

ZEB1 maintains mitochondrial fission and macrophage efferocytosis by restraining MFN2, thereby limiting inflammation and improving tendon-bone healing

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Abstract. Excessive inflammation and scar formation at the tendon-bone interface (TBI) hinder effective healing. Macrophage efferocytosis is critical for resolving inflammation, yet its regulatory mechanisms in TBI healing remain unclear. The present study investigated the role of zinc finger E-box binding homeobox 1 (ZEB1) in macrophage efferocytosis and rotator cuff repair. *Zeb1* knockdown in rats was achieved using short hairpin RNA (shRNA). Bone marrow-derived macrophages co-cultured with apoptotic Jurkat cells were used to evaluate efferocytosis efficiency. Mechanistically, ZEB1 was demonstrated to function as a critical regulator of mitochondrial dynamics by transcriptionally repressing Mitofusin-2 (MFN2), thereby maintaining the mitochondrial fission necessary for efficient efferocytosis. ZEB1-knockdown relieved MFN2 suppression, leading to excessive mitochondrial fusion and a subsequent decrease in apoptotic cell clearance. *In vivo*, ZEB1 deficiency resulted in the accumulation of secondary necrotic cells, aggravated the

inflammatory microenvironment (increased M1/decreased M2 polarization), and impaired histological and biomechanical healing of the tendon-bone interface. These findings indicate a novel ZEB1/MFN2/mitochondrial fission axis that governs macrophage efferocytosis. Targeting this axis to restore the immune microenvironment offers a potential therapeutic strategy for improving tendon-bone healing.

Introduction

Tears to the rotator cuff are a common shoulder joint injury, and postoperative chronic inflammation frequently results in fibrotic scarring, which significantly elevates the risk of re-tear and compromises the restoration of shoulder joint function (1). Due to the unique gradient structure and limited regenerative capacity of the tendon-bone interface (TBI), healing at the insertion site typically occurs via fibrovascular scar tissue formation, even after successful surgical repair (2). This fibrovascular scar connection between the tendon and bone exhibits significant mechanical impairment, resulting in high re-tear rates, particularly in elderly patients, where the re-tear rate can reach 20-90% (3). Therefore, inflammation plays a key role in the excessive scar formation in various tissues (4), and reducing excessive inflammation and scar formation may lead to improved clinical outcomes.

Neutrophils are a critical component of the inflammatory response. In the early postoperative stage, inflammatory cells rapidly accumulate at the TBI and attract monocytes/macrophages to aggregate and proliferate (5). Subsequently, most neutrophils rapidly undergo apoptosis and begin to accumulate as uncleared apoptotic cells (ACs). However, if not promptly cleared, ACs can progress to secondary necrosis, releasing intracellular self-antigens and cytotoxic compounds, thereby exacerbating the inflammatory response and leading to excessive scar formation (6). Apoptosis and the phagocytic clearance of ACs (efferocytosis) are evolutionarily conserved processes that occurs in all tissues throughout the life cycle of an organism, and they are crucial for organism development, tissue renewal and regeneration, as well as the development

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Abbreviations: TBI, tendon-bone interface; ZEB1, zinc finger E-box binding homeobox 1; BMDMs, bone marrow-derived macrophages; ACs, apoptotic cells; MFN2, Mitofusin-2; DRP1, dynamin-related protein 1

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and function of the immune system (7). However, an increasing number of chronic inflammatory diseases, such as autoimmune diseases and atherosclerosis, are caused by macrophage efferocytosis dysfunction (8). Efferocytosis not only prevents tissue necrosis and inflammation caused by secondary necrosis of dead cells but also activates anti-inflammatory signals in macrophages, which is crucial for tissue dissolution and repair after injury or inflammation (6,9). Therefore, exploring the molecular mechanisms required for efferocytosis may uncover novel therapeutic targets for the treatment or prevention of inflammatory diseases.

E-box binding homeobox-1 (ZEB1), a zinc finger transcription factor, is well-known for its role in triggering the epithelial-mesenchymal transition (EMT) program in cancer cells, which is crucial for embryonic development (neural crest migration) as well as a range of pathological conditions, such as tumor metastasis and organ fibrosis (10,11). In recent years, ZEB1 has emerged as a pivotal regulator of cellular plasticity in non-cancer cells (12,13). For instance, in macrophages infiltrating injured tissues, ZEB1 facilitates the phenotypic transition from pro-inflammatory to anti-inflammatory phenotypes (14). Additionally, a recent study demonstrated that a reduction in ZEB1 levels in macrophages increases atherosclerotic plaque formation, and targeted upregulation of *Zeb1* expression can reverse the high lipid accumulation in macrophages (15). Meanwhile, Zhang *et al* (16) revealed that ZEB1 links fragmented mitochondrial morphology with the self-renewal of hematopoietic stem cells (HSC) by inhibiting mitofusin-2 (MFN2)-mediated mitochondrial fusion. Specifically, ZEB1 represses MFN2 to induce mitochondrial fragmentation, which leads to reduced mitochondrial mass and lower reactive oxygen species (ROS) levels, which is essential to prevent HSC exhaustion and maintain their long-term self-renewal capacity.

The significant role of mitochondrial morphology changes in mitochondrial homeostasis has been highlighted in previous studies. Mitochondrial morphology alterations, also known as mitochondrial dynamics, mainly include mitochondrial fusion and fission. Wang *et al* (17) reported that mitochondrial fission plays a crucial role in promoting the continuous clearance of ACs by macrophages. Silencing the fusion mediator *Mfn1* leads to mitochondrial hyper-fragmentation and enhances efferocytosis at an ACs:macrophage ratio of 10:1, mainly due to the increase in cytoplasmic calcium concentration induced by ACs. Moreover, Hu *et al* (18) also indicated that a reduction in MFN2 leads to an increase in mitochondrial fission. The dynamic changes in mitochondrial fission and fusion are mainly regulated by fusion proteins such as MFN1 and MFN2, and fission proteins, including dynamin-related protein 1 (DRP1) and mitochondrial fission factor, which are essential for cellular functions under different conditions (8). However, the precise mechanism by which ZEB1 coordinates mitochondrial dynamics to regulate efferocytosis in macrophages remains to be elucidated. Therefore, the present study hypothesizes that ZEB1 plays a significant role in the efferocytosis process of macrophages by regulating MFN2-mediated mitochondrial dynamics, which may be closely related to the improvement of the inflammatory microenvironment at the TBI.

Therefore, exploring the potential role of ZEB1 in macrophages within the chronic inflammatory microenvironment

of rotator cuff injuries is of notable importance. The present study aimed to elucidate the regulatory mechanism of ZEB1 on mitochondrial dynamics and macrophage efferocytosis, specifically focusing on the ZEB1-MFN2 axis. To achieve this, a rat rotator cuff injury repair model and *in vitro* macrophage systems were used to investigate how ZEB1-mediated mitochondrial remodeling influences the inflammatory response and the quality of tendon-bone healing.

Materials and methods

Animal models and gene knockdown. Bone marrow-derived macrophages (BMDMs) were extracted and characterized from 10, 6-week-old male C57BL/6 mice (weighing 20-22 g). Following a one-week adaptation period, the mice were euthanized by cervical dislocation.

A total of 30 12-week-old male Sprague-Dawley (SD) rats (weighing 300-350 g) were used to establish the rotator cuff injury model for *in vivo* experiments. All animals were purchased from the Experimental Animal Center of Xi'an Jiaotong University and housed in a specific pathogen-free environment (temperature, 20-25°C; humidity, 50-60%; 12-h light/dark cycle), with *ad libitum* access to food and water, with five rats per cage. After a 7 day adaptation period, the rats were randomly divided into two groups using a random number table method.

Group 1: Rotator cuff injury negative control group (NC group; n=15); a rotator cuff injury repair model was established as described below and rats were injected with a non-targeting virus [recombinant lentivirus (rLV)-scramble short hairpin (sh)RNA]. SD rats were anesthetized via intraperitoneal injection of 0.3% pentobarbital sodium (40 mg/kg), and then shaved and disinfected. A 1-cm longitudinal incision was made in the surgical area (anterolateral aspect of the shoulder), and the subcutaneous tissue was bluntly separated. The connection between the deltoid muscle and the acromion was transected, and the supraspinatus tendon was lifted with a retractor. A 6-0 prolene suture (Ethicon, Inc.) was placed at the end of the supraspinatus tendon, and the residual fibrocartilage at the implantation site was removed. A total of two 0.5 mm bone tunnels were drilled at the joint margin of the implantation site. The suture was passed through the bone tunnels, tightened and knotted. The *in situ* fixation of the supraspinatus tendon was confirmed by exploration. The deltoid muscle and skin were sutured layer by layer.

Group 2: Rotator cuff injury control group + *Zeb1* knockdown group (shZEB1 group; n=15); in addition to the aforementioned treatment, *Zeb1* gene knockdown experiments were conducted. rLV-shRNA1-*mZeb1* suspension (1x10⁷ TU/ml; 100 μ l; Ribo-Bio) was injected locally into the affected shoulder joint immediately after surgery and at the 1st, 2nd and 3rd weeks after surgery (rats scheduled for 1-week euthanasia received injections only at the first two time points), while the NC group was injected with the same amount of rLV-scramble shRNA at the same time points. The knockdown efficiency was verified by reverse transcription-quantitative (RT-q)PCR at 48 h post-surgery (using dedicated validation samples; n=3 per group) and immunohistochemistry (IHC) at 1 week post-surgery. At 1, 4 and 8 weeks after surgery, the rats were euthanized by intraperitoneal injection of an overdose of

pentobarbital sodium (150 mg/kg). Subsequently, the rotator cuff-humeral complex specimens (the tendon-bone interface specimens, comprising the supraspinatus tendon and the greater tuberosity of the humerus) were collected for analysis. This animal experiment protocol was approved by the Animal Experiment Ethics Committee of Xi'an Jiaotong University (approval no. XJTUAE2025-2322).

