Adipose-derived Mesenchymal Stem Cell-derived Exosomes Promote Tendon Healing by Activating Both SMAD1/5/9 and SMAD2/3

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Research Article

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Abstract

Background: The use of adipose-derived mesenchymal stem cell-derived exosomes (ADSC-Exos) may become a new therapeutic method in biomedicine owing to their important role in regenerative medicine. However, the role of ADSC-Exos in tendon repair has not yet been evaluated. Therefore, we aimed to clarify the healing effects of ADSC-Exos on tendon injury.

Methods: The adipose-derived mesenchymal stem cells (ADSCs) and tendon stem cells (TSCs) were isolated from subcutaneous fat and tendon tissues of Sprague Dawley rats, respectively, and exosomes were isolated from ADSCs. The proliferation and migration of TSCs induced by ADSC-Exos were analyzed by EdU, cell scratch and transwell assays. We used western blot to analyze tenogenic differentiation of TSCs and the role of the SMAD signaling pathways. Then we explored a new treatment method for tendon injury, combining exosome therapy with local targeting using a biohydrogel. Immunofluorescence and immunohistochemistry were used to detect the expression of inflammatory and tenogenic differentiation after tendon injury, respectively. The quality of tendon healing was evaluated by Hematoxylin-eosin (H&E) staining and biomechanical testing.

Results: ADSC-Exos could be absorbed by TSCs, and promoted the proliferation, migration, and tenogenic differentiation of these cells. This effect may have depended on activation of the SMAD2/3 and SMAD1/5/9 pathways. Furthermore, ADSC-Exos inhibited the early inflammatory reaction and promoted tendon healing in vivo.

Conclusions: Overall, we demonstrated that ADSC-Exos contributed to tendon regeneration and provided proof of concept of a new approach for treating tendon injuries.

Introduction

Tendon is a dense connective tissue consisting of limited tendon cells and abundant extracellular matrix (ECM). Tendon injuries are of significant concern worldwide, with more than 30 million affected patients annually [1]. Tendon healing is slow as a result of its hypocellularity and hypovascularity, and involves three overlapping phases: inflammation, proliferation, and remodeling [2, 3]. Furthermore, the self-healing potential of any tissue depends, in part, on its endogenous resident stem cells. The viability and tenogenic differentiation of TSCs are the main mechanisms of tendon repair [4]. However, inflammation during the healing phase may compromise biomechanical function [5–7]. Therefore, it is important to enhance tendon healing by promoting anti-inflammation and the proliferation of TSCs.

Mesenchymal stem cells have demonstrated great potential in tissue healing [8]. Specifically, ADSCs are highly beneficial for clinical applications because of their abundant and conveniently accessible sources [9]. When transplanted, ADSCs are able to modulate the inflammatory environment and ECM balance to stimulate tendon regeneration [10–12]. Recent studies have demonstrated that the effectiveness of ADSCs in regenerative medicine is due to their paracrine effects [13]. Thus, ADSCs have been identified as new therapeutic agents in biomedicine [14].

Exosomes are membrane-bound extracellular vesicles that target cells by endocytosis or membrane fusion, and are important paracrine factors for stem cells [15]. In addition, exosomes play important roles in immune regulation, apoptosis, and tissue regeneration [16]. The therapeutic effect of ADSC-Exos has been demonstrated in multiple diseases. This is of great significance in the future development of tissue repair and regeneration engineering [17].

We hypothesize that ADSC-Exos promote tendon repair by regulating the biological characteristics of TSCs as well as the extracellular microenvironment. Specifically, in this study, we investigated the effects of ADSC-Exos on the proliferation, migration, and differentiation of TSCs *in vitro*, and during inflammation and regeneration situations *in vivo*.

Materials And Methods

Animals

Male Sprague Dawley rats weighing 180–230 g at 8–10 weeks of age were provided by the Experiment Center of Harbin Medical University (Harbin, Heilongjiang, China). All animals were treated according to the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the corresponding ethics committee (no. Ky2018-135).

Isolation and identification of TSCs and ADSCs

The isolation methods of ADSCs and TSCs were as performed in previous studies [18,19]. In brief, TSCs were isolated from rat tendon and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel) and 1% penicillin-streptomycin (Beyotime, Haimen, China). The multilineage differentiation potential of TSCs, as well as the identification of surface markers (CD90- and CD105-positive, and CD106- and CD11b-negative), were demonstrated in our previous study [19]. ADSCs were isolated from subcutaneous fat of rats and cultured in DMEM/F12 (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. Flow cytometry was used to identify surface markers. The adipogenic, osteogenic and chondrogenic differentiation of ADSCs was induced in differentiation medium (Cyagen, Santa Clara, CA, USA) to identify their differentiation potential.

Isolation and identification of ADSC-Exos

At 80% confluence, the culture medium of the ADSCs was changed to exosome-depleted medium (DMEM/F12 containing 10% exosome-depleted FBS (Biological Industries) and 1% penicillin-streptomycin) and incubated for 24 h. Then, the culture medium was collected without ADSCs and centrifuged at 300 × g for 10 min, 3000 × g for 10 min, 10,000 × *g* for 30 min, and 100,000 × *g* for 2 h to isolate exosomes. Exosomes attached to the bottom of the centrifuge tube were diluted with phosphate-

buffered saline. Nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and western blotting were used to identify and evaluate the collected exosomes.

Cellular internalization of ADSC-Exos

ADSC-Exos were incubated with 1 μM PKH26 (Sigma-Aldrich, St. Louis, MO, USA) in Diluent C (Sigma-Aldrich) for 5 min and excess dye was removed by ultracentrifugation. The labeled exosomes were subsequently added to serum-free medium of TSC cultures and incubated overnight. The nuclei were labeled with Hoechst 33342 (UE, China) and photos were taken with an inverted fluorescence microscope (Leica, Wetzlar, Germany).

Treatment of TSCs with ADSC-Exos

For determining the effect of ADSC-Exo treatment, 1×10^6 TSCs were seeded into six-well culture plates for 24 h and divided randomly into four groups. ADSC-Exos were added to exosome-free medium at 0, 25, 50, or 100 μg/mL and used to replace the TSC culture medium. For additional analyses, 10 nM of the TGF-β/SMAD2/3 inhibitor, SB431542, or 10 nM of the BMP/SMAD1/5/9 inhibitor, dorsomorphin, (MedChemExpress, Monmouth Junction, NJ, USA) were added to the TSCs 30 min prior to addition of the ADSC-Exos. TSCs treated with various concentrations of ADSC-Exos, with or without inhibitors, were incubated for 30 min or 24 h, then collected for analyses.

EdU assay

For the cell proliferation analysis, TSCs were incubated with 50 μM 5-ethynyl-2'-deoxyuridine (EdU) from an EdU Assay Kit (UE) for 4 h. The TSCs were then fixed with 4% paraformaldehyde and stained using the same EdU assay kit. Nuclei were labeled with Hoechst 33342 and photos were taken with an inverted fluorescence microscope.

