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Matrix cross-linking–mediated mechanotransduction promotes posttraumatic osteoarthritis

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Osteoarthritis (OA) is characterized by impairment of the load-bearing function of articular cartilage. OA cartilage matrix undergoes extensive biophysical remodeling characterized by decreased compliance. In this study, we elucidate the mechanistic origin of matrix remodeling and the downstream mechanotransduction pathway and further demonstrate an active role of this mechanism in OA pathogenesis. Aging and mechanical stress, the two major risk factors of OA, promote cartilage matrix stiffening through the accumulation of advanced glycation end-products and up-regulation of the collagen cross-linking enzyme lysyl oxidase, respectively. Increasing matrix stiffness substantially disrupts the homeostatic balance between chondrocyte catabolism and anabolism via the Rho–Rho kinase–myosin light chain axis, consequently eliciting OA pathogenesis in mice. Experimental enhancement of nonenzymatic or enzymatic matrix cross-linking augments surgically induced OA pathogenesis in mice, and suppressing these events effectively inhibits OA with concomitant modulation of matrix degrading enzymes. Based on these findings, we propose a central role of matrix-mediated mechanotransduction in OA pathogenesis.

lysyl oxidase | matrix stiffness | mechanotransduction | cartilage | osteoarthritis

The mechanics of the ECM and resulting effects on its interactions with cells regulate numerous biological functions (1). Various pathological conditions in human diseases are associated with aberrant ECM remodeling and consequent deviation from intrinsic ECM material properties (2). Mechanical perturbation of ECM affects the ways in which cells respond to externally applied mechanical forces and generate internal traction forces through cell–matrix interactions (3). Therefore, elucidation of the functional relationships between ECM mechanics and cellular transduction pathways is of critical importance.

Articular cartilage ECM consisting of a collagenous network and highly charged proteoglycans confers the unique load-bearing function to joints. The dense aggregates of negatively charged proteoglycans provide resistance to compressive loading by promoting osmotic swelling, which is counterbalanced by cross-linked collagen fibrils that confer tissue tensile strength. Disruption of this delicate balance leads to structural damage and functional failure of articular cartilage and, consequently, to development of osteoarthritis (OA), the most common arthropathy (4, 5). OA cartilage ECM undergoes extensive remodeling, characterized by a decrease in matrix compliance (6, 7). These changes occur at the level of individual collagen fibrils, although the precise mechanisms regulating matrix remodeling remain elusive. Notably, matrix remodeling precedes cartilage destruction (6, 7), suggesting that monitoring the mechanical properties of cartilage matrix could serve as an innovative diagnostic approach for early detection of OA. Significant influence of matrix stiffness on mesenchymal lineage specification has been documented, and data have been obtained on the optimal ranges of substrate rigidity promoting osteogenesis. This regulatory process requires non-muscle myosin II activity, with concomitant effects on adhesion and actin cytoskeleton structures (8).

In this study, we sought to determine molecular mechanisms leading to ECM remodeling over the course of OA development and to investigate how mechanical alterations in cartilage matrix affect chondrocyte metabolism and regulate OA pathogenesis.

Results

Aging-Associated Accumulation of Advanced Glycation End-Products Drives Matrix Stiffening. Aging is one of the most prominent risk factors contributing to OA (4, 5). Aging processes have been implicated in elevating ECM stiffness in various tissues (9). In particular, advanced glycation end-products (AGEs) are regarded as a major factor in driving nonenzymatic collagen cross-linking, thereby increasing ECM stiffness (10). Examination of AGE levels in cartilage of young and aged mice revealed significant accumulation of AGEs in aging cartilage (Fig. 1*A*). AGEs were localized in the superficial zone where the destruction of articular cartilage mediated by aging-associated OA predominantly occurs (Fig. 1*A*). Experimental elevation of AGE levels by the addition of ribose led to significantly increased elastic moduli of collagen matrices (Fig. 1*B*) in association with reduced accumulation of proteoglycan, increased matrix metalloproteinase (MMP) expression and activity, and reduced expression of cartilage ECM molecules in embedded chondrocytes (Fig. 1*C* and Fig. S1 *A* and *B*). However, ribose treatment of chondrocytes grown on a 2D culture dish had no direct regulatory effect on catabolic and anabolic factor expression (Fig. S1*C*), suggesting that the effects of AGEs are mediated primarily through matrix cross-linking and stiffening.

Significance

Osteoarthritic cartilage destruction is caused primarily by an imbalance between chondrocyte catabolism and anabolism. Various proinflammatory cytokines that disrupt this metabolic balance during osteoarthritis (OA) pathogenesis have been identified. Here, in addition to these biochemical pathways, we demonstrate that changes in the biophysical properties of the chondrocyte microenvironment triggered by cartilage matrix cross-linking play a causal role in OA pathogenesis. Two major OA risk factors, aging and mechanical stress, cause matrix stiffening via nonenzymatic and enzymatic collagen cross-linking through the accumulation of advanced glycation end-products and the upregulation of lysyl oxidase, respectively. Data from the current study illustrate the dynamic nature of physical remodeling of cartilage ECM and elucidate the key mechanotransduction pathway regulating chondrocyte metabolism and osteoarthritic cartilage destruction.