Cell culture. BMDMs were generated as described in reference through the differentiation of bone marrow cells (18). Male C57BL/6 mice (6-weeks old) were euthanized by cervical dislocation and used as the source of bone marrow. The humeri, femurs and tibiae were aseptically harvested and surface-sterilized by brief immersion in 75% ethanol for 30 sec at 20–25°C. Bone marrow was flushed from the isolated bones using ice-cold phosphate-buffered saline (PBS), and the resulting cell suspension was passed through a 70 μ m cell strainer to remove debris and obtain a single-cell suspension. The collected cells ($1\text{--}10 \times 10^5$ /cm²) were seeded in a medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 20 ng/ml recombinant macrophage colony-stimulating factor (M-CSF; PeproTech, Inc.). After 3 days of culture (37°C with 5% CO₂), the medium was replaced with fresh medium. After a total of 6 days, the cells were firmly attached to the surface of the culture plate. The expression of F4/80, a well-established and specific cell-surface marker for mature murine macrophages, was verified by immunofluorescence (IF) to verify the successful differentiation and purity of the BMDMs. Briefly, the cultured cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. Following PBS washes, the cells were blocked with 5% normal goat serum (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The cells were then incubated overnight at 4°C with a primary antibody against F4/80 (1:500; cat. no. ab300421; Abcam). Subsequently, the cells were incubated with an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. ab150081; Abcam) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI for 5 min at room temperature. Finally, the cells were observed and imaged using a fluorescence microscope (Olympus BX53) at x200 and x400 magnifications. Image analysis was performed using ImageJ software (version 1.53k; National Institutes of Health).

The *in vitro* experiment divided BMDMs into two groups: i) Control group (NC group) where cells were treated with an equal amount of rLV-scramble shRNA as the negative control group; and ii) *Zeb1*-knockdown group (sh-ZEB1 group) where 50 μ l of lentivirus (1×10^7 TU/ml, MOI=20, rLV-shRNA1-m*Zeb1*) was added to each well for *Zeb1* gene knockdown, along with polybrene (8 μ g/ml) to assist infection (19). Third-generation lentiviral particles were produced in 293T cells using a four-plasmid packaging system. The shRNA constructs were cloned into the pLKO.1-U6-Puro lentiviral vector system (Ribo-Bio). For a 10-cm dish, a total of 20 μ g of plasmid DNA was transfected, including the transfer plasmid, packaging plasmids (pMDLg/pRRE and pRSV-Rev), and the envelope plasmid (pMD2.G) at a mass ratio of 4:2:1:1. Transfection was performed at 37°C for 6–8 h using Lipofectamine 3000, followed by replacement with fresh

complete medium. Lentiviral particles were harvested from the cell supernatant at 48 and 72 h post-transfection, filtered through a 0.45 μ m filter, and concentrated. The core target sequences were as follows: Sh-*Zeb1* target sequence, 5'-ATA GAGGCTACAAGCGCTTTA-3'; scramble shRNA target sequence, 5'-CAACAAGATGAAGAGCACCAA-3'. After 12 h of infection, the medium was replaced with fresh medium and the cells were further cultured for 48 h. To establish stable cell lines, transduced cells were selected with 2 μ g/ml puromycin for 7 days and subsequently maintained in 0.5 μ g/ml puromycin. After verifying the *Zeb1* knockdown efficiency by RT-qPCR and western blotting (as described below), the cells were seeded at a density of 5×10^5 cells/well in six-well plates for subsequent experiments.

The human lymphocyte line Jurkat (clone E6-1; American Type Culture Collection) was selected as the apoptotic cell model. Logarithmic growth phase Jurkat cells (1×10^6 cells/ml) were seeded in six-well plates and exposed to ultraviolet (UV) light under uncovered conditions. A 254 nm UV light was used with an energy dose of 200 mJ/cm² for 15–20 min. Fresh RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) [supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.)] was then added and the cells were further cultured for 6 h, with an apoptosis rate of ~85% (17). Then, apoptotic Jurkat cells were labeled with propidium iodide (PI, 1 μ g/ml, incubated for 15 min, at 20–25°C, protected from light), and set aside at 37°C for 30 min for later use.

Efferocytosis assay. *In vitro* experiments were conducted using the phagocytosis of ACs assay, following the previously reported method (16). To investigate the dynamic expression of *Zeb1* during efferocytosis, BMDMs were co-cultured with ACs at a ratio of 5:1 (ACs:BMDMs) for varying durations (0, 1, 3 and 6 h). At each time point, total RNA was extracted, and *Zeb1* mRNA levels were determined by RT-qPCR and western blotting. Briefly, the aforementioned UV pre-treated apoptotic Jurkat cells were co-cultured with BMDMs that had been labeled with Mito-Tracker Green (cat. no. C1996s; Beyotime Biotechnology) for mitochondrial staining, and the mixture was incubated for the designated time period (37°C with 5% CO₂ for 6 h). Non-ingested Jurkat cells were subsequently removed by washing three times with PBS. Phagocytic activity was visualized via fluorescence microscopy, and the efferocytic index was quantified as the percentage of PI-positive macrophages as follows: Efferocytic Index (%)=(number of PI-positive macrophages/total number of macrophages) x100% (ImageJ software; version 1.53k; National Institutes of Health).

RNA isolation and RT-qPCR. Total RNA was extracted from BMDM cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.), and reverse transcribed using a AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Inc.) according to the manufacturers instructions. RNA samples were analyzed by qPCR using SYBR Green PCR premix (Applied Biosystems; Thermo Fisher Scientific, Inc.), with GAPDH as the internal reference gene. The sequences of the primers used are as follows: GAPDH forward, 5'-CTGCACCACCAA CTGCTTAG-3', and reverse, 5'-GTCTGGGATGGAAAT TGTGA-3'; inducible nitric oxide synthase (iNOS) forward,

5'-CAAGCACCTTGGAAGAGGAG-3', and reverse, 5'-AAG GCCAAACACAGCATACC-3'; TNF- α forward, 5'-CTG TAGCCACGTCGTAGC-3', and reverse, 5'-TTGAGATCC ATGCCGTTG-3'; arginase 1 (Arg-1) forward, 5'-CTCCAA GCCAAAGTCCTTAGAG-3', and reverse, 5'-AGGAGCTGT CATTAGGGACATC-3'; CD206 forward, 5'-CAAGGAAGG TTGGCATTGT-3', and reverse, 5'-CCTTTCAGTCCTTTG CAAGC-3'; ZEB1 forward, 5'-GCTGGCAAGACAACGTGA AAG-3', and reverse, 5'-GCCTCAGGATAAATGACGGC-3'. The quantification of gene expression was carried out using the $2^{-\Delta\Delta C_q}$ method relative to the internal control (20).

Western blotting. Protein extraction was performed using a standard protocol, and protein quantification was carried out with a BCA Protein Assay Kit (Beyotime Biotechnology). Mitochondrial proteins were extracted using the Cell Mitochondria Isolation Kit (cat. no. C3601; Beyotime Biotechnology). The procedure was performed strictly according to the manufacturer's instructions, involving cell homogenization followed by differential centrifugation to separate the mitochondrial fraction from the cytosolic fraction. The proteins were diluted to the same concentration with loading buffer (Beyotime Biotechnology) and then loaded onto 10% SDS-PAGE gels. After electrophoresis, the proteins (30 μ g per lane) were transferred to PVDF membranes (MilliporeSigma) by wet transfer. The PVDF membranes were blocked with 5% skim milk in TBST (0.05% Tween) for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. After washing, the membranes were incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG) at room temperature for 2 h (1:10,000; cat. no. SA00001-2; Proteintech Group, Inc.). Protein detection was performed using an ultra-sensitive enhanced chemiluminescence kit (Shanghai Yamei Biomedical Technology Co Ltd.) and using ImageJ software (version 1.53k; National Institutes of Health). The primary antibodies used were as follows: Anti- β -Actin antibody (1:5,000; cat. no. 81115-1-RR; Proteintech Group, Inc.), anti-ZEB1 antibody (1:1,000; cat. no. ab203829; Abcam), anti-MFN2 antibody (1:5,000; cat. no. ab124773; Abcam), and anti-DRP1 antibody (1:5,000; cat. no. 5391; Cell Signaling Technology, Inc.).

Flow cytometry. For purity identification, BMDMs were harvested and resuspended (5-10 $\times$ 10⁶ cells/ml) in Immunol Staining Buffer (Beyotime Biotechnology) to prevent non-specific binding and maintain cell viability. Cells were incubated with an APC-conjugated anti-mouse F4/80 antibody (cat. no. AC0988; Beyotime Biotechnology) for 30 min at 4°C in the dark. The stained cells were resuspended in PBS and analyzed immediately to prevent signal loss and ensure accurate detection of surface markers on live cells. Unstained cells served as a negative control to determine the gating strategy. The purity of BMDMs was defined as the percentage of F4/80-positive cells (number of F4/80-positive macrophages/total number of macrophages) $\times$ 100%. The flow cytometry analysis was performed using a Sino-Cyte Flow Cytometer (Beijing Sino-Cyte Scientific Co., Ltd.). Data acquisition and analysis were performed using FlowJo software (version 10; Tree Star, Inc.).

For apoptosis analysis, Jurkat cell death (early + late apoptosis) was evaluated using an Annexin V-FITC/PI Apoptosis Detection Kit (cat. no. C1062S; Beyotime Biotechnology). Briefly, after UV treatment (detailed above), Jurkat cells were collected, washed with cold PBS, and resuspended in 1 $\times$ Binding Buffer (Beyotime Biotechnology) at a density of 5-10 $\times$ 10⁶ cells/ml. Annexin V-FITC and PI were added to the cell suspension and incubated for 15 min at room temperature in the dark.

Histological analysis. The TBI specimens collected at 4 and 8 weeks post-surgery were processed for histological analysis. Serial sections were obtained from each specimen and stained with hematoxylin and eosin (H&E), safranin O-fast green (SO/FG) and Sirius Red to evaluate structural healing, fibrocartilage formation and collagen organization, respectively. Specimens were fixed in 10% neutral formalin solution for 48 h at room temperature (20-25°C). Subsequently, decalcification was performed in 10% EDTA buffer at 37°C water bath, with the EDTA solution being replaced every 3 days for ~4 weeks until the samples became soft. After decalcification, the samples were embedded in paraffin and cut into 5- μ m thick sections using an ultrathin sectioning machine along the coronal plane parallel to the shoulder joint. The interface region between the tendon graft and bone tissue was observed by H&E staining (sections were stained with hematoxylin for 5 min and eosin for 2 min at room temperature). SO/FG staining was performed according to the Safranin O-Fast Green Stain Kit (cat. no. C0621S; Beyotime Biotechnology) instructions to evaluate the formation and integration of fibrocartilage and bone tissue. For Sirius Red staining, the sections were incubated with Sirius Red solution at room temperature (20-25°C) for 1 h (cat. no. C0190S; Beyotime Biotechnology), followed by rapid dehydration in absolute ethanol and clearing in xylene to assess collagen organization. The histological scoring system applied in this study was adopted the TBI healing assessment method described by Ide *et al* (21). The TBI scoring system assigned a maximum score of 16 points for normal tissue morphology, with higher scores indicating better healing outcomes. To ensure objectivity and reduce subjective bias, the histological grading was conducted by two independent observers who were blinded to the experimental groups. The inter-rater reliability was assessed using Cohen's κ coefficient (with $\kappa > 0.8$ considered as excellent agreement). The H&E and SO/FG stained sections were scanned using a Panoramic Digital Slide Scanner (3DHitech, Ltd.), and digital images were analyzed using CaseViewer 2.4 software (3DHitech, Ltd.). Subsequently, Sirius Red-stained sections were examined under a polarizing light microscope (Eclipse E800; Nikon Corporation) to assess collagen fiber composition. Strong yellow-to-red birefringence was indicative of type I collagen fibers, whereas weak green birefringence represented the presence of type III collagen. Digital images were captured under polarized light, and ImageJ software (version 1.53k; National Institutes of Health) was utilized to quantify the mean intensity (calculated as the arithmetic mean of pixel gray values) of type I collagen across experimental groups for comparative analysis.

