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Original Article

RPL35 downregulated by mechanical overloading promotes chondrocyte senescence and osteoarthritis development via Hedgehog-Gli1 signaling

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ABSTRACT

Objectives: To investigate the potential role of Ribosomal protein L35 (RPL35) in regulating chondrocyte catabolic metabolism and to examine whether osteoarthritis (OA) progression can be delayed by overexpressing RPL35 in a mouse compression loading model.

Methods: RNA sequencing analysis was performed on chondrocytes treated with or without 20 % elongation strain loading for 24 h. Experimental OA in mice was induced by destabilization of the medial meniscus and compression loading. Mice were randomly assigned to a sham group, an intra-articular adenovirus-mediated overexpression of the negative group, and an intra-articular adenovirus-mediated overexpression of the RPL35 operated group. The Osteoarthritis Research Society International score was used to evaluate cartilage degeneration. Immunostaining and western blot analyses were conducted to detect relative protein levels. Primary mouse chondrocytes were treated with 20 % elongation strain loading for 24 h to investigate the role of RPL35 in modulating chondrocyte catabolic metabolism and regulating cellular senescence in chondrocytes.

Results: The protein expression of RPL35 in mouse chondrocytes was significantly reduced when excessive mechanical loading was applied, while elevated protein levels of RPL35 protected articular chondrocytes from degeneration. In addition, the RPL35 knockdown alone induced chondrocyte senescence, decreased the expression of anabolic markers, and increased the expression of catabolic markers *in vitro* in part through the hedgehog (Hh) pathway.

Conclusions: These findings demonstrated a functional pathway important for OA development and identified intra-articular injection of RPL35 as a potential therapy for OA prevention and treatment.

The translational potential of this article: It is necessary to develop new targeted drugs for OA due to the limitations of conventional pharmacotherapy. Our study explores and demonstrates the protective effect of RPL35 against excessive mechanical stress in OA models in vivo and in vitro in animals. These findings might provide novel insights into OA pathogenesis and show its translational potential for OA therapy.

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1. Introduction

Osteoarthritis (OA) is the most common chronic joint disease in the elderly [1]. Knee OA prevalence is expected to increase globally due to the growing aging population and obesity [2]. OA is characterized by degenerative changes in articular cartilage and loss of cartilage matrix in the affected joints, leading to chronic pain, motion limitation, and ultimately reduced quality of life [3,4]. Articular cartilage is mainly composed of chondrocytes and extracellular matrix (ECM), such as collagens and proteoglycans, including aggrecan [5,6]. The cartilage matrix degeneration during OA development eventually leads to the failure of the articular cartilage-bearing function [7]. Degradation of articular cartilage is caused by major matrix-degrading enzymes, such as members of the disintegrin and metalloproteinase (MMP) with thrombospondin motifs family and MMPs [8]. Cellular senescence is another factor that correlates closely with ECM degradation and chondrocyte dysfunction [9]. Chondrocytes may de-differentiate, lose their functional phenotype, and advance toward senescence during pathological states [10,11]. Senescent cells undergo morphological changes, including flattening, vacuolization, and stress granule accumulation. Senescent cells produce a bioactive senescence-associated secretory phenotype caused by growth arrest, resistance to cell death, and altered gene expression [12–14]. During culture, chondrocyte senescence and dedifferentiation are accompanied by decreased expression of chondrocyte-specific proteins, such as type II collagen and glycoproteins [15].

Chondrocytes maintain cartilage morphology and are mechanically sensitive. Mechanical loading within the physiological range is necessary for preserving chondrocyte homeostasis [16,17]. However, abnormal loading caused by obesity, joint instability, overuse, or trauma can lead to cartilage degeneration and is a significant risk factor associated with the development of OA. Single shock and cyclic joint loading leads to increased catabolism, chondrocyte necrosis and apoptosis, and destruction of the collagen network in a dose-dependent manner [18].

