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Krüppel-like factors-4 and -2 are important regulators of joint tissue cells and protect against tissue destruction and inflammation in osteoarthritis

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Abstract

Objectives: Analyzing expression patterns of Krüppel-like factor (KLF) transcription factors in normal and osteoarthritis (OA) human cartilage, and determining functions and mechanisms of KLF4 and KLF2 in joint homeostasis and OA pathogenesis.

Methods: Experimental approaches included human joint tissues cells, transgenic mice, and mouse OA model with viral KLF4 gene delivery to demonstrate therapeutic benefit in structure and pain improvement. Mechanistic studies applied global gene expression analysis and chromatin immunoprecipitation sequencing (ChIP-seq).

Results: Several KLF genes were significantly decreased in OA cartilage. Among them, KLF4 and KLF2 were strong inducers of cartilage collagen genes and Proteoglycan-4. Cartilage-specific deletion of *Klf2* in mature mice aggravated severity of experimental OA. Transduction of human chondrocytes with Adenovirus (Ad) expressing KLF4 or KLF2 enhanced expression of major cartilage extracellular matrix (ECM) genes and SRY-box transcription factor-9, and suppressed mediators of inflammation and ECM-degrading enzymes. Ad-KLF4 and Ad-KLF2 enhanced similar protective functions in meniscus cells and synoviocytes, and promoted chondrocytic differentiation of human mesenchymal stem cells. Viral KLF4 delivery into mouse knees reduced severity of OA-associated changes in cartilage, meniscus and synovium, and improved pain behaviors. ChIP-seq analysis suggested that KLF4 directly bound cartilage signature genes. Ras-

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related protein-1 signaling was the most enriched pathway in KLF4-transduced cells, and its signaling axis was involved in upregulating cartilage ECM genes by KLF4 and KLF2.

Conclusions: KLF4 and KLF2 may be central transcription factors that increase protective and regenerative functions in joint tissue cells, suggesting that KLF gene transfer or molecules upregulating KLFs are therapeutic candidates for OA.

Keywords

Osteoarthritis; Chondrocytes; Inflammation

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease.[1] Despite substantial progress in identifying mechanisms of OA pathogenesis and molecular targets for intervention[2], there have thus far not been any successful clinical trials and there are no approved pharmacological treatments to prevent the disease onset or progression. A potential explanation is that more important molecular mechanisms than those previously targeted are involved in OA.

Degradation and loss of articular cartilage is a major factor of OA pathogenesis.[2] Cartilage extracellular matrix (ECM) molecules, including type-2 and type-11 collagen (COL2A1 and COL11A2), aggrecan (ACAN) and cartilage oligomeric matrix protein (COMP), are regulated by SRY-box transcription factor-9 (SOX9) cooperating with SOX5 and SOX6.[3–7] Proteoglycan-4 (PRG4) also known as lubricin is dominantly expressed in the superficial zone of articular cartilage, and is essential for homeostasis of articular joints to prevent damage to the articular surface.[8] While several transcription factors are reported to regulate PRG4[9–11], PRG4 is not subject to regulation by SOX9, which is a different regulatory mechanism compared to other cartilage ECM genes described earlier.[5 12] A transcription factor that upregulates all these cartilage signature genes would be a promising therapeutic for cartilage engineering and in treatment of OA.

Activation of catabolic and inflammatory events is another key mechanism in OA.[2 13 14] Therefore, suppressing catabolic and inflammatory genes, including a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS5), matrix metalloproteinase-3 (MMP3), MMP13, interleukin-6 (IL6), prostaglandin-endoperoxide synthase-2 (PTGS2), and nitric oxide synthase-2 (NOS2), will also be important for therapeutic intervention in OA.

We previously performed RNA-seq analysis of normal and OA human knee cartilage and found that expression of several members in the Krüppel-like factor (KLF) family of transcription factors was suppressed in OA.[15] The KLF family includes 17 zinc-finger transcription factors that are typically categorized into several groups based on similarities in structure and transcriptional activity: (1) KLF3, −8 and −12 serving as transcriptional repressors; (2) KLF1, −2, −4, −5, −6 and −7 functioning predominantly as transcriptional activators; (3) KLF9, −10, −11, −13, −14 and −16 having repressor activity; KLF15 and KLF17 have not been classified because their interaction motifs have yet to be determined.[16]

While the zinc-finger domains are highly conserved among the KLFs, the non-DNA-binding regions are highly divergent, and modular activation and repression domains have been suggested to regulate transcriptional activity.[16] KLFs are involved in various biological and pathological mechanisms, including differentiation, apoptosis and tumorigenesis.[16] However, there is limited knowledge about potential roles of KLFs in cartilage and OA pathogenesis.

Here, we analyzed functions of KLF4 and KLF2 in cells from human joint tissues and OA models in vivo and determined mechanisms by which KLF4 and KLF2 protected joint tissues from OA-associated damage.

RESULTS

Expression of KLF family genes in cartilage and regulation of cartilage ECM genes by KLFs

We utilized our previous RNA-seq dataset of human knee cartilage tissues[15], to determine which KLF genes were highly expressed in normal cartilage and differentially expressed between normal and OA cartilage (supplementary figure 1; supplementary table 1). *KLF1*, *KLF14* and *KLF17* were excluded from further analyses because of their low expression levels. KLF genes with log2(counts per million reads mapped [CPM]) >5 in normal cartilage were considered to be expressed. Among those KLFs, *KLF2*, *KLF4*, *KLF9*, *KLF10* and *KLF15* were significantly decreased in OA cartilage, which suggested that these five KLFs might be associated with the compromised maintenance of cartilage homeostasis in OA.

We transduced TC28a2 human chondrocyte cells[17] with the five KLFs (figure 1A; supplementary figure 2). Overexpression of KLF2 and KLF4 upregulated expression of *COL2A1* and *COL11A2*. Notably, these two KLFs also increased expression of *PRG4*. Based on these findings, we focused subsequent studies on KLF4 and KLF2.

Decreased expression of KLF4 and KLF2 in cartilage with OA and aging

Among human primary joint cells and bone marrow-derived mesenchymal stem cells (BMSCs), chondrocytes had the highest expression levels of *KLF4* and *KLF2* (supplementary figure 3). Then, we examined whether there were zone-specific expression patterns of *KLF4* and *KLF2* in articular cartilage, utilizing our DNA microarray dataset for three zones (superficial, middle and deep) of human knee cartilage tissues.[18] Expression levels of *KLF4* and *KLF2* were not significantly different among the zones (supplementary figure 4). Six-month-old mouse knee joints were analyzed with immunohistochemistry (IHC), and there were no differences in positive cell rates of Klf4 and Klf2 between articular cartilage and subchondral bone, and between weight-bearing and non-weight-bearing regions of articular cartilage (supplementary figure 5).

Next, we examined how expression of KLF4 and KLF2 would change with OA and aging in human and mouse cartilage. Positive cell rates of both KLF4 and KLF2 were decreased in human OA cartilage compared to those in normal cartilage (figure 1B and C). IHC of Klf4 and Klf2 was also performed in knee joints from 6- and 24-month-old mice, and mice 4 or 8 weeks after OA induction by surgical destabilization of the medial meniscus (DMM)[19]

(figure 1D and E). Positive cell rates of both Klf4 and Klf2 in 24-month-old mice and in mice after DMM surgery were decreased when compared to 6-month-old control mice. *KLF4* and *KLF2* in human normal chondrocytes were down-regulated by proinflammatory cytokine IL-1β (supplementary figure 6), which is compatible with previous studies.[20 21] Meanwhile, treatment of human OA chondrocytes with TGF-β3 upregulated *KLF4* and *KLF2* (supplementary figure 7). Collectively, these findings indicated OA- and aging-associated suppression of KLF4 and KLF2 in cartilage, and that mediators of joint tissue catabolism and inflammation were potential mechanisms of this suppression.

