Cellular Physiology and Biochemistry
Cell Physiol Biochem 2016;38:319-329
DOI: 10.1159/000438632
Published online: January 27, 2016
Accepted: December 07, 2015
Open access

1421-9778/16/0381-0319$39.50/0
www.karger.com/cpb

Original Paper

TOB1 Deficiency Enhances the Effect of Bone Marrow-Derived Mesenchymal Stem Cells on Tendon-Bone Healing in a Rat Rotator Cuff Repair Model

Yulei Gao Yinquan Zhang Yanghu Lu Yi Wang Xingrui Kou Yi Lou Yifan Kang

Department of Orthopedics, Changhai Hospital, Second Military Medical University, Shanghai, China

Key Words
Tendon-bone healing • Mesenchymal stem cells • TOB1 • Gene therapy

Abstract
Background/Aims: This study investigated the effect of silencing TOB1 (Transducer of ERBB2, 1) expression in bone marrow-derived mesenchymal stem cells (MSCs) on MSC-facilitated tendon-bone healing in a rat supraspinatus repair model. ***Methods:*** Rat MSCs were transduced with a recombinant lentivirus encoding short hairpin RNA (shRNA) against TOB1. MSC cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The effect of MSCs with TOB1 deficiency on tendon-bone healing in a rat rotator cuff repair model was evaluated by biomechanical testing, histological analysis and collagen type I and II gene expression. An upstream regulator (miR-218) of TOB1 was determined in MSCs. ***Results:*** We found that knockdown of TOB1 significantly increased the proliferative activity of rat MSCs *in vitro*. When MSCs with TOB1 deficiency were injected into injured rat supraspinatus tendon-bone junctions, the effect on tendon-bone healing was enhanced compared to treatment with control MSCs with normal TOB1 expression, as evidenced by elevated levels of ultimate load to failure and stiffness, increased amount of fibrocartilage and augmented expression of collagen type I and type II genes. In addition, we found that the TOB1 3′ untranslated region is a direct target of miR-218. Similar to the effect of TOB1 deficiency, overexpression of miR-218 effectively promoted tendon-bone healing in rat. ***Conclusion:*** These results suggest that TOB1 may play a negative role in the effect of MSCs on tendon-bone healing, and imply that expression of TOB1 may be regulated by miR-218.

Y. Gao, Y. Zhang and Y. Lu contributed equally to this work.

Dr. Yifan Kang
Department of Orthopedics, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, (China)
Tel. +86-21- 31161698, E-Mail yifankangch@sina.com

Introduction

Tendon-bone junction injuries are common in both the workplace and in sports. Rotator cuff tendon-to-bone healing occurs by formation of a scar tissue interface after repair, which makes it prone to failure [1]. Currently, surgical treatment of rotator cuff defects is one of the most common orthopedic surgeries in the United States, with over 250,000 repairs performed each year [2, 3]. However, as many as 94% of rotator cuff tendons fail to heal back to the bone after repair [4, 5]. Given the relatively high failure rate of surgical repair, there is a strong need for new repair strategies to improve rotator cuff tendon-bone healing.

Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent adult stem cells that are capable of differentiating into multiple cell types including osteoblasts, chondrocytes and adipocytes *in vitro* [6]. Several animal model-based studies have found that these cells are transplanted to the injured sites and have the potential to augment the regeneration of bone, cartilage, muscle, ligament, and tendon *in vivo* [7-10], which makes MSCs an ideal tool for engineered tissue repair and cell therapy in different disease types.

TOB1 (transducer of ERBB2, 1) is a member of the TOB/B-cell translocation gene (BTG) family, which includes the proteins TOB2, BTG1, PC3/TIS21/BTG2, ANA/BTG3 and PC3/PC3K [11]. Overexpression of TOB/BTG family proteins can inhibit cell cycle progression in several cell types [12]. TOB1 has been implicated in diverse biological activities such as dorsal development of embryos and T cell activation [13-15]. It is well known that bone morphogenetic protein (BMP)/Sma and mad related family (Smad) signaling can effectively improve osteoblast differentiation and tendon-bone healing [16-18]. TOB1 is a negative regulator of BMP/Smad signaling and negatively regulates osteoblast proliferation and differentiation by suppressing the activity of receptor-regulated Smad proteins [19]. Therefore, we hypothesized that TOB1 also negatively regulates tendon-bone healing.

In the present study, we determined if transplantation of MSCs with TOB1 deficiency improved tendon-bone healing after rotator cuff injury. Considering that little is known about upstream regulators of TOB1, we further investigated if miR-218, a potent osteo-miR expressed in MSCs, could regulate TOB1 expression and affect tendon-bone healing.

Material and Methods

Isolation and Culture of MSCs

MSCs were isolated from the tibia and femur of 12 week old healthy male Sprague-Dawley rats (purchased from the Experimental Animal Department of Shanghai Medical College of Fudan University) as previously described [20]. Briefly, after euthanasia, the hind limbs were aseptically removed and the tibia and femur were dissected free of soft tissues. Bone marrow was flushed from the dissected bones with complete medium consisting of Dulbecco's modified Eagle's medium (DMEM), 1% penicillin/streptomycin and 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The cells were seeded in 10-cm dishes in a humidified 5% CO_2 atmosphere at 37°C. The medium was replaced every 2 – 3 days and non-adherent cells were removed. Cells at passage 3–5 were used for experiments.