Ribosomal proteins play pivotal roles in organismal growth and development, as well as in the regulation of cellular homeostasis. Previous studies have shown that lncNB1 and its binding ribosomal protein L35 (RPL35) induce the proliferation and survival of neuroblastoma cells, while high levels of RPL35 correlate with tumor prognosis [19]. In addition, RPL35 can interact with eEF2 to regulate CSN2 synthesis translation elongation [20]. It is known that RPL35 plays an essential role in protein synthesis as a small subunit of ribosome 60s. However, ribosomal proteins have other extra-ribosomal functions besides constituting ribosomes and participating in the basic process of translation [21]. RPL35 promotes aerobic glycolysis through HIF1α upregulation via the RPL35/ERK/HIF1α axis [22]. The present study elaborated on the relationship between mechanical stress and cellular senescence. Further, it was determined that a reduction in RPL35 protein expression serves as a pivotal mediator in chondrocyte senescence promoted by excessive mechanical stress. Specifically, RPL35 protein expression in mouse chondrocytes was significantly reduced under excessive mechanical loading, while elevated protein levels of RPL35 protected articular chondrocytes from degeneration. Moreover, the knockdown of RPL35 alone induced chondrocyte senescence, decreased the expression of anabolic markers, and increased the expression of catabolic markers *in vitro*, partly through the Hedgehog (Hh) pathway. Hh signaling modulates the growth and differentiation of normal chondrocytes during development and postnatally [23]. However, elevated signaling from Indian hedgehog and Sonic hedgehog has been associated with OA phenotypes, characterized by reduced articular cartilage thickness, proteoglycan loss, and upregulated MMP13 expression. Inhibition of the Hh signaling pathway has been suggested to mitigate the severity of OA [24,25]. Our current study indicates that exogenous RPL35 supplementation can mitigate Hh pathway activation induced by stress loading. This suggests the potential of intra-articular injections of RPL35 as a therapeutic approach for OA.

2. Methods

2.1. Primary chondrocyte culture and treatment

Primary chondrocytes from the knee joint were isolated from newborn offspring of C57 mice (*Mus musculus*) obtained from the Laboratory Animal Center of Southern Medical University. These primary chondrocytes were subsequently cultured in a maintenance medium consisting of DMEM/F12 (Procell Life Science & Technology Co., Ltd, Wuhan, China) as previously described [26]. The primary chondrocytes were treated with 5 or 10 ng/mL interleukin-1 (IL-1β) (Thermo Fisher Scientific, USA) for 24 h. Primary mouse chondrocytes prepared from ventral rib cages were planted into silicon stretch chambers. After 48 h, 5 %, 10 %, or 20 % cyclic tensile stress at 0.5 Hz was applied for 24 h using a FLEXCELL-5000 mechanical stretch system in a CO_2 incubator. Control cells were cultured in the same plate but were not subjected to stress stimulation. Primary chondrocytes were transfected with siRNA from RIBOBIO (Guangzhou, China) targeting the gene encoding ribosomal protein L35 (RPL35) and casein kinase 1 epsilon (CK1ε) using Lipofectamine 3000 (Invitrogen, USA). The medium was replaced 6 h after transfection. Primary chondrocytes were treated with RPL35 siRNA and 10 μM hedgehog pathway inhibitor cyclopamine (MCE, Monmouth Junction, NJ, USA). Overexpression vector CK1ε (CK1ε plasmid) and the controls were purchased from GeneChem (Shanghai, China). The CK1ε plasmid was transfected into chondrocytes for 48 h following the manufacturer's guidelines.

2.2. RNA isolation, cDNA synthesis, and quantitative PCR (qPCR) analysis

Cells at 90 % confluence in six-well plates were treated with TRIzol reagent (Life Technologies) to isolate total RNA. After obtaining RNA from different samples, RNA purity and concentration were determined using a spectrophotometer. Diethyl pyrocarbonate water was added to adjust the RNA concentration to approximately 300 ng/μL. Subsequent experiments were carried out using RNA samples with optical density values falling within the range of 1.8–2.0. To quantify the mRNA expression levels of RPL35, P16, P21, Col2, MMP13, Gli-1, and SMO, genomic DNA removal and reverse transcription (Vazyme) were performed on the total RNA samples. Based on the aforementioned RNA concentration, we utilized 3.3 μL of the RNA solution for each sample, ensuring an approximate total RNA input of 1000 ng, to perform reverse transcription for cDNA synthesis. Next, qPCR was conducted using 2 × ChamQ SYBR qPCR Master Mix with 2 μL of cDNA sample and 0.4 μL of both upstream and downstream primers. A Roche real-time fluorescence amplification instrument was employed following a specified protocol, with GAPDH serving as the reference gene for relative quantification analysis. The primer sequences were as follows:

RPL35 forward 5′- AGCTGTTGAAACAACTGGACG-3′;
reverse 5′- TTGCGAACGACTCGTATCTTG-3′.
SMO forward 5′- CCCTGCTGTGTGCTGTCTAC-3′.
reverse 5′- GTGTGCAACGCAGAAAGTCAG-3′.
Csnk1e forward 5′- CGCATCGAGTACATACACTCC-3′
reverse 5′- TGCCGGTCAGGTTCTTGTTTT -3′;
Arrb2 forward 5′- GGCAAGCGCGACTTTGTAG-3′;
reverse 5′- GTGAGGGTCACGAACACTTTC -3′;
P16 forward 5′- ACATCAAGACATCGTGCGATATT-3′;
reverse 5′- CCAGCGGTACACAAAGACCA-3′;
P21 forward 5′- CCTGGTGATGTCCGACCTG -3′;
reverse 5′- CCATGAGCGCATCGCAATC -3′;
Col2a1 forward 5′- GGGAATGTCCTCTGCGATGAC-3′;
reverse 5′- GAAGGGGATCTCGGGGTTG-3′;
MMP-13 forward 5′- CTATCCCTTGATGCCATTACCAG -3′;
reverse 5′- ATCCACATGGTTGGGAAGTTC -3′;
GAPDH forward 5′- AAATGGTGAAGGTCGGTGTGAAC-3′;
reverse 5′- CAACAATCTCCACTTTGCCACTG-3′.

2.3. Western blotting

After treatment, protease and phosphatase inhibitors were added to the cells, which were then lysed immediately for 5 min with radioimmunoprecipitation assay buffer. Proteins extracted from cell lysates were separated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and transferred to nitrocellulose membranes (Beyotime) according to the manufacturer's instructions. After blocking in 5 % milk, the membrane was probed with primary antibodies against MMP13, Col2 (Abcam, Cambridge, UK), P16, P21 (Proteintech Group, Inc., USA), Gli-1, SMO (Proteintech, Rosemont, IL, USA), and PTCH1, Csnk1e (Abclonal, Woburn, MA, USA) overnight and incubated with species-matched secondary antibody coupled with Horseradish Peroxidase. Western blotting (WB) experimental results were normalized to the GAPDH control and the resulting values were plotted.

2.4. Human knee cartilage samples

The present study was approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University. Five tibial plateaus were obtained from patients with osteoarthritis (OA) who underwent total knee arthroplasty. The medial portion of each tibial plateau served as the OA group, whereas the lateral tibial plateau with less cartilage degeneration served as the control group. Human cartilage was stained using Safranin O/Fast Green and immunohistochemistry.

2.5. Mice knee joint mechanical loading and OA model

All C57BL/6J mice (10–12-week-old, male) were purchased from the Laboratory Animal Center of Southern Medical University (Guangzhou, China). At the time of the experiments, the average mouse weight was 20.1 ± 0.9 g. Mice were anesthetized using 10 % chloral hydrate (0.1 mL/100 g). To achieve mechanical overload-induced OA, right knee joints (n = 5) were subjected to 60 cycles of 4-, 9-, and 13.5-N axial compressive loads as previously described [27]. Sham-operated mice (n = 5) were placed within the test frame and their knees were subjected to a static axial compressive load of 0.5 N for 10 min. Destabilization of the medial meniscus (DMM) surgery (n = 5) was then performed to induce OA. The protocol for DMM surgery has been published previously [28]. The mice were euthanized eight weeks post-surgery and knee specimens were collected. The Southern Medical University Animal Care and Use Committee approved all procedures involving mice.

