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Transcription factor Mohawk and the pathogenesis of human anterior cruciate ligament degradation

Hiroyuki Nakahara[1], **Akihiko Hasegawa**[1], **Koji Otabe**[1], **Fumiaki Ayabe**[1], **Tetsuya Matsukawa**[1], **Naoko Onizuka**[2], **Yoshiaki Ito**[3], **Toshifumi Ozaki**[4], **Martin K. Lotz**[1], and **Hiroshi Asahara**[1,2,3]

[1]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, U.S.A.

[2]National Research Institute for Child Health and Development, Tokyo, Japan

[3]Department of Systems Bio Medicine, Tokyo Medical and Dental University, Tokyo, Japan

[4]Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

Abstract

Objective—To investigate the expression and function of Mohawk (MKX) in human adult anterior cruciate ligament (ACL) tissues and ligament cells from normal and osteoarthritis-affected knees.

Methods—Knee joints were obtained at autopsy within 24-48 hours postmortem from 13 normal donors (age 36.9±11.0 years), 16 OA donors (age 79.7±11.4 years) and 8 old donors without OA (age 76.9±12.9 years). All cartilage surfaces were graded macroscopically. MKX expression was analyzed by immunohistochemistry and quantitative PCR. ACL-derived cells were used to study regulation of *MKX* expression by IL-1β. MKX was knocked down by siRNA to analyze function of MKX in extracellular matrix (ECM) production and differentiation in ACL-derived cells.

Results—The expression of *MKX* was significantly decreased in ACL-derived cells from OA knees compared with normal knees. Consistent with this finding, immunohistochemistry showed that MKX positive cells were significantly reduced in ACL tissues from OA donors in particular in cells located in disorientated fibers. In ACL-derived cells, IL-1β strongly suppressed *MKX* gene expression and reduced ligament ECM genes, *COL1A1* and *TNXB*. On the other hand, *SOX9*, chondrocyte master transcription factor, was up regulated by IL-1β treatment. Importantly, knock down of MKX expression by siRNA upregulated SOX9 expression in ACL-derived cells, whereas the expression of COL1A1 and *TNXB* were decreased.

Conclusion—Reduced expression of MKX is a feature of degenerated ACL in OA-affected joints and this may be in part mediated by IL-1β. MKX appears necessary to maintain the tissue specific cellular differentiation status and ECM production in adult human tendons and ligaments.

Keywords

Mohawk; anterior cruciate ligament; osteoarthritis

Corresponding author: Hiroshi Asahara, MD, PhD The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037 asahara@scripps.edu.

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INTRODUCTION

Osteoarthritis (OA) is the most common musculoskeletal disease caused by age-related changes in the homeostatic balance between anabolic and catabolic mechanisms (1). The main OA pathogenesis mechanisms are cartilage degradation induced by excessive mechanical stress, age-related changes in cells and extracellular matrix (ECM), mediated by the production of ECM-degrading enzymes and inflammatory cytokines (2, 3). While articular cartilage damage is central to the OA process, it involves all other joint tissues. The anterior cruciate ligament (ACL) is critical to the biomechanical stability and function of the knee joint. Traumatic ACL injury can lead to cartilage damage and OA. Aging-associated OA also leads to structural changes in the ACL, which can contribute to disease progression (4, 5). In this regard, we recently reported that the cartilage degradation and ACL degeneration show parallel progression in OA knee joints (6), suggesting the importance of ACL degradation and regeneration molecular mechanisms in OA pathogenesis.

The ECM of ACL consists of predominantly type I collagen, with small amounts of other collagens, proteoglycans and other glycoproteins, including aggrecan, decorin, tenascins and fibromodulin (7, 8). Collagen fibers provide tensile strength, and proteoglycans resistance to compression stress. ACL ruptures usually occur in the mid substance of the femoral side (6).

Cell populations in the ACL are generally referred to as fibroblast-like cells although they are heterogeneous and also include subsets of progenitor cells (9, 10). Degraded ACL are characterized by changes in cell organization, cell death and proliferation and abnormal differentiation, most notably chondrocyte-like cell morphology and gene expression (6, 11). Understanding mechanisms that govern survival, differentiation and ECM production by ligament cells is essential to developing new concepts for pathogenesis and therapeutic approaches.

Mohawk (Mkx) and Scleraxis (Scx) are the two transcription factors with relative specificity for tendon/ligament. Scx is a helix-loop-helix (bHLH) transcription factor, which regulates the differentiation of tendon/ligament progenitors during skeletal development (12, 13). However, its expression level is low in mature ligament and tendon cells, suggesting that it may not play a major role in mature tendon/ligament homeostasis. Mkx transcription factor is also important during tendon and ligament development (14-16). Mkx deficient mice have hypoplastic tendons throughout the body and deficient type I collagen production in tendon cells (14, 16). Importantly, we found that in mice, Mkx expression is maintained in mature tendon/ligament cells, suggesting a potential role of Mkx in tendon/ligament tissues homeostasis and regeneration.

Based on this observation, the present study examined expression patterns and function of MKX in human adult ligament and tendon tissues in the context of OA pathogenesis.

