Hypoxia promotes tenocyte differentiation of mesenchymal stem cells

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Research

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Abstract

Background: Tendon injury is a common but tough medical problem. Unsatisfactory clinical results have been reported in tendon repair using mesenchymal stem cells (MSCs) therapy, creating a need for a better strategy to induce MSCs to tenogenic differentiation. This study was designed to investigate the role of hypoxia in the tenogenic differentiation of MSCs in vitro and in vivo and to compare the tenogenic differentiation capacities of different MSCs under hypoxia condition in vitro. Methods: Adipose tissue-derived MSCs (AMSCs) and bone marrow-derived MSCs (BMSCs) were isolated and characterized by the expression of MSC-specific markers and tri-lineage differentiation. The expression of hypoxia induced factor-1 alpha (Hif-1α) and the proliferation of AMSCs and BMSCs were examined in order to confirm the establishment of hypoxia condition. qRT-PCR, western blot, and immunofluorescence staining were used to evaluate the expression of tendon-associated marker Col-1a1, Col-3a1, Dcn, and Tnmd in AMSCs and BMSCs under hypoxia and/or Tgf-β1 condition. In vivo, a patellar tendon injury model was established. Normoxic and hypoxic BMSCs were cultured and implanted. Histological, biomechanical and transmission electron microscopy analyses were performed to assess the improved healing effect of hypoxic BMSCs on tendon injury. Results: Hypoxia remarkably increased the expression of Hif-1α and the proliferation of AMSCs and BMSCs. Our in vitro results detected that hypoxia not only promoted a significant increase in tenogenic markers in both AMSCs and BMSCs compared with the normoxia group, but also showed higher inductility compared with Tgf-β1. In addition, hypoxic BMSCs exhibited higher potential of tenogenic differentiation than hypoxic AMSCs. Our in vivo results demonstrated that hypoxic BMSCs possessed better histological and biomechanical properties than those of normoxic BMSCs, as evidenced by histological scores, quantitative analysis of immunohistochemical staining for Col-1a1 and Tnmd, the range and average of collagen fibril diameters and patellar tendon biomechanical tests. Conclusions: These findings suggested that hypoxia may be a practical and reliable strategy to induce tenogenic differentiation of BMSCs for tendon repair and could enhance the effectiveness of MSCs therapy in treating tendon injury.

Introduction

Tendons are special connective tissues that transmit the force from muscles to bones, playing a key role in musculoskeletal system. Tendon injury is a very common soft tissue injury, afflicting about 30 million people each year and accounting for up to 50% of all sport-related injuries [1, 2]. Not only is tendon injury prevalent, it is also a tough clinic problem, due to its hard healing process because of hypovascularity and hypocellularity [3]. Many attempts, such as autologous grafting, application of growth factors and gene therapy, have been made to repair the injury, but often following the dysfunction of the donor site and a long and poor recovery [4, 5]. Therefore, the treatment strategy to repair tendon injury still needed to be explored.

Mesenchymal stem cells (MSCs) therapy has shown promise in treating tendon injury [6]. However, the effect of undifferentiated MSCs on repairing tendon is not satisfactory. No significant differences were found between the untreated group and the adipose tissue-derived mesenchymal stem cells (AMSCs)

group in all biomechanical variables at the 2 and 4 weeks after surgery in rotator cuff injury model [7]. Another study found that there was a significant increase in maximum load and stiffness in bone marrow-derived mesenchymal stem cells (BMSCs) group compared with the control group only at 2 weeks after surgery [8]. In addition, ectopic bone should be paid attention when undifferentiated MSCs were used to treat tendon injury. 28% of fluorescent-labeled BMSC-treated tendons was found ectopic bone compared to “natural healing” controls. On periphery of bone spicules, representative cells had fluorescence and were alkaline phosphatase positive [9].

Tenogenically differentiated MSCs could improve tendon repair in both histological scores and biomechanical properties. Many findings have shown that transforming growth factor-β1 (Tgf-β1) can promote tenogenic differentiation of MSCs and thus improve tendon repair. BMSCs transfected with the Tgf-β1 gene showed higher concentrations of collagen type I protein, larger fiber bundles and more rapid matrix remodeling [10]. Recent study found that tenocyte induced tenogenic differentiation of BMSCs by secreting exosomes containing Tgf-β1 [11]. BMP-induced tenogenic differentiation of AMSCs enhanced the expression of collagen type I and scleraxis and exhibited improved histological score and collagen fiber dispersion range compared to undifferentiated AMSCs [12]. Although the induction capacities were low, insulin-like growth factor 1 and basic fibroblast growth factor could still promote BMSCs to differentiate into tenocytes by upregulating collagen type I/III and scleraxis [13, 14]. However, healed tendon rarely achieves complete functionality equal to the pre-injured state. The final biomechanical properties of repaired tendon may be reduced by more than 30% [15]. As a result, the study on looking for new strategy to induce tenogenic differentiation is imperative.

Hypoxia is the microenvironment of both tenocytes and MSCs. The oxygen tension has been shown to be 1–10% in tendon [16], 1–2% in bone marrow and approximately 3% in adipose tissue [17]. Previous findings have shown that hypoxia could promote the replication and stemness of MSCs [18]. However, whether hypoxic MSCs could increase their differentiation potential to adipocyte, osteocyte and chondrocyte in vitro and in vivo has been controversial [16, 19]. As for tenogenic differentiation, only few evidences showed that the mRNA levels of Tenomodulin and Tenascin-C were increased under hypoxia condition [16, 20]. In addition, normoxic BMSC resulted in better ultrasound echogenicity score and up-regulation of Collagen type I, Decorin and Tenascin-C mRNAs than normoxic AMSC in vivo [21, 22]. However, there is no report on tenogenic differentiation capacities of AMSCs and BMSCs in hypoxia induction, which limits the knowledge for treatment of tendon injury.

The purpose of this study was to investigate the role of hypoxia in tenogenic differentiation of AMSCs and BMSCs compared with Tgf-β1, and the tenogenic differentiation capacities of AMSCs and BMSCs under hypoxia condition. Our results suggested that hypoxia could promote tenogenesis of AMSCs and BMSCs more effectively than Tgf-β1, and that hypoxic BMSCs showed higher tenogenic differentiation capacities than hypoxic AMSCs.

Materials And Method

Animals

A total of thirty New Zealand rabbits were used in this experiment (2.5–3.5 kg, 3–5 months old). Six rabbits were used for isolating AMSCs and BMSCs, and the rest were randomly divided into the phosphate buffer saline (PBS) group, the normoxia group, the hypoxia group and the control (normal) group. Before experiment, each rabbit was checked for general health. During the following experiment, each rabbit was housed in a commercial animal cage (49 cm×35 cm×32 cm) with 12 h/12 h light/dark cycle, kept at room temperature with free access to food and water.

Isolation and culture of AMSCs and BMSCs

AMSCs were isolated from the inguinal adipose tissue digested by DME/F12 medium containing 0.2% collagenase type I (Sigma, USA) in 37 °C for 2 h. The tissue residues were filtered by 200-mesh sieve, centrifuged at 350 x g for 5 min, resuspended in DME/F12 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% CO_2. The medium was changed every 2 days and the cells (passage 0, P0) were subcultured when 90% confluence was reached. BMSCs were collected from the bone marrow extruded by inserting a 22-gauge needle into the shaft of the femora and flushed out with DME/F12 medium containing 15% FBS and 1% penicillin/streptomycin/amphotericin B. After repetitively pipetting, the medium containing the whole bone marrow was then plated onto 10-cm cell culture dishes at 37 °C with 5% CO_2. After 48 h, half of the initial culture was changed by fresh culture medium. The cells (P0) typically reached 80-90% confluence within 9 days and were harvested for subcultures. Under hypoxia condition, cells were cultured in a tri-gas incubator maintained 1% O_2, 5%CO_2, and 94% N_2 (MCO-5M, SANYO, Japan). Under Tgf-β1 condition, cells were cultured in complete culture medium contained 10ng/ml Tgf-β1 (Sigma, USA). The P2-P5 cells were used in the experiment.

Flow Cytometry

Flow cytometry analysis was taken to confirm surface antigen marker of MSCs. 1×10^6 cells at passage 3 (P3) in the logarithm growth period were collected, washed twice with 1% pre-cooled FBS/PBS, and centrifuged at 350 x g for 5 min. Cells were incubated with anti-CD34-PE (GeneTex, USA), anti-CD45-APC (Invitrogen, USA), anti-CD44-APC (Novus Biologicals, USA), and anti-CD29-FITC (Invitrogen, USA) for 30 min at 4 °C in the dark, respectively. Labeled cells were washed twice, examined using the FACScan flow cytometry system (BD, Franklin Lakes, USA). Data were analyzed with the FlowJo software (TreeStar, Ashland, OR, USA). PBS solution was used for negative control staining.

