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Original Article

NSUN2 facilitates tenogenic differentiation of rat tendon-derived stem cells via m5C methylation of KLF2

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ABSTRACT

Introduction: Tendon-derived stem cells (TDSCs) play a critical role in tendon repair. N5-methylcytosine (m5C) is a key regulator of cellular processes such as differentiation. This study aimed to investigate the impact of m5C on TDSC differentiation and the underlying mechanism.
Methods: TDSCs were isolated from rats and identified, and a tendon injury rat model was generated. Tenogenic differentiation *in vitro* was evaluated using Sirius red staining and quantitative real-time polymerase chain reaction, while that *in vivo* was assessed using immunohistochemistry and hematoxylin–eosin staining. m5C methylation was analyzed using methylated RNA immunoprecipitation, dual-luciferase reporter assay, and RNA stability assay.
Results: The results showed that m5C levels and NSUN2 expression were increased in TDSCs after tenogenic differentiation. Knockdown of NSUN2 inhibited m5C methylation of KLF2 and decreased its stability, which was recognized by YBX1. Moreover, interfering with KLF2 suppressed tenogenic differentiation of TDSCs, which could be abrogated by KLF2 overexpression. Additionally, TDSCs after NSUN2 overexpression contributed to ameliorating tendon injury *in vivo*. In conclusion, NSUN2 promotes tenogenic differentiation of TDSCs via m5C methylation of KLF2 and accelerates tendon repair.
Conclusions: The findings suggest that overexpression of NSUN2 can stimulate the differentiation ability of TDSCs, which can be used in the treatment of tendinopathy.

1. Introduction

Tendinopathy is a chronic degenerative disorder that is prevalent among the general population, especially athletes, characterized by the degeneration of tendon tissue and functional impairment. This condition not only affects the mechanical properties of the tendons but also leads to clinical symptoms such as pain, swelling, and functional limitations [1]. Numerous treatment options, such as drug, physical, and exercise therapies and even surgery, are proposed for the treatment of tendinopathy [2]. However, longer medical leave is necessary to facilitate the recovery of the damaged tendons, and if proper protection is not maintained post-treatment, the condition is highly susceptible to recurrence [3]. Tendon-derived stem cells (TDSCs) are pivotal in promoting tendon growth, sustaining its integrity, and facilitating repair, serving as the therapeutic agent and acting as a healing indicator [4,5]. They possess multidirectional differentiation potential, clonogenicity, and non-immunogenicity, offering distinct advantages in tendon tissue engineering, which can promote the regeneration of tendons [6]. However, due to the influence factors such as the complexity of tendon structure and tenogenic heterogeneity, clinical therapeutic approaches based on TDSCs still face significant challenges [7]. Therefore, efficient utilization of TDSCs to facilitate the regeneration and repair of tendons is essential for the treatment of tendinopathy. It is imperative to further elucidate the regulatory mechanisms of TDSCs.

The N5-methylcytosine (m5C) RNA modification, as an integral component of RNA epigenetics, is extensively distributed in the mRNA, tRNA, and non-coding RNA of various organisms [8]. It dynamically regulates RNA stability, translational efficiency, and

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interactions with proteins through specific enzymatic reactions [9]. M5C modification has emerged as a crucial regulator of cellular processes, such as proliferation, differentiation, stress response, and cell death [10]. The NOP2/Sun RNA methyltransferase family member 2 (NSUN2) is a well-known m5C methyltransferase on various RNA substrates. NSUN2 is a nucleolar protein that is involved in tissue homeostasis and embryogenesis. Notably, NSUN2 is abnormally expressed in the disease and is involved in the progression of the disease [11]. Recent studies have highlighted the involvement of NSUN2 in the regulation of stem cell differentiation [12,13]. However, its role in tendon biology and TDSC differentiation remains unexplored.

Kruppel-like factor 2 (KLF2) is a transcription factor known for its critical functions in vascular biology, inflammatory processes, and cellular differentiation [14]. It has been reported that KLF2 regulates the development of diseases [15]. Moreover, KLF2 has been implicated in the regulation of cellular differentiation through its interaction with epigenetic modifiers and transcriptional co-regulators [16,17]. Nevertheless, whether KLF2 can influence tendon differentiation and its m5C modification remains unclear.

In the present study, we aimed to effect of NSUN2-mediated m5C methylation on the tenogenic differentiation of TDSCs isolated from rats. Moreover, we identified the regulation of NSUN2 on the m5C methylation of KLF2. This study will shed light on a novel epigenetic mechanism for tenogenic differentiation and provide a promising target for tendon repair.

2. Materials and methods

2.1. Isolation of rat TDSCs

Sprague–Dawley (SD) rats (male, 4-5-week-old) were purchased from KaiXue Biotechnology Co., LTD (Shanghai, China). The Achilles tendons were collected from the limbs of the rats under sterile conditions and cut into about 1 mm^3 pieces. These tissue pieces were digested with 3 mg/mL type I collagenase (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 2 h and then filtered by a 70 μm cell strainer to acquire single-cell suspension. After washing with PBS and centrifuging at 2000 g for 15 min, the sediments were cultured at Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin at 37 °C with 5% CO_2. The culture medium was changed every 3 days. The cells in passages 3 to 6 were used in functional experiments.