Immunofluorescence, immunohistochemical (IHC) and TUNEL staining. The preparation process of immunofluorescence (IF) staining sections is consistent with that of H&E staining. The sections were deparaffinized in xylene and

rehydrated through a descending ethanol series (100, 95, 80 and 75%). Antigen retrieval was performed by heating the sections in citrate buffer (pH 6.0) at 95–100°C for 15 min. After cooling to room temperature, the sections were permeabilized with 0.1% Triton X-100 for 15 min to facilitate antibody penetration for intracellular antigens. Subsequently, the sections were treated with normal goat serum (cat. no. C0265; Beyotime Biotechnology) at room temperature for 30 min to reduce non-specific staining. Then, the sections were incubated overnight at 4°C with primary antibodies against iNOS (1:50; cat. no. 18985-1-AP), CD206 (1:50; cat. no. 18704-1-AP) and CD68 (1:50; cat. no. 28058-1-AP) (Proteintech Group, Inc.) according to the manufacturer's instructions. The sections were incubated with the corresponding fluorescent secondary antibodies for 2 h at room temperature in the dark. Specifically, FITC-conjugated goat anti-rabbit IgG (1:200; cat. no. GB22404; Wuhan Servicebio Technology Co., Ltd.) was used for iNOS detection, while Cy3-conjugated goat anti-rabbit IgG (1:300; cat. no. GB21303; Wuhan Servicebio Technology Co., Ltd.) was used for CD206 and CD68. Finally, the sections or smears were treated with DAPI staining agent at room temperature for 5 min to visualize nuclei. Digital images were acquired using a Panoramic Digital Slide Scanner (3DHitech, Ltd.) and analyzed using CaseViewer 2.4 software (3DHitech, Ltd.).

For IHC, the procedures for slice preparation, dewaxing and antigen retrieval were performed in accordance with the protocols used for immunofluorescence (IF). The tissue sections were treated with 3% hydrogen peroxide (H_2O_2) solution at room temperature for 15 min to suppress endogenous peroxidase activity. Following this, the sections were incubated with 5% normal goat serum for 30 min at room temperature (20–25°C) to block non-specific binding sites. Subsequently, the sections were incubated overnight at 4°C with appropriately diluted primary antibodies against SOX9 (1:50; cat. no. GB14171), RUNx2 (1:50; GB125631) and α -SMA (1:500; cat. no. GB111364). After thorough washing with PBS, the sections were incubated with the corresponding HRP-conjugated goat anti-rabbit IgG secondary antibodies (1:300; cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.) at 37°C for 30 min. Thereafter, the sections were stained with diaminobenzidine (DAB) at room temperature (20–25°C) for 2–5 min, with the degree of color development strictly monitored under a microscope. After washing with distilled water, the sections were counterstained with hematoxylin at room temperature for 3 min to visualize nuclear morphology. Subsequently, the sections were differentiated in 1% acid alcohol, blued in running tap water, dehydrated through an ascending graded ethanol series, cleared in xylene, and mounted with a neutral resinous medium. Finally, the dried sections were examined under a light microscope, and representative images were captured.

Apoptosis detection was carried out using the One-Step TUNEL Apoptosis Assay Kit (cat. no. C1086; Beyotime Biotechnology), following the manufacturer's instructions. Briefly, samples were fixed in 4% paraformaldehyde at 4°C for 24 h and permeabilized. After incubation with the TUNEL reaction mixture, nuclei were counterstained with DAPI (1 μ g/ml) at room temperature for 5 min. Sections were mounted with Antifade Mounting Medium (Beyotime

Biotechnology). The sections were then scanned using a Panoramic Digital Slide Scanner (3DHitech, Ltd.). Digital images from 5 random fields of view per sample were captured and analyzed using CaseViewer 2.4 software. Each section was incubated with 50 μ l of TUNEL detection reagent at 37°C in the dark for 60 min. Following staining, the proportion of positively stained areas for each marker was quantitatively analyzed using Image analysis software.

Micro-CT analysis. The rotator cuff-humeral head complexes were harvested at 4 and 8 weeks postoperatively. After being fixed in 4% paraformaldehyde for 24 h at 4°C, they were scanned using a micro-computed tomography (micro-CT, NMC-200; Pingseng Medical Technology) with the following parameters: 80 kV, 270 μ A and an effective pixel size of 18 μ m. After scanning, three-dimensional images were reconstructed. A region of interest (ROI) with a diameter of 1.6 mm was selected in the greater tuberosity area of the humerus. Bone mineral density (BMD), trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) of the selected ROI were analyzed.

Biomechanical test. At 4 and 8 weeks post-operation, 3 rats were randomly selected from each group and sacrificed. The rotator cuff-humerus complex specimens were retrieved, with only the intact connection structure between the supraspinatus tendon and the humerus retained. The cross-sectional area of the middle part of the supraspinatus tendon was measured using a digital caliper (Tajima Industries, Ltd.). The proximal end of the humerus was fixed with polymethyl methacrylate at room temperature (20–25°C) for ~15 min, and the supraspinatus tendon was covered with polyester cloth and fixed by a lock-stitch suture. The biomechanical testing was performed using a Shimadzu AG-IS Universal Testing Machine (Shimadzu Corporation). After a 0.1 Newtons (N) pretreatment (20–25°C), the specimens were subjected to uniaxial tensile loading at a constant speed of 10 mm/min until the tendon failed at the bone repair site. The ultimate failure load was determined as the peak force recorded during the tensile test. Stiffness (N/mm) was calculated as the slope of the linear portion of the load-deformation curve.

Grip strength test and animal gait analysis. Grip strength tests were conducted at 4 and 8 weeks postoperatively in each group of rats to assess the biomechanical recovery following rotator cuff repair. During the test, the rat's tail was gently held and its forelimbs were placed on the metal grid of the grip strength meter. As the rat instinctively grasped the grid, the tester maintained the animal's body parallel to the grid and gradually pulled the tail until the forelimbs released. The peak force exerted at the moment of release was recorded by the device. Each rat performed the test in triplicate, and the mean value was calculated for subsequent statistical analysis.

At 4 and 8 weeks postoperatively, gait analysis was conducted on each group of rats using the Greenwalk system (22) to assess the gait condition after rotator cuff repair. The experimental setup consisted of a transparent glass runway and a high-resolution camera below. The rats were placed at one end of the runway in a quiet environment and walked freely through the entire channel. The camera

simultaneously captured the footprints and pressure distribution. Each rat completed ≥ 3 effective walks, and the average value was used for analysis. The analysis parameters included: i) Maximum contact area-the largest area of contact between the forepaws and the glass plate; ii) stride length-the distance between consecutive positions of the forepaws during movement; iii) running speed-the length of the glass plate divided by the time taken to pass through; and iv) mean intensity-the average pressure exerted by the forelimbs on the glass plate. A larger maximum contact area, longer stride length, faster speed and greater pressure indicated a greater healing effect.

Statistical analysis. All data were analyzed using IBM SPSS Statistics 24.0 software (IBM Corp.) and GraphPad Prism 8.0 software (GraphPad; Dotmatics). Experimental data were expressed as ($\bar{X} \pm s$) or percentages. All *in vitro* experiments were performed with at least three independent biological replicates. Normality tests (Shapiro-Wilk test) and homogeneity of variance tests (Levene's test) were first conducted for each group of data. If the data were normally distributed and had homogeneity of variance, independent sample Students t-tests were used for inter-group comparisons; For data that did not meet the assumptions of normality, the Mann-Whitney U test was utilized. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of BMDMs. After 6 days of M-CSF-induced differentiation, the identity and purity of the obtained BMDMs were verified. Immunofluorescence staining showed robust expression of the macrophage-specific marker F4/80 (green) (Fig. 1A). Consistently, flow cytometry analysis further confirmed that the proportion of F4/80-positive cells reached a high level of purity ($>85\%$), ensuring that the cells were suitable for subsequent functional assays (Fig. 1B).

ZEB1 is involved in the regulation of macrophage efferocytosis. To explore the potential role of ZEB1 in efferocytosis, BMDMs were co-cultured with ACs at a ratio of 5:1 for varying durations (0, 1, 3, and 6 h). RT-qPCR results demonstrated that *Zeb1* mRNA levels increased in a time-dependent manner during the clearance of ACs (Fig. 1C). This trend was further confirmed at the protein level using western blotting; as shown in Fig. 1D, ZEB1 protein expression significantly increased following co-culture. Specifically, while no significant change was observed at 1 h, ZEB1 levels increased ~ 1.5 -fold at 3 h and 3-fold at 6 h compared with the baseline. These data suggested that ZEB1 was involved in the biological response of macrophages during efferocytosis.

ZEB1-knockdown impairs macrophage efferocytosis. To investigate whether ZEB1 regulates macrophage polarization-a process closely linked to mitochondrial metabolism (23)-the present study first evaluated the effect of *Zeb1*-knockdown on macrophage markers under baseline conditions. BMDMs were transfected with shRNA targeting *Zeb1*, and the knockdown efficiency was validated. Compared with the NC group, *Zeb1* expression was reduced by $\sim 53\%$ at the mRNA level (Fig. 1F) and 51% at the protein level (Fig. 1G). Notably,

RT-qPCR analysis showed no significant differences in the mRNA levels of M1 (TNF α , iNOS) and M2 (CD206, Arg-1) markers between the NC and sh-ZEB1 groups under baseline conditions (Fig. 1E), indicating that *Zeb1*-knockdown does not spontaneously alter macrophage polarization.

