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Leucine rich repeat containing 32 accelerates tenogenic differentiation of tendon-derived stem cells and promotes Achilles tendon repair in rats

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Abstract: Although many surgical or non-operative therapies have been developed to treat Achilles tendon injuries, the prognosis of which is often unsatisfactory. Recently, biologic approaches using multipotent stem cells like tendon-derived stem cells (TDSCs) pose a possible treatment option. To evaluate whether the Leucine rich repeat containing 32 (*Lrrc32*) affects the tenogenic differentiation of TDSCs and thus promotes Achilles tendon healing. TDSCs were infected with the recombinant *Lrrc32*-overexpressing lentivirus (LV-*Lrrc32*) and then locally injected into the injured site of rat. Four weeks after surgery, the Achilles tendon tissue (~0.5 cm) around the injured area was harvested for analysis. Pathological results showed that *Lrrc32*-overexpressing TDSCs significantly improved the morphological changes of the injured tendons. Specifically, the increased collagen-I expression and hydroxyproline content in extracellular matrix, and more orderly arrangement of the regenerated collagen fibers were observed in the *Lrrc32* overexpression group. Moreover, 4 weeks after injection of *Lrrc32*-overexpressing TDSCs, the expression of tenocyte-related genes such as tenomodulin (*Tnmd*), scleraxis (*Scx*) and decorin (*Dcn*) were upregulated in the area of the healing tendon. These findings indicated that *Lrrc32* promoted the tenogenic differentiation of TDSCs *in vivo*. Additionally, *Lrrc32* overexpression also increased the expression of TGF-β1 and p-SMAD2/3, suggesting that the beneficial effects of *Lrrc32* on tendon repair might be associated with the expression of TGF-β1 and p-SMAD2/3. Our findings collectively revealed that *Lrrc32*-overexpressed TDSCs promoted tendon healing more effectively than TDSCs alone.

Key words: Achilles tendon healing, Leucine rich repeat containing 32 (*Lrrc32*), tendon-derived stem cells (TDSCs), tenogenic differentiation

Introduction

Achilles tendon injuries are most common injuries that are mainly related to sport activities [1]. As a general rule, most of the vessels within tendons are venules and thus tendons have the less blood supply than its adjacent tissue, such as tendon sheaths and metabolically active muscles [2, 3]. Blood supply is an important issue that relates to the normal function of tendon cells and the intrinsic healing capacity of tendons [2]. This is why the injured tendons heal slowly, and never fully recover their native biomechanical biochemical and ultrastructural properties. Currently, non-surgical interventions (bracing, splinting or plaster fixation) or surgical repair remains the main options for Achilles tendon injuries [4]. However, traditional surgical treatment was associated with a high risk of complications such as infection, muscle wasting and adhesions.

Mesenchymal stem cell (MSC)-based therapies to enhance tendon restoration have been proposed and used since 1993 [5]. Previous studies have shown positive effects of the traditional bone marrow-derived stem cells

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(BMSCs), embryonic stem cells-derived MSCs (ESC-MSCs) or adipose-derived stem cells (ASCs) on tendon repair [5–7]. In recent years, there was considerable evidence to suggest that tendon-derived stem cells (TDSCs) significantly enhanced tendon healing in the rodent tendon defect models [8, 9]. These resident stem cells in tendons possess universal stem cell characteristics such as clonogenicity, proliferative capacity and differentiation potential. It has been reported that the isolated TDSCs could regenerate tendon-like tissue after implantation *in vivo* [9]. A previous study also suggested the transplanted TDSCs might contribute to tendon healing by producing paracrine factors that promoted collagen production [8]. Achilles tendons are composed of a dense fibrous connective tissue which includes mostly of type I collagen (hierarchical architecture containing fibrils, fibers and fiber bundles) and small-molecule matrix (proteoglycans) [2, 10]. Achilles tendon injury is believed to cause biological changes in tenocytes and extracellular matrix (ECM), often accompanied by the tenocyte apoptosis and a decrease in collagen content [10, 11]. Qi Tan *et al.* has reported that TDSCs express higher tenogenic markers such as tenomodulin (*Tnmd*), scleraxis (*Scx*), collagen 1α1 and decorin (*Dcn*) than BMSCs [12]. Thus, it might be a better cell source than BMSCs for tendon repair and regeneration.

Leucine rich repeat containing 32 (*Lrrc32*), also known as glycoprotein A repetition predominant (GARP), is a type I transmembrane cell surface docking receptor. Researchers have recently found that down-regulation of *Lrrc32* results in the attenuation of proliferation and differentiation of the BMSCs [13, 14]. However, the potential function of *Lrrc32* in TDSCs remains largely unknown at present. TGF-β1 is a pleiotropic cytokine and its important role in regulation of wound healing and tendon injury has been widely studied [15–17]. Treatment of TGF-β1 directly induced the tenogenic differentiation of TDSCs, and thus promoted tendon healing [15]. It is of interest to note that *Lrrc32* has emerged as a crucial regulator of latent TGF-β1 activation [18, 19]. Therefore, we hypothesized that *Lrrc32* might play a critical role in inducing TDSCs to undergo the tenogenic differentiation.

In the present study, the *Lrrc32*-overexpressing TDSCs were generated with a lentivirus-mediated gene delivery system, and the tenocytic differentiation properties of which were evaluated in Achilles tendon injury model of rats. Moreover, we also investigated the mechanism of action of *Lrrc32* on tendon healing.