2.2. Identification of TDSCs

To identify the TDSCs, the multilineage differentiation capabilities were determined. To evaluate adipogenesis, TDSC culture and Oil Red O staining were performed using the OriCell rat TDSC lipogenesis induction differentiation kit (OriCell, Guangzhou, China). The osteogenic differentiation was analyzed using the OriCell rat TDSC osteogenic induction differentiation kit (OriCell). The chondrogenesis was evaluated using the OriCell rat TDSC chondroblast induction differentiation kit (OriCell).

Flow cytometry was performed to identify the surface markers (CD90, CD44, CD45, and CD34) of stem cells. Briefly, TDSCs were suspended in a serum-free medium and incubated with antibodies against CD90, CD44, CD106, and CD11B at 4 °C for 0.5 h in the dark. The levels of these markers were measured using a flow cytometer.

2.3. Induction of tenogenic differentiation

TDSCs were cultured in a low-glucose medium containing 5 ng/mL transforming growth factor β1 (TGF-β1; MCE, Monmouth Junction, NJ, USA) for two weeks to induce tenogenic differentiation.

2.4. Sirius red staining assay

TDSCs were fixed using 4% paraformaldehyde for 20 min. Sirius red dye (Solarbio, Beijing, China) was diluted using saturated picric acid solution (0.1 g–100 mL) and added to incubate with the cells for 30 min. After washing with water, the cell slices were dyed and sealed. The results were observed under a microscope.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from TDSCs or Achilles tendon tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Next, RNA was reverse transcribed to synthesize the first chain of cDNA using PrimeScript RT reagent kit (Takara, Dalian, China). Subsequently, qPCR was conducted to measure mRNA expression using the TB Green Premix Ex Taq II (Takara). The results were calculated using the $2^{-\Delta\Delta Ct}$ method. *GAPDH* was used as the housekeeping gene.

2.6. Dot blotting

Total RNA was loaded onto Hybond-N+ membranes. The membranes were cross-linked at UV at 254 nm for 1 min and blocked with 5% skim milk for 1 h. After incubating with primary antibody targeting m5C (ab214727, Abcam, Cambridge, MA, USA) at 4 °C for one night, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (ab205718, Abcam) for 1 h. The signals were observed using an enhanced chemiluminescence substrate kit (Abcam).

2.7. Cell transfection

Short hairpin RNA plasmids targeting NSUN2 (sh-NSUN2), sh-YBX1, their negative control (sh-NC), KLF2 overexpression plasmids (oe-KLF2), and its negative control (oe-NC) were obtained from Sangon (Shanghai, China). TDSCs were transfected with these plasmids using Lipofectamine 2000 (Invitrogen) for 48 h in line with the manufacturer's instructions.

2.8. Methylated RNA immunoprecipitation (Me-RIP)

The m5C levels of KLF2 were measured using the GenSeq m5C MeRIP kit (cloud-Seq, Shanghai, China). In brief, total RNA was fragmented at 70 °C and transferred to a new EP tube. The samples were centrifuged at 15,000 *g* for 25 min, and the sediments were collected. PGM beads were washed with IP buffer and incubated with m5C antibody for 1 h. Then, the beads were incubated with fragmented RNA and IP buffer for 1 h. After washing with the beads, RNA was purified, and KLF2 expression was measured using qPCR.

2.9. RIP

An Imprint RIP kit (Sigma–Aldrich) was utilized to assess the binding relationship. TDSCs were lysed using complete RIP lysis buffer on ice for 15 min. The lysate was acquired via centrifugation at 16,000*g* for 10 min at 4 °C. Protein A magnetic beads were incubated with 1 μg of anti-NSUN2, anti-YBX1, or anti-IgG to prepare magnetic beads for immunoprecipitation. The lysates were incubated with magnetic beads at 4 °C overnight. After washing with the beads using the wash buffer, RNA was isolated and KLF2 expression was measured using qPCR.

2.10. Dual-luciferase reporter experiment

M5C sites in KLF2 were predicted using the RNAm5Cfinder database (http://www.rnanut.net/rnam5cfinder/). To evaluate the combination of NSUN2 or YBX1 and KLF2, dual-luciferase reporter analysis was performed. In brief, wild-type (WT) KLF2 sequences were inserted into the pGL3-basic vectors (Promega, Madison, WI, USA) to construct WT reporter plasmids. Additionally, the sequences of KLF2 with each m5C site mutation (MUT) were also cloned into the pGL3-basic vectors to construct MUT reporter plasmids. TDSCs were seeded into 24-well plates. WT or MUT reporter plasmids, sh-NC or sh-NSUN2/sh-YBX1, and pRL-TK vectors were transfected into the cells using Lipofectamine 2000 for 48 h. The luciferase activities were measured using the dual-luciferase reporter assay system (Promega).

2.11. RNA stability assay

TDSCs were seeded into six-well plates and exposed to 5 μg/mL actinomycin D (Sigma–Aldrich) for 0, 4, 8, and 12 h. At each time point, RNA was isolated and KLF2 expression was measured by qPCR.

