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Stress-activated miR-204 governs senescent phenotypes of chondrocytes to promote osteoarthritis development

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Donghyun Kang[1,2]*, Jungkwon Shin[1,2]*, Yongsik Cho[1,2], Hyeon-Seop Kim[1,2], Young-Ran Gu[1,2], Haedong Kim[1,2], Kwon Tae You[1,2], Moon Jong Chang[3], Chong Bum Chang[3], Seung-Baik Kang[3], Jong-Seo Kim[1,2], V. Narry Kim[1,2], Jin-Hong Kim[1,2,4]†

A progressive loss of cartilage matrix leads to the development of osteoarthritis (OA). Matrix homeostasis is disturbed in OA cartilage as the result of reduced production of cartilage-specific matrix and increased secretion of catabolic mediators by chondrocytes. Chondrocyte senescence is a crucial cellular event contributing to such imbalance in matrix metabolism during OA development. Here, we identify miR-204 as a markedly up-regulated microRNA in OA cartilage. miR-204 is induced by transcription factors GATA4 and NF-κB in response to senescence signals. Up-regulated miR-204 simultaneously targets multiple components of the sulfated proteoglycan (PG) biosynthesis pathway, effectively shutting down PG anabolism. Ectopic expression of miR-204 in joints triggers spontaneous cartilage loss and OA development, whereas miR-204 inhibition ameliorates experimental OA, with concomitant recovery of PG synthesis and suppression of inflammatory senescence-associated secretory phenotype (SASP) factors in cartilage. Collectively, we unravel a stress-activated senescence pathway that underlies disrupted matrix homeostasis in OA cartilage.

INTRODUCTION

Articular cartilage contains abundant sulfated proteoglycans (PGs), composed of glycosaminoglycan (GAG) chains bound to a core protein (*1*). Sulfated PGs in cartilage mostly exist in an aggregated form by further interacting with hyaluronic acid (HA). Aggrecan is a major core protein essential for the formation of PG aggregates in cartilage. Aggrecan has three distinct domains: a nonsulfated globular region that forms the HA binding site, a relatively short region that provides attachment sites for keratan sulfate, and a chondroitin sulfate (CS)–rich region that comprises most of the length of the core protein. CS, consisting of a repeating disaccharide structure of *N*-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA), is sulfated in the 4- or 6-position of GalNAc (*2*), which is responsible for the high density of negative charges of cartilaginous PGs. The anionic nature of sulfated PGs strongly attracts osmotically active cations and retains water molecules in the cartilage matrix, conferring a unique load-bearing function on articular joints (*3*, *4*). Cartilage matrix homeostasis is regulated by chondrocytes, the major resident cell type in cartilage.

Osteoarthritis (OA), the most common form of arthritis, is primarily characterized by the progressive loss of the cartilage matrix but also involves pathological changes in other joint components such as subchondral bone sclerosis, osteophyte formation, and synovial inflammation (*5*). The pathogenesis of OA is multifactorial, involving systemic factors such as age (*6*) and local factors such as mechanical stress resulting from excess weight and joint instability (*7*). There is a growing body of evidence that both aging (*8*) and aberrant mechanical stress (*9*, *10*) promote accumulation of oxidative stress in chondrocytes, which in turn can activate multiple downstream pathways resulting in cellular senescence (*11*), dedifferentiation (*12*), and apoptosis (*13*). Oxidative stress generally results from the production of reactive oxygen species (ROS) that exceeds the cellular antioxidant capacity. Both preclinical and clinical studies indicate that an increase in the amount of ROS in articular cartilage is closely linked to OA development (*14*, *15*).

These OA risk factors lead to the cellular senescence of chondrocytes, a critical cellular event contributing to matrix metabolism imbalance during OA development (*16*, *17*). Recent studies indicate that removal of damaged or senescent chondrocytes attenuates disease progression in a posttraumatic OA mouse model (*18*, *19*), underscoring the causal role of chondrocyte senescence in OA pathogenesis. However, the molecular pathway governing the senescent phenotypes of chondrocytes is not well characterized, and how these phenotypes can be controlled in OA cartilage remains elusive.

The pathogenesis of OA is generally associated with low-grade, but often chronic, inflammation. Various extracellular matrix (ECM) fragments (*20*–*22*) resulting from cartilage destruction and complement pathway activation (*23*) could elicit inflammatory processes in the joint environment. Accumulating evidence indicates that senescent chondrocytes alter their microenvironment through the secretion of proinflammatory cytokines and proteases, referred to as senescence-associated secretory phenotype (SASP) factors (*16*, *24*). Chronic inflammation as the result of onset of SASP not only directly promotes matrix catabolism in cartilage but also recruits immune cells and triggers inflammatory processes in synovial fibroblasts and macrophages, further aggravating inflammation and OA progression (*16*, *25*). Therefore, accumulated senescent chondrocytes could serve as a source for chronic inflammatory response that transitions OA from an indolent to an aggravating phase.

MicroRNAs (miRNAs) are endogenous noncoding RNAs that regulate gene expression by base pairing with the 3′ untranslated region

[1]Center for RNA Research, Institute for Basic Science, 08826 Seoul, South Korea. [2]Department of Biological Sciences, College of Natural Sciences, Seoul National University, 08826 Seoul, South Korea. [3]Department of Orthopedic Surgery, Seoul National University College of Medicine, Boramae Hospital, 07061 Seoul, South Korea. [4]Interdisciplinary Program in Bioinformatics, Seoul National University, 08826 Seoul, South Korea.
*These authors contributed equally to this work.
†Corresponding author. Email: jinhkim@snu.ac.kr

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(3′UTR) of target mRNAs through their seed sequence (*26*). Gene regulation by miRNAs plays a critical role in many essential biological processes such as development, and its dysregulation is associated with various human diseases (*27*). Differential expression patterns of miRNAs have been noted during OA pathogenesis (*28*, *29*), which led to the identification of several key miRNAs that target OA-associated genes such as matrix-degrading enzymes and proinflammatory cytokines. For instance, miR-140 targets *Adamts5*, a key aggrecanase in articular cartilage, and its overexpression in chondrocytes effectively protects the cartilage from destruction (*30*). miR-27b represses the expression of matrix metalloproteinase 13 (MMP13), a major collagenase degrading type II collagen (*31*). miR-149 directly suppresses *TNFA* and *IL1B* and was down-regulated in human OA cartilage (*32*). miR-140 not only targets inflammatory cytokines such as *IL1B* and *IL6* but also inhibits the expression of syndecan-4 that regulates the ADAMTS-5 activation pathway in human chondrocytes (*33*). Here, we elucidate a stress-activated senescence pathway that involves miR-204 and underlies disrupted matrix homeostasis in OA cartilage, and develop a potential strategy to foster a regenerative microenvironment.

RESULTS

miR-204 is up-regulated in human and mouse OA cartilage

We sought to identify a previously unknown, senescence-associated signaling pathway in chondrocytes linked to major OA cartilage manifestations such as PG loss and cartilage degeneration. The deleterious effects of prolonged normoxia on primary cultured cells have been well documented (*34*–*36*). The physiological niches from which chondrocytes are isolated are at low oxygen tensions (0.5 to 5%) (*37*). We noted that primary mouse chondrocytes cultured at an oxygen concentration higher than the physiological concentration showed extensive oxidative stress as evidenced by the elevated amount of 4-hydroxynonenal (4-HNE), a lipid peroxidation product (Fig. 1A and data file S1). Chondrocytes exposed to prolonged normoxia exhibited a DNA damage response, indicated by strong γ-H2AX nuclear foci, and various senescence phenotypes such as induction of $p16^{INK4a}$, up-regulation of *Cdkn1a* and *Cdkn2a*, down-regulation of *Lmnb1*, and increased senescence-associated β-galactosidase (SA-β-Gal) positivity (Fig. 1, B to E, and data file S1). PG content was also decreased under this condition (Fig. 1F).

Noting the crucial role of miRNA-directed gene silencing in various senescence contexts (*38*), we performed small RNA sequencing in those chondrocytes undergoing serial passaging under normoxia (fig. S1A). Forty-one miRNAs were differentially up-regulated with the onset of senescence under this condition (fig. S1B). miR-204 was a marked outlier in terms of fold change (18.1-fold increase) and false discovery rate (FDR; 1.66×10^{-60}) (Fig. 1G and fig. S1C). The target transcripts of the 41 differentially up-regulated miRNAs were predicted using the TargetScan algorithm (*39*) and compared with the transcriptomes of human OA and aged cartilage. The predicted targets of miR-204-5p, along with those of three other miRNAs—miR-24-3p, miR-27b-3p, and miR-30a-3p—were highly enriched in down-regulated gene sets from both OA and aged cartilage (Fig. 1H). Further experimental screening revealed that miR-204—but not miR-24, miR-27b, or miR-30a—caused suppression of de novo sGAG synthesis in chondrocytes, an OA-associated phenotype observed in senescent chondrocytes (Fig. 1I). Therefore, we explored a possible association between miR-204 and OA pathogenesis based on the marked up-regulation of miR-204 in the context of cellular senescence and its inhibitory effect on matrix synthesis.

