



## ■ BONE BIOLOGY

# TGF- $\beta$ 1 derived from macrophages contributes to load-induced tendon-bone healing in the murine rotator cuff repair model by promoting chondrogenesis

L. Wang,  
S. Li,  
H. Xiao,  
T. Zhang,  
Y. Liu,  
J. Hu,  
D. Xu,  
H. Lu

From Xiangya Hospital,  
Central South University,  
Changsha, China

## Aims

It has been established that mechanical stimulation benefits tendon-bone (T-B) healing, and macrophage phenotype can be regulated by mechanical cues; moreover, the interaction between macrophages and mesenchymal stem cells (MSCs) plays a fundamental role in tissue repair. This study aimed to investigate the role of macrophage-mediated MSC chondrogenesis in load-induced T-B healing in depth.

## Methods

C57BL/6 mice rotator cuff (RC) repair model was established to explore the effects of mechanical stimulation on macrophage polarization, transforming growth factor (TGF)- $\beta$ 1 generation, and MSC chondrogenesis within T-B enthesis by immunofluorescence and enzyme-linked immunosorbent assay (ELISA). Macrophage depletion was performed by clodronate liposomes, and T-B healing quality was evaluated by histology and biomechanics. In vitro, bone marrow-derived macrophages (BMDMs) were stretched with CELLOAD-300 load system and macrophage polarization was identified by flow cytometry and quantitative real-time polymerase chain reaction (qRT-PCR). MSC chondrogenic differentiation was measured by histochemical analysis and qRT-PCR. ELISA and qRT-PCR were performed to screen the candidate molecules that mediated the pro-chondrogenic function of mechanical stimulated BMDMs.

## Results

Mechanical stimulation promoted macrophage M2 polarization in vivo and in vitro. The conditioned media from mechanically stimulated BMDMs (MS-CM) enhanced MSC chondrogenic differentiation, and mechanically stimulated BMDMs generated more TGF- $\beta$ 1. Further, neutralizing TGF- $\beta$ 1 in MS-CM can attenuate its pro-chondrogenic effect. In vivo, mechanical stimulation promoted TGF- $\beta$ 1 generation, MSC chondrogenesis, and T-B healing, which were abolished following macrophage depletion.

## Conclusion

Macrophages subjected to appropriate mechanical stimulation could polarize toward the M2 phenotype and secrete TGF- $\beta$ 1 to promote MSC chondrogenesis, which subsequently augments T-B healing.

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**Keywords:** Mechanical stimulation, Tendon-bone healing, Macrophage polarization, Mesenchymal stem cell chondrogenesis, Transforming growth factor- $\beta$ 1

Correspondence should be sent to  
Hongbin Lu; email:  
hongbinlu@hotmail.com

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## Article focus

■ Our primary aim in this study was

to identify the effect of macrophage-mediated mesenchymal stem cell (MSC)

chondrogenesis in load-induced tendon-bone (T-B) healing, and uncover the underlying mechanism behind this.

### Key messages

- The benefits of mechanical stimulation in T-B healing may be related to its regulation of macrophage polarization, which mediated MSC chondrogenesis by transferring transforming growth factor (TGF)- $\beta$ 1.

### Strengths and limitations

- Our study revealed the effect of mechanical load on the cellular and molecular cascade of T-B healing, and further demonstrated that targeting macrophage polarization is a potential target for T-B healing.
- Limitations were: an acute rotator cuff (RC) injury model cannot accurately mimic the chronic RC tears in clinic; Prx1+ cells may not represent all of the MSCs that are involved in T-B healing; and superficial investigation of molecular mechanisms of macrophage in MSC chondrogenesis within enthesis and T-B healing under mechanical stimulation.

### Introduction

Tendon-bone interface (TBI), known as enthesis, is histologically categorized into four continuous tissue layers: tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone.<sup>1</sup> This gradient structure connects ligaments/tendons with bone and transfers muscular forces to skeleton.<sup>2</sup> Therefore, TBI injury is common and it is difficult for restoration of injured TBI to the native form due to the slow and incomplete regeneration of the junctional fibrocartilage layer at the healing site.<sup>3,4</sup> Therapeutic strategies have been attempted to accelerate the tendon-bone (T-B) healing and promote fibrocartilage regeneration, among which postoperative loading exercise is an important part.

The musculoskeletal system is highly sensitive to its mechanical environments.<sup>5,6</sup> The reduction in muscle loading delays the development of the TBI by restraining mineralized bone accumulation and fibrocartilage formation.<sup>7</sup> Moreover, a lack of mechanical stimulation could result in worse outcomes after TBI repair.<sup>8</sup> There is growing evidence that appropriate mechanical stimulation is beneficial for the biological parameters and mechanical property of repaired TBI,<sup>9,10</sup> yet the mechanisms underpinning this are still unknown.

During the early phase of T-B healing, numerous inflammatory cells rapidly infiltrate to the injured site, which regulate the healing process. In general, the main effector myeloid cells, including monocytes and macrophages, are the cells activated first following tendon injury.<sup>11</sup> Macrophages are typically classified into two main subtypes, M1- and M2-like macrophages; the former induces an inflammatory response, while the latter suppresses inflammation and is generally considered to be favourable for tissue repair.<sup>12</sup> The balance between M1 and M2 macrophages is essential for tissue

regeneration.<sup>13</sup> Importantly, recent findings have indicated that macrophages exhibit mechanically sensitive characterization and that their polarization state could be regulated by mechanical cues,<sup>14</sup> which may account for load-induced T-B healing.

Mesenchymal stem cells (MSCs) are regarded as a critical factor for T-B healing, as they pass through holes drilled at the TBI and infiltrate around the injured site, thereby participating in the healing process.<sup>15</sup> Some treatment that augments MSC chondrogenesis could benefit fibrocartilage regeneration at the repaired interface.<sup>16,17</sup> Previous studies have demonstrated that different macrophage phenotypes have a unique effect on the cellular behaviour of MSCs, and M2-like macrophages could promote the chondrogenic differentiation of MSCs.<sup>12,18</sup>

Therefore, we hypothesized that mechanical stimulation could modulate tissue macrophages to polarize towards M2 phenotype, and then the biological behaviour of MSCs may be augmented in response to the paracrine signals from mechanically stimulated macrophages to promote fibrocartilage regeneration and T-B healing. In this study, we aimed to determine the role of macrophage-mediated MSC chondrogenesis in T-B healing induced by mechanical stimulation, and to uncover the underlying mechanism.

### Methods

**Surgical procedure.** Eight-week-old male mice were used to establish rotator cuff (RC) repair model as described in previous literature.<sup>9</sup> Briefly, mice were anaesthetized with 0.3% pentobarbital, and the skin and deltoid muscle on their left shoulder were then incised to expose the supraspinatus tendon (SST). After the 6-0 suture placement, the SST was detached from the insertion site on the humerus and the remaining fibrocartilage was scraped off. A bone tunnel was transversely drilled on the distal greater tuberosity. Next, the suture was passed through bone tunnels and was then tightened to fix the RC to its original footprint. Then, the skin incision was closed. Next, the mice with supraspinatus insertion injury were randomly assigned to one of six groups (Table I). Specimens of the SST-humeral head complex were harvested for further study at two, four, or eight weeks after the surgery.

