

Mkx Mediates Tenogenic Differentiation But Incompletely Inhibits The Proliferation of Hypoxic MSCs

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Research Article

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Abstract

Background: Hypoxia has been shown to be able to induce tenogenic differentiation of mesenchymal stem cells (MSCs) which lead hypoxia-induced MSCs to be a potential treatment for tendon injury. However, little is known about the mechanism underlying the tenogenic differentiation process of hypoxic MSCs, which limited the application of differentiation-inducing therapies in tendon repair. This study was designed to investigate the role of Mohawk homeobox (Mkx) in tenogenic differentiation and proliferation of hypoxic MSCs.

Methods: Adipose-derived MSCs (AMSCs) and bone marrow-derived MSCs (BMSCs) were isolated, identified and cultured as our previous study. qRT-PCR, western blot, and immunofluorescence staining were performed to evaluate the expression of Mkx and other tendon-associated markers in AMSCs and BMSCs under hypoxia condition. Small interfering RNA technique was applied to observe the effect of Mkx levels on the expression of tendon-associated markers in normoxic and hypoxic BMSCs. Hypoxic BMSCs infected with Mkx-specific short hair RNA (shRNA) or scramble were implanted into the wound gaps of injured patellar tendons to assess the effect of Mkx levels on tendon repair. In addition, cell counting kit-8 and colony formation unit assays were adopted to determine the proliferation capacity of normoxic or hypoxic BMSCs infected with or without Mkx-specific shRNA.

Results: Our data showed that the expression of Mkx significantly increased in hypoxic AMSCs, and increased much more in hypoxic BMSCs. Our results also detected that the expression of tenogenic differentiation markers after down-regulation of Mkx were significantly decreased not only in normoxic BMSCs, but also in hypoxic BMSCs which paralleled the inferior histological evidences, worse biomechanical properties and smaller diameters of collagen fibrils in vivo. In addition, our in vitro data demonstrated that the optical density values and the clone numbers of both normoxic and hypoxic BMSCs were significantly increased after knockdown of Mkx, and were also significantly enhanced in both AMSCs and BMSCs in hypoxia condition under which the expression of Mkx was up-regulated.

Conclusions: These findings strongly suggested that Mkx mediated hypoxia-induced tenogenic differentiation of MSCs, but could not completely repress the proliferation of hypoxic MSCs.

Introduction

Tendon is composed of collagen fibers interspaced by little vessel [1, 2]. The forces transmission from muscles to bones in body movement and the hypovascularity make tendons tend to be subject to chronic injury, which accounts for the fact that tendon injury is a common but challenging medical problem, especially in athletes [2-4]. Tendon injury is also prevalent in the old people with the increasing incidence along the aggravation of global aging [3-5].

The self-repair of tendon injury is difficult because of the poor differentiation ability of tendon cells [1, 4, 6]. However, differentiation-inducing therapy of MSCs has been reported to be potential in treating tendon injury [2, 6, 7]. Among the various differentiation-inducing therapies, hypoxia has not only been found to

promote the proliferation of MSCs [8-11], but also has shown to be an efficient inducer in tenogenic differentiation of MSCs in repairing tendon injury [8, 12, 13]. Many studies have found that hypoxia increased the number of MSCs and augmented the formation of colonies [8, 9]. The improved effect of hypoxia on the tenogenesis of MSCs has also been confirmed. The expression of tenocyte-related genes, such as tenomodulin (Tnmd) [8] and tenascin C (Tnc) [12, 13], were greater under hypoxic culture condition compared with those under normoxic culture condition. An animal study has also found that MSCs from a hypoxic culture have shown better biomechanical and histological evidences than normoxic group in Achilles tendon repair [14]. In addition, our previous study has demonstrated that hypoxic MSCs possessed stronger tenogenic differentiation capabilities in vitro and promoted patellar tendon repair in vivo [15]. However, the mechanism underlying the process of hypoxia-induced tenogenesis of MSCs is still unclear, which largely impedes the advance of differentiation-inducing therapy of MSCs in tendon injury.

Mkx gene was first found in the developing mouse embryo [16] and was widely accepted as a transcription factor in tenogenic differentiation of MSCs [1, 17, 18]. Few studies have found that Mkx was a repressor in the proliferation of MSCs. Colony-forming unit showed that Mkx-expressing MSCs significantly decreased their self-renewal capacities [19]. Many studies reported the positive correlation between the expression of Mkx and tendon-associated markers in MSCs. The tensile strength of Achilles tendon and the type I collagen (Col-1a1) productivity in Achilles tendon and tail tendon of *Mkx*^{-/-} mice [20] and *Mkx*^{-/-} rats [21] were significantly lower than those of wild-type, respectively. In addition, MSCs overexpressed Mkx displayed a significantly higher level of Col-1a1 [17], Tnmd [17], Tnc [17] and Decorin (Dcn) [21, 22]. However, whether Mkx was involved in hypoxia-induced tenogenic differentiation of MSCs has not been reported.

Hence, this study was designed to investigate the role of Mkx in hypoxia-induced tenogenic differentiation and proliferation of MSCs. Our study found that hypoxia enhanced the expression of Mkx in both AMSCs and BMSCs, and that knockdown of Mkx reduced not only the tenogenic differentiation of normoxic BMSCs but also the tenogenesis of hypoxic BMSCs in vitro and in vivo. Our results also demonstrated that Mkx remarkably repressed the proliferation of both normoxic and hypoxic BMSCs, but could not completely inhibit the effect of hypoxia on proliferation of BMSCs.

Materials And Method

Animals

Eighteen male New Zealand white rabbits were used in this study (2.5–3kg, 3–4 months old). Before the study, all rabbits were examined for general health. AMSCs and BMSCs were collected from six rabbits. The rest rabbits were randomly allocated to either the scramble group or the short hair RNA (shRNA) group. In the following experiment, a commercial animal cage (49 cm×35 cm×32 cm) with free access to water and food was used for each rabbit which was housed in an animal room kept at room temperature and a 12 h:12 h light-dark cycle. This study was conducted according to the Guideline of Animal Care and

Use Committee of the Fourth Military Medical University and was approved by the Ethics Committees of the Fourth Military Medical University. All efforts were made to minimize the number and the discomfort of the rabbits.

Isolation and culture of AMSCs and BMSCs

AMSCs and BMSCs were isolated and cultured according to our previous study [15]. Briefly, AMSCs from the inguinal adipose tissue were collected and digested by type I collagenase (0.2%, Sigma, USA), filtered by 200-mesh sieve, centrifuged at 350 x g for 5 min, resuspended in DME/F12 complete medium containing 15% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin/amphotericin B (Cellmaxin plus, Gendepot, USA), and plated onto 10-cm cell culture dishes at 37 °C with 5% CO₂. BMSCs were isolated from bone marrow of the femora which was flushed out with the DME/F12 complete medium. After repetitively pipetting, the bone marrow was plated onto 10-cm cell culture dishes at 37 °C with 5% CO₂. The medium was changed every 2 days and the cells (passage 0, P0) were subcultured when 80-90% confluence was reached. The P3 cells were used in the experiment.

In hypoxic condition described in our previous study [15], MSCs were cultured in DME/F12 complete medium for 7 consecutive days in a tri-gas incubator maintained 1% O₂, 5% CO₂, and 94% N₂ (MCO-5M, SANYO, Japan). Whereas in Tgf-β1 induction, MSCs were cultured in complete medium containing 10ng/ml Tgf-β1 (Sigma, USA) for 7 consecutive days. The complete medium was changed every 2 days.

