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Exosomes Derived from Bone Marrow Stromal Cells (BMSCs) Enhance Tendon-Bone Healing by Regulating Macrophage Polarization

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Background: Inflammation after tendon-bone junction injury results in the formation of excessive scar tissue and poor bio-mechanical properties. Recent research has shown that exosomes derived from bone marrow stromal cells (BMSCs) can modulate inflammation during tissue healing. Thus, our study aimed to enhance tendon-bone healing by use of BMSC-derived exosomes (BMSC-Exos).


Material/Methods: The mouse tendon-bone reconstruction model was established, and the mice were randomly divided into 3 groups: the control group, the hydrogel group, and the hydrogel+exosome group, with 30 mice in each group. At 7 days, 14 days, and 1 month after surgery, tendon-bone junction samples were harvested, and the macrophage polarization and tendon-bone healing were evaluated based on histology, immunofluorescence, and quantitative RT-PCR (qRT-PCR) analysis.

Results: In the early phase, we observed significantly higher numbers of M2 macrophages and more anti-inflammatory and chondrogenic-related factors in the hydrogel+BMSC-Exos group compared with the control group and the hydrogel group. The M1 macrophages and related proinflammatory factors decreased. Cell apoptosis decreased in the hydrogel+BMSC-Exos group, while cell proliferation increased; in particular, the CD146+ stem cells substantially increased. At 1 month after surgery, there was more fibrocartilage in the hydrogel+BMSC-Exos group than in the other groups. Biomechanical testing showed that the maximum force, strength, and elastic modulus were significantly improved in the hydrogel+BMSC-Exos group.

Conclusions: Our study provides evidence that the local administration of BMSC-Exos promotes the formation of fibrocartilage by increasing M2 macrophage polarization in tendon-to-bone healing, leading to improved biomechanical properties. These findings provide a basis for the potential clinical use of BMSC-Exos in tendon-bone repair.

MeSH Keywords: **Bone-Patellar Tendon-Bone Grafts • Exosomes • Inflammation • Macrophages • Mesenchymal Stromal Cells**

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Background

Tendon-bone healing is still a challenge in orthopedics and sports medicine research and clinical practice. The normal tendon-bone junction is a complex and heterogeneous structure that includes bone, mineralized fibrocartilage, nonmineralized fibrocartilage, and tendon [1,2]. This structure plays a crucial role in force conduction from the muscle to the skeleton and preventing stress concentration. Once this structure is injured, excessive scar tissue forms in the tendon-bone junction as the injury heals. The loss of normal mechanical transition results in decreased biomechanical properties [3]. This phenomenon may be the main reason why the re-tear rate is as high as 20–94% after tendon-bone reconstruction surgery [4–6]. Therefore, research on tendon-bone healing is important.

Recent research on tendon-bone healing has mainly focused on stem cells, cytokines, and tissue engineering materials [7], and some progress has been made. At the early stage of tendon-bone healing, the recruited inflammatory cells release high levels of proinflammatory factors that drive excessive scar tissue formation, as in other tissues [8]. Persistent inflammation hinders the inherent repair process; in particular, macrophages play an important role in inflammatory changes. Studies have increasingly demonstrated that the application of mesenchymal stem cells (MSCs) *in vivo* can regulate inflammation and thus promote tissue repair [9]. However, problems with immunogenicity and safety issues prevent MSCs from being used in clinical applications.

Recent research suggests that MSCs promote tissue repair through the paracrine pathway, which may be realized through MSC-secreted exosomes [10,11]. Furthermore, research indicates that BMSC-Exos regulate inflammation and affect cell apoptosis during tissue repair [12,13]. Therefore, we speculated that BMSC-Exos could modulate inflammation and promote tendon-bone healing. To test this hypothesis, we isolated exosomes from BMSCs and applied them with hydrogels at the tendon-bone healing site and investigated the effects of BMSC-Exos on macrophages and tendon-bone healing.

Material and Methods

Animals

Eight-week-old C57BL/6 male mice (body weight 20–25 g) were purchased from the Laboratory Animal Center of Army Military Medical University and raised in individual cages in a pathogen-free environment. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of the Army Military Medical University.

Cell isolation, culture, and characterization

BMSCs were isolated using a previously reported method [14]. In brief, the mouse femur was obtained, and the marrow cavity was washed with culture medium. The washing fluid was centrifuged and resuspended, and cells were inoculated into the culture dish. BMSCs were identified by flow cytometry, and antibodies used for flow cytometry were: PE anti-mouse/human CD44 (BioLegend, 103007, 1: 20), APC anti-mouse stem cell antigen 1 (Sca-1; BioLegend, 108111, 1: 80), PerCP/Cyanine5.5 anti-mouse CD34 (BioLegend, 128608, 1: 20), and PE/Cyanine7 anti-mouse CD45 (BioLegend, 103114, 1: 80). All experiments involved cells at passages 3–5.

Bone marrow-derived macrophages (BMDMs) were isolated and treated according to a previous method [15]. Typically, BMDMs were isolated using the same method of BMSCs isolation. The cell number was determined after resuspension, and the cell concentration was adjusted to 0.5×10^6 /ml. Then, 20 ng/ml macrophage colony stimulating factor (M-CSF; PeproTech, 315-02-10, America) was added to the culture medium. The nonadherent cells were removed after 3 days, and fresh M-CSF-containing culture medium was added. Four days later, the mature macrophages were harvested for subsequent experiments.

Isolation and identification of exosomes

After the cells reached 80% confluence, serum-free culture medium was added, and the supernatants were collected after culturing for 24 h. Then, the exosomes were isolated from the supernatants through traditional ultracentrifugation: 2000×g for 30 min to remove the cells and debris, centrifugation at 10 000×g for 30 min to remove the subcellular components, and centrifugation at 100 000×g for 70 min to obtain the exosomes. Finally, the exosomes were resuspended in 0.01M PBS, centrifuged at 100 000×g for 70 min for purification, and preserved in a freezer at -80°C . A Zeta View system (Particle Metrix, Germany) was used to measure the exosome concentration and size distribution, and transmission electron microscopy (TEM; Philips-Tecnal, Netherlands) was used to detect exosome morphology. The exosome surface markers were analyzed by Western blotting.

