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The transcription factor Mohawk controls tenogenic differentiation of bone marrow mesenchymal stem cells in vitro and in vivo

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Abstract

Purpose—Mohawk homeobox (MKX) has been demonstrated as a tendon/ligament specific transcription factor. The aim of this study was to investigate the role of MKX in ligament/tenogenic differentiation of bone marrow derived mesenchymal stem cells (BMMSCs).

Methods—Human BMMSCs were treated with 50 ng/ml BMP-12 or transduced with MKX or scleraxis (SCX) adenoviral vector. Gene expression analysis was performed by quantitative reverse transcribed polymerase chain reaction (qRT-PCR). Rat BMMSCs were seeded in a collagen scaffold and transplanted into a rat Achilles tendon defect model. Tenogenesis related gene expressions and histological features were analyzed.

Results—BMP-12 induced tenogenesis in BMMSCs as indicated by increased COL1a1, TNXB, DCN and SCX mRNA and MKX expression increased simultaneously. Rat BMMSCs enhanced defect repair and were still detectable 3 weeks after transplantation. Increased expressions of COL1a1, TNC and TNMD in vivo were also correlated with upregulated MKX. Adenoviral MKX promoted expression of COL1a1, TNXB and TNMD in BMMSCs.

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The authors declare no conflicts of interest.

Conclusions—This study demonstrated that MKX gene expression is enhanced during the tenogenic differentiation of BMMSCs *in vitro* and *in vivo*, and the adenoviral overexpression of MKX increases tendon extracellular matrix gene expression and protein production. Thus, MKX is a key factor for tenogenic differentiation of MSCs.

Keywords

Mesenchymal stem cells; Mohawk; Scleraxis; tenogenesis

INTRODUCTION

Tendons and ligaments are collagenous fibrous tissues, which connect bones and muscles, and transmit mechanical forces efficiently [1,2]. Tendon and ligament injuries are treated surgically or non-surgically, and both methods yielded overall good recovery rates [3,4]. However, repaired tendon tissues are often histologically unorganized and usually have inferior mechanical strength, which can lead to re-rupture [5,6]. Lack of stability resulting from incomplete healing is a risk factor for the recurrent tear or the development of osteoarthritis [7,8].

Improved understanding of the cell differentiation pathways and the factors which critically regulate tenocyte phenotype have the potential to lead to novel approaches to pharmacologically enhanced tendon healing or tendon tissue-engineering. The upregulation of growth factors at specific stages of the tendon and ligament healing process have been studied [9]. Recent discoveries suggest that transcription factors homeobox Mohawk (MKX) [10,11], Scleraxis (SCX) [12], and Early growth response factor-1 and 2 (EGF1 and 2) [13,14] as well as the growth factors such as Growth and Differentiation Factor 7 (GDF7, also known as BMP-12) [15] are important regulators of tenogenic differentiation. In particular, MKX knockout mice have defective Achilles tendon and collagen type I expression [10], and SCX gene knock out leads to severe disruption of tendons [16]. Thus, MKX and SCX are thought to be the key regulators of tenogenic differentiation.

Mesenchymal stem cells (MSCs) have potential to differentiate into several mesenchymal tissues *in vitro* [17] and are involved in tissue development, homeostasis and remodeling [18]. There have been several attempts to apply MSCs in tendon injury models and these studies actually suggested that MSCs could differentiate into tenocyte-like cells and improved histological features of regenerated tissue [19,20].

The purpose of this study was to test effects of tendon-specific transcription factors on bone marrow-derived mesenchymal stem cells (BMMSCs) *in vitro* and *in vivo*. We treated BMMSCs with BMP-12 and adenoviral MKX and SCX gene vectors to examine the effects on expression of tenogenesis related genes and ECM related genes. We analyzed gene expression patterns following *in vivo* transplantation of BMMSCs in an animal model of tendon injury. Our results suggest that MKX promotes tenogenic differentiation of BMMSCs and indicate the potential of this approach for novel regenerative therapy.

METHODS

Human BMMSCs and anterior cruciate ligament (ACL) cells

Human BMMSCs were purchased from Lonza (Walkersville, MD, USA) and cultured in Mesenchymal Stem Cell Growth Medium (MSCGM) (Lonza). The cells were re-plated at optimal initial cell density, cultured, and harvested with 0.25% Trypsin/EDTA solution (Mediatech Manassas, Herndon, VA, USA) every 14 days. Bi-lineage (osteogenic and adipogenic) differentiation abilities were tested by hMSC Osteogenic BulletKit (Lonza), and hMSC Adipogenic BulletKit (Lonza), respectively. Human ACL cells were prepared from cadaveric knees provided by tissue banks. Minced ACL tissues were digested with 5 mg/ml collagenase V (SIGMA-ALDRICH, St. Louis, MO, USA), and filtered by 70 μm mesh. Cells were then collected, cultured in MSCGM, and passaged every 14 days. Passage 2 (P2) cells were used in the experiments.