Flow Cytometry

To confirm surface marker of MSCs, flow cytometry analysis was applied according to the manufacturer's instructions. 1×10⁶ cells at P3 in the logarithm growth period were collected. After washing with 1% pre-cooled FBS/PBS and centrifuging at 350 x g for 5 min, these cells were incubated with anti-CD45-APC (Invitrogen, USA), anti-CD29-FITC (Invitrogen, USA), and anti-CD44-APC (Novus Biologicals, USA) in the dark at 4 °C for 30 min, respectively. Labeled cells were washed twice and examined using the FACScan flow cytometry system (BD, Franklin Lakes, USA). FlowJo software (TreeStar, Ashland, OR, USA) was used to analyze the data. PBS solution was used as negative control.

Immunofluorescence staining

3.5-cm laser confocal dishes were used to culture and fix MSCs when cells reached 60% confluence. After blocking with Immunol Staining Blocking Buffer (containing Triton X-100 for permeabilization; Beyotime, China) for 1 h, the cells were incubated with Mxk (1:200, Aviva Systems Biology, San Diego, USA) and Tnmd (1:400, Bioss, Beijing, China) primary antibodies solution overnight at 4°C. After washing 3 times with PBS, the cells were incubated with Cy3-goat anti-rabbit IgG (Beijing ComWin Biotech Co., Ltd. China) at room temperature for 1 h in dark place. For counterstaining, DAPI was used for 5 min to visualize cell nuclei. The prepared samples were examined under laser scanning confocal microscope (Nikon A1R, Japan). The images (each sample for at least 3 fields) were analyzed with Image Pro Plus version 6.0 (Media Cybernetics, Inc) as described previously [15]. The average optical density (AOD) was equal to

integrated optical density over area. For CD molecular identification, the cells were blocked with normal goat serum and incubated with the following antibodies: CD45-APC (Invitrogen, USA), CD29-FITC (Invitrogen, USA) and CD90-FITC (BioLegend, USA) overnight at 4 °C in dark place.

Multipotent Differentiation

The differentiation potential of AMSCs and BMSCs towards the chondrogenic, adipogenic and osteogenic lineages was assessed as described previously [15]. Briefly, 3×10^5 MSCs were collected and centrifuged to be a pellet which was cultured in chondrogenic induction medium in the 15-mL tube for 28 days. 2×10^4 cells/cm² were seeded in 6-well plates, first cultured in DME/F12 complete medium and then cultured in adipogenic induction medium A for 72 h and finally cultured in adipogenic induction medium B for 24 h. The cells were cultured in turn in adipogenic induction medium A and B and were stained after repeating 5 times. For osteogenic differentiation, 2×10^4 cells/cm² were seeded and cultured in DME/F12 complete medium in 6-well plates which were covered by 0.1% gelatin. The culture medium was changed with osteogenic induction medium after the cells were reached 70% confluence. Alcian Blue 8GX solution, Oil Red O solution and Alizarin Red solution were used for chondrogenic, adipogenic and osteogenic staining, respectively.

Cell counting kit-8 (CCK-8) assay

AMSCs and BMSCs were seeded in 96-well plates at a density of 2×10^3 cells/well, respectively, and cultured in the DME/F12 complete medium at 37°C for 7 consecutive days under normoxia condition and hypoxia condition. The culture medium was changed every 2 days. After the addition of 10µl CCK-8 assay solution (Dojindo, Japan) in each well and the incubation for 1 h, the optical density (OD) value was measured using an Infinite M200 Pro Multifunctional microplate reader (Tecan (Shanghai) Trading Co., Ltd., China) at a wavelength of 450 nm.

Colony formation unit assay

AMSCs (500 cells/well) and BMSCs (1000 cells/well) were seeded into 6-well plates, respectively, and cultured at 37°C under normoxia condition and hypoxia condition. The culture medium was changed every 3 days. After 7 days, the cells were washed twice with PBS, fixed in 4% methanol for 15 min, stained with 1% crystal violet dye (Sigma, USA) for 30 min at room temperature and washed with PBS. The images of 6-well plates were captured to count the colonies.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The mRNA levels of Mx and other tendon-specific genes were measured with qRT-PCR using the same condition as described previously [15]. Total RNA was extracted according to the manufacturer's protocol using RNAiso plus (TaKaRa, Japan). qRT-PCR analysis was conducted using the CFX96 Real-Time PCR Detection System (Rotor-Gene Q 2plex, Germany). The primer sequences of Mx synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) were: forward: CCAGAGTGCGTGTGCTACAG; reverse:

AAATGCTACCACAGGGCTGC. Other primer sequences of tendon-specific genes were same to our previous report [15]. Specificity of primers was examined by the melting curve. Data were collected from at least five independent samples and were tested at least three times. The expression levels of tendon-specific genes relative to β -actin were determined using the $2^{-\Delta\Delta CT}$ method.

Western blot

The protein expression of Mxk and other tendon-specific genes were measured with Western blot using the same condition as described previously [15]. Proteins were extracted using RIPA Lysis Buffer (Shanghai Weiao Biotechnology Co., Ltd., China). BCA protein reagent kit (Beijing Solarbio Science & Technology Co., Ltd., China) was used to measure the concentration. 30 μ g proteins were run on SDS-PAGE gels (8%) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with 10% skim milk at room temperature for 1 h and were incubated with anti-Mxk (1:200, Aviva Systems Biology, San Diego, USA), anti-Col-1a1 (1:400, Bioss, Beijing, China), anti-Collagen type III (Col-3a1, 1:400, Bioss, Beijing, China), anti-Dcn (1:400, Bioss, Beijing, China), and anti-Tnmd (1:400, Bioss, Beijing, China) primary antibodies solution and anti- β -Actin (1:1000, BOSTER Biological Technology Co., Ltd, China) primary antibody solution overnight at 4 °C. After reacting with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (BOSTER Biological Technology Co., Ltd, China) for 1 h at room temperature, proteins were detected with ECL hypersensitive chemiluminescence kit (Shanghai Weiao Biotechnology Co., Ltd., China) according to the manufacturer's recommendations.

RNA interference

After culturing in 6-well plates for 24 h, BMSCs were transfected with 100 nmol/L Mxk-specific small interfering RNA (siRNA, Guangzhou RiboBio Co., Ltd., China) or scrambled siRNA (negative control) using lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's instruction. The siRNA sequence was 5'-GCAGCTTGTGAACCGCTA-3'. Cells were collected 72 h after transfection and analyzed for mRNA expression by qRT-PCR and protein expression by Western blot.

Construction of lentiviral vectors and stable infection

To stably knock down the expression of Mxk, a specific lentiviral vector (Hanbio Biotechnology Co., Ltd., Shanghai, China) containing green fluorescent protein (GFP) and stably expressed Mxk-specific shRNA was constructed with the same sequence used in siRNA. The scramble sequence was 5'-TTCTCCGAACGTGTACGTAA-3' (Hanbio Biotechnology Co., Ltd., Shanghai, China). BMSCs were cultured in 6-well plates for 24 h and were transduced with lentiviral particles using 8 μ g/ml polybrene at a multiplicity of infection of 50 according to the manufacturer's instructions. The expression of GFP was observed at 72 h after transduction using a fluorescence microscope (Olympus, Japan). Cells were cultured in complete medium containing 2 μ g/ml puromycin (Solarbio, China) for selection.

Tendon Injury Model

Bilateral patellar tendon injury model was established in the scramble group or the shRNA group following a protocol previously described [15]. Briefly, after making a longitudinal skin incision over the patellar tendon and the removal of subcutaneous fascia, the middle part of the patellar tendon was transversely severed. 50 µl PBS containing hypoxic BMSCs (1×10^6) transduced with Mx-scramble or Mx-shRNA was injected into the wound gap followed by suturing the subcutaneous fascia and the skin. The rabbits were replaced to their own cages with plaster casts on bilateral legs after the operation, and were given an intramuscular injection of cefazolin sodium (0.1 g/kg, q.d) for 3 consecutive days. The plaster casts were removed after 3 weeks of immobilization.