2.12. Establishment of tendon injury rat model

Male SD rats (8-week-old) were randomly divided into four groups: control, model, lentivirus (LC)-NC, and LV-NSUN2, with six rats in each group. To establish a tendon injury model, 30 μL of 10 mg/mL type I collagenase was injected into both Achilles tendons. To investigate the role of NSUN2, TDSCs were infected with LV-NSUN2 and LV-NC. Twenty-four hours and 14 days after collagenase 1 injection, these TDSCs (10^6 cells) were injected into the injured Achilles tendon in the same location as collagenase 1. Four weeks after collagenase 1 injection, all rats were euthanatized. The Achilles tendon tissues were collected.

2.13. Immunohistochemistry and hematoxylin–eosin (HE) staining

Achilles tendon tissues were immobilized in 4% paraformaldehyde for 24 h and then were made into paraffin sections. For immunohistochemistry, the sections were blocked with bovine serum albumin, incubated with primary antibodies against collagen type 1 (ab270993, Abcam) and Thbs4 (ab263898, Abcam) at 4 °C for one night, and subsequently incubated with secondary antibody (HRP-conjugated goat anti-rabbit IgG, ab205718, Abcam) for 1 h at 37 °C. The results were photographed under a microscope, and the percentage of stained area was quantified using the ImageJ software. For HE staining, the sections were stained with hematoxylin (Sigma–Aldrich) for 5 min. After differentiating in 1% hydrochloric acid alcohol for 30 s, the sections were stained with eosin (Sigma–Aldrich) for 3 min. The results were observed using a microscope.

2.14. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 7.0 software, and the results are presented as mean ± SD. Differences were assessed using Student's t-test and ANOVA and were considered at $P < 0.05$.

3. Results

3.1. Identifications of rat TDSCs

To analyze the role of TDSCs, we first isolated TDSCs from the limbs of healthy SD rats. Then, the characteristics of TDSCs were identified. CD90 and CD44 were positively expressed, while CD106 and CD11B were negatively expressed in the cells (Fig. 1A). In addition, the cells could differentiate into adipocytes, osteoblasts, and chondrocytes (Fig. 1B), suggesting multidirectional differentiation potential. Thus, we confirmed that we successfully isolated TDSCs.

3.2. NSUN2-mediated m5C methylation is increased in TDSCs after tenogenic differentiation

Next, TGF-β1 was used to treat TDSCs to induce tenogenic differentiation, which was confirmed using Sirius red staining and qPCR. The results of Sirus red staining showed that the collagen levels were higher in the differentiated cells than that in the control cells (Fig. 2A). Besides, differentiate-related genes, including TNMD, SCX, and COL1A1, were highly expressed in the differentiated cells, compared with the control group (Fig. 2B). These results suggest that we successfully induce tenogenic differentiation from TDSCs. m5C methylation has been reported to regulate cellular biological behaviors; however, whether m5C modification is involved in tenogenic differentiation remains unknown. Here, dot blotting results showed that total m5C levels were increased in differentiated cells (Fig. 2C). The levels of m5C "writers" were measured by qPCR, and we found that only NSUN2 and NSUN4 levels were upregulated after tenogenic differentiation (Fig. 2D). Taken together, the results demonstrated that m5C methylation is increased in tenogenic differentiated TDSCs. The higher expression of NSUN2 was selected for follow-up experiments.

3.3. Knockdown of NSUN2 inhibits tenogenic differentiation of TDSCs

To explore the effect of NSUN2 on tenogenic differentiation, we first knocked down NSUN2 expression via transfection sh-NUSN2, compared with sh-NC transfection (Fig. 3A). After interfering with NSUN2 expression, tenogenic differentiation of TDSCs was inhibited (Fig. 3B), accompanied with the downregulation of TNMD, SCX, and COL1A1 (Fig. 3C). The results demonstrated that interfering with NSUN2 inhibits tenogenic differentiation.

3.4. NSUN2 stabilizes KLF2 mRNA via the promotion of m5C methylation

KLF2 is a crucial regulator of cell differentiation. Moreover, KLF2 expression is increased in tendon stem/progenitor cells post-tendon rupture [18]. Here, we found that knockdown of NSUN2 decreased the expression of KLF2 (Fig. 4A), suggesting that NSUN2 serves its functions by regulating KLF2 expression. Hence, we focused on the role of KLF2 in this study. As a m5C "writer", we investigated the regulation of NSUN2 on the m5C methylation of KLF2. Following NSUN2 knockdown, the m5C levels of KLF2 were decreased (Fig. 4B). The RIP results showed that NSUN2 could interact with KLF2 (Fig. 4C). Then, potential m5C sites in KLF2 were predicted. The top three sites with the highest confidence are shown in Fig. 4D. To confirm which sites could be modified by m5C, dual-luciferase reporter assay was performed. Knockdown of NSUN2 reduced the luciferase activity when these sites were WT. Once these sites were mutated, NSUN2 could not affect the luciferase activity (Fig. 4E–G). The findings suggest that all these three sites can be modified by m5C. The stability of KLF2 was evaluated, and the results indicated that interfering with NSUN2 declined the mRNA stability of KLF2 (Fig. 4H). Together, silencing of NSUN2 inhibits m5C methylation and reduces the stability of KLF2.