RNA isolation and quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR)

BMMSCs were collected 1, 2 and 7 days after transduction with adenoviral vector or 5 days after 50ng/ml BMP-12 treatment for RNA collection. Total RNAs were prepared by using TRIzol Reagent (Life Technologies, Grand Island, NY, USA). They were reverse transcribed into cDNA with TaqMan® Reverse Transcription Reagents (Life Technologies). Quantitative-PCR analysis was conducted on LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN, USA) with up to 45 cycles using TaqMan Gene Expression Assay probes (Life Technologies). The amounts of mRNA were calculated as relative quantities in comparison to β-actin mRNA. Ct value of 35 was considered as minimum gene expression.

TaqMan Gene Expression Assay probes were rat ACTB (Rn00667869_m1), rat MKX (Rn01755203_m1), rat SCX (Rn01504576_m1), rat COL1A1 (Rn01463848_m1), rat TNC (Rn01454947_m1), rat TNMD (Rn00574164_m1), human ACTB (Hs99999903_m1), human MKX (Hs00543190_m1), human SCXA (Hs03054634_g1), human COL1A1 (Hs00164004_m1), human decorin (DCN) (Hs00754870_s1), human tenascin C (Hs01115665_m1), human tenascinXB (TNXB) (Hs00372889_g1), human Egr1 (Hs00152928_m1), human Egr2 (Hs00166165_m1), and human tenomodulin (TNMD) (Hs00223332_m1).

Adenoviral vector construction

Adenoviral vectors were constructed by using Adeno-X™ Expression System 1 (Clonetech, Mountain View, CA, USA). Flag-tagged MKX and SCX DNA plasmids driven by a constitutively active CMV promoter were provided by Yoshiaki Ito at Department of Systems BioMedicine, Tokyo Medical and Dental University. These constructs were cloned into adenoviral construct vector. Successful viral vector constructions were confirmed by DNA sequencing, by LacZ Detection Kit for tissues (InvivoGen, San Diego, CA, USA) and western blotting. Viral titers were calculated and diluted to the same titer (200 pfu/ml) between the vectors, according to the vendor’s manual.

Western blotting

BMMSCs were cultured for 7 days after transfection of adenoviral vectors. Cell extracts were prepared with RIPA Lysis and Extraction Buffer (Fisher, Waltham, MA, USA). Protein concentrations were measured by BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Western blotting was performed with the LiCor Odyssey immunofluorescence detection system (LI-COR Biosciences, Lincoln, NE, USA). Equal amounts of protein were separated on 4% to 20% SDS PAGE gels and transferred to nitrocellulose membranes. As primary antibodies, we used rabbit anti-MKX (Lifespan Biosciences Inc, Seattle, WA, USA; 1:5000 dilution), rabbit anti-Scleraxis (SCX) (AP53819PU-N, Acris Antibodies, Inc, San Diego, CA, USA; 1:500 dilution), rabbit anti-FLAG (DYKDDDDK) (Kamiya Biomedical Company, Seattle, WA, USA; 1:5,000 dilution), rabbit anti-Collagen I (ab292, Abcam, Cambridge, MA, USA; 1:5000 dilution), and mouse anti-GAPDH (AM4300, Ambion, Austin, TX, USA; 1:5,000 dilution). As secondary antibodies, we used goat anti-mouse-IRDye 680 (LI-COR Biosciences; 1:10,000) and goat anti-rabbit-IRDye 800 (LI-COR Biosciences; 1:10,000 dilution).

Tenogenesis *in vivo*

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee. Bone marrow from 3 months old Wister rats (Charles River, San Diego, CA, USA) were flushed with PBS containing 1000U/ml heparin (Millipore, Billerica, MA, USA) and cells were cultured in 1g/L glucose DMEM (Mediatech, Manassas, MA) with 10% heat inactivated serum (Life Technologies) and 5% Antibiotic Antimycotic Solution (Mediatech). The medium was changed after 1 day and unattached cells were removed. P2 cells were tested for bilineage differentiation potential. For the *in vivo* model, we modified the methods previously established [19]. Briefly, 1×10^6 cells in 200μl DMEM were labeled by CellTracker CM-DiI (Life Technologies) and seeded into bovine deep flexor tendon collagen scaffolds-CollaCote® (Zimmer Dental, Carlsbad, CA, USA). Bovine collagen scaffolds soaked with DMEM only and containing no cells served as controls. About 5 mm longitudinal partial defects were created on the lateral border of the bilateral Achilles tendons of 3 month old rats under anesthesia with isoflurane and scaffolds with or without cells were implanted into the defects. After 3 weeks, the tendon repair tissues were removed and analyzed for gene expressions and histology.