MATERIALS AND METHODS

Tissue procurement, macroscopic and histological analysis

Intact human knee joints were obtained at autopsy within 24-48 hours postmortem from 13 normal donors (age 36.9±11.0 years), 16 OA donors (age 79.7±11.4 years) and 8 old donors without OA (age 76.9±12.9 years) (Table 1). Tissue collection was approved by the Scripps Human Subjects Committee. Articular cartilage in all knee compartments was graded macroscopically as described previously (17). Briefly, we established a detailed scoring method based on the International Cartilage Repair Society knee map (18) by dividing the cartilage into 39 regions, which were graded macroscopically using a modified Outerbridge scoring system (19). Each area was scored on a scale of 1–4, where 1=intact surface,

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2=fibrillation, 3=fissuring, and 4-erosion. The total knee cartilage scores ranged from 39 (normal) to 156 (maximum severity) (6). The total knee cartilage scores were translated into grades 0–IV, where grade 0=normal (total score 39), grade I=minimal change (total score 40–58), grade II=mild change (total score 59–78), grade III=moderate change (total score 79–97), and grade IV=severe change (total score 98).

ACL were examined macroscopically and histologically as described previously (6). In brief, the ACL was resected at the insertion sites on the femur and tibia. The macroscopic appearance was graded as normal, abnormal (thinner than normal and sclerotic), or ruptured (complete disappearance of the ligament or persistence of only a few fibers) (21). For histologic analysis, the samples were immediately fixed and each specimen was cut transversely at the proximal one-third of the ligament and longitudinally through the center of the ligament from the proximal one-third of the ACL substance and femur attachment site, where ACL tears frequently occur (20, 21). The ACL sections were stained with hematoxylin and eosin (H&E) and graded histologically using a modification of previously described scoring systems (6, 22). The following categories were examined and scored for each ligament: inflammation in the ACL substance, mucoid degeneration, chondroid metaplasia, cystic changes, and orientation of collagen fibers. The highest possible summed score for ligament degeneration (total ACL score) was 15.

OA grade 0 or I and age <60 were included in the normal group. The aging group included donors age ≧60 that had no history of OA and no or only minimal cartilage degradation with OA grade 0 or I. Knees with grade II to IV constituted the OA group in this study. We obtained 18 ACLs from normal group, 16 ACLs from OA group and 8 ACLs from aging group. Seven ACLs from normal group and 8 ACLs from the OA group were used for gene expression assays. Eight ACLs respectively from the normal group, OA group and aging group were used for immunohistochemistry. Six ACLs from normal group were used for cell isolation and in vitro studies on MKX expression and function (Table 1).

Immunohistochemistry

Immunohistochemistry (IHC) was performed to investigate the expression pattern of MKX in human ACL tissue. Paraffin-fixed samples were first deparaffinized in xylene substitute Pro-Par Clearant (Anatech) and rehydrated in graded ethanol and water. For antigen retrieval, sections were incubated with 10% trypsin and kept at 37°C for 30 minutes. Following a wash with phosphate-buffered saline (PBS), sections were blocked with 10% normal goat serum for 30 min at room temperature. Rabbit anti-human MKX polyclonal antibody (1:1000 dilution, LS-C30267, Lifespan Bioscience) was applied and incubated overnight at 4°C. After washing with PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:200 dilution, Vector Laboratories) or biotinylated goat anti-mouse secondary antibody (1:200 dilution, Vector Laboratories) for 30 minutes at room temperature, and then incubated using Vectastain ABC-AP kit (Vector Laboratories) for 30 minutes. Slides were washed, and sections were incubated with an alkaline phosphatase substrate (Vector Laboratories) for 15 minutes. The slides were rinsed in tap water and counterstained with Hematoxylin.

For the quantification of positive cells for MKX, at least six pictures were randomly taken under 40x magnification, showing the mid substance of ACL without severely degenerated regions with chondroid metaplasia or extensive mucoid degeneration. The total cell number and total number of positive cells in dense collagenous tissue were counted in each microscopic field by two different readers.

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RNA isolation and real time RT-PCR

Total RNA was isolated from ACL tissues or cells by extracting the homogenate in TRIzol (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction (qPCR) was performed using LightCycler 480 instrument (Roche Diagnostics) and the TaqMan Gene Expression Assay probes for transcription factors-*MKX* (Hs00543190_m1), *SCX* (Hs03054634_g1) and *SOX9* (Hs00165814_m1); extracellular matrix components-*aggrecan* (*ACAN* (Hs00202971_m1)), α1 chain of collagen types I, II and III (*COL1A1* (Hs00164004_m1), *COL2A1* (Hs00264051_m1) and *COL3A1* (Hs00943809_m1)), *decorin* (*DCN* (Hs00754870_s1)), *fibromodulin (FMOD* (Hs00157619_m1)), *tenomodulin* (*TNMD* (Hs00223332_m1)), *tenascinXB* (*TNXB* (Hs00372889_g1)); *Matrix metalloproteinase 13* (*MMP13* (Hs00233992_m1)), *Interleukin-6* (*IL6* (Hs00985639_m1)) according to the manufacturer's protocol (Applied Biosystems). Gene expression levels were assessed relative to *GAPDH* (Hs99999905_m1).

Human ACL cell isolation

Human ACL cells were isolated from mid substance of ACL from normal donors and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) as described previously (23). Experiments with ACL-derived cells were performed at passage 1 or 2.

Treatment with interleukin-1β (IL-1β)

Human ACL-derived cells were plated in 12-well plates containing DMEM with 2% calf serum and 1% penicillin/streptomycin. Cells were treated with recombinant human IL-1β (5 ng/ml; PeproTech) for 6 hours when total RNA was isolated with TRIzol reagent. Quantitative-PCR was performed with the TaqMan Gene Expression Assays (24).