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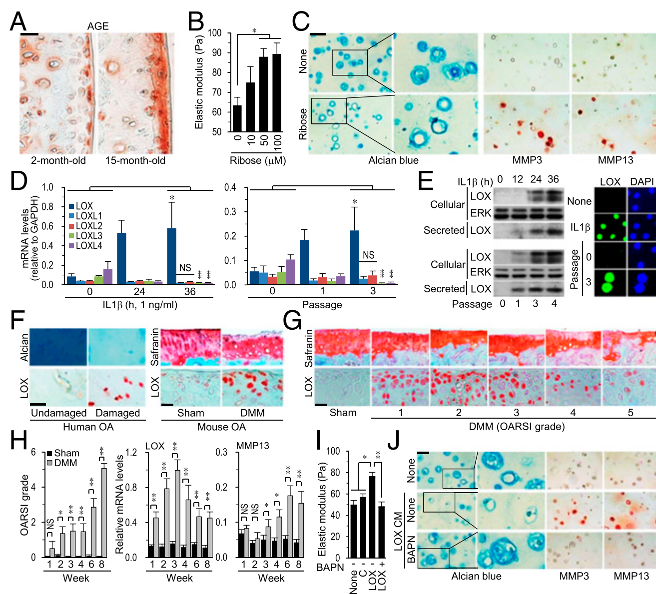

Fig. 1. AGE- and LOX-mediated ECM cross-linking promote cartilage matrix stiffening. (*A*) Immunostaining of AGE in knee cartilage sections of 2-mo-old and 15-mo-old mice. (*B* and *C*) Chondrocytes in collagen gels were treated with the indicated dose of ribose or were left untreated. (*B*) Rheometric analysis of elastic moduli of collagen gels ($n = 5$). (*C*) Proteoglycan staining and immunostaining of MMPs in collagen gel sections. (*D* and *E*) mRNA levels of LOX members quantified using qRT-PCR (*D*; $n \geq 6$) and Western blot and immunostaining of LOX (*E*) in mouse chondrocytes treated with IL-1β or differentiated via serial subculture. (*F*, *Left*) LOX immunostaining and Alcian blue staining in damaged and undamaged regions of human OA cartilage. (*Right*) Safranin-O staining and LOX immunostaining in DMM-induced mouse OA cartilage. (*G* and *H*) Safranin-O staining and LOX immunostaining (*G*) and scoring of cartilage destruction and mRNA levels of LOX and MMP13 (*H*) in cartilage tissue determined at the indicated weeks after sham or DMM operation ($n \geq 7$). (*I* and *J*) Chondrocytes in collagen gels were treated with LOX-CM with or without the LOX inhibitor BAPN (200 μM). (*I*) Rheometric analysis of elastic moduli of collagen gels ($n = 5$). (*J*) Proteoglycan staining and immunostaining of MMPs in collagen gel sections. (Scale bars: 50 μm.) Values are means ± SEM; $^*P < 0.05$, $^{**}P < 0.001$; NS, not significant.

Lysyl Oxidase Up-Regulated in Degenerating Cartilage Drives Enzymatic Matrix Cross-Linking and Stiffening. Another major risk factor for OA is mechanical stress associated with joint instability and injury. These traumatic events trigger the early onset of OA, suggesting that matrix cross-linking in posttraumatic OA is likely to occur independently of significant AGE accumulation. To obtain further molecular insights into OA-associated cartilage matrix remodeling, we examined the expression of collagen-modifying enzymes in degenerating chondrocytes. Hypoxia-inducible factor (HIF)-2α (encoded by the gene endothelial PAS domain-containing protein 1, *Epas1*) was previously identified as a transcription factor whose chondrocyte-specific expression is sufficient to drive OA pathogenesis in mice (11). The expression profiles of collagen-modifying genes were examined via microarray analysis in chondrocytes overexpressing HIF-2α (Fig. S2*A*). Among the enzymes examined, the collagen cross-linking enzyme lysyl oxidase (LOX), exhibited the highest fold increase (Fig. S2*A*). Indeed, HIF-2α overexpression in chondrocytes via adenoviral infection increased mRNA level of LOX, presumably attributable to the five consensus HIF-binding sites proximal to the transcription start site (Fig. S2 *B* and *C*). Notably, LOX was markedly up-regulated in chondrocytes treated with the OA-associated proinflammatory cytokine IL-1β and those dedifferentiated by serial subculture (Fig. 1*D*). However, these OA-associated stimuli did not elicit up-regulation of other LOX family members despite the presence of HIF-binding sites in their promoter regions (Fig. 1*D* and Fig. S2 *B* and *C*). Consequently, LOX was the predominantly expressed family member in each of these pathological conditions. All stimuli induced an increase in both cellular and secreted forms of LOX. Cellular LOX localized mainly to the nucleus (Fig. 1*E* and Fig. S2 *D* and *E*). Although LOX-mediated collagen cross-linking provides tensile strength and structural integrity to tissues, abnormally elevated LOX expression has been linked to various human diseases (12, 13). LOX expression was markedly elevated in OA-damaged regions of human cartilage compared with paired samples of undamaged cartilage from the same patients (Fig. 1*F*). Increased LOX levels also were observed in mouse OA cartilage induced by destabilization of the medial meniscus (DMM) surgery (Fig. 1*F*) or by intraarticular (IA) injection of HIF-2α expressing adenovirus (Ad-*Epas1*) (Fig. S2*F*) (11). By further increasing the detection sensitivity of immunohistochemical analysis, we were able to detect secreted LOX throughout the matrix as well as the cellular fraction in mouse OA cartilage (Fig. S1*D*). Additionally, DMM-induced LOX expression preceded both MMP13 expression and cartilage destruction (Fig. 1 *G* and *H*), suggesting a possible link between LOX-mediated ECM cross-linking and stiffening with OA cartilage destruction. Treatment of collagen matrices with LOX-conditioned medium (LOX-CM) led to significantly increased elastic moduli (Fig. 1*I*). Chondrocytes embedded in LOX-CM–treated matrices exhibited markedly reduced proteoglycan accumulation, increased MMP expression and activity, and reduced cartilage ECM expression (Fig. 1*J* and Fig. S1 *E* and *F*), whereas supplementation with β-aminopropionitrile (BAPN), a specific inhibitor of the LOX family (13), reversed these changes completely (Fig. 1 *I* and *J*).