Statistics

Comparisons were performed using the Steel-Dwass multiple comparison test after the Kruskal Wallis test, or Dunnet multiple comparison test after one-way ANOVA for 3 or more groups. Comparison between two groups was performed with Mann Whitney U test. Differences at $p < 0.05$ were considered significant. Results are expressed as mean ± S.D.

RESULTS

MKX expression in BMMSCs *in vitro*

To examine the expression patterns of MKX and other tendon/ligament related genes, we compared MKX, TNXB, and TNMD mRNA levels in human ACL cells with BMMSCs.

The expression of MKX, TNXB and TNMD in human ACL cells were higher than BMMSCs (Fig. 1A).

To test whether MKX is enhanced during tenogenic differentiation, we treated human BMMSCs with 50 ng/ml BMP-12 for 5 days and performed qRT-PCR for MKX and other tenogenic markers. Consistent with previous reports [19,21,22], BMP-12 stimulation significantly increased COL1A1, TNXB, DCN and SCX mRNA levels. This was correlated with increased MKX expression (Fig. 1B).

These data suggest basal expression level of MKX is low in undifferentiated BMMSCs, and increased MKX expression is associated with tenogenic differentiation of MSCs *in vitro*.

MKX expression in BMMSCs *in vivo*

To further examine MKX expression during tenogenic differentiation of MSCs, we prepared rat BMMSCs and confirmed osteogenic and adipogenic differentiation abilities (Fig. 2A, 2C), similar to human BMMSCs (Fig. 2B, 2D).

Rat BMMSCs were seeded onto collagen scaffolds and implanted in longitudinally half-dissected Achilles tendons of 3 month old rats (Fig. 3A). After 3 weeks, repaired tendon tissues were removed. BMMSCs stained with CellTracker CM-DiI were still clearly detected by fluorescence in the cross-sectioned tendon specimens 3 weeks after transplantation (Fig. 3B). Histologically, in BMMSCs-transplanted group, the cells showed more spindle-shaped morphology, compared with the scaffold only group (Fig. 3C), similar to results in previous reports [19,23]. BMMSCs transplanted group showed higher COL1a1, TNC, TNMD and MKX gene expression levels than scaffold only control (Fig. 3D).

These data suggest tenogenic differentiation of MSCs *in vivo* is associated with increased MKX expression.

Effect of MKX on tenogenesis in BMMSC

BMMSCs were transfected by Ad-LacZ and adenoviral vector transduction was verified by LacZ staining (Fig. 4A). Western blotting showed exogenous MKX and SCX protein production in adenovirus-transduced BMMSCs (Fig. 4B). Upon Ad-MKX transduction of BMMSCs and culture for 1 week, the expression of COL1a1, TNXB and TNC were increased significantly ($p<0.05$, $n=9$). TNMD, a marker of late stage tenogenic differentiation, was expressed at higher levels in both Ad-MKX and Ad-SCX groups than in Ad-LacZ (Mock) group ($p<0.05$, $n=9$) (Fig. 5A). Egr1 is a recently identified transcription factor that is involved in tenogenesis [14], but Egr1 and 2 expression levels were not increased following adenoviral overexpression of MKX and SCX in BMMSCs (data not shown). Western blotting revealed collagen type I protein production was enhanced in Ad-MKX treated group as compared to other groups (Fig. 5B).

DISCUSSION

There is still very limited knowledge on putative tenogenic factors for MSCs [24]. This is the first report to investigate MKX expression and function in human BMMSCs.

The present findings demonstrated that basal expression of MKX in BMMSCs is much lower compared to ligament cells. The ability of BMP-12 to promote tenogenic differentiation of BMMSCs has been reported [19,21,25-28]. In our present studies, BMP-12 treatment of BMMSCs induced COL1a1, TNXB, DCN and SCX expression and this was associated with increased MKX. These results are similar to a recent study on BMP-12 treatment of adipose derived mesenchymal stem cells [28]. Although the cell source (adipose and bone marrow), dose and evaluating time points were different, the two studies showed similar 1.5-3 fold increased in SCX after 10-100ng/ml BMP-12 treatment at day 3-7. We could not detect any significant change of TNMD expression levels between BMP-12 treated group and control group (data not shown) while in adipose-derived mesenchymal stem cells, significant but subtle changes for TNMD expression levels after BMP-12 treatment were observed [28]. This discrepancy may be due to the lower dose of BMP-12 and shorter time interval after BMP-12 treatment in our study.

Collagen type I is a major component of tendon and ligament extracellular matrix, and collagen metabolism and composition are critically important for connective tissue strength [29]. Scar tissue that forms in injured ligament contains significantly decreased amount of collagen I [30], indicating that higher collagen type I production will be favorable for tissue repair. TNX is a member of the tenascin family of extracellular matrix glycoproteins and its function is associated with collagen fibril maturation. TNX deficiency in both mice and humans is associated with impaired tendon strength [31]. DCN is a class I small leucine-rich proteoglycan (SLRP) and has been associated with increased collagen fibril assembly and crosslink in developing tendon [32]. DCN deficient mice showed decreased mechanical strength after patella tendon injury [33]. Additionally, biglycan and decorin expression levels were increased in repair tissue following Achilles tendon injury [34]. Tendon development and tissue repair share common features in many aspects [2] and MKX might play an important role not only in developmental tenogenesis as demonstrated before [10,11], but also in tenogenic differentiation of adult BMMSCs.