We characterized the expression of miR-204 in human and murine OA cartilage. miR-204 was up-regulated in OA-affected human cartilage but was barely detectable in undamaged regions of arthritic cartilage (Fig. 1J and data file S1). Similarly, $p16^{INK4a}$, a biomarker of cellular senescence (*40*, *41*), was up-regulated in the OA-affected regions of human cartilage (Fig. 1J and data file S1). In an aged mouse model of knee OA, miR-204 and $p16^{INK4a}$ were markedly elevated (Fig. 1K, fig. S2, A and B, and data file S1). We also used DMM surgery as a mouse model of posttraumatic OA (*42*, *43*). An earlier study indicated that mechanical instability in joints causes chondrocyte senescence (*19*). Consistently, we detected a marked increase in $p16^{INK4a}$ and miR-204 expression after the surgical induction of OA (Fig. 1L, fig. S2C, and data file S1). GSEA revealed that the predicted target genes of miR-204 were negatively enriched in the whole transcriptomes of OA cartilage, suggesting that miR-204 up-regulation acts to suppress its target genes under OA conditions (Fig. 1M).

Senescence-eliciting stresses induce the expression of miR-204

To investigate the molecular mechanisms by which miR-204 is up-regulated in OA cartilage, we explored the upstream signals responsible for induction of miR-204 transcription. Because oxidative stress is known to be involved in OA and miR-204 expression was elevated in a culture condition that accompanies high oxidative stress (Fig. 1, A and G, and fig. S1C), we explored the role of ROS in regulating miR-204 expression in chondrocytes. In primary cultured mouse chondrocytes, however, acute treatment with menadione, a free radical generator (*44*), or hydrogen peroxide (H_2O_2) (*11*, *45*) did not affect miR-204 expression, ruling out the short-term redox signaling as an upstream regulator (Fig. 2A). Long-term exposure to H_2O_2 markedly induced miR-204 expression, along with the accumulation of DNA damage and the induction of senescence markers (Fig. 2, B to E, fig. S3, and data file S1). We then questioned whether other senescence-eliciting stimuli also affect miR-204 expression. Ionizing radiation (IR), which causes DNA damage and cellular senescence of chondrocytes (Fig. 2, F and G, and fig. S4), robustly induced miR-204 expression (Fig. 2H). DNA-damaging chemical agents, such as bleomycin or doxorubicin, similarly promoted senescence phenotypes and miR-204 expression (Fig. 2, I to L, and fig. S5).

p53 is activated in response to DNA damage and is responsible for senescence-induced cell cycle arrest (*46*). OA-inducing conditions such as mechanical stress cause DNA damage and activate p53 (*47*). Therefore, we tested whether p53 acts as an upstream regulator of miR-204. In chondrocytes, DNA-damaging agents markedly promoted the expression of *Cdkn1a*, indicating p53 activation (fig. S6A). However, nutlin-3a–induced p53 activation did not increase miR-204 expression (fig. S6B). Consistently, siRNA-mediated knockdown of p53 had no effect on IR-induced miR-204 expression (fig. S6, C and D). Similarly, another major senescence-associated cell cycle arrest regulator $p16^{INK4a}$ (*41*) did not regulate miR-204 expression (fig. S6, E and F).

In addition to cell cycle arrest, cellular senescence causes a proinflammatory response termed SASP (*48*). As previously reported (*49*, *50*), SASP developed relatively slowly, appearing several days after the onset of senescence-mediated cell cycle arrest. miR-204 expression coincides with that of SASP factors such as *Il6* and *Mmp3* (Fig. 2M), suggesting that miR-204 and SASP might be controlled by

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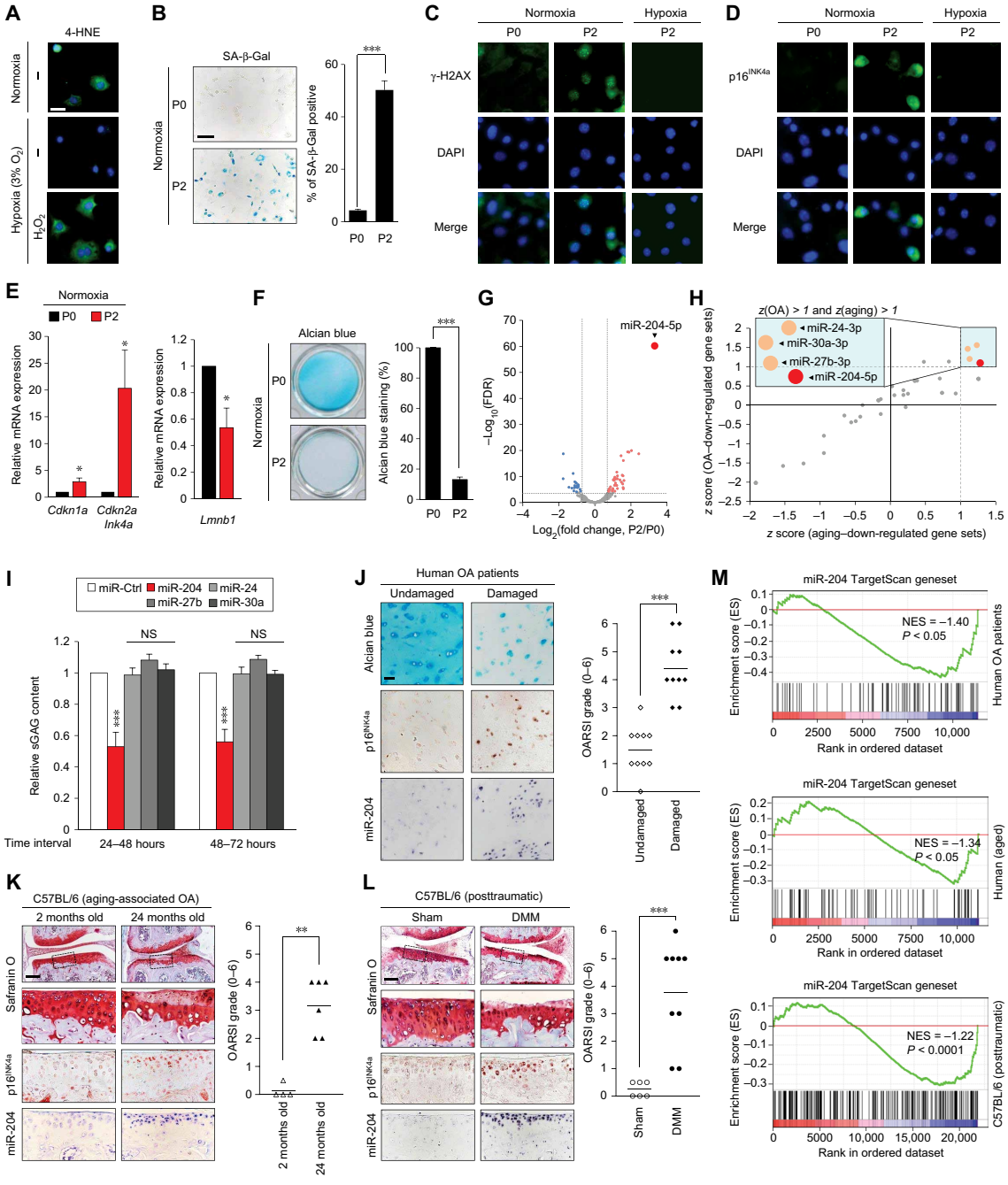

Fig. 1. miR-204 is up-regulated in OA cartilage. (**A**) Immunofluorescence staining of 4-HNE in primary mouse chondrocytes cultured under normoxia (20% O_2) or hypoxia (3% O_2) in the absence or presence of H_2O_2 (200 μM). Scale bar, 25 μm. (**B**) Mouse chondrocytes were exposed to normoxia at passage 0 (P0) and underwent two additional passages under this condition (P2). SA-β-Gal staining and quantification of SA-β-Gal positivity in P0 and P2 chondrocytes ($n = 5$). Scale bar, 100 μm. (**C** and **D**) Immunofluorescence staining of (C) γ-H2AX and (D) $p16^{INK4a}$ in P0 chondrocytes cultured under normoxia or P2 chondrocytes cultured under normoxia or hypoxia. DAPI, 4′,6-diamidino-2-phenylindole. (**E**) Relative mRNA expression of cyclin-dependent kinase (CDK) inhibitors or *Lmnb1* in P0 and P2 chondrocytes cultured under normoxia ($n = 6$). (**F**) Alcian blue staining and absorbance quantification of P0 and P2 chondrocytes ($n = 4$). (**G**) Volcano plot of $-\log_{10}$(FDR) against $\log_2$(fold change). The plot represents the difference in miRNA expressions between P0 and P2 chondrocytes. (**H**) The extent of target transcript enrichment in down-regulated gene sets of human OA and aged cartilage evaluated for 41 differentially up-regulated miRNAs. The predicted target genes of the miRNAs were determined using the TargetScan algorithm. (**I**) Sulfated GAG (sGAG) release of chondrocytes transfected with designated miRNAs measured using sGAG assay ($n \geq 5$). (**J**) Cartilage sections from the undamaged or damaged region of human OA cartilage were stained with Alcian blue, immunohistochemistry of $p16^{INK4a}$, and in situ hybridization with a probe detecting miR-204, and the extent of cartilage destruction was graded ($n = 10$). Scale bar, 50 μm. OARSI, Osteoarthritis Research Society International. (**K** and **L**) Staining of cartilage sections with safranin O, immunohistochemistry of $p16^{INK4a}$, and in situ hybridization of miR-204 from (K) 2- or 24-month-old mice ($n \geq 4$) or (L) control (sham) or posttraumatic OA cartilage [8 weeks after destabilization of the medial meniscus (DMM)] in mice ($n \geq 6$). The inset in the images is shown at magnified images in the bottom row. Scale bars, 200 μm. (**M**) Gene set enrichment analysis (GSEA) of predicted target genes of miR-204 in human OA (top), human aged (middle), and mouse posttraumatic OA cartilage (bottom) as compared to their respective controls. NES, normalized enrichment score. Data represent means ± SEM using Student's *t* test in (B), (E), and (F), analysis of variance (ANOVA) followed by post hoc test in (I), and Mann-Whitney *U* test in (J) to (L). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NS, not significant.