Immunofluorescence staining

MSCs were cultured on laser confocal dishes, fixed with 4% formalin for 15 min when they reached 60% confluence, blocked with Immunol Staining Blocking Buffer (containing Triton X-100 for permeabilization; Beyotime, China) for 1 h, followed by incubating with hypoxia induced factor-1 alpha (Hif-1α, 1:300, Bioss, Beijing, China) and Tenomodulin (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, followed by counterstaining with DAPI for 5 min. The prepared samples were observed 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). For CD molecular identification, the cells were blocked with normal goat serum prior to incubation with the following antibodies: CD34-PE (GeneTex, USA), CD45-APC (Invitrogen, USA), CD29-FITC (Invitrogen, USA) and CD90-FITC (BioLegend, USA) overnight at 4 °C in dark place.

Chondrogenic, adipogenic and osteogenic differentiation

For chondrogenic differentiation, 3×10^5 MSCs were collected and washed 3 times with chondrogenic induction medium (RBXMX-90042, Cyagen, China) under centrifugation at 350 x g for 5 min. The pellet was cultured in 0.5ml chondrogenic induction medium in the 15-mL tube. After 24 h, the pellet was suspended by slightly knocking the bottom of the tube. The medium was replaced every 2 days and the pullet was cultured for 28 days. For the staining, the pellet was fixed with 4% formalin, embedded, dewaxed, dehydrated and stained with Alcian Blue 8GX solution for 30 min. For adipogenic differentiation, the cells were seeded at a density of 2×10^4 cells/cm^2 and cultured in DME/F12 complete medium until they reached 100% confluence. Then the culture medium was changed with adipogenic induction medium A (RBXMX-90031, Cyagen, China). After 72 h, the medium was changed with adipogenic induction medium B (RBXMX-90031, Cyagen, China). After 24 h, the medium was changed with adipogenic induction medium A. After repeating 5 times, the cell were cultured with adipogenic induction medium B for 7 days. For the staining, the cells was fixed with 4% formalin for 30 min, washed and stained with Oil Red O solution for 30 min. For osteogenic differentiation, the bottom of the culture dish was covered by 0.1% gelatin in order to prevent cell shedding. MSCs were seeded at a density of 2×10^4cells/cm^2 and cultured in DME/F12 complete medium until they reached 60%-70% confluence. Then the culture medium was changed with osteogenic induction medium (RBXMX-90021, Cyagen, China). The medium was replaced every 2 days during the induction process which lasted for 28 days. For the staining, the cells was fixed with 4% formalin for 30 min, washed and stained with Alizarin Red solution for 5 min.

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 or hypoxia condition. The culture medium was changed every 2 days. After adding 10 μl CCK-8 assay solution (Dojindo, Japan) in each well and incubating 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 (CFU) assay.

AMSCs (500 cells/well) and BMSCs (1000 cells/well) were seeded into 6-well plates, respectively, and cultured at 37 °C under normoxia or 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 tenogenic genes were measured using quantitative real-time PCR (qRT-PCR). Total RNA was extracted using RNAiso plus according to the manufacturer's protocol (TaKaRa, Japan). cDNA was synthesized using a reverse transcription kit (TaKaRa, Japan). qRT-PCR analysis was performed using the CFX96 Real-Time PCR Detection System (Rotor-Gene Q 2plex, Germany). The conditions of qRT-PCR were as follows: 95 °C for 30 s, followed by 39 cycles of 95 °C for 5 s and 60 °C for 30 s, and a final extension at 95 °C for 10 s. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences of the primers were shown in Table 1. No nonspecific amplification was found by the melting curve. Data were acquired from at least five independent samples and tested at least three times. The mRNA expression levels relative to β-actin were quantified using the $2^{-\Delta\Delta CT}$ method.

Table 1. Primers used in qRT-PCR.

Gene	Primer sequences	Accession no.	Product length(bp)
Col-1a1	F:TGGCGAGCCTGGAGCTTCTG R:GCTTCTCCGTCATCTCCGTTCTTG	NC_013687.1	81
Col-3a1	F:TCCTGGTGCTATTGGTCCGTCTG R:TCCGTCGAAGCCTCTGTGTCC	NC_013675.1	160
Dcn	F:CAGTGTCACCTTCGAGTTGTCCAG R:AGGTCCAGTAGCGTCGTGTCAG	NC_013672.1	89
Tnmd	F: CGCCAGACAAGCAAGTGAGGAAG R: CACGGCGGCAGTAGCGATTG	NC_013690.1	134
β-Actin	F: CATCCTGGCCTCGCTCTCCAC R: AAAGCCATGCCAATCTCGTCT	NW_003159504.1	180

F: Forward; R: Reverse.

Western blot

Proteins were extracted using RIPA Lysis Buffer (Shanghai Weiao Biotechnology Co., Ltd., China), and the concentration was measured using the BCA protein reagent kit (Beijing Solarbio Science & Technology Co., Ltd., China). Proteins (30 μg) were separated on SDS-PAGE gels (8%), transferred onto a

polyvinylidene difluoride (PVDF) membrane, and blocked with 5% skim milk for 1 h at room temperature. The PVDF membranes were incubated with anti-Hif-1α (1:400), anti-Collagen type I (Col-1a1, 1:400), anti-Collagen type III (Col-3a1, 1:400), anti-Decorin (Dcn, 1:400), and anti-Tnmd (1:400) primary antibodies solution (Bioss, Beijing, China) and anti-β-Actin (1:1000) primary antibody solution (BOSTER Biological Technology Co., Ltd, China) overnight at 4 °C. After incubating with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (BOSTER Biological Technology Co., Ltd, China) at room temperature for 1 h, the membranes were reacted with ECL hypersensitive chemiluminescence kit (Shanghai Weiao Biotechnology Co., Ltd., China) according to the manufacturer's protocol.

Tendon Injury Model

The two hind limbs of each rabbit were operated for patellar tendon injury model (n=12 in each group). The technique was based on a previous study [23] and was modified according to our study design. Rabbit were anesthetized with chloral hydrate (500 mg/kg, i.p.). A longitudinal skin incision over the patellar tendon was made. Subcutaneous fascia was longitudinally severed (Fig. 4A). The middle part of the patellar tendon was transversely severed (Fig. 4B). The retraction of the broken end was 2 mm (Fig. 4C). 50 μl PBS without or with 10^6 MSCs was injected into the wound gap followed by suturing the subcutaneous fascia (Fig. 4D). After the skin was sutured, plaster casts were performed with the knee in full extension. Postoperatively, the rabbits were replaced to their own cages, and given an intramuscular injection of cefazolin sodium (0.1 g/kg, q.d) for 3 days. The plaster casts were removed after 4 weeks of immobilization.

Histological analysis

Rabbit were suffocated at 4 weeks after surgery for histological analysis. The surgical site (i.e. middle part) of patellar tendon was collected, fixed with 4% formalin, dehydrated using graded ethanol, vitrified using dimethylbenzene and embedded in paraffin. For hematoxylin and eosin (H&E) staining, paraffin sections with a thickness of 4 μm were dewaxed, hydrated and stained with hematoxylin and eosin. After dehydration and transparency, the sections were sealed with neutral balsam. For masson's trichrome staining, the sections were stained according to the manufacturer's protocol (Beijing Solarbio Science & Technology Co., Ltd., China). The collagen fibers and muscle fibers were stained in blue and in red, respectively. For immunohistochemical staining, paraffin sections were incubated in citrate antigen retrieval solution at 95 °C for 20 min. Endogenous peroxidase was blocked using 3% hydrogen peroxide for 15 min. After blocking with 10% normal goat serum (BOSTER Biological Technology Co., Ltd, China), the sections were incubated with antibodies to Col-1a1 and Tnmd (Bioss, Beijing, China) overnight at 4 °C. After rewarming at room temperature for 30 min, the sections were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (BOSTER Biological Technology Co., Ltd, China) for 30 min at room temperature. The diaminobenzidine (Dako, Glostrup, Denmark) working solution was used for color development. The nuclei were stained with hematoxylin.

Biomechanical Testing

Rabbit were suffocated at 4 weeks after surgery for biomechanical analysis as described in our previous study [24]. The patella-patellar tendon-tibial tubercle was harvested and stored at −80 °C. The specimens were kept at 4 °C overnight prior to the biomechanical test. After measuring the length and width of the patellar tendon, the biomechanical property was tested with the patella and the tibial tubercle mounted on aluminum clamps of a biomechanical testing machine (SPL-10 KN, Shimadzu, Japan). The patellar tendon was loaded along the vertical axis until failure at a displacement rate of 10 cm/min. The maximum load to failure, cross-sectional area, maximum stress, stiffness at failure and elastic modulus were calculated.

Transmission electron microscopy (TEM) analysis

TEM was used to analyze the diameter of collagen fibrils in regenerated tendons. The sample was first fixed in 2.5% glutaraldehyde solution at 4 °C overnight, and then was postfixed with 1% OsO4 solution for 1 h. After the sample was dehydrated by a graded series of ethanol, it was infiltrated by acetone and embedding medium, and embedded with absolute embedding medium at 37 °C overnight and then at 65 °C for 48 h. At least 3 sections with a thickness of 60 μm were obtained from each sample using ultramicrotome (Leica Leica UC7, Germany), and were stained by uranyl acetate and alkaline lead citrate for 15 min, respectively, and were observed under TEM (Hitachi HT7700, Japan).

