Research Article

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Regenerative Medicine

Adipose-derived stem cell exosomes facilitate rotator cuff repair by mediating tendon-derived stem cells

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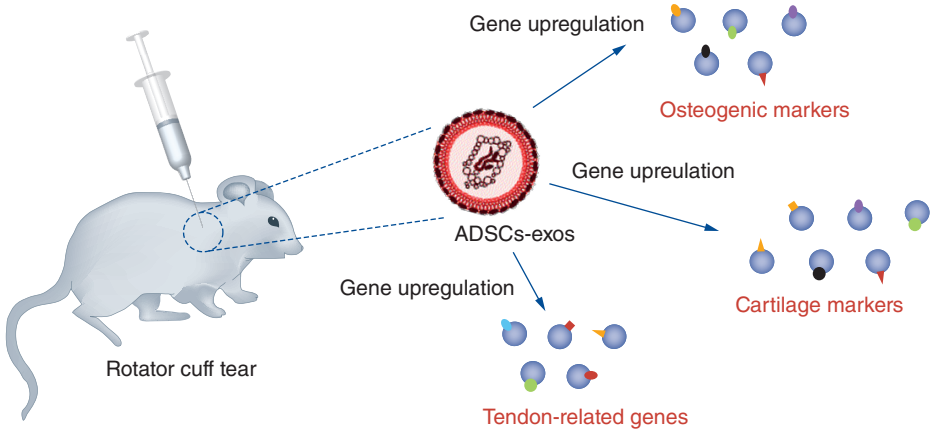
Aim: To evaluate the potential capability of adipose-derived stem cell exosomes (ADSC-exos) on rotator cuff repair by mediating the tendon-derived stem cells (TDSCs) and explored the mechanism. **Methods:** First, we investigated the growth, survival and migration of TDSCs in the presence of ADSC-exos *in vitro*. Using a rat rotator cuff injury model to analyze the ability of the ADSC-exos to promote rotator cuff healing *in vivo*. **Results:** The hydrogel with ADSC-exos significantly improved the osteogenic and adipogenesis differentiation and enhanced the expression of *RUNX2*, *Sox-9*, *TNMD*, *TNC* and *Scx* and the mechanical properties of the articular portion. **Conclusion:** The ADSC-exos have the potential to promote the rotator cuff repair by mediating the TDSCs.

Graphical Abstract:



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Keywords: exosomes • muscle regeneration • rotator cuff tear • tendon-derived stem cells

The rotator cuff tear (RCT) is a widespread problem in elderly and young people, particularly those who often exercise [1,2]. Four tendinous entheses of the rotator cuff surrounding the proximal humerus offer glenohumeral joint stability [3]. The main cause of RCT is muscle aging and tendon tearing. Clinically, arthroscopic surgeries are usually used to treat RCT. However, RCT has a high probability of recurrence, which causes great distress to patients and seriously affects quality of life [4]. The junctional fibrocartilage regeneration and new bone formation are paramount for rotator cuff healing. Although the basic and clinical research on the repair of rotator cuff are widely developed, including research on surgical repair, growth factors (GFs), grafts, biophysical therapies, mesenchymal stem cells and gene therapy, there are still many potential problems with these treatment strategies [5–7], such as biosafety, immunogenicity and efficacy.

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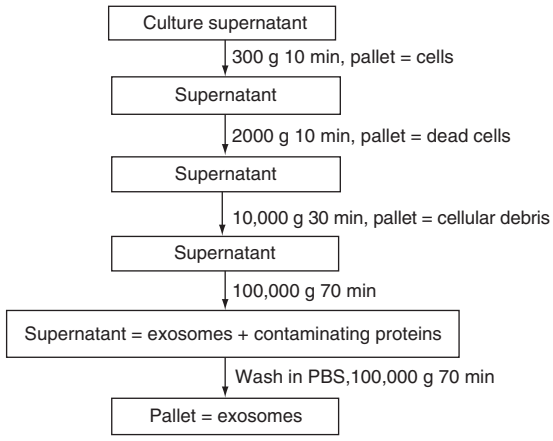

Figure 1. The isolation process of exosomes.
Data from Wang *et al.* [26].

Mesenchymal stem cells (MSCs), which are a promising cell source for various types of tissue repair and regeneration, are multipotent adult stem cells [8]. MSCs have been been considered to improve tendon healing and rotator cuff repair [9]. However, local application of MSCs has the risk of clinical safety (e.g., immune rejection, abnormal proliferation) and efficacy (e.g., rapid cell clearance) [10].

Tendon-derived stem cells (TDSCs), derived from human and mouse tendons, have clonogenicity, multipotency and self-renewal capacity [10]. It has been reported that TDSCs could differentiate into osteoblasts, chondrocytes and adipocytes *in vitro* [11,12]; nevertheless, their potential role in tendon and rotator cuff healing remains challenging in the clinical setting.

It has been documented that the ability of cartilage repair from MSCs in osteoarthritis (OA) is limited [13]. Several studies have also suggested that the beneficial results should be attributed to the MSC-derived secretome [14,15]. Extracellular vesicles (EVs) secreted by MSCs are categorized into three types based on size, apoptotic bodies, macrovesicles and exosomes [16]. Exosomes are vehicles that transfer proteins, nucleic acids and lipids and the diameter is typically 30–150 nm [14,17]. Several studies have proved that exosomes play an important role in physiology and pathological processes, for instance, inhibiting bladder cancer proliferation, accelerated cutaneous wound healing by promoting angiogenesis, eased brain ischemic injury and therapeutic potential in osteoarthritis [18–21]. Adipose-derived stem cells (ADSCs), a kind of MSC obtained from fat tissue, were reported to have a positive impact on the tendon bone interface (TBI) healing and rotator cuff repair [22]. Therefore, it is valuable to explore the effects of adipose-derived stem cells exosomes (ADSC-exos) on rotator cuff repair.

In this study, we investigated the efficacy of ADSC-exos on rotator cuff repair by mediating the TDSCs. First, we investigated the growth, survival and differentiation of TDSCs in the presence of human ADSC-exos *in vitro*. We then analyzed the ability of the ADSC-exos to promote rotator cuff healing *in vivo* using a rat rotator cuff injury model. This study is expected to reveal the mechanism of ADSC-exos to promote rotator cuff healing.