Animal model and surgical procedure

The mouse tendon-bone reconstruction model was established as described previously. Briefly, the Achilles tendon was cut off above the calcaneus, and the cartilage layer at the insertion was removed. Later, a 29-G syringe was used to drill a hole in the posterior calcaneus, a needle with 6-0 suture silk was used to pass through the bone tunnel, and the distal Achilles tendon was sutured and fixed above the calcaneus.

Table 1. Mouse primer pairs used for qRT-PCR analysis.

Gene	Forward	Reverse
IL-1 β	CAACTGTTCTGAACCTCACTG	GAAGGAAAAGAAGGTGCTCATG
IL-6	CTCCCAACAGACCTGTCTATAC	CCATTGCACAACCTCTTTCTCA
IL-10	TTCTTTCAAACAAAGGACCAGC	GCAACCCAAGTAACCCCTAAAG
TGF- β 1	CCAGATCCTGTCCAACTAAGG	CTCTTTAGCATAGTAGTCCGCT
TGF- β 3	ATCAAGAAGAAGAGGGTGAAG	GTAAGTGCAGGACCTGATAG
IGF-1	GAGGGGCTTTACTTCAACAAG	TACATCTCCAGTCTCCTCAGAT
IGF-2	GTGCTGCATCGCTGCTTAC	ACGTCCTCTCGGACTTGG
Col II	GGGAATGCCTCTGCGATGAC	GAAGGGGATCTCGGGGTTG
Acan	GTGGAGCCGTGTTCCAAG	AGATGCTGTGACTCGAACCT
Sox9	GAGCCGGATCTGAAGAGGGA	GCTTGACGTGTGGCTTGTTC
GAPDH	CAAGGTCATCCATGACAACCTTG	GTCCACCACCTGTTGCTGTAG

Col II – collagen II; Acan – aggrecan.

To promote long-term exosome retention and sustained release of exosomes at the tendon-bone healing site, we mixed the exosomes with hydrogel before implantation in the mice. The hydrogel was prepared according to the method used in our previous study [16]. A total of 90 mice were randomly divided into 3 groups: the control group, hydrogel group, and hydrogel+exosome group, with 30 mice per group. Sample sizes were chosen based on previous studies [4,8]. In each group, 14 mice were used for histochemistry and immunofluorescence analysis, 10 mice were used for qRT-PCR analysis, and 6 mice were used for biomechanical testing.

Histochemistry, immunofluorescence, and image analysis

The tendon-bone junction tissue was fixed, decalcified, and sectioned (7 μ m), and routine hematoxylin-eosin (HE) and Safranin O-Fast Green (Solarbio, China) staining were performed according to the manufacturer's protocol. The immunofluorescence staining steps were as follow. Sections were permeabilized in 0.3% Triton X-100 (Beyotime, China) for 15 min, blocked in 5% donkey serum (Solarbio, China) for 30 min, and then incubated at 4°C overnight with the following primary antibodies: inducible nitric oxide synthase (iNOS) antibody (Cell Signaling Technology, 13120S, 1: 400), Arginase-1 (Arg1) antibody (Cell Signaling Technology, 93668T, 1: 50), collagen II (Col II) antibody (Abcam, ab34712, 1: 200), proliferating cell nuclear antigen (PCNA) antibody (Cell Signaling Technology, 13110T, 1: 500), and CD146 antibody (Abcam, ab75769, 1: 200). The next day, the sections were incubated with fluorescence secondary antibody (Cell Signaling Technology, 4412S, 1: 500) at room temperature for 2 h and counterstained with DAPI (Beyotime, China). Finally, the sections were observed, and photos were taken under a laser scanning confocal microscope (LSCM; Zeiss, Germany).

qRT-PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, America) and reverse-transcribed using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, America) according to the instruction manual. qRT-PCR analysis was performed as described previously [17], and the reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad, USA) and a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The primers used for RT-PCR are listed in Table 1.

Western blotting analysis

Tendon-bone junction tissues were cut into pieces, and proteins were harvested from the tissues using ice-cold RIPA lysis. Then, the protein concentration was measured using a BCA Protein Assay kit (Beyotime, China). The protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% bovine serum albumin (BSA; Sangon Biotech, China) for 1 h. Later, primary antibody was added to incubate the membranes at 4°C overnight. Primary antibodies CD206 (ab64693, 1: 1000), CD81 (ab109201, 1: 500), CD9 (ab92726, 1: 800), and tumor susceptibility gene 101 (TSG101; ab125011, 1: 1000) were purchased from Abcam, and Arg1 (93668S, 1: 1000) was purchased from Cell Signaling Technology. Then, the membranes were incubated with HRP-conjugated secondary antibody (Beyotime, A0208, 1: 1000) at 37°C for 2 h. Finally, the reaction solution was added for exposure analysis.

