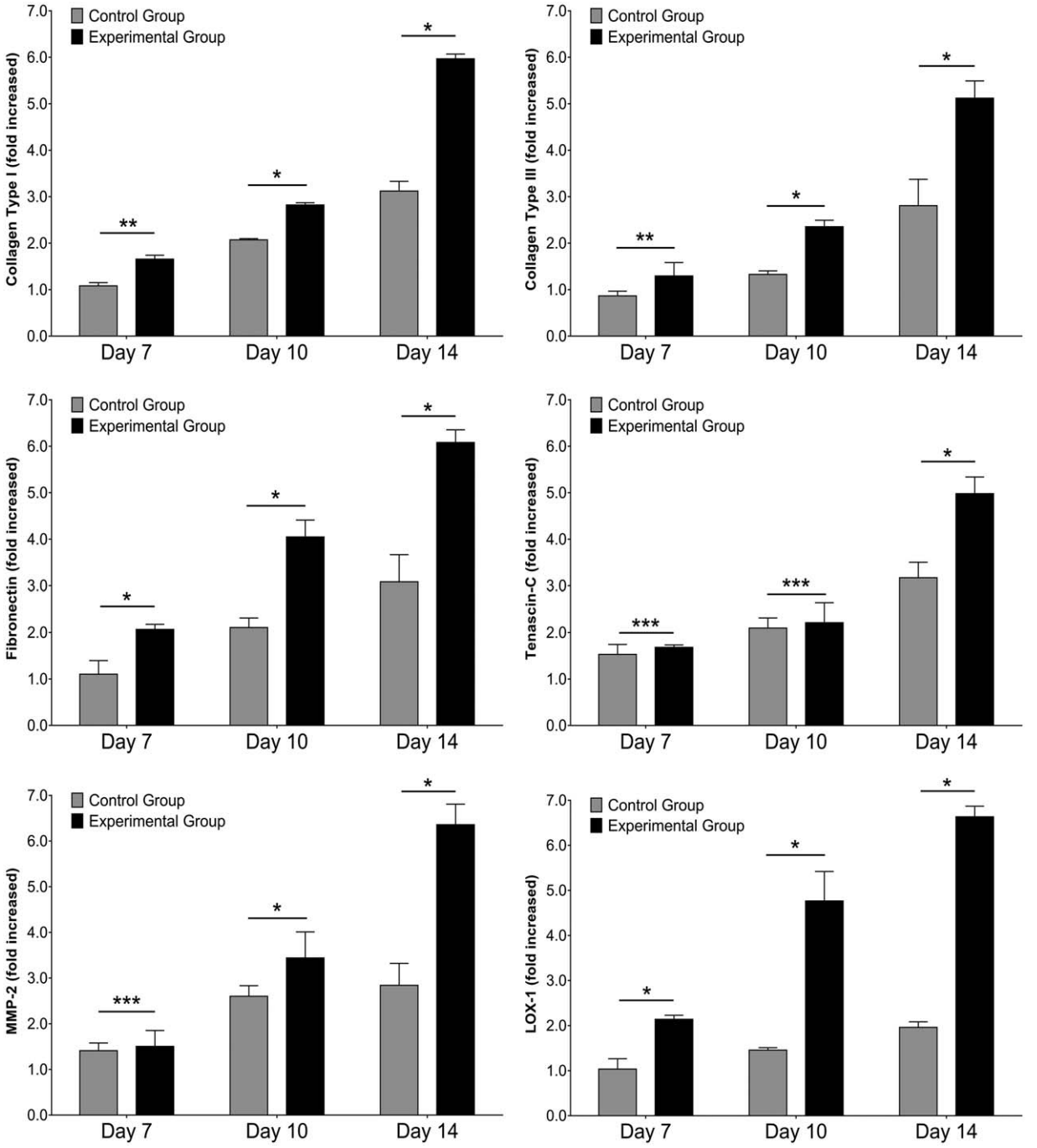

Figure 8. Expression levels of ligament-related mRNAs in hACLFs in the groups on days 7, 10, and 14. The collagen type I and III, fibronectin, tenascin-C, MMP-2, and LOX-1 mRNA expression levels in hACLFs in the two groups on days 7, 10, and 14 was examined by reverse transcription quantitative real-time polymerase chain reaction. Compared with the control group, the experimental group showed significantly increased expression of the collagen types I, III, fibronectin, tenascin-C, MMP-2, and LOX-1 genes on days 7, 10, and 14. The experimental group showed the greatest increase in the expression of ligament fibroblast-related mRNAs on day 14. All data are expressed as the mean and standard deviations. ($^{*}P < .01$, $^{**}P < .05$, $^{***}P > .05$).

vitro. Based on these properties and findings, bFGF is a growth factor that efficiently regulates cell proliferation and upregulates the expression of ligament fibroblast-related genes.