common upstream regulators. To explore this hypothesis, we first attempted to identify the putative promoter regulating transcription of miR-204. miR-204 is located in an intronic region of the *Trpm3* host gene, and expression of miR-204 and *Trpm3* are co-regulated (*51*). *Trpm3* mRNA was induced by IR in both dose- and time-dependent manner, coinciding with the miR-204 expression pattern (Fig. 2H and fig. S7, A and B). Genomic analysis indicated that *Trpm3* has two evolutionary conserved transcription start sites (TSSs), TSS1 and

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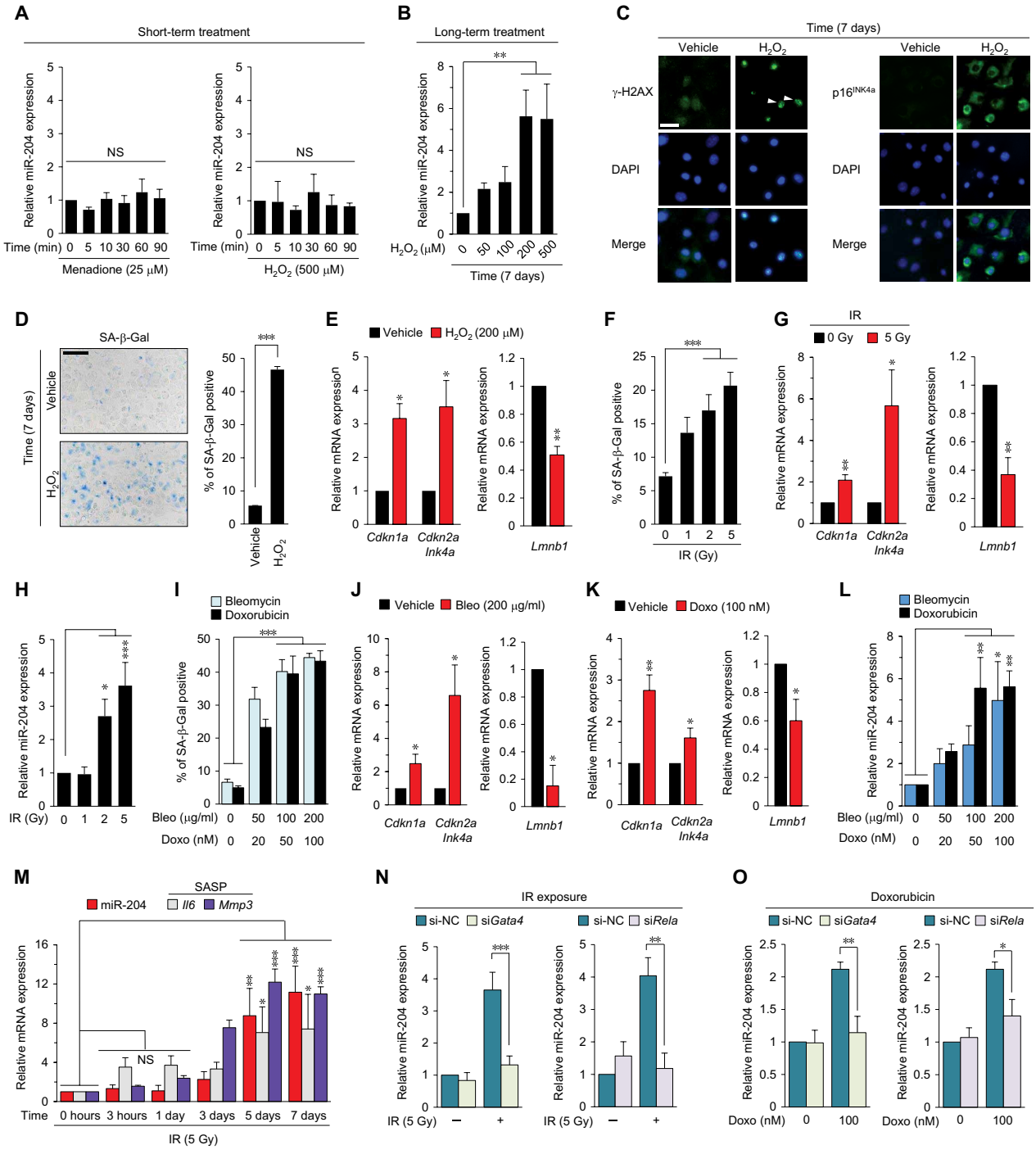

Fig. 2. Cellular senescence of chondrocytes induces miR-204 expression. (**A**) Relative miR-204 expression at indicated time points in mouse chondrocytes after menadione (25 μM; $n = 9$) or H_2O_2 (500 μM; $n = 4$) treatment. (**B**) Relative miR-204 expression in chondrocytes after 7 days of H_2O_2 treatment at indicated doses ($n = 3$). (**C**) Immunofluorescence staining of γ-H2AX (foci indicated by white arrowheads) and $p16^{INK4a}$ in vehicle- or H_2O_2-treated (200 μM) chondrocytes. Scale bar, 25 μm. (**D**) SA-β-Gal staining and quantification of SA-β-Gal positivity in chondrocytes after vehicle or H_2O_2 treatment (200 μM; $n = 3$). Scale bar, 100 μm. (**E**) Relative mRNA expression of CDK inhibitors and *Lmnb1* in chondrocytes after vehicle or H_2O_2 treatment ($n = 4$). (**F**) Quantification of SA-β-Gal positivity in chondrocytes 5 days after IR exposure at indicated doses ($n = 4$). (**G**) Relative mRNA expression of CDK inhibitors and *Lmnb1* in IR-irradiated chondrocytes ($n = 6$). (**H**) Relative miR-204 expression in chondrocytes 5 days after IR exposure ($n = 6$). (**I**) Quantification of SA-β-Gal positivity in chondrocytes after treatment with bleomycin ($n = 4$) or doxorubicin ($n = 6$) at indicated doses. (**J** and **K**) Relative mRNA expression of CDK inhibitors and *Lmnb1* in chondrocytes treated with (J) bleomycin (bleo) ($n = 6$) or (K) doxorubicin (doxo) ($n = 6$). (**L**) Relative miR-204 expression in chondrocytes after bleomycin ($n = 4$) or doxorubicin ($n = 4$) treatment. (**M**) Relative expression of miR-204, *Il6*, and *Mmp3* at indicated time points in chondrocytes after IR exposure [5 gray (Gy); $n \geq 4$]. (**N** and **O**) Relative miR-204 expression in chondrocytes after (N) IR exposure ($n \geq 3$) or (O) doxorubicin treatment ($n = 3$), following transfection with negative control small interfering RNA (siRNA) (si-NC) or siRNAs targeting *Gata4* or *Rela*. Data represent means ± SEM using ANOVA followed by post hoc test in (A), (B), (F), (H), (I), and (L) to (O) and Student's *t* test in (D), (E), (G), (J), and (K). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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TSS2 (fig. S7C). We generated two reporter constructs reflecting transcription from TSS1 and TSS2. The TSS1 reporter, but not the TSS2 reporter, was responsive to IR exposure (fig. S7D). Through analysis of transcription factor binding sites using the TRANSFAC database (*52*), GATA binding protein 4 (GATA4) (*49*) and nuclear factor κB (NF-κB) (*53*), transcription factors implicated in SASP regulation, were predicted to bind upstream of the TSS1 promoter (fig. S7E). miR-204 expression induced by IR exposure or DNA-damaging agent was effectively abolished by siRNAs targeting *Gata4* or *Rela* (Fig. 2, N and O, and fig. S7, F and G).

miR-204 impairs sulfated PG synthesis in chondrocytes and promotes OA pathogenesis in mice

We then explored the pathophysiological role of up-regulated miR-204 in chondrocytes. We directly delivered miR-Ctrl or miR-204 to chondrocytes and cultured them in three-dimensional (3D) matrices (*54*). Matrix-embedded miR-Ctrl–transfected chondrocytes deposited pericellular matrix abundant in PGs. In contrast, chondrocytes transfected with miR-204 exhibited reduced ECM deposition (Fig. 3A). Consistently, miR-204 delivery reduced the extracellular release of sGAG in chondrocytes (Fig. 3B and fig. S7H).