BMMSCs transplantation itself improves tendon injury repair according to former studies [19,23]. Besides, collagen scaffolds are useful in both tendon [19,35] and ligament [36] injury models. Notably, decellularized tendon collagen matrices induced tenogenic differentiation of MSCs *in vivo* but collagen from other sources did not [35]. We found that when BMMSCs were transplanted with bovine tendon collagen, cells survived after 3 weeks and expression of COL1a1, TNC, TNMD and MKX was enhanced, suggesting a role of MKX in the differentiation of BMMSCs *in vivo*.

We demonstrated that MKX is induced during tenogenic differentiation of MSCs *in vitro* and *in vivo*. The increased expression of MKX during tenogenic differentiation from MSCs prompted us to test the notion that ectopic expression of MKX by itself may promote tenogenic gene expression in MSCs. We first revealed that overexpression of MKX gene induced tenogenesis related genes (COL1a1, TNXB, TNMD and TNC) and protein (collagen type I) expression in BMMSCs. TNXB is an important ECM component of ligamentous tissues, and prior studies from our group showed that TNXB gene expression was suppressed by MKX siRNA in cultured ligament cells [37]. The presence of potential MKX binding motifs [38] are presumed in the TNXB promoter region [39].

We also used adenoviral transduction to overexpress SCX, also a tendon-specific transcription factor [12], but this did not increase MKX RNA levels (Fig. 5A). In addition, MKX expression also did not increase SCX expression (Fig. 5A). Shukunami et al. found that SCX positively regulates TNMD expression in chick developmental model [40]. But TNMD expression could also be detected in some SCX negative regions, suggesting other unknown factors involved in tendon development. A recent study revealed that both SCX and MKX bind to Smad3 which transmits TGFβ signaling [41], and Smad3-deficient mice manifested decreased mechanical strength and irregular collagen deposition after tendon injury [42]. These findings and our data suggest that although SCX is the dominant inducer of TNMD, MKX might be one of the factors which can induce TNMD expression without the depending on SCX, at least in human BMMSCs. Thus, MKX and SCX seem to function independently, although they may have similar effects on BMMSCs at later stage of tenogenic differentiation.

We also tested co-transfection of Ad-MKX and Ad-SCX and compared to the control vector at the same viral titer, but found no beneficial effect on tenogenic gene expression (data not shown). As MKX is thought to function at later stage of tenogenesis than SCX [10,24], sequential co-transfection of MKX and SCX at optimal time interval should be tested in future experiments.

When the overexpression of MKX gene was compared with the overexpression of SCX gene in BMMSCs, both factors induced similar TNMD gene expression but MKX induced COL1a1 mRNA and protein expression more efficiently. TNMD induction by SCX in BMMSCs was previously shown [40,43] and SCX gene transfer augmented the effect of BMMSCs transplantation in a rotator cuff injury model and improved mechanical properties [20]. It will be of interest to test the potential benefit of MKX overexpression *in vivo* tendon/ligament regenerative model.

In conclusion, MKX is a key factor for tenogenic differentiation of BMMSCs and plays an important role for homeostasis in tendons and ligaments. In this study, we demonstrated the usefulness of MKX as a strong inducer of tenogenic differentiation of BMMSCs.