Next, the impact of ZEB1 on the phagocytic capacity of macrophages was assessed. Target apoptotic Jurkat cells were prepared via UV radiation; Fig. 1I shows a representative flow cytometry plot confirming that the majority of Jurkat cells were in the early/late apoptotic stage (Annexin V-positive) prior to co-culture. BMDMs (labeled with Mito-Tracker Green) were then co-incubated with PI-labeled ACs (red) for 6 h. Fluorescence microscopy revealed that in the NC group, numerous macrophages successfully engulfed or bound to ACs (indicated by arrows showing overlapping signals). By contrast, the sh-ZEB1 group exhibited a significant reduction in phagocytic activity; the percentage of PI-positive macrophages was significantly lower in the *Zeb1* knockdown group compared to the NC group (Fig. 1H). Collectively, these results demonstrate that ZEB1 knockdown significantly impaired the efferocytosis efficiency of macrophages.

ZEB1 knockdown leads to the reduction of mitochondrial fission. Since mitochondrial fission promotes the continuous clearance of ACs by macrophages, and it has been reported that its inhibition can reduce macrophage efferocytosis (16), the present study investigated whether ZEB1 knockdown affects macrophage efferocytosis by inhibiting mitochondrial fission. To explore the effect of *Zeb1*-knockdown on mitochondrial morphology, mitochondria were stained with Mito Tracker Deep Red, images were collected with a confocal microscope and the mitochondrial morphology was analyzed. Mitochondria in the NC group exhibited a more punctuated and fragmented network with fewer branches compared with the sh-ZEB1 group (Fig. 2A), indicating that *Zeb1*-knockdown inhibited the fission of mitochondria.

ZEB1-knockdown leads to an increase in the levels of MFN2 in mitochondria. Mitochondrial morphology is closely related to the process of fusion and fission, mainly regulated by fusion and fission proteins. A previous study has shown that ZEB1 is a transcriptional repressor of MFN2 (15). However, it is also known that DRP1 plays a dominant role in mitochondrial fission (24). Therefore, the present study extracted mitochondrial proteins from *Zeb1*-knockdown primary macrophages using a cell mitochondrial isolation kit and mainly detected the protein levels of MFN2 and DRP1 by western blotting. The results showed that after *Zeb1*-knockdown, compared with the NC group, the expression level of DRP1 protein did not change significantly, but the expression level of MFN2 protein increased significantly (Fig. 2B and C). Therefore, the expression levels of MFN2 in mitochondria were further detected by immunofluorescence colocalization technology. The results showed that MFN2 exhibited distinct colocalization with the mitochondrial marker Tomm20 in the sh-ZEB1 group (Fig. 2D), confirming its mitochondrial localization. These results indicated that ZEB1 affected macrophage efferocytosis by regulating the expression of MFN2 on mitochondria. In summary, ZEB1 regulated

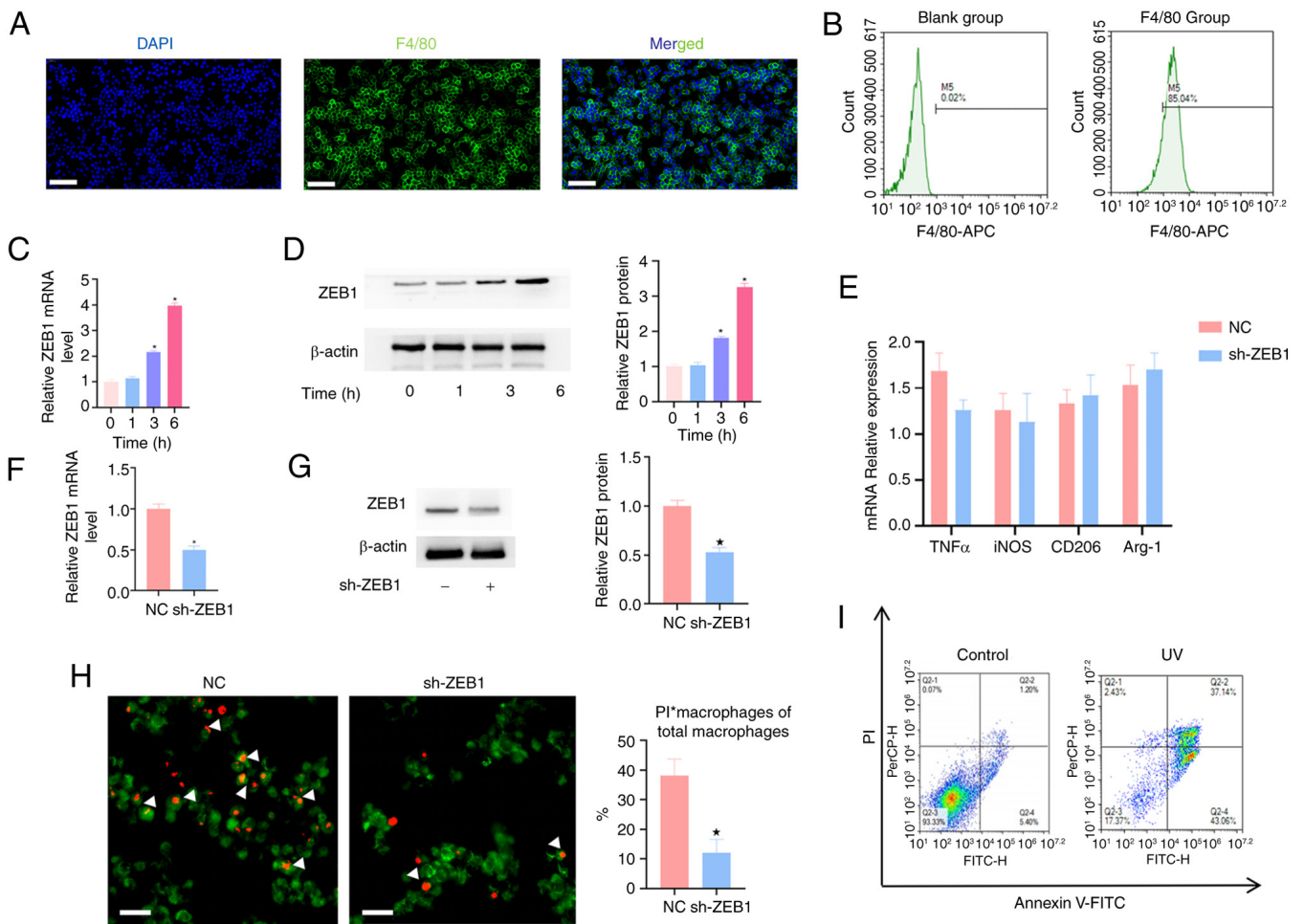


Figure 1. ZEB1 knockdown affects the cell efferocytosis efficiency of macrophages. (A) After 6 days of induction culture with M-CSF, the expression level of F4/80 (green) was detected by immunofluorescence staining (scale bar, 20 μ m). (B) Flow cytometry was performed to detect the proportion of F4/80-positive cells to verify the purity of macrophages. (C) The mRNA expression levels of ZEB1 in macrophages at different time points (0, 1, 3 and 6 h) of co-culture were determined by RT-qPCR. (D) After co-incubation of BMDMs with ACs for 0, 1, 3 and 6 h, the protein expression of ZEB1 was detected by western blotting and semi-quantitative analysis of ZEB1 expression was conducted. (E) The mRNA expressions of TNF α , iNOS, CD206 and Arg-1 were detected by RT-qPCR. (F) The expression of ZEB1 mRNA was detected by RT-qPCR to evaluate the knockout efficiency. (G) Detection of ZEB1 protein expression by WB and semi-quantitative analysis of ZEB1 expression. (H) BMDMs were labeled with Mito-Tracker (green label), and then co-cultured with PI-labeled apoptotic Jurkat cells at a ratio of 5:1 of apoptotic cells to macrophages for 6 h. After removing the non-phagocytic apoptotic cells, the proportion of PI-positive macrophages (indicated by arrows) among the total macrophages was determined by fluorescence microscopy (Scale bar, 20 μ m). (I) The apoptosis rate of cells induced by UV radiation was detected by flow cytometry (Annexin V-FITC/PI double staining) to prepare target cells for the efferocytosis assay. n=3. *P<0.05 vs. NC or 0 h groups. NC, negative control; sh, short hairpin; ZEB1, zinc finger E-box binding homeobox 1; BMDMs, bone marrow-derived macrophages; ACs, apoptotic cells; M-CSF, macrophage colony-stimulating factor; RT-qPCR, reverse transcription-quantitative PCR; iNOS, inducible nitric oxide synthase.

mitochondrial dynamics by inhibiting MFN2 expression, thereby regulating macrophage efferocytosis, which is characterized by the inhibition of mitochondrial fusion.

ZEB1-knockdown exacerbates the early inflammatory response at the TBI. To evaluate the effect of ZEB1 on the microenvironment of the TBI post-surgery, the knockdown efficiency *in vivo* was first verified. At 48 h post-operation, RT-qPCR analysis of the tendon tissues confirmed a significant reduction in *Zeb1* mRNA levels in the sh-ZEB1 group compared with the NC group (Fig. 3A). Consistently, IHC staining performed 1 week post-surgery demonstrated that ZEB1 protein expression was significantly decreased at the interface (Fig. 3B). Furthermore, ZEB1 deficiency led to a upregulation of MFN2 at both mRNA (Fig. 3C) and protein levels (Fig. 3D), as evidenced by RT-qPCR and IHC analysis, respectively.

The impact of *Zeb1* knockdown on cellular apoptosis and macrophage behavior was then investigated. TUNEL staining at 1 week post-operation revealed a significantly higher density of ACs (green fluorescence) at the TBI in the sh-ZEB1 group compared with the NC group (Fig. 3E), indicating that the efficiency of recognition and clearance of ACs by macrophages at the TBI was significantly reduced after *Zeb1* knockdown. To further characterize the macrophage response, polarization phenotypes were examined using double immunofluorescence staining. At 1 week post-surgery, the sh-ZEB1 group exhibited a significant increase in the positive area of the M1 marker iNOS (green), accompanied by a marked decrease in the M2 marker CD206 (red) compared to the NC group (Fig. 3F). These results indicate that underexpression of ZEB1 shifts the macrophage population toward a pro-inflammatory M1 phenotype in the early postoperative period.