Scratch assay

TSCs at 2×10^5 cells/well were inoculated into a 6-well plate for overnight culture. A straight line wound was made in the cultured cells using a sterile 200-μL pipette tip. Serum-free medium with ADSC-Exos was then added into each well. Images were obtained at 0 and 24 h after ADSC-Exo treatment using an inverted microscope with an Axiocam 506 camera and ZEN 2011 software (Zeiss, Oberkochen, Germany).

Transwell assay

TSCs at 1 × 10^5 cell/well were inoculated into the Transwell upper chamber, and ADSC-Exos were added into the lower compartment. After culturing for 24 h, the TSCs were fixed with absolute ethanol, then stained with crystal violet. Images were obtained under a light microscope.

Western blot analyses

Experimental protocols and surgical procedures

A total of 63 Sprague-Dawley rats were divided into three groups of 21: (1) Control: animals that underwent surgery for partial resection of the patellar tendon; (2) Gelatin methacryloyl (GelMA): animals that underwent surgery for patellar tendon partial resection and were inoculated with 30 μL GelMA (EFL-GM-60, 10% w/v) over the tendon defect; and (3) ADSC-Exos: animals for which the injured patellar tendon was treated with 30 μL GelMA containing 200 μg of ADSC-Exos. The exosome content was determined according to previous studies [19]. Rats were anesthetized with 0.3% sodium pentobarbital (30 mg/kg). The right patellar tendon was surgically exposed and the central 1/3 of the tendon tissue removed as in previous studies [20]. GelMA was then inoculated into the lesion and cross-linked into a gel state by ultraviolet light. The skin incision was closed using 4-0 sutures. The modeling process is shown in Figure 1. Animals were sacrificed on days 7, 14, or 28 post-surgery and the tendons were collected.

Histopathological and immunohistochemical analyses

Paraffin-embedded tendon tissues were sectioned at a thickness of 4 μm. The tissues were then stained with H&E for histopathological analysis. The stained patellar tendons were evaluated using light microscopy.

For immunohistochemical analyses, paraffin sections of tendon tissues were incubated with Immuno-Block reagent for 30 min after being deparaffinized and rehydrated. The sections were then incubated with the rabbit primary antibodies (Abcam,): anti-CD146 (monoclonal; 1:250), anti-TNMD (polyclonal; 1:100), anti-collagen I (polyclonal; 1:100), anti-SCXA (polyclonal; 1:100), anti-ALP (polyclonal; 1:200), and anti-Runx2 (monoclonal; 1:1000). Horseradish peroxidase–conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, Ely, UK) was used as the secondary antibody. After counterstaining with hematoxylin, the sections were dehydrated and fixed.

For immunofluorescence analyses, sections of tendon tissues were incubated with the rabbit primary antibodies (Abcam): anti-CCR7 (monoclonal; 1:200), anti-CD163 (monoclonal; 1:100), anti–IL-6 (monoclonal; 1:100), and anti-IL-10 (monoclonal; 1:100). The sections were then incubated with secondary antibodies (1:200, Proteintech, Rosemount, IL, USA) for 1 h. Nuclei were labeled with 4',6-diamidino-2-phenylindole and photos were taken with a DM4 B microscope (Leica). Three fields per section were selected randomly for statistical analysis.

Biomechanical testing

Two bony ends of a healing tendon were fixed on a universal material testing machine (Zwick, Roell, Germany). The tissues were investigated using a standard failure test with a testing speed of 5 mm/min. Failure load (N) and stiffness (N/mm) were obtained by the software of the testing machine. Young's modulus ($N \times 10^3/mm^2$) was calculated after measuring the cross-sectional area (mm^2) of the tendon with a vernier caliper.

Statistical analyses

All values are expressed as means ± standard deviation. Quantitative data for each group were analyzed by a one-way analysis of variance followed by the Tukey–Kramer test. $P < 0.05$ was considered statistically significant.

Results

Characterization of ADSCs

ADSCs exhibited long fusiform morphology (Figure 2A), and differentiated into adipocytes, osteoblasts and chondroblasts *in vitro* (Figure 2B). Flow cytometric analysis of ADSC surface markers revealed that the cells were CD90- and CD105-positive, and CD34-, CD45- and CD11b- negative (Figure 2C).

Characterization and internalization of ADSC-Exos

Transmission electron microscopy showed that ADSC-Exos were round or elliptical vesicular structures (Figure 3A). The NTA revealed the mean diameter of ADSC-Exos to be 109.6 nm (Figure 3B). Western blot analyses confirmed that the ADSC-Exo surface markers, CD9, TSG101, and HSP70, were positively expressed (Figure 3C). Finally, ADSC-Exos were internalized by TSCs and showed red fluorescence (Figure 3D).

ADSC-Exos promoted the proliferation, migration, and tenogenic differentiation of TSCs

We first measured the effect of ADSC-Exos on the proliferation and migration of TSCs. The EdU assay showed that ADSC-Exos promoted TSC proliferation (Figure 4A, B). The Transwell assay confirmed that ADSC-Exos promoted TSC migration with increasing concentrations of exosomes (Figure 4C, D). The scratch test showed results consistent with these findings (Figure 4E, F). Then, we investigated whether ADSC-Exos affected the differentiation of TSCs. Western blot analyses showed ADSC-Exos significantly increased the protein expression of TNMD, collagen I, and SCX, but had no effect on ALP or Runx2 (Figure

4G-L). These results suggest that ADSC-Exos promote the tenogenic differentiation ability of TSCs but have no effect on osteogenic differentiation.

ADSC-Exos activated the TGF-β/SMAD2/3 and BMP/SMAD1/5/9 pathways

SMAD signaling pathways play vital roles in regulating stem cell activity. TGF-β/SMAD2/3 and BMP/SMAD1/5/9 are two typical SMAD signaling pathways. Therefore, we examined changes in these two pathways after ADSC-Exo uptake by TSCs. Western blot analyses showed that ADSC-Exos increased p-SMAD2/3 and p-SMAD1/5/9 expression in TSCs (Figure 5A-C), suggesting that the uptake of ADSC-Exos by TSCs activated the TGF-β/SMAD2/3 and BMP/SMAD1/5/9 signaling pathways. Furthermore, we pretreated TSCs with the TGF-β/SMAD2/3 inhibitor, SB431542, or the BMP/SMAD1/5/9 inhibitor, dorsomorphin, for 30 min. Western blot analyses showed that SB431542 and dorsomorphin inhibited the phosphorylation of SMAD2/3 and SMAD1/5/9, respectively (Figure 5D-F).

ADSC-Exos regulated TSC proliferation, migration, and tenogenic differentiation by activating TGF-β/SMAD2/3 and BMP/SMAD1/5/9 signaling pathways

To investigate the regulatory effect of ADSC-Exos, we evaluated their effects on the proliferation, migration, and tendon differentiation of TSCs by pretreating them with SB431542 or dorsomorphin. As expected, the proliferation (Figure 5G, H) and migration (Figure 5I-L) of TSCs were significantly decreased in the ADSC-Exos + SB431542 and ADSC-Exos + dorsomorphin groups compared with that in the ADSC-Exos only group. Similarly, western blot analyses showed that pretreatment with SB431542 or dorsomorphin significantly decreased expression of the tenogenic differentiation genes, TNMD, collagen I, and SCX, in TSCs (Figure 5M-P).