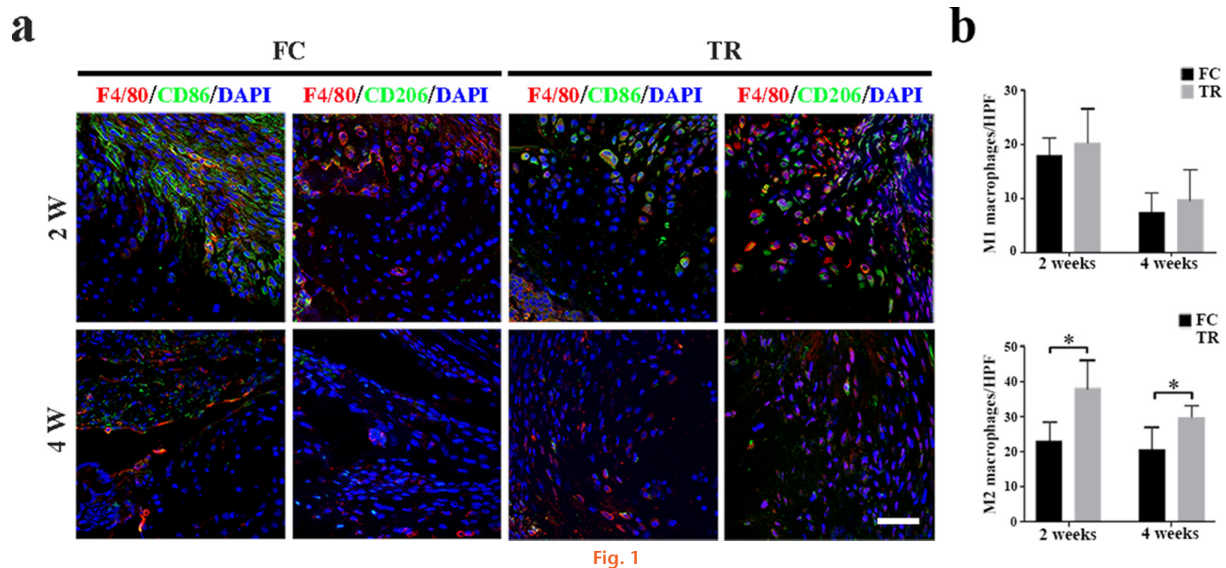
**Macrophage depletion.** Macrophage depletion was catalyzed by the clodronate liposomes (Clo-Lipo) as previously described.<sup>19</sup> Briefly, at one and two days before surgery, mice were intraperitoneally injected with Clo-Lipo or phosphate-buffered saline liposomes (PBS-lipo) (100  $\mu$ l/10 g; Liposoma B.V., Netherlands), and an additional 100  $\mu$ l Clo-Lipo or PBS-lipo were injected directly into the injury site at the time of surgery. For continued macrophage depletion, animals then received intraperitoneal injections with the same dose of Clo-Lipo every third day throughout the study.

**Histology.** The paraffin-embedded samples were cut into 5  $\mu$ m thick sections. Then, the slices were stained with haematoxylin and eosin (H&E) for general histology and with Toluidine Blue/Fast Green (TB&FG) to

**Table 1.** Groups and corresponding postoperative intervention.

Group	Postoperative intervention
FC	Free cage activities
TR	Treadmill running
FC-PBS	Free cage activities + intraperitoneal injection of PBS liposomes
FC-Clo	Free cage activities + intraperitoneal injection of clodronate liposomes
TR-PBS	Treadmill running + intraperitoneal injection of PBS liposomes
TR-Clo	Treadmill running + intraperitoneal injection of clodronate liposomes

PBS, phosphate-buffered saline.

**Fig. 1**

The effects of mechanical stimulation on macrophage polarization during rotator cuff (RC) tendon-bone (T-B) healing. **a**) Immunofluorescent staining of total macrophages biomarker, F4/80 (red), and M1-like macrophage biomarker, CD86 (green) or M2-like macrophage biomarker, CD206 (green) in the repaired RC tendon-bone interface (TBI) at two and four weeks after surgery. Scale bar: 100  $\mu$ m. **b**) Quantification of M1 (F4/80+CD86+ cells) and M2 macrophages (F4/80+CD206+ cells) in Fig. 1a. N = 6 per group. \* $p < 0.05$ , two-way analysis of variance followed by Tukey's multiple comparisons test. DAPI, 4',6-diamidino-2-phenylindole; FC, free cage activities group; HPF, high-power field; TR, treadmill running group.

assess fibrocartilage regeneration and glycosaminoglycan (GAG) accumulation. Histological staining was semi-quantitatively analyzed in a blinded fashion by two independent observers (HX and TZ) using a modified tendon-to-bone maturing score as previously described.<sup>20</sup>

**Biomechanical test.** The analysis of biomechanical property of repaired SST enthesis including failure load and stiffness was performed by a mechanical testing machine (WD-T; Shanghai Zhuoji Instrument Equipment, China). The humerus shaft was carefully gripped by a lower clamp, while the tendon was secured in the upper clamp using sandpaper and glue. Finally, the specimen was loaded to failure at a rate of 1 mm/min.

**Cell isolation and identification.** Primary bone marrow-derived macrophages (BMDMs) and MSCs were isolated from the femur and tibia of mice as previously reported.<sup>21,22</sup> The expression of the surface markers (including CD11b, F4/80) on BMDMs and the surface markers (including CD29, CD90, stem cell antigen-1 (Sca-1), CD34, CD45, and CD11b) on MSCs was detected by flow cytometry. All antibodies were purchased from BioLegend (USA). In addition, differentiation media (Cyagen

Biosciences, China) was used to determine the multipotent differentiation potentials of the isolated cells based on the manufacturer's protocol.

**Quantitative real-time polymerase chain reaction.** Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described.<sup>23</sup> All details of the primers employed in the current study are listed in Supplementary Table i. The  $\beta$ -actin gene was used for normalization.

**Statistical analysis.** Data were presented as mean (standard deviation) and analyzed with GraphPad Prism (GraphPad, USA). Statistical analysis of the data at a single timepoint was performed using an independent-samples *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistical analysis of the data at multiple timepoints was performed using two-way ANOVA followed by Tukey's multiple comparisons test. Statistical significance was set at  $p < 0.05$ .