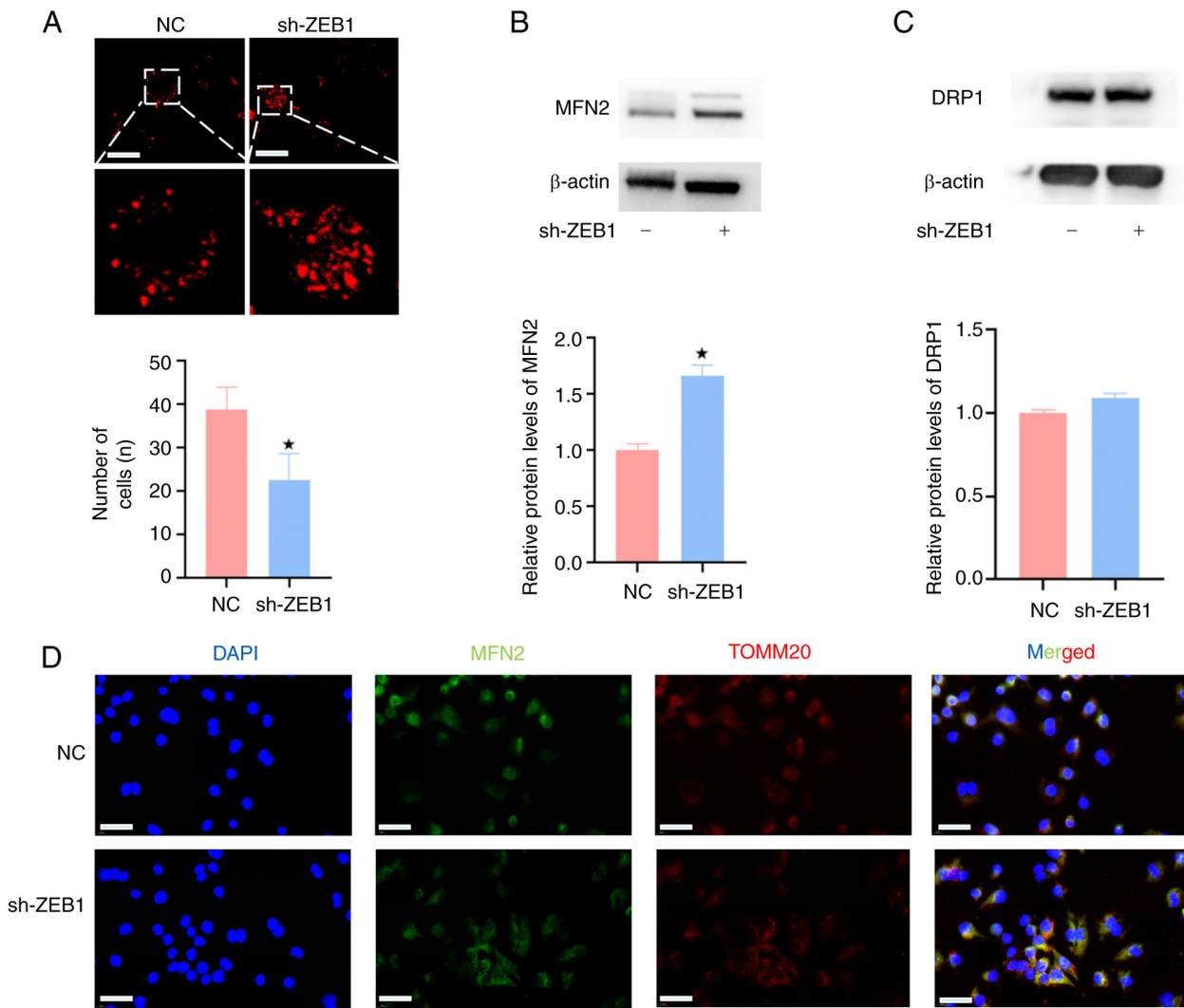


Figure 2. ZEB1 affects mitochondrial fission by regulating MFN2. (A) Confocal microscopy results of mitochondrial morphology in macrophages. Mitochondria were labeled with Mito-Tracker Red fluorescent probe (scale bar, 10 μ m; lower panels, x200 magnification). Western blotting analysis of (B) MFN2 and (C) DRP1 protein expression in macrophages after ZEB1 knockdown. Quantitative density analysis data are presented as mean $\pm$ standard deviation (n=3 independent experiments). (D) Immunofluorescence colocalization analysis of MFN2 localization in mitochondria. Nuclei were stained with DAPI (blue), MFN2 protein was labeled with Alexa Fluor 488 (green) and mitochondrial membrane protein TOMM20 was labeled with Alexa Fluor 594 (red) (scale bar, 10 μ m). n=3, *P<0.05 vs. NC. DRP1, dynamin-related protein 1; MFN2, Mitofusin-2; ZEB1, zinc finger E-box binding homeobox 1; NC, negative control; sh, short hairpin.

Finally, to clarify whether this inflammatory shift was linked to defective efferocytosis, co-localization analysis of macrophages (CD68, red) and ACs (TUNEL, green) was performed. Immunofluorescence results showed that ZEB1 deficiency significantly impaired the physical association and clearance of ACs by macrophages at the TBI (Fig. 3G). Collectively, these findings suggest that ZEB1 is needed to maintain efficient efferocytosis and to modulate macrophage polarization during the early stages of tendon-to-bone healing.

ZEB1 deficiency delays the healing of the TBI tissue. At 4 and 8 weeks postoperatively, the structure of the TBI was examined by H&E staining and SO/FG staining (Fig. 4A and B) in order to capture the remodeling and maturation phases of healing. Histological healing of the TBI was evaluated using Ide histological scoring system. A maximum score of 16 indicates normal TBI, with higher scores reflecting superior healing quality. As the healing process of the rotator cuff injury progressed, the continuity of the TBI was relatively well

restored at 8 weeks postoperatively. Compared with the 4-week time point, there was a marked increase in fibrocartilage cells and proteoglycan matrix at the interface, and the fiber arrangement exhibited a more organized and aligned pattern. In comparison to the sh-ZEB1 group, the NC group demonstrated a significantly thicker fibrocartilage layer, along with improved chondrocyte polarity and maturation. The inter-rater reliability analysis demonstrated excellent agreement between the two independent observers, with a Cohen's κ coefficient of 0.83. Based on the Ide scoring system, the histological quantification score in the NC group was significantly higher than that in the sh-ZEB1 group (Fig. 4D), with statistically significant differences at 4 and 8 weeks.

To investigate the formation of fibrous scar tissue and the expression of osteogenic and chondrogenic markers during the repair of rotator cuff injuries in rats, IHC staining was performed to assess the expression levels of α -SMA (a fibrotic scar marker), RUNx2 (an osteogenic marker) and SOX9 (a chondrogenic marker) (Fig. 4C). The results demonstrated