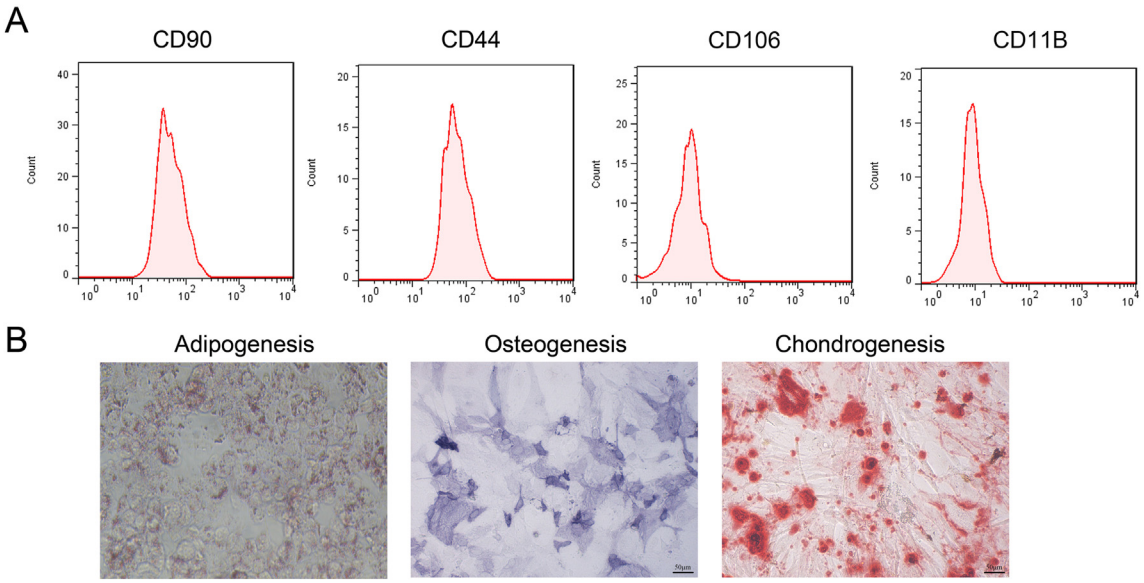

Fig. 1. Identifications of rat TDSCs. (A) The stem cell surface markers including CD90, CD44, CD106, and CD11B were measured using flow cytometry. (B) The multidirectional differentiation capabilities including adipogenesis, osteogenesis, and chondrogenesis were analyzed.

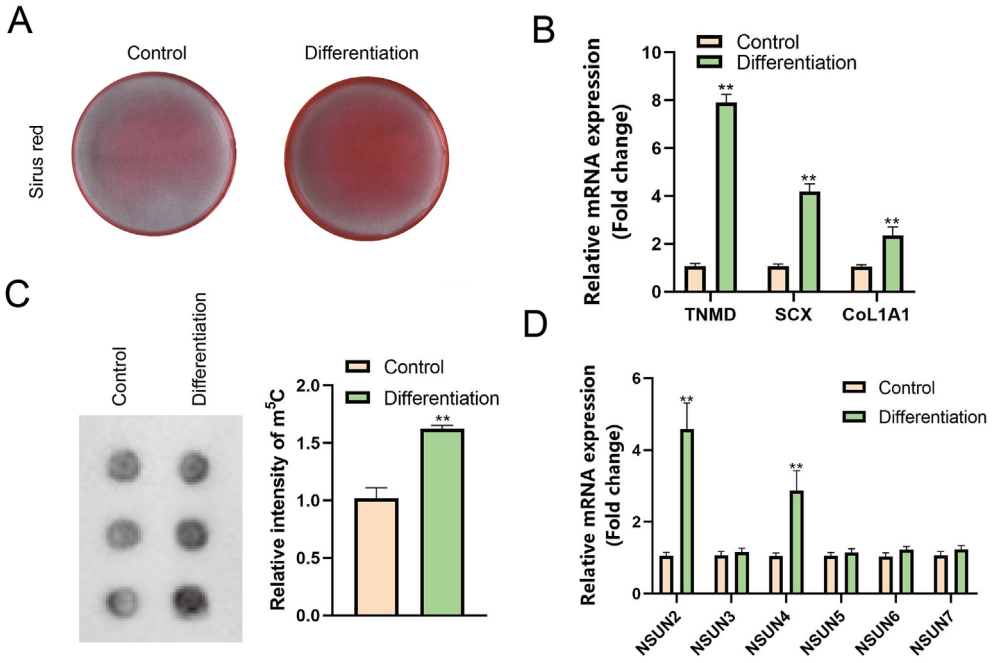

Fig. 2. NSUN2-mediated m5C methylation is increased in TDSCs after tenogenic differentiation. (A) Tenogenic differentiation was evaluated using the Sirius red staining. (B) qPCR was performed to measure the levels of differentiation-related factors including TNMD, SCX, and COL1A1. (C) Total m5C levels were analyzed using dot blotting. (D) The levels of m5C-related writers were measured by qPCR. **P < 0.01.