Histological analysis

Rabbits were suffocated at 4 weeks after surgery for histological analysis. According to our previously described protocol [15], after the repaired patellar tendons were harvested, the staining of hematoxylin and eosin (H&E), masson's trichrome and immunohistochemistry (IHC) for Col-1a1 and Tnmd were performed and the histological scores of H&E and IHC were evaluated. For frozen sections, the repaired patellar tendons were fixed and embedded with OCT compound and were sectioned using thermostatic freezing slicer (CryoStar NX50, Thermo).

Biomechanical Testing

Rabbits were suffocated at 4 weeks after surgery for biomechanical analysis as described in our previous study [15]. The patella-patellar tendon-tibial tubercle was harvested. After measuring the length and width of the patellar tendon, the patella and the tibial tubercle were fixed on aluminum clamps of a biomechanical testing machine (SPL-10 KN, Shimadzu, Japan). The patellar tendon was loaded until failure along the vertical axis at a displacement rate of 10 cm/min. The maximum load to failure, stiffness at failure, maximum stress, cross-sectional area, and elastic modulus were calculated.

Transmission electron microscopy (TEM) analysis

The preparation of samples and the analysis of TEM sections were conducted following our previous protocol [15]. Briefly, after fixing, the samples were dehydrated, infiltrated and embedded with absolute [embedding medium](#). Three sections, obtained using ultramicrotome (Leica Leica UC7, Germany) from each sample with a thickness of 60 µm, were stained. The cleanest section was chose for TEM analysis (Hitachi HT7700, Japan). The software-Image Pro Plus version 6.0 (Media Cybernetics, Inc) was applied to measure the fiber diameter.

Statistical analysis

SPSS version 16.0 was applied in this study. Homogenous variances and normal distribution of data were examined using the Levene's test and the Shapiro-Wilk test, respectively. All data were presented as the mean \pm standard deviation (SD). Independent-samples t-test was used and $P < 0.05$ was considered to be statistically significant.

Results

Identification of AMSCs and BMSCs using flow cytometry, immunofluorescence staining and commercial induction medium of tri-lineage differentiation kit was shown in Supplementary Figure 1.

Hypoxia increased the expression level of Mxk in AMSCs and BMSCs.

In our previous study, Tgf- β 1 induction was demonstrated to be a good comparison in tenogenic differentiation. In the present study, Tgf- β 1 was also adopted to compare with hypoxia in term of the expression level of Mxk.

As shown in Fig. 1a, the mRNA level of Mxk in both AMSCs and BMSCs was significantly increased in hypoxia induction than that in normoxia. Although significantly higher mRNA level of Mxk was found in Tgf- β 1 induction compared to normoxia in both MSCs, the mRNA level of Mxk in Tgf- β 1 induction was significantly lower than that in hypoxia induction in BMSCs. However, the mRNA level of Mxk in hypoxia and Tgf- β 1 induction in both MSCs was lower than that in hypoxia induction alone but higher than that in Tgf- β 1 induction alone. Similar results were found in the protein expression of Mxk in western blot analysis shown in Fig. 1b, c (Supplementary Figure 2-4). Furthermore, the protein level of Mxk in all groups in BMSCs was significantly higher than that in the same group in AMSCs, respectively.

In addition, immunofluorescence staining of Mxk in both MSCs showed the similar results to western blot analysis (Fig. 1d, e). The quantitative analysis of immunofluorescence staining detected that the AOD of Mxk was significantly higher in hypoxia induction compared with the Tgf- β 1 induction in AMSCs (Fig. 1e).

Downregulation of Mxk repressed the expression level of tenogenic differentiation markers in both normoxic BMSCs and hypoxic BMSCs.

As shown in Fig. 2a, b, the mRNA and protein levels of Mxk in BMSCs were significantly higher than those in AMSCs (Supplementary Figure 5). Therefore, BMSCs were selected to knock down the expression of Mxk using Mxk-specific siRNA sequences in order to investigate the role of Mxk in tenogenic differentiation in vitro.

After downregulating the mRNA levels of Mxk (Fig. 2c), the mRNA levels of Mxk (Fig. 2d) and tenogenic differentiation markers, such as Col-1a1 (Fig. 2e), Col-3a1 (Fig. 2f), Dcn (Fig. 2g) and Tnmd (Fig. 2h) were significantly lower than those in the scramble group in hypoxia induction. Similarly, the protein levels of the above tenogenic differentiation markers in the siRNA group were also significantly lower than those in the scramble group in both normoxia condition and hypoxia condition (Fig. 2i, Supplementary Figure 6-15). As shown in Fig. 2j-i, the quantitative analysis of immunofluorescence staining detected that the AOD of Mxk and Tnmd were both significantly decreased in the hypoxia and siRNA group compared with those in the hypoxia and scramble group.

Downregulation of Mxk inhibited patellar tendon repair treated by hypoxic BMSCs.

In order to study the role of Mxk in tenogenesis of hypoxic BMSCs in vivo, a specific lentiviral vector containing GFP and stably expressed Mxk-specific shRNA was constructed with the same sequence used in siRNA. The transfection efficiency was shown in Supplementary Figure 16.

Gross inspection and fluoroscopic inspection. As shown in Fig. 3a, there was a remarkable defect in the patellar tendon at 4 weeks after surgery when seeded hypoxic BMSCs with Mxk-specific shRNA infection in the wound of patellar tendon. However, when applied hypoxic BMSCs with scramble infection into wound gap, the injured patellar tendon recovered better under gross observation. The frozen sections of the hypoxia and shRNA group and the hypoxia and scramble group were observed under fluorescence microscopy, and the results showed that fluorescence could be seen at 4 weeks after surgery.

Masson staining and H&E staining. The histological difference between the hypoxia and shRNA group and the hypoxia and scramble group was evaluated by Masson staining and H&E staining. To detect the formation of tendon-like tissues, Masson staining was adopted. As shown in Fig. 3a, less fibrous matrix stained in blue and more empty spaces were found in the hypoxia and shRNA group, compared to those in the hypoxia and scramble group. In H&E staining (Fig. 3b), similar large empty spaces and relatively fewer number of tendon cells were detected in the hypoxia and shRNA group with disorder collagen fibers and bold vessels. However, in the hypoxia and scramble group, the collagen fibers were arranged more regularly, within which less empty spaces and more cells were observed. In the following quantitative analysis (Fig. 3c), the histological scores of the hypoxia and shRNA group was significantly higher than that of the hypoxia and scramble group.

IHC staining. In order to determine the difference of tenogenic differentiation, IHC staining of tendon-specific markers Col-1a1 and Tnmd was performed (Fig. 3b). The staining of Col-1a1 and Tnmd was lighter in the hypoxia and shRNA group, and became deeper in the hypoxia and scramble group. The average optical density of Col-1a1 and Tnmd in the hypoxia and shRNA group was significantly lower than that in the hypoxia and scramble group, respectively (Fig. 3c).

Biomechanical properties of repaired patellar tendons. The maximum load (Fig. 4a), stiffness (Fig. 4b), maximum stress (Fig. 4c) and elastic modulus (Fig. 4e) were significantly lower in the hypoxia and shRNA group than those in the hypoxia and scramble group. However, the cross-sectional area was significantly higher in the hypoxia and shRNA group than that in the hypoxia and scramble group (Fig. 4d).

Ultrastructural morphology of repaired patellar tendons. The diameters of collagen fibrils in repaired patellar tendons at 4 weeks after surgery were observed under transmission electron microscopy, as shown in Fig. 5a-e. After calculating the range of fibrils diameters, the results revealed that the majority of fibrils diameters ranged from 39 to 58 nm in the hypoxia and shRNA group (Fig. 5a, b) and 46 to 78 nm in the hypoxia and scramble group (Fig. 5c, d). As shown in Fig. 5e, the average diameter of collagen fibrils in the hypoxia and shRNA group was significantly smaller than that in the hypoxia and scramble group.

