Original Paper

Up-Regulation of TGF- β Promotes Tendon-to-Bone Healing after Anterior Cruciate Ligament Reconstruction using Bone Marrow-Derived Mesenchymal Stem Cells through the TGF- β /MAPK Signaling Pathway in a New Zealand White Rabbit Model

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Key Words

TGF- β • Tendon-to-bone healing • Anterior cruciate ligament reconstruction • Bone marrow-derived mesenchymal stem cell • TGF- β /MAPK signaling pathway

Abstract

Background/Aims: This study aimed to explore the role of TGF- β in tendon-to-bone healing after anterior cruciate ligament (ACL) reconstruction using bone marrow-derived mesenchymal stem cells (BMSCs) through the TGF- β /MAPK signaling pathway in a New Zealand white rabbit model. **Methods:** A total of 72 healthy male New Zealand white rabbits were selected for these experiments. Flow cytometry and immunofluorescence were used to detect the expression of BMSC surface markers, and qRT-PCR was performed to detect TGF- β mRNA expression. The ACL reconstruction model was established with autografts. The rabbits were randomly divided into the following groups: inhibition of TGF- β (inhibition), over-expression of TGF- β (over-expression), empty vector and untreated (n = 18 per group). Hematoxylin-eosin (HE) staining, toluidine blue staining and Masson trichrome staining were conducted to observe any chondrocyte-like cell growth, and biomechanical tests were used to calculate the maximum load and rigidity. Three-dimensional CT imaging and Western blotting were applied to detect changes in bone tunnel size and bone density and the expression levels of TGF- β /MAPK signaling pathway-related proteins, respectively. **Results:** CD90 and CD44 were positively expressed, while CD11b was not detected. Compared with the empty vector and untreated groups, TGF- β mRNA expression was significantly decreased in the inhibition group but increased in the over-expression group; the latter group had a larger number of fibroblasts, a tighter tendon-bone interface, an increased number of chondrocyte-like cells and fibrochondrocytes, and more collagen fibers than the inhibition, empty vector and

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untreated groups. Compared with the empty vector and untreated groups, the maximum load and rigidity; the CT values of bone tunnel and bone tunnel margin; and the protein expression levels of TGF- β , p-ERK1/2, p-p38, p-JNK, c-jun and c-myc were significantly down-regulated in the inhibition group but up-regulated in the over-expression group. **Conclusion:** Our study indicated that up-regulating TGF- β expression in BMSCs from New Zealand white rabbits could promote tendon-to-bone healing after ACL reconstruction by regulating the TGF- β /MAPK signaling pathway.

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Introduction

The anterior cruciate ligament (ACL) is a vital component in the knee and plays an important role in protecting the stability of knee joint and maintaining its normal function. Tearing of the ACL injury is a severe and common injury during sports activities, leading to functional impairment along with disabilities due to knee joint laxity, reduced quadriceps strength, meniscal injuries and poor knee joint loading [1]. The key healing process after ACL reconstruction primarily relies on the integration of the graft with the host bone. Unfortunately, slow and incomplete healing of the tendon-to-bone interface can manifest from inappropriate treatment regimens, which results in knee joint instability, movement dysfunction, cartilage damage and intra-articular meniscus and ultimately leads to inferior functional rehabilitation as well as worsening osteoarthritis of the knee [2, 3]. Recently, surgical reconstruction coupled with soft tissue autograft or allograft, including the patellar tendon and hamstring tendon, has become the standard treatment for a torn ACL [4, 5]. Generally, these autografts can restore initial knee stability, but there remain several associated issues. Approximately a quarter of patients have dissatisfactory long-term results, with clinical outcomes indicative of osteoarthritic changes in the knee joint [1]. Since the success and failure of surgery are largely dependent on the biological healing between the bone tunnel and graft, and the early connection of tendon-bone interface has been shown to be the weakest link [6], it is pivotal to investigate better strategies to improve the healing of tendon-to-bone interface.

Stem cell therapy has been proposed as having promising biological potential for tendon-bone healing following ACL reconstruction [7]. Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent stem cells and have become an increasingly important source of cells for engineered cell therapy and tissue repair [8]. BMSCs can differentiate into diverse cells *in vitro*, such as osteoblasts and chondrocytes, and the osteogenic capabilities augment the regeneration of bone, muscle, tendon and ligament *in vivo* [9,10]. The osteogenic differentiation capacity of BMSCs has been characterized in several *in vitro* studies [11, 12]. Additionally, experiments based on small animal models have implied the ability of BMSCs to form bone *in vivo* when implanted with biodegradable scaffolding, which highlights a great prospect for therapeutic application [13, 14]. Interestingly, transforming growth factor-beta (TGF- β) is believed to induce stem cell differentiation towards both ligamentous and bone tissue at the end and the central region of the biomaterial [15]. However, the role of TGF- β in BMSCs is not completely understood regarding tendon-to-bone healing after ACL. Our study aims to explore the role of the TGF- β /MAPK signaling pathway in tendon-to-bone healing after ACL reconstruction using BMSCs in a New Zealand white rabbit model.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University and was conducted in strict accordance with the standard of Instructive Notions with Respect to Caring for Laboratory Animals published by the Ministry of Science and Technology of the People's Republic of China in 2006.