Methods

Preparation of human ADSC-exos

ADSCs were isolated from human abdominal adipose tissue [23]. In short, the cells were extracted from human subcutaneous adipose tissue by liposuction surgery (six healthy women; age range 35–55 years) in the Tongji University affiliated Shanghai East Hospital. The cells were cultured in serum-free medium (NC0103; Yocon Biology, Beijing, China) and incubated at 37°C in a humidified atmosphere with 5% CO_2. After second- and third-generation ADSCs culturing, ADSC-exos were isolated according to differential ultracentrifugation described previously [24,25]. Briefly, ADSCs were rinsed with phosphate-buffered saline (PBS) when the adherent cells reached 80–90% confluency and were then cultured in fresh serum-free medium (25 ml/dish) for 48 h to obtain culture supernatants. To discard the cells and cellular debris, the medium was centrifuged at 300*g* for 10 min and 2000*g* for 10 min at 4°C, followed by centrifugation at 10,000*g* for 30 min. The supernatant was ultracentrifuged at 100,000*g* for 70 min. Pellet at the bottom of the centrifuge tube were resuspended in PBS and centrifuged at 100,000*g* for 70 min to eliminate contaminating proteins and acquire ADSC-exos pellets (Figure 1).

The morphology of ADSC-exos was characterized by transmission electron microscopy (TEM; FEI Tecnai G2 Spirit, FEI, OR, USA). The size distribution was measured with NanoSight NTA. The expression of exosomal

markers CD9 (1:1000; Abcam, Cambridge, England), HSP70 (1:1000; Proteintech, IL, USA) and TSG-101 (1:1000; Proteintech) were analyzed by western blotting, and the Calnexin (1:1000; Proteintech) was used as a negative exosomal marker (Supplementary Figure 1).

Isolation & culture of TDSCs

TDSCs were isolated from tendon tissue of human [26]. After taking the tissue from tendon tissue of human, the tissue was immediately put it into a 50-ml centrifuge tube containing tissue protection solution (Hanks' balanced salt solution containing 1% s double antibody), stored at 4°C, and transferred to the laboratory for primary cell separation. In the ultra-clean table, the tissue was removed and placed in a 10-cm cell-culture dish. The tendon tissue was cut into small pieces of 1–3 mm^3. The tissues were digesting for 3 h at 37°C after mincing. The cell suspension was obtained by passage through a cell strainer. The cells were washed in PBS by centrifugation and resuspended in medium. The obtained cells were vaccinated and cultured at 37°C, 5% CO_2. Suspended cells were removed by washing with PBS after culturing for 2 days. Primary cells were obtained by digesting with trypsin after culturing for 8 days. Culture medium was changed every 2 days for TDSCs (Supplementary Figure 2).

Multidifferentiation assessment

Osteogenic differentiation assays: TDSCs were incubated in osteogenic medium for 28 days [26]. Then, 1 nmol dexamethasone, 50 mmol ascorbic acid and 20 mmol β-glycerolphosphate were added to the medium. The cells were seeded at a density of 6000 cells/well in 96-well plates and cultured with medium supplemented with 0.3 mg/ml of exosomes for exo-0.3 group (each well was cultured with 200 µl of medium and 200 µl/well PBS solution of exosomes) or 200 µl/well of PBS for control group (each well was cultured with 200 µl of medium). Under the action of this medium, the culture medium and exosomes were changed every 3 days and cultured at 37°C and 5%$C0_2$ for 2 weeks. The medium was removed. The pellets were rinsed twice with PBS, fixed with 4% formaldehyde for 30 min and stained with Alizarin Red.

Adipogenic differentiation assays: the incubation of TDSCs was the same as osteogenic differentiation, and the medium was replaced with adipogenic medium [27], which was supplemented with 500 nmol dexamethasone, 0.5 mmol isobutylmethylxanthine, 50 µmol indomethacin and 10 µg/ml insulin. The cells were seeded at a density of 6000 cells/well in 96-well plates and cultured with medium supplemented with 0.3 mg/ml of exosomes for exo-0.3 group (each well was cultured with 200 µl of medium and 200 µl/well PBS solution of exosomes) or 200 µl/well of PBS for the control group (each well was cultured with 200 µl medium). The medium and exosomes were changed every 3 days and cultured at 37°C and 5%$C0_2$ for 2 weeks. The pellets were rinsed twice with PBS, fixed with 4% formaldehyde for half an hour and stained with Oil Red O.

Chondrogenic differentiation assays: for chondrogenic differentiation, approximately 6×10^5 cells were placed in a 15-ml polypropylene tube (Becton Dickinson, NJ, USA) and centrifuged at 450*g* for 10 min. The pellet was cultured in chondrogenic medium (low-glucose Dulbecco's modified eagle's medium [Invitrogen, CA, USA] supplemented with 500 ng/ml BMP-2, 10 ng/ml TGF-b3 [R&D Systems, MN, USA], 10^{-7} M dexamethasone [Sigma-Aldrich, MO, USA], 50 mg/ml ascorbate-2-phosphate, 40 ug/ml proline, 100 µg/ml pyruvate and 50 mg/ml ITS+Premix [Becton Dickinson, NJ, USA]) and 0.3 mg/ml of exosomes for exo-0.3 group (each well was cultured with 200 µl medium and 200 µl/well PBS solution of exosomes) or 200 µl/well of PBS for control group (each well was cultured with 200 µl medium). The medium and exosomes were replaced every 3–4 days and cultured at 37°C and 5% $C0_2$ for 21 days. For microscopy, the pellets were embedded in paraffin, cut into 4-mm sections and stained with toluidine blue (Supplementary Figure 3) [27].