Hypoxia promoted the proliferation of AMSCs and BMSCs.

At the second day, the OD value of hypoxic AMSCs was significantly higher than that of normoxic AMSCs according to the CCK-8 assay shown in Fig. 6a. The significant increase in OD value of hypoxic AMSCs lasted 5 days until the end of CCK-8 test. Similarly, the OD value of hypoxic BMSCs was significantly higher than that of normoxic BMSCs from the third day to the seventh day (Fig. 6b). After 7 consecutive days of hypoxic culture, the number of colony formation was significantly higher than that of normoxic culture in both AMSCs and BMSCs, as shown in Fig. 6c-e.

Downregulation of Mxk promoted the proliferation of BMSCs in normoxia and hypoxia condition.

As shown in Fig. 7a, the OD value of shRNA group was significantly higher than that of the scramble group at the second day according to the CCK-8 assay in normoxia condition. The significant increase in OD value of the shRNA group lasted 5 days until the end of CCK-8 test. Similarly, the OD value of shRNA group was significantly higher than that of scramble group from the second day to the seventh day in hypoxia condition (Fig. 7b). After culturing for 7 consecutive days, the number of colony formation in shRNA group was significantly higher than that in scramble group in both normoxia and hypoxia condition, as shown in Fig. 7c-e.

Discussion

Although the improved effects of hypoxia on tenogenic differentiation of MSCs have been reported [8, 12, 13, 15], none of them conducted research about the underlying mechanism of tenogenesis in hypoxia condition. In the present study, we detected that the expression level of Mxk was significantly enhanced in hypoxic AMSCs and hypoxic BMSCs in comparison to that of normoxic MSCs, respectively, and that the hypoxia and shRNA group showed significantly lower expression of tendon-specific markers in vitro and worse histological and biomechanical properties in vivo than those of the hypoxia and scramble group in BMSCs. Our in vitro results further showed that the OD values and the clone numbers of BMSCs were significantly increased after downregulation of Mxk in both normoxia and hypoxia condition, and were also significantly enhanced in AMSCs and BMSCs in hypoxia condition under which the expression of Mxk was promoted.

Many studies have proved that transcription factors Scleraxis (Scx) played an important role in promoting tenogenic differentiation of MSCs [19, 23]. However, Mxk has been shown to be more effective than Scx in inducing tenogenic differentiation of MSCs and could dramatically enhance the expression level of Scx [19]. In our study, we first found that the Mxk content of BMSCs was significantly higher than that of AMSCs, and that the protein expression of Mxk in hypoxic BMSCs was also significantly higher than that in hypoxic AMSCs. Our results further showed that the expression of tendon-specific markers, such as Col-1a1, Col-3a1, Dcn, Tnmd were all decreased in normoxic BMSCs when Mxk was down-regulated. This was similar to previous results which showed that decreased type I collagen productivity was found in Mxk mutant mice [20] and Mxk mutant rats [21]. Similarly, we detected that the expression levels of the above tendon-specific markers were also reduced in the hypoxia and shRNA group in BMSCs, compared with the hypoxia and scramble group. This indicated that Mxk was crucial for hypoxia-induced

tenogenesis of BMSCs. In addition, as a comparative inducer of tenogenesis used in our previous study [15], Tgf- β 1 was found to promote the expression of Mxk as well, which may be a reason for its improved effect on tenogenic differentiation of MSCs.

In order to better examine the formation of tendon-like tissues directly from tenogenesis of the implanted BMSCs in our study, the GFP-labeled hypoxia-induced BMSCs with Mxk-specific shRNA or scramble were seeded in the wound gap of patellar tendons. At 4 weeks after the surgery, we found that the green GFP fluorescence could still be seen in the frozen sections of the hypoxia and shRNA group and the hypoxia and scramble group under fluorescence microscopy. This paralleled a previous finding which revealed that GFP-labeled MSCs can be observed at 4 weeks after transplantation [24]. In addition, the worse histological evidences and the lower scores of Col-1a1 and Tnmd after IHC staining in the hypoxia and shRNA group were corresponded to the following inferior biomechanical properties and the smaller fibril diameters. This indicated that our in vivo findings validated the role of Mxk in hypoxia-induced tenogenic differentiation of BMSCs in vitro.

Tnmd was considered to be an important and specific collagen matrix in tendon formation [8, 15]. In our previous study, Tnmd was found to be the highest expression gene of MSCs in mRNA and protein level under hypoxia condition [15]. In the present study, we also examined the expression of Tnmd after the downregulation of Mxk and found that almost no red fluorescence (Tnmd) were seen in the hypoxia and shRNA group in BMSCs after immunofluorescence staining, compared with the hypoxia and scramble group. The result demonstrated that hypoxia improved tenogenic differentiation of MSCs, which was mediated by Mxk, mainly by promoting the expression of Tnmd.

Many studies have reported that hypoxia prompted the self-renewal of MSCs [8-11]. This was consistent with our findings which showed that the significantly higher OD values and clone numbers were detected in both AMSCs and BMSCs in the hypoxia group, compared with those in the normoxia group. Previous study reported that Mxk has been shown to be able to inhibit the colony formation numbers of MSCs [19]. This finding was identical with our results which showed that the OD values and clone numbers of the shRNA group were significantly higher than that of the scramble group in both normoxia and hypoxia condition. However, the remarkably increased level of Mxk and the significantly higher OD values and clone numbers in the hypoxic BMSCs, compared with the normoxic BMSCs in our study, indicated that Mxk could not completely inhibit the effect of hypoxia on proliferation.

Conclusion

In conclusion, we have investigated the role of Mxk in hypoxia-induced tenogenic differentiation and proliferation of MSCs. Our data showed that the expression of Mxk significantly enhanced in AMSCs and BMSCs under hypoxia condition in vitro, and that Mxk mediated hypoxia-induced tenogenic differentiation of BMSCs in vitro and in vivo, but incompletely inhibited the proliferation of hypoxic BMSCs in vitro.

Abbreviations

AMSCs: Adipose-derived MSCs; AOD: Average optical density; BMSCs: Bone marrow-derived MSCs; CCK-8: Cell counting kit-8; CFU: Colony formation unit; Col-1a1: Collagen type I; Col-3a1: Collagen type III; Dcn: Decorin; FBS: Fetal bovine serum; GFP: green fluorescent protein; H&E: Hematoxylin and eosin; IHC: immunohistochemistry; Mx: Mohawk homeobox; MSCs: Mesenchymal stem cells; OD: Optical density; P: Passage; PBS: Phosphate buffer saline; PVDF: Polyvinylidene difluoride; qRT-PCR: Quantitative real-time polymerase chain reaction; Scx: Scleraxis; SD: Standard deviation; shRNA: short hair RNA; siRNA: small interfering RNA; TEM: Transmission electron microscopy; Tgf- β 1: Transforming growth factor- β 1; Tnc: tenascin C; Tnmd: Tenomodulin.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committees of the Fourth Military Medical University and were conducted according to the Guideline of Animal Care and Use Committee of the Fourth Military Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

Guanyin Chen and Qiang Hao conceived and designed the study. Guanyin Chen and Dong Fan performed the analysis, wrote the paper and contributed equally to the manuscript. Zhaohui Liu and Yingqi Zhang supervised the experiments and reviewed the draft. Guanyin Chen, Wangqian Zhang and Shuning Wang conducted cytological experiments. Guanyin Chen, Jintao Gu, Yuan Gao and Lei He performed animal

experiments. Guanyin Chen, Weina Li, Cun Zhang and Meng Li carried out molecular biology experiments. All authors read and approved the final manuscript.