SiRNA knock down of MKX in ACL-derived cells

MKX specific siRNA and negative-control siRNA were purchased from Qiagen (SI04222064 and SI04346909). ACL-derived cells were transfected with 100nM siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were incubated for 48 hours after transfection and then harvested for quantitative-PCR and Western blot analyses.

Western blotting

Cell extracts were prepared with SDS lysis buffer. Protein concentrations were measured by BCA protein assay kit (Bio-Rad). Equal amounts of protein were applied. Western Blot (WB) analysis was performed using anti-MKX antibody (1:5,000 dilution, LS-C30267, Lifespan Bioscience), anti-Collagen I antibody (1:5,000 dilution, ab292, Abcam) and anti-SOX9 antibody (1:1,000 dilution, AB5535, Millipore). Anti-Rabbit IgG ECL Antibody, HRP Conjugated (NA9340, GE health care) or Anti-Mouse IgG ECL Antibody, HRP Conjugated (NA9310, GE Health Care) were used as secondary antibody (1:5000 dilution for 1hr).

Statistical analysis

Mean values are presented with standard error of the mean (SEM) in all figures. Wilcoxon signed-rank test was used to calculate the significance of differences in mean values for comparisons between two groups. Differences among three groups were analyzed using a one-way analysis of variance (ANOVA). Post-hoc comparisons were performed using the Tukey test. A value of $p<0.05$ was considered statistically significant.

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RESULTS

Gene expression differences between ACL from normal and OA knees

To determine changes in gene expression patterns in ACL from OA knee joints as compared to normal knees, we performed using qPCR for the transcription factors *MKX*, *SCX* and *SOX9*, the extracellular matrix components *ACAN*, *COL1A1*, *COL2A1*, *COL3A1*, *DCN*, *FMOD*, *TNMD*, *TNXB* and *MMP13*. The expression of *MKX* was significantly lower in the OA group. (Figure 1). For *SOX9*, there was a trend towards increased expression in the OA group. The *COL1A1* and *TNXB* expression were significantly lower in OA group (Figure 1). The expression of *COL2A1*, *COL3A1*, *DCN*, *FMOD* and *TNMD* showed no significant difference between ACL from normal and OA knees (Figure 1).

MKX expression pattern in human ACL tissue

Immunohistochemistry was used to examine the expression pattern of MKX on sagittal sections of human ACLs (Figure 2). Cells and collagen fibers of normal group were smooth and well arranged (Figure 2A-C). ACLs in the OA group showed collagen fibril and cellular irregularity, angiogenesis and areas with increased cell density (Figure 2D-F). In the aging group, disorientation of collagen fibers was more frequently observed and cell density was lower compared with the normal group (Figure 2G-I). MKX was robustly expressed by ACL cells in normal knees, and this was reduced in OA and aging groups. In normal ACL, 77.8±9.1% (mean± SD) of cells were MKX positive and the percentage of positive cells decreased in OA to 28.6±12.1 and in the aging group to 49.6±10.1. In the OA group, collagen fibers were frequently disoriented and fibroblast like cells in disoriented fibers or small round cells were usually negative for MKX. In contrast, cells in well-arranged collagen fibers remained positive to MKX even in OA or aging group. We previously reported that disorientation of collagen fibers was observed in 105 (89.7%) of 117 ACLs and chondroid metaplasia was observed in 40 (34.2%) of 117 ACLs. However, chondroid metaplasia was not observed in any of the ACLs from normal knees from young donors, disorientation of collagen fibers was observed in 6 of 9 ACLs from young normal donors (6). In present study, extensive chondroid metaplasia was observed in the OA group (n=3) and the percentage of MKX positive cells in these chondrocyte like cells was 69.1±4.7%.

IL-1β suppresses *MKX* gene expression in human ACL-derived cells

To explore the premise that pro-inflammatory cytokines, such as IL-1β, which are associated with ACL degeneration, may down-regulate *MKX* expression, primary cultures of ACL-derived cells from normal knees were treated with IL-1β and expression of MKX, SCX, SOX9, ACAN, COL1A1, COL2A1, COL3A1, DCN, FMOD, TNMD, TNXB, IL6 and *MMP13* were analyzed. The expression of *MKX* was significantly decreased by IL-1β. The expression of *SCX* was also decreased, while *SOX9* was significantly increased (Figure 3A). *IL-6* and *MMP13* expression were significantly increased in ACL cells after IL-1β treatment (Figure 3B). Among ECM genes, the expression of *COL1A1*, *COL3A1* and *TNXB* were decreased significantly after IL-1β (Figure 3C).

Knockdown of MKX expression in human ACL-derived cells

To investigate the function of MKX in regulating ECM genes and other transcription factors, ACL-derived cells were transfected with either control siRNA or siRNA specific for MKX (siMKX). The expression of *MKX* was reduced to about 30% (Figure 4A). As a result, *SOX9* expression was significantly increased, while the expression of *SCX* was unaffected (Figure 4A). Inflammation related genes such as *IL6* or *MMP13* were also unaffected in ACL-derived cells transected with siMKX (Figure 4B). Among the ECM related genes we examined, only *TNXB* was decreased significantly (Figure 4C).

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In addition, we examined protein level expression of MKX, SOX9 and type I collagen in ACL derived cells using Western blot analysis (Figure 4D). The SOX9 protein expression was significantly elevated by siMKX. In contrast, type I collagen was significantly reduced (Figure 4D and E).