Experimental animals

A total of 72 healthy male New Zealand white rabbits (aged 4 ~ 6 months; weight 2.1 ~ 2.9 kg) and 2 newborn male New Zealand white rabbits were obtained from the Animal Experiment Center of Tianjin Hospital. All rabbits were in good condition and did not have any other related diseases based on examination.

Collection of platelet rich-plasma (PRP)

PRP was prepared by using the Landesberg two-step centrifugation method. Whole blood (10 mL) was collected into trisodium citrate (an anticoagulant) from the central artery near the ear margin after the rabbits were anesthetized. The blood was centrifuged at $200 \times g$ for 10 min to separate the supernatant (3 mm under the interface), which was transferred into another tube and centrifuged again at $200 \times g$ for 10 min. The liquid in the centrifuge tube was separated into two layers; the upper supernatant comprised platelet-poor plasma. Approximately 75% of the supernatant was removed, and the remaining fluid was collected as PRP.

Isolation, culture and identification of BMSCs

A 10-mL injector was washed with 0.2 mL heparin (3000U; Sigma-Aldrich Chemical Company, St Louis, MO, USA) and used to obtain 5 mL of bone marrow from two 3-day-old male New Zealand white rabbits. The marrow was washed with phosphate-buffered saline (PBS) and centrifuged at 1200 r/min for 10 min, after which the supernatant was discarded. The cell pellet was resuspended in Dulbecco's modified Eagle medium (DMEM)/F-12 medium (Sigma-Aldrich Chemical Company, St Louis, MO, USA) supplemented with penicillin, streptomycin and 15% fetal bovine serum (FBS), and the suspension was slowly added into the same volume of Percoll lymphocyte separation medium (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). After centrifugation at 2500 r/min for 20 min, the bone marrow mononuclear cells were separated, and the cells above the interface were collected and moved into new sterile centrifuge tube for a second centrifugation to collect the pellet. The remaining cells were resuspended in DMEM/F-12 medium and then cultured in a 37°C incubator containing 5% CO₂, and the medium was changed every three days. When the cells reached 100% confluency, trypsin was added to lift the cells. The common surface markers of BMSCs, including CD44, CD90 and CD11b, were detected by flow cytometry and immunofluorescence. Third-generation BMSCs were seeded into a 24-well plate with a cover slip and cultured overnight. After the medium was discarded, the cells were fixed in 4% paraformaldehyde (Shanghai Suran Chemicals Co. Ltd., Shanghai, China), sealed with confining liquid and washed with PBS (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The cells were then incubated with primary antibodies against CD11b (No. ab75476) and CD44 (No. ab157107, both diluted at 1:500; Abcam Inc., Cambridge, MA, USA) followed by treatment with a fluorescent secondary antibody targeting the aforementioned primary antibodies or the addition of CD90-FITC antibody (No. ab225; diluted at 1:500; Abcam Inc., Cambridge, MA, USA). The cells were dyed with Hoechst (Roche Holding AG, Basel, Switzerland), sealed with glycerium, observed under the fluorescence microscope (Nikon Eclipse 80i, Nikon Inc, Tokyo, Japan) and photographed. After a 30 min incubation in the dark condition and the addition of a goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC), the cells were centrifugated at 1000 r/min for 5 min, after which the supernatant was removed, and 200 μ L of 1% polyoxyethylene was added. The cells underwent flow cytometry (BD FACSAria; Becton, Dickinson and Company, USA) to detect BMSCs.

Plasmid construction and cell transfection

Based on the TGF- β DNA sequences provided by the National Center of Biotechnology Information (NCBI), primers targeting TGF- β were designed (Forward: GCCCTGGACACCAACTACTG; Reverse: TCAGCTGCACTTGCAGGAG). The targeted DNA fragment was obtained by amplifying DNA from rabbit blood followed by cloning and sequencing, through which the full-length sequence of TGF- β mRNA was obtained. The Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. was authorized to construct the inhibitory and over-expression plasmid vectors with a pcDNA3 backbone by using the antisense RNA sequences. After three passages, the BMSCs were digested with pancreatin, seeded into a 6-well plate and cultured for 24 h until a monolayer formed. The medium was then removed, and the cells were divided into the inhibition group (inhibition of TGF- β), over-expression group (over-expression of TGF- β), empty vector group and untreated group. Suspensions (200 μ L) of inhibitory TGF- β plasmid, over-expressing TGF- β