Because the diminished capacity of cartilage matrix synthesis is a major manifestation of OA chondrocytes, we tested whether intra-articular delivery of miR-204 elicits OA-associated phenotypes in vivo in mice (Fig. 3C). The effective delivery of small RNAs to mouse knee cartilage using transfection reagents, particularly variants of cationic polymers, has been previously reported (*55*, *56*). We experimentally confirmed that chondrocytes within the cartilage ECM are the target of transfection by intra-articular injection of jetPEI and the fluorescein isothiocyanate (FITC)–labeled miRNA (FITC-miRNA) complex. FITC-miRNAs were predominantly found in chondrocytes located in the superficial and middle zones of articular cartilage (fig. S8A). In addition, the injected miRNAs were also found in the synovial lining cells of joints.

miR-204 delivery to mouse knee joint tissues triggered marked PG loss and spontaneous cartilage destruction, as manifested by fibrillation or fissures, whereas the delivery of miR-Ctrl had no apparent effect on the cartilage (Fig. 3, D to F). miR-204–treated mice also exhibited a moderate degree of subchondral bone sclerosis; osteophyte development and synovitis were not observed. Immunohistochemistry analysis showed increased MMP13 expression in articular chondrocytes of miR-204–injected mice, indicating a degenerative process in the cartilage (fig. S8B and data file S1). We speculate that reduced PG synthesis caused by miR-204 may have impaired the load-bearing function of the cartilage matrix and augmented the mechanical stress imposed on the articular cartilage, thereby eliciting catabolic factor expression (Fig. 3, E and F, and fig. S8B). In contrast, despite the efficient delivery of miR-204 to synovial cells, the expression of interleukin-1β (IL-1β), tumor necrosis factor–α (TNF-α), or MMP13 was not detected in the synovium, coinciding with no sign of synovitis (fig. S8, C and D). Together, this ruled out the possibility that an inflammatory response in the synovial lining secondarily contributed to matrix catabolism in the articular cartilage.

We then examined how miR-204 expression affects the progression of posttraumatic OA in mice. Compared with miR-Ctrl delivery, miR-204 delivery markedly accelerated OA progression in DMM-induced OA (Fig. 3, G and H) by augmenting all examined OA manifestations, including cartilage destruction, subchondral bone sclerosis, osteophyte maturation, and synovitis.

miR-204 regulates multiple components of the PG biosynthesis pathway in chondrocytes

To comprehensively elucidate the underlying mechanisms through which miR-204 disrupts cartilage homeostasis, we performed RNA sequencing from chondrocytes transfected with miR-Ctrl and miR-204. We attempted to determine the functional modules that are sets of genes implicated in a biological process (*57*) and controlled by miR-204. We conducted hierarchical clustering to arrange differentially down-regulated genes based on their coexpression patterns. This clustering revealed four distinct gene sets enriched with sequence-based miR-204 targets (fig. S9, A and B). We hypothesized that these gene sets reflect functional modules inactivated by miR-204 up-regulation. Pathway analysis indicated that the identified gene sets are associated with annotations related to "proteoglycan synthesis," "cell cycle," or "programmed cell death" (fig. S9, C and D). Because these annotations are closely related to cellular senescence (*58*, *59*), we tested the possibility that miR-204 serves as a driver of cellular senescence in chondrocytes. However, delivery of miR-204 did not induce senescence-associated phenotypes in chondrocytes (Fig. 4, A to D).

Among the four functional gene modules, cluster 2 was most highly enriched with predicted miR-204 targets and exhibited a strong association with terms related to PG biosynthesis pathways (fig. S9C). Therefore, we postulated that the primary miR-204 targets are genes implicated in the chondrocyte PG synthesis pathway. The most abundant GAG chains of sulfated PGs in cartilage consist of CS (*4*). Therefore, we examined how miR-204 affects the genes in PG biosynthesis pathway including those involved in PG backbone assembly, elongation of the CS chain, and sulfate conjugation on CS chains, derived from the database in IPA (Fig. 4E) (*60*). Upon miR-204 transfection in chondrocytes, there was an overall down-regulation of the genes comprising the PG biosynthesis pathway; 11 genes involved in the pathway contained putative miR-204 binding sites in their 3′UTR, and seven of them were actually down-regulated by miR-204 transfection in chondrocytes.

The seven genes regulated by miR-204 have crucial functions in PG biosynthesis and assembly. SLC35D1 is a transporter that carries uridine 5′-diphosphate (UDP)–GlcA and UDP-GalNAc, the building blocks of CS disaccharide repeats (*61*). CHSY1 and CSGALNACT2 are involved in the elongation of the CS chain (*62*, *63*), and CHST11 and CHST15 are the enzymes required for the sulfation of GalNAc in the chain (*64*, *65*). HAS2 and HAPLN1 are required for the assembly of the HA and core protein PG backbone (*66*, *67*). In addition, we used real-time PCR analysis to verify that the mRNA expression of these genes is repressed by miR-204 in both primary cultured mouse chondrocytes and a human chondrosarcoma cell line, SW1353 (Fig. 4F and fig. S10).

To test the possibility that these seven genes are direct targets of miR-204, we constructed 3′UTR reporters with and without mutations in the putative miR-204 binding site (Fig. 4G) (*68*). Introduction of miR-204 effectively repressed reporter gene expression, whereas mutation of the miR-204 binding sequences abolished these effects (Fig. 4, H to J). In vivo efficacy of miR-204–mediated down-regulation of these targets was examined by immunohistochemistry using the sections of mouse cartilage to which either miR-Ctrl or miR-204 was delivered. The expression of SLC35D1, CHSY1, CHST11, and HAPLN1 was suppressed in miR-204–delivered cartilage (Fig. 4K, fig. S11A, and data file S1). Consistently, the overall CS positivity in articular cartilage was reduced after miR-204 delivery (Fig. 4K and figs. S11B and S12), supporting the notion that miR-204 negatively

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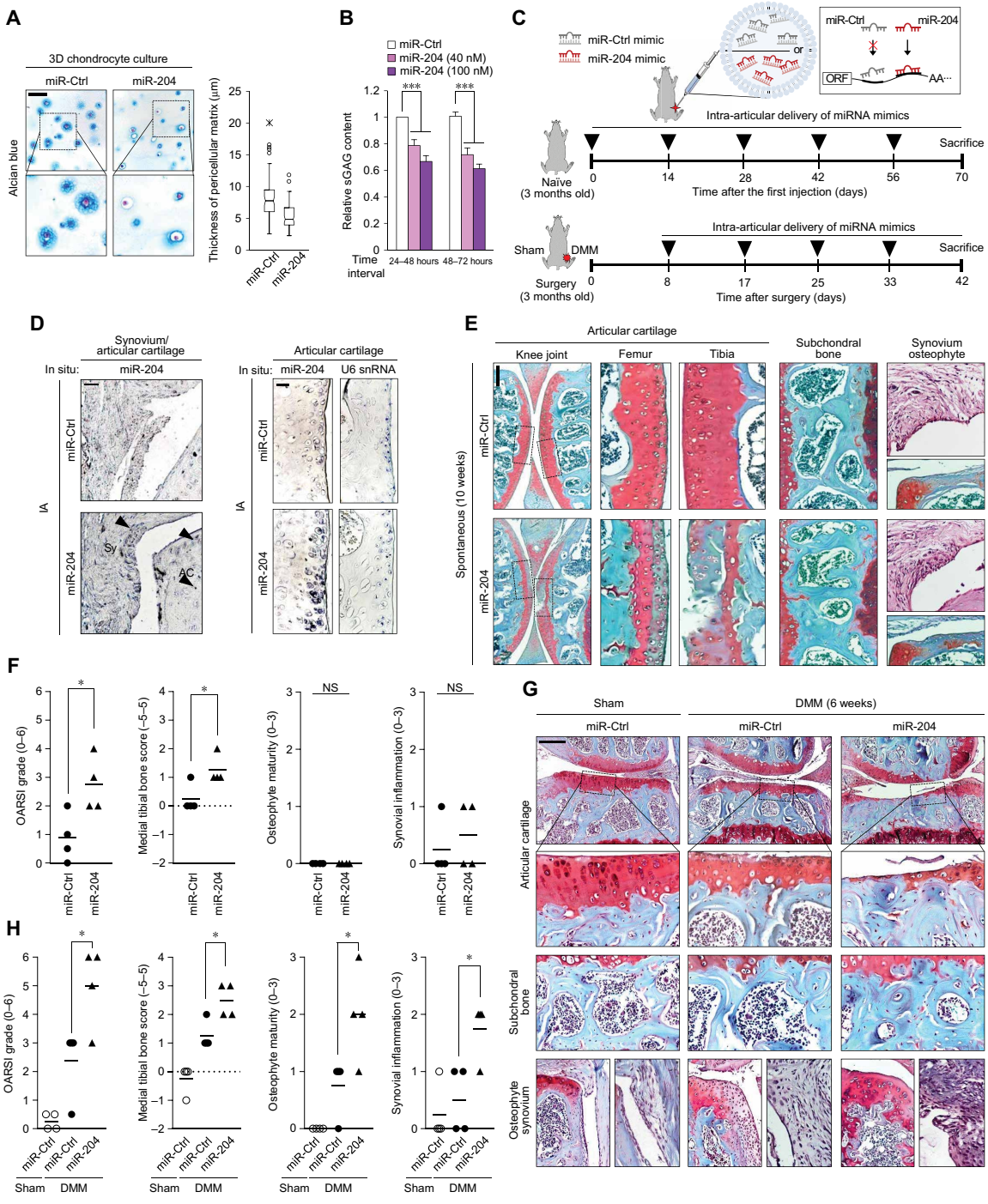