Matrix Stiffness Regulates Chondrocyte Catabolism and Anabolism via the Rho–Rho Kinase–Myosin Light Chain Axis. To ascertain the role of ECM stiffness in OA development, we examined chondrocyte catabolism and anabolism on substrata having physiologically relevant compliance similar to cartilage matrix (6). Chondrocytes were grown on type II collagen-coated polyacrylamide gels with variations in physical stiffness (4–31 kPa) while maintaining identical adhesion ligand composition. Increasing substrate stiffness shifted the homeostatic balance toward catabolism in chondrocytes through up-regulation of essential catabolic effector molecules of matrix degradation and down-regulation of cartilage ECM molecules (Fig. 2*A* and Fig. S3*A*). Sex-determining region Y-box 9 (SOX9) localizes to the nucleus on soft surfaces. Increasing matrix stiffness disrupted this nuclear localization, resulting in decreased SOX9 transcriptional activity (Fig. 2*A* and Fig. S3*B*). Additionally, chondrocytes on relatively soft substrates (≤7 kPa) exhibited immature adhesion to underlying matrices with round morphology, whereas stiffer surfaces (12–31 kPa) promoted focal adhesion and stress fiber formation (Fig. S3*B*).

Because morphological changes associated with substratum stiffening are related to the mechanotransduction pathway comprising the Rho–Rho kinase–myosin light chain (Rho–ROCK–MLC) axis (2, 14, 15), we examined whether this pathway is regulated in chondrocytes by matrix compliance and under OA pathological conditions. Among the components of the Rho–ROCK–MLC axis (Fig. S4*A*), Rho activity was markedly augmented in chondrocytes grown on stiff substrates (Fig. 2*B*). ROCK activity was similarly increased in chondrocytes grown on stiff substrates, as determined based on phosphorylation of MLC at Ser19 (14) (Fig. 2*B*). Notably, phosphorylated MLC (pMLC) levels were increased substantially in OA cartilage of humans and mice (Fig. 2*C*). Next, we examined the role of the Rho–ROCK–MLC axis in matrix stiffness-induced modulation of the expression of catabolic and anabolic factors. Inhibition of Rho with C3 transferase, of ROCK with Y27632, and of myosin II ATPase with blebbistatin or disruption of F-actin with cytochalasin D abolished stiffening-mediated focal adhesion and stress fiber formation and restored SOX9 nuclear localization (Fig. S4*B*). Consistently, inhibition of the Rho–ROCK–MLC axis abolished stiffening-mediated up-regulation of matrix-degrading enzymes, down-regulation of collagen, type II, alpha 1 (COL2A1), aggrecan, and SOX9 (Fig. 2*D* and Fig. S4*C*), and inhibition of SOX9 activity (Fig. 2*E*).

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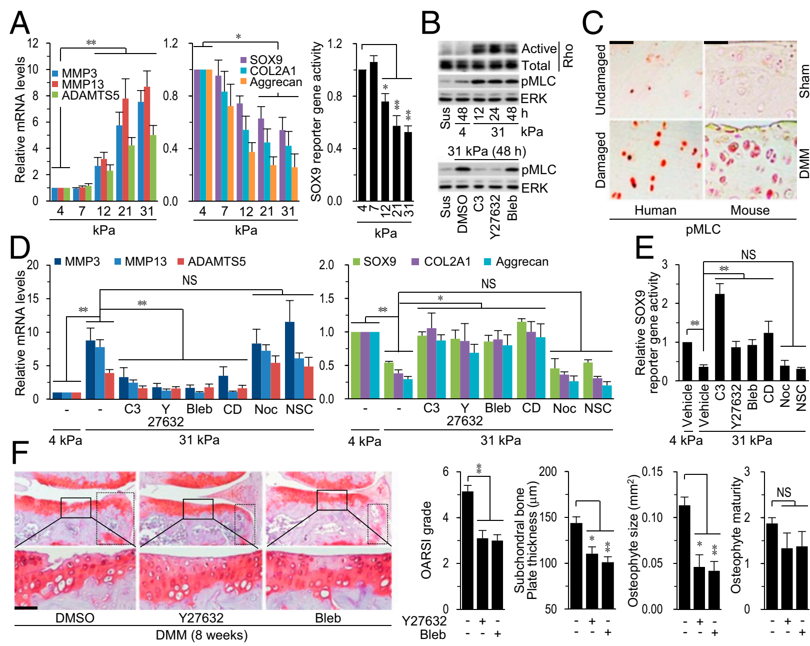