Moreover, based on our Western blot data, the expression of collagen type I and III was significantly increased in hACLFs cultured with bFGF compared with hACLFs cultured without bFGF. Thus, bFGF might improve the secretion of collagen type I and III proteins, moderate protein loss during hACLF culture in vitro, and optimize the repair ability and stringency of hACLFs, as demonstrated through the unchanged proportion of collagen proteins cultured without bFGF.

Our study has some limitations. First, the primary goal of our study was to examine the effect of bFGF on the expression of specific genetic markers of hACLFs and the secretion of other

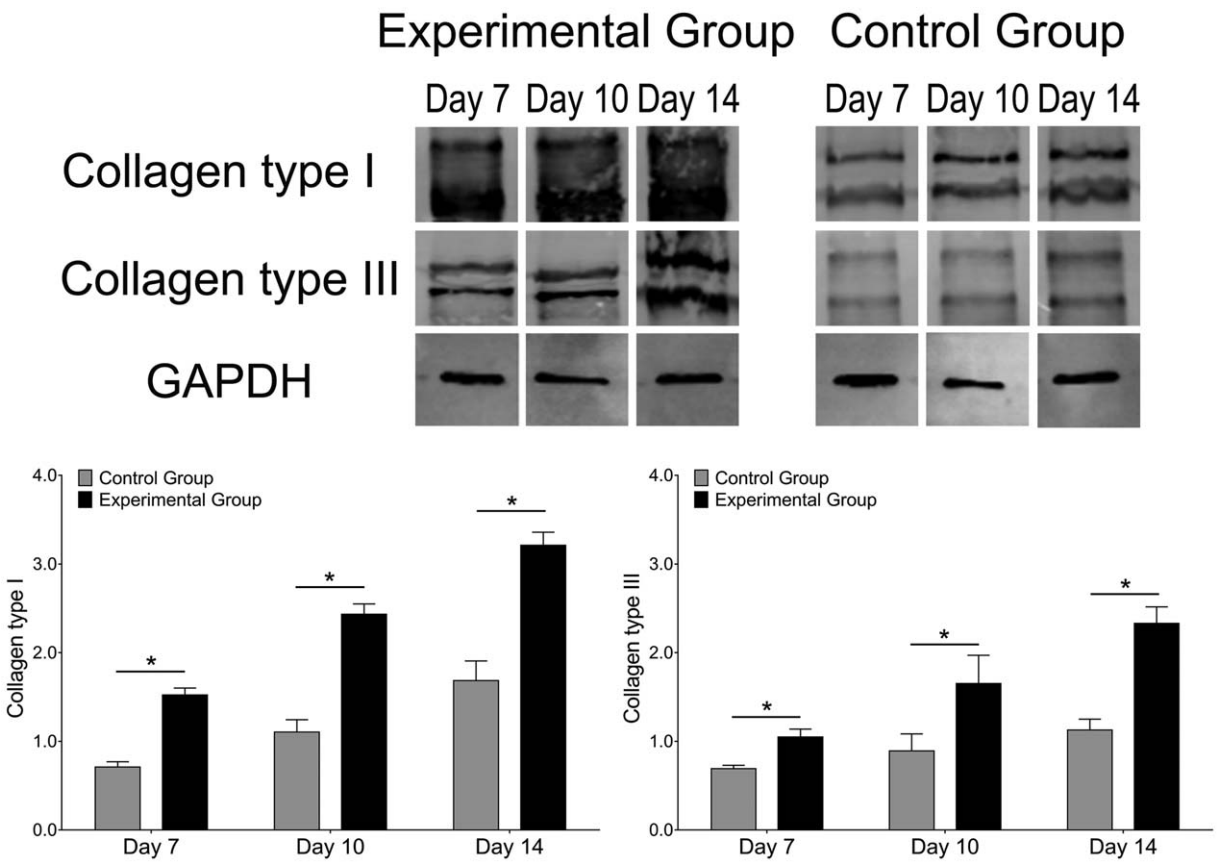

Figure 9. Protein expression of collagen types I and III in hACLFs in the different groups. The protein expression levels were determined by Western blotting. The expression level of collagen types I and III after cultivation with bFGF for 7, 10, and 14 days was determined by Western blotting. The experimental group expressed the highest levels of collagen types I and III on day 14. All data are expressed as the means and standard deviations. ($^{*}P < .05$).