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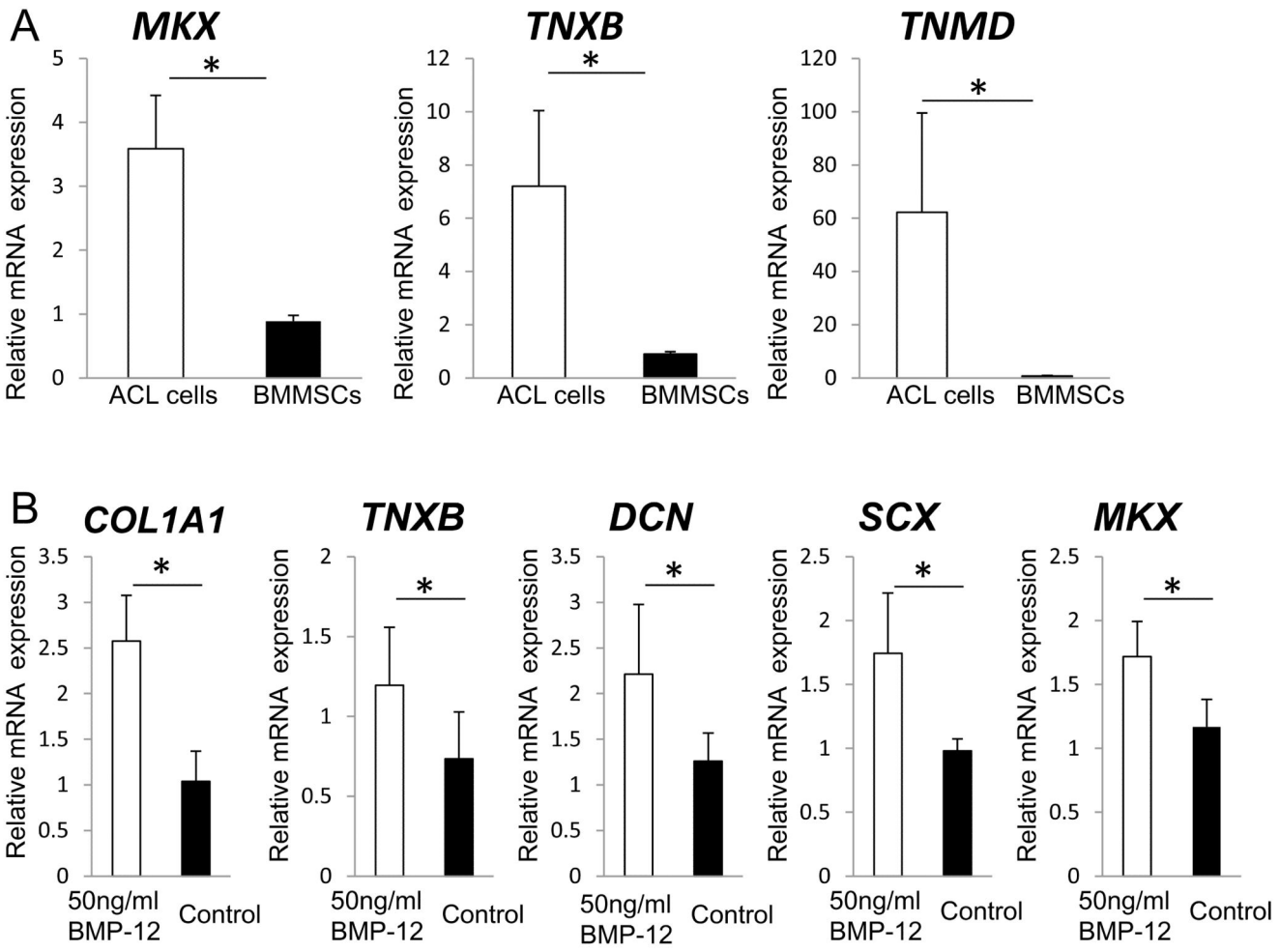

Figure 1.

MKX expression in BMMSCs before and after BMP-12 induced tenogenesis.

A, Basal mRNA expression of MKX, TNXB, and TNMD were higher in ACL cells than human BMMSCs. Bars show the mean SD (n=4 from 4 donors) * = $p < 0.05$ by Mann Whitney U test. **B,** BMP-12 treatment induced tenogenic related gene expression including MKX after 5 days. Bars show the mean SD (n=6 from 2 donors). * = $p < 0.05$, by Mann Whitney U test.