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Figures

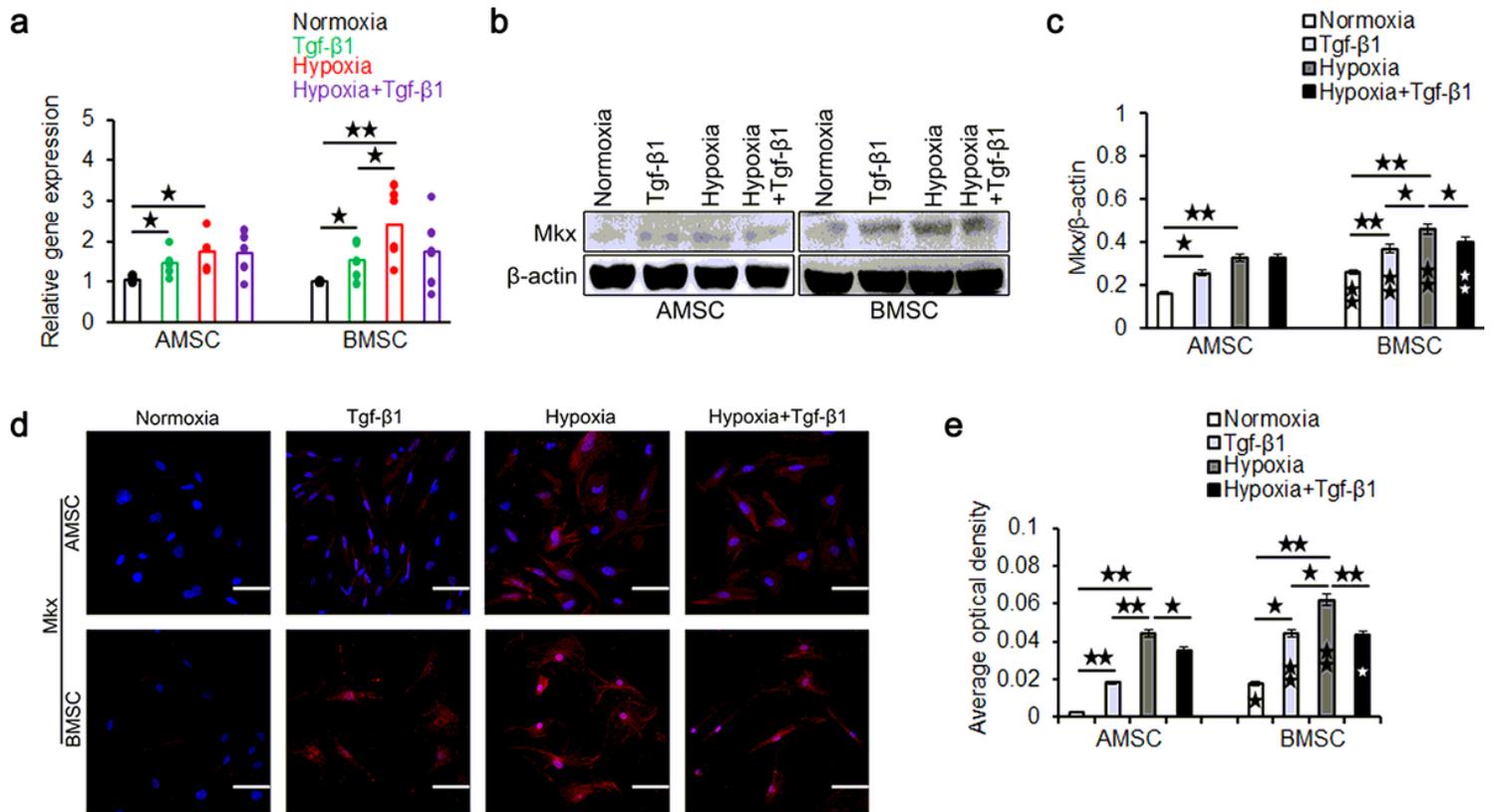


Figure 1

The mRNA and protein expressions of Mxk in AMSCs and BMSCs at 7 days after induction. a: qRT-PCR analysis of gene expression of Mxk under normoxia, Tgf- β 1, hypoxia, and hypoxia+Tgf- β 1 condition in AMSCs and BMSCs. b-c: Representative western blots (b) and quantification of protein expression (c) of Mxk under normoxia, Tgf- β 1, hypoxia, and hypoxia+Tgf- β 1 condition in AMSCs and BMSCs. Asterisks in the column in BMSCs indicated significantly different from the same condition in AMSCs. d-e: Representative immunofluorescence staining (d) of Mxk (red) and DAPI-labeled nuclei (blue), and quantification data (e) under normoxia, Tgf- β 1, hypoxia, and hypoxia+Tgf- β 1 condition in AMSCs and BMSCs. Asterisks in the column in BMSCs indicated significantly different from the same condition in AMSCs. Scale bar = 50 μ m; Magnification: \times 400. Data were shown as mean \pm SD. $\star p < 0.05$; $\star\star p < 0.01$.