Material and Methods

Animal and ethics statement

One hundred and two male Sprague-Dawley (SD, 6–8-week-old) rats were used as recipients or donors in this study. Six SD rats, which did not undergo an operation, were the source of TDSCs. The remaining 96 rats were divided into 4 groups (24 rats per group): non-treated control group, Achilles tendon injury and PBS-applied negative control group (NC group), Achilles tendon injury and TDSCs+LV-NC-applied group (TDSCs+LV-NC group), and Achilles tendon injury and TDSCs+LV-*Lrrc32*-applied group (TDSCs+LV-*Lrrc32* group). All rats were lodged in a climate-controlled environment (12 h light/12 h dark photoperiod, temperature, 22 ± 1°C; relative humidity, 45–55%) with free access to water and food. All studies were reviewed and approved by the Ethics Committee of the Third Hospital of Hebei Medical University (number: Z 2020-009-1).

Isolation and culture of rat TDSCs

The isolation and culture of rat TDSCs were performed as described previously [20]. Briefly, Achilles tendon tissue from 6–8-week-old SD male rats was collected and peritendinous connective tissue was removed carefully. The tissue was cut into 1~2 mm^3 and then washed three times with PBS. The tissue fragments were cultured in Dulbecco's Modified Eagle Medium (Servicebio, Wuhan, China) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells adhered to the culture surface were digested with trypsin (Sigma-Aldrich, St. Louis, MO, USA) and seeded at a density of 2 cells/cm^2 to form colonies. After 10~12 days, the colonies were trypsinized and passaged. Cells from passages 3~5 were designated as TDSCs and used in the following experiments.

Characterization of TDSCs *in vitro*

Herein, the isolation and culture of TDSCs were performed as described by Chen *et al.* [20]. Accordingly, the cell surface markers including CD29, CD90, CD45 and CD44 were used to confirm the phenotype of TDSCs. Briefly, TDSCs in suspension were incubated with the specific antibodies towards CD29 (BioLegend, San Diego, CA, USA), CD90 (BioLegend), CD45 (Multisciences (LIANKE) Biotech, Co., Ltd., Hangzhou, China) or CD44 (proteintech, Chicago, IL, USA) for 30 min at 4°C. Then, the cells were incubated with Fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG (proteintech) for 30 to 45 min under dark. After centrifugation, the cells were re-suspended in 500 μl staining buffer. The samples were analyzed using a No-

voCyte flow cytometer (ACEA Biosciences Inc. San Diego, CA, USA). Further, in order to analyze the multilineage differentiation potential of TDSCs, cells were seeded in a 6-well plate and cultured in complete culture medium until the cells reached confluence. Then, they were incubated in osteogenic medium, adipogenic medium or chondrogenic medium for 14 days to induce differentiation. The calcium nodule formation in TDSCs was detected using Alizarin red staining (Yuanyebio., Shanghai, China) assay. Positive induction of adipogenesis was confirmed by Oil Red O staining (Leagene, Beijing, China). Safranin O staining (Solarbio, Beijing, China) was performed to confirm the induction of chondrogenesis. The sections were observed under a microscope (OLYMPUS, Tokyo, Japan).

Infection of TDSCs

The recombinant *Lrrc32* overexpression lentivirus (LV-*Lrrc32*) and the negative control lentivirus (LV-NC) were prepared and titered to 10^8 TU/ml. The lentiviral vector (Fenghui Biotechnology Co., Ltd., Hunan, China) carried an enhanced green fluorescent protein (eGFP) sequence. Before infection, TDSCs were seeded at a density of 10^5 cells per well in a 6-wells culture plate and incubated under 5% CO_2 at 37°C, and then 40 μl/well LV-*Lrrc32* or LV-NC particles were added in culture medium. The plate was gently swirled to mix and incubated in 5% CO_2 at 37°C for 72 h. Subsequently, TDSCs with LV-*Lrrc32* or empty LV vector were harvested for the next experiments.

Rat Achilles tendon injury model and study design

To establish an intra-tendinous incompletely Achilles tendon injury model, surgery was performed as previously described with minor modifications [21]. Briefly, a 1.5~2 cm longitudinal skin incision was made with a sharp blade to expose Achilles tendon (Fig. 1a). Then, surgical blades were used to make an intra-tendinous incision (~0.5 mm in width) in the tendon, approximately 0.5 cm from the calcaneus (triangle). The skin was sutured after damage. At 24 h and day 14 post-surgery, TDSCs with LV-*Lrrc32* or empty LV vector were locally injected into the injury site of rats in the TDSCs+LV-*Lrrc32* group or TDSCs+LV-NC group, respectively. At the same time, a PBS injection was performed on the injury site of rats in the NC group. Total 1×10^6 TDSCs were injected at each time point. The effective concentration of TDSCs was determined based on the previous studies [15, 21]. All rats were sacrificed at week 4 post-surgery, and the Achilles tendon tissue (~0.5 cm) around the injured area was harvested for subsequent examinations.

Hematoxylin and eosin (H&E) and Masson's trichrome staining

The treated rats were sacrificed and the Achilles tendon tissue (~0.5 cm) around the injured area was harvested. For histological evaluations, the isolated Achilles tendon tissue was dehydrated in an ethanol series, embedded in paraffin and cut into sections with a thickness of 5 μm. The sections were stained with H&E to examine the general morphology of the healing tendon. Also, Masson's trichrome staining was performed according to the instructions to analyze the distribution and content of collagen. The sections were observed under a microscope (OLYMPUS).