Klf2 functions in joint homeostasis and OA pathogenesis

When KLF2 and KLF4 were knocked down using small interfering RNAs (siRNAs) in human normal chondrocytes, expression levels of *COL2A1*, *COL11A2*, *PRG4* and *SOX9* were suppressed, while *IL6*, *MMP3*, *PTGS2*, *ADAMTS5* and *MMP13* were upregulated (supplementary figure 8).

We obtained $Klf2^{flox/flox}$ mice[22] and $Aggrecan\text{-}Cre^{ERT2}$ knock-in mice[23], and created $Aggrecan\text{-}Cre^{ERT2};Klf2^{flox/flox}$ (conditional knock-out, cKO) mice to analyze roles of Klf2 in maintenance of mature articular cartilage and in OA pathogenesis. *Klf2* deletion in articular cartilage of skeletally mature mice was confirmed using knee cartilage of 12-week-old cKO mice after tamoxifen injection (figure 2A). We performed DMM surgery to induce OA in cKO and littermate control mice and harvested knee joints 8 weeks after surgery for histological analysis (figure 2B). Severity of OA in cKO mice was significantly higher than in control mice, as shown by the Osteoarthritis Research Society International (OARSI) scores[24] (figure 2C and D). In cKO mice, meniscus histopathological scores[25], synovitis scores[26] and bone scores[9] were also higher than in control mice (figure 2E and F; supplementary figure 9). These results demonstrated that Klf2 deletion in articular cartilage increased severity of OA.

KLF4 and KLF2 regulation of cartilage signature genes

To investigate functions of KLF4 and KLF2 in regulation of joint tissue homeostasis, we used adenovirus (Ad) to overexpress KLF4 and KLF2 in several different cell types. In human OA chondrocytes, Ad-KLF4 increased expression of *COL2A1*, *COL11A2*, *COMP*, *PRG4* and *SOX9* (figure 3A; supplementary figure 10A), and Ad-KLF2 upregulated *COL2A1*, *COL11A2*, *PRG4* and *SOX9* (figure 3B; supplementary figure 10B). Human meniscal cells transfected with Ad-KLF4 showed higher mRNA levels of *COL2A1*, *COL11A2*, *COMP* and *SOX9* (figure 3C; supplementary figure 10C). Transduction of KLF4 also upregulated scleraxis (*SCX*) and tenascin-B (*TNXB*), which are reported to be highly expressed in the meniscus.[27] In Ad-KLF2-transduced meniscal cells, expression of *COL2A1*, *COL11A2*, *SOX9* and *TNXB* was increased (figure 3D; supplementary figure 10D).

We further studied KLF4 and KLF2 in chondrogenesis of human BMSCs. In BMSCs under monolayer culture, Ad-KLF4 increased *COL2A1*, *COL11A2*, *ACAN*, *COMP*, *PRG4*, *SOX5* and *SOX9* (supplementary figure 11), while Ad-KLF2 upregulated expression of *COL2A1*, *COL11A2* and *PRG4* (supplementary figure 12). During 2-week pellet culture of BMSCs,

KLF4 upregulated *COL2A1*, *COL11A2* and *PRG4* (figure 3E; supplementary figure 13A), and transduction of KLF2 increased *COL2A1*, *PRG4* and *SOX5* (figure 3F; supplementary figure 13B). Collectively, KLF4 and KLF2 upregulated various chondrogenic and anabolic genes in human chondrocytes, meniscal cells and BMSCs.

Suppression of inflammatory and catabolic genes by KLF4 and KLF2

We examined regulation of genes related to inflammation and ECM degradation by the KLFs. When OA chondrocytes were treated with IL-1β, Ad-KLF4 down-regulated *IL6*, *MMP3*, *NOS2*, *PTGS2*, *ADAMTS5* and *MMP13*, and Ad-KLF2 decreased expression levels of *IL6*, *MMP3*, *NOS2*, *ADAMTS5* and *MMP13* (figure 4A; supplementary figure 14). In IL-1β stimulated meniscal cells, *IL6*, *MMP3*, *PTGS2*, *ADAMTS5* and *MMP13* were down-regulated by KLF4- or KLF2-transduction (figure 4B; supplementary figure 15). Similarly in synoviocytes, Ad-KLF4 and Ad-KLF2 suppressed expression levels of *IL6*, *MMP3*, *PTGS2*, *ADAMTS5* and *MMP13* under IL-1β stimulation (figure 4C; supplementary figure 16C). These findings suggested that KLF4 and KLF2 suppressed catabolic and inflammatory genes in joint tissue cells.

Therapeutic effects of KLF4 gene delivery using adeno-associated virus

To directly confirm therapeutic effects against OA, mice with DMM or sham surgery received intraarticular injection of adeno-associated virus (AAV) expressing KLF4 (figure 5A; supplementary figure 17A), AAV-EGFP (supplementary figure 17B) or phosphate-buffered saline (PBS) at 2 and 4 weeks after surgery. Knee joints were harvested at 8 weeks postoperatively, as illustrated in figure 5B. We performed von Frey test[28 29] preoperatively, and at 4 and 8 weeks after surgery to evaluate mechanical allodynia (figure 5C; supplementary figures 18 and 19). AAV-KLF4 injected mice with DMM surgery showed significantly decreased numbers of paw withdrawals at 4 and 8 weeks. Histological analyses revealed that AAV-KLF4 injection significantly improved the OARSI scores, meniscus histopathological scores, synovitis scores and bone scores (figure 5D–G; supplementary figure 20). These results clearly demonstrated that KLF4 reduced OA-associated joint damage and mechanical allodynia.

Global analysis of KLF4-regulated genes

To study genes regulated by KLF4 comprehensively and to elucidate regulatory mechanisms of KLF4, we performed global expression profiling by digital RNA with pertUrbation of Genes (DRUG-seq) analysis[30] of Ad-KLF4-, Ad-EGFP- and non-transduced TC28a2 cells with and without IL-1β stimulation (supplementary figure 21). For differential expression analysis, we compared cells that were treated with various combinations of IL-1β, Ad-EGFP and Ad-KLF4. Lists of all significantly upregulated genes (URGs) and down-regulated genes (DRGs) in each comparison are shown in supplementary tables 3–10, where genes with a false discovery rate (FDR) of <0.05 and a |log2(fold change [FC])| >1 were considered to be significantly differentially expressed genes.

Ad-KLF4 significantly upregulated cartilage ECM genes such as *COL2A1*, *COL11A2*, *ACAN*, *COMP* and *PRG4* (Fig. 6A and supplementary table 7). Notably, a number of genes which are anabolic or protective against OA were also significantly upregulated,

including tissue inhibitor of metalloproteinases-1 (*TIMP1*), *TIMP2*, *TIMP3*, cluster of differentiation-24 (*CD24*), *COL6A1*, *COL6A2*, *COL9A2*, *COL9A3*, nuclear factor of activated T cells-1 (*NFATC1*), *NFATC2*, forkhead box O1 (*FOXO1*), *FOXO3* and fibroblast growth factor-18 (*FGF18*)[9 31–38] (supplementary table 7).