Lentivirus infection

Lentivirus was generated by co-transfection of lentiviral vectors encoding the TOB1 silencing sequence or miR-218 precursor with two other plasmids coding for the lentiviral envelope (pCMV-VSV-G) and packaging proteins (pCMV delta R8.2) into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The lentiviral vectors encoding miR-218 and shRNA for TOB1 were purchased from Hanbio (Shanghai, China). MSCs were transduced with lentiviral supernatants containing the above vectors according to the manufacturer protocols. After 72 h, transduction efficiency was monitored by real-time PCR or by western blotting for the transduced targets.

miRNA transfection

MSCs were transfected with miR-218 mimic, miR-218 inhibitor or scramble sequence as a negative control (Ruibo, Guangdong, China) using siPORT NeoFX transfection reagent (Life Technologies, Carlsbad,

CA, USA) according to the manufacturer protocols. After 48 h of transfection, cells were harvested for further analysis.

Rotator Cuff Repair Model of Rat

All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai, China. Male Sprague-Dawley rats (12 weeks old, n = 72) were used in this study. The surgical procedure consisted of detachment and repair of the supraspinatus tendon as described previously [21]. Briefly, the deltoid was split and the supraspinatus was detached from its footprint. A 0.5 mm bone tunnel was drilled anterior to posterior through the greater tuberosity. The supraspinatus tendon was sharply detached from its insertion in the greater tuberosity. Sutures in the tendon were then passed through the tunnels using a modified Mason-Allen technique. Before tying the sutures to complete the repair, the rats were randomly assigned into groups of 18 rats, each of which received one of three different treatments at the interface between tendon and bone: fibrin glue carrier plus MSCs (2×10^6 cells), fibrin glue carrier plus shRNA-TOB1-transduced MSCs, or fibrin glue carrier plus miR-218-transduced MSCs. The model group without insertion of MSCs was used as the control group. Animals were sacrificed at 4 and 8 weeks, and the tissues were analyzed using histologic and biomechanical testing.

Biomechanical testing

Three animals per group were euthanized at 4 and 8 weeks for biomechanical testing as previously described [22]. Briefly, each specimen was preloaded to 0.1 N, then loaded to failure at a rate of 14 mm/s, corresponding to approximately 0.4% strain. The ultimate load to failure and the failure site were recorded. Stiffness was calculated by determining the slope of the linear portion of the load-displacement curve.

Histological analysis

Three animals per group were euthanized at 4 and 8 weeks for histologic examination as previously described [1]. In brief, specimens were fixed in 10% formalin, decalcified with Immunocal, and embedded in paraffin. Sections were cut at 5 μm thickness in the coronal plane through the repaired tendon-bone interface. The slides were then stained with hematoxylin and eosin (H&E) for observation by light microscopy.

Real-Time PCR

Total RNA was isolated from cells or tendon tissues with the use of TRIzol reagent (Invitrogen) and then quantified using a NanoDrop Spectophotometer (Thermo Scientific, DE, USA). For microRNA detection, reverse transcription of RNA was performed with specific RT primers. For determination of mRNA expression level, complementary DNAs (cDNAs) were synthesized using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). Real-time PCR reactions were performed using the SYBR Premix Ex Taq II kit (TaKaRa) and detected on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA served as internal normalized references for quantitation of mRNA and miRNAs, respectively.

MTT assay

Cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assays as described previously [23]. In brief, 72 h after infection, cells were trypsinized and resuspended in DMEM (1×10^6 cells/mL). The cells were seeded into 96-well plates in 100 μL total volume per well in triplicate. After 24, 48 and 72 h incubation, the cells were stained with 20 μL MTT dye (5 g/L) for 4 h. The absorbance at 490 nm of each well was measured using a microplate reader.

Luciferase Assay and constructs

The 3′-untranslated region (UTR) of TOB1 was amplified by PCR from total rat cDNA and subcloned into the pGL3 control vector (Promega, Fitchburg, WI, USA). The corresponding mutant constructs were created by mutating the seed region of the miR-218 binding site using overlap extension PCR. HEK293T cells were transfected with 100 ng of pGL3 vectors, 100 nM of either miR-218 mimic or a scrambled sequence, and 1 ng of *Renilla* luciferase pRL-TK vector (Promega) using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, luciferase assays were performed using the dual luciferase assay system (Promega) according to the manufacturer's protocol. *Renilla* luciferase was used for normalization.

Western blot analysis

Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% nonfat milk in phosphate-buffered saline, incubated with primary antibodies against TOB1 or GAPDH (Sigma–Aldrich, St. Louis, MO, USA) for 2 h at room temperature, washed and hybridized for 1 h with secondary antibodies conjugated to horseradish peroxidase. Detection was performed with an ECL chemiluminescence kit (Santa Cruz, Dallas, TX, USA). Band intensities were quantitated by Image J software. The intensities of the bands corresponding to TOB1 were normalized to those corresponding to GAPDH.

Statistical analysis

Data are presented as mean ± standard deviation (SD). All statistical analyses were performed with the use of SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical differences among groups were analyzed by one-way analysis of variance. Statistical differences between two groups were determined by the Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Knockdown of TOB1 increases the proliferative activity of rat MSCs in vitro

To investigate the role of TOB1 in tendon-bone healing, a recombinant lentiviral vector, encoding shRNA against TOB1 was constructed and used to infect MSCs. After 72 h, TOB1 expression was examined by western blotting. As shown in Fig. 1A, shRNA-TOB1 virus, but not the control virus, efficiently reduced the expression of TOB1 protein in MSCs. Subsequently, cell viability was determined by MTT assay. We found that knockdown of TOB1 significantly increased the proliferative activity of MSCs on the second and third days (Fig. 1B). These results suggest that loss of TOB1 has no obvious detrimental effect on cell viability.