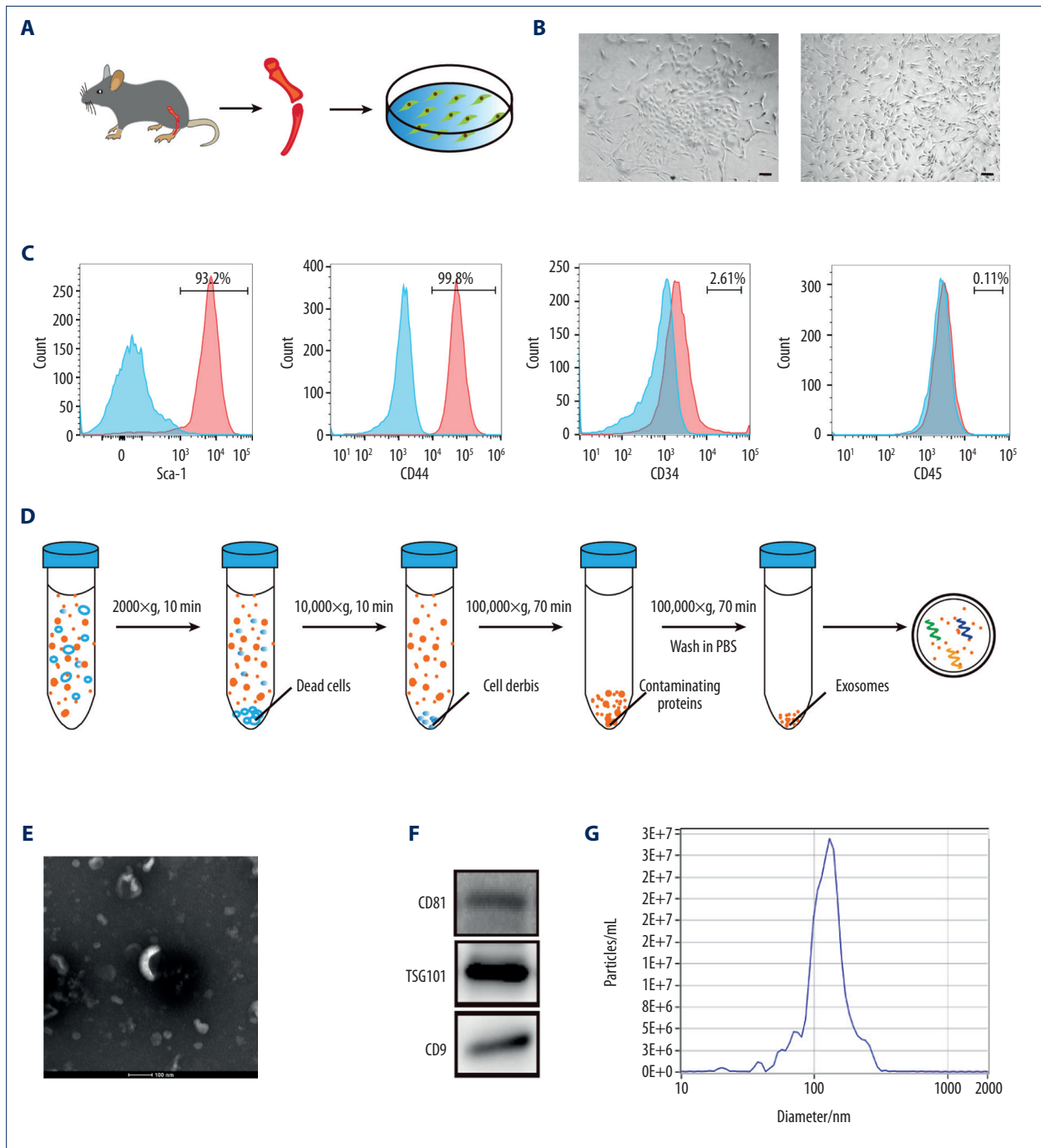


Figure 1. Isolation and identification of BMSCs and BMSC-Exos. **(A)** Flow chart for the isolation of the BMSCs. **(B)** Morphology of the BMSCs, cell clonal growth at P0, and a representative spindle-like morphology at P3. Scale bar: 100 μ m. **(C)** Flow cytometric analysis of the expression of cell surface markers in the cultured BMSCs. **(D)** Schematic diagram of the isolation of the BMSC-Exos. **(E)** TEM image of the BMSCs-Exos, scale bar: 100 nm. **(F)** Surface markers of the BMSC-Exos characterized by Western blotting. **(G)** The particle size distribution of the BMSC-Exos analyzed by NTA.

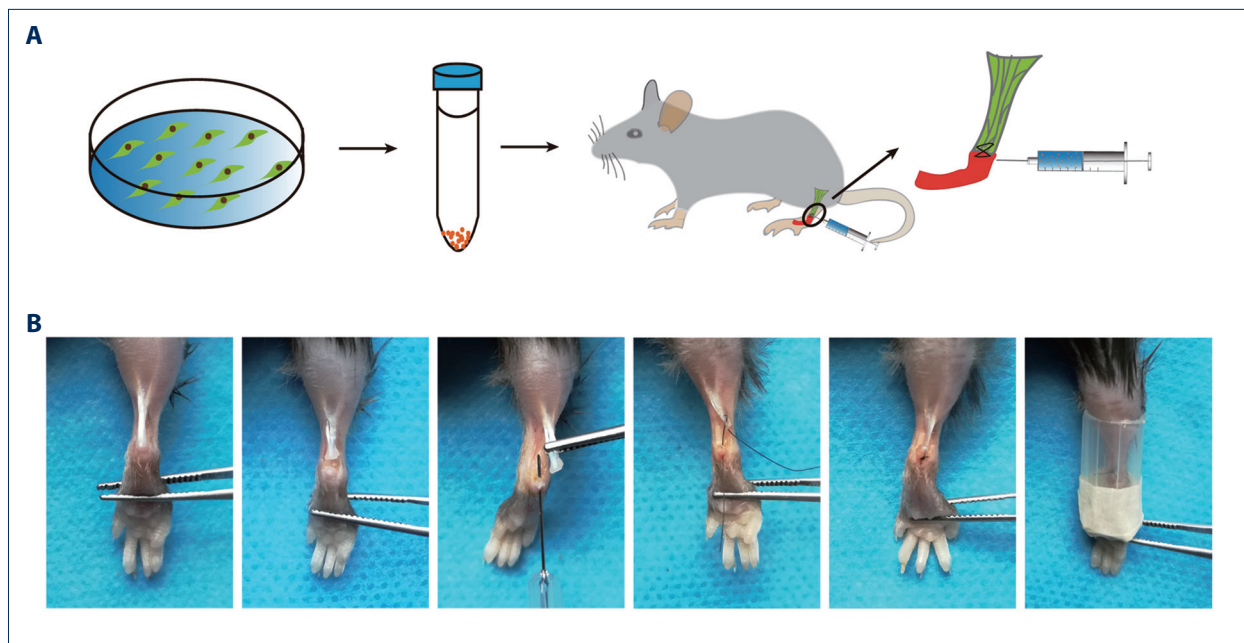


Figure 2. The mouse tendon-bone reconstruction model and surgical procedure. **(A)** Schematic diagram of the exosomes injection into tendon-bone reconstruction site. **(B)** Surgical procedure mouse tendon-bone reconstruction.

Biomechanical testing

Tendon-bone junction samples were collected, and the biomechanical properties were assessed using a microcomputer-controlled electronic universal testing machine (Rigal Instrument Co., Shenzhen, China). Our biomechanical testing was based on a previous study [8]. The cross-sectional area of the Achilles tendon at the point of insertion into the calcaneus was measured using a vernier caliper. The calcaneus was fixed at the clamp below, and the proximal Achilles tendon was sutured with the 3-0 silk and fixed at the clamp above. After preloading at 1 N, the samples were loaded to failure at a rate of 10 mm/min. Biomechanical data were recorded with the Rigal Test Control System (Rigal Instrument Co., Shenzhen, China). Then, the maximum force was calculated the maximum force at the tendon-bone junction failure. Elastic modulus was automatically obtained through the Rigal Test Control System. Stiffness was calculated from the slope of the linear portion of the load-displacement curve. Strength was calculated by dividing the maximum force by the cross-sectional area of tendon-bone junction [4].