In the presence of IL-1β, Ad-KLF4 significantly suppressed *IL6*, *MMP3*, *PTGS2*, *ADAMTS5*, *MMP13*, and also other genes associated with OA pathogenesis, including fibronectin-1 (*FN1*), periostin (*POSTN*), cellular communication network factor-2 (*CCN2*), leukemia inhibitory factor (*LIF*), gremlin-1 (*GREM1*), *SOX4*, IL-1 receptor type 1 (*IL1R1*), *IL1B, IL11*, *IL33*, *ADAMTS1*, *ADAMTS12* and *MMP1*[14 39–49] (figure 6B; supplementary table 10). These findings suggested that KLF4 suppressed key mediators of OA pathogenesis.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses[50] were performed using URGs and DRGs. Significantly enriched pathways are shown in supplementary tables 11–16. In the URGs by KLF4, “ECM-receptor interaction”, where cartilage ECM genes such as *COL2A1*, *COL11A2* and *COMP* are annotated, was significantly enriched (figure 6C; supplementary table 13). “Rap1 signaling pathway” was the top pathway, and several signaling pathways including “Calcium signaling pathway” and “MAPK signaling pathway” were highly ranked, suggesting their regulation by KLF4. Among the DRGs by KLF4 on IL-1β stimulation, “TNF signaling pathway” was the second most enriched pathway (figure 6D; supplementary table 16).

Genome-wide analysis of KLF4-DNA association in KLF4-transduced cells

Chromatin immunoprecipitation sequencing (ChIP-seq) was performed using TC28a2 cells transfected with a vector expressing KLF4 with HA tag (HA-KLF4) or an empty vector (supplementary table 17). Non-specific peaks were removed, and only specific peaks were used for subsequent analyses (supplementary figure 22). In gene ontology analysis, terms related to chondrocyte differentiation were significantly enriched, such as “growth plate cartilage chondrocyte differentiation”, and “regulation of chondrocyte development” (supplementary table 18). Next, *de novo* motif analysis was performed for the obtained ChIP-seq peaks. A KLF motif (figure 7A) was top-ranked among the significantly recovered motifs (supplementary figure 23), and this motif was highly enriched at the predicted centers of the peaks (figure 7B). These findings supported direct binding of KLF4 to DNA as well as integrity of the ChIP-seq dataset. Then, we examined peak signals of the ChIP-seq dataset around cartilage signature genes, as well as the top four URGs by KLF4 in the DRUG-seq analysis (supplementary table 7). Large numbers of peaks were detected in the putative regulatory regions of *SOX9*, *COL2A1*, *COL11A2*, *PRG4*, *ACAN* and *COMP*, and most of these peak regions contained the recovered KLF motif, while similar findings were found for the top URGs (figure 7C and D; supplementary figures 24–31). These data suggested that KLF4 directly bound to DNA around major chondrogenic genes, indicating that KLF4 was an important regulator of chondrogenesis.

Regulation of chondrogenesis by KLFs via PKA-RAP1-MEK-CREB signaling axis

In addition to the function of KLF4 as a transcription factor to directly upregulate the cartilage signature genes, we focused on relation between Ras-related protein-1 (RAP1) signaling and KLFs, since “Rap1 signaling pathway” was the top-ranked enriched pathway in the KEGG pathway analysis (figure 6C; supplementary tables 13 and 15). It is reported that activation of RAP1 promotes chondrogenesis.[51] While KLF4 and KLF2 did not increase expression levels of *RAP1A* and *RAP1B* in OA chondrocytes (supplementary figure 32) or in TC28a2 cells (supplementary table 19), KLF4 directly interacts with RAP1 protein.[52] Our results showed that treatment of KLF4- and KLF2-transfected TC28a2 cells with Rap1 inhibitor GGTI-298 diminished or eliminated upregulation of cartilage ECM genes, *COL2A1*, *COL11A2*, *ACAN*, *COMP* and *PRG4* (figure 8A; supplementary figure 33).

RAP1 is a small GTPase[53] and a member of the PKA (protein kinase A)-RAP1-MEK (mitogen-activated protein kinase kinase)-CREB (cyclic-AMP [cAMP] response element-binding protein) signaling axis.[54–56] Fluid flow shear stress induces expression of Prg4 in articular cartilage via activation of this signaling pathway[11], and treatment of TC28a2 cells with the cAMP inducer forskolin increased expression levels of cartilage ECM genes (supplementary figure 34). We thus hypothesized that KLF4 and KLF2 might upregulate cartilage ECM genes via the PKA-RAP1-MEK-CREB signaling axis. To test this hypothesis, we treated KLF4- and KLF2-transfected TC28a2 cells with inhibitors of several members in the signaling axis; PKA inhibitor H89, MEK inhibitor U0126, and CREB inhibitor 666–15. Upregulation of the cartilage ECM genes was attenuated or cancelled by each of these inhibitors (figure 8B–D; supplementary figure 35). Collectively, these findings demonstrated that KLF4 and KLF2 upregulated major cartilage ECM genes, not only by direct regulation as a transcription factor but also via the PKA-RAP1-MEK-CREB signaling axis (supplementary figure 36).

DISCUSSION

This is the first detailed analysis of KLFs in the context of cartilage biology, joint homeostasis and OA pathogenesis. We focused on KLF4 and KLF2 as they were the strongest inducers of cartilage collagen genes and *PRG4*, and as their expression was suppressed in OA cartilage. KLF4 and KLF2 are closely related members within the KLF family.[16 57]

KLF4 and KLF2 down-regulated mediators of inflammation and ECM-degrading enzymes in human joint tissue cells, which are compatible with previous studies showing that they suppressed NF-κB activity.[58 59] Previous reports show KLF4 binding to the promoter proximal region of *COL2A1* gene[60] and KLF4 colocalization and interaction with SOX9 in chondrocytes.[61] The present study suggested that KLF4 and KLF2 were important regulators of chondrogenesis in joint tissue cells because they upregulated cartilage signature genes with different regulatory mechanisms; *SOX9*, a master regulator of chondrogenesis, SOX9-regulated genes such as *COL2A1*, *COL11A2*, *ACAN* and *COMP*[3–7], and *PRG4* which is not subject to regulation by SOX9.[5 12] ChIP-seq analysis supported the model of KLF4 as a central transcription factor of chondrogenesis. KLF4 bound to large numbers of the putative regulatory regions of major chondrogenic genes, and many of the bound

sites included the KLF motif. All these findings indicated direct regulation of the cartilage signature genes by KLF4. We also revealed a novel link of KLF4 with RAP1 signaling. The PKA-RAP1-MEK-CREB signaling axis is involved in modulation of various types of genes[54–56], and Prg4 expression in articular cartilage is induced by fluid flow shear stress via this signaling pathway.[11] Our results showed that the KLF4- or KLF2-effects on cartilage ECM genes were dependent on each molecule in the PKA-RAP1-MEK-CREB axis.

Because KLF4 upregulated more chondrogenic and anabolic genes, and down-regulated more inflammatory and catabolic genes than KLF2 in our in vitro experiments, we assumed KLF4 to be more promising as a therapeutic target for OA. Intraarticular Ad-KLF2 had previously shown to protect against cartilage damage induced by monoiodoacetate.[21] Therefore, we tested intraarticular injection of AAV-KLF4 in the mouse DMM model. AAV-KLF4 improved pain behaviors, and reduced severity of OA histopathological changes in cartilage, meniscus and synovium. These results indicated that KLF4 had therapeutic and protective effects against OA-associated tissue damage and pain.

Several limitations should be noted in the present study. First, all mouse experiments were done with only male animals, and sex differences were not addressed. Second, aging-associated spontaneous OA model was not performed in our study. Third, only experiments using *Aggrecan-Cre^{ERT2};Klf2$^{flox/flox}$* mice were performed, because *Klf4* deletion in articular cartilage of *Aggrecan-Cre^{ERT2};Klf4$^{flox/flox}$* mice was not sufficient. Finally, because KLF4-overexpressed cells were used in our ChIP-seq analysis, more sites could be detected as peaks than in ChIP-seq of endogenous KLF4.