ADSC-Exos regulated the early inflammatory response during tendon healing

We investigated the in vivo effect of ADSC-Exos on early healing of tendon injury. At week 1 after injury, the level of CCR7 (M1 macrophage marker) decreased in the ADSC-Exo group while the level of CD163 (M2 macrophage marker) increased (Figure 6A, B). Furthermore, IL-10 (an anti-inflammatory factor) increased, and IL-6 (a pro-inflammatory factor) decreased (Figure 6C, D). Quantitative analyses showed there were more $CD163^+$ and $IL\text{-}10^+$ cells in the ADSC-Exo group, while $CCR7^+$ and $IL\text{-}6^+$ cells predominated in the control and GelMA groups (Figure 6E).

ADSC-Exos improved the healing of tendon injury

We next assessed whether ADSC-Exos contributed to the healing of patellar tendon injury in rats. H&E staining showed the ADSC-Exo group had significantly higher cell density and more longitudinal fibrous tissue in the defect area at week 2 compared with the other groups (Figure 7A). At week 4, the collagen fiber alignment in the ADSC-Exo group was more compact than other groups (Figure 7H).

Immunohistochemical analyses showed higher expression of TNMD, collagen I, and SCX in the ADSC-Exo group at week 2 than in the control and GelMA groups (Figure 7B-D). At week 4, the expression of these three genes remained high in the ADSC-Exo group (Figure 7I-K). Furthermore, ALP and Runx2 expression was unchanged among the three groups at both week 2 and 4 (Figure 7E, F, L, M). The results of the quantitative analyses are shown in Figure 7G, N.

Biomechanical testing showed that the failure load, stiffness, and Young's modulus of the patellar tendon in the ADSC-Exo group were significantly increased compared with the control and GelMA groups (Figure 7O-R).

ADSC-Exos promoted TSC proliferation during tendon healing

To investigate the mechanism by which ADSC-Exos promoted tendon healing in vivo, we measured the number of TSCs in tendon tissue during early healing. CD146 was used as a marker of TSCs [21]. Immunohistochemical staining showed that the number of $CD146^+$ TSCs in the injured tendon increased with extension of the healing time. Meanwhile, as expected, the number of $CD146^+$ TSCs increased significantly in the ADSC-Exo group (Figure 8A, B).

Discussion

Improving the quality of healing after tendon injury remains a major medical challenge. TSCs play an important role in tendon healing [22]. However, Zhang et al. reported that culture-expanded TSCs were prone to lose their phenotypic characteristics and exhibited reduced regeneration ability [23]. Therefore, activating the proliferation and differentiation of TSCs is key to improving tendon healing.

We first studied the influence of ADSC-Exos on TSCs in vitro. The results revealed that ADSC-Exos were internalized into TSCs and promoted their proliferation, migration, and tenogenic differentiation. Implantation of TSCs improves tendon healing in rats [24–27] and the activity of TSCs determines the quality of this healing. It is well-known that the SMAD family of signaling pathways play important roles in regulating stem cell functions, with two typical SMAD signaling pathways, TGF-β/SMAD2/3 and BMP/SMAD1/5/9, having potential significance in regulating the activity of TSCs [28–30]. Accordingly, we hypothesized that ADSC-Exos promoted the proliferation, migration, and tenogenic differentiation of

TSCs by activating SMAD family signaling pathways. As expected, ADSC-Exos increased the phosphorylation of SMAD2/3 and SMAD1/5/9 in TSCs, which was later found to be attenuated by the inhibitors, SB431542 and dorsomorphin, respectively. We also found that application of these two inhibitors blocked the effects of ADSC-Exos on the activity of TSCs. These results support the hypothesis that ADSC-Exos enhanced the proliferation and migration of TSCs by promoting activation of the TGF-β/SMAD2/3 and BMP/SMAD1/5/9 signaling pathways.

Tenogenic differentiation is a complex process. SCX is a key molecule in the early development of tendons. It is responsible for the differentiation of TSCs into tenocytes and the positive regulation of TNMD expression [31, 32]. Subsequently, the TNMD gene is necessary for tendon maturation and has a positive effect on the self-renewal of TSCs [33]. In addition, the expression of collagen I determines the strength of tendons [34]. Because abnormal ossification during tendon healing affects normal tendon functions, we hypothesized that ADSC-Exos would be able to promote tenogenic differentiation and inhibit osteogenic differentiation of TSCs. The results showed that, indeed, ADSC-Exos increased TNMD, collagen Ⅰ, and SCX expression in TSCs via activation of the TGF-β/SMAD2/3 and BMP/SMAD1/5/9 pathways. However, ADSC-Exos did not affect the expression of ALP or Runx2 in TSCs. This suggests that ADSC-Exos could effectively promote tenogenic differentiation of TSCs, but not inhibit osteogenic differentiation.

Scar formation caused by inflammation after tendon injury is a major cause of histological changes affecting tendon healing prognosis [35]. Therefore, inhibiting the early inflammatory response of tendon injury is beneficial to early healing. Macrophage infiltration and the release of pro-inflammatory cytokines are characteristics of early inflammation after tendon injury [36, 37]. Shen et al. found that ADSC-Exos reduced the early inflammatory response after tendon injury by regulating macrophages, while Heo et al. confirmed that ADSC-Exos were able to modulate macrophages from an M1 to M2 phenotype [10, 38]. In the current study, we found that $CD163^+$ M2 macrophages increased significantly in the ADSC-Exo group. In addition, the M2-stimulating factor, IL-10, was increased in the ADSC-Exo group. Therefore we suggest that ADSC-Exos were able to alleviate early inflammation after tendon injury by modulating macrophages.

Tissue integrity is the standard for evaluating the quality of tendon healing. We used the central 1/3 patellar tendon injury rat model to evaluate tendon healing. H&E staining showed that collagen fibers in the ADSC-Exo group were more regular compared to those in the control and GelMA groups. In addition, the biomechanical properties of tendon tissues in the ADSC-Exo group were significantly improved at 4 weeks. We also investigated the regulatory effect of ADSC-Exos on TSCs in vivo. Immunohistochemical analyses showed that ADSC-Exos promoted the expression of tenogenic differentiation genes in vivo but did not inhibit the expression of osteogenic differentiation genes in the injured area.

In previous reports, CD146 has been used as a surface marker of TSCs; $CD146^+$ TSCs switch to an activated state during tendon-injury healing and increase their proliferation, migration, and tenogenic differentiation ability [39]. Our results showed that the expression of $CD146^+$ TSCs in the ADSC-Exo group

was the highest among three groups. This indicated that ADSC-Exos promoted the proliferation ability of $CD146^+$ TSCs.