3.5. YBX1 is essential for the recognition of KLF2 m5A methylation

RNA methylation is dependent on the recognition of "readers". YBX1 can bind to m5C-modified mRNAs to enhance mRNA stability [19]. In this study, we identified whether YBX1 could recognize the m5C methylation of KLF2. The expression of KLF2 was down-regulated after knocking down YBX1 (Fig. 5A). Next, YBX1 was found to bind with KLF2 (Fig. 5B and C). Additionally, silencing of YBX1 decreased the mRNA stability of KLF2 (Fig. 5D).

3.6. Silencing of NSUN2 inhibits tenogenic differentiation by downregulating KLF2

Rescue experiments were carried out the assess both the function of NSUN2 and KLF2. After TDSCs were transfected with NSUN2, the levels of NSUN2 and KLF2 were reduced, while overexpression of KLF2 reversed the reduction of KLF2 expression but failed to affect NSUN2 expression (Fig. 6A and B). Then, the results of Sirius red showed that knockdown of NSUN2 suppressed tenogenic

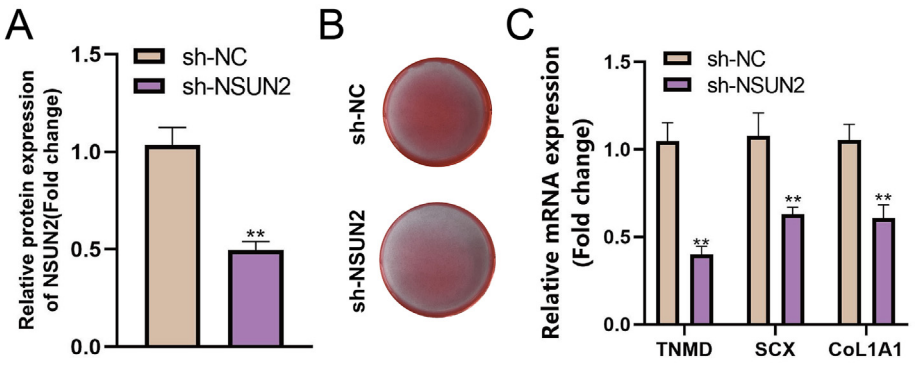

Fig. 3. Knockdown of NSUN2 inhibits tenogenic differentiation of TDSCs. (A) qPCR was performed to measure NSUN2 expression after sh-NSUN2 or sh-NC transfection. (B) Tenogenic differentiation was evaluated using the Sirius red staining. (C) The expression of TNMD, SCX, and COL1A1 was examined using qPCR. **P < 0.01.