Fig. 2. The matrix stiffness-induced mechanotransduction pathway causes an imbalance between chondrocyte catabolism and anabolism. (*A*) mRNA levels of the indicated targets and SOX9 reporter gene activity ($n \geq 6$) in chondrocytes cultured for 120 h on type II collagen-coated gels of varying stiffness (4–31 kPa). (*B*) Western blot of active and total Rho and pMLC in chondrocytes isolated in suspension (Sus) or cultured on soft (4 kPa) or stiff (31 kPa) substrates following 48 h treatment with various inhibitors of components of the Rho–ROCK–MLC axis. (*C*) pMLC immunostaining in OA cartilage sections of humans and mice. (*D* and *E*) Chondrocytes grown on type II collagen-coated gels for 72 h were treated with various inhibitors of components of the Rho–ROCK–MLC axis for an additional 48 h. mRNA levels of the indicated targets (*D*) and SOX9 reporter gene activity (*E*) were quantified ($n \geq 5$). (*F*) Safranin-O staining and scoring of OA parameters ($n \geq 8$) in DMM-operated mice injected IA with the ROCK inhibitor Y27632 or with the MLC inhibitor blebbistatin. (Scale bar: 50 μm.) Values are means ± SEM; $*P < 0.01$, $**P < 0.001$; NS, not significant.

In contrast, neither inhibition of Rac1 nor disruption of microtubules affected stiffening-associated changes in chondrocytes (Fig. 2 *D* and *E* and Fig. S4 *A–C*).

Next, we examined the effects of the contractility inhibitors on chondrocytes grown in AGE– or LOX–cross-linked collagen gels. Imbalances between chondrocyte catabolism and anabolism caused by ribose or LOX-CM treatment were ablated effectively by both Y27632 and blebbistatin (Fig. S4*D*), indicating that cellular contractility has a key role in transducing the microenvironmental changes elicited by controlled matrix cross-linking. BAPN treatment similarly inhibited the effects of LOX-CM on chondrocyte catabolism and anabolism (Fig. S4*E*). Under these conditions, blebbistatin did not have a synergistic effect (Fig. S4*E*), supporting the theory that LOX-mediated collagen cross-linking occurs upstream of the contractility pathway. To examine this notion directly, chondrocytes cultured on 2D polyacrylamide soft and stiff gels were treated with BAPN or blebbistatin. The stiffness of these 2D gels is determined essentially by bisacrylamide-mediated cross-linking of polyacrylamide gel rather than by collagen cross-linking. Blebbistatin treatment effectively alleviated the homeostatic imbalance between catabolism and anabolism induced by stiff matrix, but BAPN had no significant effects on chondrocytes grown under similar conditions (Fig. S4*F*). Taken together, these results corroborate our findings that the effects of AGE- and LOX-mediated collagen cross-linking occur primarily through the mediated matrix stiffening and the resulting contractility pathway comprising the Rho–ROCK–MLC axis. We further characterized in vivo regulation of OA by the Rho–ROCK–MLC axis via IA injection of Y27632 or blebbistatin. Treatment with either of the inhibitors significantly reduced DMM-induced cartilage destruction, subchondral bone sclerosis, and osteophyte development (Fig. 2*F*), indicating that the matrix-mediated mechanotransduction pathway has an essential role in OA pathogenesis.

Matrix Cross-Linking in Cartilage Tissue Causes OA Pathogenesis in Mice. Next, we focused on the mechanisms by which AGE- and LOX-mediated matrix cross-linking regulate OA pathogenesis in vivo. First, the contribution of AGE-mediated ECM cross-linking to OA pathogenesis was evaluated following IA injection of ribose into the knee joints of mice. Ribose-mediated AGE accumulation enhanced all manifestations of DMM-induced OA (Fig. 3 *A* and *B*), further highlighting the deleterious effects of excessive collagen cross-linking and matrix stiffening on cartilage homeostasis. The in vivo function of LOX in OA pathogenesis was evaluated through genetic modulation of *Lox* in mice. LOX overexpression was induced in mouse knee joint tissue via IA injection of Ad-*Lox*. Consistent with previous demonstrations of effective local gene delivery by adenoviral systems (11, 16), Ad-*Lox* injection triggered LOX overexpression in cartilage, meniscus, and synovium (Fig. S5*A*). At 3 wk postinjection, Ad-*Lox* caused cartilage destruction (Fig. 3*C*) and induced synovial inflammation (Fig. S5*B*), consistent with the report that up-regulation of collagen cross-linking enzymes is associated with OA-related fibrosis (17). Subchondral bone sclerosis and osteophytes were not evident at this time but were detected clearly after 8 wk of Ad-*Lox* injection (Fig. 3*D*).

To elucidate the mechanisms underlying LOX activity in OA, we examined the effects of LOX overexpression in chondrocytes. Ad-*Lox* infection, which enhanced the levels of both secreted and nuclear-localized cellular LOX (Fig. 3*E*), resulted in up-regulation of MMP3, MMP13, and ADAMTS5 (a disintegrin and metalloproteinase with thrombospondin motifs 5) (Fig. 3*F*) and down-regulation of COL2A1, aggrecan, and SOX9 (Fig. 4*F* and Fig. S5*C*). Therefore, LOX in articular chondrocytes induced simultaneous up-regulation of catabolic factors and down-regulation of anabolic factors. LOX overexpression in fibroblast-like synoviocytes (FLS) similarly induced enhanced expression of various catabolic factors (Fig. S5*D*). Consistent with the in vitro effects, IA injection of Ad-*Lox* in mice caused up-regulation of MMPs and down-regulation of SOX9 in cartilage tissue (Fig. 3*G*).