Exosomes are generally used to repair tissues by intravenous or local injection. However, due to difficulty in their local retention, exosomes are unable to exert their full biological efficacy. GelMA is a photosensitive biohydrogel with excellent biocompatibility and degradability, and is widely used in various tissue engineering applications [40, 41]. GelMA exists in a liquid state at 37°C and becomes cross-linked under ultraviolet light to form a gel state with ECM properties. Because of its mild response to environmental conditions, GelMA has great advantages for use in biomedicine and is expected to be applicable for various clinical treatments [42]. For instance, Aubin et al. attempted to change the proliferative arrangement of different cells using micropatterned GelMA to provide a theoretical basis for constructing functional tissues in vitro [43]. Zou et al. used GelMA to construct biomimetic bone with a trabecular bone structure and Hu et al. used GelMA microspheres loaded with small extracellular vesicles to promote cartilage regeneration [44, 45]. In the current study, GelMA was used as a carrier of ADSC-Exos to provide a good microenvironment for exosome storage and their gradual absorption. Therefore, ADSC-Exos loaded into GelMA is a promising treatment for tendon injury.

The current study does have some limitations. First, we selected only one time point to analyze phosphorylation in TSCs. Phosphorylation is a continuous process and the 30-min time point selected may not be optimal to detect TSC phosphorylation. Second, we only evaluated short-term tendon healing. The long-term therapeutic effect of ADSC-Exos on tendon healing (scar formation) requires further study. In addition, which specific substance in exosomes activates the SMAD pathways still needs further exploration.

Conclusions

Overall, our results showed that ADSC-Exos were absorbed by TSCs and promoted their proliferation, migration, and tenogenic differentiation via the TGF-β/SMAD2/3 and BMP/SMAD1/5/9 signaling pathways. In addition, ADSC-Exos alleviated early inflammation and promoted tendon healing. These findings suggest the potential clinical value of ADSC-Exos in treating tendon defects and provide a new approach for the treatment of tendon injuries.

Abbreviations

ADSCs: Adipose-derived mesenchymal stem cells; ADSC-Exos: Adipose-derived mesenchymal stem cell derived exosomes; TSCs: Tendon stem cells; ECM: extracellular matrix; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; NTA: Nanoparticle tracking analysis; TEM: Transmission electron microscopy; EdU: 5-ethynyl-2'-deoxyuridine; H&E: Hematoxylin-eosin; TNMD: Tenomodulin; SCXA: Scleraxis; ALP: Alkaline phosphatase; Runx2: Runt-related transcription factor 2; GelMA: Gelatin methacryloyl

Declarations

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Authors’ contributions

HCL and MZZ contributed to cytology experiment, animal experiments, data acquisition, data analysis, and manuscript writing, MYS, TTZ, WJL and SLY provided experimental technical support and final approval of manuscript; QBC and ZZL took part in the experimental design, text revision, and final approval of manuscript.

Author’s information

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The experimental protocol about animals was approved by the Harbin Medical University Ethics Committee.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflicting interests.

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Figures

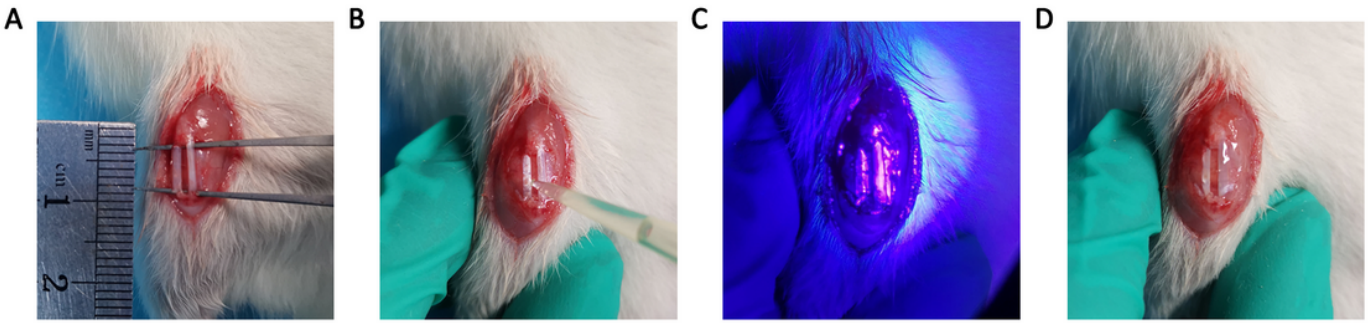

Figure 1

Patellar tendon injury model. A Exposure and removal of central 1/3 right patellar tendon tissue. B ADSC-Exos-loaded GelMA injected into the location of the patellar tendon defect. C Radiation GelMA with 405nm light source for 30s. D Photocross-linking under radiation forms a gel state.