TDSCs uptake of ADSC-exos

The ADSC-exos were labeled with PKH26 fluorescent labeling kit. First, the TDSCs were incubated with the labeled exosomes at 37°C for 10 h. To confirm the TDSC uptake of the labeled exosomes, the treated cells were fixed and imaged. The ADSC-exos were taken off the ice to melt, then mixed with 200 µl diluent C in tube A. A 2′ dye solution (4 × 10-6 M) in 300 µl diluent C was prepared by adding 4 ml of the PKH26 ethanolic dye solution (cat. no. P7333) to 1.5 ml Ep for tube B. Tube A and B were mixed and incubated at room temperature for 5 min and then 600 µl 1% bovine serum albumin was added to terminate dyeing. Volume mixture was increased to 20 ml with 1% bovine serum albumin, the mixture was ultracentrifuged at 120,000*g* for 60 min. Pellets at the bottom of the centrifuge tube were resuspended in PBS to prepare PKH26- exosomes. TDSCs were washed with PBS three times, then exosome-free fetal bovine solution and a fresh preparation of PKH26- exosomes were

added at a concentration of ~10 μg/ml. The control group was using aseptic PBS incubation with TDSCs. All the TDSCs were incubated at 37.5°C, 5% CO_2 for 12 h. After incubation, the TDSCs were washed three times with preheated PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. Then, the TDSCs were washed three times with PBS and treated with 0.1% Triton X-100 PBS at room temperature for 5 min. The TDSCs were washed three times with PBS and diluted with rhodamine phyllophin at a ratio of 1:800. TDSCs uptake of ADSC-exos were observed by a fluorescence microscope.

Cellular proliferation

The ability of cellular proliferation was performed by cell counting kit-8(CCK-8, 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2 H-tetrazolium sodium salt, WST-8) assay. The cells were seeded at a density of 8000 cells/well in 96-well plates and cultured with different medium supplemented with 0.075, 0.15 and 0.30 mg/ml PBS solution of exosomes (each well was cultured with 200 μl of medium and 200 μl/well PBS solution of exosomes), corresponding to the Exo-0.075, Exo-0.15 and Exo-0.3 groups in the cell proliferation curve, respectively. The control group refers to the component without PBS solution of exosomes. The PBS group was cultured with 200 μl of medium and 200 μl/well of PBS solution, and the tendon-derived stem cells were normal. The cells were incubated with the WST-8 mixture for 30 min after 1, 2, 3, 4 and 5 days of culture. The optical density (OD) values were measured using a microplate reader at 450 nm.

Animals & experimental design

Forty-eight SD rats (8-10 weeks, n = 96 samples) were anaesthetized by 4% chloral hydrate. The surgical site was shaved and disinfected. The rats' supraspinatus tendon was cut off on bilaterial shoulders and cartilage was worn down (Figure S4). 4 weeks later, surgery was performed again to repair the rotator cuff insertion (modified Mason-Allen suture) with a No. 5–0 non-absorbable suture (Ethicon, NJ, USA). And the rats were randomly divided into four groups: group A, in which PBS solution was injected in the shoulder (100 μl, n = 24); group B, in which hydrogel (VitroGel 3D Kit, Shanghai, China) was injected in the shoulder (100 μl, n = 24); group C ADSC-exos–hydrogel complex (EHC) was injected in the shoulder at a a concentration of ADSC-exos of 0.3 mg/ml, and the ADSC-exos to hydrogel ratio was 1:3 (100 μl, n = 24); and group D, which was the control group and unprocessed (n = 24). At 4 and 8 weeks after implantation, six rats from each group were sacrificed with CO_2. Muscle around the humerus as well as humerus bone samples were harvested for histological analysis, PCR and biomechanical testing. All experiments were approved by the Institutional Animal Care and Use Committee of the Tongji University, School of Medicine, Shanghai.

Histological examination

Tissue samples were fixed with 4% neutral buffered formalin and embedded in paraffin after decalcification. The samples (n = 3, per group) were cut to a thickness of 4 μm and stained with hematoxylin and eosin and Masson for histological examination.

Real-time reverse-transcription PCR

The gene expression from tissue samples was quantified and compared by PCR. The RNA, isolated from tendon-to-bone tissue samples (n = 3, per group), was measured with a Nanodrop. mRNA was extracted by using High Capacity Reverse Transcription kit(Vazyme, Nanjing, China) and reversely transcripted to cDNA. Quantitative PCR was conducted by real-time PCR system. The relative mRNA expression of tested gene in each sample (such as Osteogenic marker *RUNX2*, chondrogenic markers *Sox-9*, tenogenesis markers *TNC*, *TNMD* and *Scx* in each group) was normalized against GAPDH mRNA, and calculated using the 2-ΔΔCT method.

Biomechanical testing

Biomechanical testing was measured by a tensile testing machine (Instron 3345; Bluehill 2.0, Instron Corporation, MA, USA). The specimens (n = 6, per group) were immersed in PBS to avoid drying out. Specimens were preconditioned from 0 to 10 N for three cycles. Ramp to failure was then performed at a rate of 0.3%/s [28]. The tensile strength of each specimen was maximum tensile force at fracture. There are three replications in each group.