## Results

**Mechanical stimulation induced M2 macrophage polarization during T-B healing.** Immunofluorescent staining was

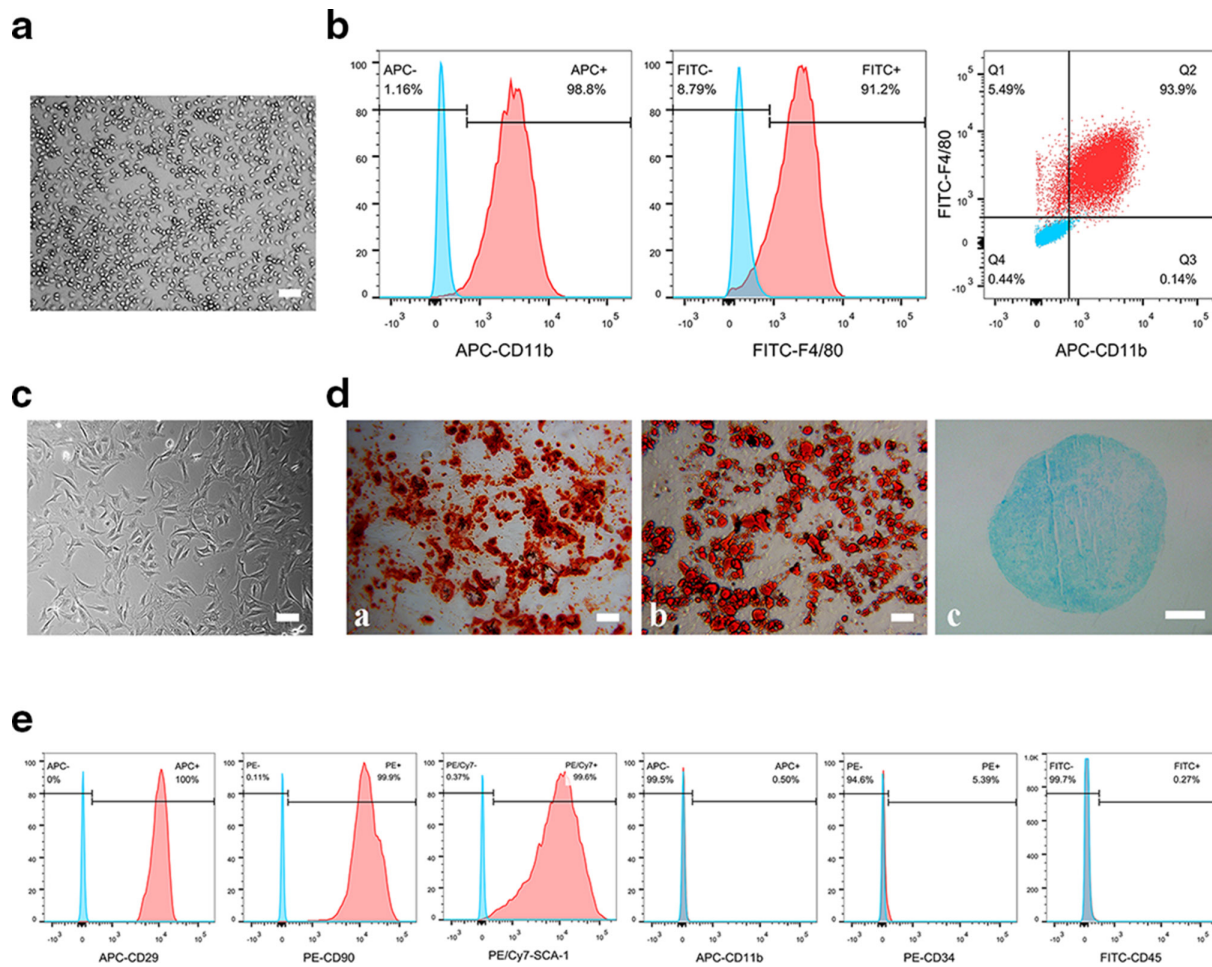


Fig. 2

Morphology and identification of bone marrow-derived macrophages (BMDMs) and mesenchymal stem cells (MSCs). a) Representative morphology of BMDMs. Scale bar: 100  $\mu$ m. b) Flow cytometry for BMDM. c) Representative morphology of MSCs. Scale bar: 100  $\mu$ m. d) The osteogenic, chondrogenic, and adipogenic differentiation of MSCs confirmed by Alizarin red staining (D-a; Scale bar: 100  $\mu$ m), Oil Red O staining (D-b; Scale bar: 100  $\mu$ m), and Alcian Blue staining (D-c; Scale bar: 200  $\mu$ m). e) Flow cytometry for MSCs. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; SCA-1, stem cell antigen-1.

performed to examine macrophages' phenotype at the healing site. As indicated in Figure 1, M2-like macrophages (F4/80+ CD206+ cells) within SST entheses in the TR group were more than that in FC group mice at postoperative weeks 2 and 4 ( $p < 0.001$  and  $p = 0.027$ ). However, there was no significant difference in M1-like macrophages (F4/80+ CD86+ cells) within SST entheses between the FC group and TR group ( $p > 0.05$ , all two-way ANOVA followed by Tukey's multiple comparisons test).

**Characterization of BMDMs and MSCs.** BMDMs appeared at approximately four days after initial plating and exhibited typically polygonal or spindle shapes. Flow cytometry analysis demonstrated that BMDMs highly expressed CD11b and F4/80 (Figures 2a and 2b). The colonies of MSCs formed at approximately five days and showed a spindle-like morphology. MSCs can differentiate into osteoblasts, adipocytes, and chondrocytes under different culture conditions, as confirmed by Alizarin red, Oil Red O, and Alcian Blue staining. The results of flow cytometry

revealed that MSCs were highly positive for CD29, CD90, and Sca-1, but negative for CD11b, CD34, and CD45 (Figures 2c to 2e).

**Mechanical stimulation modulated BMDMs to polarize towards M2 phenotype in vitro.** The phenotype switch of BMDMs was measured by flow cytometry. After being treated with 5% cyclic stretch, the percentages of CD206+ cells (M2 macrophage) increased, while the percentages of CD86+ cells (M1 macrophage) decreased. Furthermore, 10% and 15% cyclic stretch also increased the expression of CD206 and decreased the expression of CD86 in BMDMs, but the rate was lower than that in the 5% stretch groups. The gene expression of M1-specific markers (tumour necrosis factor (TNF)- $\alpha$ , inducible nitric oxide synthase (iNOS), and chemokine receptor type 7 (CCR7)) and M2-specific markers (Arg, interleukin (IL)-10, and CD206) in BMDMs was detected by qRT-PCR. As expected, the expression of M2-specific markers was significantly upregulated and the expression of M1-specific