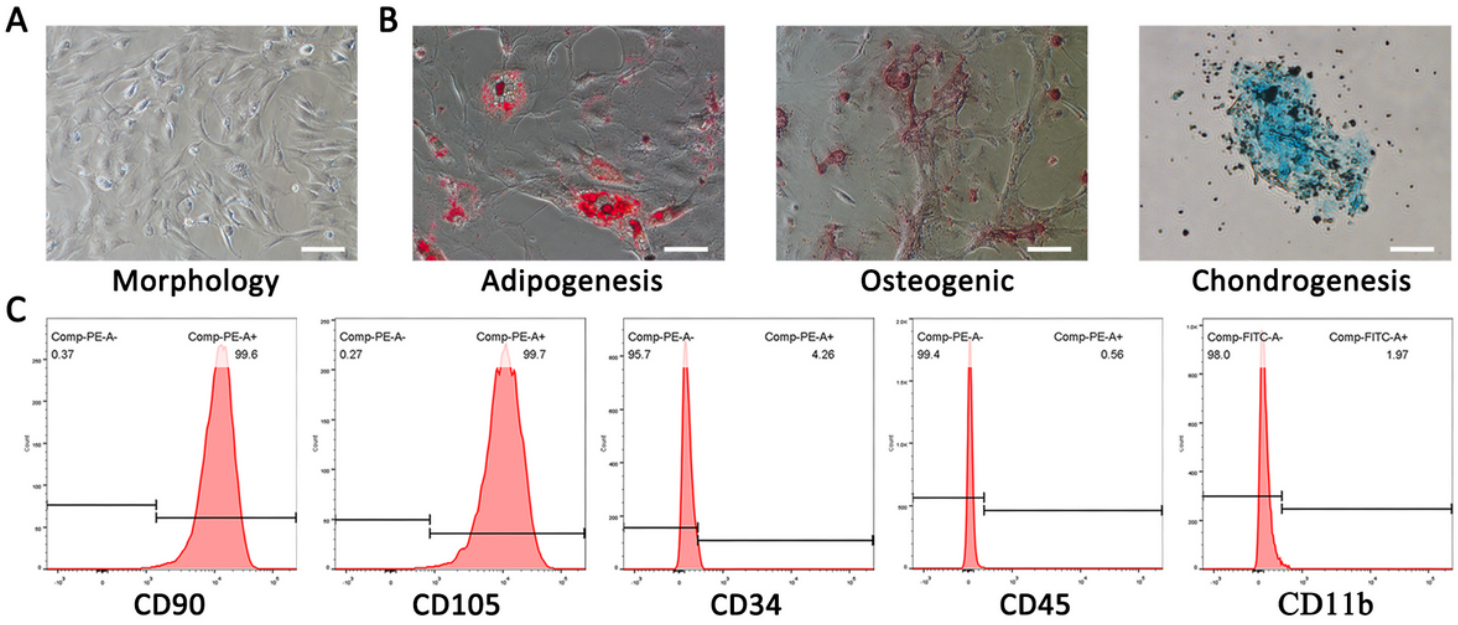

Figure 2

Characterization of ADSCs. A Morphology of ADSCs. B Adipogenic, osteogenic and chondrogenic differentiation of ADSCs. C Flow cytometry for detection of ADSC surface markers. Bars, 100μm

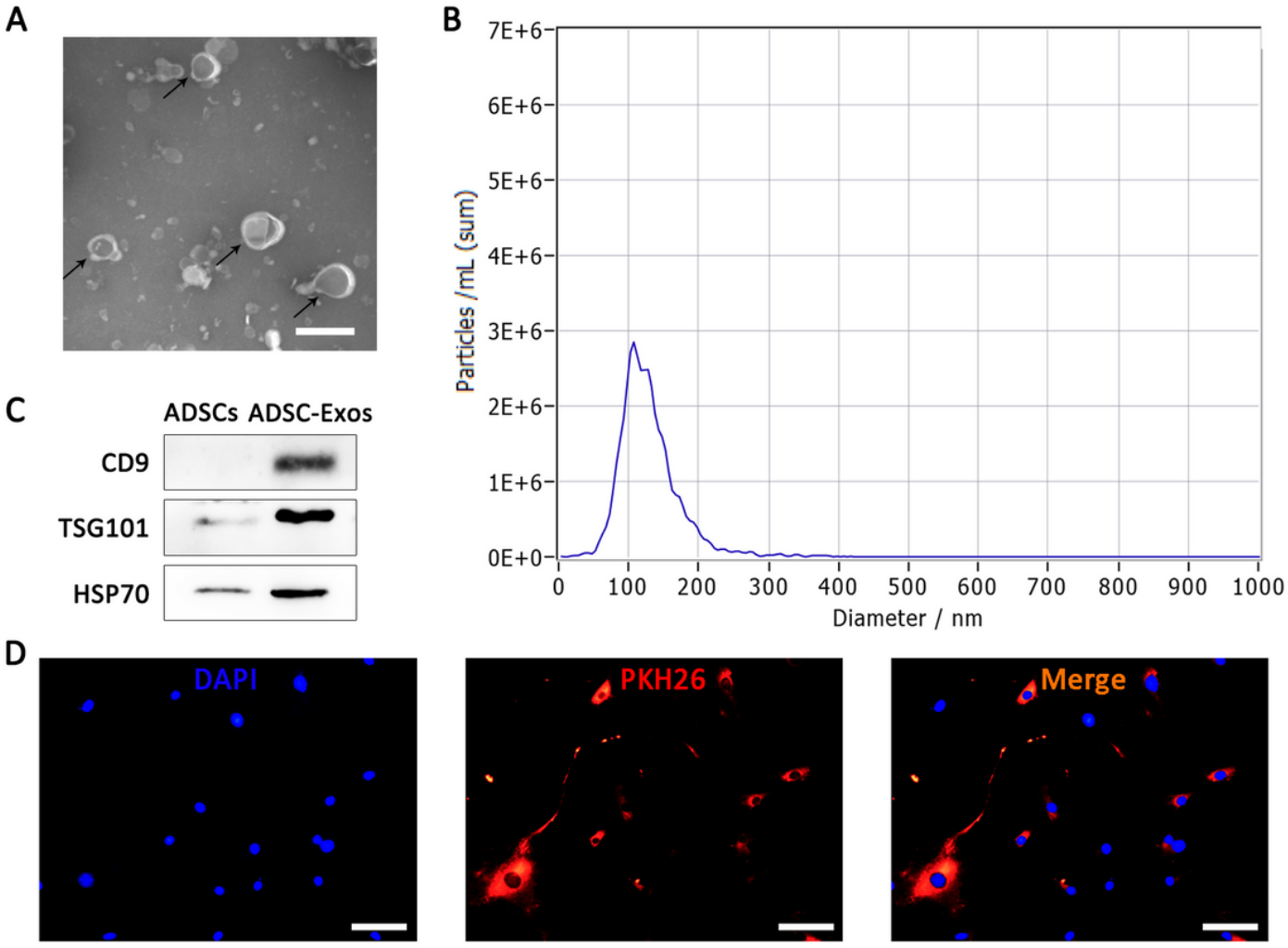

Figure 3

Characterization of ADSC-Exos. A Morphology of ADSC-Exos under transmission electron microscope. B Particle size distribution. C Western blot was used to detect exosome surface markers. D PKH26-labeled ADSC-Exos internalization by TSCs. Bars (ADSC-Exos) 100nm; Bars (ADSCs), 100μm