plasmid and empty vector were added into the respective cell groups. No plasmid was added into the blank group. In addition, 200 μ L culture solution of serum-free DMEM (Gibco Company, Grand Island, NY, USA) was supplemented into the 6-well plate, followed by the addition of 1 mL DMEM culture solution containing 10% FBS (HyClone (Thermo Scientific Inc.), Logan, UT, USA) 2 ~ 3 h later. Cell growth was observed under a microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA in the cells from the four groups was extracted using a TRIzol kit following the manufacturer's instructions and quantified by ultraviolet spectrophotometry. Approximately 0.5 μ g of total RNA was used for RT-PCR. The sequence of the upstream-specific PCR primer (sense strand) was 5'-CCAACTATTGCTTCAGCTCCA-3', and that of downstream PCR primer (antisense strand) was 5'-GTGTCCAGGCTCCAAATGT-3'. The length of PCR product was 157 bp. GAPDH was used as the internal reference (sense, 5'-AATGCATCCTGCACCACCA-3'; antisense 5'-GTAGCCATATTCATTGTCATA-3') and produced a PCR product 515 bp in length. The primers targeting GAPDH and the genes of interest were amplified in the same tube. The reaction condition of PCR were as follows: 1 cycle at 94°C for 20 min; 25 cycles at 94°C for 10 s, 58°C for 30 s and 60°C for 40 s; 15 cycles at 94°C for 10 s, 55°C for 30 s and 72°C for 40 s; and 1 cycle at 72°C for 5 min. The sample was subsequently maintained at 4°C until further use. A negative control tube was prepared at each time of amplification. The PCR products (5 μ L) were separated on a 2% agarose gel using electrophoresis. A QualityOne system was applied to detect and analyze the density of the protein bands. The cell transfection efficiency in each group was assessed by measuring the TGF- β mRNA levels detected by using qRT-PCR.

Establishment of an ACL reconstruction model and rabbit grouping

To establish an ACL reconstruction model, 2.5% sodium pentobarbital solution (1 mL/kg) was injected into the ear vein of rabbits as anesthetic after they were weighed. The skin around the knee was prepared, disinfected and covered with towels. The flexor digitorum longus tendon of the hind limb was obtained as the autograft for ACL reconstruction, and its diameter (2.5 ± 0.3 mm) and length (6.2 ± 0.3 mm) were measured. The two ends of tendon were stitched with No. 2 braided wire, and a 10-cm suture was left in the seam as the traction thread. The unilateral knee joint and medial patellar retinaculum were separated in the medial parapatellar approach, but a lateral dislocation was performed to expose the knee joint [16]. After complete removal of the ACL (no remnant preservation for ACL insertion at the top and bottom ends), the anterior drawer and Lachman tests were used to test positive expression. Then, a bone tunnel ($d = 2.5$ mm) was created from the inside of the tibial eminence to the original tibial insertion point of the ACL. The hole ($d = 2.0$ mm) that was drilled at the attachment point of ACL was used as the tunnel between the tibia and femur. The soft tissue around the hole was removed, and the tendon was passed through the tibial and femoral tunnels led by the perforated traction thread. The press-fit technique was applied to affix the femoral tendon on the soft tissue. After repeated stretches, the knee joint was bent at 30° to strain the tendon and allow for the tibial tendon to be affixed. The joint cavity was washed, and 0.4 mL of 3% PRP and transfected BMSCs (inhibition, overexpression, empty vector, or untreated; $n=18$ per group) were injected into the bone tunnel and graft tendon cavity, respectively [17]. The patella was restored, and the incision was sutured. The diseased limb was not fixed after operation. Postoperatively, animals were injected with penicillin (20 mU/day) for a week. The rabbits were permitted to engage in all activities, and plaster immobilization was performed at the end of the hind legs. The rabbits were all in good condition, and no significant differences were found regarding age and weight among four groups (all $P > 0.05$).

Knee-joint specimen collection and general anatomical observation

At either 6 or 12 weeks after ACL reconstruction, 9 rabbits in each group were euthanized, and the air embolism method was employed to intravenously inject approximately 15 mL air. Before dissection, the crack of the knee joint incision, the inflammation and infection of the knee joint, and the tension and slackness of the tendon graft were observed. After the dissection, the following parameters were recorded: the presence of edema, necrosis and rupture of the joint, osteophyte formation in the femoral and tibial bones, hyperplasia of the synovium, closure status of internal and external interfaces of the femoral and tibial bone tunnels, conditions of the meniscus, the presence of scar tissue in the internal and external interfaces of the bone tunnels, the growth of osteoid tissues and injury to the cartilage in the articular cavity.