Immunohistochemistry (IHC) staining for collagen-I

The treated rats were sacrificed and the Achilles tendon tissue (~0.5 cm) around the injured area was harvested. For IHC analysis, the paraffin-embedded sections (5 μm) were deparaffinized by xylene, treated with a graded series of ethanol. Antigen retrieval for collagen-I was performed through incubation with Proteinase K (Servicebio) antigen retrieval solution for 15 min at room temperature. After washing and blocking, the sections were incubated with anti-collagen-I (AF7001, Affinity Biosciences, Jiangsu, China) overnight at a dilution of 1:100. HRP-conjugated Goat Anti-Rabbit IgG secondary antibody (31460, 1:500, ThermoFisher, Waltham, MA, USA) were added for an hour, followed by Diaminobenzidine (DAB, Fuzhou New Step Biotechnology Development Co., Ltd., Fujian, China). Afterwards, the sections were counterstained in hematoxylin and finally examined by microscope (OLYMPUS).

ELISA

Tendon tissue was collected by removing the Achilles tendon (~0.5 cm) around the injured area. The content of hydroxyproline was quantified by Hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the vendor's instructions. Briefly, 1 ml of hydrolysate was added to 100 mg of the tendon tissue. The tissue was hydrolyzed under a boiling water bath for 20 min and then cooled using flowing waters. Following adjustment of the pH value around 6.0~6.8, the designated reaction reagents were added and treated for 15 min under a 60°C water bath. After centrifugation, the supernatant was harvested for determination of hydroxyproline. The absorbance was read at 550 nm by using a UV-752N ultraviolet spectrophotometer (Shanghai Youke Instrument Co., Ltd., Shanghai, China).

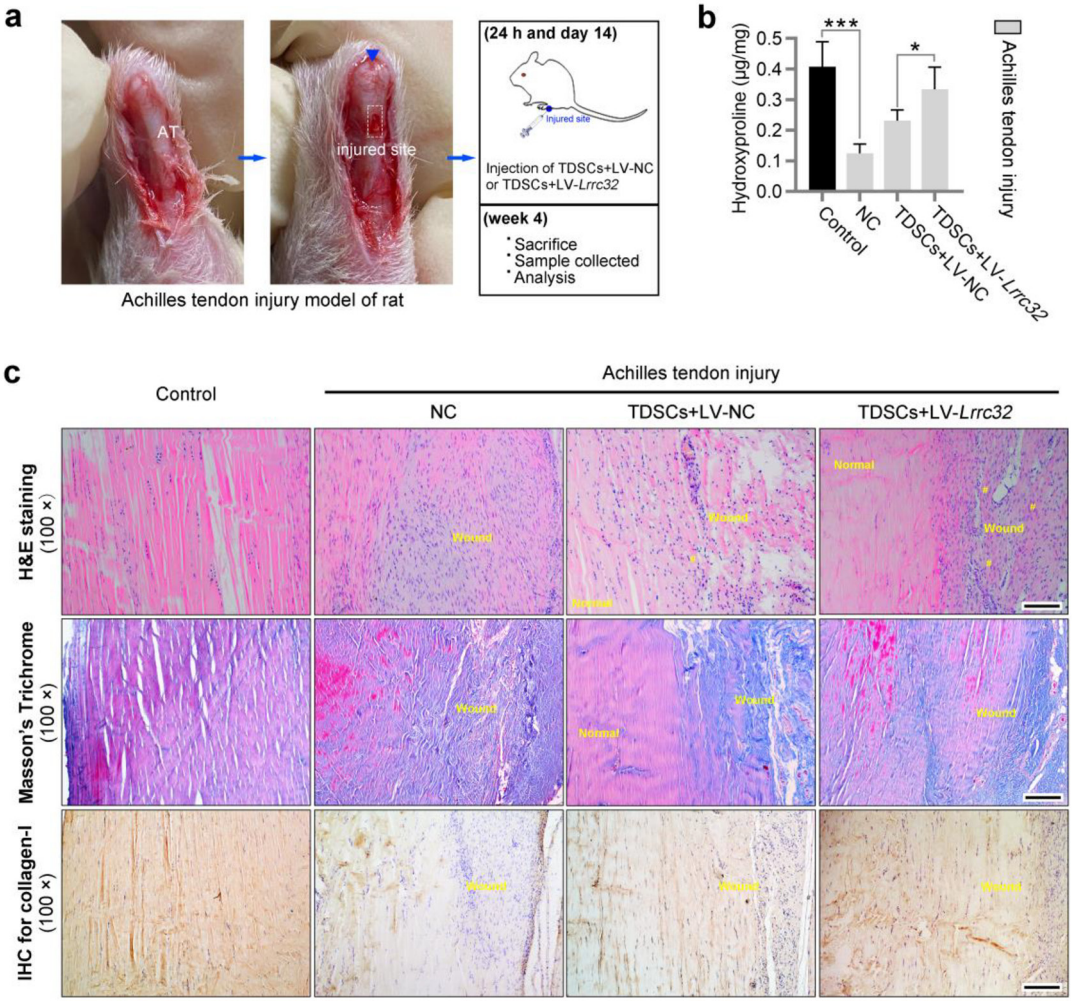

Fig. 1. *Lrrc32* overexpression promoted tendon repair *in vivo*. (a) Achilles tendon injury model of rat. Triangle: calcaneus. (b) Quantification of hydroxyproline by ELISA (n=6 per group). (c) Histological evaluation of the area of the healing tendon (6 rats per group were used for histology analysis): hematoxylin and eosin (H&E) staining (the upper row); Masson's trichrome staining (the middle row); immunohistochemical staining for collagen-I (the lower row). #: healing tendon cells. Magnification 100×, scale bar=200 μm. TDSCs: tendon-derived stem cells; *Lrrc32*: Leucine rich repeat containing 32; Control: Normal rat without Achilles tendon injury; NC: Achilles tendon injury and PBS-applied group; TDSCs+LV-NC: Achilles tendon injury and TDSCs+LV-NC-applied group; TDSCs+LV-*Lrrc32*: Achilles tendon injury and TDSCs+LV-*Lrrc32*-applied group. **P*<0.05, ****P*<0.001.