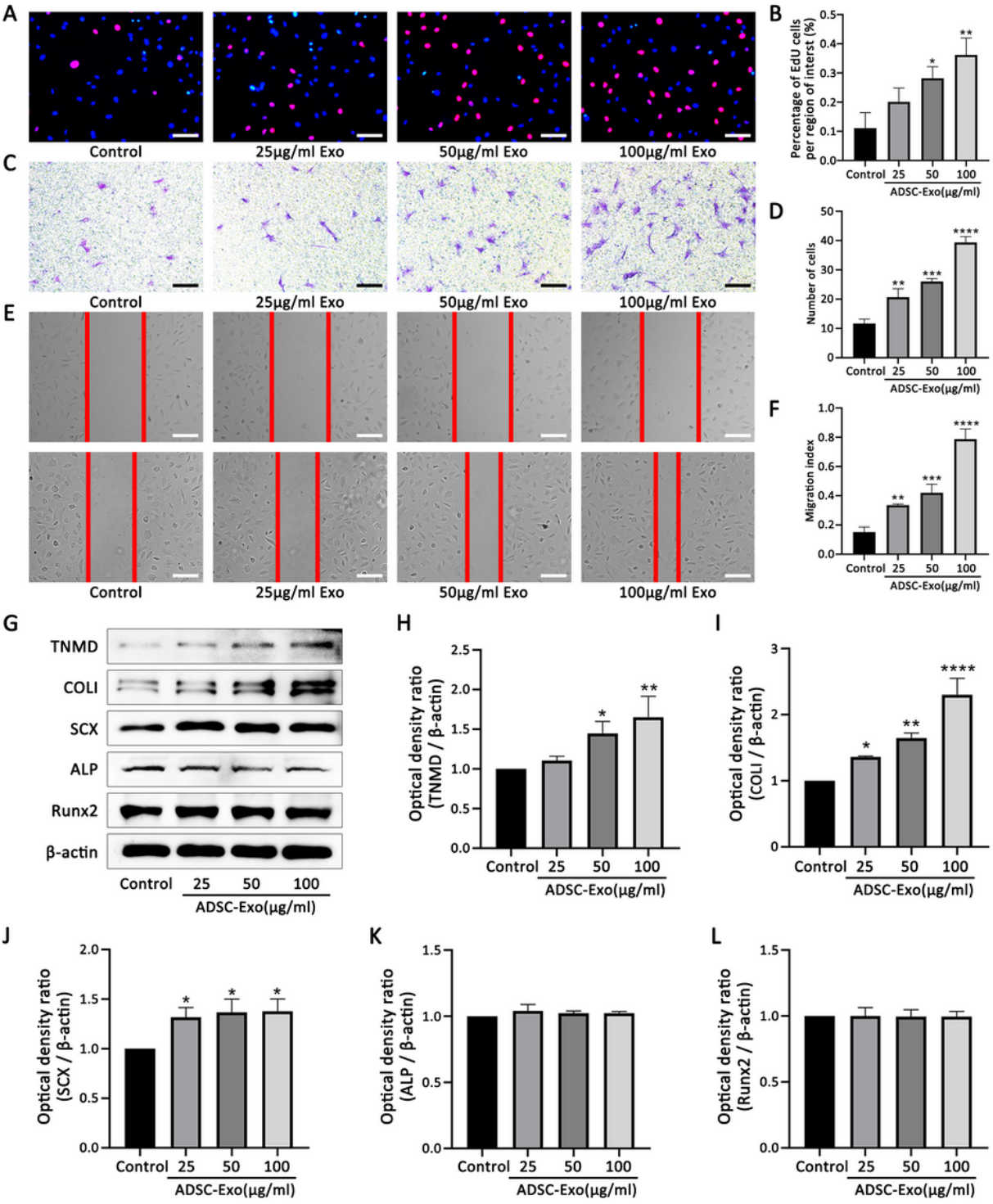

Figure 4

ADSC-Exos promote the proliferation, migration, and tenogenic differentiation of TSCs. A, B Effect of different concentrations of ADSC-Exos on the proliferation of TSCs by EdU assays. Effect of different concentrations of ADSC-Exos on the migration of TSCs by C, D transwell assays and E, F scratch assays. G-L Western blot analysis of protein levels of TNMD, Collagen Ⅰ, SCX, ALP, and Runx2 induced by different

concentrations of ADSC-Exos. Bars, 100μm. Data are represented as mean ± SD. *, vs Control group; n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

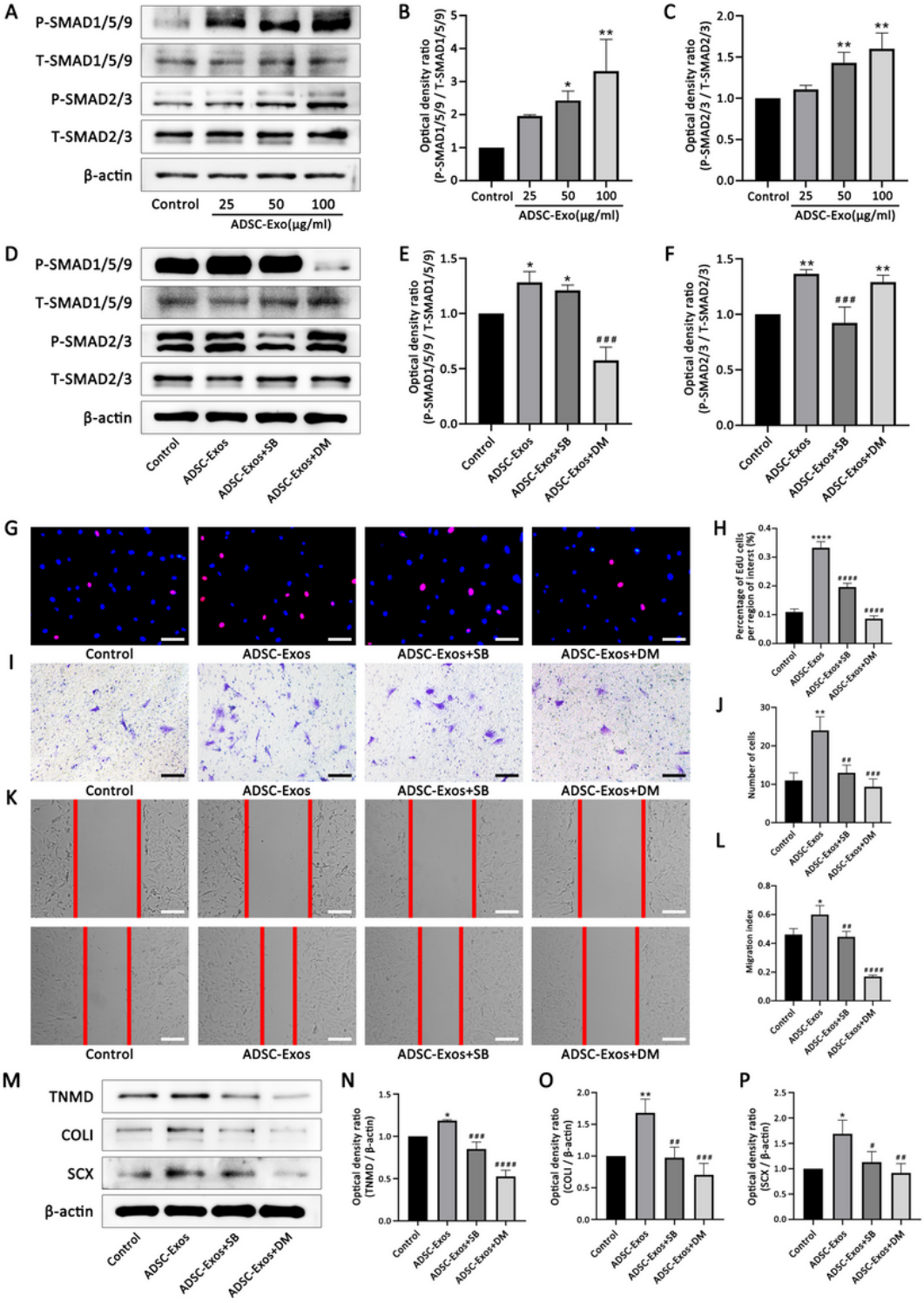

Figure 5

ADSC-Exos promote the proliferation, migration, and tenogenic differentiation of TSCs via the TGF-β/SMAD2/3 and BMP/SMAD1/5/9 signaling pathways. A-C Western blot analysis of protein levels of p-SMAD2/3 and p-SMAD1/5/9 induced by different concentrations of ADSC-Exos. D-F SB431542 and

dorsomorphin inhibit the activation of TGF-β/SMAD2/3 and BMP/SMAD1/5/9 induced by ADSC-Exos, respectively. G, H EdU assay showed that ADSC-Exos-mediated TSC proliferation was suppressed by inhibitors SB431542 and dorsomorphin. I, J Transwell assay and K,L scratch assays showed that ADSC-Exos-mediated TSC migration was suppressed by inhibitors SB431542 and dorsomorphin. M-P Western blot analysis of protein levels of TNMD, Collagen Ⅰ, and SCX promoted by ADSC-Exos was inhibited by inhibitors SB431542 and dorsomorphin. Bars, 100μm. Data are represented as mean ± SD. *, vs Control group; #, vs ADSC-Exos group; n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001