regulatory factors. bFGF increased the mRNA levels of these markers. However, the proliferation and protein secretion responses rely on several factors, including the number of specific cells involved, growth factors and cytokines. Second, we did not evaluate the optimal concentration of bFGF for cultured hACLFs or other factors influencing proliferation and protein secretion in vitro. Third, as shown in our in vitro analyses, bFGF increased hACLF proliferation and the secretion of collagen type I and III as well as related ligament proteins. However, the effect of bFGF on hACLFs in vivo has not been defined, and future studies should focus on a more complete evaluation of the use of hACLFs cultured with bFGF-loaded scaffolds for the treatment of ligament injuries in vivo.

In conclusion, this study showed that the in situ culture of tissue pieces enhanced the proliferation and improved the morphology of isolated hACLFs, and bFGF application improved the proliferation of cultured hACLFs and upregulated their expression of ligament-related genes and proteins. Consequently, hACLFs might play a vital role in ACL regeneration, and bFGF can be considered an appropriate growth factor for constructing tissue-engineered ligaments in vivo. However, the therapeutic capacity of hACLFs cultured with bFGF-loaded scaffolds in vivo should be evaluated in long-term studies to completely elucidate the specific regenerative and healing responses during the treatment of ligament injuries.

Acknowledgments

The authors sincerely thank Dr. Chenghao Zhang from the University of Pennsylvania for manuscript editing and submission consultation.

Author contributions

Conceptualization: Gang Zou, Yi Liu, Wei Huang.
Data curation: Ziming Liu, Yuwan Li, Jibin Yang, Shengmin Wang, Huazhang Xiong, Yi Liu, Wei Huang.
Formal analysis: Ziming Liu, Yuwan Li, Ying Jin, Xizhong Zhu.
Funding acquisition: Yi Liu, Wei Huang.
Investigation: Ziming Liu, Yuwan Li, Youliang Ren, Ying Jin, Shengmin Wang, Xizhong Zhu, Huazhang Xiong, Gang Zou.
Methodology: Ziming Liu, Yuwan Li, Ying Jin, Shengmin Wang, Gang Zou, Yi Liu.
Project administration: Ziming Liu, Yuwan Li.
Resources: Ziming Liu, Jibin Yang, Huazhang Xiong, Gang Zou, Wei Huang.
Software: Youliang Ren, Jibin Yang, Shengmin Wang, Xizhong Zhu, Huazhang Xiong, Gang Zou.
Supervision: Yi Liu, Wei Huang.
Visualization: Ziming Liu.
Writing – original draft: Ziming Liu.
Writing – review & editing: Yi Liu, Wei Huang.

References

[1] Smith SE, White RA, Grant DA, et al. Gold and hydroxyapatite nanocomposite scaffolds for anterior cruciate ligament reconstruction: in vitro characterization. J Nanosci Nanotechnol 2016;16:1160–9.

[2] McLean SG, Beaulieu ML. Complex integrative morphological and mechanical contributions to ACL injury risk. Exerc Sport Sci Rev 2010;38:192–200.

[3] Wierer G, Runer A, Gföller P, et al. Extension deficit after anterior cruciate ligament reconstruction: is arthroscopic posterior release a safe and effective procedure? Knee 2017;24:49–54.

[4] Janssen RP, Reijman M, Janssen DM, et al. Arterial complications, venous thromboembolism and deep venous thrombosis prophylaxis after anterior cruciate ligament reconstruction: a systematic review. World J Orthop 2016;7:604–17.

[5] Battaglia MJ, Lenhoff MW, Ehteshami JR, et al. Medial collateral ligament injuries and subsequent load on the anterior cruciate ligament: a biomechanical evaluation in a cadaveric model. Am J Sports Med 2009;37:305–11.

[6] Cooper JAJr, Bailey LO, Carter JN, et al. Evaluation of the anterior cruciate ligament, medial collateral ligament, Achilles tendon and patellar tendon as cell sources for tissue-engineered ligament. Biomaterials 2006;27:2747–54.