Fig. 3. miR-204 suppresses PG synthesis and promotes OA progression. (**A**) Alcian blue staining (left) and notched box plot of the thickness of pericellular matrix (right) in mouse chondrocytes transfected with miR-Ctrl or miR-204 and grown in 3D matrices for 14 days ($n = 68$ chondrocytes). The inset in the images is shown at higher magnification in the bottom row. The box plot represents the median and the first to third interquartile range. The notch represents the 95% confidence interval around the median. Scale bar, 100 μm. (**B**) sGAG release of chondrocytes transfected with miR-Ctrl or miR-204 ($n = 8$). (**C**) Schematic illustration of miRNA delivery schedules in spontaneous OA model mice (top) or posttraumatic OA model mice (bottom). ORF, open reading frame; AA, polyadenylation. (**D**) In situ hybridization of miR-204 (black arrowheads) in sections of synovium (left) or cartilage (right) in which miR-Ctrl or miR-204 was delivered intra-articularly (IA). Scale bars, 25 μm. snRNA, small nuclear RNA. (**E**) miR-Ctrl or miR-204 was intra-articularly delivered to mouse knee joints using an in vivo transfection reagent, in vivo-jetPEI (0.4 mg/kg of miRNA; once every 2 weeks for 10 weeks). Joint sections were stained with safranin O, fast green, and hematoxylin. The inset in the images is shown at magnified images in the right panels. Scale bar, 200 μm. (**F**) Cartilage destruction, subchondral bone sclerosis, osteophyte formation, and synovial inflammation were determined by safranin O/hematoxylin staining and scored ($n = 4$). (**G**) miR-Ctrl or miR-204 was intra-articularly delivered to sham- or DMM-operated mice (0.4 mg/kg; once every 10 days for 6 weeks). Joint sections were stained with safranin O, fast green, and hematoxylin. The inset in the images is shown at magnified images in the bottom row. Scale bar, 200 μm. (**H**) Cartilage destruction, subchondral bone sclerosis, osteophyte formation, and synovial inflammation were determined by safranin O/hematoxylin staining and scored ($n = 4$). Data represent means ± SEM using ANOVA followed by post hoc test in (B), Mann-Whitney *U* test in (F), and Kruskal-Wallis test followed by the Mann-Whitney *U* test in (H). $^*P < 0.05$, $^{***}P < 0.001$.

regulates PG synthesis (Fig. 3, A and B) and disrupts cartilage matrix homeostasis (Fig. 3, E to H).

Given the net effects of miR-204 on PG synthesis, we examined the possibility that miR-204 may regulate the expression of SRY (sex determining region Y)–box 9 (SOX9) and aggrecan despite the absence of miR-204 canonical binding seed sequences on the 3′UTR of these genes. However, neither the mRNA expression nor the 3′UTR reporter activity of *Sox9* and *Acan* was affected by miR-204

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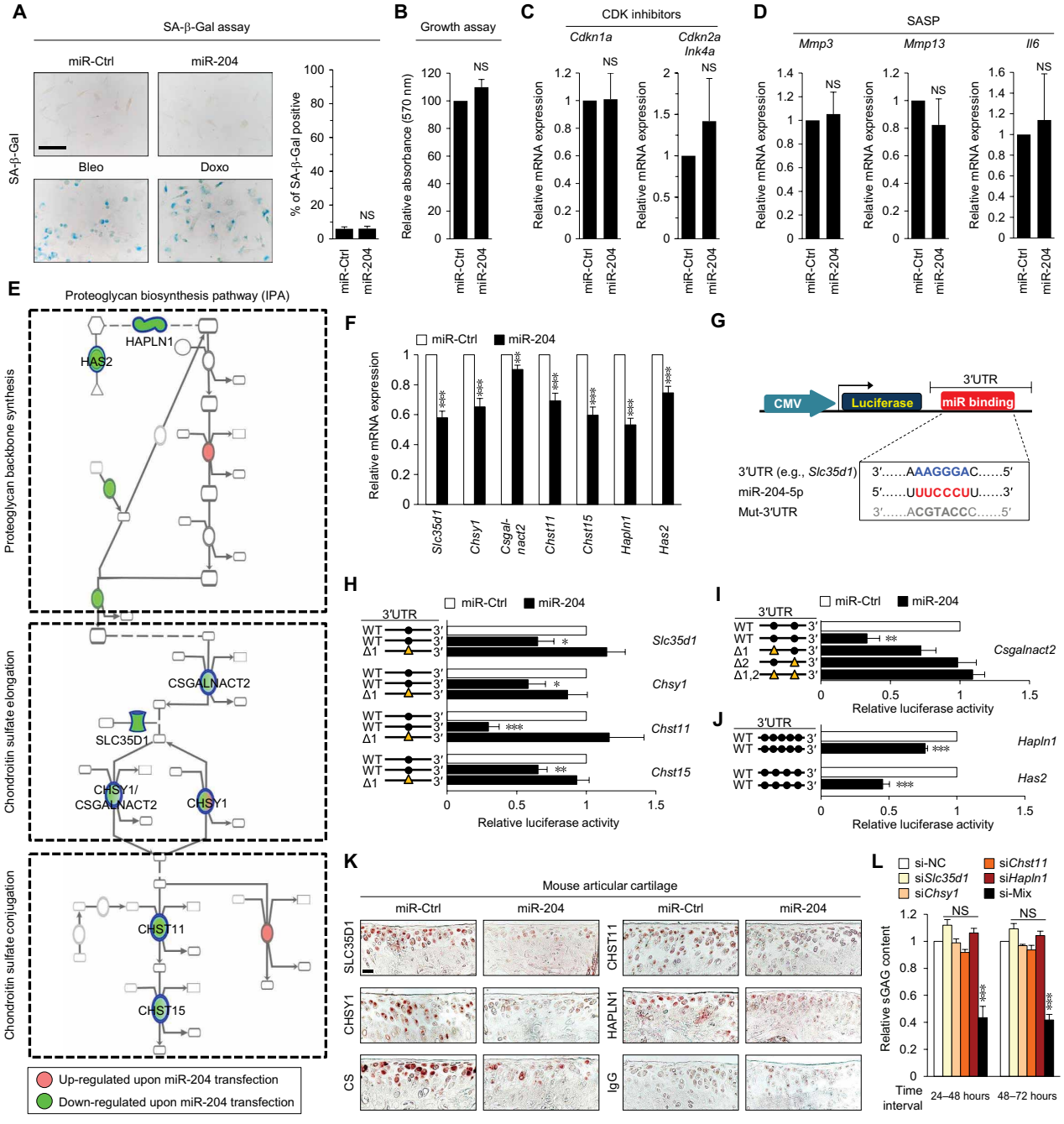

Fig. 4. miR-204 collectively targets multiple components of cartilage PG biosynthesis pathway. (**A**) Representative images of SA-β-Gal staining of mouse chondrocytes transfected with miR-Ctrl or miR-204 or those treated with bleomycin (200 μg/ml) or doxorubicin (100 nM) and quantification of SA-β-Gal positivity in chondrocytes transfected with miR-Ctrl or miR-204 ($n = 3$). Scale bar, 100 μm. (**B**) Growth assay of chondrocytes transfected with miR-Ctrl or miR-204 ($n = 6$). (**C** and **D**) Relative mRNA expression of (C) CDK inhibitors or (D) SASP factors in chondrocytes transfected with miR-Ctrl or miR-204 ($n \geq 4$). (**E**) PG biosynthesis pathway was constructed from the relevant pathways obtained from Ingenuity Pathway Analysis (IPA). Fold changes in the gene expression after miR-204 transfection as compared with miR-Ctrl are indicated as the intensity of green (down-regulated) to red (up-regulated) color based on RNA sequencing data. (**F**) Relative mRNA expression of genes down-regulated in (E) determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) ($n = 8$). (**G**) A design scheme of 3′UTR reporter vector for predicted miR-204 target gene. The 6-mer seed binding site of miR-204, AAGGGA, was mutated to CGTACC in mutant 3′UTR reporters. (**H** and **I**) Relative luciferase activity of wild-type (WT) or mutant 3′UTR reporters of (H) *Slc35d1*, *Chsy1*, *Chst11*, *Chst15*, and (I) *Csgalnact2* in chondrocytes transfected with miR-Ctrl or miR-204 ($n \geq 3$). (**J**) Relative luciferase activity of 3′UTR reporters of *Hapln1* and *Has2* in chondrocytes transfected with miR-Ctrl or miR-204 ($n = 3$). (**K**) SLC35D1, CHSY1, CHST11, HAPLN1, and CS were immunostained in cartilage sections from mice after intra-articular delivery of miR-Ctrl or miR-204. Scale bar, 25 μm. IgG, immunoglobulin G; SLC35D1, solute carrier family 35 member D1; CHSY1, chondroitin sulfate synthase 1; CHST11, carbohydrate sulfotransferase 11; HAPLN1, hyaluronan and proteoglycan link protein 1. (**L**) sGAG release of chondrocytes transfected with 25 nM siRNAs targeting *Slc35d1*, *Chsy1*, *Chst11*, or *Hapln1* or a mixture of these siRNAs. The total amount of siRNAs transfected was adjusted to 100 nM using negative control siRNA ($n = 6$). Data represent means ± SEM using Student's *t* test in (A) to (D), (F), (H) to (J), and (L). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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in chondrocytes (fig. S13, A and B). Similarly, SOX9 and aggrecan expression in whole-cell lysates was not affected, ruling out the possibility of their regulation at the translational level by miR-204 (fig. S13C). We also examined the expression of SOX9 and aggrecan in cartilage sections obtained from mice that were intra-articularly injected with miR-Ctrl or miR-204. Although the SOX9 expression was unaffected, that of aggrecan was moderately decreased in miR-204–injected cartilage (fig. S13D and data file S1). We speculate that the reduction in aggrecan is likely associated with the overall loss of sulfated PGs in cartilage during OA progression (Fig. 3E). Similarly, the increased expression of MMP13 in articular cartilage upon miR-204 injection (fig. S8B) was likely not due to a regulatory role of miR-204 in MMP13 expression (Fig. 4D). Presumably, reduced PG synthesis caused by miR-204 may have impaired the load-bearing function of cartilage matrix and augmented the mechanical stress imposed on the articular cartilage, thereby eliciting catabolic factor expression, such as expression of MMP13.