Statistical analysis

Data are expressed as $\bar{x} \pm s$. The independent-samples *t* test and one-way analysis of variance (ANOVA) were utilized for comparisons between 2 groups and among multiple groups, respectively. The significance level was set at $P < 0.05$. SPSS 22.0 software was used for all statistical analyses.

Results

Isolation and identification of the BMSCs and BMSC-Exos

First, we obtained the mouse BMSCs according to the methods described in previous reports [18] (Figure 1A). Microscopic observation showed that primary BMSCs gradually showed colony-like growth after 7 days (Figure 1B). Then, the BMSC surface antigens were detected through flow cytometry, and the results indicated that stem cell markers (CD44, Sca-1) were highly expressed in the cells, while the CD34 and CD45 expression levels were relatively low (Figure 1C).

Subsequently, we harvested exosomes from the BMSC supernatants through ultracentrifugation (Figure 1D), and experiments were carried out to determine the characteristics of the exosomes (Figure 1E, 1G). TEM data showed that these vesicles mostly had a typical spherical double-membrane structure with a diameter of approximately 100 nm (Figure 1E). Then, the protein expression of the exosome membrane was detected by Western blotting, and the results indicated that the exosome membrane proteins CD81, TSG101, and CD9 were expressed (Figure 1F). Nanoparticle tracking analysis (NTA) demonstrated that these vesicles had an average diameter of 120.3 nm, and the main peak of size distribution was located at 127.1 nm (Figure 1G). In summary, the above results revealed that the mouse BMSCs produced many exosome-like vesicles.

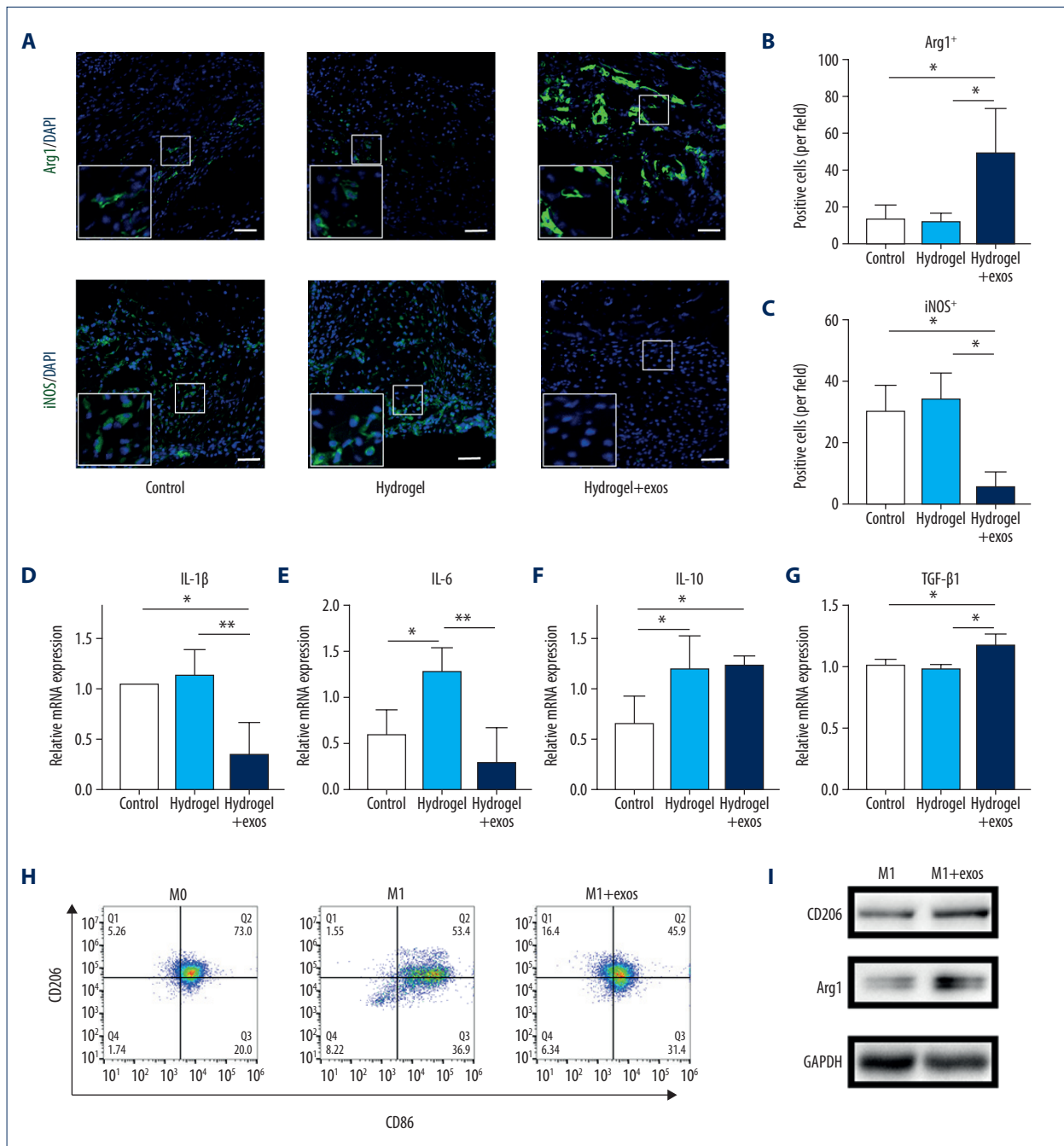


Figure 3. BMSC-Exos induce M2 macrophage polarization during tendon-bone healing. **(A)** Immunofluorescence analysis of the Arg1+ and iNOS+ cells in the tendon-bone junction region (n=5). Scale bar: 100 μ m. **(B, C)** Quantification of the percentage of Arg1+ and iNOS+ cells in total cells. * P<0.05. **(D-G)** qRT-PCR analysis of macrophage-related inflammatory cytokines (IL-1 β , IL-6, IL-10, and TGF- β 1) in tendon-bone healing tissue (n=5). * P<0.05; ** P<0.01. **(H)** Flow cytometric analysis of the altered macrophage polarization after treatment with the BMSC-Exos. **(I)** Western blotting analysis of the alterations in macrophage polarization after treatment with the BMSC-Exos.