DISCUSSION

Mkx has recently been identified as a tendon/ligament specific transcription factor regulating expression of tendon/ligament ECM genes, including Col1a1, tenomodulin and/or fibromodulin during skeletal development in mice (14, 16); however, MKX expression and function in human tendon/ligament cells have not been elucidated. In this study, we demonstrated that MKX is expressed in adult human ligament and MKX expression is clearly decreased in ligaments from patients with OA and this correlates with deficient expression important ECM genes such as *COL1A1* and *TNXB*.

Prior studies examined the relationship between ACL degeneration and OA (6, 25-27). Typically, the degenerative changes in ACL start with collagen fiber disorientation, followed by mucoid degeneration, inflammatory cell infiltration and /or neovascularization. IL-1β is among the critical pro-inflammatory cytokines involved in cartilage degradation during OA pathogenesis and also in ACL degeneration (28, 29). IL-1β stimulation in human tendon derived cells significantly reduces the expression of collagen I, collagen III, tenomodulin and SCX and promotes expression of genes associated with ACL degeneration, such as *ACAN, COX2, MMP-1, -3, -13, ADAMTS4* and *IL-6* (30, 31). Here we show that MKX is also down-regulated by IL-1β stimulation in human ligament cells. In addition, the down regulation of MKX by siRNA in human ACL-derived cells decreases the expression of *TNXB*. These results support our notion that MKX down regulation is involved in ligament degeneration in OA, and that MKX maintains ligament function and prevents degeneration.

As previously reported, Mkx regulates type I collagen in tendon/ligament development (14, 16). In this study, MKX knock down in human ACL-derived cells did not significantly reduce the expression of *COL1A1* mRNA. However, western blotting analysis indicated that type I collagen protein expression was reduced by siMKX treatment. This discrepancy between mRNA and protein levels could be caused by indirect effect of MKX knockdown via reduced *TNXB* expression. TNXB is a member of tenascin family in ECM glycoproteins that contribute to matrix structure, and regulate collagen fibrillogenesis via directly binding with type I collagen (32). Studies of cultured dermal fibroblasts showed that *Tnxb*–/– fibroblasts failed to deposit type I collagen into cell-associated matrix, although synthesis of type I collagen by *Tnxb*–/– and wild type cells was similar (33).

Interestingly, the expression of the chondrogenic transcription factor *SOX9*, was increased in both IL-1β stimulation and MKX siRNA knockdown in human ACL-derived cells. Also the immunohistochemical analysis of human ACL tissues demonstrated that SOX9 positive cells were increased in ACL from OA knee compared with normal ACL. In this regard, we and others previously reported that chondroid metaplasia was observed in ACLs from cartilage degenerated knees but not in ACLs with normal cartilage (6, 10, 34). Several reports indicated that ACL-derived cells possess high chondrogenic capacity and SOX9 drives the differentiation from tenocyte to chondrocyte (10, 35). It thus appears that ligament/tendon may contain a substantial proportion of progenitor cells. In OA, progenitor cells are activated, abnormally expressing SOX9 and differentiated to chondrocyte like cells (27, 36). Though these abnormal cells in degenerated ACL are frequently positive to SOX9, other chondrogenic makers such as type II collagen or ACAN are not expressed by all of these cells. In our recent study, the expression of collagen II or ACAN was seen only in a

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subset of cells even in areas with chondroid metaplasia (36). This is consistent with our in vitro observations where increased *SOX9* expression was not associated with other chondrogenic makers such as *COL2A1* and *ACAN*. Moreover, several studies on chondrogenesis of human MSCs indicated that the increasing *SOX9* expression precedes *COL2A1* or *ACAN*(37).

In this study we also aimed at distinguishing changes that are related to normal aging from OA-related changes. We included a set of donors with no history of OA and minimal articular cartilage changes on macroscopic examination of the knees. In the present study, the mean age of OA and normal aging group were similar but there was a significantly lower level of MKX expression in the aging group compared in normal group. Therefore, the effect of aging on the suppression of MKX is evident. This ACL from OA-affected knees had even lower levels of MKX, indicating and OA-related mechanisms also contribute to the changes in MKX. The observations that IL-1β suppressed MKX in ACL-derived cells offer a potential mechanism.

In conclusion, this study demonstrates that MKX is expressed in cells of normal adult human ACL. MKX expression is reduced in ACL from OA-affected joints. In vitro, IL-1β suppresses MKX and enhances SOX9. These observations support the novel concept that loss of MKX, in part driven by pro-inflammatory cytokines such as IL-1β, leads to abnormal chondrocyte-like differentiation of ligament cells and production of ECM with deficient biomechanical properties. Correcting abnormal MKX expression can be pursued as a new approach to address tissue repair after injury and during chronic processes such as OA.