Knockdown of TOB1 enhances the effect of MSCs on tendon-bone healing in a rat rotator cuff repair model

To investigate if knockdown of TOB1 could enhance the effect of MSCs on tendon-bone healing *in vivo*, Sprague-Dawley rats underwent detachment and repair of the supraspinatus tendon. A group of 18 rats received MSCs in a fibrin glue carrier, and another 18 rats received shRNA-TOB1-transduced MSCs. Three animals per group were euthanized at 4 weeks and 8 weeks for biomechanical testing. As shown in Fig. 2A, at 4 weeks the shRNA-TOB1 group exhibited significantly higher ultimate load to failure compared to the MSC or the control group. The ultimate load of the MSC group was higher than that of the control group. However, there were no differences at 4 weeks among the three groups in terms of stiffness (Fig. 2B). At 8 weeks, the ultimate load to failure was greater in the shRNA-TOB1 group compared to the MSC or control group. The maximum load of the MSC group was higher than that of the control group. The stiffness of the shRNA-TOB1 group was significantly higher than that of the MSC or control group. There was also an increase in stiffness in the MSC group compared to the control group.

We then carried out H&E staining of the control group, MSCs group and shRNA-TOB1 group at 4 and 8 weeks, after surgery. At 4 weeks after surgery, the control group specimens showed a loose fibrovascular interface between tendon and bone consisting mainly of fibroblasts (Fig. 3A). No cartilage or collagen fibers were observed in the zone. In the MSCs group and shRNA-TOB1 group, the interface was narrower, and small numbers of cartilage-like cells were visible at the tendon-bone junction (Fig. 3B and 3C). However, there are no significant differences at 4 weeks between the MSCs group and shRNA-TOB1 group. At 8 weeks after surgery, sparse and unorderly collagen fibers were observed in the control group (Fig. 3D). In the MSCs group, there was more perpendicular collagen fibers resembling Sharpey's fibers compared to the control group. Small numbers of chondrocytes were aslo observed at the interface between tendon and bone in the MSCs group at 8 weeks

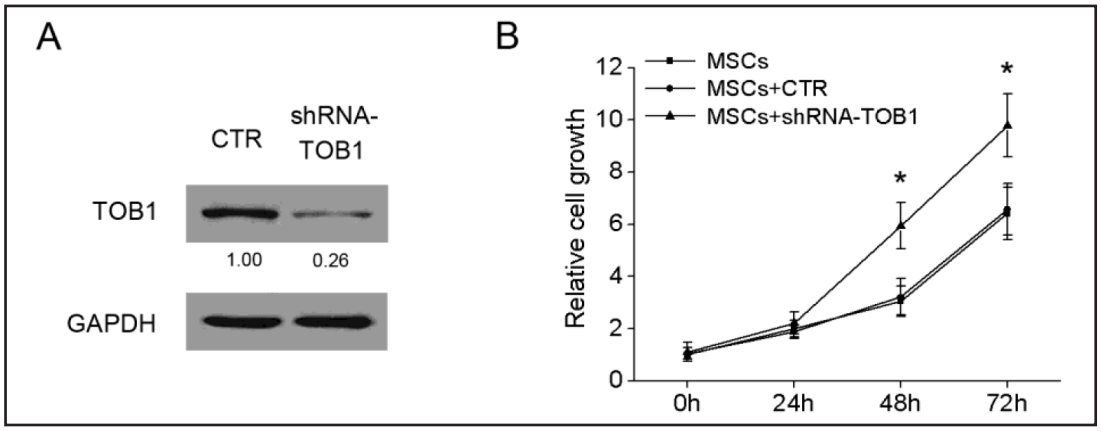

Fig. 1. Knockdown of TOB1 enhances the proliferative activity of MSCs. MSCs were transduced with shRNA-TOB1 lentivirus or control lentivirus. (A) After 72 h, TOB1 expression was examined by western blotting. (B) Cell viability was determined by MTT assay as described in Materials and Methods. Values represent mean ± SD (n = 3). * $p < 0.05$ *vs*. MSC group.

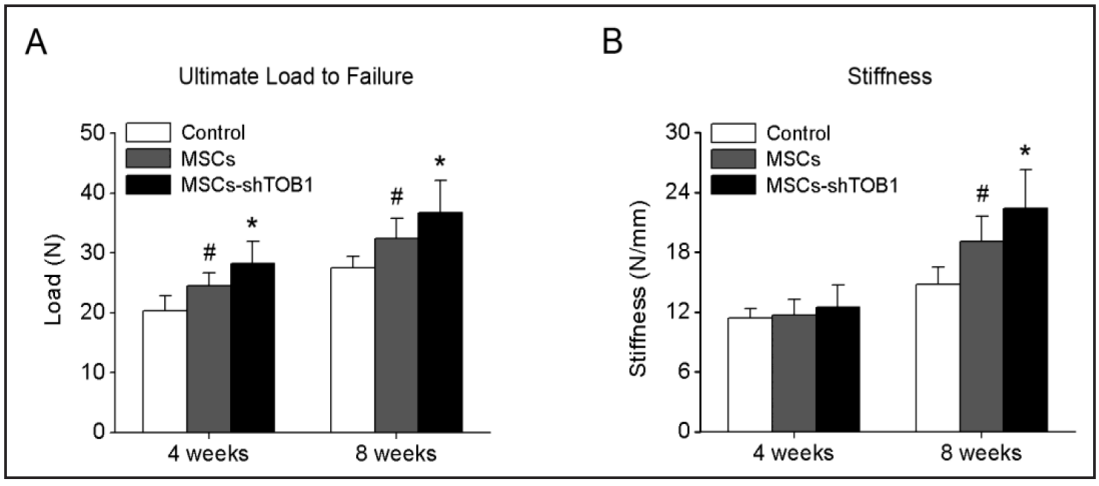

Fig. 2. Biomechanical testing of the control, MSCs and shRNA-TOB1 groups at 4 and 8 weeks after surgery. (A) Ultimate load to failure; (B) Stiffness. Values represent mean ± SD (n = 3). # $p < 0.05$ *vs* control group; * $p < 0.05$ *vs*. MSCs group. The model group without insertion of MSCs was used as the control group.