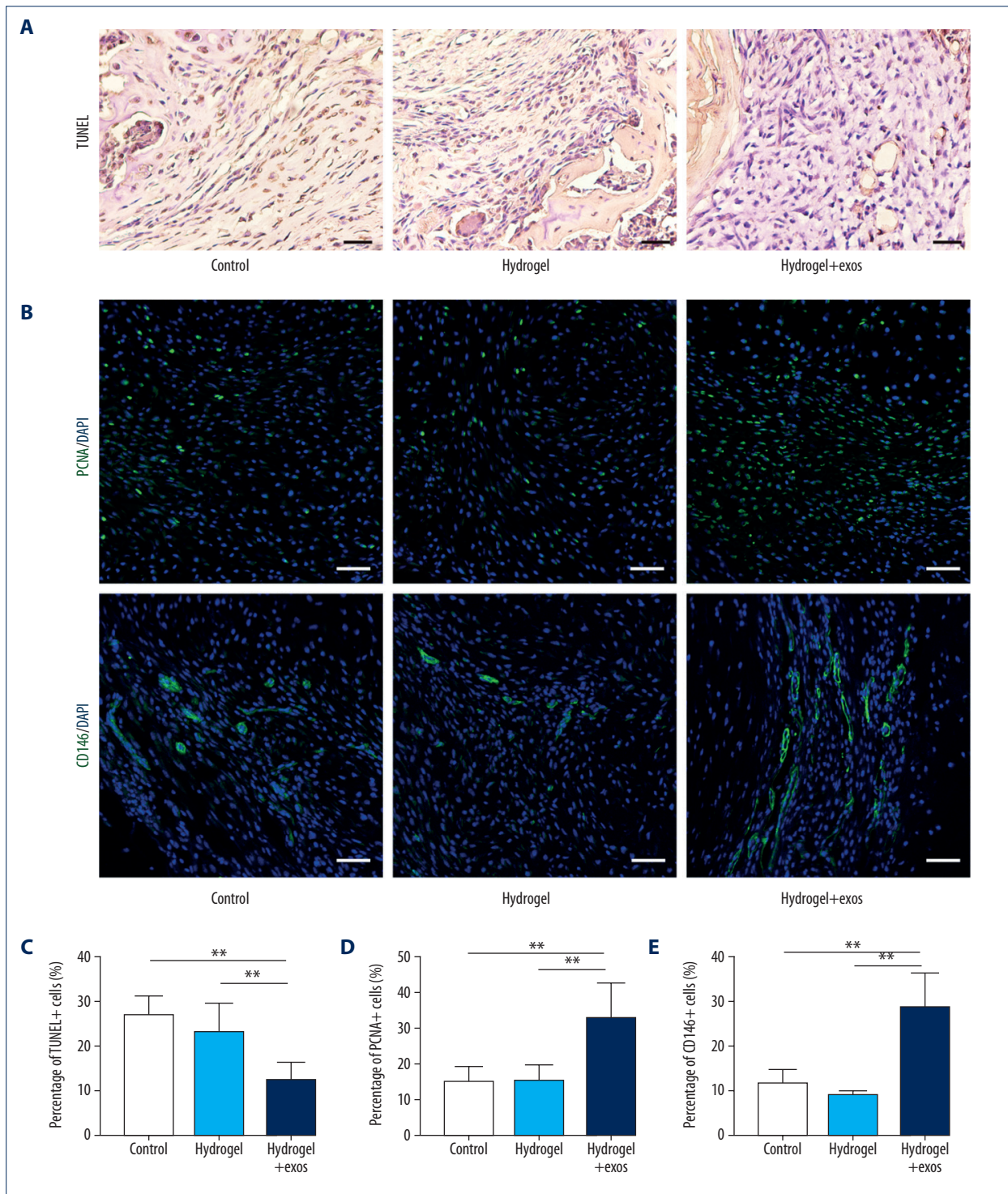


Figure 4. BMSC-Exos inhibit cell apoptosis and promote proliferation during tendon-bone healing. **(A, C)** Immunohistochemistry analysis of the TUNEL-positive cells in the tendon-bone junction region (n=5). Scale bar: 100 μ m. **(B, D, E)** Immunofluorescence analysis of the PCNA-positive cells and the CD146+ cells in the tendon-bone junction region (n=5). Scale bar: 100 μ m. * P<0.05; ** P<0.01; *** P<0.001.