Total RNA isolation and qRT-PCR

Total RNA (control group: 301.5 ng/μl; NC group: 198.6 ng/μl; TDSCs+LV-NC group: 225.4 ng/μl and TDSCs+LV-*Lrrc32* group: 174.3 ng/μl) was extracted by TRIpure (BioTeke, Beijing, China), according to the manufacturer's instructions, and cDNA was synthesized from ~1 μg of total RNA using BeyoRT II M-MLV reverse transcriptase (Beyotime Institute of Biotechnology, Shanghai, China). qRT-PCR was performed using the ExicyclerTM 96 PCR system (BIONEER, Daejeon, Korea) in a 20 μl reaction mixture containing 0.3 μl of SYBR Green (Solarbio), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer (10 μM), 10 μl of 2×Taq PCR MasterMix (Solarbio), and 1 μg of cDNA. The PCR conditions were 94°C for 5.25 min, then 60°C for 25 s, and termination at 72°C for 30 s. The condition of 40 cycles was as follows: 72°C for 5.5 min, then 40°C for 2.5 min, and 60°C to 94°C with a heating rate of 1°C/s. The following primers were used: *Lrrc32*(forward, 5′-AGGACGGTGAACAAGGAG-3′; reverse, 5′-GGTGGCGAAGTGCTGTAT-3′), *Tnmd*(forward, 5′-GCCGCACCAGACAAGCAA-3′; reverse, 5′- AAGGCATGATGACACGACAGAT-3′), *Scx*(forward, 5′-CTGGGCAACGTGCTACTGG-3′; reverse, 5′-TTGGGCTGGGTGTTCTCG-3′), *Dcn*(forward, 5′-TGGCAGTCTGGCTAATGT-3′; reverse, 5′-ACTCACGGCAGTGTAGGA-3′), β-ACTIN (forward, 5′-GGAGATTACTGCCCTGGCTCCTAGC-3′; reverse,

5′-GGCCGGACTCATCGTACTCCTGCTT-3′). The relative mRNA level was calculated by using the $2^{-\Delta\Delta CT}$ Method and the β-ACTIN expression was used for normalization.

Immunofluorescent (IF) staining

Tendon tissue was collected by removing the Achilles tendon (~0.5 cm) around the injured area. The sections of Achilles tendon tissue (5 μm) were incubated with the primary antibody *Tnmd* (Bs-7525R, 1:100, Beijing Biosynthesis Biotechnology, Beijing, China) overnight at 4°C, followed by incubation with the secondary antibody Cy3-labeled Goat anti-Rabbit IgG (A27039, 1:200, Invitrogen, Carlsbad, CA, USA) for 60 min at 37°C. Nuclei were stained with DAPI (Aladdin, Shanghai, China) and visualized under a microscope (OLYMPUS).

Western blot analysis

The treated rats were sacrificed and the Achilles tendon tissue (~0.5 cm) around the injured area was harvested. The samples were homogenized with cell lysis buffer (Beyotime Institute of Biotechnology) containing 1mM PMSF and then centrifuged at 4°C, 10,000 g for 5 min. A BCA protein assay kit (Beyotime Institute of Biotechnology) was used to detect the protein concentration of supernatant. Equal amount of proteins (40 μg) from each sample were subjected to SDS-polyacrylamide gel, and then transferred to PVDF membranes. After blocking with 5% (M/V) skimmed milk for 1 h, the membranes were probed with the primary antibodies to *Lrrc32* (A16858, 1:1,000, ABclonal Technology Co., Ltd., Wuhan, China), TGF-β1 (WL02998, 1:500, wanleibio, Shenyang, China), SMAD2/3 (A18674, 1:1,000, ABclonal Technology Co., Ltd.) or p-SMAD2/3 (AP0548, 1:1,000, ABclonal Technology Co., Ltd.). Afterwards, the membranes were incubated with HRP-labeled Goat Anti-Rabbit IgG (A0208, 1:5,000) or Goat Anti-Mouse IgG (A0216, 1:5,000) secondary antibodies (all from Beyotime Institute of Biotechnology) at 37°C for 45 min. The proteins were visualized using ECL reagent (Beyotime Institute of Biotechnology), and band density was analyzed with Gel-Pro-Analyzer software (WD-9413B, Liuyi Biology, Beijing, China) by using β-ACTIN (sc-47778, 1:1,000, Santa Cruz, TX, USA) as reference.

Statistical analysis

All quantitative data were expressed as mean ± SD. Comparison of the 2 groups (Control group vs. NC group) was performed with the Two-tailed unpaired Student's *t* tests. Unless otherwise stated, One-way ANOVA with Tukey's multiple comparisons test was performed to assess statistical significance of results among groups. All the statistical analyses were performed by GraphPad prism 8.0. A *P*-value less than 0.05 were accepted as statistically significant.