During the dissection, the joint capsule was opened through the previous incision, and the muscular fascia and periosteal tissue were removed. Subsequently, the thighbone was truncated by a hacksaw at 3 cm above the knee, and knee-joint and joint specimens were obtained. The left knee-joint specimen was used for the biomechanical test, and the right knee-joint specimen was used for histological inspection.

Histological observation

Hematoxylin-eosin (HE) staining, toluidine blue staining and Masson trichrome staining were conducted on paraffin sections of the specimens to observe the growth of chondrocyte-like cells. For HE staining, the specimens were fixed in 10% formaldehyde, conventionally dehydrated, embedded in paraffin, sectioned, deparaffinized, stained with HE, transparentized, sealed and observed under an optical microscope to identify histomorphological changes in the joint. The nuclei were stained blue, and the cytoplasm was stained red. The image analysis software Image-Pro Plus was used to count the number of fibroblasts. The mean was calculated from three randomly selected visual fields. For toluidine blue staining, tissue sections were deparaffinized with water, soaked in a toluidine blue ($C_{15}H_{16}N_3$, Biofer S.p.A) and ethanol solution (Shanghai Zhongshi General Chemical Company, Shanghai, China), washed with water, dehydrated by using an alcohol gradient, transparentized with xylene, sealed up by using neutral balsam and observed under a microscope. For Masson trichrome staining, the tissue sections were deparaffinized with water, treated with a Masson staining fluid (Fuzhou Maixin Biotech. Co., Ltd., Fuzhou, China) for 5 min, washed with distilled water, incubated with aniline blue, differentiated in a differentiation liquid, washed in distilled water again, dehydrated by using an alcohol gradient, transparentized with xylene, sealed up by using neutral balsam and observed under a microscope.

Biomechanical test

The left knee-joint specimen obtained at either 6 or 12 weeks after ACL reconstruction was used for the biomechanical test. A 4-cm section on the upper and lower ends of the left knee joint was preserved, and all other soft tissues except the anterior cruciate ligament were removed. The fixture was installed on both sides of the joint, which was fixed on a mechanical testing machine (Scientific & Technical Instruments, Norwood, Massachusetts, UK). Initially, laxity in the tendon was eliminated with a preloading force (2 N). After measuring the tendon length, the tensile test was conducted, in which the tendon was stretched at the speed of 1 mm/s until rupture. The experimental data were recorded by a machine to calculate the maximum load and rigidity.

Three-dimensional CT imaging

At 12 weeks after ACL reconstruction, the rabbits were anesthetized and euthanized to obtain the knee joints. Three-dimensional CT images of the knee joints were collected, and changes in the bone tunnel size and density were observed.

Western blotting

At 12 weeks after the model was established, the white rabbits in the inhibition, over-expression, empty vector and untreated groups were euthanized. Blood (3 mL) from the auricular vein of rabbits was collected into a vacuum tube containing ethylenediamine tetraacetic acid (EDTA)-K2 anticoagulant, stored at 4°C for 2 h, and centrifuged at 4000 r/min for 5 min. Then, 50 μ L of serum was transferred into a 0.5 mL centrifuge tube with 200 μ L of lysate and centrifuged for 15 min at 13000 r/min at 4°C. The bicinchoninic acid (BCA) protein assay system (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was applied to quantify the proteins in the resultant supernatant. After loading buffer was added to total protein (20 μ g per well), the samples were boiled for 5 min for protein degeneration and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel under a constant voltage of 90 V until the bromophenol blue reached the top of separation gel followed by a constant voltage of 120 V until the bromophenol blue reached the bottom of separation gel. The proteins were electrophoretically transferred at a constant current of 100 mA. The membranes were blocked with 5% skim milk in Tris-buffered saline containing Tween (TBST) at room temperature for 2 h. Primary antibodies against TGF- β (No. ab66043), p-ERK1/2 (No. ab17942), p-p38 (No. ab31828), p-JNK (No. ab124956), c-jun (No. ab32385) and c-myc (No. ab51156) (Abcam Inc., Cambridge, MA, USA) were all diluted at 1 : 800 and incubated with the membranes at 4°C overnight. After 3 subsequent washes in TBST (10 min/wash), the membrane was treated with

secondary antibody at room temperature for 1 h and washed with TBST 3 times (10 min/wash). Finally, the membranes were subjected to chemiluminescence prior to development on X-ray film and photographic imaging to determine the results.

Statistical analysis

Data were analyzed using SPSS 21.0 (International Business Machine, Armonk, NY, USA). Measurement data were displayed as the mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups, and the least significant difference method (LSD) was applied for comparisons between two groups. $P < 0.05$ was regarded as statistically significant.