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References

1. Lotz MK, Carames B. Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. Nat Rev Rheumatol. 2011; 7(10):579–87. [PubMed: 21808292]
2. Hashimoto S, Ochs RL, Komiya S, Lotz M. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum. 1998; 41(9):1632–8. [PubMed: 9751096]
3. Martin JA, Brown TD, Heiner AD, Buckwalter JA. Chondrocyte senescence, joint loading and osteoarthritis. Clin Orthop Relat Res. 2004; (427 Suppl):S96–103. [PubMed: 15480082]
4. Sah RL, Yang AS, Chen AC, Hant JJ, Halili RB, Yoshioka M, et al. Physical properties of rabbit articular cartilage after transection of the anterior cruciate ligament. J Orthop Res. 1997; 15(2):197–203. [PubMed: 9167621]
5. Nelson F, Billinghurst RC, Pidoux I, Reiner A, Langworthy M, McDermott M, et al. Early post-traumatic osteoarthritis-like changes in human articular cartilage following rupture of the anterior cruciate ligament. Osteoarthritis Cartilage. 2006; 14(2):114–9. [PubMed: 16242972]
6. Hasegawa A, Otsuki S, Pauli C, Miyaki S, Patil S, Steklov N, et al. Anterior cruciate ligament changes in the human knee joint in aging and osteoarthritis. Arthritis Rheum. 2012; 64(3):696–704. [PubMed: 22006159]
7. Petersen W, Tillmann B. Structure and vascularization of the cruciate ligaments of the human knee joint. Anat Embryol (Berl). 1999; 200(3):325–34. [PubMed: 10463347]
8. Laurencin CT, Freeman JW. Ligament tissue engineering: an evolutionary materials science approach. Biomaterials. 2005; 26(36):7530–6. [PubMed: 16045982]

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9. Cheng MT, Yang HW, Chen TH, Lee OK. Isolation and characterization of multipotent stem cells from human cruciate ligaments. Cell Prolif. 2009; 42(4):448–60. [PubMed: 19489981]
10. Furumatsu T, Hachioji M, Saiga K, Takata N, Yokoyama Y, Ozaki T. Anterior cruciate ligament-derived cells have high chondrogenic potential. Biochem Biophys Res Commun. 2010; 391(1): 1142–7. [PubMed: 20006576]
11. Levy YD, Hasegawa A, Patil S, Koziol JA, Lotz MK, D'Lima DD. Histopathological changes in the human posterior cruciate ligament during aging and osteoarthritis: correlations with anterior cruciate ligament and cartilage changes. Ann Rheum Dis. 2013; 72(2):271–7. [PubMed: 22872023]
12. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development. 2007; 134(14):2697–708. [PubMed: 17567668]
13. Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Developmental Biology. 2006; 298(1):234–47. [PubMed: 16876153]
14. 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(23):10538–42. [PubMed: 20498044]
15. Kimura W, Machii M, Xue X, Sultana N, Hikosaka K, Sharkar MT, et al. Irxl1 mutant mice show reduced tendon differentiation and no patterning defects in musculoskeletal system development. Genesis. 2011; 49(1):2–9. [PubMed: 21254332]
16. Liu W, Watson SS, Lan Y, Keene DR, Ovitt CE, Liu H, et al. The atypical homeodomain transcription factor Mohawk controls tendon morphogenesis. Mol Cell Biol. 2010; 30(20):4797–807. [PubMed: 20696843]
17. Allain J, Goutallier D, Voisin MC. Macroscopic and histological assessments of the cruciate ligaments in arthrosis of the knee. Acta Orthop Scand. 2001; 72(3):266–9. [PubMed: 11480602]
18. Nakahara H, Misawa H, Yoshida A, Hayashi T, Tanaka M, Furumatsu T, et al. Bone repair using a hybrid scaffold of self-assembling peptide PuraMatrix and polyetheretherketone cage in rats. Cell Transplant. 2010; 19(6):791–7. [PubMed: 20573298]
19. Outerbridge RE. The etiology of chondromalacia patellae. J Bone Joint Surg Br. 1961; 43-B:752–7. [PubMed: 14038135]
20. Kennedy JC, Weinberg HW, Wilson AS. The anatomy and function of the anterior cruciate ligament. As determined by clinical and morphological studies. J Bone Joint Surg Am. 1974; 56(2):223–35. [PubMed: 4452683]
21. Wang IE, Mitroo S, Chen FH, Lu HH, Doty SB. Age-dependent changes in matrix composition and organization at the ligament-to-bone insertion. J Orthop Res. 2006; 24(8):1745–55. [PubMed: 16779829]
22. Mullaji AB, Marawar SV, Simha M, Jindal G. Cruciate ligaments in arthritic knees: a histologic study with radiologic correlation. J Arthroplasty. 2008; 23(4):567–72. [PubMed: 18514876]
23. Mok SS, Masuda K, Hauselmann HJ, Aydelotte MB, Thonar EJ. Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. Identification of two distinct metabolic matrix pools. J Biol Chem. 1994; 269(52):33021–7. [PubMed: 7806530]
24. Miyaki S, Nakasa T, Otsuki S, Grogan SP, Higashiyama R, Inoue A, et al. MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. Arthritis & Rheumatism. 2009; 60(9):2723–30. [PubMed: 19714579]
25. Hovis KK, Alizai H, Tham SC, Souza RB, Nevitt MC, McCulloch CE, et al. Non-traumatic anterior cruciate ligament abnormalities and their relationship to osteoarthritis using morphological grading and cartilage T2 relaxation times: data from the Osteoarthritis Initiative (OAI). Skeletal Radiol. 2012; 41(11):1435–43. [PubMed: 22366737]
26. Cushner FD, La Rosa DF, Vigorita VJ, Scuderi GR, Scott WN, Insall JN. A quantitative histologic comparison: ACL degeneration in the osteoarthritic knee. J Arthroplasty. 2003; 18(6):687–92. [PubMed: 14513440]