The role of LOX was investigated further using chondrocyte-specific *Lox* transgenic (TG) mice. TG mice exhibited normal skeletal development with markedly higher levels of LOX in cartilage tissue (Fig. S6 *A–C*). Notably, high levels of the secreted fraction of LOX were detected throughout the cartilage matrix of LOX TG mice (Fig. S6*D*). The mechanical instability caused by DMM surgery significantly enhanced OA phenotypes in *Lox* TG mice, compared with their WT littermates (Fig. 4 *H–J*). However, spontaneous cartilage destruction was not observed in 18-mo-old mice (Fig. S6*E*), suggesting that LOX overexpression in chondrocytes is not sufficient to trigger aging-associated OA.

Reduced LOX Expression or Activity in Joint Tissues Suppresses OA Pathogenesis in Mice. We further explored the in vivo role of LOX via knockout with adenoviral shRNA targeting *Lox* (Ad-sh*Lox*) or

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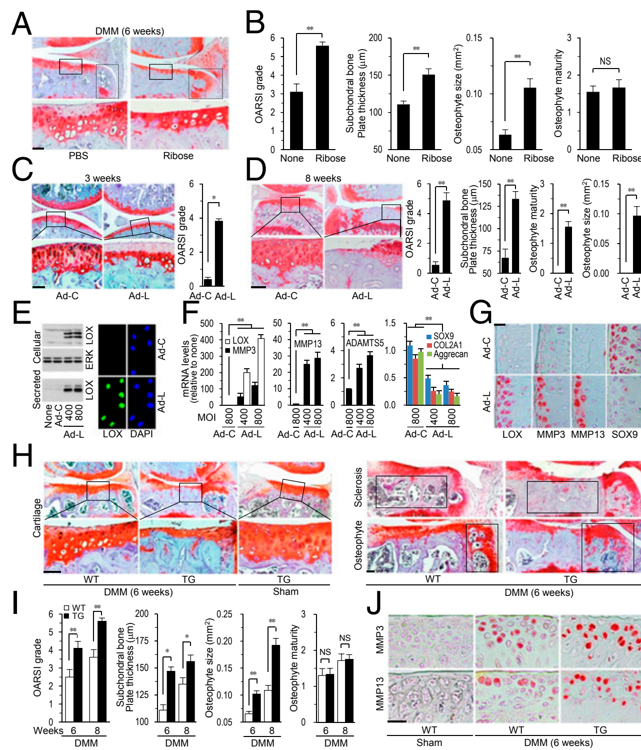

Fig. 3. Posttraumatic OA pathogenesis is promoted by increased AGE accumulation and LOX expression. (*A* and *B*) Safranin-O staining (*A*) and scoring of OA parameters (*B*) ($n \geq 8$) in DMM-operated mice IA injected with PBS or ribose. (*C* and *D*) Safranin-O staining and scoring of OA parameters in mice 3 wk (*C*) or 8 wk (*D*) after IA injection of 1×10^9 pfu of Ad-C or Ad-*Lox* ($n = 11$). (*E* and *F*) Western blot and immunostaining of LOX (*E*) and mRNA levels of the indicated targets (*F*) in chondrocytes infected with Ad-C or Ad-*Lox* at the indicated MOI ($n \geq 6$). (*G*) Immunostaining of the indicated proteins in cartilage sections of mice 3 wk after IA injection of Ad-C or Ad-*Lox* ($n = 11$). (*H*–*J*) Safranin-O staining (*H*), scoring of OA parameters (*I*), and immunostaining of MMPs (*J*) in DMM-operated WT and *Lox* TG mice ($n \geq 10$). (Scale bars: 50 μm.) Values are means ± SEM; $*P < 0.05$, $**P < 0.005$; NS, not significant.

by inhibiting its activity with BAPN in DMM-operated mice. IA injection of BAPN or Ad-sh*Lox* effectively suppressed DMM-induced cartilage destruction (Fig. 4*A*), with a concomitant decrease in the expression of the matrix-degrading enzymes in cartilage tissue (Fig. 4*B*). Subchondral bone sclerosis and osteophyte development also were suppressed in LOX-suppressed mice (Fig. 4*C*). These data indicate that the inhibition of LOX expression or activity is sufficient to block DMM-induced OA pathogenesis. Together with the results of gain-of-function studies, our findings suggest that LOX plays a critical role in posttraumatic OA pathogenesis.