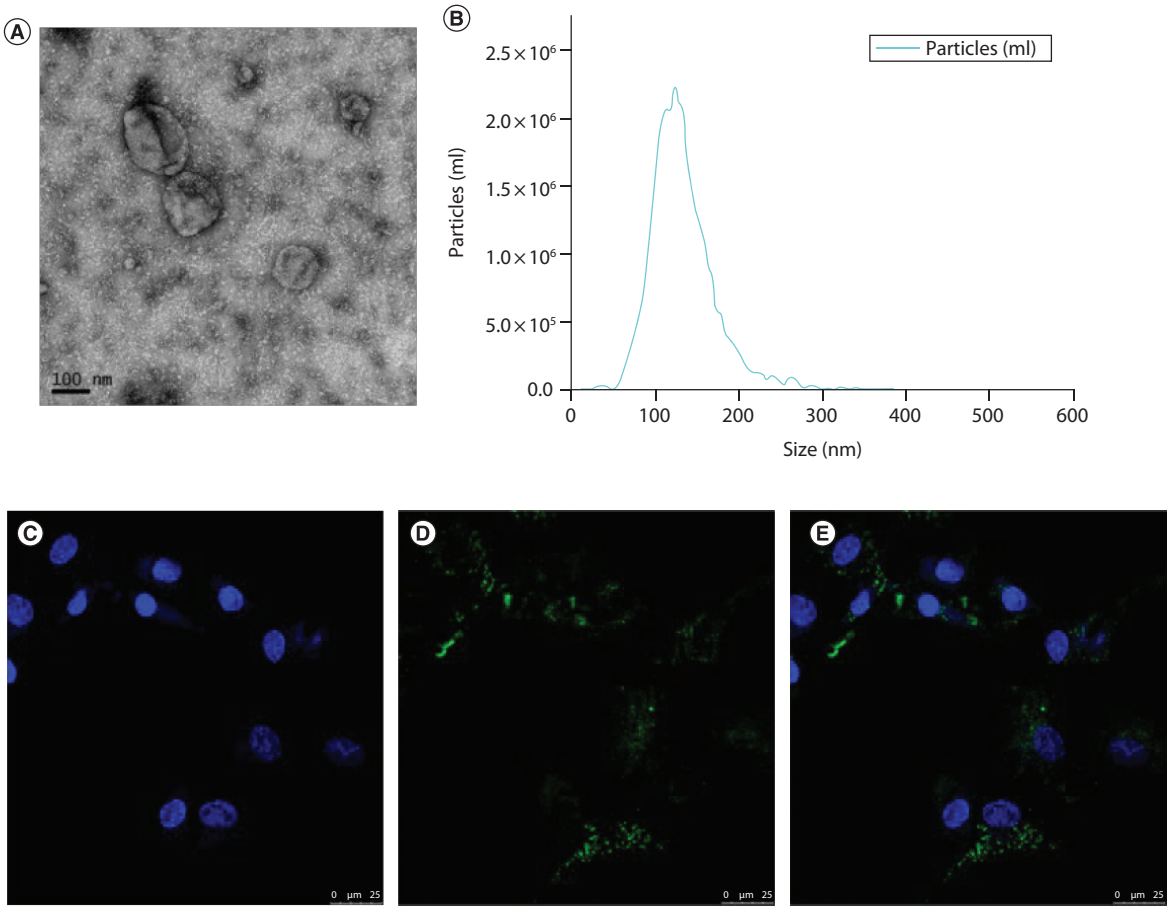

Figure 2. Characterization of adipose-derived stem cells exosomes and exosome uptake. (A) Morphology of adipose-derived stem cells exosomes (ADSC-exos) was characterized by transmission electron microscopy. **(B)** Particle size distribution of ADSC-exos, **(C)** tendon-derived stem cells, **(D)** labeled ADSC-exos and **(E)** tendon-derived stem cells uptake of ADSC-exos were observed by fluorescence microscope.

Statistical analysis

All data were reported as mean ± standard deviation (SD). The cell proliferation curves, real-time PCR analysis and biomechanical testing data were analyzed using SPSS for one-way analysis of variance. Differences of $p < 0.05$ were considered statistically significant.

Ethics statement

Sprague Dawley rats were obtained from Shanghai Slacker Laboratory Animal Co, Ltd. (Shanghai, China) and housed in rooms maintained at constant temperature and humidity with a 12 -h light cycle. Animals were allowed food and water ad libitum. For this study, organs were obtained from mice that had to be killed because of excessive breeding. Animal handling was performed according to strict governmental and international guidelines, and all experiments were approved by the Institutional Animal Care and Use Committee of the Tongji University, School of Medicine, Shanghai.

Results

Characterization of ADSC-exos & exosome uptake

The cup-shaped morphology of the exosomes was observed by transmission electron microscopy (Figure 2A). The size distribution of most ADSC-exos, ~30–150 nm, was measured with NanoSight NTA (Figure 2B). The TDSCs were incubated with labeled exosomes to evaluate whether ADSC-exos interacted directly with TDSCs. The fluorescent of TDSCs was monitored by fluorescence microscope at 5 h, which indicated that TDSCs endocytosed exosomes (Figure 2E).

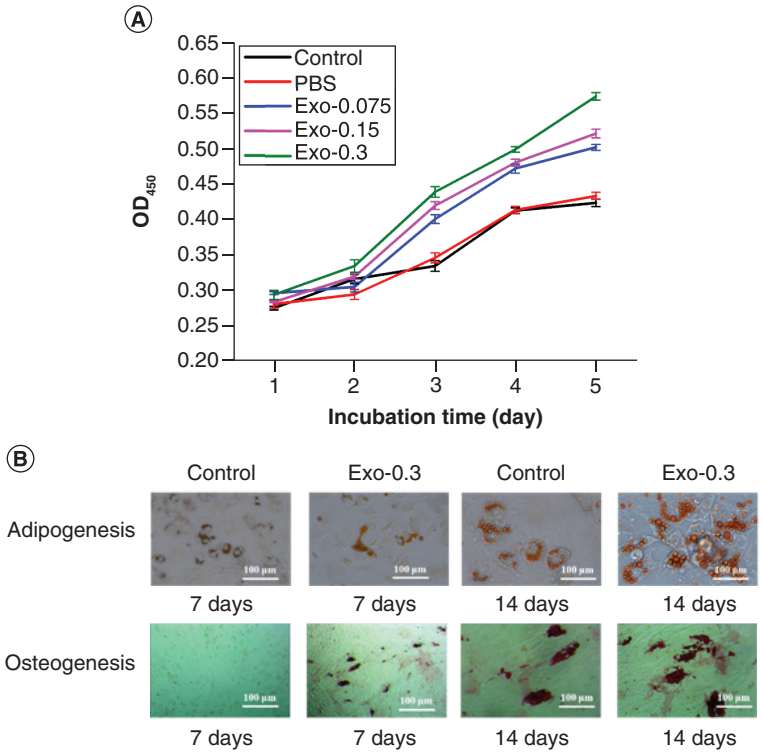

Figure 3. The tendon-derived stem cells proliferation & differentiation enhanced by adipose-derived stem cells exosomes. (A) Cell proliferation curves (n = 3), **(B)** differentiation potential of tendon derived stem cells after 7 and 14 days of differentiation. Adipogenic differentiation was examined using Oil Red O staining (the first line), and osteogenic differentiation was examined using Alizarin Red S staining (the second line).

The ADSC-exos enhance proliferation & differentiation potential

Figure 3A shows the TDSCs proliferation curves incubated with different concentrations of ADSC-exos. All five groups exhibited increased cell proliferation. For the control group or PBS group, cell proliferation rate was lower than that with ADSC-exos. After the cultivation time exceeded 3 days, there was a significant difference between the treatment group and the control or PBS groups. The Exo-0.3 group (cells incubated with 0.3 mg/ml ADSC-exos) was significantly higher than the other groups. The results indicated that the high dose of ADSC-exos (0.3 mg/ml) had better efficacy. Therefore, histopathological and differentiations studies were conducted to further explore their mechanism. The ADSC-exos on multidifferentiation potential of TDSCs is shown in Figure 3B. The results of Oil Red O and Alizarin Red S staining clearly indicated a significant increase in adipogenesis and osteogenesis with ADSC-exos-treated TDSCs.