Statistical analysis

SPSS version 16.0 was used in this study. Data were identified as homogenous variances and normal distribution when $P > 0.1$ using the Levene's test and the Shapiro-Wilk test. All data were presented as the mean ± standard deviation (SD). Data with homogenous variances and normal distribution were compared using one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Identification of AMSCs and BMSCs

Analysis of AMSC and BMSC characteristics was performed using flow cytometry and immunofluorescence staining. Flow cytometry analysis showed that both MSCs expressed a series of markers which are considered to be specific to mature MSCs, including CD29 (95.8% in AMSCs, 95.5% in BMSCs), CD44 (95% in AMSCs, 99.5% in BMSCs), but did not express hematopoietic lineage markers CD34 (0.94% in AMSCs, 0.36% in BMSCs) and CD45 (0.31% in AMSCs, 0.22% in BMSCs) (Fig. 1A). Laser scanning confocal microscopic images of immunofluorescence-labeled AMSC and BMSC detected positive signals for MSC specific markers CD29 and CD90 and no signal for CD34 and CD45 (Fig. 1B). To examine the multipotential differentiation capability, AMSCs and BMSCs were cultured in commercial induction medium in order to induce differentiation towards chondrogenic, adipogenic and osteogenic lineages. Both MSCs exhibited fibroblast-like morphology under light microscope. Treated with

chondrogenic differentiation media, both AMSCs and BMSCs contained plenty of cartilage specific acid mucopolysaccharide stained with Alcian Blue solution. Under adipogenic induction medium, they contained red lipid droplet after Oil Red O staining. Upon osteogenic induction treatment, they were able to mineralize and deposit red calcium nodules, as identified by Alizarin Red staining (Fig. 1C).

Hypoxia promotes the expression of Hif-1α and the proliferation of AMSCs and BMSCs

The expression of Hif-1α. To establish hypoxia condition, we examined the expression level of Hif-1α by western blot and immunofluorescence staining using laser scanning confocal microscope in BMSCs at 7 days after hypoxic induction. Western blot showed Hif-1α was nearly not expressed in normoxic BMSCs in the vicinity of 120 kDa, whereas it was abundantly expressed in hypoxic BMSCs (Fig. 2A). Immunofluorescence staining showed the same results to western blot analysis (Fig. 2B). Quantitative analysis detected that the average optical density (AOD) of Hif-1α was significantly higher under hypoxia condition compared with the nomoxia condition (Fig. 2C).

The proliferation of AMSCs and BMSCs. To investigate the role of hypoxia in the proliferation of AMSCs and BMSCs, CCK-8 and CFU assays were used. The results of CCK-8 assay revealed that hypoxia promoted the proliferation of both MSCs (Fig. 2D-E). From the 3rd day, the OD values in both AMSCs and BMSCs were significantly increased in hypoxia induction than those in normoxia. In addition, hypoxia enhanced the colony-forming ability of both MSCs (Fig. 2F). CFU assays detected that hypoxic AMSCs and hypoxic BMSCs remarkably increased their clone number, which were 2-fold higher than that of normoxic AMSCs and normoxic BMSCs, respectively (Fig. 2G-H).

Hypoxia increases the expression level of tenogenic differentiation markers in both MSCs in vitro.

Col-1a1, Col-3a1, Dcn and Tnmd were used as tenocyte-lineage markers. Tenogenic differentiation was assessed based on the expression of these markers at both mRNA and protein levels at 7 days after induction.

Hypoxia promotes tenogenic differentiation of AMSCs and BMSCs. As shown in Fig. 3A-D, the mRNA levels of all four tenogenic genes in both MSCs were significantly increased in hypoxia induction than those in normoxia. In addition, the mRNA levels of Tnmd in hypoxia induction was the highest among the four genes in both MSCs. Significantly upregulated protein expression levels of all four tenogenic markers in both MSCs were also observed in hypoxia induction than those in normoxia (Fig. 3E-I). Similar results were found in immunofluorescence staining, which showed that the AOD of Tnmd in hypoxic AMSCs and hypoxic BMSCs was significantly higher than that in normoxic AMSCs and normoxic BMSCs, respectively (Fig. 3J-K).

Hypoxic BMSCs exhibited the higher potential of tenogenic differentiation than hypoxic AMSCs. As shown in Fig. 3A-D, the mRNA levels of all four tenogenic genes in hypoxic BMSCs were higher than hypoxic AMSCs, although the differences were not significant. However, when the protein levels of the four tenocyte-lineage markers were tested, all but Col-31a1 were significantly higher in hypoxic BMSCs

than those in hypoxic AMSCs (Fig. 3E-I). The protein level of Tnmd examined by western blot was similar to that detected by laser scanning confocal microscope, under which the AOD of Tnmd in hypoxic BMSCs was significantly higher than that in hypoxic AMSCs (Fig. 3J-K).

Hypoxia shows the higher inductility compared with Tgf-β1. As shown in Fig. 3A-D, the mRNA levels of all four tenogenic genes in both MSCs were higher in hypoxia induction than those in Tgf-β1 induction, although only the difference of Tnmd was significant. However, western blot analysis showed that the expression of protein level of the four tenocyte-lineage markers in both MSCs were significantly higher in hypoxic induction than those in Tgf-β1 induction (Fig. 3E-I). Immunofluorescence staining detected that the AOD of Tnmd in both MSCs was significantly higher in hypoxia induction than that in Tgf-β1 induction, which was similar to the western blot analysis (Fig. 3J-K).

Hypoxia and Tgf-β1 don't induce synergistically MSCs to tenogenic differentiation. Tgf-β1 enhanced the mRNA expression of Col-1a1 in BMSCs (Fig. 3A), the protein expression of Col-3a1 in AMSCs (Fig. 3G), and the mRNA and protein expression of Dcn and Tnmd in AMSCs and BMSCs (Fig. 3C-D, H-I). Hypoxia promoted the ability of Tgf-β1 to induce tenogenic differentiation, as shown by the mRNA and protein levels of all four tenogenic genes in both MSCs were higher in hypoxia and Tgf-β1 induction than those in Tgf-β1 induction alone (Fig. 3A-I), except for the mRNA expression of Col-3a1 in AMSCs (Fig. 3B). However, Tgf-β1 inhibited the ability of hypoxia to induce tenogenic differentiation, as shown by the mRNA and protein levels of all four tenogenic genes in both MSCs were lower in hypoxia and Tgf-β1 induction than those in hypoxia induction alone (Fig. 3A-I).

Hypoxia increases the expression level of tenogenic differentiation markers in BMSCs in vivo.

The inductility of tenegenosis under hypoxia condition was examined in vivo. Normoxic and hypoxic BMSCs were cultured for 7 days, and then were injected into the wound gap of patellar tendon after surgery (Fig. 4A-D), respectively. After 4 weeks, the repaired tendons with patella and tibial tubercle were removed for further analysis.

H&E staining. As shown in Fig. 4E-H, there was a remarkable defect in the PBS group at 4 weeks after surgery. However, when applied normoxic BMSCs into wound gap, the injured tendon was repaired better than that in the PBS group under gross observation. The repair was further improved in the hypoxia group, which was still obviously different with the normal tendon. The difference between the four groups in tenogenic differentiation was evaluated by H&E staining. As shown in Fig. 5A, there were large empty spaces and relatively fewer cells within irregularly arranged collagen fibers in the PBS group. In addition, bold vessels extended into empty spaces were observed. In the normoxia group, the collagen fiber became compact, and fewer empty spaces and more cells were found. The cellularity of repaired tendon continuously increased in the hypoxia group. The arrangement of collagen fiber was further improved, as shown by few vessels and empty spaces. However, the histological properties of the hypoxia group were still remarkably different to those in the control group, which had regularly arranged fibers and fewer cells and vessels. Quantitative analysis showed that the histological score of the normoxia group was significantly lower than that in the PBS group, but was significantly higher than that in the hypoxia group.

The histological score of the hypoxia group was significantly higher than that in the control group (Fig. 5B).

Masson's trichrome staining. Masson's trichrome staining was used in order to evaluate the formation of tendon-like tissues. As shown in Fig. 5A, few formation of collagen (shown in blue) was seen in the PBS group. By contrast, a large amount of muscle fibers (shown in red) was observed instead. In the normoxia group, more tendon-like tissues were deposited, but were apparently fewer than that in the hypoxia group. In the control group, the patellar tendon was abundant in fibrous matrix which was stained in blue, whereas very few muscle fibers occurred in the tendon.