Results

Characterization of TDSCs and lentivirus mediated overexpression of *Lrrc32*

To characterize the parental TDSCs, we first assessed the surface CD markers expression of TDSCs. As shown in Fig. 2a, the TDSCs derived from Achilles tendon tissue showed similar expression profile of CD markers as MSCs [20]. Namely, the positive ratio of TDSCs for the CD29 was 98.27%, the CD44 84.84%, the CD90 90.25%, while the hematopoietic marker CD45 (0.13%) was negative. Furthermore, as shown in Fig. 2b, a spindle shape and typical fibroblast-like appearance of TDSCs could be seen under the light microscopy. The multilineage differentiation capacity is one of the universal characteristics of stem cells. After induction for 14 days, the Alizarin red staining showed the matrix mineralization in TDSCs was significant increase, suggesting that the cells could differentiate into osteo-lineage. Moreover, the cells also had the capacity to undergo adipogenic differentiation as demonstrated by using Oil Red O staining. And the appearance of ovoid cells as shown in Safranin O staining confirmed the chondrogenic differentiation of the cells. To further investigate the bioactivities of *Lrrc32*, lentivirus vector encoding rat *Lrrc32* gene was constructed and infected into TDSCs. Seventy-two hours after infection, the *Lrrc32*-overexpressing TDSCs was verified by qRT-PCR and western blot analysis. Clearly, TDSCs with LV-*Lrrc32* expressed higher mRNA (Fig. 2c) and protein levels (Fig. 2d) of *Lrrc32*, in comparison with the cells infected with empty LV-NC vector.

Lrrc32 overexpression promoted Achilles tendon repair

Next, a rat Achilles tendon injury model was used to evaluate the effects of *Lrrc32* on Achilles tendon repair. According to the study protocol (Fig. 1a), the *Lrrc32* stable TDSCs (TDSCs+LV-*Lrrc32*) and TDSCs with LV-NC vector (TDSCs+LV-NC) were locally injected into the injured site. 4 weeks after surgery, the hydroxyproline content was detected to evaluate the total collagen level. Compared to the control group, a lower level of hydroxyproline was observed in the Achilles tendon injured group (NC group). However, *Lrrc32* overexpression in TDSCs resulted in a marked increase in the content of hydroxyproline (Fig. 1b). Next, we further evaluated the morphological changes in the injured

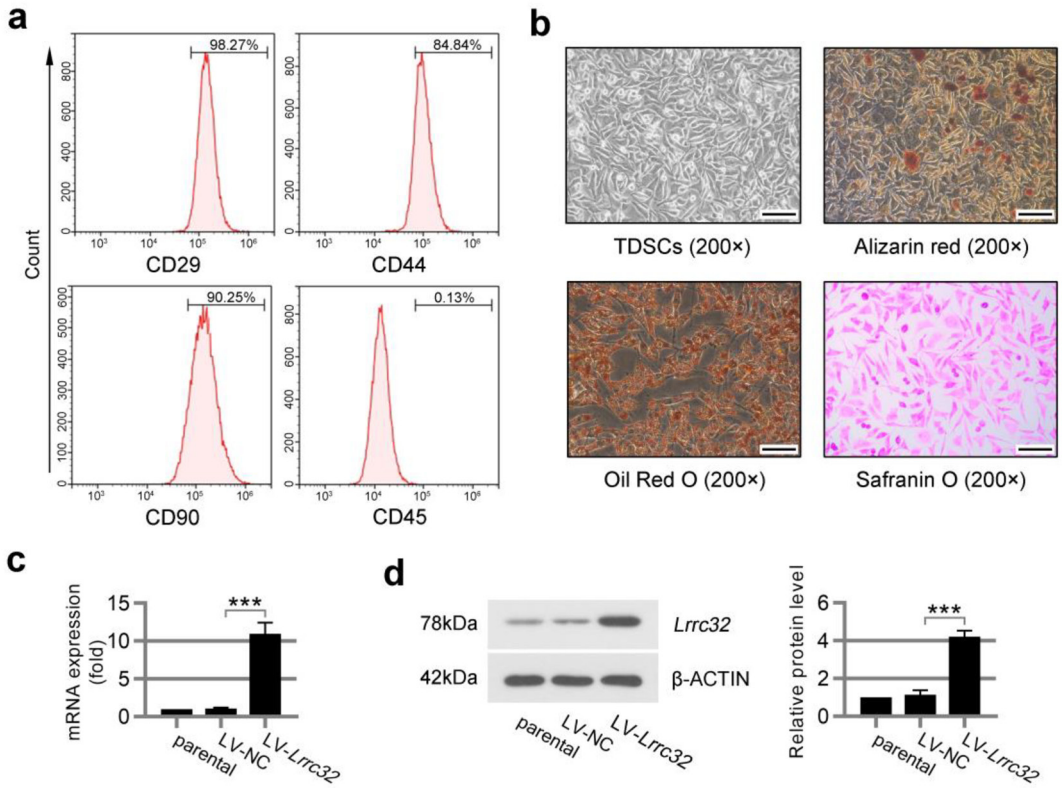

Fig. 2. Characterization of parental TDSCs and lentivirus mediated *Lrrc32* overexpression in TDSCs. (a) MSCs surface maker (CD29, CD44, CD90 and CD45) expression of TDSCs was detected by flow cytometer. (b) Light microscopy observation of the morphology of normal TDSCs (parental), and the differentiation assays were performed to analyze the osteogenic (Alizarin red staining), adipogenic (Oil Red O staining) and chondrogenic (Safranin O staining) differentiation of TDSCs. Magnification 200×, scale bar=100 μm. *Lrrc32* mRNA and protein levels were analyzed by (c) qRT-PCR and (d) western blotting (n=6). TDSCs: tendon-derived stem cells; *Lrrc32*: Leucine rich repeat containing 32. ***P<0.001.