Histological analysis

All groups were evaluated by histological analysis every 4 weeks for the condition of wounded rotator cuff muscles of rats. Figure 4 shows that the injured rotator cuff muscles of rats still have more defective areas in the control and the PBS groups at 4 weeks. The intercellular space between cells and irregular muscle shrinkage was found in the control and PBS groups at 4 weeks. However, at 4 and 8 weeks, the hydrogel and the EHC groups showed less inflammation than the other two groups. The blue collagen fibers, red cytoplasm, muscle fibers and blood cells could be observed using Masson staining (Figure 4). We observed that the collagen fibers were defective and arranged in disorder in the control group and PBS groups at 4 and 8 weeks. Whether at 4 or 8 weeks, the collagen fiber and muscle bundles showed more regular alignment in the EHC group compared with other groups. These results indicate that EHC has the potential to promote the healing of rotator cuff repairs.

Real-time PCR was used to quantify the mRNA expression levels of each gene. EHC treatment significantly induced upregulation of osteogenic marker *RUNX2* and chondrogenic marker *Sox-9* (Figure 5 A–D). Figure 5E–J shows that the mRNA expression levels of tenogenesis markers *TNC, TNMD* and *Scx* in each group was upregulated at 4 and 8 weeks. EHC treatment significantly induced upregulation of *TNC*. There is a significant difference between the treatment and control groups. Compared with the control group, the hydrogel group also increased the corresponding genes, which indicates that the hydrogel also promotes bone formation. However, compared with the hydrogel group, the EHC group has more significant upregulation of various genes, which indicates that the EHC group has superior bone formation potential.

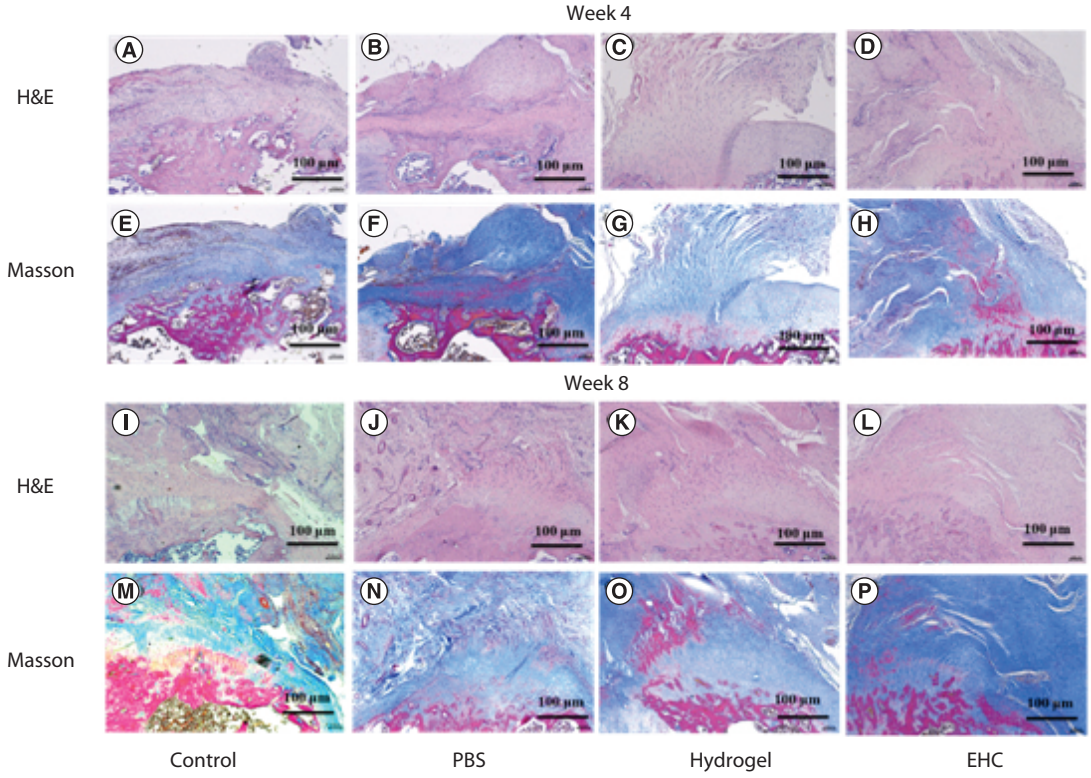

Figure 4. Histological analysis of different treatment groups with hematoxylin and eosin and Masson staining. H&E staining of histological analysis every 4 weeks for the **(A)** control, **(B)** PBS, **(C)** hydrogel and **(D)** EHC groups. Masson staining of histological analysis every 4 weeks for the **(E)** control, **(F)** PBS, **(G)** hydrogel and **(H)** EHC groups. H&E staining of histological analysis every 8 weeks for the **(I)** control, **(J)** PBS, **(K)** hydrogel and **(L)** EHC groups. Masson staining of histological analysis every 8 weeks for the **(M)** control, **(N)** PBS, **(O)** hydrogel and **(P)** EHC.
H&E: Hematoxylin and eosin; EHC: Adipose-derived stem cell exosome–hydrogel complex.

Biomechanical analysis

Biomechanical analysis of the RCT at 4 and 8 weeks after treatment is shown in Figure 6. The maximum tensile forces at the fracture of PBS (20.04 N), hydrogel (26.32 N) and EHC (30.24 N) groups was significantly lower than that of the normal group (40.15 N) at 4 weeks. The EHC injection (23.3 N) promoted the increase in biomechanical properties of RCT compared with other groups, even though the biochemical properties of the EHC group were lower than those of the normal group. At 8 weeks, maximum tensile forces at the fracture of the PBS (20.92 N), hydrogel (30.48 N) and the EHC (40.01N) groups were also lower than the normal group (41.89 N). All raw data of biomechanical analysis are listed in Supplementary Table 1. There are significant differences between the EHC and hydrogel groups. Statistics show that EHC can significantly improve the RCT healing biomechanically. The fundamental mechanism of tendon healing has not been well investigated, but the EHC induced upregulation for the expression of genes encoding tendon-associated molecule and significantly improved the biomechanical RCT healing.

Discussion

RCT is a widespread problem that seriously affects quality of life. The surgical approach is to reconstruct the rotator cuff tendons to the humeral footprint. Due to the high retear rate, some other therapeutic strategies, such as application of ADSCs and bone-marrow-derived MSCs, have been explored to treat RCT [29,30].