Immunohistochemical staining. As shown in Fig. 5A, immunohistochemical staining of Col-1a1 and Tnmd was applied to examine the difference of tenogenic differentiation in the four groups. Both Col-1a1 and Tnmd were slightly stained in the PBS group. The staining of Col-1a1 and Tnmd was deeper in the normoxia group, and became more deeply in the hypoxia group. Quantitative analysis of the average optical density (AOD) of Col-1a1 (Fig. 5C) and Tnmd (Fig. 5D) found that the PBS group showed the lowest AOD among the four groups. The AOD of the hypoxia group was significantly higher than that of the nomoxia group. The staining of Col-1a1 and Tnmd was the deepest in the control group, which had the highest AOD in the four groups.

Ultrastructural morphology of collagen fibrils. The diameters of collagen fibrils in repaired tendon determined the biomechanical properties of the tendon. As a result, transmission electron microscopy (TEM) was used to analyze the diameters of the fibrils at 4 weeks after surgery. We calculated the range of collagen fibril diameters, and found that most diameters of collagen fibrils ranged from 25 to 46 nm in the PBS group, 45 to 55 nm in the normoxia group, 53 to 73 nm in the hypoxia group and 159 to 256 nm in the control group (Fig. 6A). The average diameter of collagen fibrils in the normoxia group was significantly larger than that in the PBS group, but was significantly smaller than that in the hypoxia group. However, the average diameter of collagen fibrils in the hypoxia group was remarkably smaller than that in the control group (Fig. 6B).

Biomechanical properties of repaired tendons. The maximum load to failure (Fig. 6C), stiffness at failure (Fig. 6D), maximum stress (Fig. 6E) and elastic modulus (Fig. 6G) were significantly higher in the normoxia group, compared with those in the PBS group, but were significantly lower than those in the hypoxia group, which were remained significantly lower than those in the control group except for stiffness. However, the cross-sectional area (Fig. 6F) was significantly larger after surgery, with the largest in the PBS group, the smallest in the hypoxia group which was still significantly larger than that in the control group. The maximum load to failure, stiffness at failure, maximum stress and elastic modulus in the PBS group at 4 weeks after surgery exhibited 32.8%, 31.5%, 20.3%, and 22.3%, respectively, of those in the control group, whereas those in the normoxia group exhibited 56%, 49.3%, 38.9%, and 38.45%, respectively. The biomechanical properties were improved persistently in the hypoxia group, evidenced by the finding that the maximum load to failure, stiffness at failure, maximum stress and elastic modulus exhibited 80.2%, 87.5%, 50.9%, and 63.5%, respectively, of those in the control group.

In addition, the relationship between histological score and elastic modulus was evaluated. As shown in Fig. 6H, the results from each group demonstrated that there was a direct but negative relationship between the decreasing scores and the increasing biomechanical property. Histological scores greater than 4 (the PBS group and the normoxia group) correlated with elastic modulus less than 40% of that of a normal control. By contrast, histological scores below 4 (the hypoxia group) were associated with elastic modulus greater than 60% of that of a normal control. Regression analysis detected that the coefficient of determination (R^2) was 0.9813, which indicated that there was a strong correlation between the histological appearances and the biomechanical characteristics in regenerated tendons.

Discussion

Although different findings have been reported in tri-lineage differentiation of MSCs in hypoxia induction [16, 19], few, if any, studies have conducted the potential of tenogenic differentiation of different MSCs under hypoxia condition. In this study, we showed that the associated markers of tenogenesis, such as Col-1a1, Col-3a1, Dcn and Tnmd, were significantly increased in hypoxic AMSCs and hypoxic BMSCs in vitro, compared with those in normoxic MSCs., and the higher expression of Col-1a1 and Tnmd in hypoxic group in vivo was similar to a previous finding showing that transplantation of hypoxic MSCs may be a more effective treatment than normoxic MSCs for Achilles tendon ruptures [23]. In addition, hypoxic BMSCs had greater potential of tenogenic differentiation than hypoxic AMSCs. Our data further demonstrated that the inductivity of hypoxia in tenogenic differentiation of AMSCs and BMSCs was significantly greater than that of Tgf-β1.

Few tendon-specific markers have been identified. Col-1a1 and Col-3a1 are considered to be the main tendon collagen in matrix [5], especially Col-1a1 which has often been used as a tendon-associated marker in tenogenic differentiation study [13, 25]. Tnmd is a member of type II transmembrane glycoprotein. The reduced cell numbers and the altered structure of collagen fibrils in adult tendons of Tnmd-deficient mice suggested Tnmd was a marker of differentiated tenocytes in mature tendon [26]. Dcn is the most abundant proteoglycan in tendon tissue. The fact that decorin-deficient mice showed aberrant collagen fibrils indicated that decorin binding was required for appropriate assembly of collagen fibrils [27]. In our study, we analyzed the expression characteristic of those tenogenic markers in hypoxia induction, and detected that Tnmd had the highest mRNA expression, which showed nearly 5-fold to control in AMSCs and more than 5-fold to control in BMSCs, whereas Col-1a1, Col-3a1, and Dcn all exhibited less than 2-fold to control. Our findings were similar to a previous study which has found that compared with E11.5 tendon progenitor cells, Tnmd was the second most differentially expressed gene, displaying a 376-fold change in the top 100 upregulated genes in E14.5 differentiated tendon cells, whereas Dcn and Col-1a1 displayed a 36-fold change and a 14-fold change, respectively [28]. Considering the also significantly increased protein levels of Tnmd in hypoxia induction shown in western blot analysis and confocal scanning, we believed hypoxia promoted tenogenic differentiation of MSCs mainly by enhancing the expression of Tnmd.

The biological characteristic of AMSC and BMSC has been reported to be significantly different. The concentration of AMSCs in adipose tissue is about 2%, while BMSCs constitute very little proportion of the cells, about 0.01–0.001% in bone marrow [29, 30]. AMSCs possessed the higher proliferation potential and colony frequency [29, 31], and exhibited greater tolerance to apoptosis [32], compared with BMSCs. The fact that regenerated tendon treated with BMSCs exhibited an earlier improvement and a higher mRNA expression of Col-1a1, Col-3a1, Dcn and Tnmd compared with AMSCs indicated the potential of tenogenic differentiation of BMSCs was significantly stronger than that of AMSCs [21]. Our results demonstrated the similar result that the potential of tenogenic differentiation of hypoxic BMSCs was also significantly stronger than that of hypoxic AMSCs.

In order to better examine the role of hypoxia in tenogenesis, we compared the inductivity of tenogenic differentiation between hypoxia and Tgf-β1, and revealed that the protein levels of Col-1a1, Col-3a1, Dcn and Tnmd under hypoxia condition in both AMSCs and BMSCs were all significantly higher than those in the presence of Tgf-β1. Previous study has found that the mRNA expression levels of Tnmd in BMSCs after 7 days in Tgf-β1 induction did not show any significant difference with that at 3 days after Tgf-β1 induction [33]. This result suggested that the potential of tenogenic differentiation of MSC did not increase with induction time of Tgf-β1. Recent data showed that after Tgf-β1 administrations in a rat rotator cuff repair model, the mRNA levels of Col-1a1, Col-3a1, and Tnmd in tendon formation were slightly increased at all time points after surgery, but with no significantly difference compared with the normal tendon. However, the expression of matrix metalloproteinase-9 (MMP-9) and MMP-13 was significantly decreased at 2 weeks postoperatively [34]. These findings indicated that Tgf-β1 increased collagen accumulation by inhibiting MMP-9 and MMP-13 expression to enhance tendon formation at the healing site. These may be possible explanations why Tgf-β1 had a relative low inductivity in comparison to hypoxia, which directly promoted the expression of tenogenic markers of MSCs and thus resulted in higher expression of these markers and better recovery of injuried tendon in our study.

In addition, we showed that hypoxia significantly promoted proliferation of both AMSCs and BMSCs. This was consistent with previous reports demonstrating that hypoxia increased clonogenicity and encouraged self-renewal capacity of MSCs [18]. Our study did not seed fluorescent-labeled MSCs in the wound gap to investigate the role of hypoxia in tenogenesis in vivo. However, previous study reported that BrdU-labeled MSCs can be observed at 4 weeks after transplantation, and the percentages of the hypoxic MSCs were increased, compared to the normoxic MSCs [23]. This indicated that transplanted MSCs can be able to survive and proliferate in vivo at least at 4 weeks postoperatively, and thus contributed to a corresponding role in tendon healing. Therefore, there were valid reasons to believe that the histological and biomechanical analyses in the present study, which also lasted 4 weeks after surgery, were the results came from the role of the transplanted MSCs. Further studies are needed to investigate the mechanisms underlying tenogenic differentiation of MSCs in hypoxia induction.

Conclusion

In conclusion, we have investigated the role of hypoxia in tenogenic differentiation of AMSCs and BMSCs and conducted histological and biomechanical analyses in patellar tendon injury model. Our data showed that hypoxia can significantly enhance the expression of Col-1a1, Col-3a1, Dcn and Tnmd in AMSCs and BMSCs in vitro. Hypoxic BMSCs possessed higher levels of tenogenic markers than hypoxic AMSCs. In addition, the expression levels of tenogenic markers in both MSCs in hypoxia were significantly higher than those in Tgf-β1. In vivo, hypoxic BMSCs exhibited improved histological appearances and biomechanical properties and thus produced superior healing compared to normoxic BMSCs.