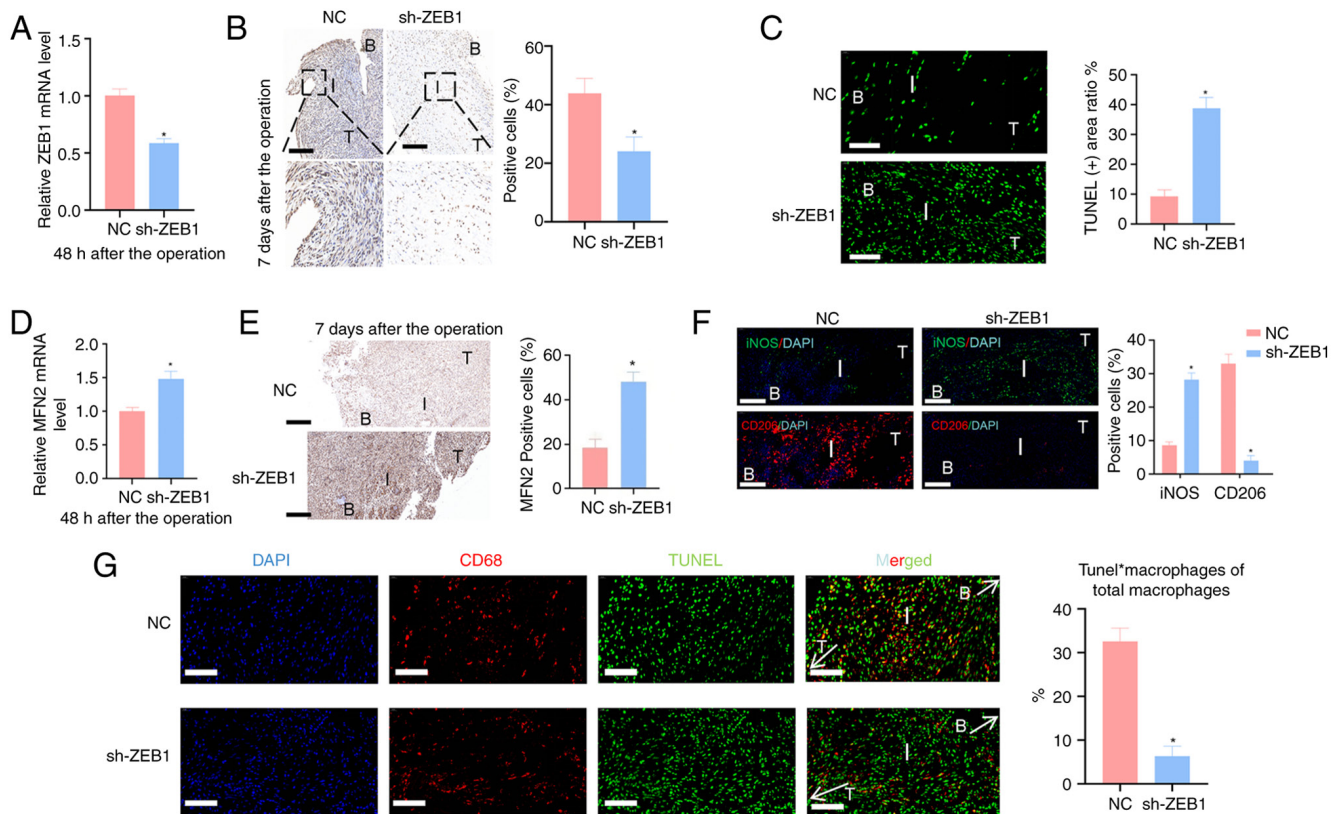


Figure 3. ZEB1 knockdown aggravates the inflammatory response at the TBI. (A) The expression level of ZEB1 was detected by RT-qPCR 48 h after the operation. (B) The expression of ZEB1 protein was detected by IHC 1 week after the operation, and the optical density value was semi-quantitatively analyzed by ImageJ software (n=3 high-power fields) (scale bar, 50 μ m; lower panels, x50 magnification, scale bar, 20 μ m). (C) Apoptotic cells at the TBI were detected by TUNEL method in the early postoperative period (1 week), with green fluorescence (FITC labeled) indicating apoptotic cells with DNA fragmentation (scale bar, 20 μ m). (D) The expression level of MFN2 was detected by RT-qPCR 48 h after the operation. (E) The expression of MFN2 protein was detected by IHC 1 week after the operation, and the optical density value was semi-quantitatively analyzed by ImageJ software (scale bar, 50 μ m). (F) At 1 week after the operation, double immunofluorescence staining was used to detect the polarization phenotype of macrophages. The cell nuclei were stained with DAPI (red), M1 macrophages were labeled with iNOS (green), and M2 macrophages were labeled with CD206 (red) (scale bar, 20 μ m). (G) At 1 week after the operation, co-localization analysis of apoptotic cells (Tunel, green) and macrophages (CD68, red) at the TBI was performed by immunofluorescence. The cell nuclei were stained with DAPI (blue) (scale bar, 20 μ m). The labels in the figure are as follows: T=tendon, B=bone tissue, I=tendon-bone interface. n=3. *P<0.05 vs. NC group. TBI, tendon-bone interface; ZEB1, zinc finger E-box binding homeobox 1; RT-qPCR, reverse transcription-quantitative PCR; IHC, immunohistochemistry; MFN2, Mitofusin-2; iNOS, inducible nitric oxide synthase; NC, negative control; sh, short hairpin.

that ZEB1 deficiency during the healing process significantly promoted the formation of fibrous scar in the TBI while suppressing the development of bone and cartilage tissues, with statistically significant differences (Fig. 4E).

Furthermore, at 4 weeks post-surgery, collagen fibers in the sh-ZEB1 group exhibited a more disorganized arrangement compared to those in the NC group, and the yellow-red birefringent areas indicative of type I collagen were reduced. Semi-quantitative analysis based on the intensity of birefringence under polarized light microscopy revealed no significant difference between the two groups. By 8 weeks post-surgery, collagen fibers in both groups displayed a more regular and aligned structure with enhanced birefringence, although the NC group showed superior healing outcomes. Semi-quantitative analysis further confirmed that the NC group had significantly higher levels of type I collagen compared to the sh-ZEB1 group (Fig. 5D).

Micro-CT scanning was performed at 4 and 8 weeks postoperatively to evaluate bone formation at the tendon-bone healing interface. Three-dimensional reconstruction of the TBI specimens was conducted, and quantitative analysis of the ROI

was carried out (Fig. 5A). The three-dimensional morphological data revealed the growth and remodeling of subchondral bone at the TBI in rats. At 4 weeks post-surgery, minimal bone ingrowth into the subchondral region was observed, whereas by 8 weeks, new bone formation was evident at the interface. ROI-based quantitative analysis demonstrated that, at both 4 and 8 weeks postoperatively, the NC group exhibited significantly higher values of BV/TV, Tb.Th, BMD, and Tb.N in the subchondral bone at the TBI compared with the sh-ZEB1 group.

As the healing period progressed, biomechanical parameters such as failure load and stiffness gradually increased. Biomechanical analysis (Fig. 5B) revealed no significant differences in the cross-sectional area of the TBI among groups at 4 and 8 weeks post-surgery. However, at both time points, the NC group exhibited significantly higher failure load and stiffness at the TBI compared to the sh-ZEB1 group. In parallel, grip strength measurements (Fig. 5C) demonstrated that the NC group displayed significantly greater grip strength than the sh-ZEB1 group at both 4 and 8 weeks postoperatively.

Collectively, these findings indicated that ZEB1 deficiency during the healing of rotator cuff injuries in rats impaired

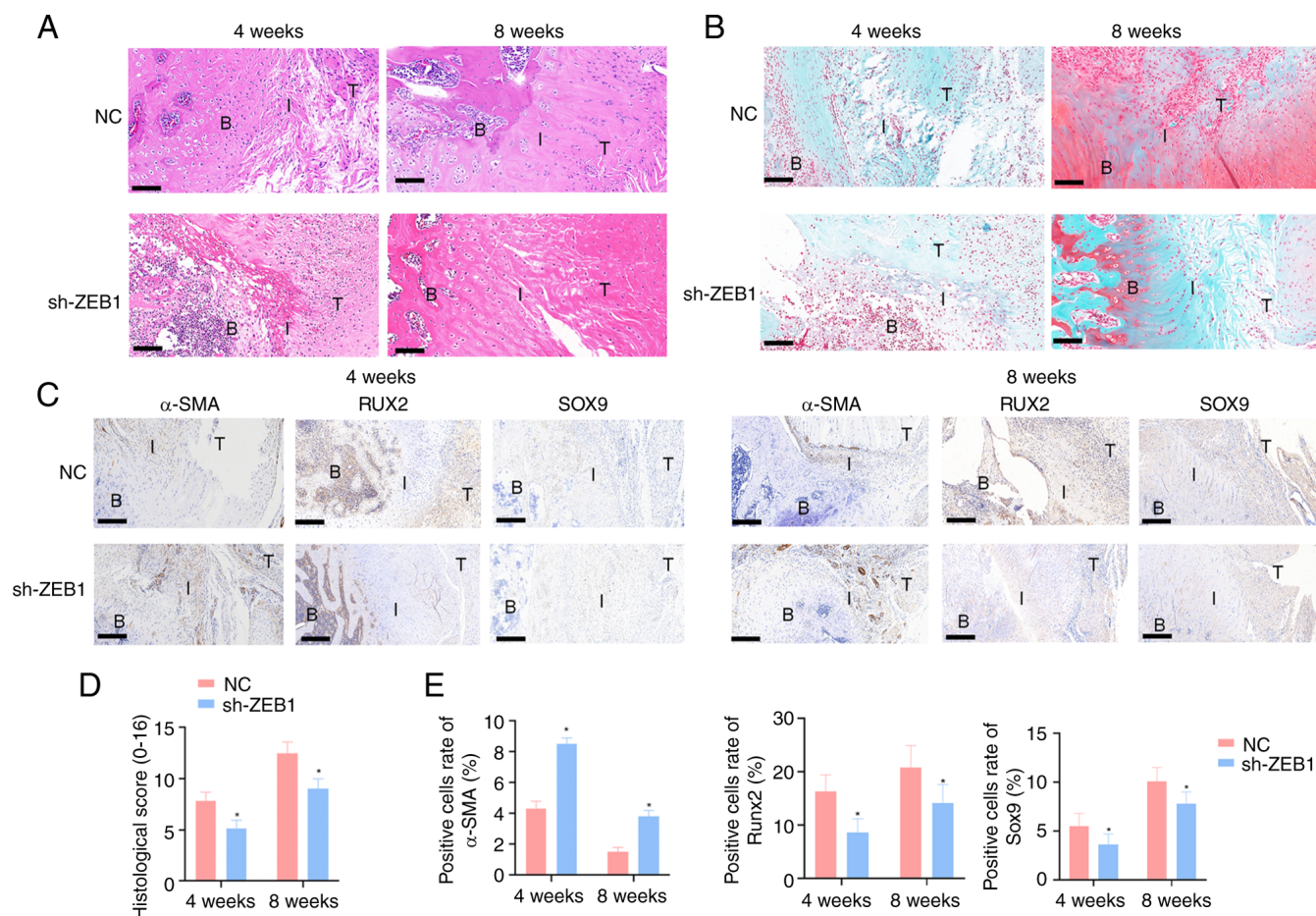


Figure 4. ZEB1 deficiency impairs the morphological healing of the TBI tissue. (A) H&E and (B) SO/FG staining were used to show the histological changes at the TBI at 4 weeks and 8 weeks postoperatively (scale bar, 50 μ m). (C) Immunohistochemical detection of the expression and distribution of α -SMA, RUNX2 and SOX9 at the TBI. Positive signals are brownish-yellow granules, and the cell nuclei are counterstained with hematoxylin (blue) (scale bar, 50 μ m). (D) Results of histological quantitative scoring. (E) Bar charts showing the histological scores and quantitative analysis of the positive cell rates of α -SMA, RUNX2 and SOX9 at 4 and 8 weeks postoperatively. The labels in the figure are as follows: T=tendon, B=bone tissue, I=tendon-bone interface. Data are presented as mean $\pm$ standard deviation. $n=3$. * $P<0.05$ vs. NC. TBI, tendon-bone interface; ZEB1, zinc finger E-box binding homeobox 1; H&E, hematoxylin and eosin; SO/FG, safranin O-fast green; NC, negative control; sh, short hairpin.

histological repair of the TBI and compromised the regeneration and remodeling of subchondral bone, ultimately resulting in reduced biomechanical integrity and functional grip performance.

ZEB1-knockdown impairs postoperative gait recovery in rats. The Greenwalk system was used to evaluate the walking movement ability of rats after rotator cuff injury repair, in order to obtain detailed quantitative analysis of motor function. The footprints of the rats were successfully captured by a high-resolution camera placed under a transparent glass plate (Fig. 6A), and a three-dimensional volcano plot was generated based on pressure intensity (Fig. 6B). At 4 and 8 weeks after surgery, the maximum contact area of swing, stride length, running speed and average pressure of the NC group were all significantly higher than those of the sh-ZEB1 group (Fig. 6C).

Discussion

The present study aimed to investigate the impact of ZEB1 deficiency on macrophage efferocytosis and its subsequent effects for rotator cuff healing. To the best of our knowledge,

this is the first study to systematically elucidate the interrelationships among macrophage ZEB1 expression, efferocytosis, polarization and tendon-bone junction repair within an animal model of rotator cuff injury. The key findings demonstrated that ZEB1 deficiency significantly impaired macrophage efferocytosis. Mechanistically, our results demonstrate that ZEB1 modulates MFN2-mediated mitochondrial dynamics, a process critically involved in the regulation of efferocytosis. These effects may be closely associated with the modulation of the inflammatory microenvironment at the TBI. Therefore, targeting ZEB1 may represent a promising therapeutic strategy for enhancing tendon-bone healing.