However, stem cell therapies have not yielded satisfactory results [31–34]. MSCs (from bone marrow or adipose tissue) were applied in an animal tendon defect model (rotator cuff or Achilles' tendon) to evaluate the biomechanical and histological properties of the repair. The result revealed that the application of MSCs in the animal tendon repair model did not improve the biomechanical and histological properties of the tendon-to-bone healing. Recently, some extracellular vesicles have been regarded as the therapeutic potential of MSCs [35–37]. However, exosomes

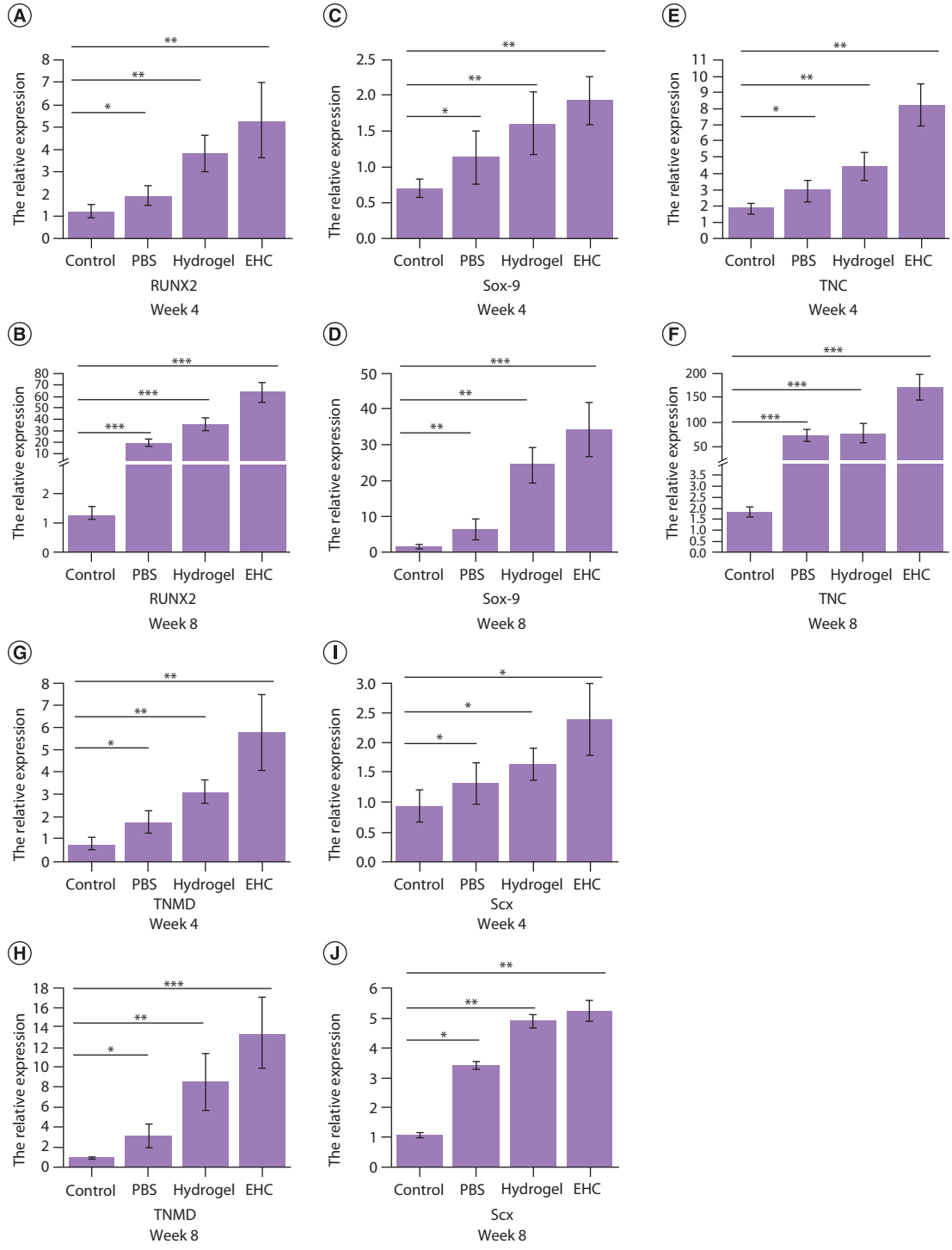

Figure 5. Real-time PCR analysis showed the regulation of genes associated with differentiation. (A & B) RUNX2, **(C & D)** Sox9, **(E & F)** tenascin, **(G & H)** tenomodulin and **(I & J)** scleraxis by ADSC-EHC treatment. Data represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with control ($n = 8$).
EHC: Adipose-derived stem cell exosome–hydrogel complex.