Abbreviations

AMSCs: Adipose tissue-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; H&E: Hematoxylin and eosin; Hif-1α: Hypoxia induced factor-1 alpha; MMP: Matrix metalloproteinase; MSCs: Mesenchymal stem cells; OD: Optical density; P: Passage; PBS: Phosphate buffer saline; PVDF: Polyvinylidene difluoride; qRT-PCR: Quantitative real-time polymerase chain reaction; SD: Standard deviation; TEM: Transmission electron microscopy; Tgf-β1: Transforming growth factor-β1; 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

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GYC designed the study, performed the experiments, and wrote the manuscript. GYC, WQZ, JTG and YG isolated and identified MSCs, carried out qTR-PCR, WB and immunofluorescence assays and analyzed the data. LH, SNW, WNL and CZ conducted the animal experiment, carried out histological and biochemical tests and analyzed the data. ML, WZ, QH, YQZ supervised the study, reviewed the data, and edited the manuscript. All authors read and approved the final manuscript.

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References

1. Maffulli N, Wong J, Almekinders LC. Types and epidemiology of tendinopathy. Clin Sports Med. 2003;22:675-92.
2. Kannus P. Tendons–a source of major concern in competitive and recreational athletes. Scand J Med Sci Sports. 1997;7:53-4.
3. Lui PP. Stem cell technology for tendon regeneration: current status, challenges, and future research directions. Stem Cells Cloning. 2015;8:163-74.
4. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71.
5. Lipman K, Wang C, Ting K, Soo C, Zheng Z. Tendinopathy: injury, repair, and current exploration. Drug Des Devel Ther. 2018;12:591-603.
6. Docheva D, Muller SA, Majewski M, Evans CH. Biologics for tendon repair. Adv Drug Deliv Rev. 2015;84:222-39.
7. Valencia MM, Antuna AS, Garcia AM, Carrascal MT, Barco R. Application of adipose tissue-derived stem cells in a rat rotator cuff repair model. Injury. 2014;45 Suppl 4:S22-7.
8. Degen RM, Carbone A, Carballo C, Zong J, Chen T, Lebaschi A, et al. The Effect of Purified Human Bone Marrow-Derived Mesenchymal Stem Cells on Rotator Cuff Tendon Healing in an Athymic Rat. Arthroscopy. 2016;32:2435-43.
9. Harris MT, Butler DL, Boivin GP, Florer JB, Schantz EJ, Wenstrup RJ. Mesenchymal stem cells used for rabbit tendon repair can form ectopic bone and express alkaline phosphatase activity in constructs. J Orthop Res. 2004;22:998-1003.
10. Hou Y, Mao Z, Wei X, Lin L, Chen L, Wang H, et al. The roles of TGF-beta1 gene transfer on collagen formation during Achilles tendon healing. Biochem Biophys Res Commun. 2009;383:235-9.

11. Xu T, Xu M, Bai J, Lin J, Yu B, Liu Y, et al. Tenocyte-derived exosomes induce the tenogenic differentiation of mesenchymal stem cells through TGF-beta. Cytotechnology. 2019;71:57-65.
12. Norelli JB, Plaza DP, Stal DN, Varghese AM, Liang H, Grande DA. Tenogenically differentiated adipose-derived stem cells are effective in Achilles tendon repair in vivo. J Tissue Eng. 2018;9:1544426705.
13. Rajpar I, Barrett JG. Optimizing growth factor induction of tenogenesis in three-dimensional culture of mesenchymal stem cells. J Tissue Eng. 2019;10:1543389112.
14. Madhurakkat PS, Lee J, Ahmad T, Kim EM, Byun H, Lee S, et al. Harnessing biochemical and structural cues for tenogenic differentiation of adipose derived stem cells (ADSCs) and development of an in vitro tissue interface mimicking tendon-bone insertion graft. Biomaterials. 2018;165:79-93.
15. Sharma P, Maffulli N. The future: rehabilitation, gene therapy, optimization of healing. Foot Ankle Clin. 2005;10:383-97.
16. Zhang J, Wang JH. Human tendon stem cells better maintain their stemness in hypoxic culture conditions. Plos One. 2013;8:e61424.
17. Kowalski TJ, Leong NL, Dar A, Wu L, Kabir N, Khan AZ, et al. Hypoxic culture conditions induce increased metabolic rate and collagen gene expression in ACL-derived cells. J Orthop Res. 2016;34:985-94.
18. Griffon DJ, Cho J, Wagner JR, Charavaryamath C, Wei J, Wagoner JA. Effects of Hypoxia and Chitosan on Equine Umbilical Cord-Derived Mesenchymal Stem Cells. Stem Cells Int. 2016;2016:2987140.
19. Yu Y, Lin L, Zhou Y, Lu X, Shao X, Lin C, et al. Effect of Hypoxia on Self-Renewal Capacity and Differentiation in Human Tendon-Derived Stem Cells. Med Sci Monit. 2017;23:1334-9.
20. Lee WY, Lui PP, Rui YF. Hypoxia-mediated efficient expansion of human tendon-derived stem cells in vitro. Tissue Eng Part A. 2012;18:484-98.
21. Romero A, Barrachina L, Ranera B, Remacha AR, Moreno B, de Blas I, et al. Comparison of autologous bone marrow and adipose tissue derived mesenchymal stem cells, and platelet rich plasma, for treating surgically induced lesions of the equine superficial digital flexor tendon. Vet J. 2017;224:76-84.
22. Dai L, Hu X, Zhang X, Zhu J, Zhang J, Fu X, et al. Different tenogenic differentiation capacities of different mesenchymal stem cells in the presence of BMP-12. J Transl Med. 2015;13:200.
23. Huang TF, Yew TL, Chiang ER, Ma HL, Hsu CY, Hsu SH, et al. Mesenchymal stem cells from a hypoxic culture improve and engraft Achilles tendon repair. Am J Sports Med. 2013;41:1117-25.
24. Chen G, Zhang H, Ma Q, Zhao J, Zhang Y, Fan Q, et al. Fresh-frozen Complete Extensor Mechanism Allograft versus Autograft Reconstruction in Rabbits. Sci Rep. 2016;6:22106.
25. Ito Y, Toriuchi N, Yoshitaka T, Ueno-Kudoh H, Sato T, Yokoyama S, et al. The Mohawk homeobox gene is a critical regulator of tendon differentiation. Proc Natl Acad Sci U S A. 2010;107:10538-42.

26. Docheva D, Hunziker EB, Fassler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol. 2005;25:699-705.
27. Juneja SC, Veillette C. Defects in tendon, ligament, and enthesis in response to genetic alterations in key proteoglycans and glycoproteins: a review. Arthritis. 2013;2013:154812.
28. Havis E, Bonnin M, Olivera-Martinez I, Nazaret N, Ruggiu M, Weibel J, et al. Transcriptomic analysis of mouse limb tendon cells during development. Development. 2014;141:3683-96.
29. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24:1294-301.
30. Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA, Ferrero-Gutierrez A, Fernandez-Rodriguez MA, Gala J, et al. Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. Transplant Proc. 2013;45:434-9.
31. Dahl JA, Duggal S, Coulston N, Millar D, Melki J, Shahdadfar A, et al. Genetic and epigenetic instability of human bone marrow mesenchymal stem cells expanded in autologous serum or fetal bovine serum. Int J Dev Biol. 2008;52:1033-42.
32. Peng L, Jia Z, Yin X, Zhang X, Liu Y, Chen P, et al. Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. Stem Cells Dev. 2008;17:761-73.
33. Yin Z, Guo J, Wu TY, Chen X, Xu LL, Lin SE, et al. Stepwise Differentiation of Mesenchymal Stem Cells Augments Tendon-Like Tissue Formation and Defect Repair In Vivo. Stem Cells Transl Med. 2016;5:1106-16.
34. Arimura H, Shukunami C, Tokunaga T, Karasugi T, Okamoto N, Taniwaki T, et al. TGF-beta1 Improves Biomechanical Strength by Extracellular Matrix Accumulation Without Increasing the Number of Tenogenic Lineage Cells in a Rat Rotator Cuff Repair Model. Am J Sports Med. 2017;45:2394-404.