Cellular LOX Promotes OA Pathogenesis by Modulating Chondrocyte Catabolism and Anabolism via Heat Shock Factor 1 and NF-κB. Unexpectedly, we observed nuclear-localized cellular LOX in OA chondrocytes (Figs. 1*E* and 3*E* and Fig. S2*E*). In addition to its extracellular role, LOX is reported to act intracellularly to modulate various cellular processes, including epigenetic modification and signaling pathways (12, 18, 19). For example, hydrogen peroxide, produced as a by-product of LOX activity, modulates cellular functions (19). However, in our experiments removal of hydrogen peroxide with catalase did not affect Ad-*Lox*–induced modulation of catabolic or anabolic factor expression in chondrocytes (Fig. S7*A*). Because cellular LOX localizes predominantly to the nucleus, we examined the effects of the nuclear LOX on the activities of various transcription factors. Activities of six transcription factors [octamer-binding transcription factor 4 (OCT4), heat shock factor 1 (HSF1), NF-κB, STAT3, STAT1/2, and CCAAT/enhancer binding protein (C/EBP)] were increased more than twofold following Ad-*Lox* infection (Fig. 5*A*). Inhibition or knockdown of OCT4, STAT3, STAT1/2, C/EBPα, or C/EBPβ had no effect on LOX-induced regulation of catabolic or anabolic factors (Fig. S7*B*). In contrast, inhibition of HSF1 with KRIBB11 or of NF-κB with SC-514 blocked LOX-induced up-regulation of catabolic enzymes, and inhibition of NF-κB specifically abrogated LOX-mediated attenuation of anabolic factors (Fig. 5 *B* and *C* and Fig. S7*B*). Furthermore, overexpression of HSF1 or p65 was sufficient to trigger chondrocyte catabolism, with concomitant suppression of anabolism (Fig. 5 *D* and *E* and Fig. S7 *C* and *D*). Consistently, cartilage destruction (Fig. 5*F*) and synovial inflammation (Fig. S7*E*) elicited by Ad-*Lox* injection were suppressed effectively upon coinjection of the HSF1 or NF-κB inhibitor. These results collectively indicate that the cellular fraction of LOX has a significant role in regulating cartilage homeostasis and OA cartilage destruction.

We further explored whether the contractility pathway triggered by matrix stiffening contributes to HSF1 and NF-κB pathway activation. However, our results disclosed no effects of matrix stiffness on transcriptional activities of HSF1 or NF-κB (Fig. S8*A*). Similarly, treatment with Y27632 or blebbistatin did not affect their transcriptional activity (Fig. S8*A*), suggesting that LOX-mediated activation of the HSF1/NF-κB pathway is independent of the contractility pathway. We further examined whether activation of HSF1/NF-κB by nuclear LOX affects the contractility pathway. Inhibition of the HSF1 pathway with KRIBB11 or of the NF-κB pathway with SC-514 did not affect cellular contractility on stiff surfaces (Fig. S8*B*). Based on these findings, we conclude that the contractility pathway activated by stiff substratum is independent of HSF1/NF-κB activation by nuclear LOX.

Based on our current findings that IL-1β and HIF-2α trigger LOX expression and previous reports that IL-1β induces HIF-2α expression via NF-κB activation (11, 20), we examined whether IL-1β–mediated NF-κB activation induces HIF-2α, which

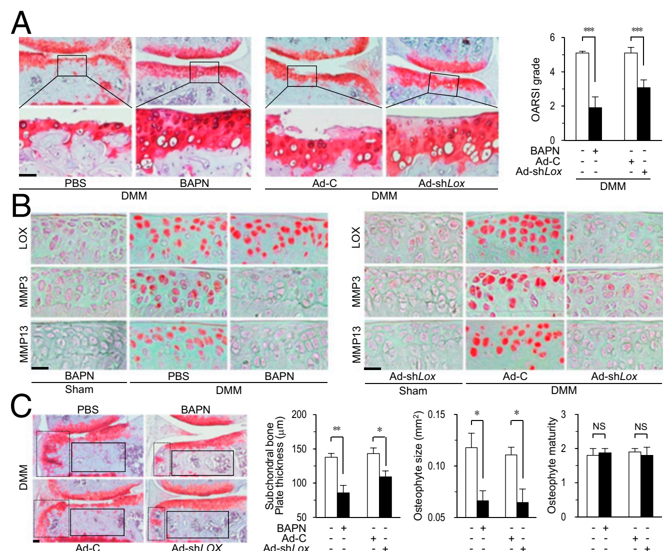

Fig. 4. OA pathogenesis is attenuated by inhibition or knockdown of LOX in mouse joint tissues. DMM-operated mice were IA-injected with PBS or BAPN or alternatively with Ad-C or Ad-sh*Lox*. (*A*) Cartilage destruction was determined with safranin-O staining and was scored via Osteoarthritis Research Society International (OARSI) grade. (*B*) Representative images of LOX and MMP immunostaining in cartilage sections. (*C*) Subchondral bone sclerosis and osteophyte formation detected with safranin-O and hematoxylin staining and scoring of these parameters ($n \geq 10$). (Scale bars: 50 μm.) Values are means ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; NS, not significant.

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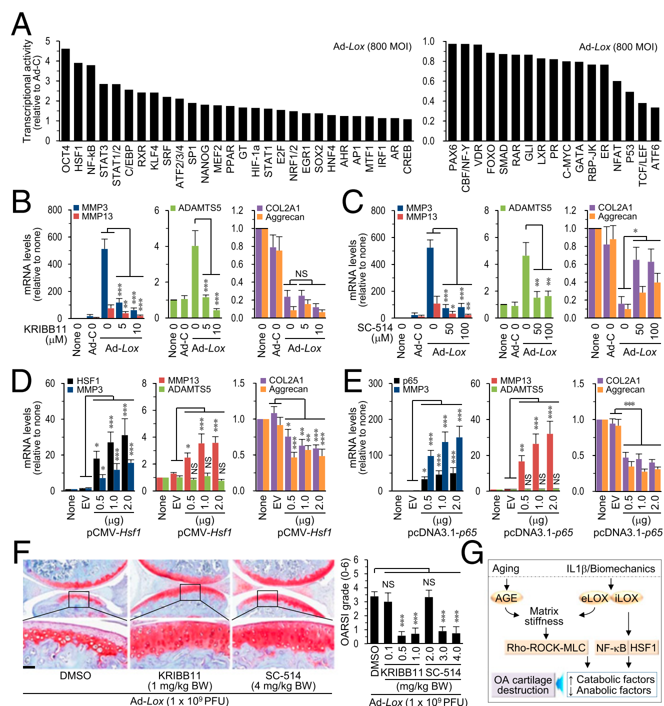