In conclusion, this study identifies KLF4 and KLF2 as new transcription factors that are important regulators of the chondrocyte phenotype, and that also have effects on other joint tissues by upregulating ECM components and by suppressing ECM-degrading enzymes and inflammatory mediators. KLF4 and KLF2 thus have a spectrum of biological activities with promise in treatment of joint diseases such as OA.

MATERIALS AND METHODS

Experimental procedures are provided in the supplementary materials and methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement:

The GEO accession numbers for all the datasets utilized in the present study are available in the supplementary materials and methods.

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Key messages:

What is already known about this subject?

- We previously performed RNA-seq analysis of normal and OA human knee cartilage and found that several members in the Krüppel-like factor (KLF) family of transcription factors are suppressed in OA.

What does this study add?

- The suppression of KLF4 and KLF2 seen in OA does have functional consequences for the phenotype of cells in joint tissues, leading to increased OA severity.
- KLF4 and KLF2 upregulate major cartilage extracellular matrix and chondrogenic genes, and they also suppress mediators of inflammation and ECM-degrading enzymes.
- Delivery of KLF4 gene into mouse knee joints reduces severity of experimental OA-associated changes in cartilage, meniscus and synovium and improves pain behaviors.

How might this impact on clinical practice or future developments?

- KLF4 and KLF2 are central transcription factors regulating protective functions in joints, and are promising therapeutic targets for joint diseases such as OA.

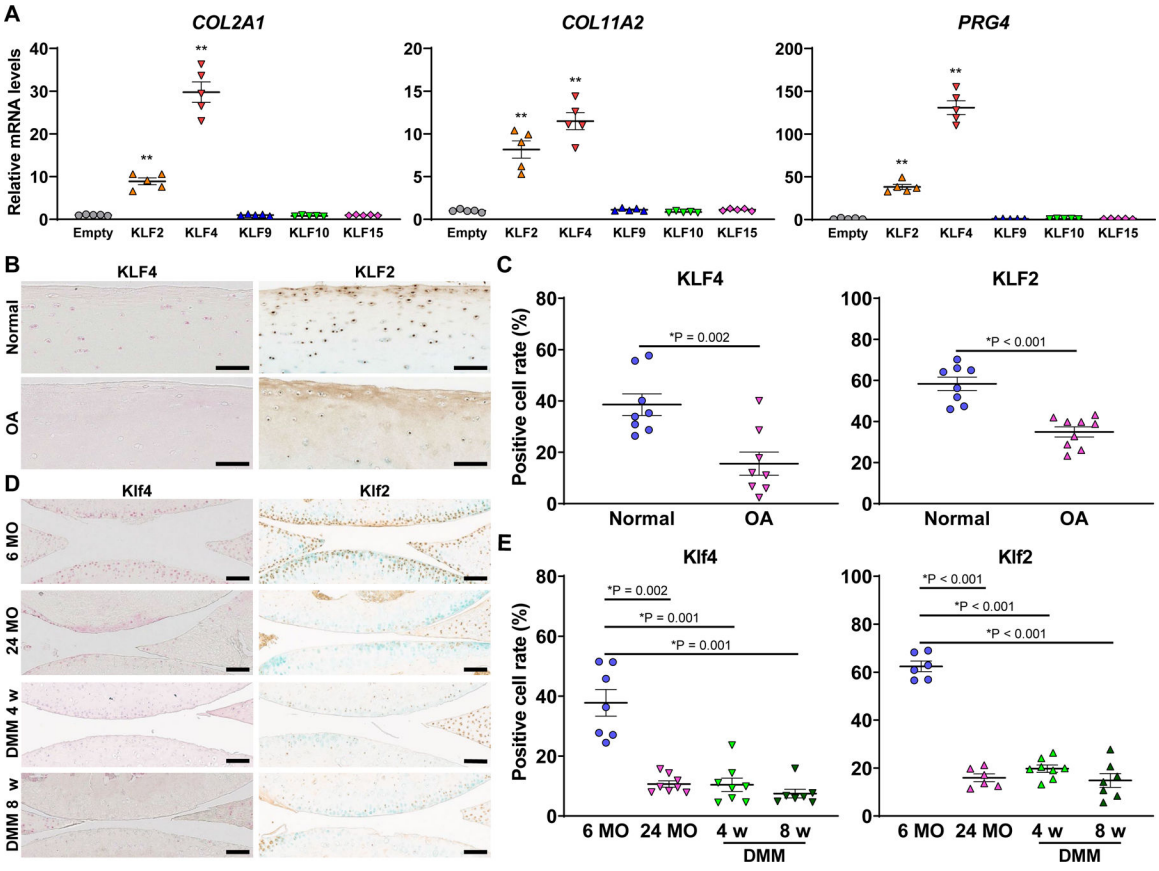

Figure 1. Regulation of cartilage collagen genes and *PRG4* by KLFs, and expression of KLF4 and KLF2 in human and mouse cartilage tissues.

(**A**) TC28a2 cells were transfected with an empty vector, or expression vectors for either KLF2, KLF4, KLF9, KLF10 or KLF15, and RNA was collected 48 hours after transfection (n=5 from five independent experiments). mRNA levels relative to empty vector are shown. *P<0.05, **P<0.01 versus empty vector, Dunnett's test. (**B** and **C**) Immunohistochemistry (IHC) for KLF4 and KLF2 in human knee cartilage. For KLF4, normal cartilage samples were from 8 donors (5 males and 3 females; age 22–48, mean 33±3), while osteoarthritis (OA)-affected cartilage samples were from 8 donors (4 males and 4 females; age 29–90, mean 65±7). For KLF2, normal cartilage samples were from 8 donors (5 males and 3 females; age 18–53, mean 38 ± 4), while OA-affected cartilages were from 9 donors (3 males and 6 females; age 51–90, mean 70±5). *P<0.05, Welch's t-test in (C). (**D** and **E**) IHC for Klf4 and Klf2 in mouse knee cartilage (n=6–8 per condition). While 6 MO samples were from knees of 6-month-old mice, 24 MO indicates samples from knees of 24-month-old mice. DMM 4 w and DMM 8 w samples were from knees of 6-month-old mice 4 and 8 weeks after destabilization of the medial meniscus (DMM) surgery. *P<0.05 versus 6 MO, Dunnett's T3 test in (E). All quantitative data are expressed as means±SE, and results of omnibus tests for multiple comparisons are shown in supplementary table 2. Scale bars, 100 μm.