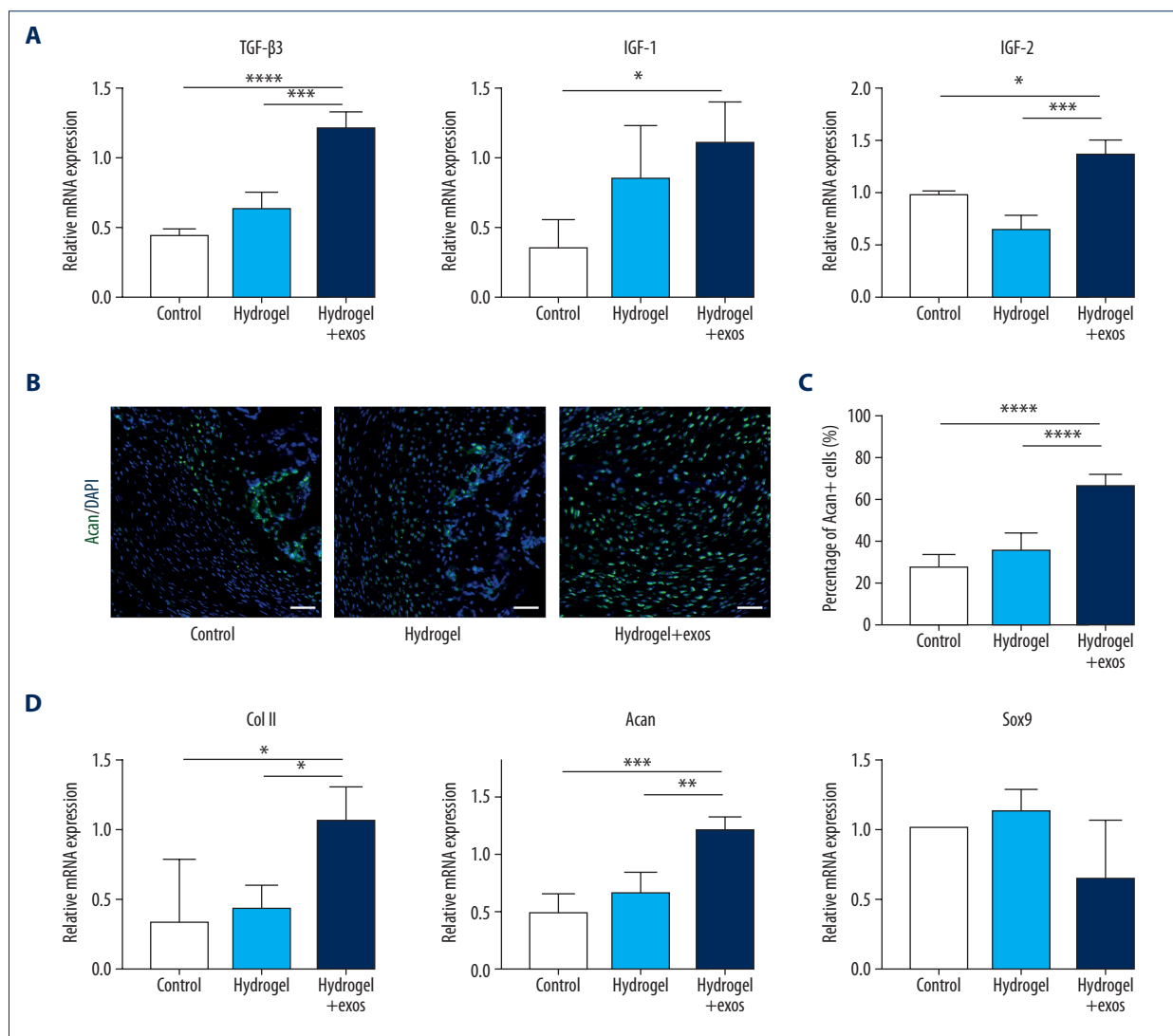


Figure 5. BMSC-Exos change the expression of chondrogenic-related factor and enhances early chondrogenesis. **(A)** The gene expressions of chondrogenic-related factor, includes TGF-β3, IGF-1 and IGF-2 (n=5). **(B, C)** Immunofluorescence analysis of aggrecan in the sections of the tendon-bone junction region (n=5). Scale bar: 100 μm. **(D)** qRT-PCR analysis of cartilage-related genes (Col II, Acan and Sox-9) (n=5). * P<0.05; ** P<0.01; *** P<0.001.

BMSC-Exos induced M2 macrophage polarization during tendon-bone healing

To evaluate the effect of the BMSC-Exos on inflammation during tendon-bone healing, we generated a mouse tendon-bone reconstruction model, and hydrogel loaded with exosomes was administered at the injury site (Figure 2A, 2B). First, we analyzed the effect of the exosomes *in vivo* on macrophage polarization at the tendon-bone healing site. Seven days after surgery, M2 macrophages (Arg1+) increased in the hydrogel+exosome group compared with the control group and the hydrogel alone group, whereas M1 macrophages (iNOS+) decreased (Figure 3A–3C), suggesting that the exosomes polarized the proinflammatory macrophages to anti-inflammatory macrophages. Moreover, to

verify the impact of the BMSC-Exos on the polarization of macrophages, we isolated macrophages, cultured them *in vitro* and induced them into M1 macrophages using LPS. Then, the macrophages were treated with the BMSC-Exos and assessed with flow cytometry; the results indicated that the BMSC-Exos substantially enhanced the proportion of CD206-positive M2 macrophages and reduced that of CD86-positive M1 macrophages (Figure 3H). The Western blotting results showed that the BMSC-Exos induced M1 to M2 macrophage polarization (Figure 3I). Overall, the above results suggested that the local administration of BMSC-Exos enhanced the polarization of macrophages to M2 macrophages at the tendon-bone healing site and secreted excessive anti-inflammatory factors.