Results

Expression of BMSC surface markers

According to the flow cytometry results, CD90 and CD44 were prominently expressed (97.3% and 74.9% positive, respectively), while CD11b was barely expressed (0.11% positive) (Fig. 1A). The immunofluorescence assay showed that CD44 and CD99 were expressed as indicated by the red and green fluorescence, respectively, whereas CD11b was negatively expressed (Fig. 1B). The blue fluorescence highlights the round nuclei of the BMSCs. These results indicated that BMSCs were successfully separated and cultured.

Growth conditions of rabbit BMSCs

After they were seeded, the primary BMSCs were round in shape with bright soma, good refractivity and an oval cell nucleus. The cells started to adhere to the surface after 4 h. After 12 h, the cells gradually changed into a short fusiform shape with a few cell protrusions and large and oblate cell nuclei. After 48 h, the medium was changed, and most of the suspension cells were aspirated leaving the short fusiform cells left on the wall. Three days later, cell colonies started to form in long fusiform and polygonal shapes (Fig. 2A). The fusiform cell protrusions became longer, cell proliferation accelerated, cell colonies enlarged and increased, and cells presented clone-like growth and became confluent. The cells were arranged in an orderly fashion along the longitudinal axis of the soma in a school-of-fish, spiral or radial arrangement (Fig. 2B). Cells digested by trypsin changed from a long prismatic shape into spherical and showed good refractivity upon suspension in the culture solution. Several hours after they were passaged, most cells adhered to the wall and grew evenly with uniform morphology, appearing as long fusiform fibroblast cells (Fig. 2C).

Fig. 1. Expressions of BMSCs surface markers detected by flow cytometry and immunofluorescence. Note: (A) expressions of BMSCs surface markers detected by flow cytometry; (B) expressions of BMSCs surface markers detected by immunofluorescence ($\times 100$); BMSCs, bone marrow-derived mesenchymal stem cells.

Fig. 2. Growth condition of primary and passaged BMSCs ($\times 100$). Note: (A) primary BMSCs growth in short fusiform shape (the 3rd day); (B) primary BMSCs growth in fish school, spiral or radial shapes (the 6th day); (C) the uniform and stable third generation BMSCs in long fusiform shape (the 6th day); BMSCs, bone marrow-derived mesenchymal stem cells.

Fig. 3. Expressions of TGF- β mRNA in the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups detected by qRT-PCR. Note: *, $P < 0.05$ compared with the empty vector and blank groups; #, $P < 0.05$ compared with the over-expressed TGF- β group; qRT-PCR, quantitative real-time polymerase chain reaction.

Comparisons of the TGF- β mRNA expression levels among the four groups

The TGF- β mRNA expression levels in the inhibition, over-expression, empty vector and untreated groups were detected by qRT-PCR. As shown in Fig. 3, TGF- β mRNA expression in the inhibition group was significantly decreased, but the levels in the over-expression group were significantly increased compared with the empty vector and untreated groups (all $P < 0.05$). The results suggested that the transfections were successful.

Comparisons of the general anatomical observations of the knee joints among the four groups at 6 and 12 weeks after operation

The experimental rabbits recovered well and behaved normally after ACL reconstruction, and neither death nor infection was found. Six weeks after the operation, no significant differences were found in the knee joints among the inhibition, over-expression, empty vector and untreated groups. A small amount of scar tissue and bone tissue were observed in the internal opening of the femoral and tibial tunnels, and a lack of necrosis of the fracture and some synovium hyperplasia were also noted. Twelve weeks after the operation, there was decreased scar tissue near the joint tunnel opening, increased chondroid and osteoid tissues, a fully closed bone tunnel, an intact meniscus and generally stable tissue observed in the over-expression group. Compared with the over-expression group, the cell growth and recovery were slower in the empty vector group, which also presented neither an obvious decrease in the scar tissue nor a fully closed bone tunnel, but the meniscus was in good condition. The joint growth and recovery in the inhibition group were the slowest, and increased scar tissue and bone tissue in the internal opening of the femoral and tibial tunnels as well as fracture necrosis were observed.

Fig. 4. HE staining in the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups ($\times 100$) 6 and 12 weeks after operation. Note: HE, hematoxylin and eosin.

Table 1. Comparisons of the fibroblast number at the tendon-bone junction among the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups 6 and 12 weeks after operation. Note: *, $P < 0.05$ compared with the empty vector and blank groups; #, $P < 0.05$ compared with the over-expressed group

Group	Case	6 weeks	12 weeks
The inhibited TGF- β group	9	$6.69 \pm 0.99^{*#}$	$5.14 \pm 0.81^{*#}$
The over-expressed TGF- β group	9	14.24 ± 1.05	12.96 ± 1.01
The empty vector group	9	13.36 ± 1.13	11.84 ± 1.11
The blank group	9	13.05 ± 0.96	11.19 ± 1.02

Comparisons of the HE, toluidine blue and Masson trichrome staining among the four groups at 6 and 12 weeks after the operation