Fig. 5. Cellular LOX promotes OA via activation of NF-κB and HSF1 transcription factors. (*A*) Primary cultured chondrocytes were infected with Ad-C or Ad-*Lox* at an MOI of 800 for 2 h and were incubated for an additional 24 h. Transcriptional activities of the indicated transcription factors were determined using a transcription factor array kit ($n = 4$). (*B* and *C*) Chondrocytes infected with Ad-C or Ad-*Lox* were treated with KRIBB11 (*B*) or SC-514 (*C*). mRNA levels were quantified by qRT-PCR ($n \geq 7$). (*D* and *E*) mRNA levels of the indicated targets ($n \geq 5$) in chondrocytes transfected with *Hsf1* (*D*) or *p65* (*E*) expression vectors. (*F*) Safranin-O staining and scoring of cartilage destruction in mice IA-injected with Ad-C or Ad-*Lox* with or without KRIBB11 or SC-514 ($n \geq 8$). (*G*) Proposed model of matrix stiffness-mediated mechanotransduction in OA development. (Scale bar: 50 μm.) Values are means ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant.

subsequently promotes LOX expression. Inhibition of NF-κB blocked the IL-1β–induced increase in HIF-2α and LOX expression (Fig. S8*C*). However, knockdown of HIF-2α did not ablate IL-1β–induced LOX expression (Fig. S8*D*). Similarly, IL-1β–mediated induction of HIF-2α did not require LOX (Fig. S8*D*), suggesting that LOX and HIF-2α are likely to be parallel downstream targets of IL-1β–NF-κB axis. Meanwhile, LOX overexpression was sufficient for transcriptional activation of NF-κB (Fig. 5*A*). Because NF-κB previously had been shown to induce IL-1β expression in FLS and HIF-2α expression in chondrocytes (11, 20, 21), we further examined whether LOX-mediated activation of NF-κB is capable of inducing IL-1β and HIF-2α. Neither LOX nor HIF-2α induced IL-1β expression in chondrocytes (Fig. S8*E*). In contrast, LOX-mediated activation of NF-κB clearly triggered HIF-2α expression (Fig. S8 *F* and *G*). Similarly, HIF-2α overexpression activates LOX expression in an NF-κB–dependent manner (Fig. S8*G*). Therefore, our data indicate a reciprocal activation of LOX and HIF-2α pathways via NF-κB transcription factor (Fig. S8*H*).

Discussion

OA is characterized primarily by cartilage destruction caused by the up-regulation of matrix-degrading enzymes and/or the down-regulation of cartilage-specific ECM molecules in chondrocytes. Several key cellular pathways regulating these catabolic and anabolic processes have been identified, including the HIF-2α transcriptional network (11, 20), the zinc–ZIP8–MTF1 axis (16), and complement pathways (22). In contrast, molecular changes occurring in the ECM during OA pathogenesis have been relatively underexplored to date. Recent advances in nanoscopic high-resolution imaging facilitated the biophysical characterization of OA-associated ECM, revealing that cartilage matrix undergoes collagen fiber thickening and stiffening (6, 7). This finding further raised the questions of the types of molecular events that lead to this mechanical remodeling of cartilage matrix and, more importantly, whether mechanical alterations in the surrounding ECM affect chondrocyte metabolism and consequently contribute to OA pathogenesis.

In this study, we show that the matrix-mediated mechanotransduction pathway has a causal role in triggering OA. Increasing the extent of cross-linking in the underlying substrate, and consequently its stiffness, was sufficient to induce an increase in chondrocyte catabolism and a decrease in anabolism. OA-eliciting molecular changes were mediated through specific activation of the Rho–ROCK–MLC axis. Inhibition of this axis abolished stiffening-mediated modulation of catabolic and anabolic factors. Notably, matrix-mediated activation of this mechanotransduction pathway substantially disrupted the localization and transcriptional activity of SOX9. Although no available reports clearly indicate the regulation of matrix-degrading enzymes in chondrocytes by the Rho–ROCK–MLC axis, our results are in keeping with the previous finding that Rho-GTPase regulates MMP expression during tumor metastasis (23). Down-regulation of anabolic factors by the Rho–ROCK–MLC axis is consistent with the report that Rho signaling negatively regulates SOX9 expression and activity (24).

We propose that the two major OA risk factors, aging and mechanical stress, commonly promote cartilage matrix stiffening but do so through distinct molecular mechanisms. Aging articular cartilage accumulates significant amounts of AGEs in the superficial zone. AGE formation in this collagen-rich tissue facilitates cross-linking of collagen molecules, leading to loss of elasticity and subsequent reduction in tissue compliance. Indeed, elevating the AGE level in mouse knee joints sensitized the tissue to surgically induced mechanical instability to a considerable extent, augmenting OA pathogenesis. Meanwhile, in OA pathogenic conditions associated with mechanical stress, the collagen-modifying enzyme LOX was significantly up-regulated. Intriguingly, LOX expression preceded OA cartilage destruction, consistent with the finding that cartilage collagen fiber thickening and stiffening occur in early stages of OA before cartilage destruction (6). Our data confirmed that extracellularly secreted LOX induces a significant increase in Young's modulus of collagen matrices and that chondrocytes embedded in LOX-treated 3D matrices exhibit OA-associated gene expression. Similar to AGE-mediated nonenzymatic matrix cross-linking, cartilage-specific increases in LOX-mediated enzymatic matrix cross-linking did not result in spontaneous OA cartilage destruction but significantly sensitized cartilage tissue to mechanical instability, augmenting posttraumatic OA.