During the process of tendon-bone healing, inflammatory microenvironment disorder, cellular senescence and the persistent activation of myofibroblasts are all important factors for the occurrence and progression of the disease (25). As central mediators of inflammatory infiltration, macrophages play an essential role in tissue injury and repair. Therefore, the ability to effectively reduce excessive inflammatory responses and prevent excessive scar formation, and restore the gradient structure at the tendon-bone junction as much as possible (26) has become a focus of current academic research.

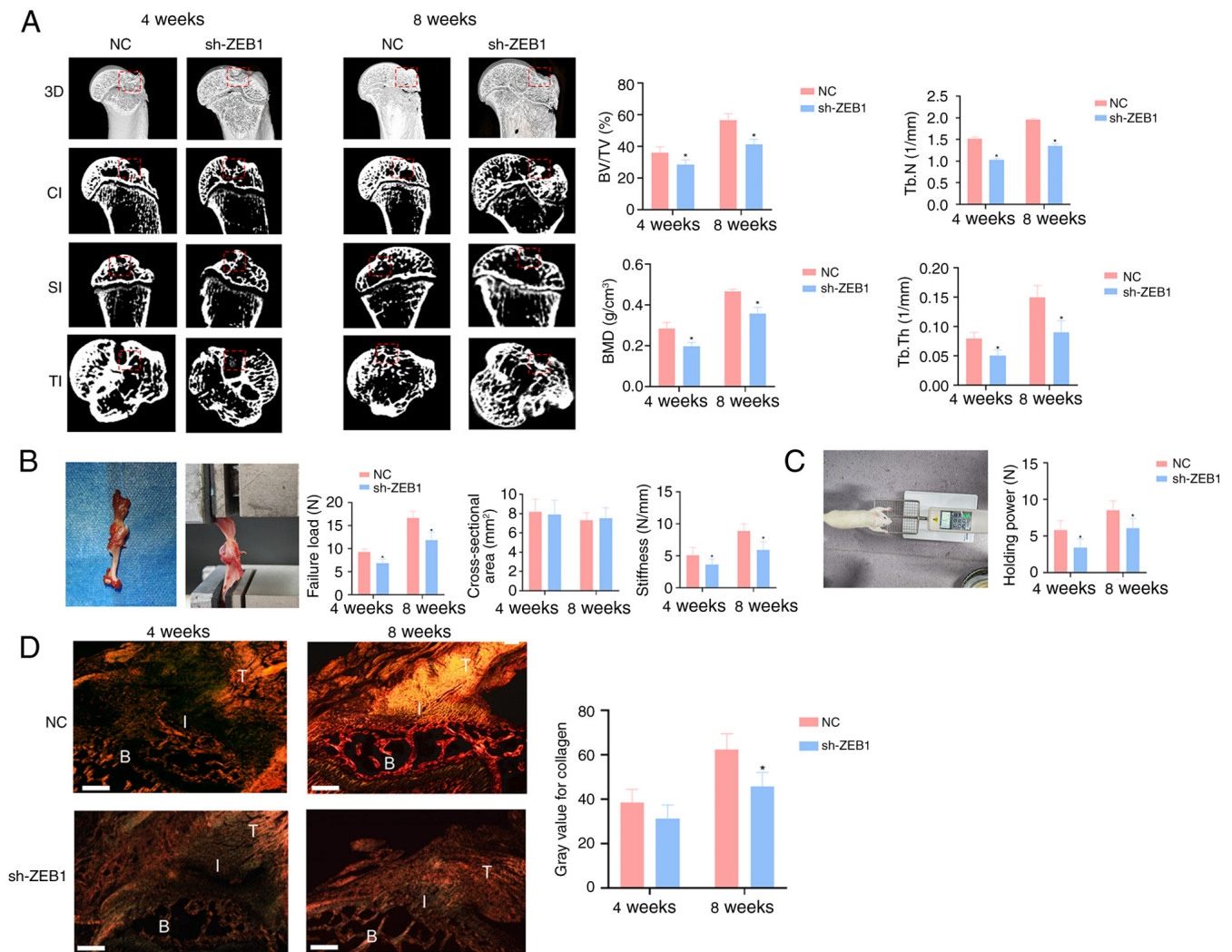


Figure 5. ZEB1 deficiency impairs bone regeneration and biomechanical properties at the tendon-bone interface. (A) Representative Micro-CT images and quantitative analysis of BV/TV, Tb.N, BMD and Tb.Th. The red dashed boxes indicate the regions of interest within the necrotic lesions of the weight-bearing area for quantitative analysis and morphological observation. (B) Gross observation of biomechanical testing along with quantitative analysis of failure load, cross-sectional area, and stiffness. (C) Gross observation and quantitative analysis of grip strength. (D) Picrosirius Red staining showing the recovery of the tendon-bone junction and semi-quantitative analysis of type I collagen content based on optical density (scale bar, 50 μ m). The labels in the figure are as follows: T=tendon, B=bone tissue, I=tendon-bone interface. n=3. *P<0.05 vs. NC. TBI, tendon-bone interface; ZEB1, zinc finger E-box binding homeobox 1; BV/TV, bone volume fraction; Tb.N, trabecular number; BMD, bone mineral density; Tb.Th, trabecular thickness; NC, negative control; sh, short hairpin; CI, coronal interface; SI, sagittal interface; TI, transverse interface.

Efferocytosis is the specialized process by which ACs are recognized, engulfed and digested by phagocytes, particularly macrophages, guided by chemokine gradients. This mechanism is mainly responsible for the clearance of ACs (27). In the absence of effective efferocytosis, ACs may release cytotoxic metabolic byproducts that can initiate excessive inflammatory responses, ultimately resulting in secondary necrosis (6,28). Efferocytosis is a highly regulated biological process that encompasses the recognition, binding, internalization and degradation of ACs. Beyond merely preventing secondary necrosis-induced damage, this process actively reprograms macrophages to secrete pro-reparative signaling molecules, thereby playing a pivotal role in tissue regeneration following injury or inflammatory insult.

In recent years, growing evidence has highlighted the critical role of macrophage polarization in tendon-bone healing (29,30). However, the relationship between macrophage efferocytosis and tendon-bone healing remains poorly

understood, with significant gaps still present in current research. The present study revealed that the expression level of ZEB1 in macrophages was time-dependent with macrophage efferocytosis. Meanwhile, knockdown of ZEB1 in macrophages led to a decrease in their efferocytic efficiency, indicating that ZEB1 was involved in the process of macrophage efferocytosis. ZEB1 has been widely studied for its role in triggering the EMT program in cancer cells and regulating stem cell functions (10,15). However, the latest research evidence indicates that it can also promote the plasticity of non-malignant cells from various tissue sources, including macrophages (14). In the context of skeletal physiology, ZEB1 serves as a pivotal regulator of mesenchymal stem cell differentiation and bone development, playing a crucial role in balancing osteogenesis and adipogenesis (13). Furthermore, the loss of ZEB1 impairs the formation of type H vessels, thereby exacerbating osteonecrosis (31). Meanwhile, a study has indicated that the downregulation of ZEB1 enhances atherosclerotic plaque

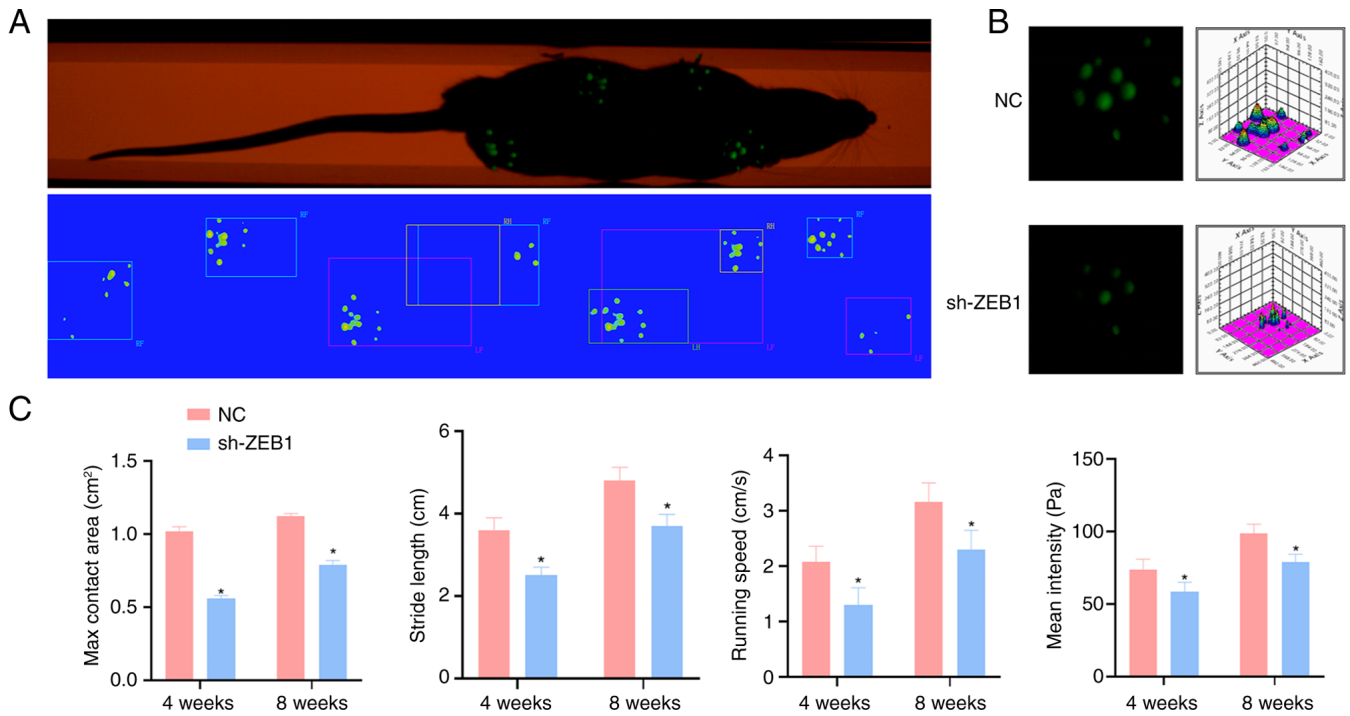


Figure 6. Gait analysis of rats after rotator cuff injury surgery. (A) Schematic diagram of the rat footprint collection device. (B) Representative images of the forelimb footprints and three-dimensional volcano plots after surgery. (C) Quantitative analysis and comparison of gait-related parameters. n=3. *P<0.05 vs. 4 weeks. NC, negative control; sh, short hairpin; ZEB1, zinc finger E-box binding homeobox 1.

formation in mice and is closely associated with the aggravation of chronic inflammation (15). The present study indicated that knocking down the homeostatic level of ZEB1 in macrophages led to poor healing at the TBI. The underlying mechanism involves the regulation of MFN2-mediated mitochondrial dynamics, which directly affects macrophage efferocytosis. The present study revealed that ZEB1 expression is essential for maintaining mitochondrial fission and preserving the efficiency of macrophage efferocytosis.