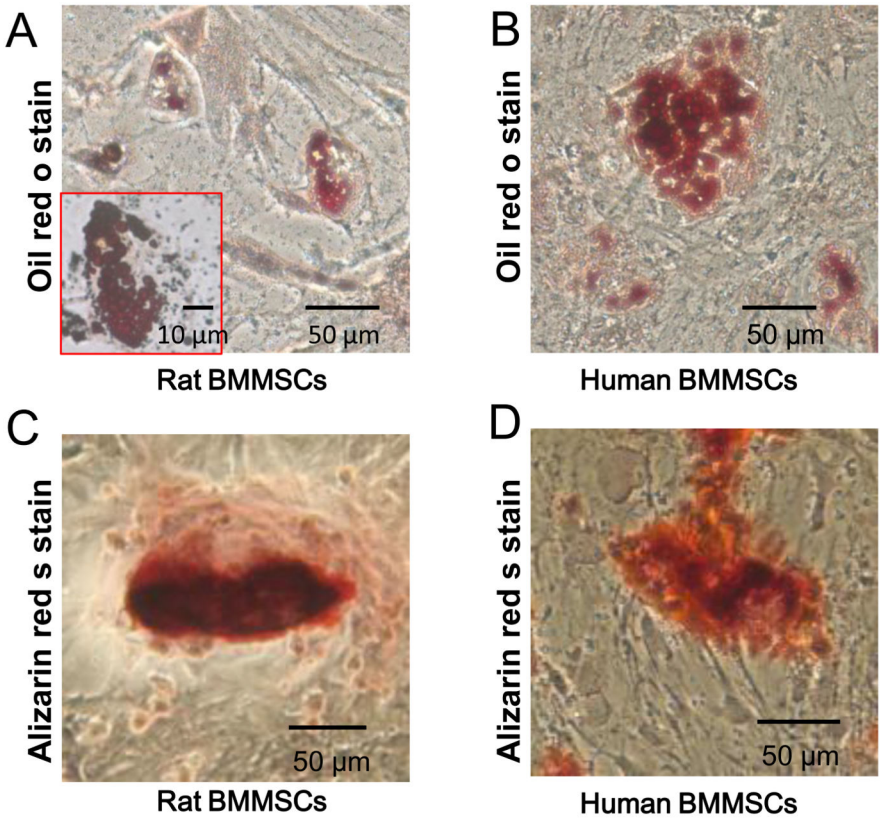

Figure 2.

Rat BMMSCs differentiation potential.

MSCs were cultured with media supplements to induce adipogenesis or osteogenesis. **A,** Rat BMMSCs with lipid droplets stained by oil red o. Red square is the enlarged image of droplets. **B,** Human BMMSCs with lipid droplets stained by oil red o. **C,** Calcified rat BMMSCs stained by alizarin red s. **D,** Calcified human BMMSCs stained by alizarin red s.

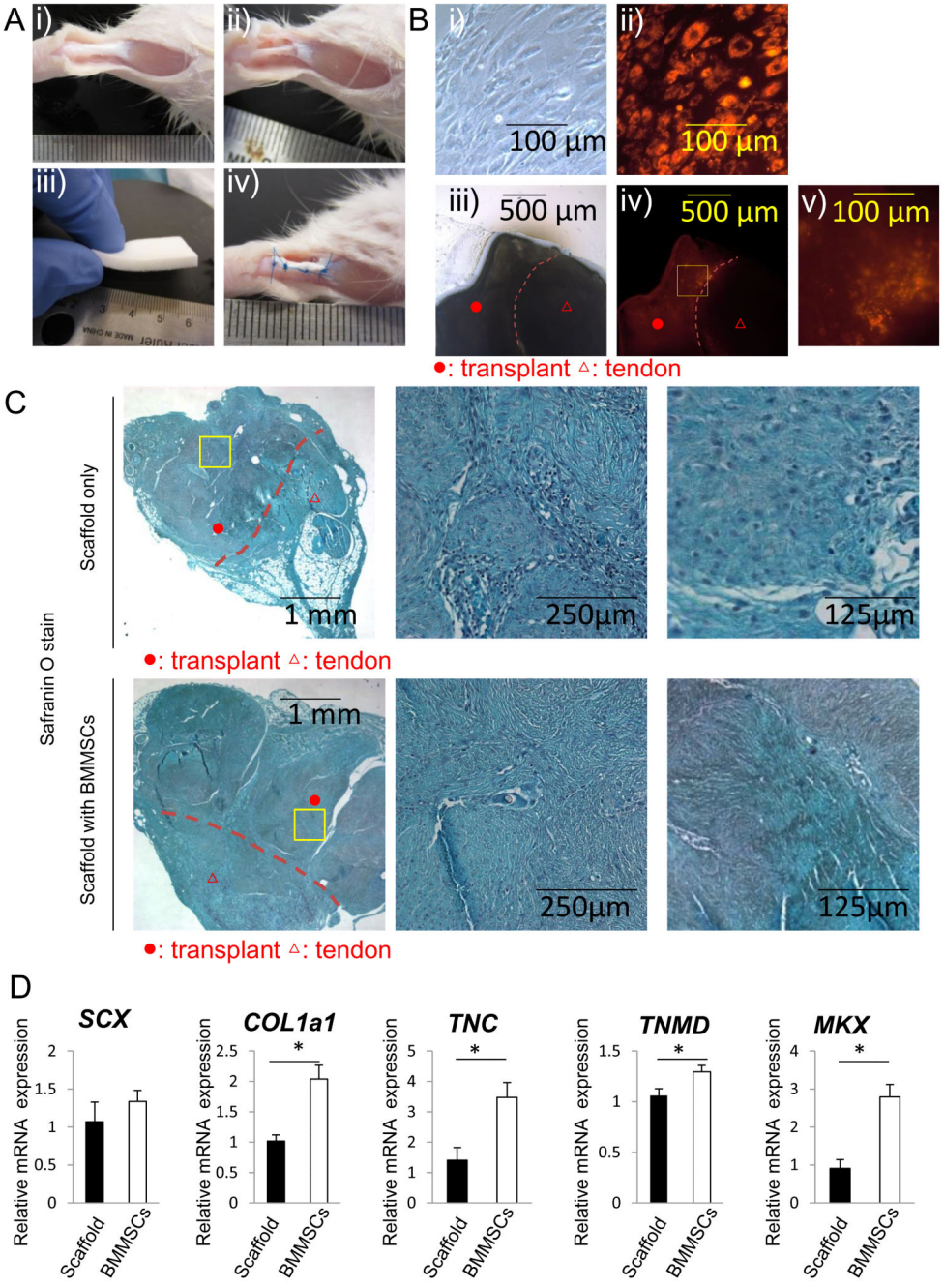

Figure 3.