In addition to the major extracellular collagen cross-linking function of LOX, intracellular localization of LOX family members has been reported, and their functions have been investigated extensively (18, 19). We observed predominantly nuclear localization of cellular LOX in chondrocytes under various OA pathogenic conditions. Nuclear-localized LOX affected the activation profiles of numerous transcription factors. Among these, HSF1 and NF-κB were associated with LOX-mediated expression of matrix-degrading enzymes. Therefore, we propose that nuclear-localized cellular LOX acts, in part, through HSF1 and NF-κB to induce the expression of matrix-degrading enzymes. Indeed, both HSF1 and NF-κB are implicated in inflammatory arthritis, and their activation by proinflammatory cytokines is well documented (25, 26). Moreover, NF-κB is a known direct transactivator of ADAMTS5 (27) and HIF-2α (11, 20); the latter directly regulates the expression of MMP targets (11). In addition to regulating catabolic factors, NF-κB is a potent suppressor of the anabolic master regulator SOX9 (28). This finding is in line with our observation that inhibition of NF-κB,

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but not HSF1, reversed LOX-mediated suppression of SOX9 and cartilage-specific ECM molecules. Thus, it appears that both extracellular LOX (eLOX) and nuclear-localized, intracellular LOX (iLOX) regulate chondrocyte catabolism and anabolism. HSF1/NF-κB pathways activated by iLOX did not affect the contractility pathway activated by matrix stiffening, whereas cellular contractility elicited by stiff ECM was not sufficient to promote the transcriptional activities of HSF1/NF-κB. Therefore, although we cannot completely rule out the possibility of an association between contractility and the HSF1/NF-κB pathway, our current data suggest that eLOX and iLOX activate the mechanotransduction and HSF1/NF-κB pathways, respectively, via distinct mechanisms.

Our experiments revealed significant roles of AGE- and LOX-mediated matrix cross-linking in the progression of posttraumatic OA in mice. IA injection of ribose or chondrocyte-specific overexpression of LOX markedly augmented OA phenotypes following DMM surgery. However, neither ribose-injected nor LOX TG mice developed spontaneous OA at age 20 wk. Further aging of LOX TG mice did not promote spontaneous OA phenotypes in the C57BL/6 mouse strain. Accordingly, we conclude that the matrix cross-linking–mediated mechanotransduction pathway plays a more determining role in posttraumatic OA than in aging-associated OA.

Based on our data, stiffness and dimensionality were identified as potentially critical biophysical parameters that regulate chondrocyte homeostasis. In a 2D context, 4–7 kPa was the optimal range of matrix stiffness promoting chondrocyte anabolism and suppressing catabolism while maintaining nascent adhesion with the underlying matrix without significant loss in cell viability. Interestingly, in a 3D microenvironment, ~100-fold lower stiffness led to optimal chondrocyte metabolism characterized by robust proteoglycan accumulation. However, parallel comparison of optimal stiffness in 2D and 3D microenvironments is difficult, because they also differ in the context of cell adhesion with surrounding and underlying matrix. Therefore, matrix stiffness and dimensionality may serve as key design parameters in developing effective cell-therapy strategies for OA.

Our results collectively highlight the pathogenic roles of the matrix-mediated mechanotransduction pathway in OA development (Fig. 5*G*). The two major risk factors of OA, aging and mechanical stress, enhance cartilage matrix cross-linking and stiffening through AGE- and LOX-mediated collagen cross-linking, respectively. The stiffened matrix in turn activates the Rho–ROCK–MLC mechanotransduction pathway and primes chondrocytes for osteoarthritic changes. Unexpectedly, we identified a nuclear-localized fraction of cellular LOX along with its transcription factor targets. These findings support the notion that modulation of the matrix stiffening-mediated mechanotransduction presents an effective therapeutic approach for OA.

Materials and Methods

The use of International Cartilage Repair Society (ICRS) grade 4 human OA cartilage sourced from individuals (aged 51–72 y) subjected to arthroplasty was approved by The Institutional Review Board of Wonkwang University Hospital. Written informed consent was obtained from all participants before the operative procedure. Mice were maintained under pathogen-free conditions, and all experiments involving mice were approved by Gwangju Institute of Science and Technology Animal Care and Use Committee.

A detailed outline of the procedures and specific materials used for human OA cartilage tissue and experimental OA in mice; histology and immunostaining; primary culture of chondrocytes and FLS; preparation of collagen-coated polyacrylamide substrates and collagen gels; infection and IA injection of adenovirus in mice; the active Rho pull-down assay; the SOX9 reporter gene assay; transcription factor array analysis; HIF-2α microarray analysis; skeletal staining; RT-PCR and siRNA transfection; Western blot analysis; MMP activity assay; and statistical analysis are provided in *SI Materials and Methods*. PCR primers and experimental conditions are summarized in Table S1.

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