Next, we questioned which of the miR-204 targets we identified serves as a limiting factor for controlling the flux through PG synthesis. Individual knockdown of *Slc35d1*, *Chsy1*, *Chst11*, or *Hapln1* did not affect the PG synthesis rate in chondrocytes (Fig. 4L and fig. S14). We instead noted the ability of miR-204 to simultaneously target multiple genes implicated in PG synthesis. Concurrent introduction of siRNAs targeting *Slc35d1*, *Chsy1*, *Chst11*, and *Hapln1* markedly diminished PG synthesis in chondrocytes (Fig. 4L). Therefore, our results suggest that these miR-204 targets, when collectively regulated, have the net effect of fine-tuning the flux into PG synthesis, underscoring the importance of the intrinsic ability of miRNAs to simultaneously repress multiple genes.

Last, because miR-204 targets include not only the transporters for CS disaccharide repeats (SLC35D1) but also the enzymes involved in the elongation (CHSY1 and CSGALNACT2) and sulfation (CHST11 and CHST15) of CS chain, we examined whether miR-204 up-regulation affects chain length or/and sulfation extents of PGs in chondrocytes. The elution profiles of size exclusion chromatography revealed that the length of CS chains of miR-204–transfected chondrocytes was relatively shorter than that of the miR-Ctrl group (fig. S15, A to C). Furthermore, miR-204 up-regulation decreased the overall sulfation extent of CS chains synthesized by chondrocytes (fig. S15D).

miR-204 antagonism ameliorates experimental OA in mice

We next investigated the effect of miR-204 antagonism on sulfated PG synthesis, chondrocyte senescence, and OA pathogenesis. Inhibition of miR-204 activity by anti–miR-204 alleviated the repression of miR-204 targets (Fig. 5A) and de novo PG synthesis in chondrocytes (Fig. 5B). Together, these results further support our finding that miR-204 regulates the sulfated PG biosynthesis pathway. Consistent with our results showing that miR-204 delivery does not cause chondrocyte senescence (Fig. 4A), DNA damage–induced senescence was not alleviated by anti–miR-204 treatment in chondrocytes (Fig. 5C and fig. S16A). However, up-regulation of SASP factors, which appears as a delayed response to DNA damage in chondrocytes, was effectively abolished by the inhibition of miR-204 activity (Fig. 5D and fig. S16, B and C), suggesting that the miR-204 expression induced over the course of senescence is necessary for the display of SASP. *Il1b* and *Adamts5*, which are the core OA-associated proinflammatory cytokine and aggrecanase, respectively, were also up-regulated by senescent chondrocytes and effectively abolished by the inhibition of miR-204 (fig. S17).

The therapeutic effect of miR-204 inhibition was examined in a surgically induced OA model in mice (Fig. 5E). Intra-articular delivery of anti–miR-204 did not affect the normal appearance of articular cartilage in knee joints in sham-operated mice (fig. S18A). DMM-operated mice injected with anti–miR-Ctrl exhibited severe cartilage destruction, subchondral bone sclerosis, osteophyte development, and a moderate degree of synovial inflammation. Intra-articular injection of anti–miR-204 ameliorated DMM-induced cartilage destruction and subchondral bone sclerosis, along with modest effects on osteophyte maturity and synovial inflammation (Fig. 5, F and G). At a molecular level, miR-204 inhibition in knee joints of DMM-operated mice caused concomitant recovery of sulfated PG synthesis and suppression of both inflammatory SASP factors and non–cell autonomous propagation of cellular senescence in cartilage (Fig. 5H, fig. S18B, and data file S1). Moreover, we measured weight distributions between the surgically (DMM or sham) treated and untreated legs as a behavioral assessment of OA-induced pain. DMM surgery with intra-articular injection of anti–miR-Ctrl caused decreased weight bearing on the injured leg. The weight imbalance caused by DMM surgery was alleviated by treatment with anti–miR-204 (Fig. 5I). Together, this indicates that miR-204 antagonism effectively ameliorates OA manifestations in mice.

Stress-activated miR-204 serves as a potential therapeutic target for human OA

Last, we evaluated the relevance of our findings to humans using primary cultured human articular chondrocytes and tissue explants from patients undergoing total knee arthroplasty. Doxorubicin, a DNA-damaging chemical agent, caused robust cellular senescence in primary cultured human articular chondrocytes as evidenced by the induction of SA-β-Gal activity and $p16^{INK4a}$ expression (Fig. 6, A and B, and data file S1). Senescence was further confirmed by the up-regulation of *CDKN1A* and *CDKN2A* and down-regulation of *LMNB1* (Fig. 6C). In those senescence-induced human articular chondrocytes, miR-204 expression was markedly increased (Fig. 6D), validating the conserved regulatory mechanism of miR-204 expression between human and mouse. Similarly, the mRNA expression of identified miR-204 target genes, which encode proteins comprising the PG biosynthesis pathway, was repressed in human articular chondrocytes transfected with miR-204 (Fig. 6E), corroborating our results from primary cultured mouse chondrocytes and a human chondrosarcoma cell line, SW1353 (Fig. 4F and fig. S10B). Last, to test the feasibility of miR-204–targeting therapy in clinical OA, we evaluated the effect of miR-204 antagonism in an explant culture of OA cartilage from patients undergoing total knee arthroplasty. miR-204 inhibition in OA-affected tissue explants augmented the amount of CS and suppressed the expression of catabolic mediators such as MMP3 and MMP13 (Fig. 6F).

DISCUSSION

OA is primarily caused by an imbalance in cartilage matrix anabolism and catabolism. Recent progress in understanding the pathophysiology of OA has led to the discovery of several key catabolic regulators contributing to cartilage destruction (*23*, *43*, *69*–*72*). Chondrocyte senescence is considered a crucial cellular event contributing to matrix catabolism during OA development (*18*, *19*), and senolytic approaches effectively prevent OA development by eradicating the source of the catabolic mediators. Another key feature of senescent

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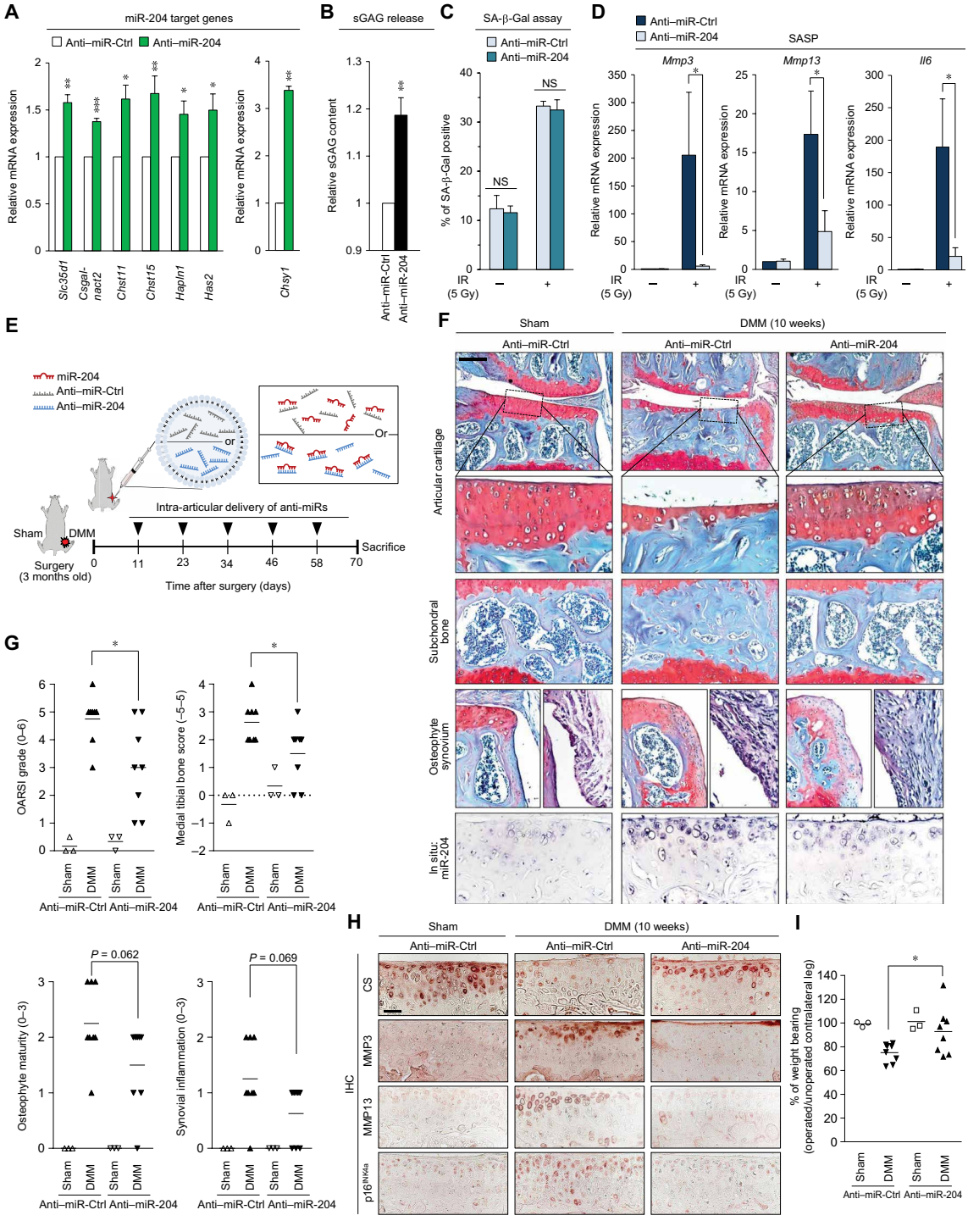