Achilles tendon. H&E staining (the upper row in Fig. 1c) showed that *Lrrc32*-overexpressed TDSCs treated group had more matrix formation in the wound region compared to the NC group. Masson's trichrome staining (the middle row in Fig. 1c) also showed the disorganized collagen orientation in the NC group. Due to TDSCs treatment, both TDSCs+LV-NC and TDSCs+LV-*Lrrc32* group were showed nascent collagen formation in the wound area. However, TDSCs+LV-*Lrrc32* treated group displayed relatively more ordered collagen fibers compared with the TDSCs+LV-NC group. At the same time, IHC staining (the lower row in Fig. 1c) showed that the wound region in TDSCs+LV-*Lrrc32* group obviously expressed more collagen-I than that in the NC group. Taken together, *Lrrc32* overexpression in TDSCs enhanced the formation of tendon-like tissue.

Lrrc32 overexpression promoted the tenogenic differentiation of TDSCs

Furthermore, qRT-PCR analysis revealed that the expression of the tenocyte lineage markers *Tnmd* and *Scx*, as well as the pericellular matrix proteoglycan *Dcn* was downregulated after tendon injury, compared with the control group. However, overexpression of *Lrrc32* significantly increased the mRNA levels of *Tnmd* (Fig. 3a), *Scx* (Fig. 3b) and *Dcn* (Fig. 3c). In addition, immunofluorescence staining was also performed for evaluation the expression of *Tnmd* on the area of the healing tendon. We found that the number of *Tnmd*-positive cells was increased in the *Lrrc32* overexpression group (Fig. 3d), which was consistent with the change of the mRNA levels of *Tnmd* (Fig. 3a). These findings collectively indicated that *Lrrc32* enhanced the tenogenic differentiation of TDSCs.

Lrrc32 overexpression increased the expression of TGF-β1 and p-SMAD2/3

TGF-β1 played a pivotal role in tenogenic differentiation of TDSCs and wound healing [8, 22], via Smad activation. Therefore, we further evaluated the effects of *Lrrc32* on the expressions of TGF-β1 and SMAD2/3 in the area of the healing tendon, at week 4 post-surgery. As shown in Figs. 4a and b, overexpression of *Lrrc32* significantly upregulated the protein levels of TGF-β1