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27. Kumagai K, Sakai K, Kusayama Y, Akamatsu Y, Sakamaki K, Morita S, et al. The extent of degeneration of cruciate ligament is associated with chondrogenic differentiation in patients with osteoarthritis of the knee. Osteoarthritis Cartilage. 2012; 20(11):1258–67. [PubMed: 22846713]
28. Kraus VB, Birmingham J, Stabler TV, Feng S, Taylor DC, Moorman CT 3rd, et al. Effects of intraarticular IL1-Ra for acute anterior cruciate ligament knee injury: a randomized controlled pilot trial (NCT00332254). Osteoarthritis Cartilage. 2012; 20(4):271–8. [PubMed: 22273632]
29. Marks PH, Donaldson ML. Inflammatory cytokine profiles associated with chondral damage in the anterior cruciate ligament-deficient knee. Arthroscopy. 2005; 21(11):1342–7. [PubMed: 16325085]
30. Tsuzaki M, Guyton G, Garrett W, Archambault JM, Herzog W, Almekinders L, et al. IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. J Orthop Res. 2003; 21(2):256–64. [PubMed: 12568957]
31. Busch F, Mobasheri A, Shayan P, Lueders C, Stahlmann R, Shakibaei M. Resveratrol modulates IL-1beta-induced PI3K and NF-kappaB signaling pathways in human tenocytes. J Biol Chem. 2012
32. Minamitani T, Ikuta T, Saito Y, Takebe G, Sato M, Sawa H, et al. Modulation of collagen fibrillogenesis by tenascin-X and type VI collagen. Exp Cell Res. 2004; 298(1):305–15. [PubMed: 15242785]
33. Mao JR, Taylor G, Dean WB, Wagner DR, Afzal V, Lotz JC, et al. Tenascin-X deficiency mimics Ehlers-Danlos syndrome in mice through alteration of collagen deposition. Nat Genet. 2002; 30(4):421–5. [PubMed: 11925569]
34. Clegg PD, Strassburg S, Smith RK. Cell phenotypic variation in normal and damaged tendons. Int J Exp Pathol. 2007; 88(4):227–35. [PubMed: 17696903]
35. Takimoto A, Oro M, Hiraki Y, Shukunami C. Direct conversion of tenocytes into chondrocytes by Sox9. Exp Cell Res. 2012
36. Hasegawa A, Nakahara H, Kinoshita M, Asahara H, Koziol J, Lotz MK. Cellular and extracellular matrix changes in anterior cruciate ligaments during human knee aging and osteoarthritis. Arthritis Res Ther. 2013; 15(1):R29. [PubMed: 23406989]
37. Suzuki S, Muneta T, Tsuji K, Ichinose S, Makino H, Umezawa A, et al. Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration. Arthritis Res Ther. 2012; 14(3):R136. [PubMed: 22676383]

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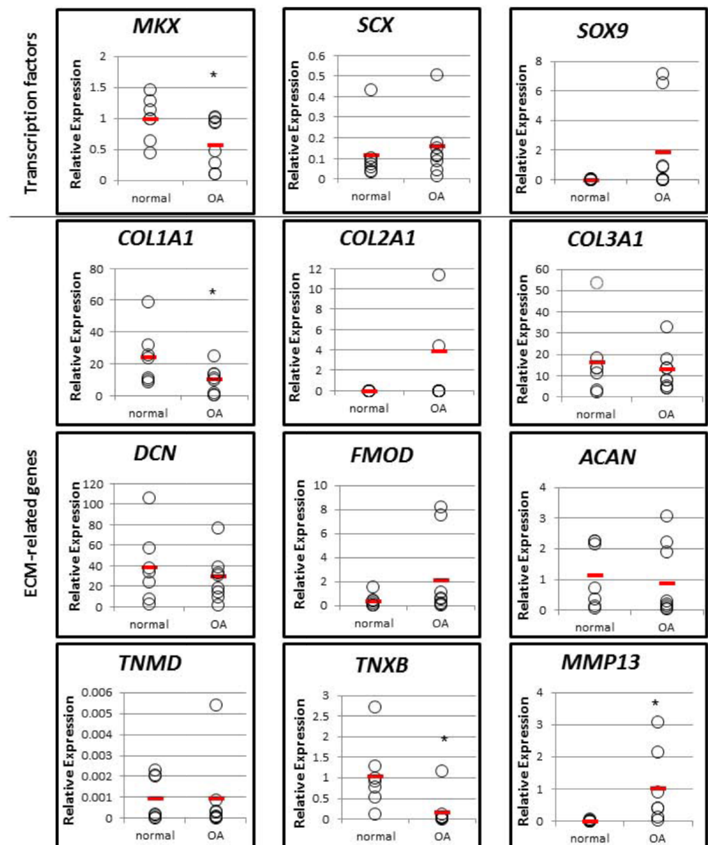

Figure 1.

Expression changes of transcription factors *MKX, SOX9* and *SCX* and ECM-related genes in ACL tissue from normal and OA knee joints.

Gene expression in mid substance of human ACLs was determined by quantitative polymerase chain reaction. The expression of *MKX* was significantly decreased in the OA group. *COL1A1* and *TNXB* expression were also decreased significantly in the OA group. *MMP13* expression was increased in the OA group. Bars show mean +/−SEM. n= Normal group 7, OA group 8. * = P < 0.05.