Fig. 5. Inhibition of miR-204 counteracts OA progression in mice. (**A**) Relative mRNA expression of PG-associated miR-204 target genes in P2 mouse chondrocytes transfected with anti–miR-Ctrl or anti–miR-204 ($n = 6$). (**B**) sGAG release of chondrocytes transfected with anti–miR-Ctrl or anti–miR-204 ($n = 5$). (**C**) Quantification of SA-β-Gal positivity in chondrocytes after IR exposure after transfection with anti–miR-Ctrl or anti–miR-204 ($n \geq 3$). (**D**) Relative mRNA expression of SASP factors in chondrocytes after IR exposure after transfection with anti–miR-Ctrl or anti–miR-204 ($n = 4$). (**E**) Schematic illustration of anti-miR therapy in posttraumatic OA model mice. (**F**) Anti–miR-Ctrl or anti–miR-204 was intra-articularly delivered to sham- or DMM-operated mice using an in vivo transfection reagent, in vivo jetPEI (0.4 mg/kg; once every 12 days for 10 weeks). Joint sections were stained with safranin O, fast green, hematoxylin, and in situ hybridization of miR-204. The inset in the images is shown at magnified images in the bottom row. Scale bar, 200 μm. (**G**) Cartilage destruction, subchondral bone sclerosis, osteophyte formation, and synovial inflammation were determined by safranin O/hematoxylin staining and scored ($n = 3$ for sham; $n = 8$ for DMM). (**H**) CS, SASP factors (MMP3 and MMP13), and senescence marker p16^{INK4a} were detected by immunohistochemistry (IHC). Scale bar, 25 μm. (**I**) The percentage of weight placed on the sham- or DMM-operated limb versus contralateral limb after intra-articular delivery of anti–miR-Ctrl or anti–miR-204. Data represent means ± SEM using Student's *t* test in (A), (B), and (I), ANOVA followed by post hoc test in (C) and (D), and Kruskal-Wallis test followed by the Mann-Whitney *U* test in (G). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

chondrocytes is a decline in chondrocyte-mediated anabolism (*8*). However, the underlying mechanism of cessation of matrix anabolism remains largely elusive.

Here, we demonstrate that senescence-mediated induction of miR-204 leads to cessation of PG anabolism in chondrocytes and may play a causal role in the development of OA by impairing the load-bearing capacity of cartilage. Moreover, miR-204 expression is necessary for the display of SASP and subsequent inflammatory responses in joint environments. Therefore, anti-miR therapy targeting miR-204 effectively ameliorates surgically induced OA in mice

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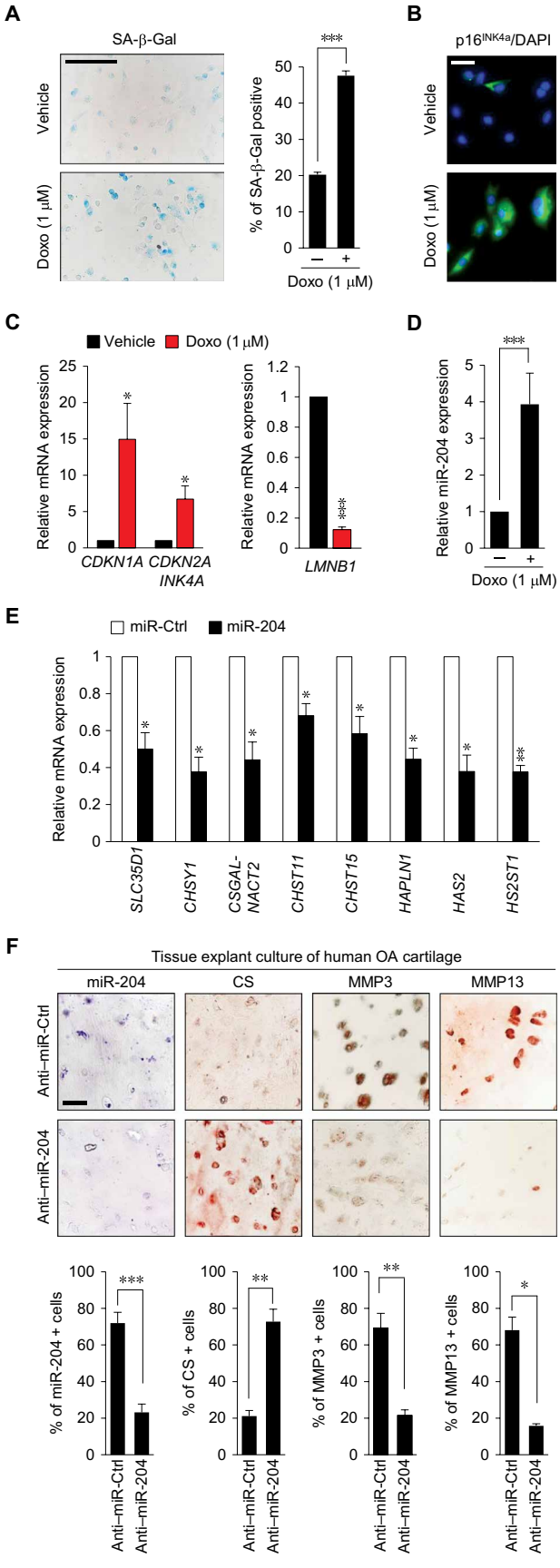
Fig. 6. Inhibition of senescence-induced miR-204 attenuates imbalance between matrix catabolism and anabolism in human OA cartilage. (**A**) Representative images and quantification of SA-β-Gal staining of primary cultured human articular chondrocytes after 14 days of doxorubicin treatment (1 μM; $n = 3$). Scale bar, 100 μm. (**B**) Immunofluorescence staining of $p16^{INK4a}$ in vehicle- or doxorubicin-treated human articular chondrocytes. Scale bar, 25 μm. (**C**) Relative mRNA expression of CDK inhibitors and *LMNB1* in vehicle- or doxorubicin-treated human articular chondrocytes ($n = 5$). (**D**) Relative miR-204 expression in human chondrocytes after vehicle or doxorubicin treatment ($n = 4$). (**E**) Relative mRNA expression of PG biosynthesis pathway genes after miR-Ctrl or miR-204 transfection was determined by qRT-PCR ($n = 3$). (**F**) Anti–miR-Ctrl or anti–miR-204 was transfected to explant culture of human OA cartilage. Cartilage explant sections were stained by in situ hybridization of miR-204 and immunohistochemistry of CS, MMP3, and MMP13. The percentage of cells positive for miR-204, CS, MMP3, and MMP13 was quantified ($n \geq 3$). Scale bar, 100 μm. Data represent means ± SEM using Student's *t* test in (A) and (C) to (F). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



by attenuating the imbalance between matrix catabolism and anabolism (fig. S19).

Our study suggests a possible alternative approach to the recently developed senolytic therapy for OA treatment. Senolytics showed promise as a potential disease-modifying therapy for OA in mice, but there are several caveats to be resolved before its translation to the clinical realm. Because chondrocytes are sparser in the human cartilage than in the murine cartilage, tolerance of human cartilage toward cell loss and potential adverse outcomes, such as tissue dystrophy, should be extensively examined (*73*). Moreover, excessive reduction of the chondrocyte population may accelerate exhaustion of stem cell populations (*74*) that potentially replace chondrocytes during OA process and contribute to cartilage repair (*75*, *76*). Another important consideration is the potential off-target effects of senolysis, which could affect not only nonsenescent cells but also beneficial senescent cells, such as those participating in wound repair (*17*, *77*). Our approach targeting miR-204 may serve as an attractive strategy to treat senescent chondrocytes by circumventing excessive or unintended removal of cell populations in aged cartilage. This miR-204–targeting therapy may enable sustained matrix anabolism and quenching of the inflammatory process (the onset of SASP expression) by senescent chondrocytes. Together, our approach may effectively prevent the transition of OA to a rapidly progressing pathological phase.

Our findings also support a link between oxidative stress and OA development. Both aging (*8*) and mechanical stress (*9*, *10*, *78*–*80*), the two major OA risk factors, have been implicated in generating ROS, thus leading to oxidative stress in articular cartilage. Oxidative stress regulates the expression of various miRNAs (*81*), for example, the H_2O_2-induced up-regulation of miR-34a in chondrocytes (*82*). miR-34a represses sirtuin 1 (SIRT1) expression by directly targeting its 3′UTR, which in turn attenuates p53 and activates the apoptotic pathway during OA pathogenesis (*83*). miRNAs may also play a regulatory role in controlling oxidative stress–induced cellular senescence in chondrocytes. For instance, miR-335, miR-494, and miR-34a that are up-regulated in senescent cells or aged tissues repress genes implicated in the antioxidant pathway, making senescent cells even more vulnerable to oxidative stress (*81*). Our results show that prolonged oxidative stress leads to gradual accumulation of DNA damage in chondrocytes and consequently facilitates their entry into cellular senescence, resulting in miR-204 expression.