Rat BMMSCs transplantation into rat tendon injury model.

A, Surgical method. i): rat intact Achilles tendon. ii): tendon severed by half. iii): bovine collagen scaffold. iv): scaffold sutured with tendon. **B,** Transplanted BMMSCs. i): monolayer BMMSCs in bright field. ii): monolayer BMMSCs with CM-DiI positive red fluorescence. iii): rat tendon specimen section in bright field. iv): rat tendon specimen section with red fluorescence. There is no apparent autofluorescence in intact tendon tissue and scaffold itself, but strong fluorescence was confirmed in transplanted cells. v): enlarged

image of yellow rectangular area of (iv), showing fluorescence positive rat BMMSCs in transplants. **C,** Representative Safranin O stain of scaffold only group and BMMSCs transplanted group. Upper: Scaffold only transplanted group, Lower: Scaffold with BMMSCs transplanted group. Enlarged images of yellow rectangular areas are shown on right. **D,** mRNA expression in transplanted tendon 3 weeks after surgery. Bars show the mean SD (n=4). * = $p<0.05$ by Mann Whitney U test.

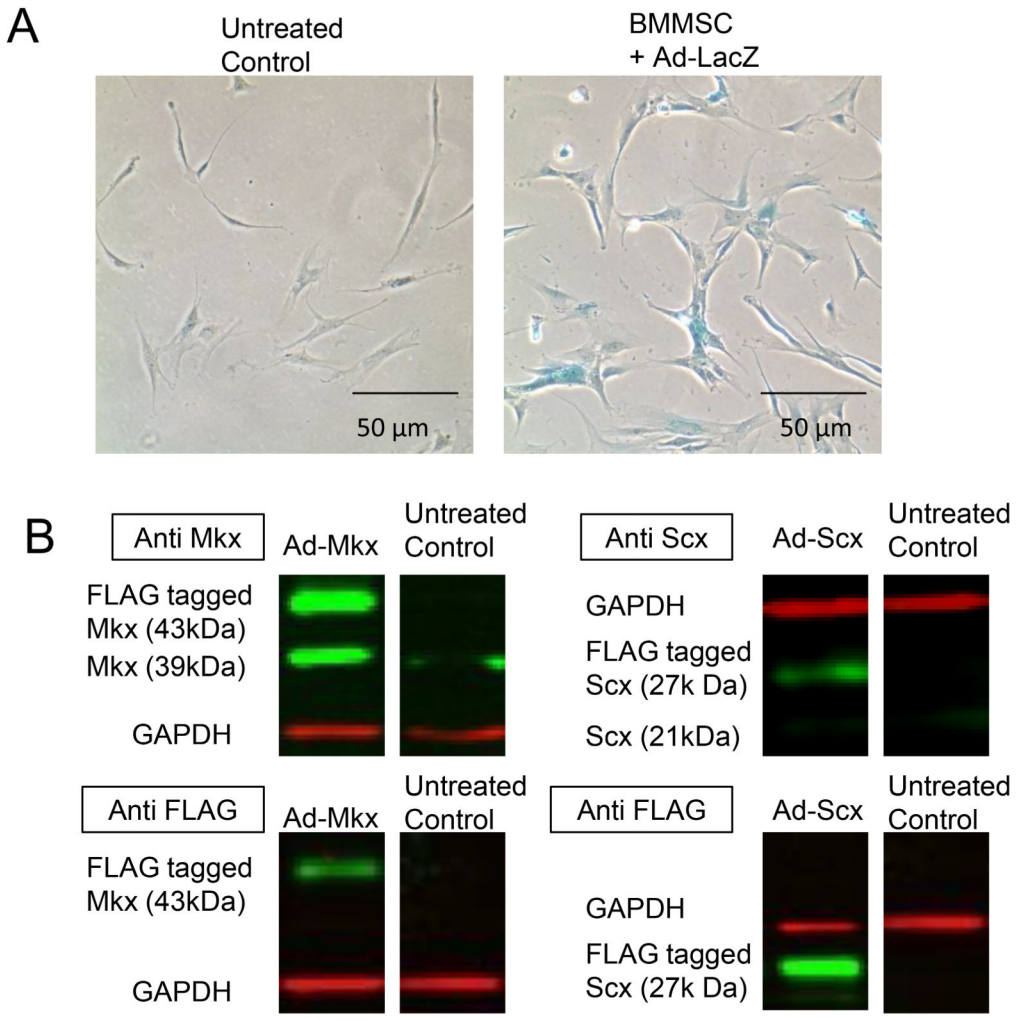

Figure 4.