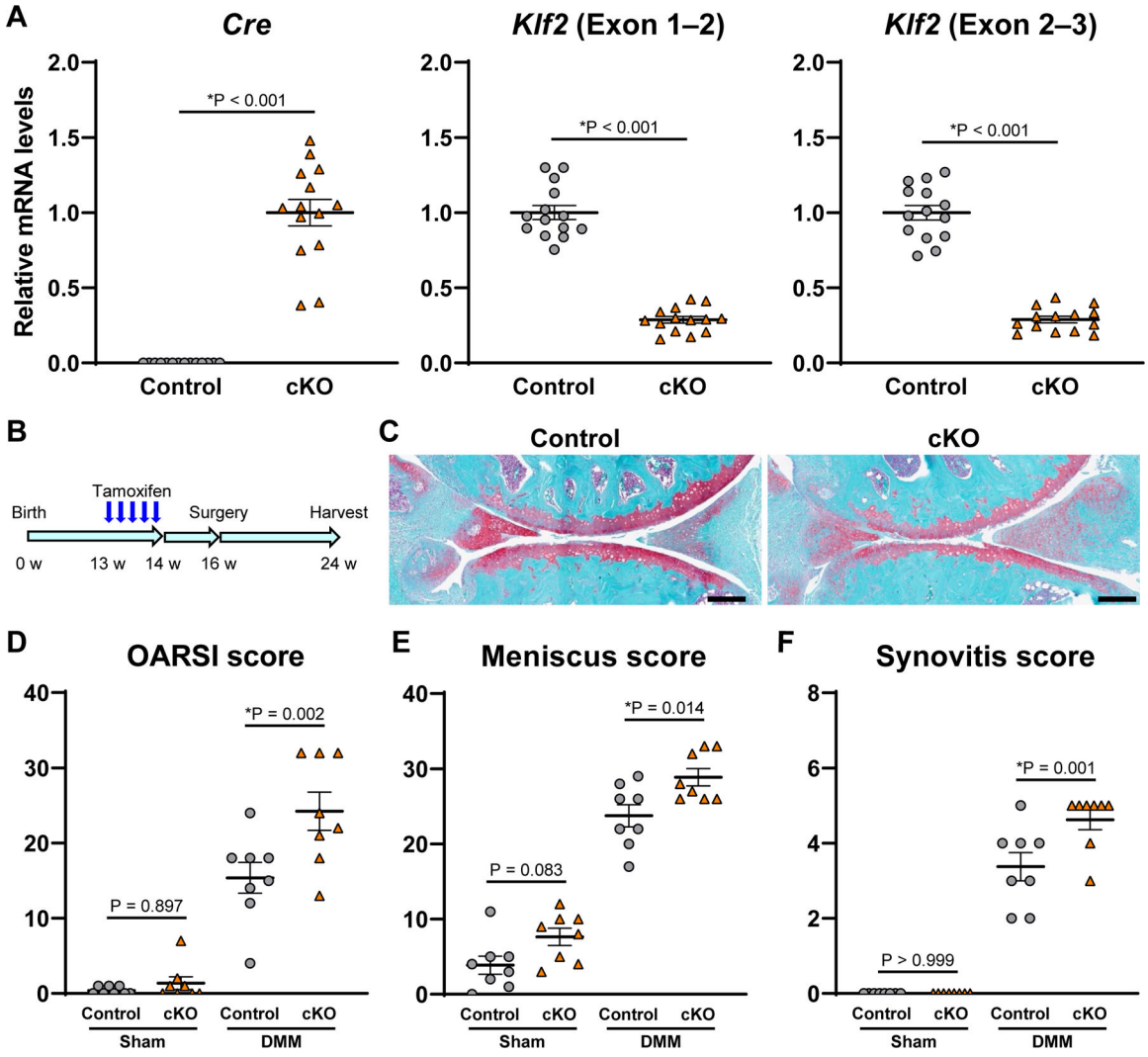

Figure 2. Postnatal deletion of Klf2 in cartilage using *Aggrecan-CreERT2* mice.

(**A**) RNA was collected from knee cartilage of 12-week-old *Aggrecan-Cre^{ERT2};Klf2$^{flox/flox}$* (cKO) and littermate control mice 2 weeks after tamoxifen injection (n=14 per group). mRNA levels are relative to cKO for *Cre*, and relative to control for *Klf2*. *P<0.05, unpaired Welch's t-test. (**B**) Sixteen-week-old cKO and littermate control mice underwent DMM or sham surgery 2 weeks after tamoxifen injection. The knees were harvested at 8 weeks postoperatively for histological analysis (n=8 per group). (**C**) Representative Safranin-O staining images of control and cKO mice with DMM surgery. Scale bars, 200 μm. (**D**) Summed Osteoarthritis Research Society International (OARSI) scores for the medial femoral condyle and tibial plateau. (**E**) Meniscus histopathological scores. (**F**) Synovitis scores. For (D–F), *P<0.05, Sidak's multiple comparison test. All quantitative data are expressed as means±SE, and results of two-way ANOVA test are shown in supplementary table 2.

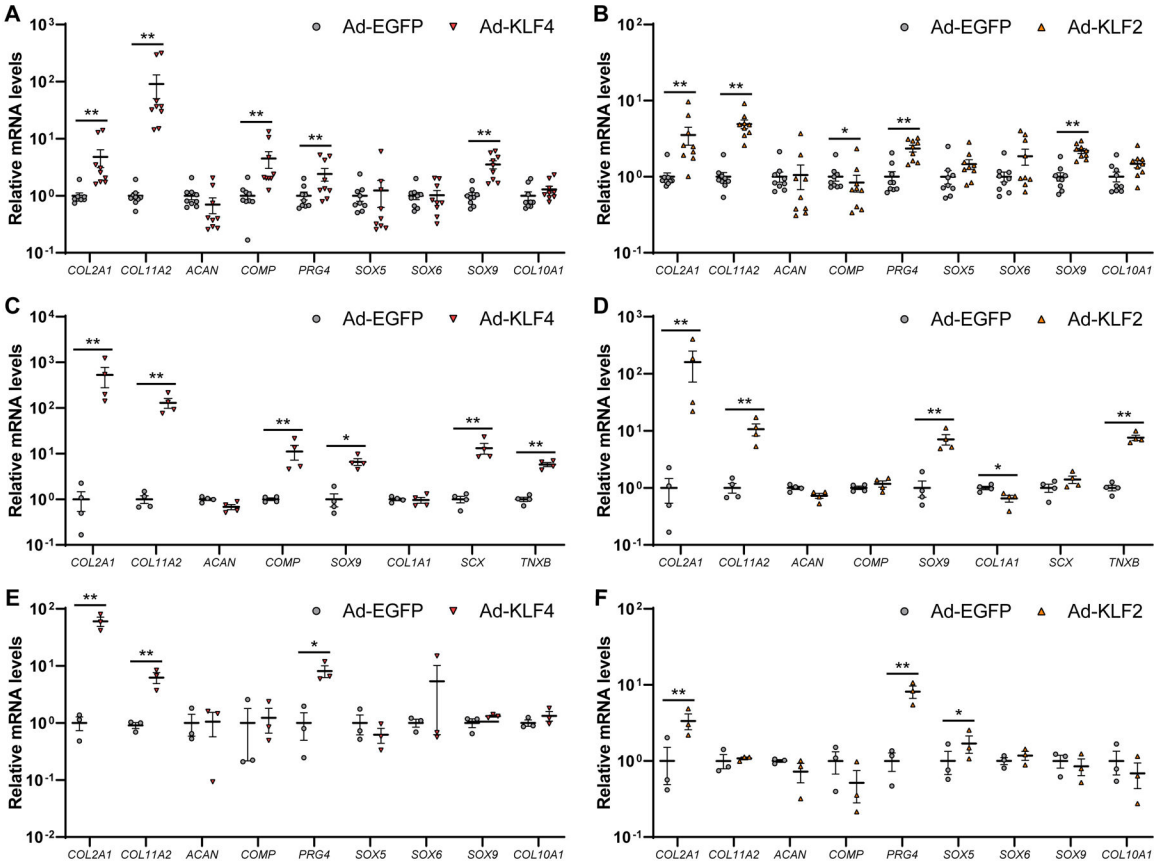

Figure 3. Regulation of chondrogenic and anabolic genes by KLF4 and KLF2 in human OA chondrocytes, meniscal cells and BMSC pellets.

(**A** and **B**) Human OA chondrocytes were transfected with adenovirus (Ad-KLF4 or Ad-EGFP for (A), and Ad-KLF2 or Ad-EGFP for (B)), and RNA was collected 48 hours after transfection (n=9 donors). (**C** and **D**) Human meniscal cells were transfected with adenovirus (Ad-KLF4 or Ad-EGFP for (C), and Ad-KLF2 or Ad-EGFP for (D)), and RNA was collected 48 hours after transfection (n=4 donors). (**E** and **F**) Human bone marrow-derived mesenchymal stem cells (BMSCs) were transfected with adenovirus (Ad-KLF4 or Ad-EGFP for (E), and Ad-KLF2 or Ad-EGFP for (F)), and were cultured in pellets. RNA was collected 2 weeks after pellet culture (n=3 donors). mRNA levels are expressed as means±SE, relative to Ad-EGFP. *P<0.05, **P<0.01, paired t-test.