Figures

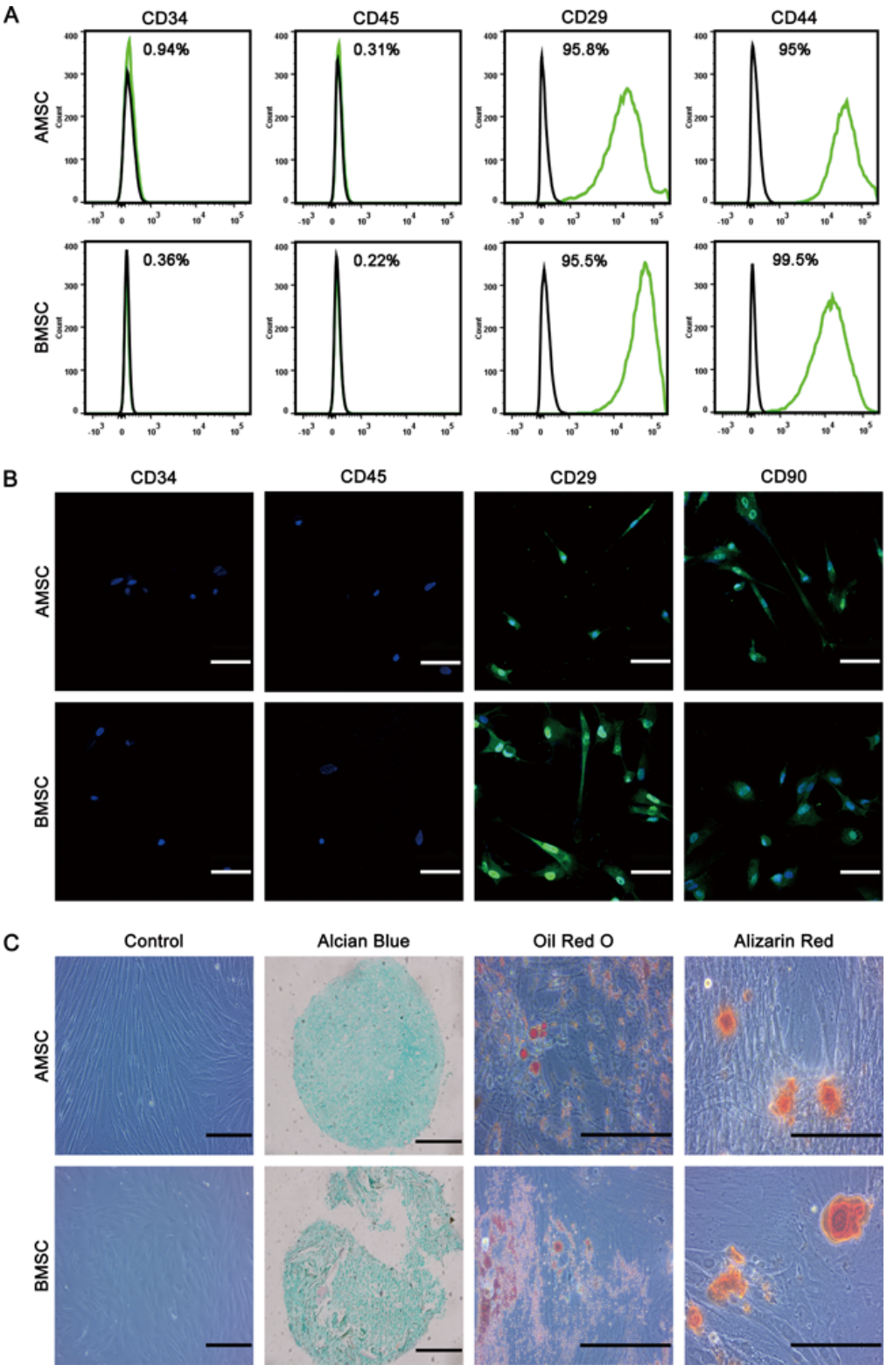

Figure 1

Identification of AMSCs and BMSCs. A: Flow cytometric analysis of immunophenotypic profiles of AMSCs and BMSCs. B: Laser scanning confocal microscopic images of immunofluorescence staining for MSCs specific markers CD29 and CD90 and hematopoietic lineage markers CD34 and CD45. Scale bar = 50 μm. Magnification: ×400. C: Light micrographs and tri-lineage differentiation of AMSCs and BMSCs to chondrocytes stained with Alcian Blue, adipocytes stained with Oil Red O and osteocytes stained with

Alizarin Red. Control and Alcian Blue staining: scale bar = 200 μm; magnification: ×200. Oil Red O and Alizarin Red staining: scale bar = 200 μm; magnification: ×400.

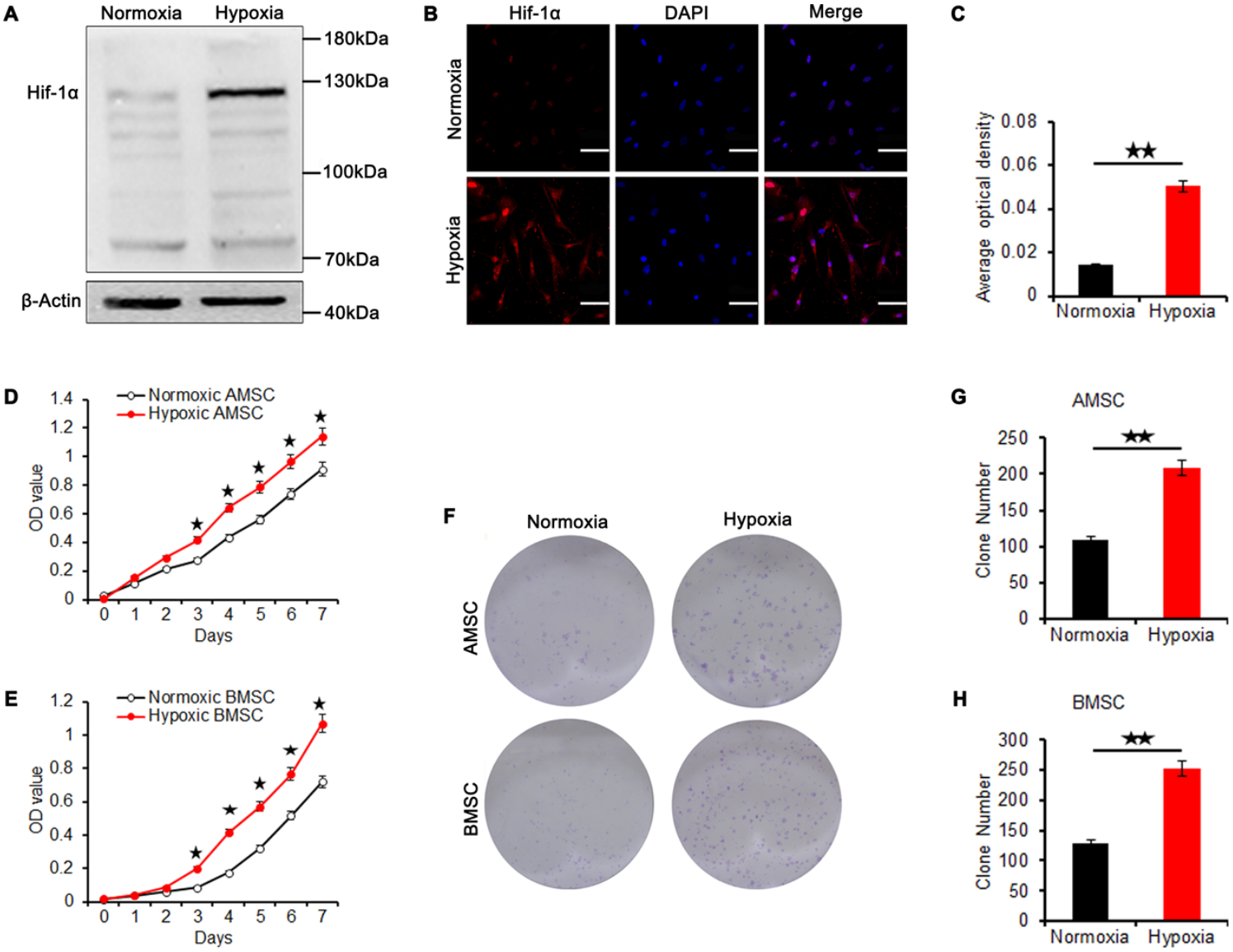

Figure 2

The expression of Hif-1α and the proliferation of AMSCs and BMSCs at 7 days after hypoxic induction. A: Representative western blots and immunofluorescence staining (B) of Hif-1α (red) and DAPI-labeled nuclei (blue), and quantification data (C) under normoxia and hypoxia condition in BMSCs. Scale bar = 50 μm; Magnification: ×400. D-E: Cell counting kit-8 assay of the proliferation of AMSCs (D) and BMSCs (E) under normoxia and hypoxia condition. OD: optical density. F-H: Colony formation unit assay (F) and quantification data of the proliferation of AMSCs (G) and BMSCs (H) under normoxia and hypoxia condition. Assays were done in triplicate. Data were shown as mean ± SD. * $p<0.05$; ** $p<0.01$, vs. the normoxia condition.