(Fig. 3E). Furthermore, more chondrocytes and fibrocartilage were observed at the tendon-bone interface in the shRNA-TOB1 group compared to the MSCs group (Fig. 3F). The zone of chondrocytes appeared more organized in the shRNA-TOB1 group. Our results showed that MSCs/shRNA-TOB1 treatment had greater potential to improve tendon-bone healing compared with MSCs treatment.

Collagen type I is a primary component of tendon and bone, and collagen type II is a marker of proliferative chondrocytes. We determined gene expression of both collagen type I and II at the injured tendon-bone junction by real-time PCR. As shown in Fig. 4, at 4 weeks the expression level of collagen type I mRNA was significantly upregulated by treatment with MSCs. Moreover, collagen type I mRNA expression was further enhanced in tendons of the shRNA-TOB1 group compared to tendons in the MSCs group (Fig. 4A). There were no significant differences at 4 weeks among the three groups in the expression of collagen type II (Fig. 4B). However, at 8 weeks, expression of both collagen type I and II mRNA in the MSCs group was significantly stronger compared to the control group, and was further increased in the shRNA-TOB1 group compared with the MSCs group. Taken together, these

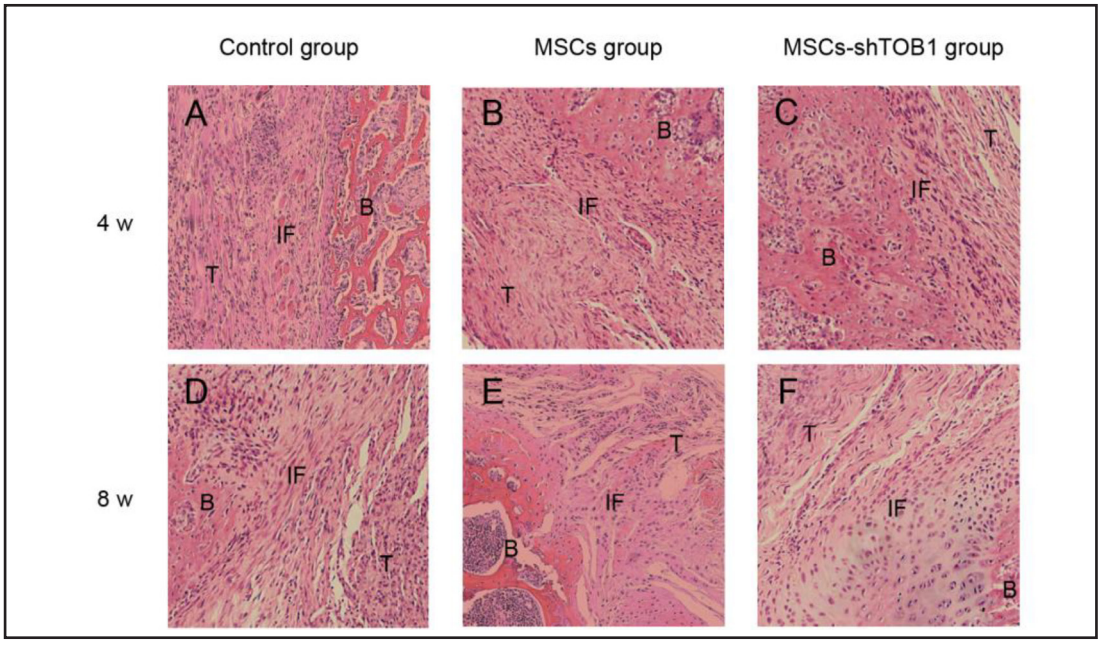

Fig. 3. Histological analysis of the tendon-bone interface. H&E staining of the control group (A, D), MSCs group (B, E) and shRNA-TOB1 group (C, F) at 4 and 8 weeks, respectively, after surgery. Sparse and unorderly collagen fibers were observed 8 weeks after surgery in the control group (D). At the same 8 week time point, a greater number of chondrocytes and more fibrocartilage were observed at the tendon-bone interface in the shRNA-TOB1 group (F) compared to the MSCs group (E) (magnification 100×). T, tendon; B, bone; IF, interface.

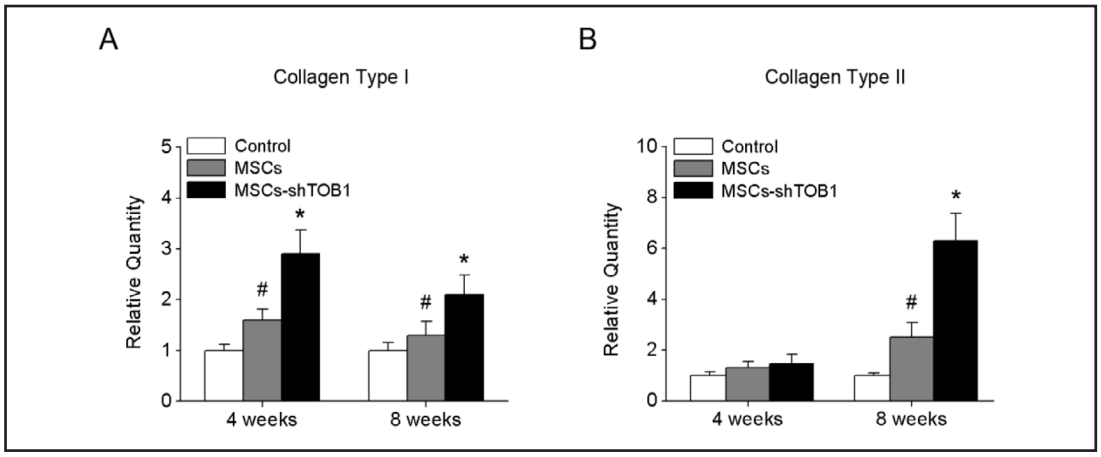

Fig. 4. *In vivo* gene expression of collagen type I and collagen type II. (A) Collagen type I and (B) collagen type II mRNA expression were quantified by real-time PCR. The values of target gene expression were normalized to the housekeeping gene GAPDH. Values represent mean ± SD (n = 3). # $p < 0.05$ *vs.* control group; * $p < 0.05$ *vs.* MSCs group. The model group without insertion of MSCs was used as the control group.