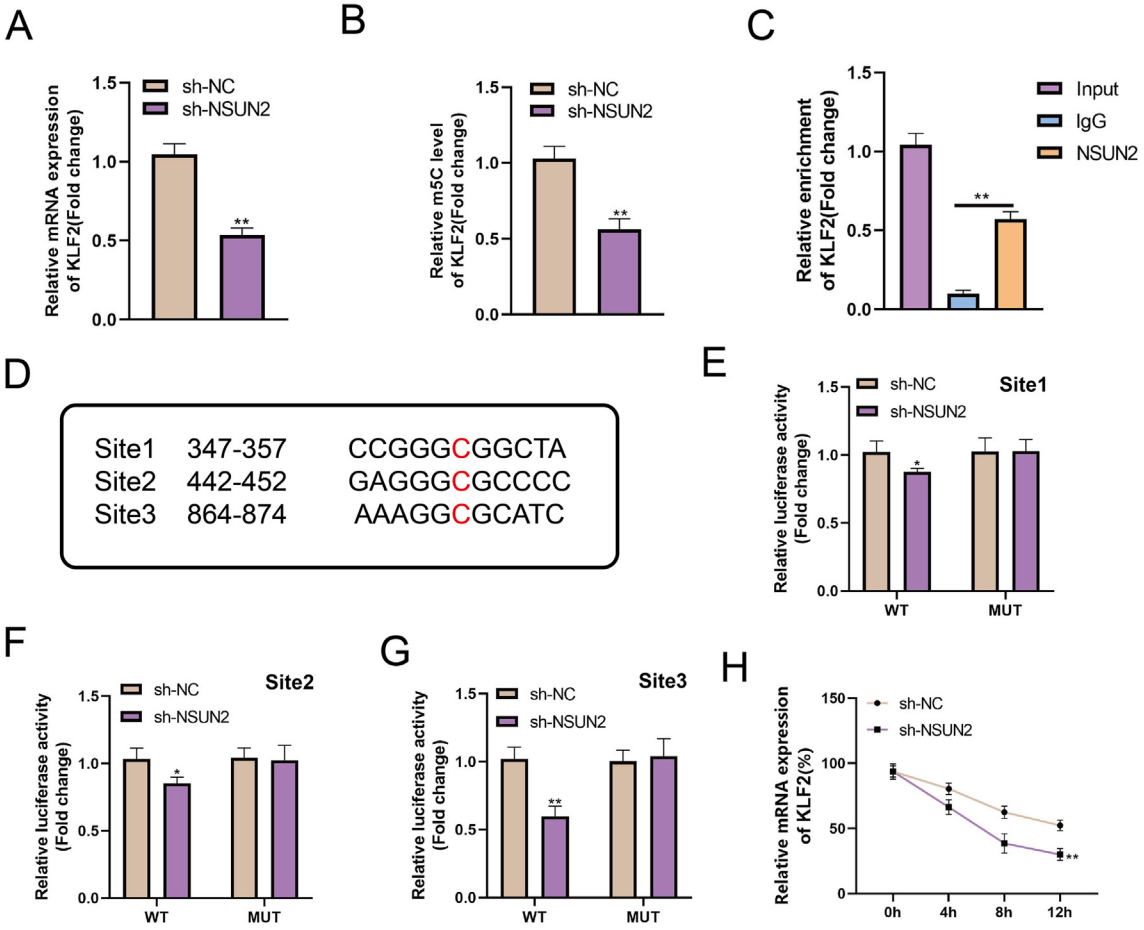

Fig. 4. NSUN2 stabilizes KLF2 mRNA via the promotion of m5C methylation. (A) The expression of KLF2 was measured after NSUN2 knockdown. (B) The regulation of m5C levels of KLF2 by NSUN2 was detected using MeRIP. (C) RIP was conducted to determine the interaction between NSUN2 and KLF2. (D) The top three m5C sites with the highest confidence were predicted. (E–G) The binding relationship between NSUN2 and each potential m5C site was analyzed by dual-luciferase reporter assay. (H) The effect of NSUN2 on the stability of KLF2. **P < 0.01. *P < 0.05.

differentiation, which was abrogated by KLF2 overexpression (Fig. 6C). The findings suggest that interfering with NSUN2 decreased KLF2 to suppress tenogenic differentiation of TDSCs.

3.7. TDSCs with NSUN2 overexpression improve tendon injury in vivo

Finally, we investigated the role of NSUN2 *in vivo*. TDSCs infected with LV-NC and LV-NSUN2 were injected into rats with tendon injury. NSUN2 expression was decreased in the Achilles tendon tissues of rats in the model group, whereas NSUN2 upregulated TDSCs counteracted the decreased expression of NSUN2 (Fig. 7A). Next, the histophathology was analyzed using HE staining. The results showed that the collagen bundles were disordered and lacerated in the Achilles tendon tissues of rats after model establishment. The histopathology of the tissues in the LV-NC group was not significantly different from that of the model group, while LV-

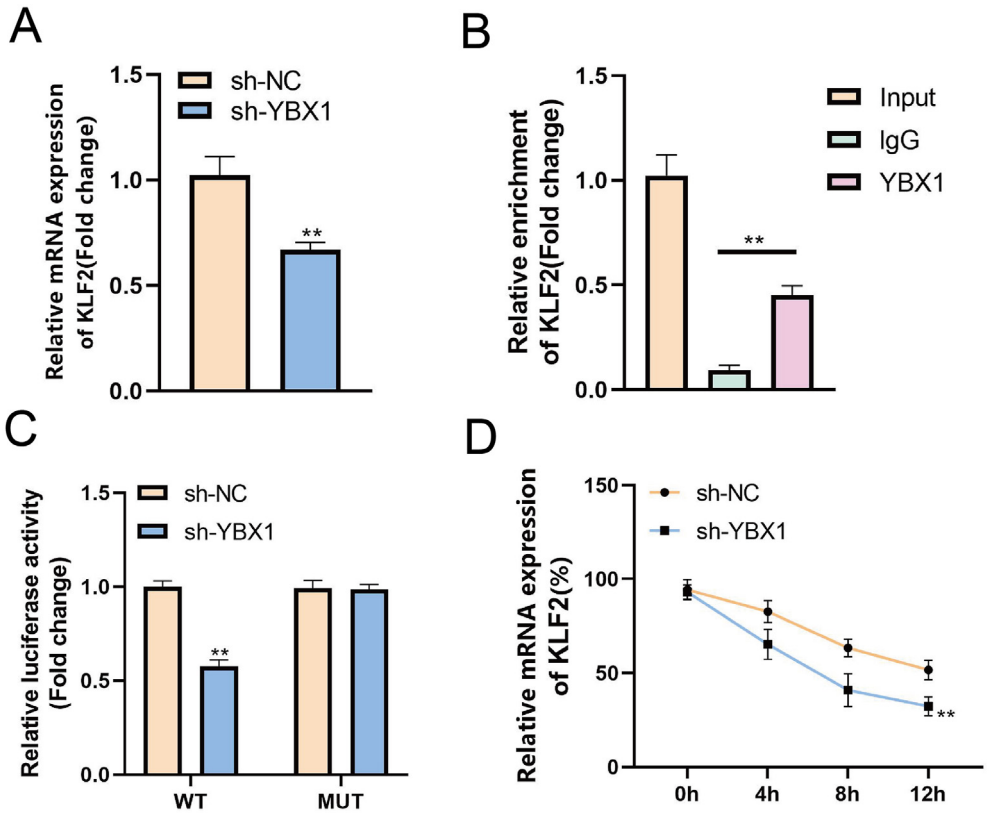

Fig. 5. YBX1 is essential for the recognition of KLF2 m5A methylation. (A) The expression of KLF2 was measured using qPCR after sh-YBX1 or sh-NC transfection. (B) RIP and (C) dual-luciferase reporter assays were used to analyze te binding relationship between YBX1 and KLF2. (D) The stability of KLF2 is mediated by YBX1. **P < 0.01.