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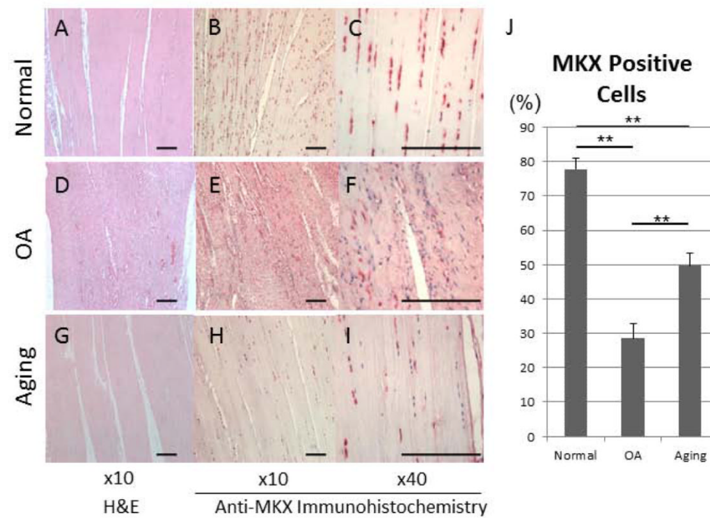

Figure 2.

MKX expression in ACL cells in situ.

Representative H&E staining of ACL from normal donor (A), OA donor (D) and aging donor (G). Anti-MKX staining of ACL from normal (B, C), OA (E, F) and aging knee joint (H, I). In normal ACL, a large majority of cells were MKX positive (B, C). Typical changes of OA group were collagen fibril and cellular irregularity, angiogenesis and increased cell density (D-F). MKX expression was decreased in cells in the ligament substance both in OA and aging groups (E, F, H, I), although MKX positive cells were present in perivascular areas (E, F). The percentages of MKX positive cells were significantly decreased in OA group and aging group (J). n=8. **P<0.01. Scale bar = 200μm.

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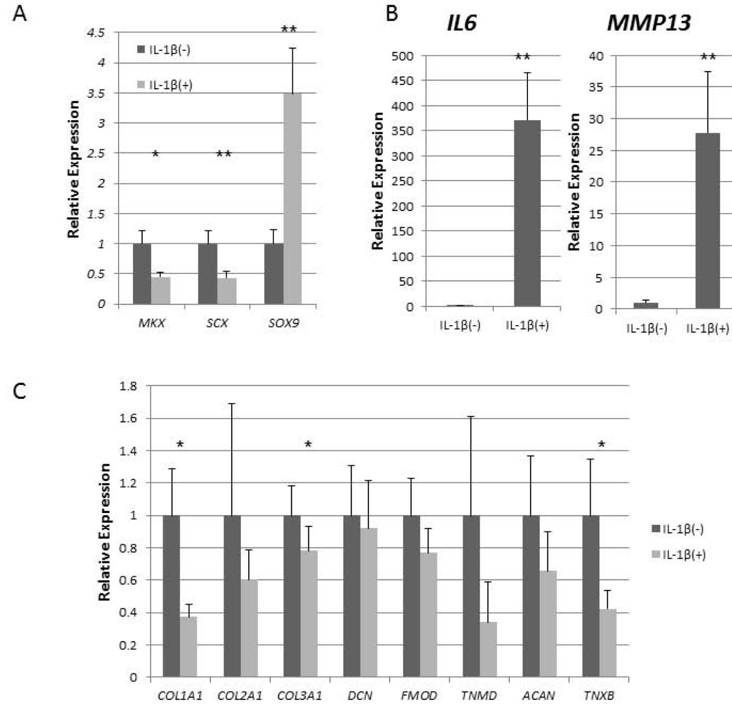

Figure 3.

Interleukin-1β (IL-1β) induced gene expression changes in human ACL-derived cells. ACL cells from normal donors were treated with IL1-β (5 ng/ml) for 6 hours. The expression of transcription factors *MKX*, *SCX* and *SOX9*; matrix components-aggrecan (*ACAN*), α1 chain of collagen types I, II and III *(COL1A1*, *COL2A1* and *COL3A1*), decorin (*DCN*), fibromodulin (*FMOD*), tenomodulin (*TNMD*), tenascinXB (*TNXB*) were analyzed by using quantitative polymerase chain reaction. IL-1β stimulation significantly decreased *MKX* and *SCX* expression and increased *SOX9* expression (A). Among ECM related genes, *COL1A1, COL3A1* and *TNXB* were decreased significantly (C). *COL2A1, DCN, FMOD, TNMD* and *ACAN* expression did not change significantly (C). *IL6* and *MMP13* were also increased significantly by IL-1β stimulation (B). n=12 (12 different preparations from 6 different donors.) * = $P < 0.05$, ** = $P < 0.01$.

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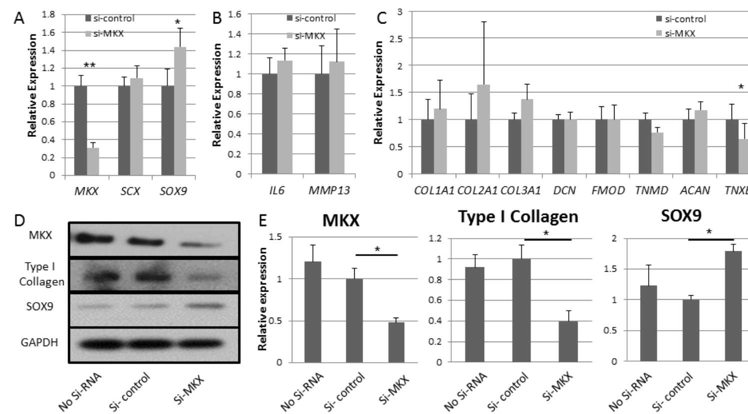

Figure 4.