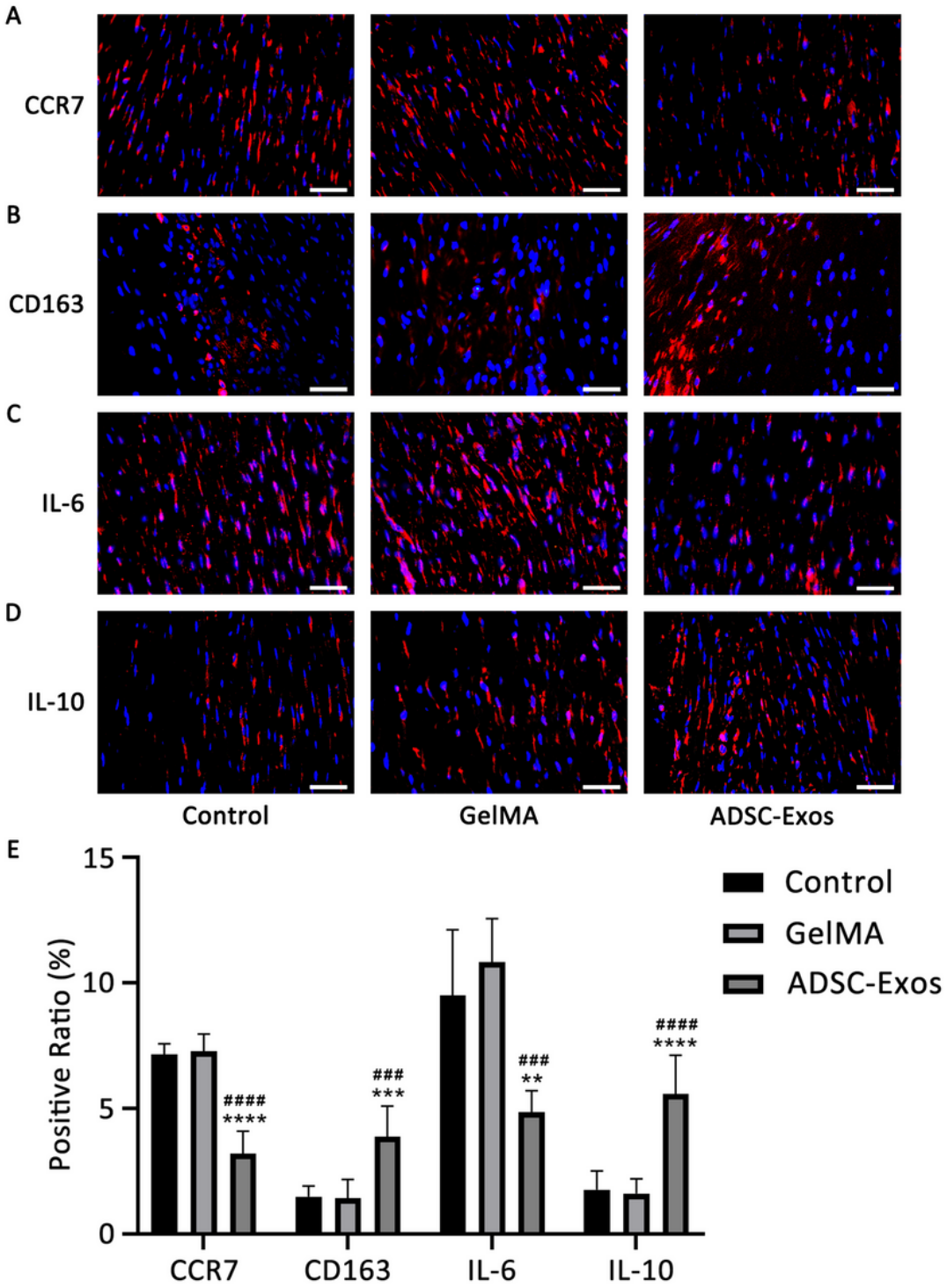

Figure 6

ADSC-Exos inhibits inflammatory expression in tendon injury. A-D The expression of CCR7+, CD163+, IL-6+, and IL-10+ cells were detected by immunofluorescence at week 1. E Positive ratio of inflammation-related factors. Bars, 50μm. Data are represented as mean ± SD. *, vs Control group; #, vs GelMA group; n = 6. **P < 0.01, ***P < 0.001, ****P < 0.0001, ###P < 0.001, ####P < 0.0001