[7] Brune T, Borel A, Gilbert TW, et al. In vitro comparison of human fibroblasts from intact and ruptured ACL for use in tissue engineering. Eur Cell Mater 2007;14:78–90. discussion 90-1.

[8] Sun X, Liu W, Cheng G, et al. The influence of connective tissue growth factor on rabbit ligament injury repair. Bone Joint Res 2017;6:399–404.

[9] Zhi Y, Liu W, Zhang P, et al. Electrospun silk fibroin mat enhances tendon-bone healing in a rabbit extra-articular model. Biotechnol Lett 2016;38:1827–35.

[10] Wang Y, Dang Z, Cui W, et al. Mechanical stretch and hypoxia inducible factor-1 alpha affect the vascular endothelial growth factor and the connective tissue growth factor in cultured ACL fibroblasts. Connect Tissue Res 2017;58:407–13.

[11] Kouroupis D, Kyrkou A, Triantafyllidi E, et al. Generation of stem cell-based bioartificial anterior cruciate ligament (ACL) grafts for effective ACL rupture repair. Stem Cell Res 2016;17:448–57.

[12] Sun L, Qu L, Zhu R, et al. Effects of mechanical stretch on cell proliferation and matrix formation of mesenchymal stem cell and anterior cruciate ligament fibroblast. Stem Cells Int 2016;2016:9842075.

[13] Parry JA, Wagner ER, Kok PL, et al. A Combination of a polycaprolactone fumarate scaffold with polyethylene terephthalate sutures for intra-articular ligament regeneration. Tissue Eng Part A 2018;24:245–53.

[14] Fu W, Li Q, Tang X, et al. Mesenchymal stem cells reside in anterior cruciate ligament remnants in situ. Int Orthop 2016;40:1523–30.

[15] Muir P, Hans EC, Racette M, et al. Autologous bone marrow-derived mesenchymal stem cells modulate molecular markers of inflammation in dogs with cruciate ligament rupture. PLoS One 2016;11:e0159095.

[16] Kato S, Saito M, Funasaki H, et al. Distinctive collagen maturation process in fibroblasts derived from rabbit anterior cruciate ligament, medial collateral ligament, and patellar tendon in vitro. Knee Surg Sports Traumatol Arthrosc 2015;23:1384–92.

[17] Leong DJ, Sun HB. Mesenchymal stem cells in tendon repair and regeneration: basic understanding and translational challenges. Ann N Y Acad Sci 2016;1383:88–96.

[18] Li Y, Liu Z, Jin Y, et al. Differentiation of human amniotic mesenchymal stem cells into human anterior cruciate ligament fibroblast cells by in vitro coculture. BioMed Res Int 2017;2017:7360354.

[19] Kondo E, Yasuda K, Tohyama H. In vivo effects of partial electrothermal shrinkage on mechanical properties of the anterior cruciate ligament in rabbits. Clin Biomech (Bristol, Avon) 2007;22:1037–44.

[20] Thomas KA, Gibbons MC, Lane JG, et al. Rotator cuff tear state modulates self-renewal and differentiation capacity of human skeletal muscle progenitor cells. J Orthop Res 2017;35:1816–23.

[21] Date H, Furumatsu T, Sakoma Y, et al. GDF-5/7 and bFGF activate integrin alpha2-mediated cellular migration in rabbit ligament fibroblasts. J Orthop Res 2010;28:225–31.

[22] MacLean SB, Gratzer PF. Effect of basic fibroblast growth factor on the cellular repopulation of decellularized anterior cruciate ligament allografts. J Tissue Eng Regen Med 2011;5:201–9.

[23] Fortino VR, Chen RS, Pelaez D, et al. Neurogenesis of neural crest-derived periodontal ligament stem cells by EGF and bFGF. J Cell Physiol 2014;229:479–88.

[24] Kimura Y, Hokugo A, Takamoto T, et al. Regeneration of anterior cruciate ligament by biodegradable scaffold combined with local controlled release of basic fibroblast growth factor and collagen wrapping. Tissue Eng Part C Methods 2008;14:47–57.

[25] Lu D, Yang C, Zhang Z, et al. Enhanced tendon-bone healing with acidic fibroblast growth factor delivered in collagen in a rabbit anterior cruciate ligament reconstruction model. J Orthop Surg Res 2018;13:301.

[26] Schmidt CC, Georgescu HI, Kwoh CK, et al. Effect of growth factors on the proliferation of fibroblasts from the medial collateral and anterior cruciate ligaments. J Orthop Res 1995;13:184–90.