HE staining of paraffin sections obtained 6 and 12 weeks after the operation are shown in Fig. 4. After 6 weeks, it was found that in the inhibition group, the number of inflammatory cells and fibroblasts was increased at the tendon-bone junction, but there was no tendon-bone connection. In the over-expression group, a cartilage transitional zone had formed at the tendon-bone junction, the neatly arranged chondrocyte-like cells had gradually matured, and a large quantity of collagen fibers were produced. In the empty vector and untreated groups, the collagen fibers were increased but not uniformly arranged, which provided a tight association between the tendon and the bone. After 12 weeks, there was no obvious recovery of the operative site and no junction between the tendon graft and bone observed in the inhibition group. However, a small amount of loose connective tissue, a few fibroblasts and new bone tissue were identified. In the over-expression group, there was obvious recovery at the operative site occurred, and many regular and compact sharp fibers and dense connective tissues had formed with a close joint junction. In the empty vector and untreated groups, the recovery was worse than that in the over-expression group but better than that in the inhibition group with regard to the tight tendon-bone junction. The number of fibroblasts in the HE-stained sections in each group 6 and 12 weeks after the reconstruction operation is recorded in Table 1. The fibroblast number in the inhibition group was significantly lower than that in the empty vector and untreated groups (all $P < 0.05$). However, no significant difference was noted between the over-expression group and either the empty vector groups or the untreated groups (all $P > 0.05$).

Toluidine blue staining of the paraffin sections collected 6 and 12 weeks after the operation are shown in Fig. 5. After 6 weeks, there were several scattered chondrocyte-like cells observed at the tendon-bone interface in the inhibition group, but there was no tendon-bone junction. In the over-expression group, there was a profound number of chondrocyte-like cells stained by toluidine blue at the tendon-bone interface. In the empty vector and untreated groups, only a small number of toluidine blue-positive chondrocyte-like cells were found. After 12 weeks, the graft tendons in the inhibition group were separated from the bone interface, and a small amount of loose connective tissue was noted. Conversely, the over-expression group experienced a rapid recovery and a plethora of chondrocyte-like cells

Fig. 5. Toluidine blue staining in the inhibited TGF- β , over-expressed TGF- β and empty vector groups 6 and 12 weeks after operation ($\times 100$).

Fig. 6. Masson trichrome staining in the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups 6 and 12 weeks after operation ($\times 100$).

Table 2. Comparisons of the mean maximum load and rigidity of the bone-ligament-bone complex among the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups 6 and 12 weeks after operation. ($\bar{x} \pm s$, $n = 9$). Note: *, $P < 0.05$ compared with the empty vector and blank groups; #, $P < 0.05$ compared with the over-expressed group; N, Newton

Group	Maximum load (N)		Rigidity (N/mm)	
	6 weeks	12 weeks	6 weeks	12 weeks
The inhibited TGF- β group	13.73 $\pm$ 1.43**	38.14 $\pm$ 3.86**	2.24 $\pm$ 1.02**	9.11 $\pm$ 0.85**
The over-expressed TGF- β group	47.15 $\pm$ 4.72*	90.74 $\pm$ 9.26*	20.33 $\pm$ 4.38*	37.84 $\pm$ 7.01*
The empty vector group	25.21 $\pm$ 3.11	56.38 $\pm$ 6.98	6.39 $\pm$ 1.79	17.96 $\pm$ 6.77
The blank group	26.72 $\pm$ 2.95	58.66 $\pm$ 7.21	6.57 $\pm$ 1.50	18.43 $\pm$ 6.18

Table 3. Comparisons of CT values of bone tunnel and bone tunnel margin among the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups 12 weeks after operation. ($\bar{x} \pm s$, $n = 6$). Note: *, $P < 0.05$ compared with the empty vector and blank groups; #, $P < 0.05$ compared with the over-expressed group; CT, computed tomography

Group	CT value of bone tunnel (HU)	CT value of bone tunnel margin (HU)
The inhibited TGF- β group	303.9 $\pm$ 23.6**	616.2 $\pm$ 42.9**
The over-expressed TGF- β group	549.8 $\pm$ 42.6*	1096.5 $\pm$ 61.3*
The empty vector group	456.8 $\pm$ 18.6#	825.7 $\pm$ 32.8#
The blank group	433.5 $\pm$ 22.4	796.6 $\pm$ 35.7

and fibrochondrocytes, which grew evenly and regularly at the tendon-bone interface. In the empty vector group, a small number of chondrocyte-like cells were produced, some loose connective tissues were formed between the graft tendon and bone tunnel, and the tendon-bone junction was loosely linked.

Masson trichrome staining of the paraffin sections obtained 6 and 12 weeks after the operation are shown in Fig. 6. After 6 weeks, the inhibition group presented a small number of fibroblasts produced between the tendon graft and bone tunnel, but no collagen fibers

Fig. 7. Three-dimensional CT images of rabbit knee joint in the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups 12 weeks after operation. Note: CT, computed tomography.