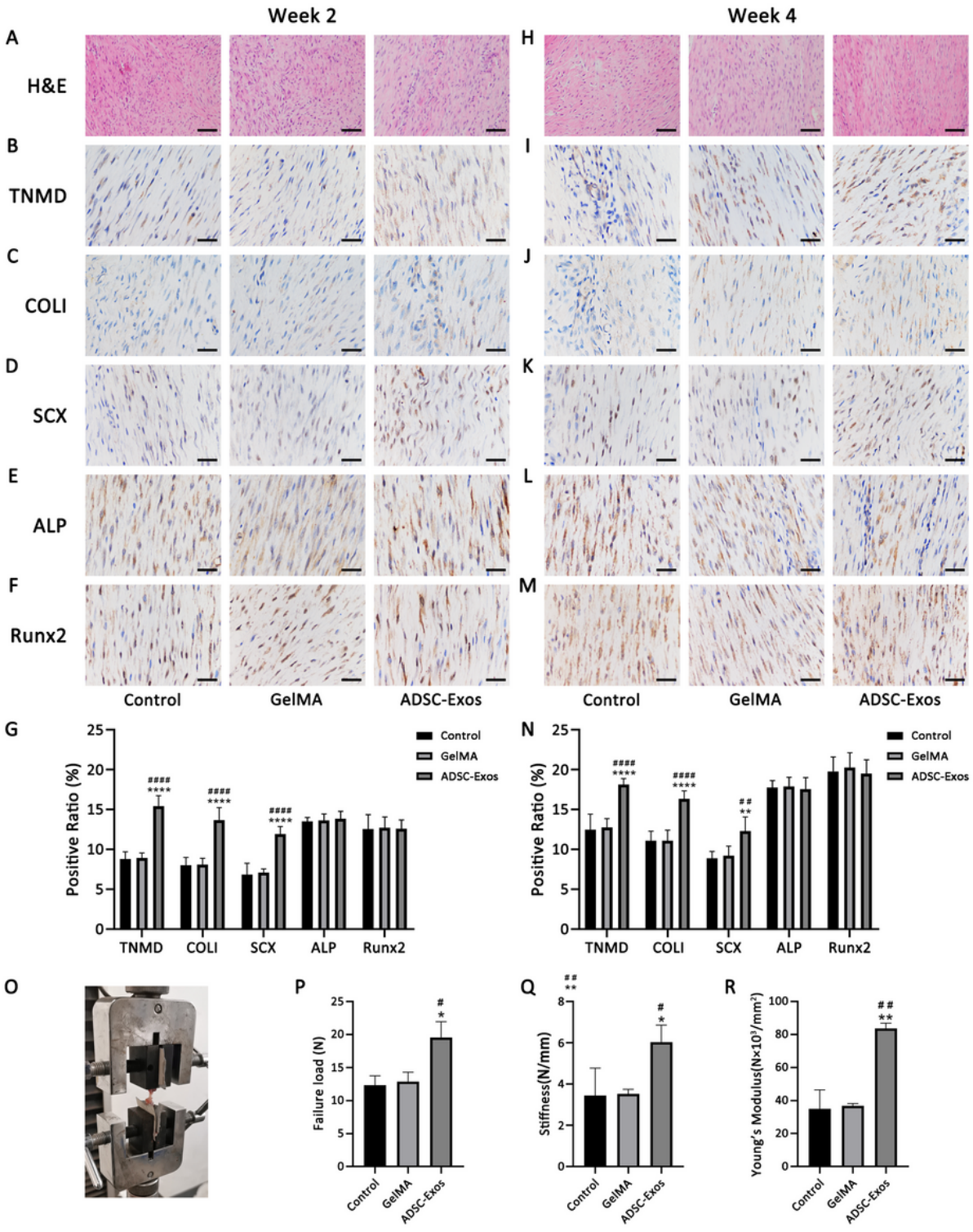

Figure 7

ADSC-Exos improved the healing of tendon injury. A, H The H&E staining of tendon injury at week 2 and week 4. B-F, I-M The expression of TNMD, Collagen Ⅰ, SCX, ALP, and Runx2 were detected by immunohistochemistry assay at week 2 and week 4. G,N Quantitative analysis of tenogenic and osteogenic related factors at week 2 and week 4. O-R Results of biomechanical tests (Failure load, Stiffness, Young's Modulus) at 4 weeks. Bars (H&E), 100μm; Bars (immunohistochemistry), 50μm. Data

are represented as mean ± SD. *, vs Control group; #, vs GelMA group; n = 6. **P < 0.01, ****P < 0.0001, ####P < 0.0001

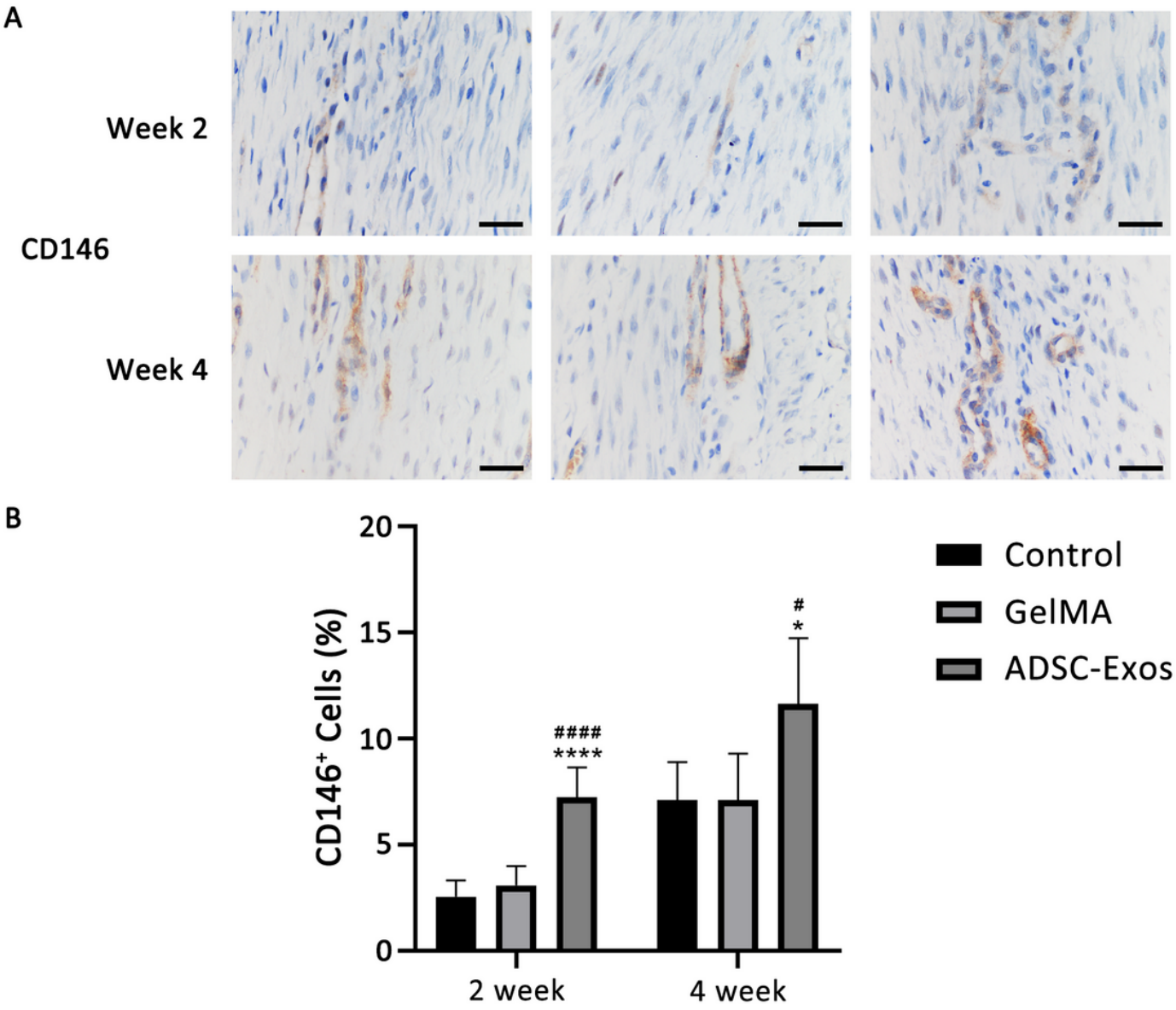

Figure 8

ADSC-Exos promoted TSC proliferation in vivo. A Cellular expression of CD146+ at week 2 and week 4 was evaluated by immunohistochemistry assay. B Ratio of CD146+ cells at week 2 and week 4. Bars, 50μm. *, vs Control group; #, vs GelMA group; n = 6 *P < 0.05, ****P < 0.0001, #P < 0.05, ####P < 0.0001

Supplementary Files

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