Mitochondria play a crucial role in cellular functions such as energy metabolism, calcium homeostasis regulation and reactive oxygen species (ROS) generation (32,33). Mitochondrial homeostasis is governed by a finely tuned equilibrium between fission and fusion processes. This dynamic balance is primarily orchestrated by DRP1 and mitochondrial fusion proteins 1 and 2 (MFN1/2), which serve as master regulators in sustaining mitochondrial structural and functional integrity (32). A number of studies have shown that efferocytosis is a process mediated by mitochondria, and mitochondrial metabolism, mitochondrial dynamics, and communication between mitochondria and other organelles all affect the clearance of ACs by macrophages (17,34). The present study demonstrated that *Zeb1* knockdown in macrophages resulted in the formation of a distinct tubular mitochondrial network, a morphological change that aligned with previous observations by Zhang *et al* (16); this study revealed that ZEB1, functioning as a transcriptional repressor, directly binds to the promoter region of MFN2 and suppresses its expression (16). This downregulation of MFN2 by ZEB1 disrupts mitochondrial fusion, leading to a fragmented mitochondrial morphology characterized by small, rounded structures. By contrast, the absence of ZEB1 promotes the formation of elongated, tubular

mitochondrial networks (15). Collectively, these findings indicate that ZEB1 depletion enhances mitochondrial fusion through the upregulation of MFN2 expression. Emerging evidence has firmly established a link between mitochondrial dynamics and phagocytic activity (17). In this study, reduction of the mitochondrial fission protein DRP1 in macrophages was shown to diminish phagocytic capacity, whereas depletion of the mitochondrial fusion protein MFN1 enhanced phagocytosis. These findings indicate a positive association between mitochondrial fission and phagocytic efficiency. Our data demonstrated that MFN2 expression was significantly upregulated in ZEB1-knockdown macrophages, and increased MFN2 levels promoted mitochondrial fusion, thereby attenuating phagocytosis. Notably, the present study observed that ZEB1 deficiency did not significantly affect DRP1 expression levels. We hypothesized that this is due to ZEB1 directly binding to the promoter region of MFN2 as a transcriptional repressor, directly inhibiting the expression of MFN2 (16). Secondly, the process of mitochondrial fission itself is positively associated with the ability of phagocytosis. When macrophages phagocytize ACs, it further enhances mitochondrial fission. Although DRP1 expression remains unchanged, enhanced mitochondrial fusion may shift the fission-fusion equilibrium, resulting in a more tubular, network-like mitochondrial morphology. Collectively, these observations support the notion that ZEB1 modulates macrophage phagocytosis-most plausibly via suppression of MFN2 expression-thereby limiting mitochondrial fusion and supporting efferocytosis.

The present study found that ZEB1 plays a crucial role in maintaining macrophage efferocytosis, and this effect could in turn promote M2 polarization, thereby forming a positive feedback cycle of an anti-inflammatory microenvironment.

A previous study has indicated that macrophage polarization phenotypes are closely associated with their capacity to clear ACs (35). M1 macrophages primarily drive the early inflammatory response by secreting IL-1 β and TNF- α . Although they possess a certain degree of phagocytic function, their efficiency in recognizing and clearing ACs is generally lower than that of the M2 phenotype. By contrast, M2 macrophages express high levels of receptors such as MerTK and CD206, exhibiting a more efficient efferocytic capacity. They are specialized in the timely clearance of ACs to prevent secondary necrosis and inflammatory leakage (36). More importantly, efferocytosis is not merely a clearance process but a key driving force for inducing M2 polarization. After macrophages engulf ACs, they activate intracellular signaling pathways that inhibit the release of pro-inflammatory mediators and instead secrete TGF- β and IL-10, thereby further promoting the conversion of macrophages to the M2 anti-inflammatory phenotype (37). Das *et al* (38) suggests that this efficient efferocytosis acts as an important 'signal' for the conversion of M0 to the anti-inflammatory M2 phenotype. Our *in vivo* experimental data show that ZEB1-knockdown leads to a significant increase in ACs at the TBI, a marked decrease in efferocytosis efficiency, a significant increase in the area of iNOS positivity and a significant decrease in the area of CD206 positivity. It is worth noting that, although our *in vitro* experimental results show that *Zeb1* knockdown does not affect macrophage polarization, this does not present a conflict. This is because our *in vitro* experiments were conducted in an environment without apoptotic cell accumulation, using isolated macrophages. The direct impact of *Zeb1* knockdown on intrinsic polarization signaling pathways may be minimal. However, in the *in vivo* tendon-bone injury environment, there is a large number of ACs. In this context, the impaired efferocytosis due to ZEB1 deficiency leads to the accumulation of uncleared ACs (secondary necrosis), which in turn drives the inflammatory response and alters macrophage polarization. Taken together, all these results indicate that *Zeb1* knockdown impairs macrophage efferocytosis, reduces M2 polarization and aggravates inflammation at the TBI. Moreover, previous studies have confirmed that inducing M2 macrophage aggregation at the TBI through biological intervention in the early postoperative period is beneficial for tendon-bone healing (39,40). In summary, these findings suggest that ZEB1 plays a key role in regulating the inflammatory microenvironment during tendon-bone healing by modulating macrophage efferocytosis and M2 polarization.

The natural gradient structure of the TBI is composed of tendon, uncalcified fibrocartilage, calcified fibrocartilage and bone (41). Following injury, this delicate architecture is frequently replaced by fibrovascular scar tissue, which possesses inferior mechanical properties. Achieving an ideal, site-specific transition interface remains a significant challenge. Currently, researchers believe that the biomechanical properties of this interface are closely related to bone ingrowth and bone integration. Notably, enhanced M2 polarization is associated with a reduced TBI width and improved fiber alignment. (29,42). Our micro-CT analysis demonstrated that all groups exhibited an increase in microstructural parameters between 4 and 8 weeks post-surgery. However, the ZEB1-knockdown group displayed significantly impaired bone formation and reduced bone ingrowth around the tunnels. This

is also similar to the previous research results of our research group, that is, the absence of ZEB1 has an important impact on vascular-dependent bone regeneration (31). Efficient clearance of ACs is pivotal for maintaining bone homeostasis and immune equilibrium. Dysfunction in this process not only disrupts normal bone metabolism but also amplifies inflammatory responses, potentially contributing to the development of inflammatory bone disorders (43). Histological evaluation revealed that at 4 weeks post-operation, the TBI in all groups was predominantly occupied by disorganized fibrovascular tissue. By the 8th week, however, the continuity of the TBI was markedly improved. Notably, compared to the sh-ZEB1 group, the NC group exhibited a significantly thicker fibrocartilage layer, with more organized and mature chondrocyte arrangement. Furthermore, the histological scoring system confirmed a significantly higher tissue quality score in the NC group relative to the sh-ZEB1 group. Furthermore, immunohistochemical and Sirius Red staining analyses demonstrated that *Zeb1* knockdown significantly exacerbated scar formation at the TBI, markedly reduced osteogenesis and chondrogenesis, and led to more disorganized collagen architecture and tissue structure. These pathological alterations ultimately resulted in a significant decline in both the maximum failure load and stiffness in the experimental animals. In addition, functional assessments of postoperative grasping force and gait recovery revealed that the sh-ZEB1 group exhibited significantly decreased grasping force, maximum contact area, stride length, running speed and average pressure compared with the control group. These findings suggest that favorable structural healing is closely associated with improved functional outcomes, highlighting the potential of targeting ZEB1 to enhance TBI regeneration as a promising therapeutic strategy for rotator cuff injuries.

The present study has several limitations that warrant consideration. First, due to technical constraints associated with surgical procedures, rats were selected as the experimental model. Future studies should employ genetically modified models, such as ZEB1-deficient or macrophage-depleted rats, to more rigorously validate the observed findings. Second, given that rodents do not fully recapitulate human pathophysiology, further investigations in large mammalian models are necessary to enhance translational relevance. Third, TUNEL staining lacks specificity for polymorphonuclear neutrophils (PMNs). To provide more definitive evidence, dual fluorescence labeling combining TUNEL with PMN-specific immunofluorescent markers should be employed for precise identification of apoptotic neutrophils. Nonetheless, the TUNEL results obtained in the present study do indicate a general trend toward improved apoptotic cell clearance. Finally, the present study focused solely on the regulatory effects of ZEB1-knockdown on macrophages and did not assess the potential benefits of ZEB1 overexpression. This was primarily due to the suboptimal transfection efficiency of the ZEB1 plasmid in macrophages. Despite testing multiple transfection reagents and optimizing DNA concentration (500-1,000 ng/ μ l), transfection efficiency remained low, and higher reagent concentrations induced notable cytotoxicity. Therefore, in our subsequent research, we plan to explore its upstream regulation, with the aim of perfecting the regulatory network of ZEB1-mitochondrial dynamics- efferocytosis. In summary, the absence of ZEB1 disrupts the delicate balance of the inflammatory microenvironment at the tendon-bone

healing interface, thereby impeding the physiological trajectory of tissue repair. This impairment is mechanistically driven by the dysregulation of MFN2-mediated mitochondrial dynamics which subsequently compromises macrophage efferocytosis. Therefore, targeting ZEB1 may be a potential strategy to improve tendon-bone healing.

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Availability of data and materials

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

LHF conceived and supervised the study. YZ and YKZ suggested experiments. YZ, TL and SQZ performed experiments. SQZ, XZ and TL performed statistical analysis. YZ, YKZ and KN did animal experimental procedures. YKZ, KN and XZ provided critical advice. YZ, YKZ and LHF wrote the paper; YZ, KN and LHF confirm the authenticity of all the raw data. All authors read and approved the final reviewed the final manuscript.

Ethics approval and consent to participate

This animal experiment protocol was approved by the Animal Experiment Ethics Committee of Xi'an Jiaotong University (approval no. XJTUAE2025-2322).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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