results indicate that MSCs with TOB1 silencing enhanced tendon-bone healing compared to MSCs alone.

miR-218 regulates TOB1 expression in vitro

Given that little is known about upstream regulators of TOB1, we further investigated if miRNA could regulate TOB1 expression and affect the tendon-bone healing. Using two different target prediction programs, TargetScan and miRanda, we derived a putative miR-

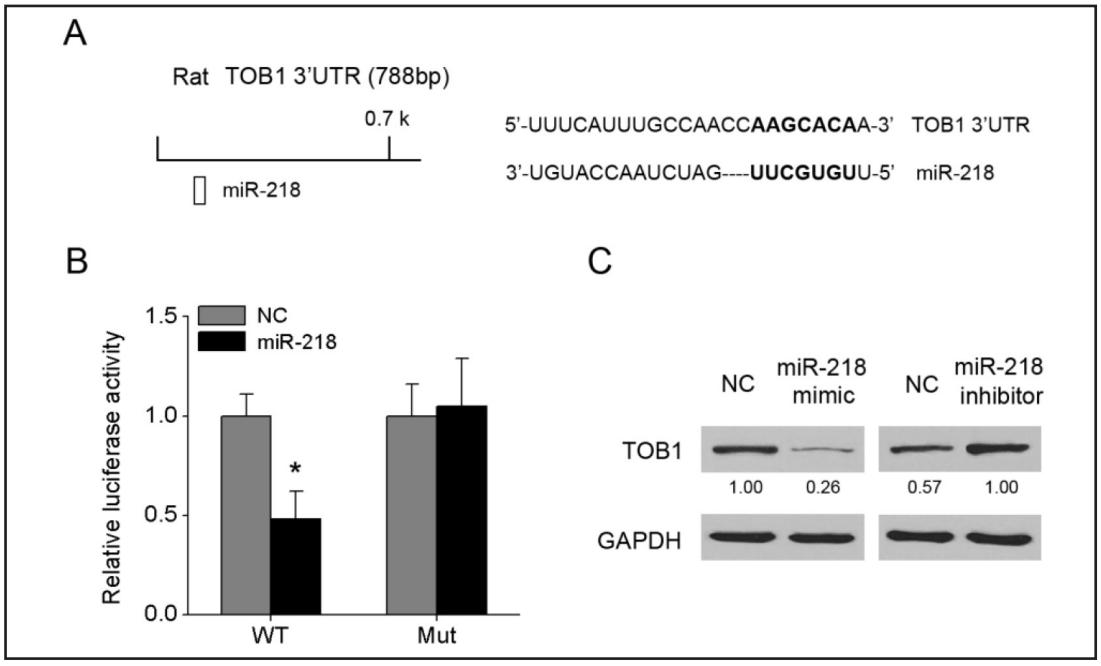

Fig. 5. TOB1 3′-UTR is a direct target of miR-218. (A) The predicted target site of miR-218 within the TOB1 3′-UTR. The putative seed region is highlighted in bold. (B) The ability of miR-218 to target wild-type (WT) and mutant (Mut) TOB1 3′-UTR was compared in dual luciferase reporter assays. (C) MSCs were transfected with miR-218 mimic, miR-218 inhibitor or negative control (NC) oligonucleotides and harvested for western blotting. TOB1 protein expression is shown and GAPDH was used as a loading control. Values represent mean ± SD (n = 3). * $p < 0.05$.

218 target site within the TOB1 3′UTR (170–177 bp). It has been shown that miR-218 is upregulated during osteoblast differentiation and is a potent osteo-miR expressed in MSCs [24]. To determine whether the putative site could indeed be targeted by miR-218, we cloned TOB1 3′UTR sequence downstream of luciferase cDNA to obtain a TOB1 3′UTR reporter construct (Fig. 5A). The corresponding mutant constructs were created by mutating the seed region of the miR-218 binding site. Luciferase reporter assays showed that after transfecting the TOB1 3′UTR reporter construct in the presence of miR-218, relative luciferase activity was significantly reduced (Fig. 5B). Furthermore, TOB1 protein was significantly downregulated by miR-218 mimic and upregulated by miR-218 inhibitor. These results suggested that TOB1 is a direct target of miR-218.

miR-218 promotes tendon-bone healing in rat

To explore whether miR-218 has functional effects on tendon-bone healing, we stably infected MSCs with lentivirus containing miR-218. The highly upregulated expression of miR-218 was confirmed by real-time PCR (Fig. 6A). The TOB1 protein level was significantly downregulated by overexpression of miR-218 (Fig. 6B). Then, before tying the sutures to complete the repair of supraspinatus tendon, the rats were randomly assigned to receive MSCs or MSCs/miR-218 treatment. An untreated group without insertion of MSCs was used as the control group. The animals were sacrificed for biomechanical and histological studies 8 weeks after surgery. As shown in Fig. 6C and 6D, overexpression of miR-218 effectively improved tendon-bone healing, as evidenced by elevated levels of ultimate load to failure and stiffness, a greater number of chondrocytes and increased amount of fibrocartilage at 8 weeks. Taken together, our results indicated that miR-218 promotes tendon-bone healing in rats.