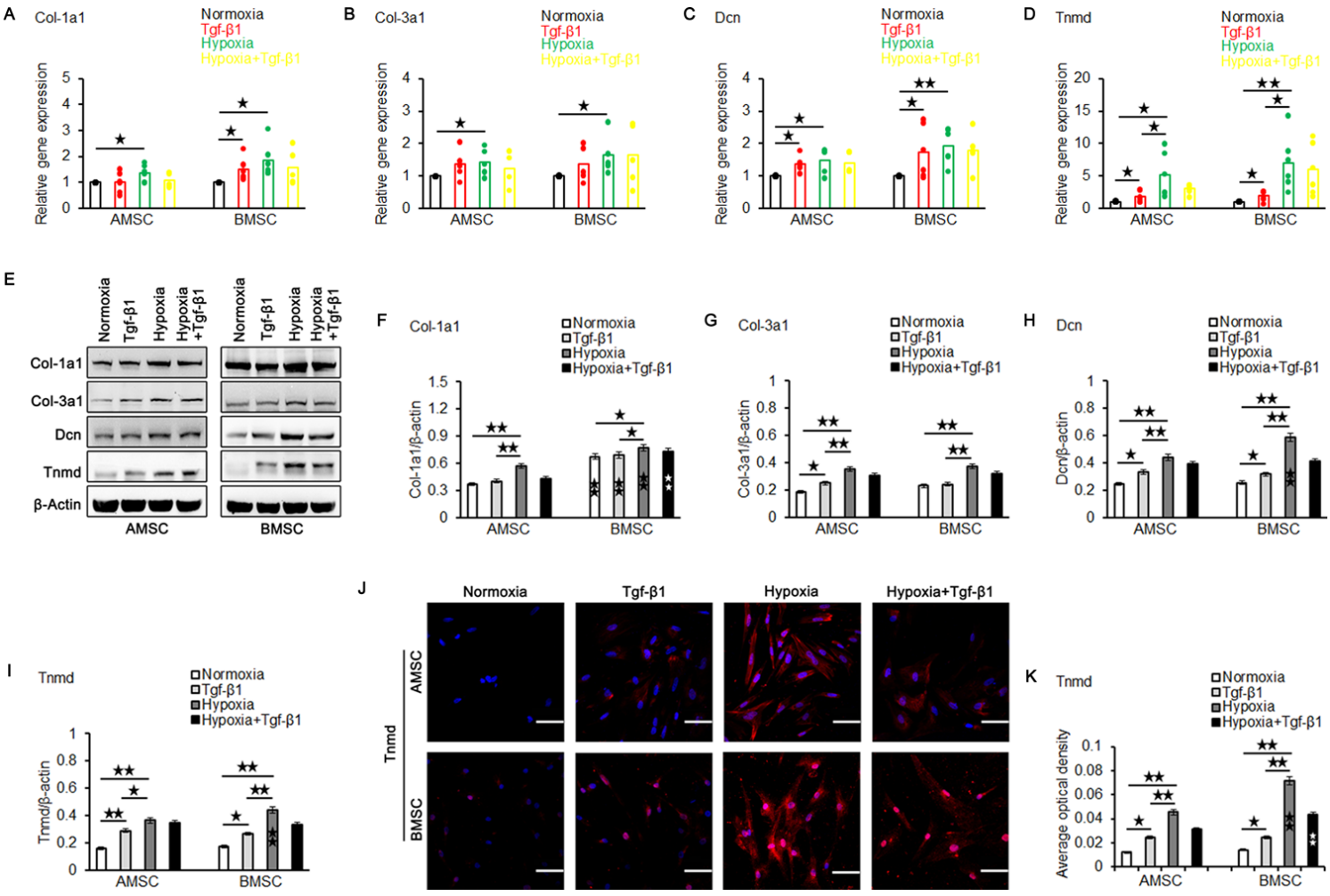

Figure 3

The mRNA and protein expressions of tenogenic markers in AMSCs and BMSCs at 7 days after induction. A-D: qRT-PCR analysis of gene expression of Col-1a1 (A), Col-3a1 (B), Dcn (C) and Tnmd(D) under normoxia, Tgf-β1, hypoxia, and hypoxia+Tgf-β1 condition in AMSCs and BMSCs. E-I: Representative western blots (E) and quantification of protein expression of Col-1a1 (F), Col-3a1 (G), Dcn (H) and Tnmd(I) under normoxia, Tgf-β1, hypoxia, and hypoxia+Tgf-β1 condition in AMSCs and BMSCs. Asterisks in the column in BMSCs indicated significantly different from the same condition in AMSCs. J-K: Representative immunofluorescence staining (J) of Tnmd (red) and DAPI-labeled nuclei (blue), and quantification data (K) under under normoxia, Tgf-β1, hypoxia, and hypoxia+Tgf-β1 condition in AMSCs and BMSCs. Asterisks in the column in BMSCs indicated significantly different from the same condition in AMSCs. Scale bar = 50 μm; Magnification: ×400. Assays were done in triplicate. Data were shown as mean ± SD. *p<0.05; **p<0.01.

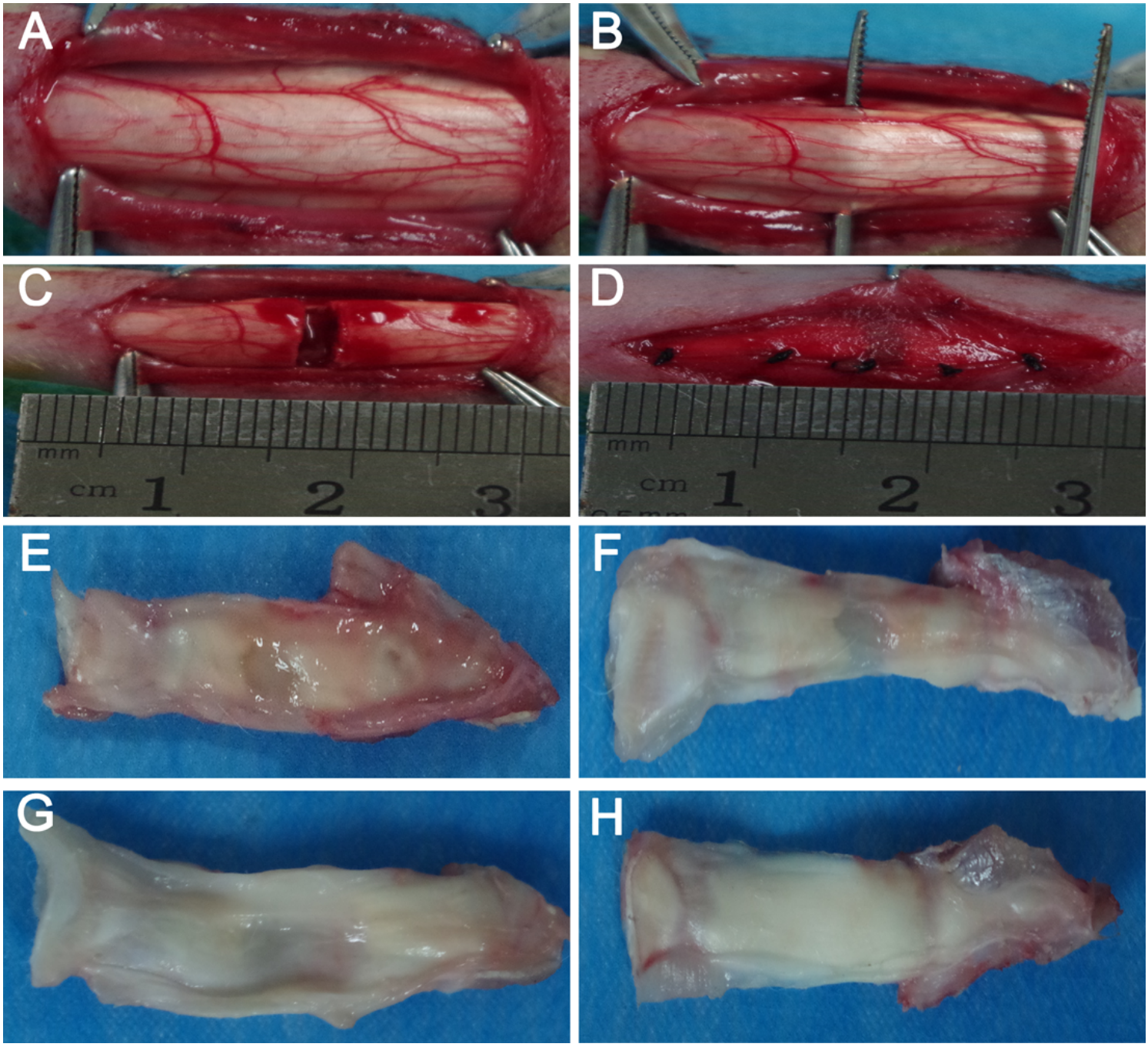

Figure 4

Surgical procedure for patellar tendon injury model and the gross observation of specimen at 4 weeks after surgery. A: Exposure of patellar tendon. B: Preparation of the middle part of patellar tendon. C: Retraction of the broken end after severing the middle part of patellar tendon. D: Sutures after implanting BMSCs. E-H: Gross observation of the PBS group (E), the normoxia group (F), the hypoxia group (G) and the control (normal) group (H).