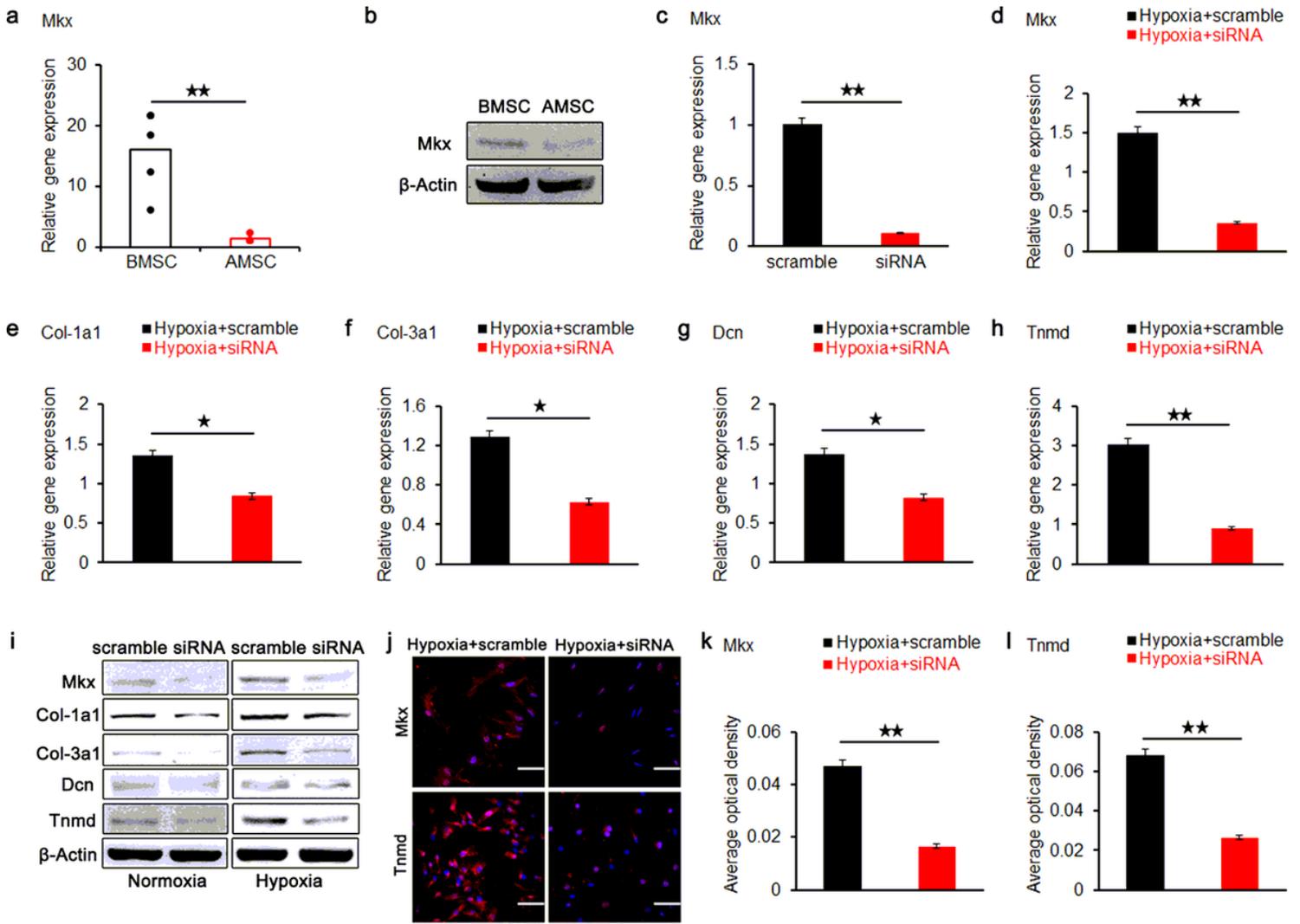


Figure 2

The Mxk content of AMSCs and BMSCs and the mRNA and protein expressions of Mxk and tenogenic differentiation markers after downregulation of Mxk by siRNA in BMSCs. a-b: qRT-PCR analysis (a) and representative western blots (b) of Mxk content in AMSCs and BMSCs. c-d: qRT-PCR analysis of gene expression of Mxk in the scramble group and the siRNA group in normoxia condition (c) and in hypoxia condition (d). e-h: qRT-PCR analysis of gene expression of Col-1a1 (e), Col-3a1 (f), Dcn (g) and Tnmd (h) in the hypoxia and scramble group and the hypoxia and siRNA group. i: Representative western blots of Mxk and tenogenic differentiation markers after siRNA application in normoxia condition and hypoxia condition. j-l: Representative immunofluorescence staining (j) of Mxk and Tnmd, and quantification data of Mxk (k) and Tnmd (l). Scale bar = 50 μ m; Magnification: \times 400. Data were shown as mean \pm SD.

\square p<0.05; $\square\square$ p<0.01.

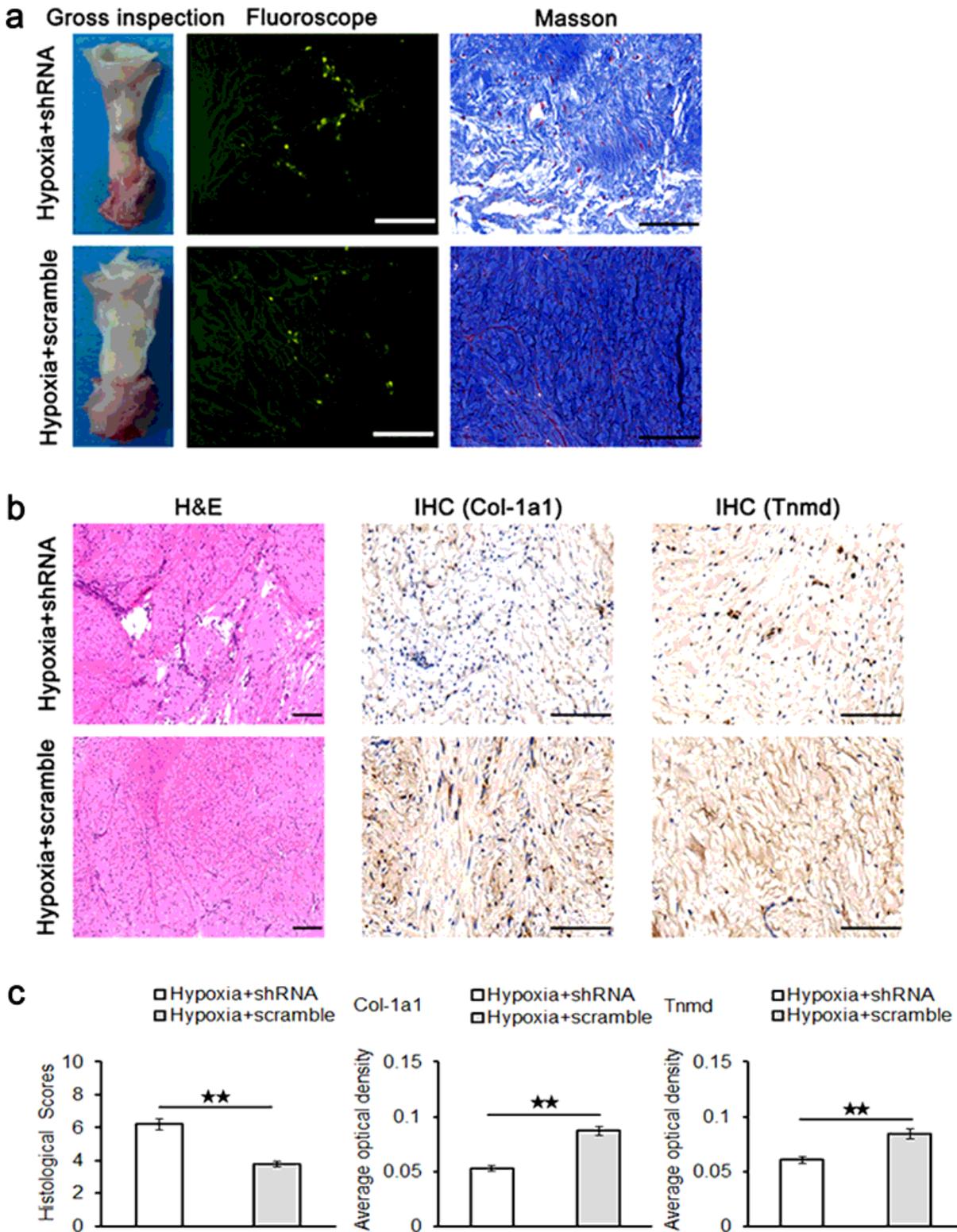


Figure 3

Gross observation and histological analysis of repaired patellar tendons at 4 weeks after surgery. a: Gross observation, frozen sections observed by fluorescence microscopy and masson's staining of the hypoxia and scramble group and the hypoxia and shRNA group. scale bar = 200 μ m; magnification: \times 400. b: H&E staining and IHC staining for Col-1a1 and Tnmd of the hypoxia and scramble group and the hypoxia and shRNA group. H&E staining: scale bar = 200 μ m; magnification: \times 200; IHC staining: scale bar

= 200 μm ; magnification: $\times 400$. c: Histological scores of H&E staining and quantification of Col-1a1 and Tnmd after IHC staining in the hypoxia and scramble group and the hypoxia and shRNA group. Data were shown as mean \pm SD. $\square\square p < 0.01$.