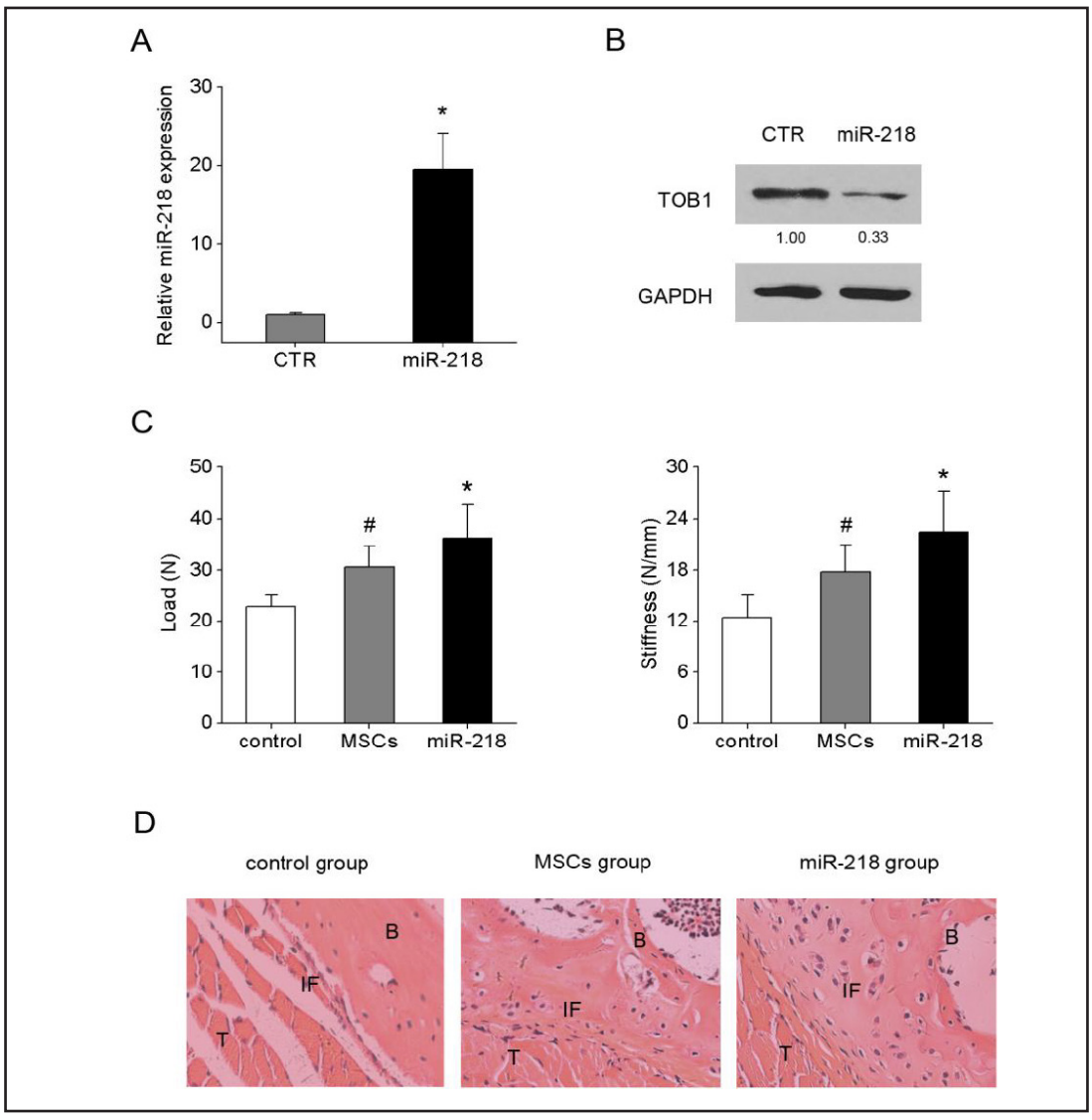

Fig. 6. miR-218 promotes tendon-bone healing in a rat model of rotator cuff repair. (A) MSCs were infected with lentivirus encoding miR-218. After 72 h, relative miR-218 expression was examined by real-time PCR. * $p < 0.05$ *vs*. CTR. (B) TOB1 protein levels were determined by western blotting. (C) Biomechanical testing and (D) H&E staining of the control group, MSCs group and miR-218 group 8 weeks after surgery (magnification 200×). Values represent mean ± SD (n = 3). # $p < 0.05$ *vs.* control group; * $p < 0.05$ *vs*. MSCs group.

Discussion

The scar tissue between transplanted tendon and bone is mechanically weaker than native tissue, and probably accounts for the high failure rate of rotator cuff repair [4]. Therefore, there is an urgent need for new repair strategies to improve rotator cuff tendon-bone healing. Recently, MSC-based biologic therapies have offered a potential way to improve rotator cuff repair. In the present study, we demonstrated that TOB1 deficiency improved the effect of MSCs on tendon-bone healing in a rotator cuff repair rat model, and showed that miR-218 was an upstream regulator of TOB1 and was involved in the process of tendon-bone healing.

MSCs are able to differentiate into multiple cell lineages and provide the potential for regenerating damaged tissues. Several studies have reported that MSC-based gene therapy

enhanced tendon-bone repair at damaged sites. Lawrence et al. [4, 21] showed that MSCs transduced with adenoviral-mediated scleraxis improved regeneration at the tendon-bone insertion site in a rat rotator cuff repair model. Mesenchymal stem cells genetically modified to overexpress the developmental gene membrane type 1-matrix metalloproteinase also augmented rotator cuff healing at 4 weeks as shown by the presence of more fibrocartilage at the insertion site and improved biomechanical strength. Dong et al. [23] found that MSCs genetically modified to overexpress BMP-2 improved tendon-bone healing in anterior cruciate ligament reconstructions. Tian et al. [25] reported that MSCs significantly elevated the levels of ultimate load to failure, stiffness and stress in the Achilles tendons of rats, the effects of which were enhanced by chemokine (C-X-C motif) ligand 13. These strategies may provide some potential methods for improving tendon-bone healing.