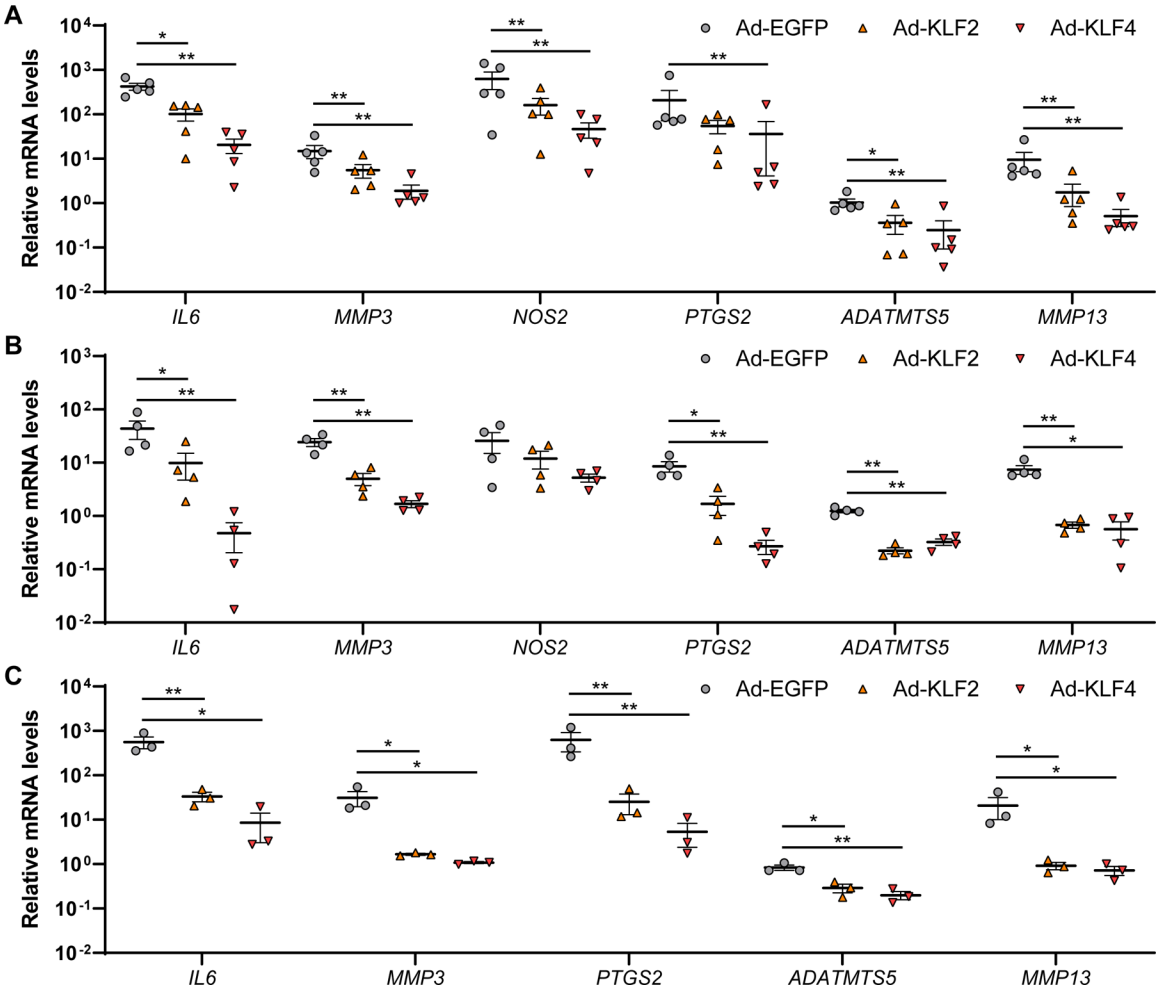

Figure 4. Regulation of inflammatory and catabolic genes by KLF4 and KLF2 in human OA chondrocytes, meniscal cells and synoviocytes on IL-1β stimulation.

Cells were transfected with Ad-EGFP, Ad-KLF2 or Ad-KLF4, and RNA was collected 48 hours after adenoviral transfection and 6 hours after treatment with 10 ng/ml of interleukin-1β (IL-1β). (**A**) n=5 donors were used for human OA chondrocytes. (**B**) n=4 donors were used for human meniscal cells. (**C**) n=3 donors were used for human synoviocytes. mRNA levels are expressed as means±SE, relative to Ad-EGFP without IL-1β as controls. *P<0.05, **P<0.01 versus Ad-EGFP, Dunnett's test. Results of one-way mixed-effects ANOVA test are shown in supplementary table 2.

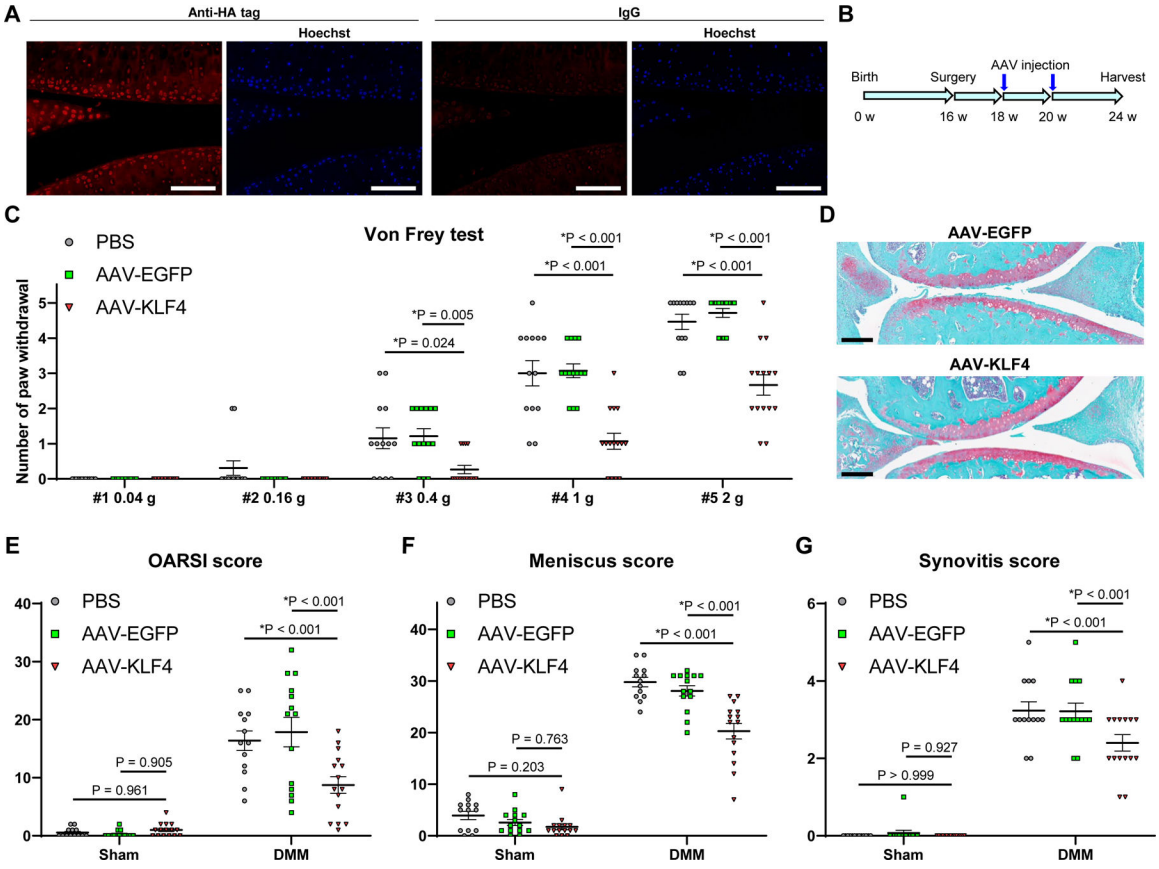

Figure 5. Therapeutic effects of OA by KLF4 gene delivery.