Adenoviral vector transduction of BMMSCs. Human BMMSCs were transduced with adenoviral MKX, SCX or control vector (LacZ). **A,** LacZ positive BMMSCs transfected by AdLacZ. **B,** Western blotting showed increased Flag-tagged MKX and SCX protein expression following adenovirus transduction to BMMSCs.

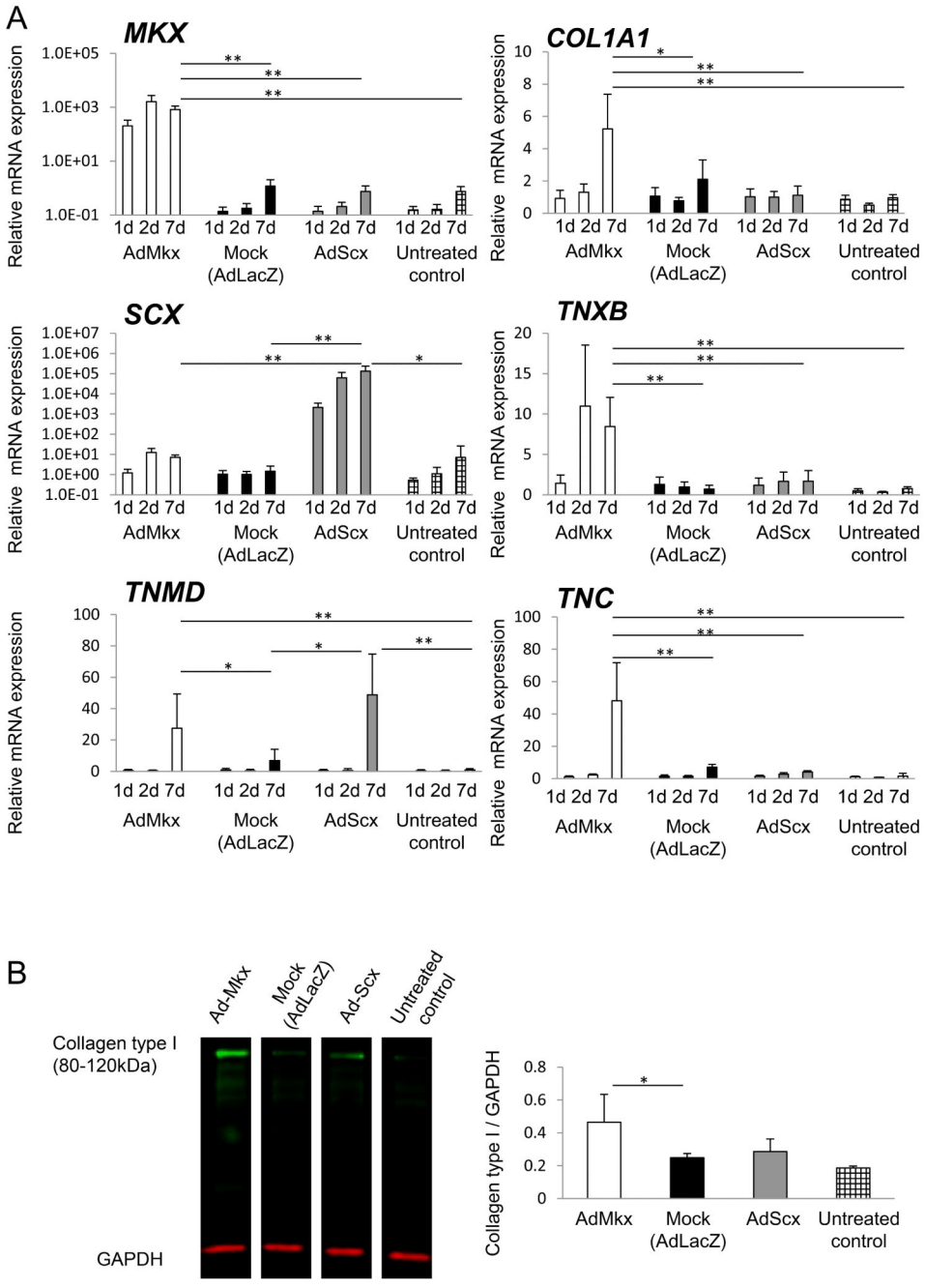

Figure 5.

Adenoviral MKX overexpression and tenogenesis in BMMSCs. **A,** Human BMMSCs were treated with adenoviruses expressing MKX, LacZ or SCX and tendon/ligament related gene expression was analyzed by quantitative PCR at days 1, 2 and 7. The values are displayed as mean ± standard deviation (n=9 from 3 donors). * = $p < 0.05$, ** = $p < 0.01$ by Steel Dwass test after Kruskal Wallis test. **B,** Western blotting showed increased collagen type I protein

level in Ad-MKX treated groups. The values are displayed as mean ± standard deviation (n=3 from 1 donors in each group), * = $p<0.05$ by Dunnet test after ANOVA.