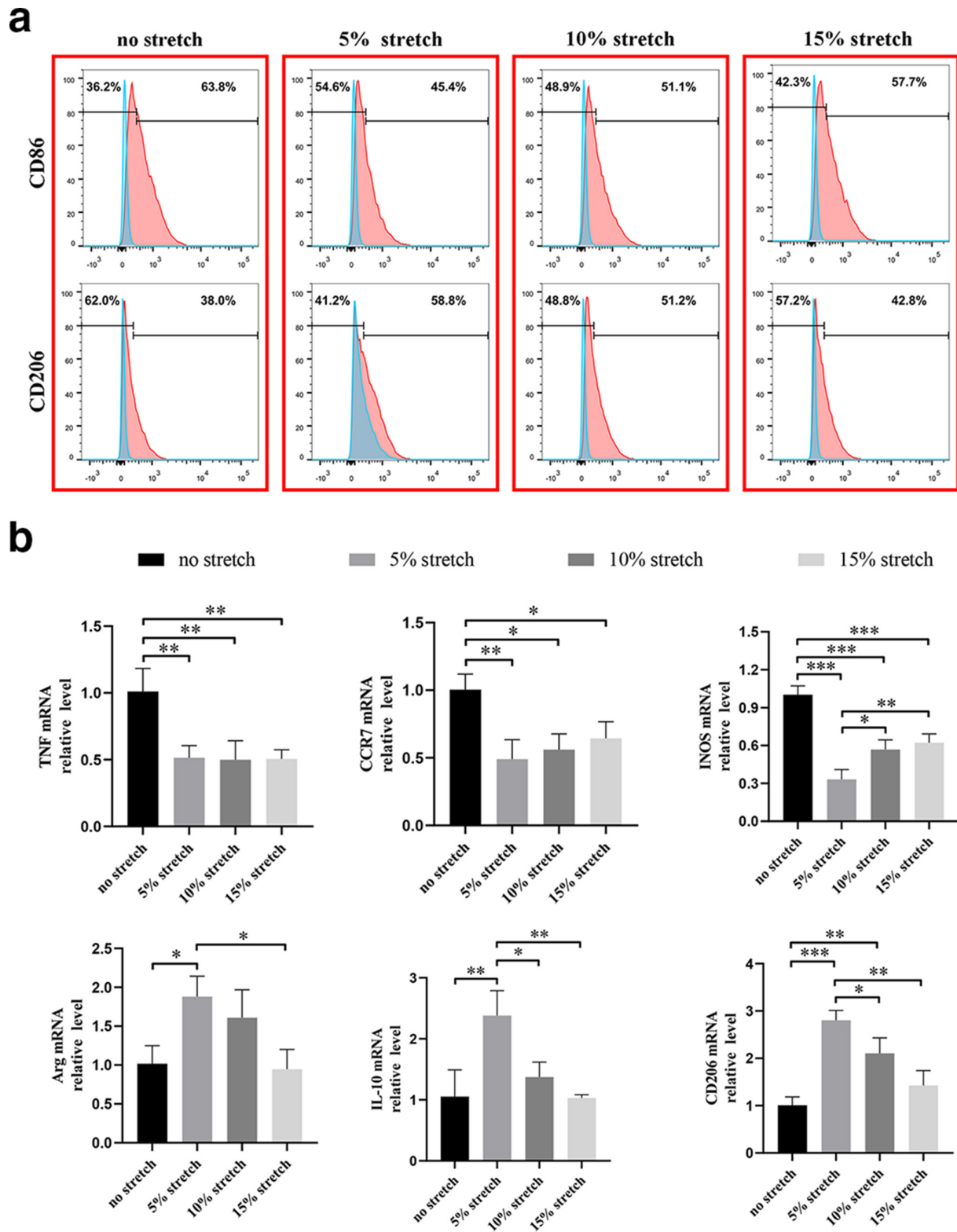


Fig. 3

The effect of mechanical stimulation on the phenotype switch of bone marrow-derived macrophages (BMDMs) in vitro. a) The expression of CD86 and CD206 in statically cultured or mechanically stimulated BMDMs was determined by flow cytometry. b) Gene expression in BMDMs under different treatments was analyzed by qRT-PCR (tumour necrosis factor (TNF)- $\alpha$ , iNOS, and CCR7 were used as M1-polarized markers; Arg, interleukin (IL)-10, and CD206 were used as M2-polarized markers;  $\beta$ -actin was used as inner reference. N = 3 per group. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ , one-way analysis of variance followed by Tukey's multiple comparisons test.

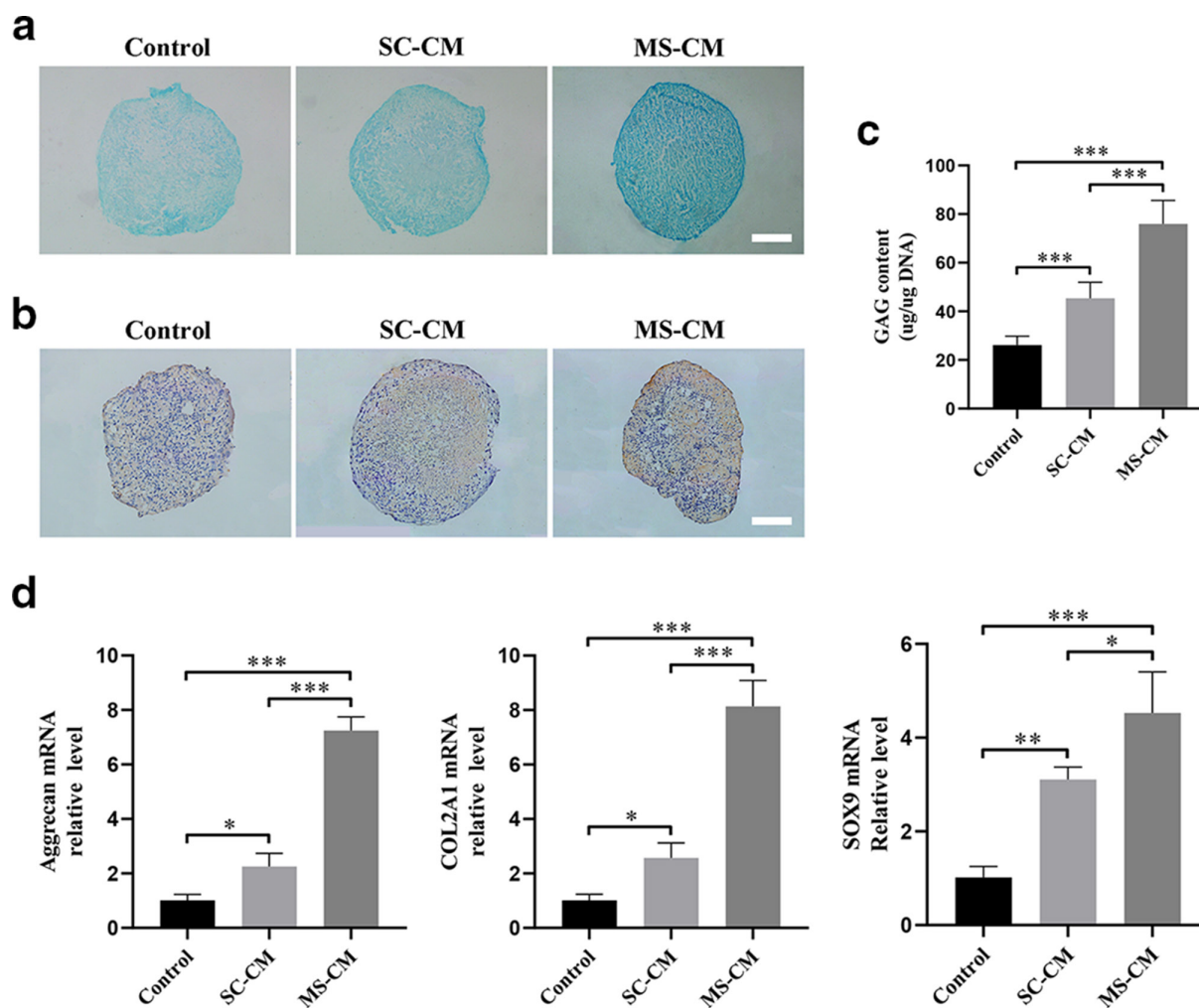


Fig. 4

Mechanically stimulated macrophages conditioned media augments the chondrogenic differentiation of mesenchymal stem cells (MSCs). a) Alcian blue staining of micromasses after culturing for 21 days. Scale bar = 200  $\mu$ m. b) Immunohistochemical staining of micromasses for collagen type II after culturing for 21 days. Scale bar = 50  $\mu$ m. c) Glycosaminoglycan (GAG) content was examined and normalized to DNA content. d) Relative messenger RNA (mRNA) expression of aggrecan, collagen type II alpha 1 (COL2A1), and SRY-related HMG box 9 (SOX9). n = 3 per group. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ , one-way analysis of variance followed by Tukey's multiple comparisons test. MS-CM, the conditioned media of mechanically stimulated BMDMs; SC-CM, the conditioned media of statically cultured BMDMs.

markers was significantly downregulated in BMDMs treated with 5% cyclic stretch when compared with those statically cultured BMDMs (tumour necrosis factor alpha (TNF- $\alpha$ ):  $p = 0.006$ ; inducible nitric oxide synthase (iNOS):  $p = 0.004$ ; CCR7:  $p < 0.001$ ; Arg:  $p = 0.023$ ; IL-10:  $p = 0.005$ ; CD206:  $p < 0.001$ , one-way ANOVA followed by Tukey's multiple comparisons test). Moreover, the expression of M2-specific markers in BMDMs treated with 5% cyclic stretch was higher than that in BMDMs treated with 15% cyclic stretch (Arg:  $p = 0.015$ ; IL-10:  $p = 0.004$ ; CD206:  $p = 0.001$ , one-way ANOVA followed by Tukey's multiple comparisons test). There was also a significant difference in the expression of IL-10 ( $p = 0.022$ ) and CD206 ( $p = 0.049$ ) in BMDMs between the 5% stretch group and the 10% stretch group, and the expression of iNOS in BMDMs in the 5% stretch group was lower than that in the 10% stretch group ( $p = 0.018$ ) and 15%

stretch group ( $p = 0.006$ , all one-way ANOVA followed by Tukey's multiple comparisons test). Throughout the remainder of the results, 5% cyclic stretch was used for further experiments (Figure 3).