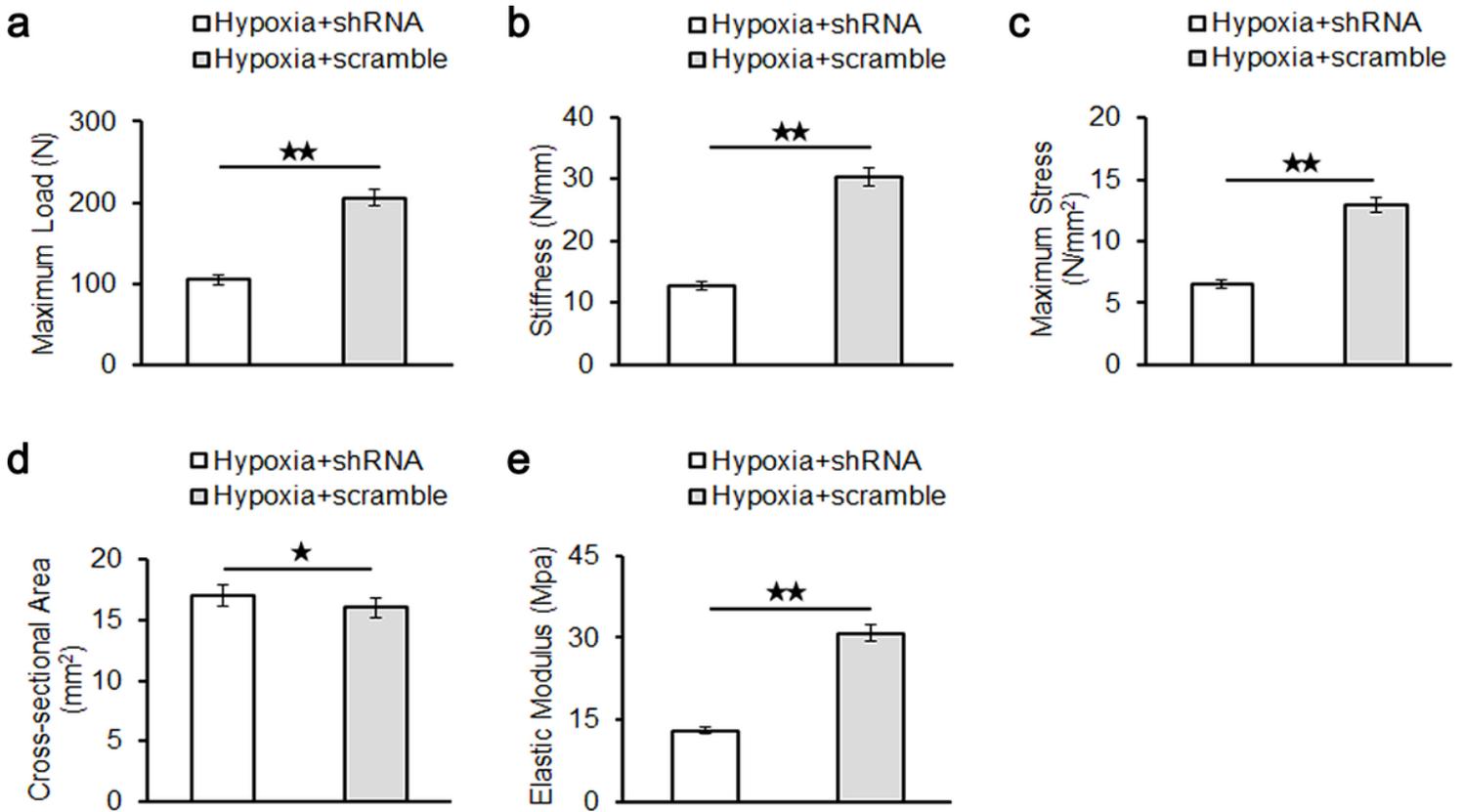


Figure 4

Biomechanical analysis of repaired patellar tendons at 4 weeks after surgery. Biomechanical analysis for maximum load to failure (a), stiffness (b), maximum stress (c), cross-sectional area (d) and elastic modulus (e) in the hypoxia and scramble group and the hypoxia and shRNA group. Data were shown as mean \pm SD. $\square p < 0.05$; $\square\square p < 0.01$.

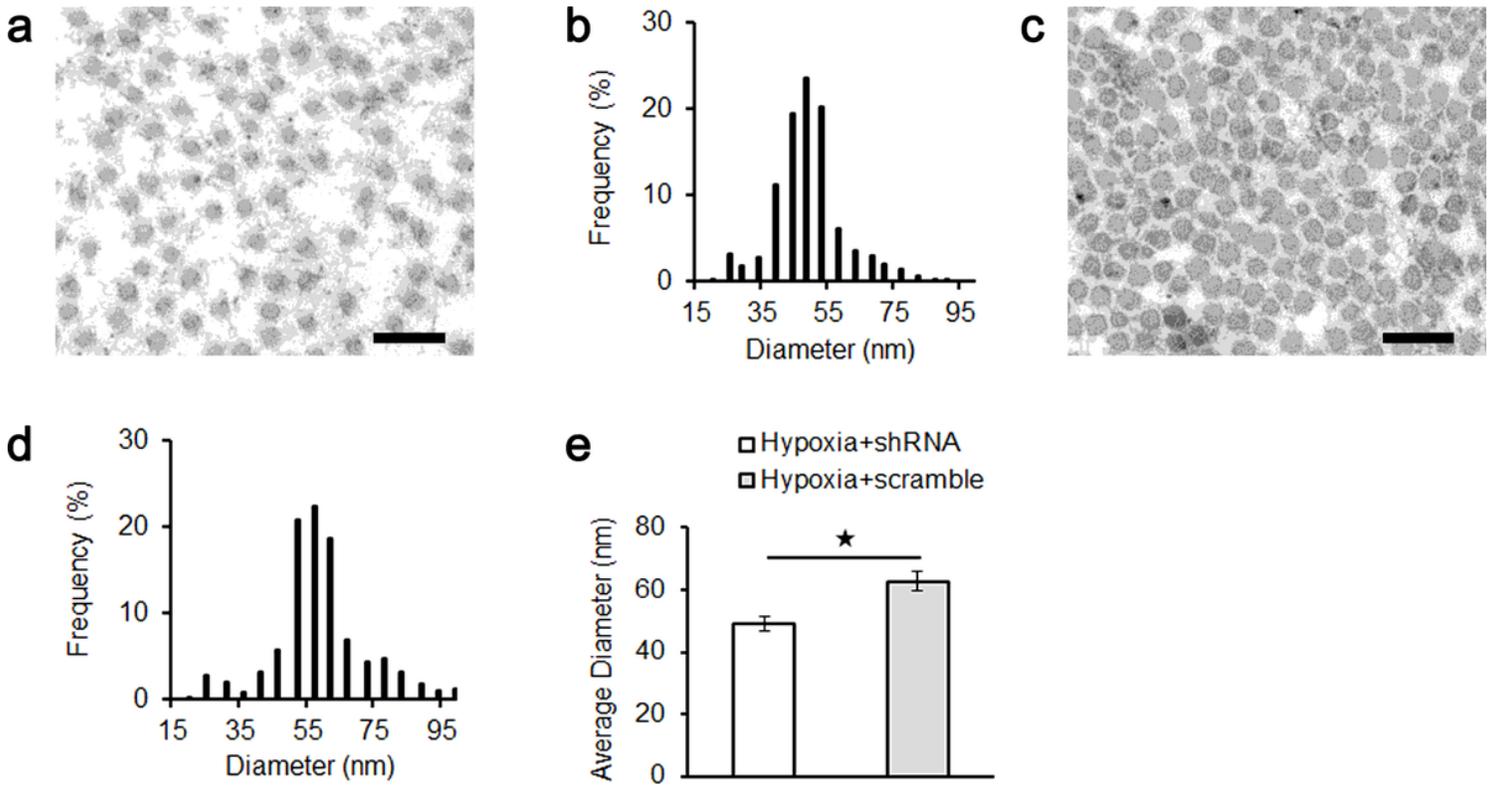


Figure 5

Ultrastructure of repaired patellar tendons at 4 weeks after surgery. Representative images of transmission electron microscopy (a) and the distribution of collagen fibril diameters (b) of the hypoxia and shRNA group. Representative images of transmission electron microscopy (c) and the distribution of collagen fibril diameters (d) of the hypoxia and scramble group. e: The average diameter of collagen fibrils in the hypoxia and scramble group and the hypoxia and shRNA group. Scale bar = 200 nm.

Magnification: $\times 15000$.