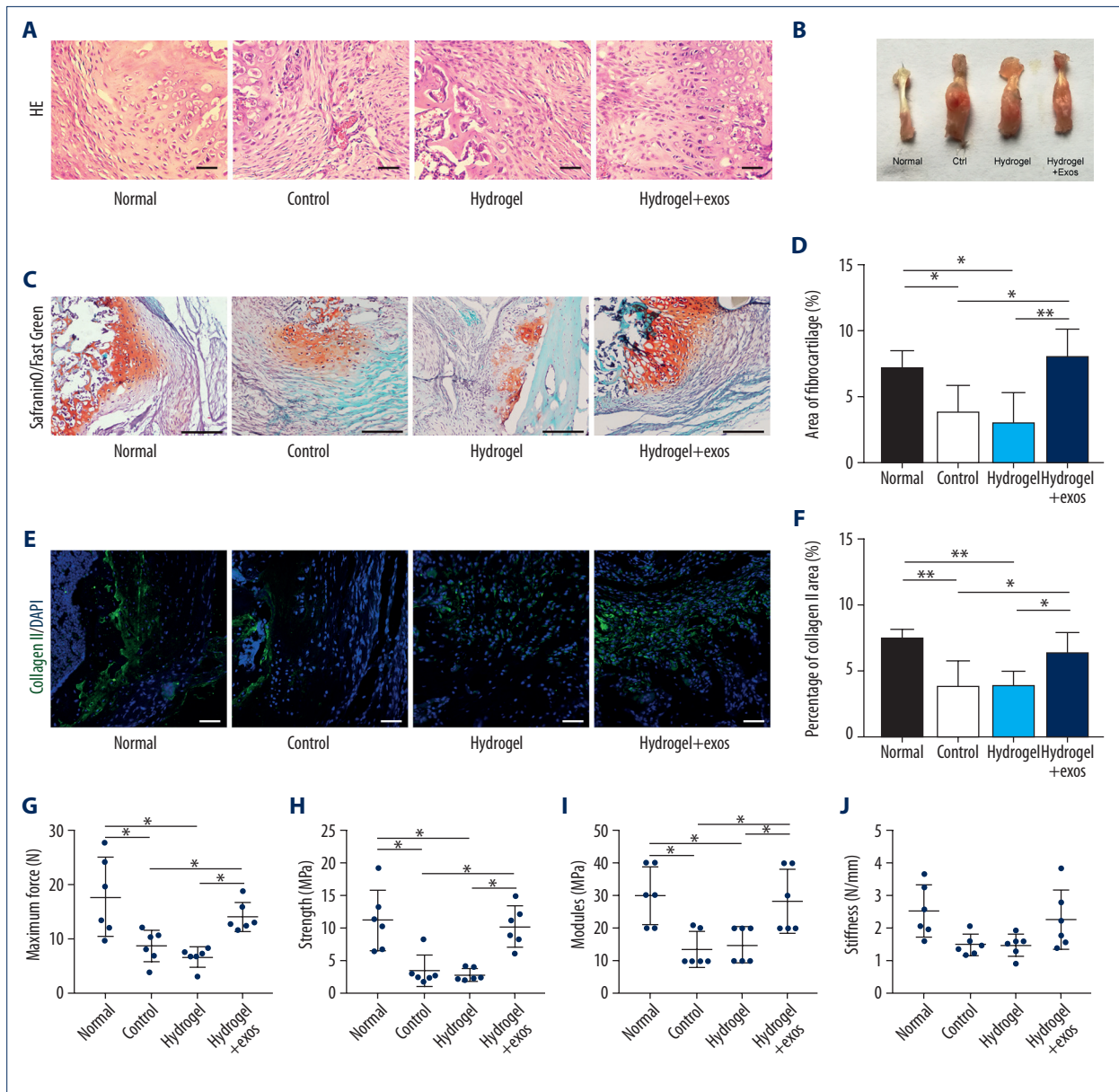


Figure 6. BMSC-Exos promote fibrocartilage regeneration and enhance the biomechanical properties in tendon-bone healing. (A, B) H&E-stained and gross morphology of the tendon-bone junctions (n=4). Scale bar: 200 μ m. (C, D) Safranin O/Fast Green-stained sections of the tendon-bone junctions (n=4). Scale bar: 200 μ m. (E, F) Immunofluorescence analysis of collagen II in the sections of the tendon-bone junction region (n=4). Scale bar: 100 μ m. (G–J) Biomechanical test of tendon-bone junctions (n=6). * $P < 0.05$; ** $P < 0.01$.

BMSC-Exos induced the inflammatory response *in vivo*

Further, we investigated the *in vivo* inflammation in the 3 groups. Seven days after the operation, the tissues at tendon-bone healing sites were collected for PCR analysis, and the results demonstrated that, compared with the control and hydrogel alone groups, the hydrogel+exosome group showed downregulated expression of proinflammatory factors (such as IL-1 β and IL-6) and upregulated expression of anti-inflammatory

factors (such as IL-10 and TGF- β 1) that promote tissue repair (Figure 3D–3G). *In vivo* inflammatory reactions revealed that the BMSC-Exos regulated the inflammatory factors at the tendon-bone healing site, which was beneficial for tissue repair.

BMSC-Exos inhibited cell apoptosis and promoted proliferation during healing

We analyzed the effects of the BMSC-Exos on cell proliferation and apoptosis at the tendon-bone healing site. First, TUNEL staining was performed to detect the apoptotic cells. At 7 days after the operation, compared with those of the control and hydrogel alone groups, the TUNEL-positive apoptotic cells at the tendon-bone healing site in the hydrogel+exosome group were markedly reduced (Figure 4A, 4C). Subsequently, immunofluorescence was carried out to analyze the proliferation of the cells. As shown in Figure 4B, 4D, the PCNA-positive proliferating cells in the hydrogel+exosome group increased relative to those in the control and hydrogel alone groups. Moreover, recent research suggests that CD146 is a novel marker of MSCs that plays an important role in tendon healing. Compared with those in the control and hydrogel alone groups, the CD146-positive stem cells in the hydrogel+exosome group apparently increased (Figure 4B, 4E). The above findings suggested that local administration of the BMSC-Exos suppressed cell apoptosis *in vivo* at the tendon-bone healing site, promoted local cell proliferation, and increased stem cell aggregation.

BMSC-Exos promoted fibrocartilage regeneration at the tendon-bone interface

Previous research has shown [1] that during the tendon-bone healing process, the regeneration of the fibrocartilage layer is the key factor that affects healing quality. To investigate whether exosome-induced macrophage polarization affected fibrocartilage regeneration during tendon-bone healing, we analyzed early chondrogenesis and late fibrocartilage formation.

In the early stages of tendon-bone healing (Day 7), the gene expression of chondrogenic-related factors such as TGF- β 3, IGF-1 and IGF-2 were significantly upregulated in the hydrogel+exosome group (Figure 5A). To investigate the early chondrogenesis during tendon-bone healing (Day 14), we carried out immunofluorescence analysis, and the result showed that there was more aggrecan (Acan) in the hydrogel+exosome group compared with the control and hydrogel groups (Figure 5B, 5C). Similarly, the gene expression level of collagen II and aggrecan were significantly increased in the hydrogel+exosome group (Figure 5D). In the middle and later stages, we evaluated fibrocartilage regeneration at the tendon-bone interface. First, gross observation of the samples 1 month after operation suggested that there was less scar hyperplasia in the hydrogel+exosome group than in the control and hydrogel groups (Figure 6B). Thereafter, HE staining demonstrated that the cells and the collagen tissues at the tendon-bone junction in the 3 groups were disordered, but a transition structure similar to tendon-bone was formed in the hydrogel+exosome group, the chondrocytes increased and

were tightly arranged, and the collagen tissues also showed an orderly arrangement (Figure 6A). Safranin O-Fast Green staining showed that the Safranin O-positive area at the tendon-bone junction in the hydrogel+exosome group was larger than those in the control and hydrogel groups (Figure 6C, 6D). Additionally, collagen II is a specific marker of fibrocartilage that was apparently upregulated in the hydrogel+exosome group (Figure 6E, 6F). In conclusion, the above findings suggested that the BMSC-Exos promoted fibrocartilage regeneration during tendon-bone healing.

BMSC-Exos enhanced the biomechanical properties of tendon-bone healing

Biomechanical properties are the ultimate index for evaluating tendon-bone healing. In this study, the samples were subjected to biomechanical testing, and all of them broke at the tendon-bone junction. These test results indicated that the maximum force, elastic modulus, and strength in the hydrogel+exosome group were substantially higher than those in the control and hydrogel groups, but there was no significant difference compared with the normal group. However, the stiffness showed no distinct differences among the various groups (Figure 6G–6J). Overall, local administration of the BMSC-Exos enhanced the biomechanical properties of tendon-bone healing.