[27] Ghebes CA, Kelder C, Schot T, et al. Anterior cruciate ligament- and hamstring tendon-derived cells: in vitro differential properties of cells involved in ACL reconstruction. J Tissue Eng Regen Med 2017;11:1077–88.

[28] Follin B, Juhl M, Cohen S, et al. Increased paracrine immunomodulatory potential of mesenchymal stromal cells in three-dimensional culture. Tissue Eng Part B Rev 2016;22:322–9.

[29] Kowalski TJ, Leong NL, Dar A, et al. Hypoxic culture conditions induce increased metabolic rate and collagen gene expression in ACL-derived cells. J Orthop Res 2016;34:985–94.

[30] Kim JH, Lee SK, Lee SK, et al. Relaxin receptor RXFP1 and RXFP2 expression in ligament, tendon, and shoulder joint capsule of rats. J Korean Med Sci 2016;31:983–8.

[31] Konopka JA, DeBaun MR, Chang W, et al. The intracellular effect of relaxin on female anterior cruciate ligament cells. Am J Sports Med 2016;44:2384–92.

[32] Ruschke K, Meier C, Ullah M, et al. Bone morphogenetic protein 2/SMAD signalling in human ligamentocytes of degenerated and aged anterior cruciate ligaments. Osteoarthritis Cartilage 2016;24:1816–25.

[33] Hu L, Li Y, Zhang X, et al. Fabrication of magnetic water-soluble hyperbranched polyol functionalized graphene oxide for high-efficiency water remediation. Sci Rep 2016;6:28924.

[34] Ye LB, Yu XC, Xia QH, et al. Erratum to: regulation of caveolin-1 and junction proteins by bFGF contributes to the integrity of blood-spinal cord barrier and functional recovery. Neurotherapeutics 2017;14:828–9.

[35] Wu J, Zhu J, He C, et al. Comparative study of heparin-poloxamer hydrogel modified bFGF and aFGF for in vivo wound healing efficiency. ACS Appl Mater Interfaces 2016;8:18710–21.

[36] Osiecka-Iwan A, Moskalewski S, Hyc A. Influence of IGF1, TGFbeta1, bFGF and G-CSF/M-CSF on the mRNA levels of selected matrix proteins, cytokines, metalloproteinase 3 and TIMP1 in rat synovial membrane cells. Folia Histochem Cytobiol 2016;54:159–65.

[37] Du M, Zhu T, Duan X, et al. Acellular dermal matrix loading with bFGF achieves similar acceleration of bone regeneration to BMP-2 via differential effects on recruitment, proliferation and sustained osteodifferentiation of mesenchymal stem cells. Mater Sci Eng C Mater Biol Appl 2017;70:62–70.

[38] Chen B, Li B, Qi YJ, et al. Enhancement of tendon-to-bone healing after anterior cruciate ligament reconstruction using bone marrow-derived mesenchymal stem cells genetically modified with bFGF/BMP2. Sci Rep 2016;6:25940.

[39] Kohno T, Ishibashi Y, Tsuda E, et al. Immunohistochemical demonstration of growth factors at the tendon-bone interface in anterior cruciate ligament reconstruction using a rabbit model. J Orthop Sci 2007;12:67–73.

[40] Tang JB, Chen CH, Zhou YL, et al. Regulatory effects of introduction of an exogenous FGF2 gene on other growth factor genes in a healing tendon. Wound Repair Regen 2014;22:111–8.

[41] Feng L, Zhang Y, Kou X, et al. Cadherin-11 modulates cell morphology and collagen synthesis in periodontal ligament cells under mechanical stress. Angle Orthod 2017;87:193–9.

[42] Xuan Y, Chi L, Tian H, et al. The activation of the NF-kappaB-JNK pathway is independent of the PI3K-Rac1-JNK pathway involved in the bFGF-regulated human fibroblast cell migration. J Dermatol Sci 2016;82:28–37.

[43] Naaldijk Y, Johnson AA, Ishak S, et al. Migrational changes of mesenchymal stem cells in response to cytokines, growth factors, hypoxia, and aging. Exp Cell Res 2015;338:97–104.

[44] Xie J, Wang C, Huang DY, et al. TGF-beta1 induces the different expressions of lysyl oxidases and matrix metalloproteinases in anterior cruciate ligament and medial collateral ligament fibroblasts after mechanical injury. J Biomech 2013;46:890–8.