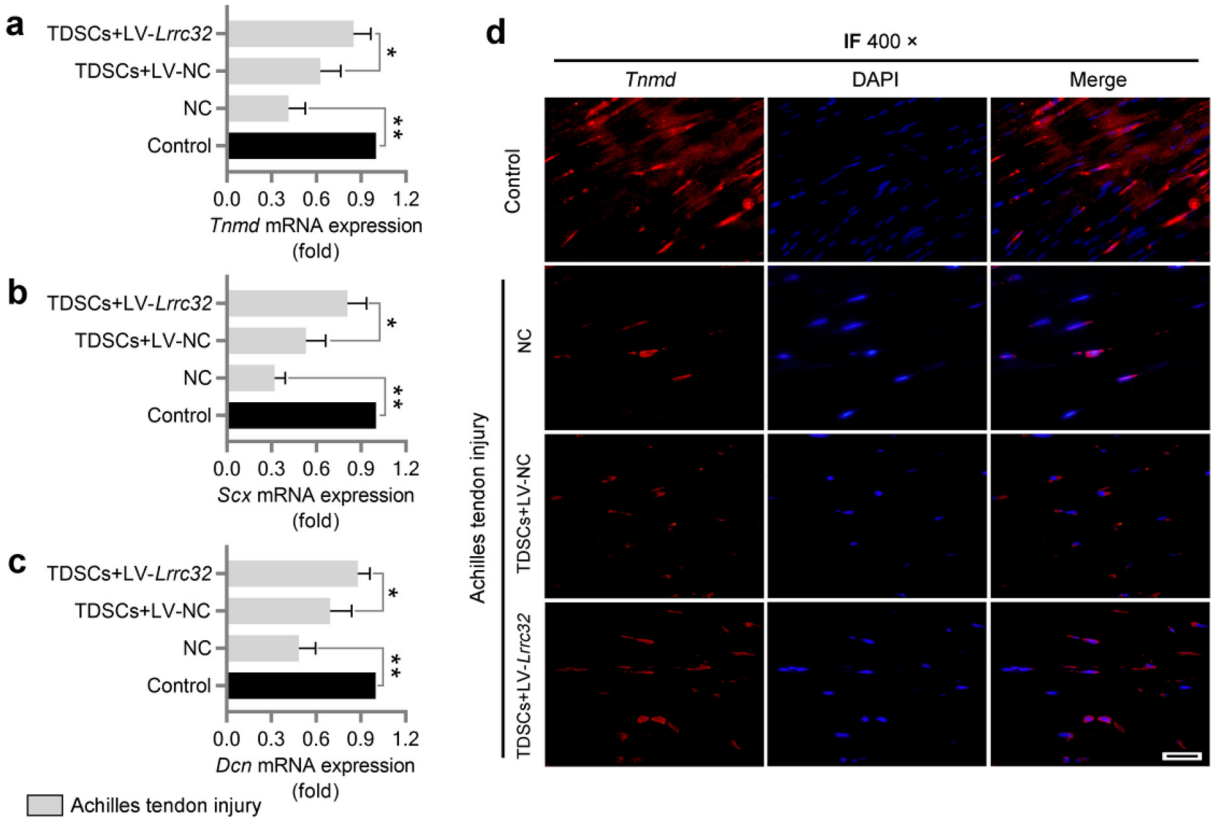

Fig. 3. *Lrrc32* overexpression promoted TDSC tenogenic differentiation. qRT-PCR analysis of transcript levels of (a)*Tnmd*, (b)*Scx* and (c)*Dcn* in Achilles tendon tissue (n=6 per group). (d) Immunofluorescent staining for *Tnmd*. Magnification 400×, scale bar=50 μm. TDSCs: tendon-derived stem cells; *Lrrc32*: Leucine rich repeat containing 32; Control: Normal rat without Achilles tendon injury; NC: Achilles tendon injury and PBS-applied group; TDSCs+LV-NC: Achilles tendon injury and TDSCs+LV-NC-applied group; TDSCs+LV-*Lrrc32*: Achilles tendon injury and TDSCs+LV-*Lrrc32*-applied group. **P*<0.05, ***P*<0.01.

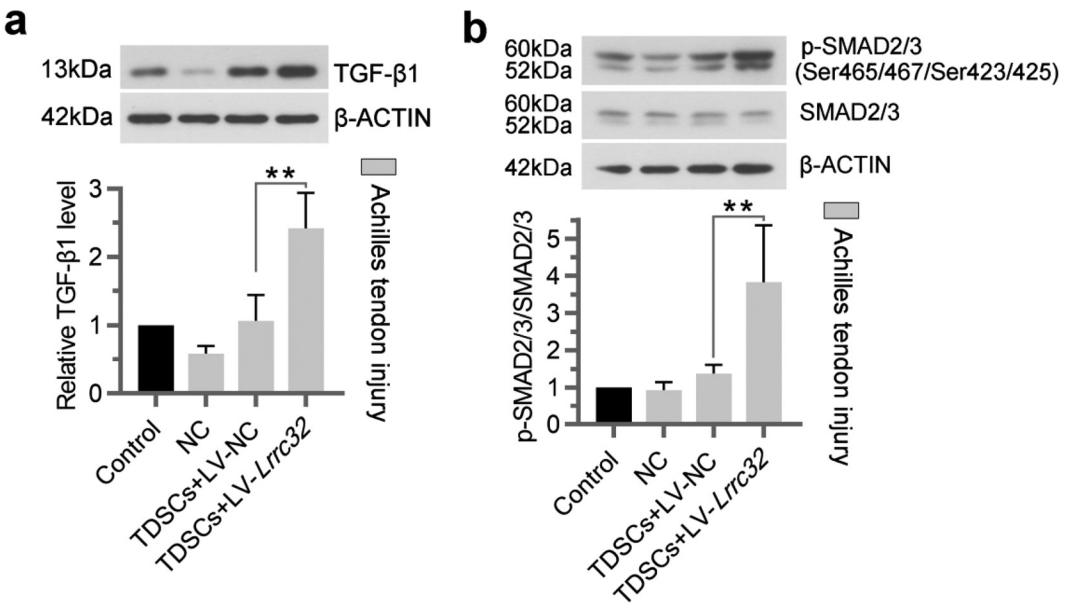

Fig. 4. *Lrrc32* overexpression increased the expressions of TGF-β1 and p-SMAD2/3. (a) TGF-β1, (b) SMAD2/3 and p-SMAD2/3 protein levels were analyzed by western blotting (n=6 per group). TDSCs: tendon-derived stem cells; *Lrrc32*: Leucine rich repeat containing 32; Control: Normal rat without Achilles tendon injury; NC: Achilles tendon injury and PBS-applied group; TDSCs+LV-NC: Achilles tendon injury and TDSCs+LV-NC-applied group; TDSCs+LV-*Lrrc32*: Achilles tendon injury and TDSCs+LV-*Lrrc32*-applied group. ***P*<0.01.

and p-SMAD2/3, which might contribute to accelerate the healing process of the injured Achilles tendon.

Discussion

Nowadays, tissue engineering based on stem cells, especially MSCs and TDSCs, become an attractive biological strategy for the tendon healing and repair [23]. In this study, we demonstrated that TDSCs with *Lrrc32* overexpression expressed high levels of tenocyte-related genes and efficiently formed tendon-like tissue *in vivo*, suggesting that *Lrrc32* contributed to tendon formation by promoting TDSC tenogenic differentiation. In addition, the positive effects of *Lrrc32* on tendon repair may be associated with the upregulation of TGF-β1 and p-SMAD2/3.