**Mechanically stimulated macrophages enhanced chondrogenic differentiation of MSCs.** Alcian blue staining and the quantitative analysis of GAG (Figures 4a and 4c) showed that MS-CM could increase GAG synthesis and matrix deposition in micromass (all  $p < 0.001$ , one-way ANOVA followed by Tukey's multiple comparisons test). Immunohistochemical staining for collagen type II (Figure 4b) showed a trend similar to those observed in the Alcian blue staining experiments mentioned above. Micromass in the MS-CM showed a higher accumulation of collagen type II in comparison with the conditioned media of statically cultured BMDMs (SC-CM) group and control group. Additionally, qRT-PCR was used to

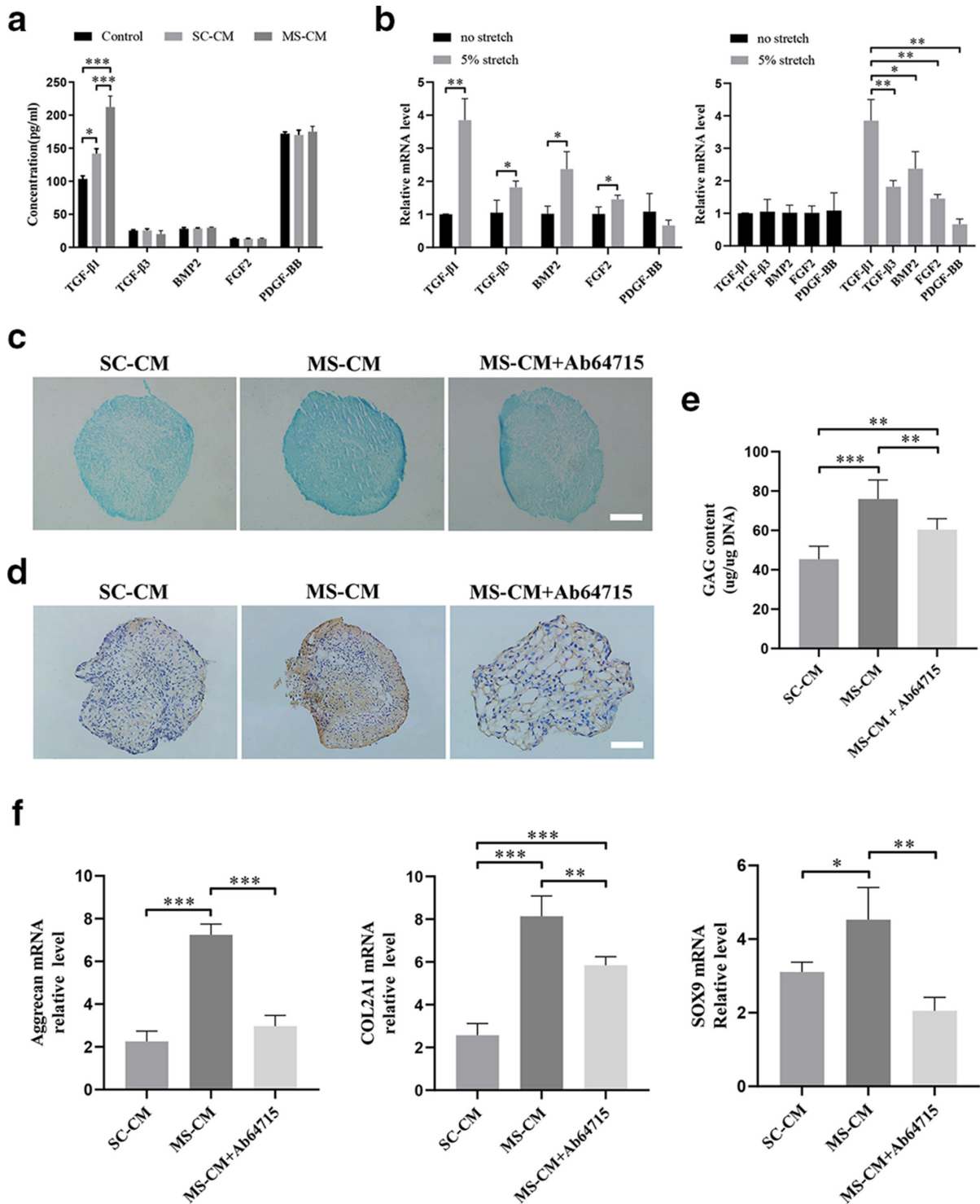


Fig. 5

Transforming growth factor (TGF)- $\beta$ 1 is involved in mechanically stimulated macrophage-mediated chondrogenic differentiation of mesenchymal stem cells (MSCs). a) Enzyme-linked immunosorbent assay for the pro-chondrogenic cytokines in conditioned media of bone marrow-derived macrophages (BMDMs) with or without mechanical stimulation and Dulbecco's Modified Eagle Medium. b) Relative messenger RNA (mRNA) expression of the chondrogenesis-related gene in BMDMs with or without mechanical stimulation (independent-samples *t*-test). c) Alcian blue staining of micromasses after culturing for 21 days. Scale bar = 200  $\mu$ m. d) Immunohistochemical staining of micromasses for collagen type II after culturing for 21 days. Scale bar = 50  $\mu$ m. e) Glycosaminoglycan (GAG) content was examined and normalized to DNA content. f) Relative mRNA expression of aggrecan, collagen type II alpha 1 (COL2A1), and SRY-related HMG box 9 (SOX9). N = 3 per group. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001, one-way analysis of variance followed by Tukey's multiple comparisons test. BMP2, bone morphogenetic protein 2; FGF2, fibroblast growth factor 2; MS-CM, the conditioned media of mechanically stimulated BMDMs; MS-CM+Ab64715, the conditioned media of mechanically stimulated BMDMs + TGF- $\beta$ 1 antibody; PDGF-BB, platelet-derived growth factor-BB; SC-CM, the conditioned media of statically cultured BMDMs; TGF- $\beta$ 1, transforming growth factor-beta 1; TGF- $\beta$ 3, transforming growth factor-beta 3.