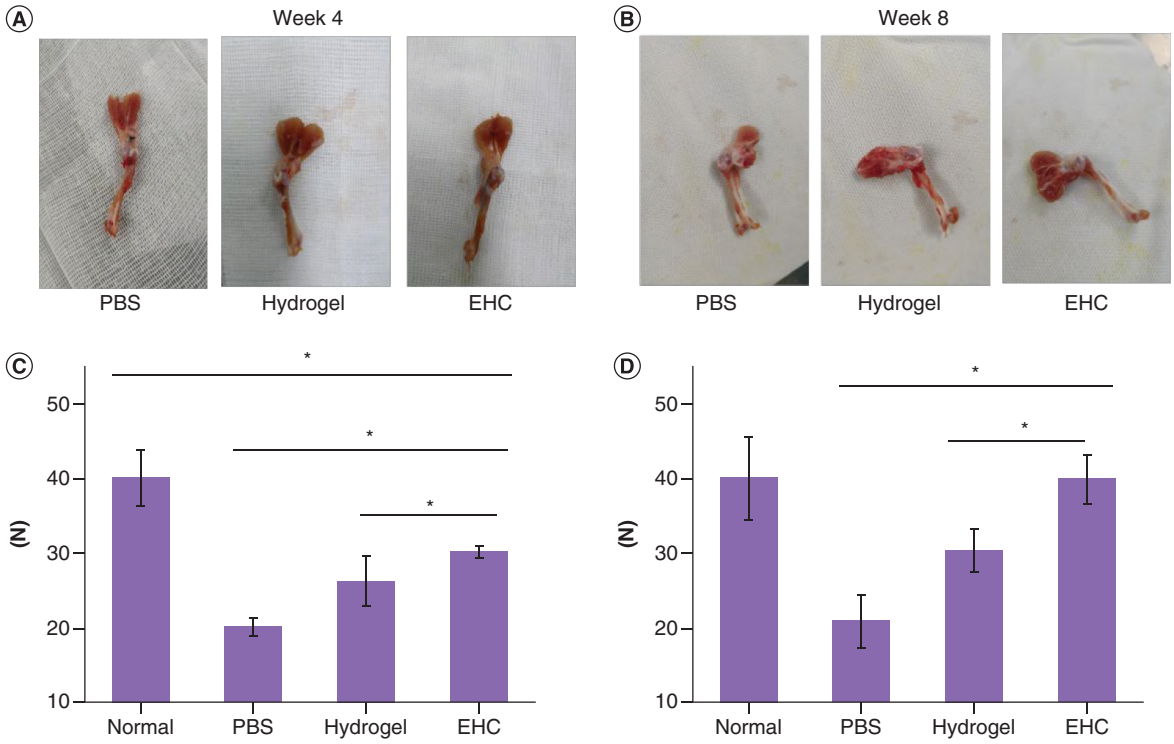

Figure 6. Biomechanical analysis of the rotator cuff tear at 4 and 8 weeks after treatment. (A) The articular portion of rat after treatment at 4 weeks. **(B)** The articular portion of rat after treatment at 8 weeks. **(C)** The maximum tensile force at the fracture of the articular portion at 4 weeks. **(D)** The maximum tensile force at the fracture of the articular portion at 8 weeks. Data represent mean ± SEM. *$p < 0.05$ ($n = 6$).
EHC: Adipose-derived stem cell exosome–hydrogel complex.

can avoid the drawbacks of MSCs, such as cell expansion *in vitro*, low survival rate and potential immunological rejection *in vivo* [31]. MSC-derived exosomes can be more stable and safe. Therefore, it is valuable to explore the effects of ADSC-exos on rotator cuff repair.

TDSCs located in tendon were identified in 2007 [17]. Then there was a growing debate about whether bone marrow MSCs promote tissue repair through their proliferation and differentiation capacity or through activation of the local stem/progenitor cells indirectly. With regard to the seeding cells in tendon tissue engineering, Sevivas *et al.* reported that MSC secretome can promote the viability of tendon cells *in vitro* and union of tendon to bone *in vivo* through tissue engineering strategy in a chronic massive RCT rat model [38], and Zhang *et al.* [39] reported that MSC-derived exosomes regulated osteochondral defects repair through increased cellular proliferation and infiltration. The conditioned medium of bone marrow MSCs promoted the proliferation, migration and viability of tenocytes *in vitro* and improved the repair effect of RCT [40]. Recently, a bone marrow MSCs and TDSC indirect coculture system revealed that bone marrow MSCs actually accelerate the proliferation and migration of TDSCs through paracrine mechanism. Moreover, by application of the bone marrow MSC-derived exosomes and fibrin gel complex into the rat patellar tendon defect area, TDSCs promoted the repair of rat patellar tendon defects by internalizing bone marrow MSC-derived exosomes and playing the roles of proliferation, migration and tenogenic differentiation [41]. These research findings have provided a theoretical basis for the preparation of a usable, cell-free, stem-cell-based therapeutic pathway that is efficient for the treatment of tendon injury. Thus, we may deduce that the ADSC-exos can mediate the TDSCs fortune in promoting tendon repair.

Therefore, we hypothesize that ADSCs might have a positive effect on TDSCs via an indirect pathway such as paracrine signaling. Herein, the results from an indirect co-culture system demonstrated that ADSCs indeed promote the proliferation and differentiation of TDSCs via a paracrine approach (Figure 3).

The cup-shaped morphology (30–150 nm) of the exosomes was observed by TEM, and the fluorescence of TDSCs confirmed that TDSCs endocytose exosomes, which indicates that ADSC-exos have a good interaction with TDSCs. Zhang *et al.* suggested that exosomes may be able to communicate with chondrocytes because the

cells endocytosed the exosomes, and the labeled exosomes mainly localized in cytoplasm of the chondrocytes [39]. The same research showed that the ADSC-exos have the potential to communicate with TDSCs. The CCK-8 confirmed that the Exo-0.3 group could significantly promote the TDSCs proliferation and the results of Oil Red O and Alizarin Red S staining, clearly indicating a significant increase in adipogenesis and osteogenesis with ADSC-exos treated TDSCs *in vitro*. Similar results were observed in Yu's report, which indicated that bone marrow MSC-derived exosomes could repair patellar tendon by facilitating the proliferation and migration of endogenous tendon stem/progenitor cells [41]. Histological analysis showed that the intercellular space between cells and irregular muscle shrinkage was found in the control and PBS groups at 4 weeks. These results indicate that it has necrocytosis and muscle degeneration in the rotator cuff [42,43]. Whether 4 or 8 weeks, the collagen fiber and muscle bundles showed more regular alignment in the EHC group compared with other groups [41]. As reported in the study of Wang *et al*. [44], muscle degeneration in RCTs should be noted. These results indicate that EHC has the potential to promote the healing of RCTs.

Most of this musculoskeletal pain in the rotator cuff is connected with tendon disorders [45]. However, the tendinopathy was generally misdiagnosed as soft tissue pain. Tendon, as a bridge to link muscle and bone, is part of the musculoskeletal system [46]. The tendinopathy usually occurs in major tendons with high loading requirements [47]. Tendon repair after injury mainly undergoes five stages: inflammation, cell proliferation, cell migration, remodeling and finally formed spatial organization of type I collagen [48]. After injury, the injured tendons and tissues release a large number of growth factors and cytokines, such as VEGF and interleukins [49]. The interleukins produced by proinflammatory M1 macrophages, and growth factors produced by anti-inflammatory M2 macrophages, which were both molecular responses during tendon repair [50]. The expression of genes encoding tendon-associated molecules including *TNMD*, *TNC* and *Scx* was also upregulated after tendon injury [51,52]. The EHC treatment significantly induced upregulation of *TNC*. The *Scx* and *TNMD* expression is enhanced in high-intensity mechanical environment, such as treadmill running [53,54]. Thus, the expression of *Scx* and *TNMD* was substantially lower than *TNC*.