Potential limitations of our study include the use of intra-articular injection–mediated delivery of miR-204 rather than the use of transgenic mice with a chondrocyte-specific promoter for miR-204

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overexpression. Although we confirmed that chondrocytes within articular cartilage are the target of transfection by the jetPEI and miRNA complex, the delivery of the injected miRNA to synovial lining cells of the joints was also detected. However, miR-204 expression in synoviocytes caused neither expression of catabolic mediators nor synovitis, diminishing the possibility that an inflammatory response in the synovial lining secondarily contributed to matrix catabolism in the articular cartilage. Nevertheless, the transgenic overexpression of miR-204 under the *Col2a1* or *Prg4* promoter would more clearly dissect the role of miR-204 in articular chondrocytes in mediating OA development in mice.

Another limitation reflects the technical hurdles to be surmounted before anti–miR-204 is successfully used as a therapeutic agent clinically. For the effective treatment of chronic diseases such as OA, repeated dosing of anti-miR oligonucleotides is required because RNA-based oligonucleotides have low stability. Therefore, development of protective vehicles or RNA modification methods will be necessary to design a therapeutic strategy with a practical injection interval for anti–miR-204. Furthermore, to minimize potential off-target effects of anti–miR-204 on noncartilaginous tissues upon intra-articular injection, development of a cartilage-specific delivery method for oligonucleotides would be beneficial. For instance, anti–miR-204 delivery using polyethylenimine conjugated to chondrocyte-affinity peptide may further improve the efficiency of miR-204 antagonism therapy for OA. Last, the efficacy of miR-204 inhibition in preclinical murine OA models should be translated cautiously. An approach to decrease miR-204 activity needs to be further tested in large animals similar in anatomy and joint mechanics to humans. Future studies involving sequential phase trials are warranted to demonstrate the ultimate safety and efficacy of miR-204 antagonism in human OA.

In summary, we show that miR-204 is a key regulator controlling PG synthesis in chondrocytes. Senescence-induced miR-204 shifts the secretory phenotypes of chondrocytes from those required for cartilage matrix construction to those accelerating degenerative processes. miR-204 antagonism effectively ameliorates OA development in mice, validating the potential therapeutic effect on OA progression in preclinical animal models. Collectively, these findings provide insights for the development of future OA therapies aimed at attenuating the imbalance between matrix synthesis and inflammatory phenotypes in senescent chondrocytes.

MATERIALS AND METHODS

Study design

The overall objective of this study was to identify miRNAs associated with OA development and to develop a therapeutic approach using miRNA antagonism. Considering the emerging significance of senescence of chondrocytes in OA development (*8*, *24*), we particularly aimed to screen miRNAs whose inhibition could effectively modulate senescent phenotypes of chondrocytes to treat OA. We conducted a paired analysis of knee joint cartilages derived from patients with OA and aging-associated and posttraumatic OA mouse models. We investigated the regulatory mechanisms of miR-204 under various stress-eliciting stimuli in primary cultured human and mouse chondrocytes. We examined the in vivo effects of miR-204 overexpression and its antagonism in surgically induced OA mouse models. DMM surgery was used to induce posttraumatic OA in 12-week-old mice. Small RNAs were delivered to mouse knee joints by intra-articular injection. Various OA manifestations including cartilage destruction, subchondral bone sclerosis, osteophyte maturity, and synovial inflammation in mice were histologically inspected. Weight distributions between the surgically treated and untreated legs were measured as a behavioral assessment of OA-induced pain. Mice used for animal studies were randomly assigned to each group with approximately equal numbers in each group, and all samples were analyzed by experienced histopathologists who were blinded to the experimental conditions. The sample sizes were determined on the basis of previous studies (*19*, *43*, *84*). For each experiment, sample size reflects the number of independent biological replicates and is indicated in the figure legend. For animal studies, persistent lameness and discomfort after surgical recovery were used as criteria for stopping further experiments with the corresponding mouse before completion. However, none of the animals met these criteria in this study. No exclusion criteria were applied for any measurements collected from all experimentations. Human OA cartilage specimens were sourced from patients with OA undergoing total knee replacement at Seoul National University (SNU) Boramae Medical Center. The Institutional Review Board (IRB) of SNU Boramae Medical Center (IRB nos. 26-2016-143 and 30-2017-48) approved the collection of materials, and the IRB of SNU (IRB nos. E1612/003-005 and E1803/003-009) approved the use of these materials. Full written informed consent was provided by all participants before the total knee replacement arthroplasty operative procedure. All animal experiments were approved by the SNU Institutional Animal Care and Use Committee (IACUC no. SNU-170914-1). The design, analysis, and reporting of animal experiments followed the Animal Research: Reporting of In Vivo Experiments guidelines. Additional information related to RT-PCR experiments and patient demographics can be found in the Supplementary Materials (fig. S20 and tables S1 to S4). Individual subject-level data are provided in data file S1.

Statistical analysis

For in vitro studies, each experiment was conducted independently at least three times. To compare the experimental groups, parametric test based on two-tailed Student's *t* test or one-way ANOVA followed by post hoc test (least significant difference) was used. For in vivo experiments, each independent trial was conducted using an individual mouse. To determine significant differences, nonparametric test based on Mann-Whitney *U* test was used. For nonparametric, multigroup comparisons, the Kruskal-Wallis test followed by the Mann-Whitney *U* test was used. Statistical significance was accepted at $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics 23.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Small RNA sequencing reveals miR-204 as a miRNA potentially associated with OA development.

Fig. S2. Validation of $p16^{INK4a}$ detection and its up-regulation in OA and aged mouse cartilage.

Fig. S3. Effect of H_2O_2 long-term treatment on the cell viability of mouse chondrocytes.

Fig. S4. Validation of cellular senescence in chondrocytes by IR.

Fig. S5. Validation of cellular senescence in chondrocytes after bleomycin or doxorubicin treatment.

Fig. S6. miR-204 expression in chondrocytes is not regulated by the senescence-associated cell cycle arrest regulators, p53 and $p16^{INK4a}$.

Fig. S7. miR-204 expression in chondrocytes is regulated by GATA4 and NF-κB.

Fig. S8. Intra-articular injection of miR-204 does not elicit synovial inflammation but induces MMP13 expression in articular cartilage.

Fig. S9. Transcriptome-wide coexpression analysis reveals that miR-204 inactivates putative functional modules related to PG synthesis, cell cycle, or programmed cell death.

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Fig. S10. miR-204 targets multiple genes involved in PG biosynthesis pathways in mouse primary chondrocytes and human chondrosarcoma cells.
Fig. S11. Negative controls for immunohistochemistry.
Fig. S12. Quantification of CS to measure the effect of miR-204 on PG accumulation in mouse articular cartilage.
Fig. S13. Effect of miR-204 on SOX9 and aggrecan expression in primary cultured mouse chondrocytes and knee joint cartilage.
Fig. S14. Knockdown efficiency of siRNAs designed for miR-204 target genes.
Fig. S15. miR-204 decreases the chain length and sulfation extent of CS chains in PGs.
Fig. S16. miR-204 antagonism does not affect SA-β-Gal activity but suppresses the SASP factors *Timp2* and *Igfbp7* in chondrocytes.
Fig. S17. miR-204 antagonism suppresses the expression of *Adamts5* and *Il1b* in senescent chondrocytes.
Fig. S18. miR-204 inhibition by anti–miR-204 does not affect the appearance of articular cartilage in sham-operated mice but suppresses $p16^{INK4a}$ expression in articular cartilage of DMM-operated mice.
Fig. S19. Schematic representation of the senescence-induced signaling pathway mediated by miR-204 in OA development.
Fig. S20. Uncropped gel images with size markers from the indicated figures.
Table S1. Descriptive characteristics of human patients with OA.
Table S2. List of siRNAs, miRNAs, and anti-miR.
Table S3. List of PCR primers.
Table S4. List of PCR primers for cloning.
Data file S1. Individual subject-level data (Excel file).
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Stress-activated miR-204 governs senescent phenotypes of chondrocytes to promote osteoarthritis development

Donghyun Kang, Jungkwon Shin, Yongsik Cho, Hyeon-Seop Kim, Young-Ran Gu, Haedong Kim, Kwon Tae You, Moon Jong Chang, Chong Bum Chang, Seung-Baik Kang, Jong-Seo Kim, V. Narry Kim and Jin-Hong Kim

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Stress, senescence, and joint health

Oxidative stress increases with aging and contributes to osteoarthritis (OA), a form of degenerative joint disease affecting cartilage and bone. Kang *et al*. investigated the role of cartilage cell (chondrocyte) senescence in OA. Oxidative stress induced DNA damage and senescence in chondrocytes. miR-204 was up-regulated in senescent chondrocytes and in aged and osteoarthritic human cartilage. Mice treated with miR-204 exhibited accelerated cartilage degeneration and maladaptive changes in extracellular matrix content, particularly a reduction in proteoglycan synthesis. Treatment with anti–miR-204 rescued cartilage catabolism in a posttraumatic OA mouse model and ex vivo human OA cartilage explants. This study identifies a pathway important for cartilage matrix homeostasis.

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