Since the discovery of *Lrrc32* in 1992 [24], the function of *Lrrc32* has been extensively studied on several aspects. For instance, *Lrrc32* was shown to be involved in maintaining Tregs-mediated peripheral tolerance [19]. Aberrant overexpression of *Lrrc32* has been shown to function in promoting Treg generation and immune escape [18, 25]. Bill X. Wu *et al.* also reported that *Lrrc32* is required for epithelial fusion during palatogenesis, a highly regulated morphogenetic process closely related to some common congenital craniofacial defect in humans, such as cleft lip or cleft palate [26]. Previous studies also found that the amplification of *Lrrc32* gene in human malignancies generally promoted oncogenesis [27, 28]. Recent evidence suggested that MSCs lacking *Lrrc32* expressed an impaired proliferative capacity [13]. In addition, knockdown of *Lrrc32* resulted in the attenuation of osteoblast differentiation of BMSCs *in vitro* [14]. Despite vast investigations, *Lrrc32* has not yet been associated with the tenogenic differentiation of TDSCs. In the present study, application of *Lrrc32*-overexpressing TDSCs significantly improved the morphological changes of the injured tendon. To further assess whether *Lrrc32* has an effect on tendon healing, we detected the ECM components including collagen-I and hydroxyproline. As the principal amino acid of collagens, hydroxyproline are considered to be an indirect quantification of total collagen content [29]. In collagenase-induced tendinopathy, collagen bundles are disrupted, and hydroxyproline content is greatly reduced [30, 31]. Here we showed that TDSCs-*Lrrc32* overexpressing treatment significantly upregulated the levels of hydroxyproline and collagen-I. Collagen-I is an extremely important protein for the formation of connective tissue in the ECM of tendons, which represents more than 90% of the total collagen content of a normal tendon [2]. As the tendon matures, collagen-I gradually increased to provide the majority tensile strength of the regenerated tendons [32]. Thus, the results suggested a beneficial role of *Lrrc32* overexpression in TDSCs against Achilles tendon injury.

Furthermore, our findings established a connection between *Lrrc32* and TDSC tenogenic differentiation by detecting the expression of tenocyte-related genes, including *Tnmd*, *Scx* and *Dcn*. *Tnmd* is an important differentiation marker of tenocytes, which belongs to the new family of type II transmembrane glycoproteins and predominantly expresses in tendons [33, 34]. *Tnmd* exerts a positive effect on TDSCs through supporting self-renewal [35]. It is reported that *Tnmd*-deficient mice displayed a severe decrease in tenocyte numbers and cell proliferation at newborn stage [36]. In addition, the expression of *Tnmd* was also closely associated with the generation of tenocytes during chick development, and was positively regulated by *Scx* in a tendon cell lineage-dependent manner [37]. *Scx* was suggested to be a marker of tendon formation and it was indispensable for the maintenance of the tendon tissue [38]. *Dcn*, a small cellular or pericellular matrix proteoglycan, is a component of connective tissue, binds to collagen-I fibrils, and plays a critical role in the deposition of a tendon-like ECM [39]. A previous study demonstrated that upregulation of the expressions of *Tnmd*, *Scx*, COL I/III and *Dcn* significantly altered TDSC stemness and further enhanced tendon formation [40]. Here, TDSCs overexpressing *Lrrc32* showed increased *Tnmd*, *Scx* and *Dcn* expressions *in vivo*, indicating that *Lrrc32* overexpressing promoted TDSC tenogenic differentiation.

TGF-β is expressed by the majority of cells and plays important roles in numerous aspects of biological processes [41, 42]. TGF-β1, TGF-β2, and TGF-β3 are three isoforms of TGF-β, yet, TGF-β1 is the most studied among the three [43]. *Lrrc32* was identified as a main docking receptor for latent TGF-β1, which concentrated latent TGF-β1 on the cell surface and promoted its activation [44]. Researchers from Sophie Lucas' laboratory revealed that *Lrrc32* increased the rate of pro-TGF-β cleavage via a furin-independent manner [45]. It has been clarified that SMAD2/3 proteins are involved in direct signaling from the TGF-β receptor [46]. Topical application of Benzyl alcohol improved the recovery process of tendon healing by enhancing the mRNA and protein expressions of TGF-β1 and p-SMAD2/3 [22]. After the extracorporeal shock waves treatment, increasing TGF-β1 expression was also observed in the early stage of tendon repair [47]. It is worth noting that TGF-β1 was differentially expressed in the distinct time courses of tendon healing. Heisterbach PE *et al.* [48] demonstrated that TGF-β1 was elevated at week 1 of tendon healing, possibly in response to the inflammation after rupture.

However, it was subsequently declined at weeks 2 and 4, which is consistent with our results. In addition, they also showed that the expression of TGF-β1 strongly increased again in week 8, probably reflecting remodeling and scar formation in the late stages of tendon healing. Since we found that *Lrrc32* overexpression dramatically upregulated the expressions of TGF-β1 and p-SMAD2/3, we suggested that *Lrrc32* might played an important role in mediating TGF-β1 and p-SMAD2/3 induced tendon healing. However, limitations of our work were the time of follow up during tendon healing. The dynamic effects of *Lrrc32* on TGF-β1 throughout Achilles tendon healing will be further explored in our future work.

Additionally, we only examined four CD markers on TDSCs in this study. Further determination of more stem cell surface markers will contribute to better characterize the TDSCs [49]. Overall, our work provides a valuable insight in understanding the role of *Lrrc32* for tendon healing, which is helpful in developing TDSCs-based tendon tissue engineering approaches for tendon repair.

Comflict of Interests

The authors declare that they have no competing interests.

Author's Contribution

Conception and design of the study: Cui L, Kang K and Gao S; Acquisition and interpretation of data: Kang K, Cui L and Zhang Q; Manuscript writing: Cui L and Kang K; Critical revision: Kang K, Cui L and Gao S; Final approval of the version to be published: Kang K, Cui L, Zhang Q and Gao S. All authors have read and approved the manuscript.

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