The fundamental mechanism of tendon healing has not been well investigated, but the EHC induced upregulation for the expression of genes encoding tenogenesis molecule and significantly improved the biomechanical RCT healing. Similar results were observed in the process of using ADSCs to prevent the muscle degeneration and augmentation of rotator cuff healing in ADSC-exos [23,26]. In the repair process, the tensile strength of the healed tendon was significantly lower than that of the normal tendon in human, which is mainly affected by the formation of scar tissue [55].

Our study has some limitations. First, the rat RCT model was conducted on healthy tendons rather than a natural degenerative process in our study, which was not in accordance with the fact that RCTs usually occur in older adults. Second, the optimal frequency of ADSC-exos injection is uncertain. Whether more injections could produce better results than a single injection is unclear. Third, the fundamental mechanism of this study should be further explored in future follow-up projects. Fourth, further exploration into whether ADSCs are the best source of exosomes is needed before we can conclude that ADSC-exos treatment is a promising frontier for rotator cuff repair. Finally, further studies are needed to compare exosomes derived from ADSCs in clinical settings (e.g., platelet-rich plasma and ADSCs) to provide the optimal choice for tendon-to-bone healing.

Conclusion

In this study, we investigated the mechanism of ADSC-exos on rotator cuff repair by mediating the TDSCs. We isolated and cultivated the ADSCs, ADSC-exos, and TDSCs. A trilineage-induced differentiation assessment was also conducted. The results of Oil Red O and Alizarin Red S staining clearly indicated that the effects of adipogenesis and osteogenesis were significantly increased in ADSC-exos treated with TDSCs. We plan to use a spectrophotometer to quantify adipogenesis and osteogenesis in our future research. The proliferation results showed that the high dose of ADSC-exos (0.3 mg/ml) had better efficacy. The treatment of the EHC group promoted the regeneration of collagen fiber and muscle bundles in RCT, which may be caused by the upregulation of tenogenesis genes (*TNC, TNMD* and *Scx*) induced by adipose stem cell exosomes. The EHC significantly induced upregulation of osteogenic marker *RUNX2* and chondrogenic markers *Sox-9*. The mRNA expression levels of tenogenesis genes (*TNC, TNMD* and *Scx*) were also upregulated. To a certain extent, the EHC could improve the RCT healing biomechanically. These results demonstrated that the ADSC-exos promoted rotator cuff repair. On the basis of this research, we propose a possible repair mechanism. ASDC-exos induce adipogenesis

and osteogenesis of tendon stem cells, resulting in upregulation of osteogenic markers (*RUNX2*), cartilage markers (*Sox-9*) and tenogenesis genes (*TNC, TNMD* and *Scx*) and also facilitate RCT repair.

Translational perspective

To treat RCT, a variety of surgical repair methods have been implemented in the clinic. However, with surgical repair alone, the injured rotator cuff cannot be fully restored, and the retear rate is high. In this study, we investigated the mechanism of ADSC-exos on rotator cuff repair by mediating the TDSCs. This trial demonstrated that the ADSC-exos had a positive effect on the treatment of RCT. ADSC-exos can mediate the local TDSCs, promote TDSC proliferation and differentiation and enhance the recovery of rotator cuff histologic and biomechanical properties. ADSC-exos therapy may represent an exciting advancement in the treatment of RCT.

Previous research has proved that the direct application of stem cells and platelet-rich plasma on the repair of RCTs have also promoted healing of the rotator cuff. Further research needs to be conducted to confirm the optimal dose and injection frequency. Also, further studies are needed to compare exosomes derived from ADSCs in clinical settings (e.g., platelet-rich plasma and ADSCs) to determine the optimal choice for tendon-to-bone healing.

Summary points

- Rotator cuff tear (RCT) is a widespread problem that seriously affects quality of life. Adipose-derived stem cells (ADSCs) have a positive impact on the tendon bone interface healing and rotator cuff repair. Therefore, it is valuable to explore the effects of ADSC exosomes (ADSC-exos) on rotator cuff repair.
- The transmission electron microscopy and fluorescence microscope were used to evaluate morphology of ADSC-exos and whether ADSC-exos interacted directly with tendon-derived stem cells (TDSCs). CCK-8 was used to detect cell proliferation rate, and immunohistochemical identification was used to explore the effects of the ADSC-exos on multidifferentiation potential of TDSCs.
- The animal model and histological analysis were used to explore the efficacy of the rotator cuff repair, real-time PCR was used to quantify the expression levels of each gene and biomechanical analysis of the RCT was used to further confirm the repair effect of ADSC-exos for rotator cuff repair.
- The cup-shaped morphology (30–150 nm) of the exosomes was observed by transmission electron microscopy, and the fluorescence of TDSCs confirmed that they endocytosed exosomes, which indicated that ADSC-exos have a good interaction with TDSCs. CCK-8 confirmed that the Exo-0.3 group could significantly promote the TDSC proliferation, and the results of Oil Red O and Alizarin Red S staining clearly indicated a significant increase in adipogenesis and osteogenesis with ADSC-exos-treated TDSCs *in vitro*.
- The animal model and histological analysis both showed that ADSC-exos–hydrogel complex (EHC) had the potential to promote healing of RCTs. The treatment of the EHC group promoted the regeneration of collagen fiber and muscle bundles in RCT. The EHC significantly induced upregulation of osteogenic marker *RUNX2* and chondrogenic markers *Sox-9*. The mRNA expression levels of tenogenesis genes (*TNC, TNMD* and *Scx*) were also upregulated.
- The biomechanical analysis of the RCT confirmed that EHC can improve the biomechanical healing of RCT to a certain extent. These *in vivo* and *in vitro* experiments have shown that the ADSC-exos could promote rotator cuff repair.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2021-0004

Author contributions

Conception and design: G Fu, F Yin; administrative support: F Yin; provision of study materials or patients: G Fu, L Lou, Z Pan, F Yin; collection and assembly of data: G Fu; data analysis and interpretation: G Fu; manuscript writing: all authors; final approval of manuscript: all authors.

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Financial & competing interests disclosure

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Ethical conduct of research

Ethics approval was given for the study by the Human Research Ethics Committee and Institutional Animal Care and Use Committee of the Tongji University, School of Medicine, Shanghai. In addition, informed consent has been obtained from the participants involved.

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