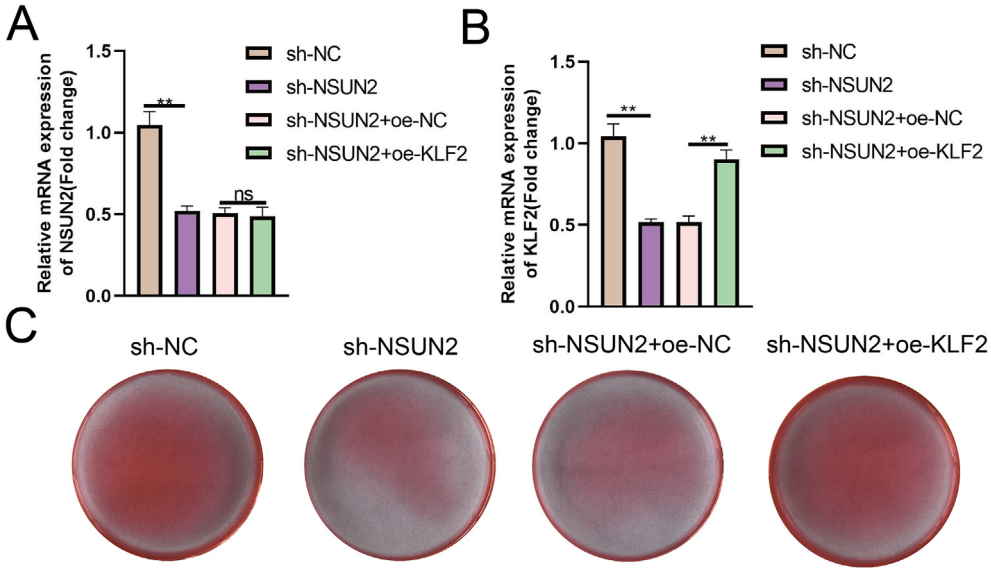

Fig. 6. Silencing of NSUN2 inhibits tenogenic differentiation by downregulating KLF2. The expression of (A) SUN2 and (B) KLF2 was measured by qPCR after NSUN2 was knocked down and/or KLF2 was overexpressed. (C) Tenogenic differentiation was evaluated using Sirius red staining. **P < 0.01. ns: no significance.

NSUN2-transfected TDSCs significantly improved the damaged collagen bundles (Fig. 7B). In addition, the downregulation of collagen type I and Thbs4 was found in tendon injury rats, which was abrogated by injecting with NSUN2 overexpression TDSCs (Fig. 7C and D). The findings suggest that TDSCs with overexpression of NSUN2 alleviate tendon injury in rats.

4. Discussion

In tendinopathy, alterations in the microenvironment and mechanical loads lead to abnormal differentiation of TDSCs, which in turn cause pathological changes such as tendon matrix fibrosis, heterotopic ossification, adipose infiltration, and tissue