Fig. 8. The serum levels of TGF- β /MAPK signaling pathway-related proteins in the inhibited TGF- β , over-expressed TGF- β , empty vector and blank groups detected by Western Blotting 12 weeks after operation. Note: (A) expression bands of TGF- β /MAPK signaling pathway-related proteins in four groups; (B & C) serum levels of TGF- β /MAPK signaling pathway-related proteins in four groups; *, $P < 0.05$ compared with the empty vector and blank groups; #, $P < 0.05$ compared with the over-expressed TGF- β group.

were observed. In the over-expression group, the collagen fibers between the tendon graft and bone tunnel were obviously increased, neatly arranged, and vertically linked to the bone tunnel wall. In the empty vector and untreated groups, fibroblast hyperplasia and the number of collagen fibers were increased, but only limited linkage to the bone tunnel wall was observed. After 12 weeks, the inhibition group showed a clear tendon-bone junction with noticeable fibroblasts and collagen fibers, but there was only a small connection with the bone tunnel. In the over-expression group, a closely linked tendon-bone tunnel was noted, and a sufficient number of chondrocytes and calcified cartilage cells were produced at the tendon-bone junction. In the empty vector and untreated groups, dense collagen fibers were present among the graft tendon, bone tunnel and sharp fibers, which were perpendicular to the bone tunnel and irregularly arranged.

Comparisons of the mean maximum load and rigidity among the four groups at 6 and 12 weeks after the operation

The mean maximum load and rigidity of the bone-ligament-bone complex at 6 and 12 weeks after the operation in the inhibition, over-expression, empty vector and untreated groups are listed in Table 2. Compared with the empty vector and untreated groups, the maximum load and rigidity were lower in the inhibition group but higher in the over-expression group (all $P < 0.05$).

Comparisons of the CT values of the bone tunnel and bone tunnel margin among the four groups 12 weeks after the operation

The CT results of the bone tunnel at 12 weeks after the operation are shown in Fig. 7 and Table 3. The CT values of the bone tunnel in the inhibition, over-expression, empty vector and untreated groups were 303.9 ± 23.6 HU, 549.8 ± 42.6 HU, 456.8 ± 18.6 HU and 433.5 ± 22.4 HU, respectively. The CT values of the bone tunnel margin in the inhibition, over-expression, empty vector and untreated groups were 616.2 ± 42.9 HU, 1096.5 ± 61.3 HU, 825.7 ± 32.8 HU and 796.6 ± 35.7 HU, respectively. Compared with the empty vector and untreated groups, the CT values of the bone tunnel and bone tunnel margin were significantly decreased in the inhibition group but remarkably increased in the over-expression group (all $P < 0.05$).

Comparisons of the expression levels of TGF- β /MAPK signaling pathway-related proteins among the four groups 12 weeks after operation

The relative protein expression levels in the inhibition, over-expression, empty vector and untreated groups at 12 weeks after establishment of the model are shown in Fig. 8. Compared with the empty vector and untreated groups, the protein expression levels of TGF- β , p-ERK1/2, p-p38, p-JNK, c-jun and c-myc were significantly down-regulated in the inhibition group (all $P < 0.05$) but remarkably up-regulated in the over-expression group (all $P < 0.05$). No significant differences were found in the protein expression of ERK1/2 and p38 among the four groups (all $P > 0.05$).

Discussion

The present study aims to clarify the role of the TGF- β /MAPK signaling pathway in tendon-to-bone healing after ACL reconstruction by using BMSCs in a New Zealand white rabbit model. This work concludes that by using BMSCs as a carrier, the over-expression of TGF- β significantly promoted the tendon-to-bone healing after ACL reconstruction. The results lay a theoretical foundation for the further understanding of the molecular mechanism involved in tendon-to-bone healing after ACL reconstruction and provide a new therapeutic strategy to accelerate the process of tendon-to-bone healing.

In our study, it was found that compared with the empty vector and untreated groups, the expression of TGF- β , p-ERK1/2, p-P38, JNK, c-jun and c-myc proteins was significantly down-regulated in the inhibition group while up-regulated in the over-expression group. The TGF- β /MAPK signaling pathway plays an important role in cell development, including differentiation, migration and proliferation [18]. ERK1/2 and ERK5, JNK1/2/3, and the p38 isoforms are members of the conventional MAPK family [19]. It was pointed out that ERK1/2 is activated by PI3K/Akt triggered by the binding of TGF- β with its receptors, indicating that TGF- β may induce MAPK proteins [20, 21]. More importantly, TGF- β can regulate MAPK (e.g., ERK, JNK, and p38) signaling in a cell-specific manner [22, 23]. Interestingly, the activation can be either slow or rapid based on different kinetics and magnitudes [24]. Largely consistent with our study, a previous study reported by Li et al. demonstrated that ERK1/2 and p38 signals are activated by TGF- β 1 in chondrogenesis and increase upon up-regulation of TGF- β 1 [25]. In addition, Atfi et al. noted that TGF- β leads to a significant and constant increase in JNK activity in a dose-dependent manner with maximal sensitization at 12 h [26].