Effects of MKX specific siRNA on gene expression and protein expression in human ACL-derived cells. Cells from normal donors in primary culture were treated with MKX specific siRNA for 48 hours. The expression of transcription factors, matrix components, *IL6* and *MMP13* were analyzed by using quantitative polymerase chain reaction. Cell lysates were prepared for analysis by Western blotting. MKX specific siRNA significantly increased expression of *SOX9* while the expression of *TNXB* was significantly reduced (C). Knock-down of MKX had not significant influence on the expression of the other genes (C). n=12 (12 different preparations from 6 different donors.). The protein expression of COL1A1 and MKX were decreased by MKX specific siRNA treatment, while SOX9 was significantly increased (D, E). Densitometry of the autoradiographs was performed with NIH Image J software (E). * = P < 0.05, ** = P < 0.01.

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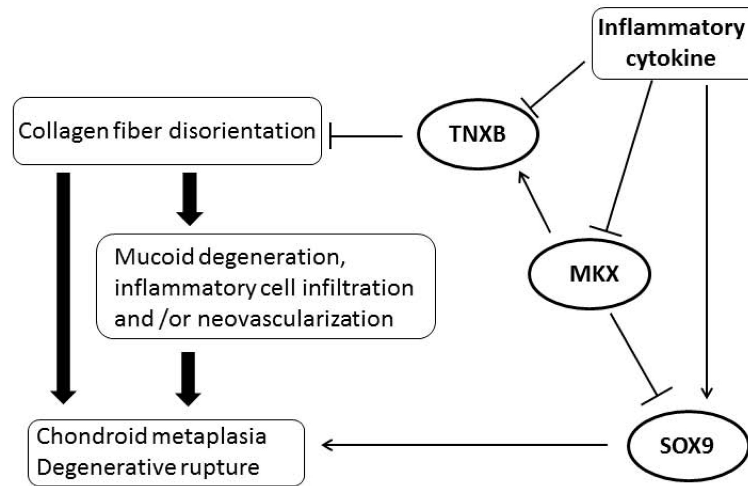

Figure 5.
Hypothesis for a role of MKX in ligament homeostasis and degeneration. MKX maintains ligament function and prevents degeneration via regulating TNXB and SOX9. Reduced expression of MKX in degenerated ligaments, mediated by proinflammatory cytokines, leads to abnormal differentiation and ECM production.

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Table 1

Donor information for ACL tissue examinations

	age	sex	Total knee cartilage score (39-156)	cartilage grade (0-IV)	Total ACL score (0-15)	ACL histology grade	applications
Normal group							
Normal 1	28	F	39	0	0.5	mild	mRNA
Normal 2	50	F	46	I	4	mild	mRNA
Normal 3	42	F	42	I	2.5	mild	mRNA
Normal 4	45	F	54	I	4	mild	mRNA
Normal 5	24	M	39	0	1.5	mild	mRNA
Normal 6	23	F	40	I	2	mild	mRNA, IHC
Normal 7	23	F	40	I	0.5	mild	mRNA, IHC, cell
Normal 8	24	M	39	0	0	normal	IHC
Normal 9	36	M	43	I	0.5	mild	IHC
Normal 10	42	F	39	0	0	normal	IHC
Normal 11	44	F	42	I	0.5	mild	IHC
Normal 12	48	F	43	I	3.5	mild	IHC
Normal 13	51	M	44	I	0	normal	IHC
Normal 14	55	M	53	I	1.5	mild	cell
Normal 15	29	M	39	0	0	normal	cell
Normal 16	29	M	39	0	0	normal	cell
Normal 17	49	F	49	I	1.5	mild	cell
Normal 18	49	F	43	I	1	mild	cell
OA group							
OA 1	76	M	75	II	5	mild	mRNA
OA 2	78	M	81	III	3	mild	mRNA
OA 3	88	F	83	III	7	moderate	mRNA
OA 4	62	F	87	III	3.5	mild	mRNA
OA 5	76	M	84	III	3	mild	mRNA
OA 6	88	F	76	II	2	mild	mRNA
OA 7	91	M	71	II	7	moderate	mRNA
OA 8	94	M	71	II	5	severe	mRNA
OA 9	64	F	69	II	5	mild	IHC
OA 10	64	M	101	IV	5	mild	IHC
OA 11	82	M	65	II	6	moderate	IHC
OA 12	85	F	100	IV	12	severe	IHC
OA 13	86	M	77	II	8	moderate	IHC
OA 14	90	F	81	III	3	mild	IHC
OA 15	92	F	81	III	6	moderate	IHC
OA 16	92	F	70	II	6	moderate	IHC
Aging group							

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	age	sex	Total knee cartilage score (39-156)	cartilage grade (0-IV)	Total ACL score (0-15)	ACL histology grade	applications
Aging 1	60	F	42	I	3.5	mild	IHC
Aging 2	63	M	45	I	1	mild	IHC
Aging 3	68	F	45	I	4	mild	IHC
Aging 4	76	M	57	I	4	mild	IHC
Aging 5	79	F	54	I	1	mild	IHC
Aging 6	81	M	53	I	0.5	mild	IHC
Aging 7	94	F	54	I	2	mild	IHC
Aging 8	94	F	58	I	3	mild	IHC

OA = osteoarthritis; mRNA = messenger ribo nucleic acid; IHC = immunohistochemistry.