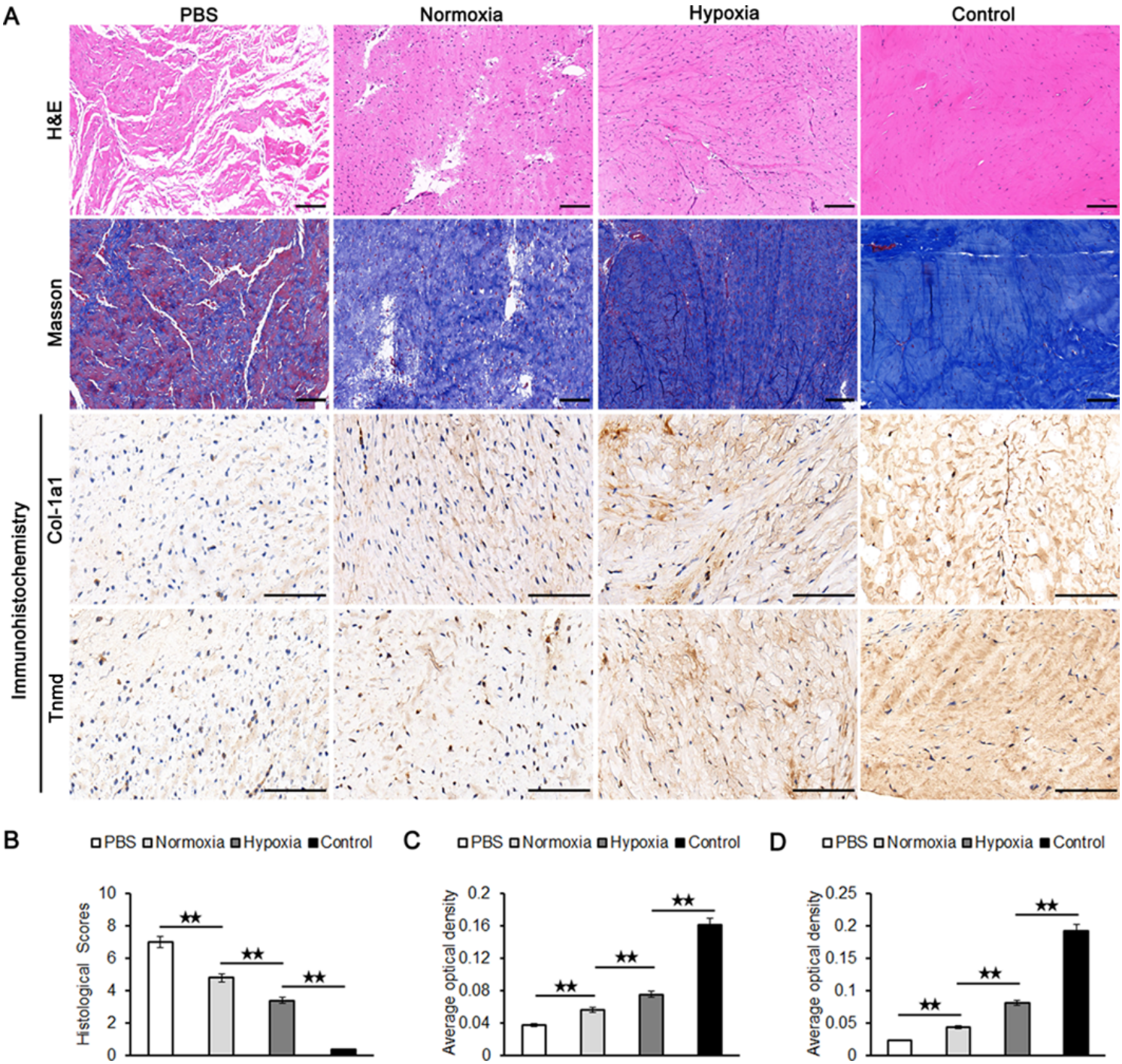

Figure 5

Histological analysis of specimen at 4 weeks after surgery. A: Hematoxylin and eosin (H&E) staining, masson's trichrome staining and immunohistochemical staining for Col-1a1 and Tnmd in the PBS group, the normoxia group, the hypoxia group and the control (normal) group. B: Quantification of histological properties after H&E staining in the PBS group, the normoxia group, the hypoxia group and the control (normal) group. C: Quantification of Col-1a1 and Tnmd (D) after immunohistochemical staining in the PBS group, the normoxia group, the hypoxia group and the control (normal) group. H&E and masson's trichrome staining: scale bar = 200 μm; magnification: ×200; Immunohistochemical staining: scale bar = 200 μm; magnification: ×400. Assays were done in triplicate. Data were shown as mean ± SD. **p<0.01.

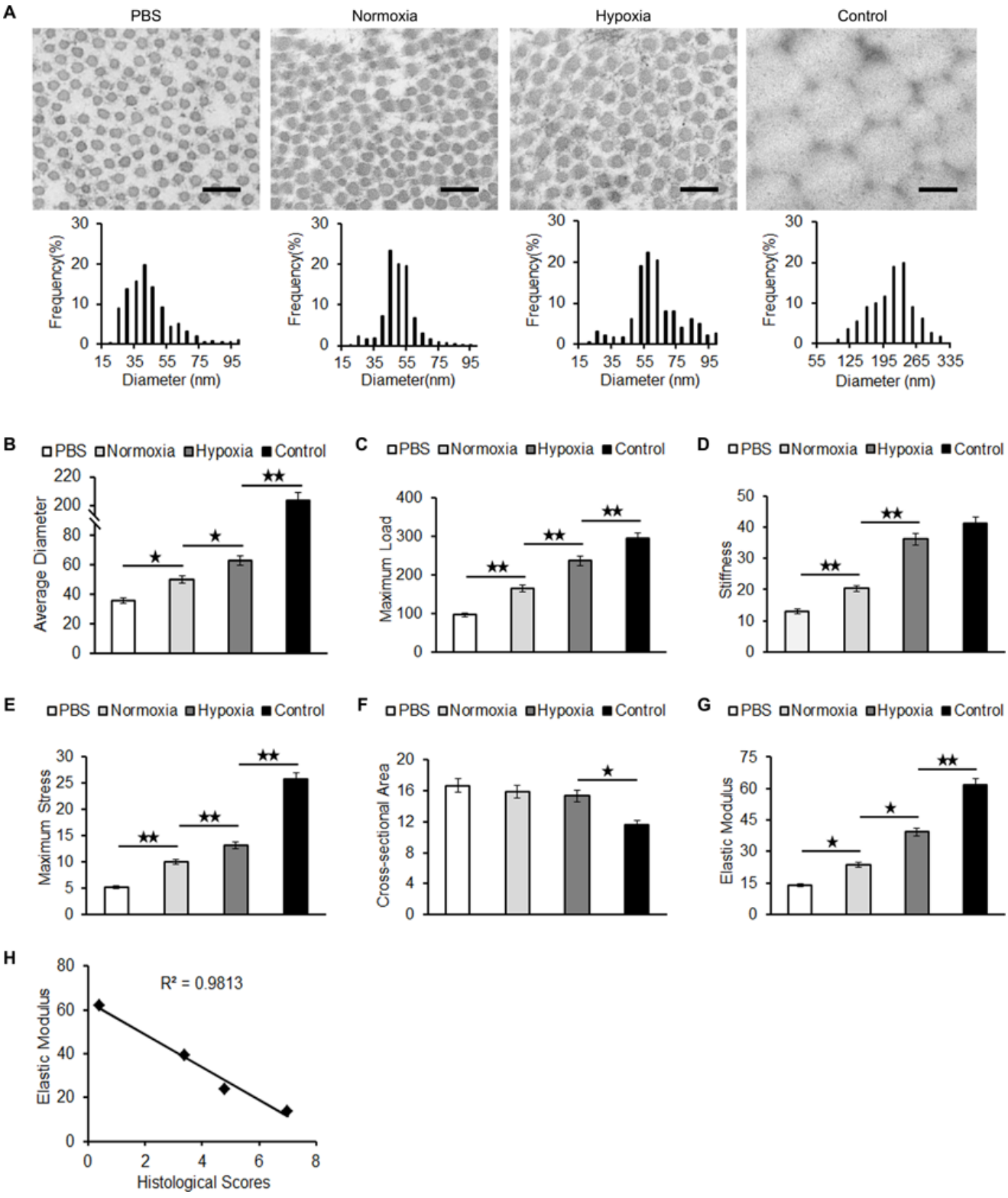

Figure 6

Ultrastructure and biomechanical analysis of specimen at 4 weeks after surgery. Representative images of transmission electron microscopy and the distribution of collagen fibril diameters (A), the average diameter of collagen fibrils (B) in the PBS group, the normoxia group, the hypoxia group and the control (normal) group. Scale bar = 200 nm. Magnification: ×15000. C-G: Biomechanical analysis for maximum load to failure (C), stiffness at failure (D), maximum stress (E), cross-sectional area (F) and elastic

modulus (G) in the PBS group, the normoxia group, the hypoxia group and the control (normal) group. H: Regression analysis of the relationship between histological score and elastic modulus. Assays were done in triplicate. Data were shown as mean ± SD. *$p<0.05$; ** $p<0.01$.