Based on general anatomical and histological observations in this study, over-expression of TGF- β was associated with a better recovery at the surgical site, a tighter tendon-bone junction, a larger number of fibroblasts, increased formation of cartilage cells and fibrochondrocytes, and elevated numbers of cartilage-like cells and collagen fibers compared with the inhibition, empty vector and untreated groups. In addition, the scar tissues near the tunnel entrances were reduced, the bone tunnel was completely sealed and the tissues stably grew. To the best of our knowledge, bone formation and tendon-to-bone healing are important factors in ACL reconstruction, and the healing of the tendon-to-bone interface is crucial to some orthopedic procedures and essential for the long-term survival of patients undergoing ACL reconstruction [27, 28]. BMSCs are considered to be multipotent cells that can differentiate into mesenchymal-lineage cell types both *in vivo* and *in vitro* and have long been identified as promising molecules for engineered tissue repair and cell therapy [6, 25]. Lim et al. noted that MSCs promote better ACL reconstruction by biologically changing the normal healing process of the hamstring tendon grafts to their ambient bone tunnels, and the application of MSCs to the tendon grafts enhances graft osteointegration and healing through the formation of an intervening chondroid layer resembling the chondral enthesis of normal ACL insertions [29]. As Ouyang et al. reveals, increased fiber formation and proliferation of cartilage-like cells at the tendon-bone interface are presented at 4 weeks after subjects received BMSCs, indicating that applying BMSCs to the bone tunnel can promote the insertion healing of tendon-to-bone in a rabbit model [30]. Li et al. also verified that BMSCs have a promising ability to improve tendon-to-bone tunnel healing at 4-8 weeks after surgery based on the results from their experiment group [31]. On the other hand, TGF- β promotes the differentiation of MSCs that may act as potential graft cells in MSC-induced therapy [32]. TGF- β 1, which is activated and released by osteoclasts, induces the migration of BMSCs to bone resorptive locations and promotes bone resorption as well as bone formation in adult mice, indicating a key role of TGF- β 1 activity in bone remodeling diseases [33]. Moreover, TGF- β 1 is the most important fibrotic inducer, which can explain the increased of collagen fibers associated with TGF- β overexpression [34]. Stanton et al. stated that the two MAPKs, ERK and p38, are also involved in TGF- β -mediated chondrocyte function, including cell proliferation and differentiation [35]. Activation of ERK and p38 MAPK signaling by TGF- β 1 in the chondrogenesis of rat BMSCs exerts complex effects on the expression of chondrogenic-specific genes, implying that ERK and p38 regulate the chondrogenesis of rat BMSCs by interaction with the TGF- β 1 signaling pathway [25]. Thus, we assume that with BMSCs as a carrier, TGF- β over-expression may promote new bone formation in the tendon-bone tunnel.

Additionally, the biomechanical test revealed that the mean maximum load and rigidity were higher in the over-expression group than in the empty vector and untreated groups, with the lowest levels in the inhibition group. Largely consistent with our study, Lim et al. found that the failure loads and rigidity in MSC-treated reconstructions were significantly higher than those in the control reconstructions at 8 weeks, and this study also proposed that MSCs can affect the biomechanical characteristics of the tendon [29]. In addition, the CT values of the bone tunnel and bone tunnel margin in the over-expression group were significantly increased, suggesting that new bone formation and the bone mineral density of tunnel is greater in the over-expression group. Three-dimensional CT is commonly used for the postoperative evaluation of bone formation and bone mineral density after ACL reconstruction, and it was found that CT depicts the tunnel more accurately than radiographs [36]. A previous study reported that bone mineral density plays a key role in the strength of tendon graft fixation during ACL reconstruction [37]. Furthermore, Månsson et al. reported that low bone mineral density is considered a main risk factor for fracture; thus, we can conclude that increasing the bone mineral density is associated with the improvement of the degree of ossification [38]. Therefore, the CT scan in this study further confirmed the proactive function of BMSCs on bone formation.

In conclusion, TGF- β over-expression in BMSCs can improve bone formation and tendon-to-bone healing in the bone tunnel after ACL reconstruction. These findings will provide a

better understanding of the TGF- β /MAPK signaling pathway in the process of tendon-to-bone healing via BMSCs. Further research is required with larger sample size and more long-term follow-up observations to identify the long-term healing effects and contribute to the development of relevant drugs for treating ACL injury.

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Disclosure Statement

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