(**A**) Immunofluorescence of HA tag with nuclear staining by Hoechst 33342 in 12-week-old mouse knee joints one week after injection of adeno-associated virus (AAV) expressing KLF4 and HA tag. Representative images from n=6 are shown. Scale bars, 100 μm. (**B**) Sixteen-week-old mice underwent DMM or sham surgery, and AAV-KLF4 (n=15), AAV-EGFP (n=14) or PBS (n=13) was injected into knee joints at 2 and 4 weeks after surgery. Knees were harvested at 8 weeks postoperatively for histological analysis. (**C**) Von Frey test in mice at 8 weeks after DMM surgery. Numbers of paw withdrawals from 5 stimuli per filament per mouse are shown. *P<0.05 versus AAV-KLF4, Dunn's test. (**D**) Representative Safranin-O staining images of AAV-EGFP- and AAV-KLF4-injected mice with DMM surgery. Scale bars, 200 μm. (**E**) Summed OARSI scores for the medial femoral condyle and tibial plateau. (**F**) Meniscus histopathological scores. (**G**) Synovitis scores. For (E–G), *P<0.05 versus AAV-KLF4, Dunnett's test. All quantitative data are expressed as means±SE, and results of omnibus tests for multiple comparisons are shown in supplementary table 2.

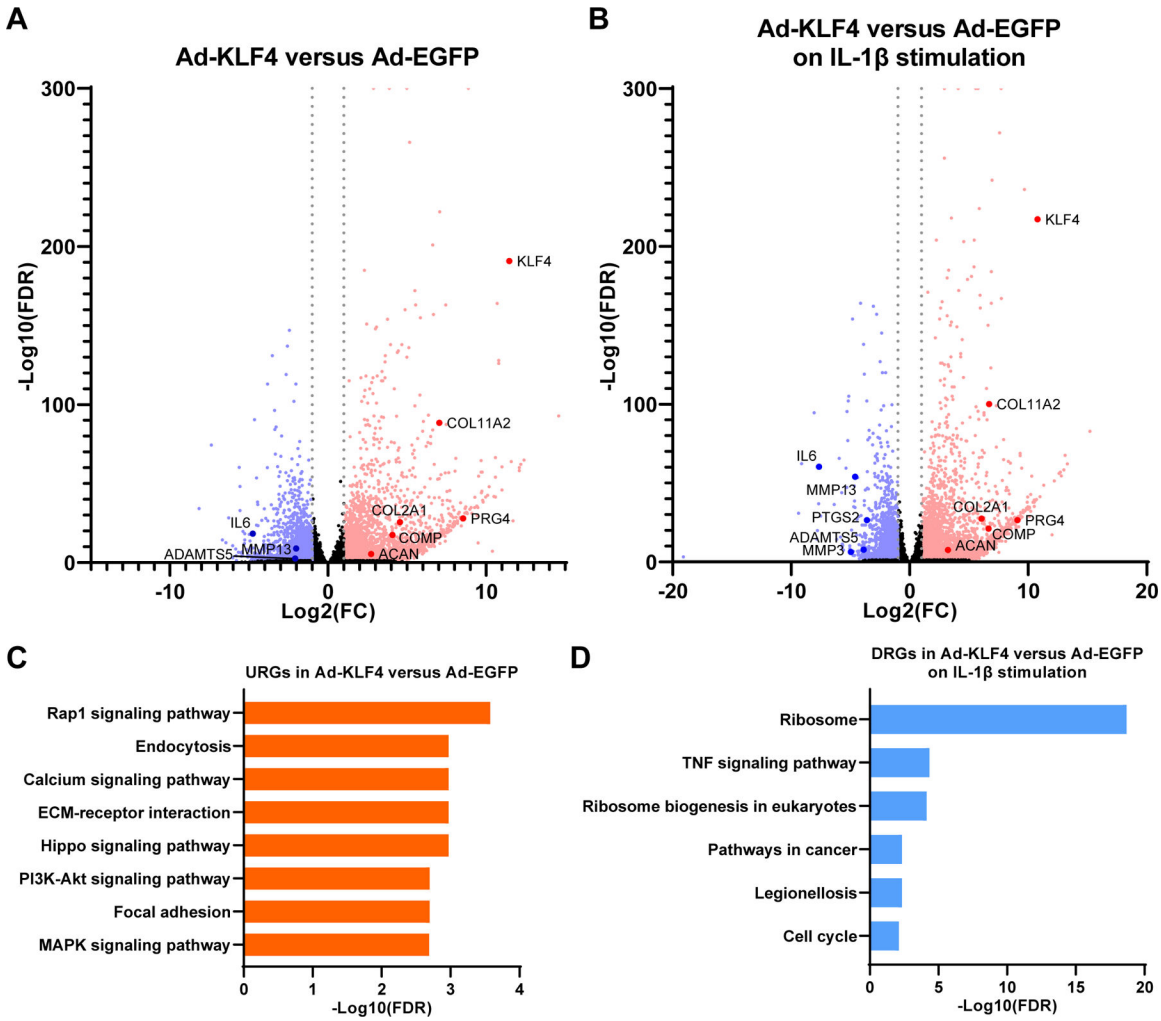

Figure 6. Digital RNA with pertUrbation of Genes (DRUG-seq) analysis of Ad-EGFP or Ad-KLF4 transfected TC28a2 cells.

In samples with IL-1β stimulation, cells were treated with 10 ng/ml of IL-1β for 6 hours before RNA collection. n=7 per condition from seven independent experiments were analyzed. (**A**) Volcano plot analysis to identify differentially expressed genes (DEGs) in Ad-KLF4 versus Ad-EGFP transfected samples. (**B**) Volcano plot analysis to identify DEGs in Ad-KLF4 versus Ad-EGFP transfected samples on IL-1β stimulation. For (A and B), grey dotted lines indicate |log2(fold change [FC])|=1. Significantly upregulated genes (URGs) are shown as red dots, while significantly down-regulated genes (DRGs) are indicated as blue dots; black dots represent non-significant DEGs. (**C**) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for URGs in Ad-KLF4 versus Ad-EGFP transfected samples. The top eight enriched pathways are shown. (**D**) KEGG pathway analysis for DRGs in Ad-KLF4 versus Ad-EGFP transfected samples on IL-1β stimulation. All significantly enriched pathways are shown. FDR, false discovery rate.