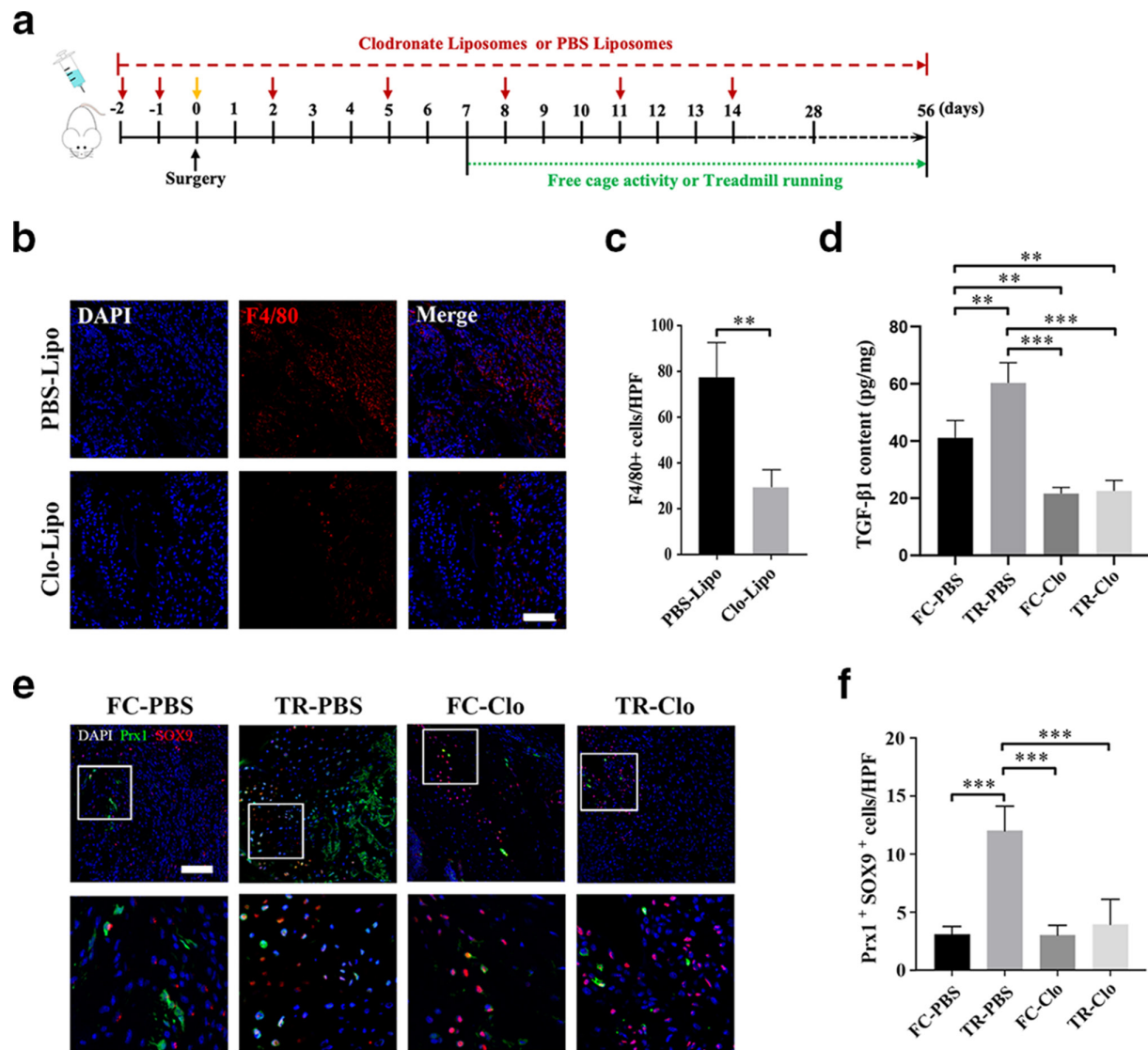


Fig. 6

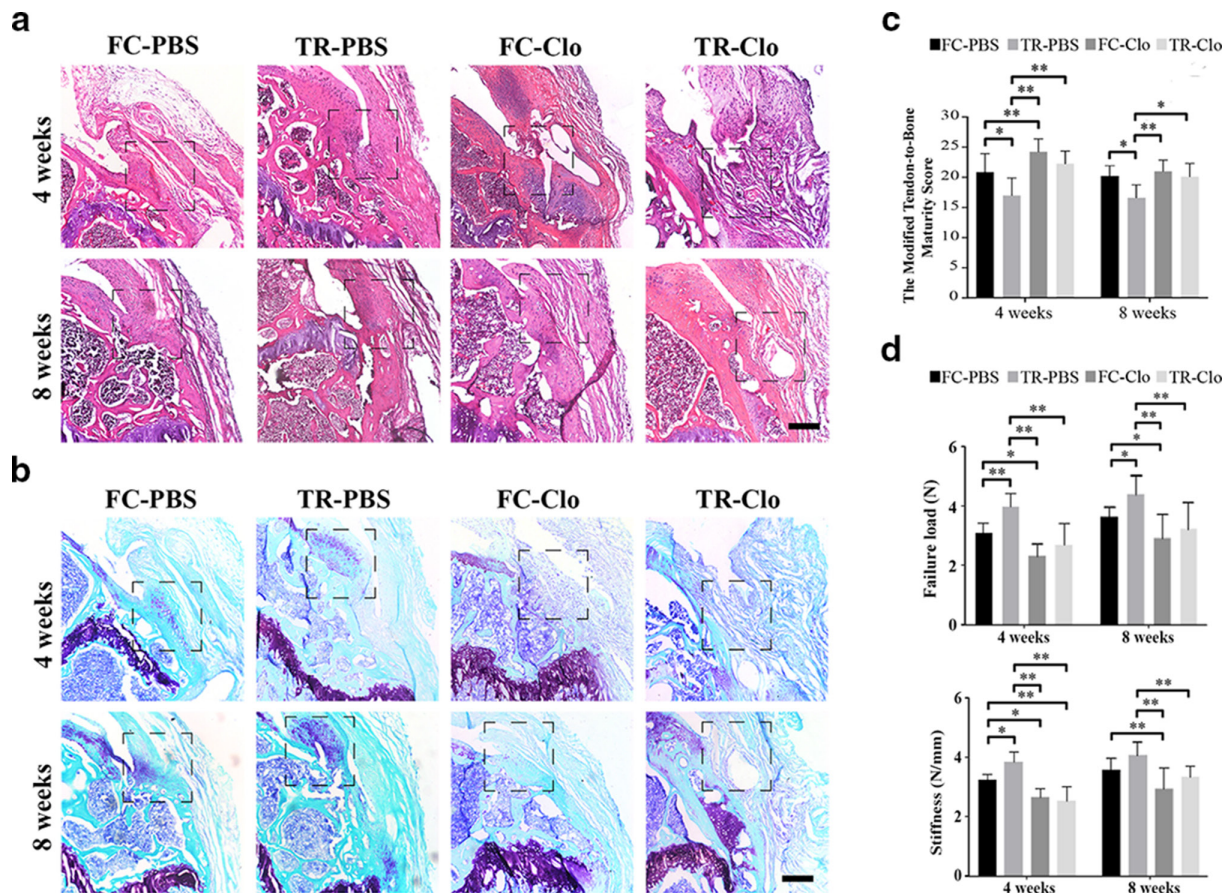
Macrophages mediated the ability of treadmill running to enhance transforming growth factor (TGF)-β1 generation and mesenchymal stem cell (MSC) chondrogenesis within entheses. a) Schematic diagram of the intervention with phosphate-buffered saline or clodronate liposomes (PBS-Lipo or Clo-Lipo). b) Immunofluorescent staining of F4/80 (red) at supraspinatus tendon (SST) entheses on seven days after surgery. Scale bar: 100 μm. c) Quantitation of macrophages (F4/80+ cells) in Fig. 6b (independent-samples *t*-test). N = 6 per group. d) TGF-β1 content (pg) of SST entheses at two weeks after surgery detected by enzyme-linked immunosorbent assay normalized against protein content (mg). N = 3 per group. e) Immunofluorescent image of green fluorescent protein (GFP) (Prx1+ cells, green) and SRY-related HMG box 9 (SOX9) (red) staining of SST entheses at four weeks after surgery. Scale bar: 100 μm. f) Quantitation of Prx1+ SOX9+ cells in Fig. 6e. N = 6 per group. \*\*p < 0.01 and \*\*\*p < 0.001, one-way analysis of variance followed by Tukey's multiple comparisons test. DAPI, 4',6-diamidino-2-phenylindole; FC, free cage activities group; HPF, high-power field; TR, treadmill running group.

determine the expression level of the chondrogenic gene (Figure 4d). After culturing for 21 days, the expression of aggrecan, collagen type II alpha 1 (COL2A1), and SRY-related HMG box 9 (SOX9) in MSCs of MS-CM was statistically higher than that in the control groups (all p < 0.001) and SC-CM (aggrecan, COL2A1: p < 0.001; SOX9: p = 0.0366, all one-way ANOVA followed by Tukey's multiple comparisons test).