BMP/Smad signaling plays a vital role in tendon-bone healing. Yu et al. [16] established an extra-articular patellar tendon-bone healing ovine model and examined the expression of BMPs and their downstream Smad signal transduction factors during tendon-bone healing. They found that BMP-2, BMP-7, Smad1, Smad4, and Smad5 were elevated at the tendon-bone interface during healing. It is well known that BMP also controls osteoblast proliferation and differentiation through Smad proteins. TOB1 is a negative regulator of BMP/Smad signaling and TOB1 deficiency efficiently induced osteogenic activity *in vivo* [14]. We hypothesized that MSCs with TOB1 deficiency might significantly promote tendon-bone healing. Our study found that knockdown of TOB1 enhanced the proliferative activity of MSCs, which is consistent with results in mouse embryonic stem cells [26]. However, a recent study found that TOB1 knockdown inhibited growth of estrogen-independent estrogen receptor positive breast cancer cells [27]. The reported variability in TOB1 function may be due to distinctive regulation of survival and cell cycle signaling in different cell types. In addition, *in vivo* experiments confirmed a significant improvement in tendon-to-bone healing when damaged interfaces were treated with MSCs with TOB1 deficiency compared to treatment with MSCs alone. Knockdown of TOB1 resulted in increased ultimate load to failure at 4 and 8 weeks, as well as greater stiffness at 8 weeks. More fibrocartilage was found in the TOB1 deficiency group compared to the MSCs and control groups at 8 weeks. These findings correlated with enhanced gene expression of collagen type I and II in the TOB1 deficiency group at 8 weeks.

miRNAs are a group of endogenous small noncoding RNAs (21–24 nucleotides) which control key components of osteogenic pathways by targeting the 3′UTRs of mRNAs [28-30]. We hypothesized that miRNAs may also be important regulators of TOB1. Here, we demonstrated that miR-218 could repress TOB1 expression *in vitro* by hybridization with TOB1 3′UTR. It was previously reported that miR-218, which is highly expressed in osteoblasts, promotes stem cell osteogenic differentiation [31, 32]. However, little is known about the role of miR-218 in tendon healing. In the present study, we found that overexpression of miR-218 effectively promoted tendon-bone healing in a rat model, similar to the effect of TOB1 deficiency.

In summary, this study showed that TOB1 deficiency enhanced the effect of MSCs on tendon-bone healing in a rat rotator cuff repair model and demonstrated that expression of TOB1 may be regulated by miR-218. These findings offer a theoretical basis for better application of MSCs to tendon-bone healing.

Acknowledgments

This study was supported by National Natural Foundation of China (No.81371974).

Disclosure Statement

The authors declare no conflict of interests.

References

1 Gulotta LV, Kovacevic D, Packer JD, Ehteshami JR, Rodeo SA: Adenoviral-mediated gene transfer of human bone morphogenetic protein-13 does not improve rotator cuff healing in a rat model. Am J Sports Med 2011;39:180-187.
2 Killian ML, Cavinatto L, Shah SA, Sato EJ, Ward SR, Havlioglu N, Galatz LM, Thomopoulos S: The effects of chronic unloading and gap formation on tendon-to-bone healing in a rat model of massive rotator cuff tears. J Orthop Res 2014;32:439-447.
3 Colvin AC, Egorova N, Harrison AK, Moskowitz A, Flatow EL: National trends in rotator cuff repair. J Bone Joint Surg Am 2012;94:227-233.
4 Gulotta LV, Kovacevic D, Montgomery S, Ehteshami JR, Packer JD, Rodeo SA: Stem cells genetically modified with the developmental gene mt1-mmp improve regeneration of the supraspinatus tendon-to-bone insertion site. Am J Sports Med 2010;38:1429-1437.
5 Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K: The outcome and repair integrity of completely arthroscopically repaired large and massive rotator cuff tears. J Bone Joint Surg Am 2004;86-A:219-224.
6 Phinney DG, Prockop DJ: Concise review: Mesenchymal stem/multipotent stromal cells: The state of transdifferentiation and modes of tissue repair--current views. Stem Cells 2007;25:2896-2902.
7 Sun Z, Tee BC, Kennedy KS, Kennedy PM, Kim DG, Mallery SR, Fields HW: Scaffold-based delivery of autologous mesenchymal stem cells for mandibular distraction osteogenesis: Preliminary studies in a porcine model. PLoS One 2013;8:e74672.
8 Torres-Espin A, Redondo-Castro E, Hernandez J, Navarro X: Bone marrow mesenchymal stromal cells and olfactory ensheathing cells transplantation after spinal cord injury--a morphological and functional comparison in rats. Eur J Neurosci 2014;39:1704-1717.
9 Yokoya S, Mochizuki Y, Natsu K, Omae H, Nagata Y, Ochi M: Rotator cuff regeneration using a bioabsorbable material with bone marrow-derived mesenchymal stem cells in a rabbit model. Am J Sports Med 2012;40:1259-1268.
10 Liu Z, Wang C, Wang X, Xu S: Therapeutic effects of transplantation of as-mir-937-expressing mesenchymal stem cells in murine model of alzheimer's disease. Cell Physiol Biochem 2015;37:321-330.
11 Jia S, Meng A: Tob genes in development and homeostasis. Dev Dyn 2007;236:913-921.
12 Matsuda S, Kawamura-Tsuzuku J, Ohsugi M, Yoshida M, Emi M, Nakamura Y, Onda M, Yoshida Y, Nishiyama A, Yamamoto T: Tob, a novel protein that interacts with p185erbb2, is associated with anti-proliferative activity. Oncogene 1996;12:705-713.
13 Xiong B, Rui Y, Zhang M, Shi K, Jia S, Tian T, Yin K, Huang H, Lin S, Zhao X, Chen Y, Chen YG, Lin SC, Meng A: Tob1 controls dorsal development of zebrafish embryos by antagonizing maternal beta-catenin transcriptional activity. Dev Cell 2006;11:225-238.
14 Usui M, Yoshida Y, Tsuji K, Oikawa K, Miyazono K, Ishikawa I, Yamamoto T, Nifuji A, Noda M: Tob deficiency superenhances osteoblastic activity after ovariectomy to block estrogen deficiency-induced osteoporosis. Proc Natl Acad Sci USA 2004;101:6653-6658.
15 Tzachanis D, Freeman GJ, Hirano N, van Puijenbroek AA, Delfs MW, Berezovskaya A, Nadler LM, Boussiotis VA: Tob is a negative regulator of activation that is expressed in anergic and quiescent t cells. Nat Immunol 2001;2:1174-1182.
16 Yu Y, Bliss JP, Bruce WJ, Walsh WR: Bone morphogenetic proteins and smad expression in ovine tendon-bone healing. Arthroscopy 2007;23:205-210.
17 Kim JG, Kim HJ, Kim SE, Bae JH, Ko YJ, Park JH: Enhancement of tendon-bone healing with the use of bone morphogenetic protein-2 inserted into the suture anchor hole in a rabbit patellar tendon model. Cytotherapy 2014;16:857-867.
18 Chamberlain CS, Lee JS, Leiferman EM, Maassen NX, Baer GS, Vanderby R, Murphy WL: Effects of bmp-12-releasing sutures on achilles tendon healing. Tissue Eng Part A 2015;21:916-927.
19 Yoshida Y, Tanaka S, Umemori H, Minowa O, Usui M, Ikematsu N, Hosoda E, Imamura T, Kuno J, Yamashita T, Miyazono K, Noda M, Noda T, Yamamoto T: Negative regulation of bmp/smad signaling by tob in osteoblasts. Cell 2000;103:1085-1097.