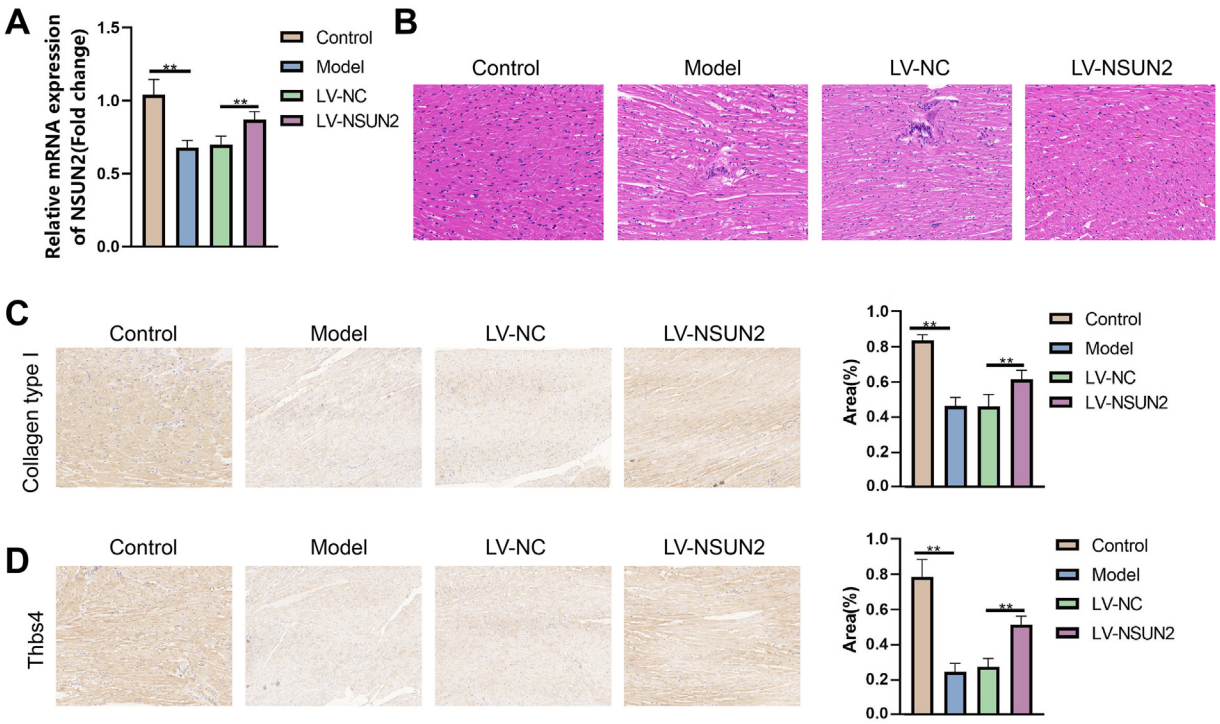

Fig. 7. TDSCs with NSUN2 overexpression improve tendon injury *in vivo*. (A) The mRNA levels of NUSN2 in Achilles tendon tissues were measured using qPCR. (B) HE staining images of the Achilles tendon tissues of rats. The levels of (C) collagen type I and (D) Thbs4 were detected using immunohistochemistry. **P < 0.01.

hyalinisation [7]. The multi-differentiation potential of TDSCs is a necessary condition for the formation of optimal tendons [20], particularly in promoting tenogenic differentiation, which is key to for TDSCs to serve their role in tendon repair. Hence, how to promote tenogenic differentiation of TDSCs is an important problem to be solved.

In this study, we found that m5C methylation levels were increased in tenogenic differentiated TDSCs, accompanied by the upregulation of NSUN2, suggesting NSUN2 is potentially involved in the tenogenic process. NSUN2 has been reported to regulate many types of differentiation, such as epidermal differentiation [21], epithelial differentiation [22], and testicular differentiation [23]. Moreover, NSUN2 is closely related to stem cell differentiation. For example, NSUN2 expression is reduced during the differentiation processes of neural stem cells, and the deficiency of NSUN2 impairs neural differentiation of the cells [12]. In addition, NSUN2 is necessary for epidermal stem cell differentiation [13]. Nevertheless, whether NSUN2 regulates the differentiation of TDSCs remains unknown. In our study, the functional role of NSUN2 in TDSC differentiation was supported by loss-of-function experiments, which demonstrated that knockdown of NSUN2 inhibited tenogenic differentiation. Moreover, the results of animal study indicated that NSUN2-overexpressed TDSCs promoted tendon repair. The findings suggest that NSUN2 may be a target for promoting tendon repair.

The identification of KLF2 as a direct target of NSUN2-mediated m5C methylation is a significant finding of this study. KLF2 has been previously implicated in various cellular processes, including differentiation. For instance, overexpression of KLF2 promotes osteoblast differentiation through its interaction with a differentiation marker Runx2 [24]. Besides, liriope muscari baily saponins C (DT-13) promotes acute myeloid leukemia cell apoptosis and differentiation by targeting KLF2 [25]. Additionally, KLF2 suppresses T follicular helper cell differentiation and production, which is involved in B cell response [26]. Among the pathological changes associated with tendinopathy, KLF2 expression is increased during the formation of heterotopic ossification. It can inhibit osteogenesis by regulating mitochondrial functions in tendon stem/progenitor cells and thus promote ectopic ossification [18]. Block the expression of KLF2 can impair the differentiation of both tenocytes and chondrocytes [27]. However, the role of KLF2 in tendon biology remains largely unknown. We found that silencing of NSUN2 inhibited m5C methylation of KLF2 and reduced KLF mRNA stability. Moreover, overexpression of KLF2 reversed the inhibition of tenogenic differentiation of TDSCs caused by NSUN2 knockdown. The findings suggest that NSUN2 facilitates tenogenic differentiation of TDSCs by m5C methylation of KLF2.

The majority of the biological roles of m5C modifications are associated with the binding protein called “reader”. Currently, ALYREF and YBX1 are widely studied m5C “readers” that bind to m5C sites to serve their functions [28]. YBX1 is a cytoplasmic mRNA m5C “reader that targets mRNA to affect stability [29]. In this study, we found that YBX1 interacted with KLF2 and was related to KLF2 stability, suggesting that m5C methylation of KLF2 mediated by NSUN2 can be recognized by YBX1. However, whether YBX1 is involved in the tenogenic differentiation of TDSCs need further to be investigated.

Our study also revealed that NSUN4 expression was upregulated in tenogenic differentiated TDSCs. However, whether NSUN4 can affect tendon differentiation in the same way as NSUN2 and its underlying mechanisms have not been investigated. We will study the role of NSUN4 in tendinopathy in our future work.

In conclusion, the results of this study demonstrate that NSUN2 promotes tenogenic differentiation of rat TDSCs to repair injured tendons. Mechanistically, NSUN2 stabilizes KLF2 mRNA by promoting its m5C methylation in a YBX1-dependent manner. These findings reveal a novel epigenetic mechanism in tendon repair and regeneration and provide a potential therapeutic target for the treatment of tendinopathy.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Taizhou Hospital in Zhejiang Province. All animal experiments should comply with the ARRIVE guidelines.

All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. W L and Z L drafted the work and revised it critically for important intellectual content; L W and Y Z were responsible for the acquisition, analysis and interpretation of data for the work; W L, Z L and H X made substantial contributions to the conception or design of the work. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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