Discussion

The injury healing process at the tendon-bone junction is similar to that of other tissues, which undergo 3 stages: inflammation, proliferation and remodeling. Research in numerous tissue fields suggests that, in the case of injury in the body, macrophage depletion *in vivo* is not favorable for tissue regeneration and repair [19–21]. During fracture healing, macrophage depletion via vein injection of clodronate liposomes has also been shown to significantly reduce endochondral ossification and delay hard callus formation [22]. Furthermore, in a medial collateral ligament transection model, macrophage depletion resulted in decreased early matrix formation and weak ligament strength [23]. Therefore, macrophages play key roles in inflammatory outcomes and tissue repair.

At the early stage of tendon-bone healing, an acute inflammatory reaction occurs in the healing area, and extensive M1 macrophages infiltration is observed [21]. M1 macrophages secrete high levels of proinflammatory factors (such as TNF- α , IL-1 β , and IL-6) to enhance local inflammatory reactions, stimulate cell proliferation, and recruit many fibroblasts to aggregate at the injury site [24,25]. IL-1 β and IL-6 have been previously confirmed to inhibit chondrogenic differentiation and cartilage matrix formation [26]. Then, macrophages are polarized into the anti-inflammatory type, namely, M2 macrophages [21].

In addition, M2 macrophages release anti-inflammatory factors (such as IL-10 and TGF- β) to reduce local inflammatory reactions and promote tissue regeneration and repair [24]. A previous study showed that TGF- β 3 is a key regulator of chondrogenic differentiation [27]. However, a strong inflammatory reaction or M1 macrophages persistently exist during tissue healing, stimulating fibroblasts to secrete excessive extracellular matrix (ECM) and leading to scar tissue formation [3,28]. The tendon-bone healing junction is similar to tendon tissues, and excessive scar tissues will be formed after injury, while excessive scar tissues hinder fibrocartilage regeneration and tissue structural remodeling at the tendon-bone junction. Consequently, regulation of the transition of macrophages from M1 to M2 reduces the inflammatory reaction and promotes repair via M2 macrophages, thus facilitating tendon-bone healing.

This hypothesis was also verified in our study, which suggested that local administration of exosomes decreased the M1 macrophages, reduced the proinflammatory factors (IL-1 β and IL-6) in local tissues, decreased cell apoptosis, increased cell proliferation, reduced ECM deposition, and suppressed excessive scar formation. Proinflammatory factors enhance cell apoptosis in injured tissues, while other studies have suggested that exosomes directly act on cells to reduce cell apoptosis in an inflammatory environment [17,18]. Thus, after local administration of exosomes during the tendon-bone healing process, exosomes may directly act on cells; alternatively, they may reduce macrophage-secreted proinflammatory factors to suppress cell apoptosis. In addition, proinflammatory factors such as IL-1 β suppress cartilage regeneration and promote degradation of the cartilage matrix [17,26]. Our study also discovered that the administration of exosomes reduced proinflammatory factors, which contributed to fibrocartilage formation at the tendon-bone junction. After local administration of exosomes, the M2 macrophages increased; accordingly, the secreted cytokines, such as IL-10, TGF- β , and IGF, increased, thus promoting tissue repair. Some research has shown that TGF- β 3 and IGF-1 can strongly promote stem cell differentiation into cartilage [27,29,30]; additionally, IL-10 not only has anti-inflammatory effects, but also possesses a strong chondrogenic capacity [31]. These cytokines secreted by M2 macrophages may promote fibrocartilage regeneration at the tendon-bone interface.

This was also confirmed in our study, as we found the expression of chondrogenic marker such as collagen II and aggrecan significantly increased on Day 14. We also noted that the gene expression of Sox-9 did not increase, which may be due to the fact that the change of Sox-9 took place in the early stages and was earlier than that of collagen II and aggrecan. Consequently, we discovered that regulating macrophage polarization is beneficial for tendon-bone healing, which promotes fibrocartilage regeneration and enhances biomechanical properties.

Recent studies have found that MSCs are not only stem cells with multipotential differentiation, but are also inflammatory modulators [9,10]; in other words, MSCs interact with multiple immune cells (such as neutrophils and macrophages) to secrete bioactive molecules to suppress lymphocyte proliferation, increase the production of anti-inflammatory M2 macrophages, and thus exert anti-inflammatory effects. Chamberlain et al. [32] reported that the administration of MSCs at the tendon injury site enhanced the number of M2 macrophages and substantially alleviated the inflammatory reactions, thereby accelerating tendon healing, but the mechanism by which MSCs regulate macrophage polarization remains unclear. Further research has shown that stem cells implanted in the body will not completely differentiate into target cells to participate in repair; more importantly, they regulate adjacent immune cells through paracrine signaling, thus enhancing tissue regeneration and repair. Stem cell-secreted exosomes are typical examples, and the administration of exosomes is more advantageous than that of stem cells; for instance, exosomes have high biocompatibility and are nonimmunogenic, and they can penetrate the tissue barrier [33]. Thus, we selected exosomes to regulate macrophages. As proven in research on numerous tissues, the administration of stem cell exosomes regulates the M2 polarization of macrophages, thus enhancing tissue repair; moreover, the administration of exosomes is more advantageous than that of stem cells [34,35]. We discovered in this study that local administration of exosomes at the tendon-bone healing site decreased M1 macrophages while apparently increasing M2 macrophages. Accordingly, excessive cytokines were secreted to promote tendon-bone healing.

In summary, this is the first study to show that the local administration of BMSC-Exos promotes the formation of fibrocartilage via increasing M2 macrophage polarization in tendon-to-bone healing, leading to improved biomechanical properties. However, the mechanism by which exosomes regulate macrophage polarization has not been fully clarified, and further research is required.

Conclusions

The findings in this study demonstrated that during tendon-bone healing, the local administration of exosomes induced M2 macrophages polarization and improved the inflammatory microenvironment to promote fibrocartilage regeneration at the tendon-bone interface and to enhance biomechanical properties. Our research provides a strategy for enhancing the tendon-bone healing effect in clinical practice.

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Conflict of interest

None.