20 Li Y, Fan L, Hu J, Zhang L, Liao L, Liu S, Wu D, Yang P, Shen L, Chen J, Jin Y: Mir-26a rescues bone regeneration deficiency of mesenchymal stem cells derived from osteoporotic mice. Mol Ther 2015;23:1349-1357.
21 Gulotta LV, Kovacevic D, Packer JD, Deng XH, Rodeo SA: Bone marrow-derived mesenchymal stem cells transduced with scleraxis improve rotator cuff healing in a rat model. Am J Sports Med 2011;39:1282-1289.
22 Zhao S, Peng L, Xie G, Li D, Zhao J, Ning C: Effect of the interposition of calcium phosphate materials on tendon-bone healing during repair of chronic rotator cuff tear. Am J Sports Med 2014;42:1920-1929.
23 Dong Y, Zhang Q, Li Y, Jiang J, Chen S: Enhancement of tendon-bone healing for anterior cruciate ligament (acl) reconstruction using bone marrow-derived mesenchymal stem cells infected with bmp-2. Int J Mol Sci 2012;13:13605-13620.
24 Hassan MQ, Maeda Y, Taipaleenmaki H, Zhang W, Jafferji M, Gordon JA, Li Z, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB: Mir-218 directs a wnt signaling circuit to promote differentiation of osteoblasts and osteomimicry of metastatic cancer cells. J Biol Chem 2012;287:42084-42092.
25 Tian F, Ji XL, Xiao WA, Wang B, Wang F: Cxcl13 promotes the effect of bone marrow mesenchymal stem cells (mscs) on tendon-bone healing in rats and in c3hiot1/2 cells. Int J Mol Sci 2015;16:3178-3187.
26 Chen Y, Wang C, Wu J, Li L: Btg/tob family members tob1 and tob2 inhibit proliferation of mouse embryonic stem cells via id3 mrna degradation. Biochem Biophys Res Commun 2015;462:208-214.
27 Zhang YW, Nasto RE, Varghese R, Jablonski SA, Serebriiskii IG, Surana R, Calvert VS, Bebu I, Murray J, Jin L, Johnson M, Riggins R, Ressom H, Petricoin E, Clarke R, Golemis EA, Weiner LM: Acquisition of estrogen independence induces tob1-related mechanisms supporting breast cancer cell proliferation. Oncogene DOI:10.1038/onc.2015.226.
28 Bartel DP: Micrornas: Target recognition and regulatory functions. Cell 2009;136:215-233.
29 Li Z, Hassan MQ, Volinia S, van Wijnen AJ, Stein JL, Croce CM, Lian JB, Stein GS: A microrna signature for a bmp2-induced osteoblast lineage commitment program. Proc Natl Acad Sci USA 2008;105:13906-13911.
30 Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB: Biological functions of mir-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 2009;284:15676-15684.
31 Xu JF, Yang GH, Pan XH, Zhang SJ, Zhao C, Qiu BS, Gu HF, Hong JF, Cao L, Chen Y, Xia B, Bi Q, Wang YP: Altered microrna expression profile in exosomes during osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. PLoS One 2014;9:e114627.
32 Zhang WB, Zhong WJ, Wang L: A signal-amplification circuit between mir-218 and wnt/beta-catenin signal promotes human adipose tissue-derived stem cells osteogenic differentiation. Bone 2014;58:59-66.