**TGF-β1 is a candidate effector of mechanically stimulated macrophages that mediated chondrogenic differentiation of MSCs.** To confirm potential functional molecules that mediated the ability of mechanically stimulated BMDMs

to promote chondrogenic differentiation of MSCs, we determined cytokine levels of transforming growth factor (TGF)-β1, TGF-β3, bone morphogenetic protein 2 (BMP2), fibroblast growth factor 2 (FGF2), and platelet-derived growth factor (PDGF)-BB in BMDM conditioned media and Dulbecco's Modified Eagle Medium (DMEM) media by enzyme-linked immunosorbent assay (ELISA) and their expression in BMDMs by qRT-PCR. The ELISA result showed that the TGF-β1 content in MS-CM was significantly higher than that of the SC-CM and control media (all p < 0.001, one-way ANOVA followed by Tukey's multiple comparisons test). Moreover, the messenger





Macrophages mediated the ability of treadmill running to promote tendon-bone (T-B) healing. a) Representative haematoxylin and eosin images of supraspinatus tendon (SST) entheses at postoperative weeks 4 and 8. Scale bar: 200  $\mu$ m. The area selected by the rectangle dashed line indicates the SST entheses. b) Representative Toluidine Blue/Fast Green images of SST entheses at postoperative weeks 4 and 8. Scale bar: 200  $\mu$ m. c) Results of the modified tendon-to-bone maturity scores. N = 8 per group. d) Biomechanical property of the rotator cuff (RC) T-B healing site at postoperative weeks 4 and 8, as expressed by failure load and stiffness. N = 10 per group. \* $p < 0.05$ , \*\* $p < 0.01$ , two-way analysis of variance followed by Tukey's multiple comparisons test. FC-Clo, free cage activities + intraperitoneal injection of clodronate liposomes group; FC-PBS, free cage activities + intraperitoneal injection of phosphate-buffered saline liposomes group; TR-Clo, treadmill running + intraperitoneal injection of clodronate liposomes group; TR-PBS, treadmill running + intraperitoneal injection of PBS liposomes group.

RNA (mRNA) expression of TGF- $\beta$ 1 ( $p = 0.002$ ), TGF- $\beta$ 3 ( $p = 0.035$ ), BMP2 ( $p = 0.015$ ), and FGF2 ( $p = 0.036$ , all independent-samples *t*-test) in mechanically stimulated BMDMs was higher than that in statically cultured BMDMs, and the rate was highest in TGF- $\beta$ 1 mRNA expression (TGF- $\beta$ 1 vs TGF- $\beta$ 3:  $p = 0.007$ ; TGF- $\beta$ 1 vs BMP2:  $p = 0.038$ ; TGF- $\beta$ 1 vs FGF2:  $p = 0.003$ , independent-samples *t*-test) (Figures 5a and 5b).

To further identify whether TGF- $\beta$ 1 directly functioned in mechanically stimulated macrophage mediated MSC chondrogenic differentiation, a specific neutralizing antibody (ab64715; Abcam, UK) was used to block the binding between TGF- $\beta$ 1 and its receptor. The Alcian blue staining, immunohistochemistry (IHC), GAG quantification, and qRT-PCR demonstrated that the TGF- $\beta$ 1 neutralizing antibody successfully impeded the pro-chondrogenic effect of mechanically stimulated macrophages on MSCs ( $p < 0.05$ , one-way ANOVA followed by Tukey's multiple comparisons test). In general, our results

suggest that TGF- $\beta$ 1 is necessary for mechanically stimulated macrophage-induced promotion of chondrogenic differentiation of MSCs (Figures 5c to 5f).

**Macrophages mediated the ability of treadmill running to enhance TGF- $\beta$ 1 generation and MSC chondrogenesis within entheses.** To further understand whether macrophages are involved in load-induced T-B healing, we depleted macrophages with Clo-Lipo one and two days prior to and immediately at RC repair surgery and every third day following surgery (Figure 6a). As expected, Clo-Lipo could significantly decrease the macrophage population (F4/80+ cells) at RC TBI ( $p < 0.001$ , independent-samples *t*-test), which is exhibited by immunofluorescence (Figures 6b and 6c). Then, an ELISA was used to measure the content of TGF- $\beta$ 1 in the regenerated interface at postoperative week 2 based on the study by Xiao et al.<sup>24</sup> TGF- $\beta$ 1 content within entheses in the TR-PBS group was higher than that in the FC-PBS group ( $p = 0.007$ , one-way ANOVA followed by Tukey's multiple comparisons

test), however there was no difference in TGF- $\beta$ 1 expression between the treadmill running + intraperitoneal injection of clodronate liposomes (TR-Clo) group and free cage activities + intraperitoneal injection of clodronate liposomes (FC-Clo) group ( $p > 0.05$ , one-way ANOVA followed by Tukey's multiple comparisons test) (Figure 6d).

At four weeks after surgery, SST enthesis from Prx1-CreER-GFP/R26R-EYFP mice with or without treadmill running was harvested to track MSC chondrogenesis by immunofluorescent staining (Figures 6e and 6f). More Prx1+ SOX9+ cells were observed at the healing site in the TR-PBS group when compared with the FC-PBS group ( $p < 0.001$ , one-way ANOVA followed by Tukey's multiple comparisons test), yet no difference in these cells at the healing site was observed between the TR-Clo group and FC-Clo group ( $p > 0.05$ , one-way ANOVA followed by Tukey's multiple comparisons test). Collectively, macrophage-mediated TGF- $\beta$ 1 release is necessary for the load-induced promotion of MSC chondrogenesis.

**Macrophages mediated the ability of treadmill running to promote TBI healing.** Based on H&E and TB&FG staining images (Figures 7a to 7c), the TBI in the TR-PBS group showed better insertion continuity, more cartilage-like cells, GAG accumulation, and lower modified tendon-to-bone maturity score than that in the FC-PBS group at four and eight weeks ( $p = 0.008$  and  $p = 0.015$ , two-way ANOVA followed by Tukey's multiple comparisons test). Macrophage depletion could impede this promoting role of mechanical stimulation on T-B healing, as no difference in structure recovery of TBI was observed between the TR-Clo group and the FC-Clo group ( $p > 0.05$ , two-way ANOVA followed by Tukey's multiple comparisons test).

Biomechanically, failure load at postoperative weeks 4 ( $p = 0.009$ ) and 8 ( $p = 0.028$ ), and stiffness at postoperative week 4 ( $p = 0.011$ ) of SST enthesis in TR-PBS group, were significantly higher than that in FC-PBS group, and there was no difference in them between TR-Clo group and FC-Clo group ( $p > 0.05$ , all two-way ANOVA followed by Tukey's multiple comparisons test). As shown above, our results suggest that the benefits of mechanical stimulation in T-B healing were largely associated with its regulation of macrophages (Figure 7d).