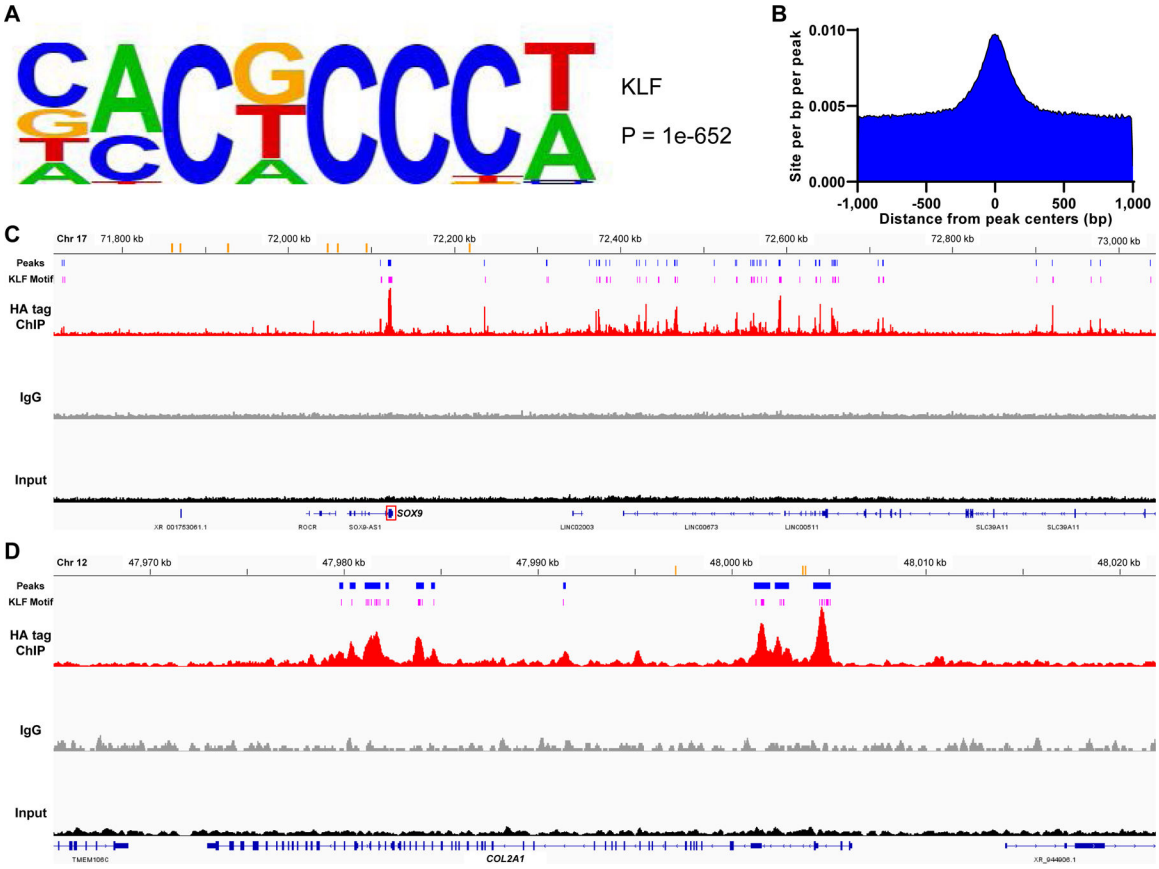

Figure 7. Chromatin immunoprecipitation sequencing (ChIP-seq) for genome-wide analysis of KLF4-DNA association in KLF4 overexpressed TC28a2 cells.

(**A**) A KLF motif recovered from ChIP-seq peaks. The motif logo displays nucleotide frequencies (scaled relative to the information content) at each position. (**B**) Distribution of the KLF motif within a 1,000 base pair (bp) window from the peak centers with a 10 bp step size. (**C** and **D**) Visualization of aligned reads for HA tag ChIP-seq, IgG and input samples around the *SOX9* gene (C) and around the *COL2A1* gene (D). Orange bars indicate previously identified enhancers for each gene. Blue bars indicate peak regions that were putative regulatory domains of *SOX9* or *COL2A1* and common between the replicates of ChIP samples. Pink bars show the predicted sites of the recovered KLF motif which were located within the peak regions. Chr, chromosome. kb, kilobase.

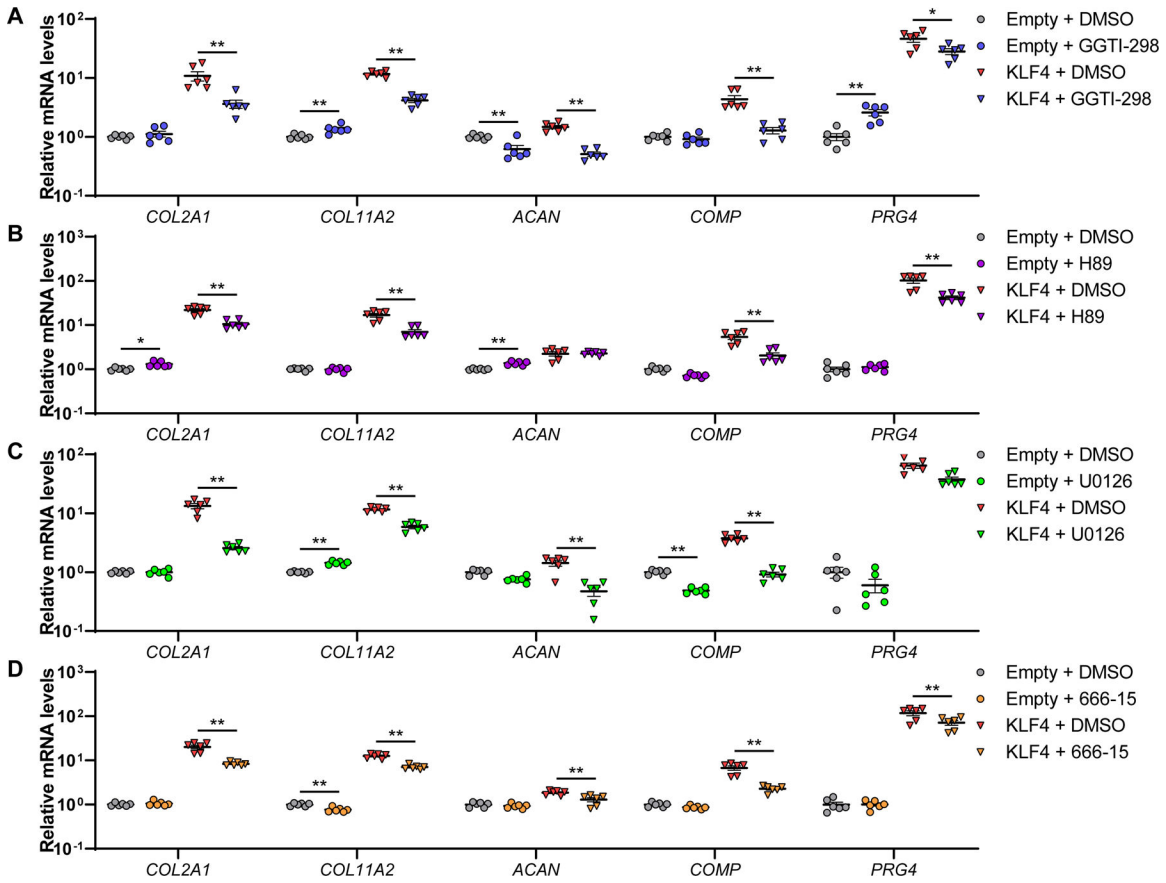

Figure 8. KLF4 regulation of cartilage ECM genes through the RAP1-related signaling axis.

TC28a2 cells were transfected with an expression vector encoding KLF4 or an empty vector. Then, cells were treated with GGTI-298 (**A**), H89 (**B**), U0126 (**C**) or 666–15 (**D**), or dimethyl sulfoxide (DMSO). RNA was collected 48 hours after transfection (n=6 from six independent experiments). mRNA levels are expressed as means±SE, relative to empty vector-transfected samples with DMSO as controls. *P<0.05, **P<0.01, Sidak's multiple comparison test. Results of two-way mixed-effects ANOVA test are shown in supplementary table 2.