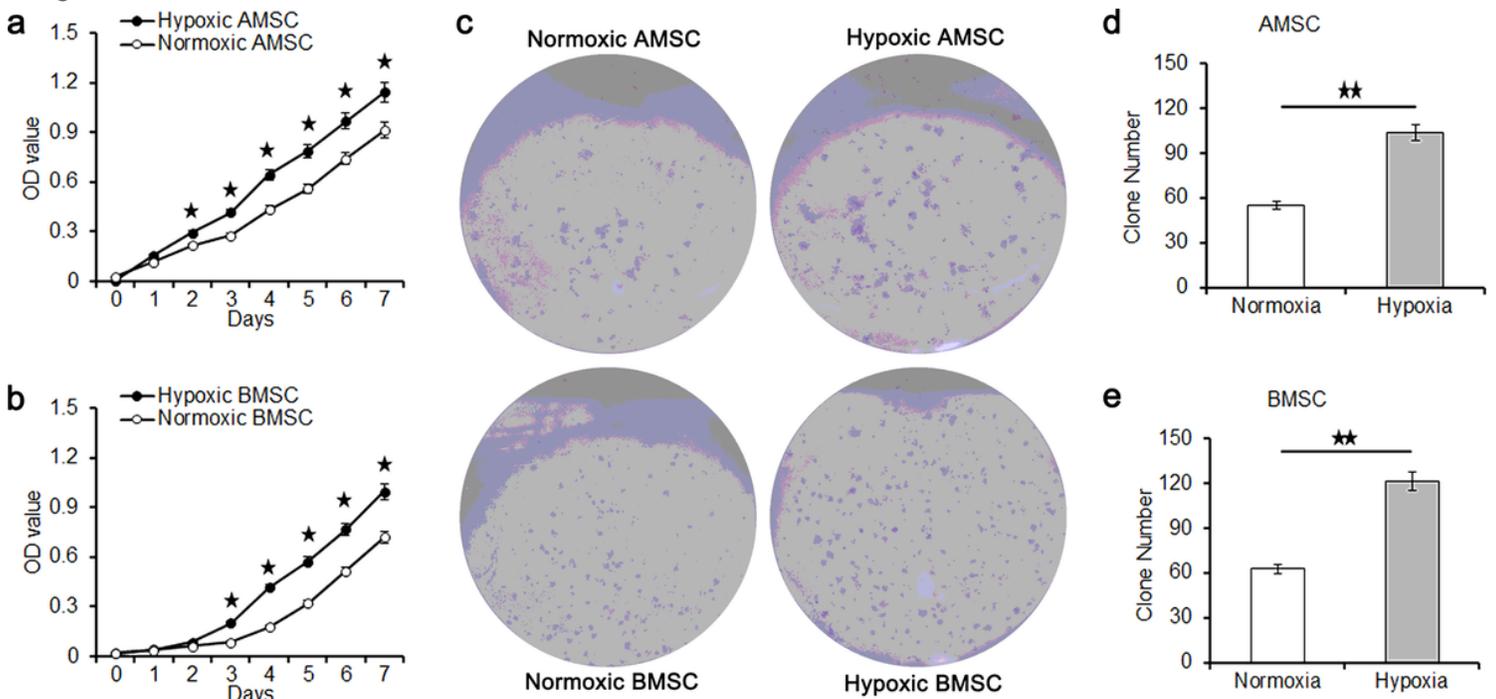


Figure 6

The OD values and the clone numbers of AMSCs and BMSCs under normoxia condition and hypoxia condition. a-b: The OD values of AMSCs (a) and BMSCs (b) under normoxia condition and hypoxia condition. c: Clone formation of AMSCs and BMSCs under normoxia condition and hypoxia condition. d-e: Clone numbers of AMSCs (d) and BMSCs (e) under normoxia condition and hypoxia condition. Data were shown as mean \pm SD. $\star p < 0.05$; $\star\star p < 0.01$.

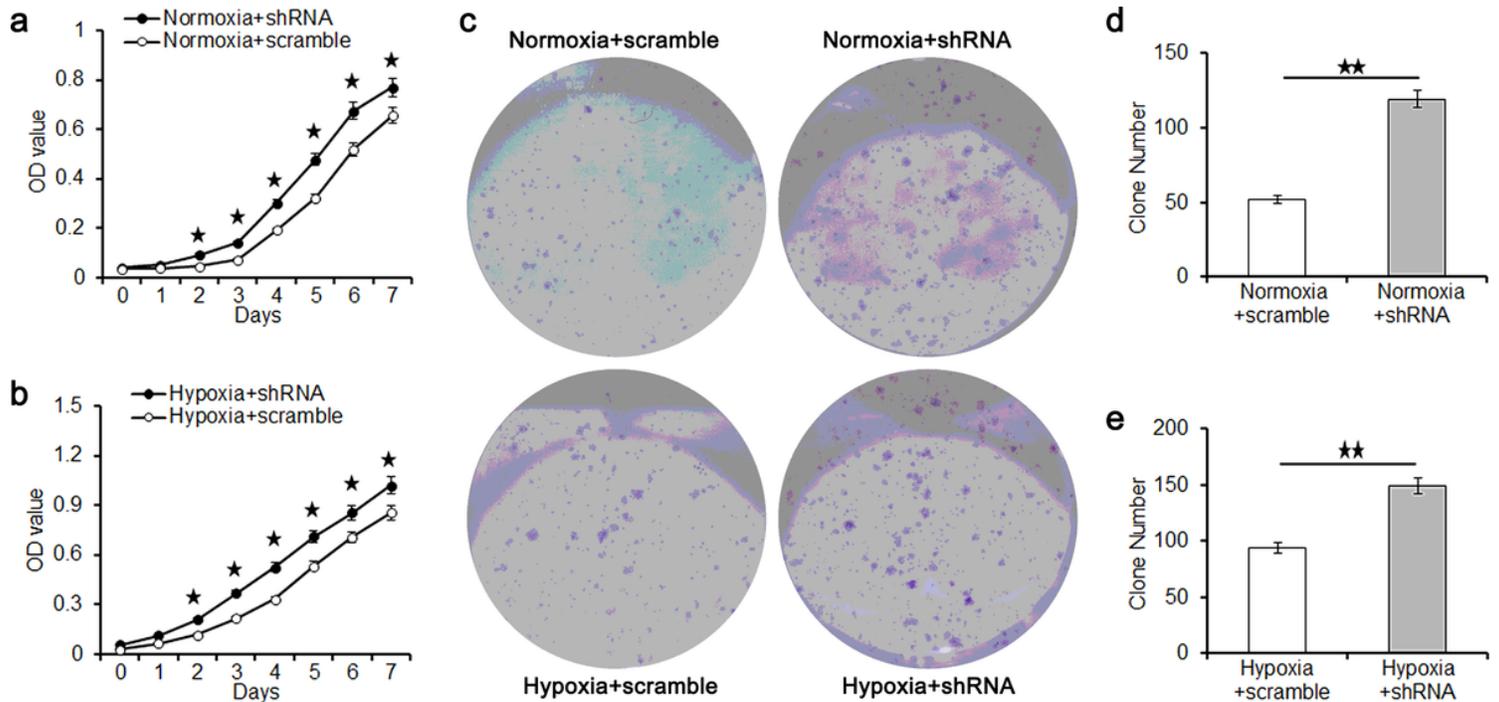


Figure 7

The OD values and the clone numbers of the scramble group and the shRNA group under normoxia condition and hypoxia condition. a-b: The OD values of the scramble group and the shRNA group under normoxia condition (a) and hypoxia condition (b). c: Clone formation of the scramble group and the shRNA group under normoxia condition and hypoxia condition. d-e: Clone numbers of the scramble group and the shRNA group under normoxia condition (d) and hypoxia condition (e). Data were shown as mean \pm SD. $\star p < 0.05$; $\star\star p < 0.01$.

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