## Discussion

This study aimed to investigate the effect of macrophages on mechanical stimulation mediated T-B healing in a murine RC repair model and its underlying mechanism. We first demonstrated that mechanical stimulation could promote macrophage M2 polarization in vivo and in vitro. Our work further indicates that mechanically stimulated macrophages could augment the chondrogenic differentiation of MSCs in a TGF- $\beta$ 1 mediated manner. Furthermore, we confirmed that the benefit of mechanical stimulation in TGF- $\beta$ 1 generation, MSC chondrogenesis within enthesis, and T-B healing was related to its regulation of macrophages by using the macrophage depletion model. To sum up, this is the first study to uncover the mechanical stimulation–inflammation–chondrogenic

differentiation–TBI healing axis, and show how macrophages respond to mechanical stimulation to promote MSC chondrogenesis and T-B healing.

Tissue injury can trigger the inflammation response and cause tissue repair.<sup>25</sup> As important immune cells, macrophages have been shown to play an important role in tissue healing.<sup>26,27</sup> It is well known that macrophages are mainly divided into M1-like and M2-like macrophages, and the regulation of the transition of macrophages from M1 to M2 phenotype can inhibit the inflammatory cascade and enhance subsequent reparative activities, thereby facilitating T-B healing.<sup>1,28,29</sup> Being consistent with previous research,<sup>14,30,31</sup> our results revealed that mechanical stimulation modulated macrophages to polarize toward the M2 phenotype both in vivo and in vitro, which highlighted the macrophages as a potential target of mechanical stimulation to manipulate T-B healing. Moreover, in vitrol assay, we found that 5% mechanical stretch exhibited superior positive role in macrophage M2 polarization when compared with 10% and 15% stretch, which indicates that the loading intensity played a crucial role in determining the polarization state of macrophages. Based on the regulation of mechanical stimulation on macrophage polarization during T-B healing, further studies could be performed to determine optimal parameters, including starting timing and magnitude of mechanical stimulation by counting M2-like macrophage at TBI.

MSCs spontaneously migrate to the injury site in response to the local inflammatory environment and participate in the process of T-B healing by their differentiation and paracrine.<sup>10,32</sup> Interplay between macrophages and MSCs is closely associated with the healing process and tissue repair.<sup>33</sup> Fine-tuning macrophage polarization may represent an effective strategy to augment the therapeutic potential of MSCs.<sup>12</sup> Many studies have shown that M2 macrophages exert a chondro-inductive effect on MSCs and promote cartilage regeneration.<sup>18,34,35</sup> Unsurprisingly, our study revealed that MS-CM can substantially increase the chondrogenic differentiation of MSCs, in which TGF- $\beta$ 1 is extremely highly expressed. Further functional assays showed that TGF- $\beta$ 1 is required for mechanically stimulated macrophage-induced promotion of chondrogenic responses of MSCs. Thus, the crosstalk between inflammatory reactions, especially macrophages and MSCs under mechanical stimulation, might contribute to chondrogenesis possibly via paracrine signalling.

Liposomes encapsulating the bisphosphonate clodronate can be used for macrophage depletion.<sup>36</sup> To investigate the role of macrophages in mechanical stimulation induced T-B healing, we used Clo-Lipo to eliminate macrophages in the mice RC repair model. Our finding showed that the mechanical stimulation could promote TGF- $\beta$ 1 regeneration, MSC chondrogenesis within enthesis, and T-B healing, yet the administration of macrophage depletion with Clo-Lipo abolished this auxo-action induced by mechanical stimulation. In addition, previous research

also indicated that local application of TGF- $\beta$ 1 neutralizing antibody at the injured site impeded mechanical stimulation induced T-B healing.<sup>24</sup> Collectively, our study suggests that the benefits of mechanical stimulation in MSC chondrogenesis and T-B healing may be related to its regulation on macrophage mediated TGF- $\beta$ 1 transferring. However, apart from promoting chondrogenic differentiation, TGF- $\beta$ 1 is also a potent fibrosis inducer. TGF- $\beta$ 1 may accelerate rotator cuff repair by scar-mediated healing.<sup>37</sup> Therefore, more research needs to be conducted to determine the appropriate working concentration of TGF- $\beta$ 1 for fibrocartilage regeneration of TBI before it can be used clinically.

There are some potential limitations in this study. First, the model established in the current study is an acute injury, however most RC tears in a clinic are chronic. Considering the differences in the micro-environment between acute and chronic injury, our findings should be validated in a RC chronic tears model. Second, due to the lack of a specific marker for MSCs, we labelled the Prx1+ cells to trace MSC chondrogenesis within enthesis as previously reported.<sup>38-40</sup> However, the Prx1+ cells may not represent all of the MSCs that are involved in T-B healing.<sup>41</sup> Third, although TGF- $\beta$ 1 was identified as the key molecule that mediated the function of macrophages to augment MSC chondrogenesis and T-B healing under mechanical stimulation, this is mainly dependent on previous experience and reported review, and may cause bias. More research should be performed to uncover the whole mechanisms and targets.

In conclusion, our findings suggest that macrophages subjected to appropriate mechanical stimulation polarize toward the M2 phenotype and secrete high levels of TGF- $\beta$ 1 to promote MSC chondrogenesis, which subsequently augments T-B healing.

### Supplementary material



Supplementary methods and a table showing a list of primer sequences used in this study.

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#### Author information:

- L. Wang, MMed, Researcher
- H. Xiao, MD, Researcher
- T. Zhang, MMed, Researcher
- Y. Liu, BS, Researcher
- D. Xu, MD, PhD, Orthopaedic Surgeon, Researcher
- H. Lu, MD, PhD, Head of Department, Researcher  
Department of Sports Medicine, Xiangya Hospital, Central South University, Changsha, China; Key Laboratory of Organ Injury, Aging and Regenerative Medicine of Hunan Province, Changsha, China; National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China.
- S. Li, MD, Researcher, Orthopaedic Surgeon, Department of Sports Medicine, Xiangya Hospital, Central South University, Changsha, China; Key Laboratory of Organ Injury, Aging and Regenerative Medicine of Hunan Province, Changsha, China; National Clinical Research Center for Geriatric Disorders, Xiangya Hospital,

Central South University, Changsha, China; Department of Orthopedics, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China.

- J. Hu, MD, PhD, Researcher, Orthopaedic Surgeon, Key Laboratory of Organ Injury, Aging and Regenerative Medicine of Hunan Province, Changsha, China; National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China; Department of Spine Surgery and Orthopaedics, Xiangya Hospital, Central South University, Changsha, China.

#### Author contributions:

- L. Wang: Methodology, Project administration, Writing – original draft.
- S. Li: Methodology, Project administration, Writing – review & editing.
- H. Xiao: Formal analysis, Writing – review & editing.
- T. Zhang: Formal analysis, Writing – review & editing.
- Y. Liu: Formal analysis.
- J. Hu: Conceptualization, Supervision.
- D. Xu: Funding acquisition, Supervision.
- H. Lu: Conceptualization, Funding acquisition, Supervision.
- L. Wang and S. Li contributed equally to this work.

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- All authors declare no conflicts of interest.

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- The datasets generated and/or analyzed during the current study are not publicly available due to the research design, but are available from the corresponding author on reasonable request.

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