2.5. Intra-articular administration of RPL35 overexpression adenovirus or RPL35 siRNA treatment

Adenovirus-mediated overexpression of RPL35 (Ad-RPL35), sourced from HanBio (Shanghai, China), was intra-articularly injected into C57BL/6J mice (n = 5). This injection targeted osteoarthritis induced by mechanical overload, with a regimen of once per week for 4–6 weeks. Concurrently, a dose of 5 nmol of RPL35 siRNA from RIBOBIO (Guangzhou, China) for animal use was administered twice a week to the knees of C57BL/6J mice (n = 5) as well as those with osteoarthritis caused by excessive mechanical stress (n = 5). Upon completion of the treatment, mice were euthanized for histological analyses. The control groups were treated with negative control (n = 5) for the same periods.

2.6. Histological analysis

All mice were sacrificed eight weeks after DMM surgery and two, four, and six weeks after mechanical loading. Knee joints were removed, decalcified for four weeks, and then embedded in paraffin. Serial mid-sagittal sections were cut at a thickness of 4 μm. Hematoxylin, eosin, and Safranin O-Fast Green staining were also performed.

2.7. Immunohistochemical (IHC) and immunofluorescence (IF) staining

All sections were prepared following the methods described above. RPL35 and Gli-1 (Proteintech, Rosemont, IL, USA) primary antibodies were used for IHC analysis and incubated overnight at 4 °C. After washing three times in phosphate-buffered saline (PBS), the slides were incubated with a secondary antibody mixture for 1 h. Then IHC slides were stained with diaminobenzidine (ZSGB-Bio, Beijing, China) chromogen. For IF staining, the primary antibodies were against Col2a, MMP13 (Proteintech, Rosemont, IL, USA), P16, P21 (Abcam, Cambridge, UK), and Csnk1e (Abclonal, Woburn, MA, USA). After three washes with PBS, the slides were incubated with species-matched Alexa-488 or 594-labeled secondary antibodies for 1 h. Nuclei were stained with 0.4 μg/mL of DAPI. The images were acquired using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

2.7. Senescence-associated β-galactosidase assay (SA-β-Gal)

The SA-β-Gal assay was performed using cytochemical staining of SA-β-Gal with the SA-β-Gal staining kit (G1580). Chondrocytes were seeded into a six-well plate, fixed at room temperature for 10 min, washed, and incubated with a staining solution at 37 °C overnight.

2.8. Adenovirus transfection assay

Adenovirus-mediated overexpression of RPL35 (Ad-RPL35) was achieved by Hanbio Biotechnology (Shanghai, China) for infection into mouse chondrocytes and ad-green fluorescent protein was used as a negative control (Ad-NC). The adenovirus titer was 2.5×10^{12} plaque-forming units mL^{-1} and MOI was 500:1. After adenovirus infection for 24 h, the cells were treated with excessive mechanical loading for 24 h and harvested for further analysis.

2.9. Statistical analysis

Unpaired Student's t-test was used when comparing two experimental groups for qPCR and WB data. One-way analysis of variance (ANOVA) was carried out for the comparison of three or more groups, such as for the Osteoarthritis Research Society International scores and immunohistochemistry analysis of RPL35 in articular cartilage of mice treated with multiple loading episodes. A two-way analysis of variance was used for four groups to consider the effect of two factors, such as for RPL35 and Hh inhibitor as the two factors in the pathway inhibition experiment. The statistical analysis was conducted using GraphPad Prism software GraphPad Prism software (Version 6.0, GraphPad Software, Inc., La Jolla, CA, USA). All data were reported as the mean ± SD. $P < 0.05$ indicated statistical significance.

3. Results

3.1. RPL35 down-regulation in senescent chondrocytes in vitro was induced by excessive mechanical loading

To identify genes important for chondrocytes treated with excessive mechanical loading, which is considered an excellent model for studying the physiological features of osteoarthritis (OA), RNA sequencing analysis was performed on chondrocytes treated with or without 20 % cyclic tensile strain for 24 h. Differential expression analysis revealed 459 differentially expressed genes between treatments. Gene expression of RPL35 showed the most pronounced down-regulation [Fig. 1(A)]. To further validate the association between RPL35 and chondrocytes treated with excessive mechanical loading, the expression of a series of genes in 20 % elongation strain-loaded chondrocytes was examined. Consistent with previous results, the quantitative PCR (qPCR) results revealed that excessive mechanical stress not only promoted catabolic effects but also stimulated chondrocyte senescence [Fig. 1(B)]. The

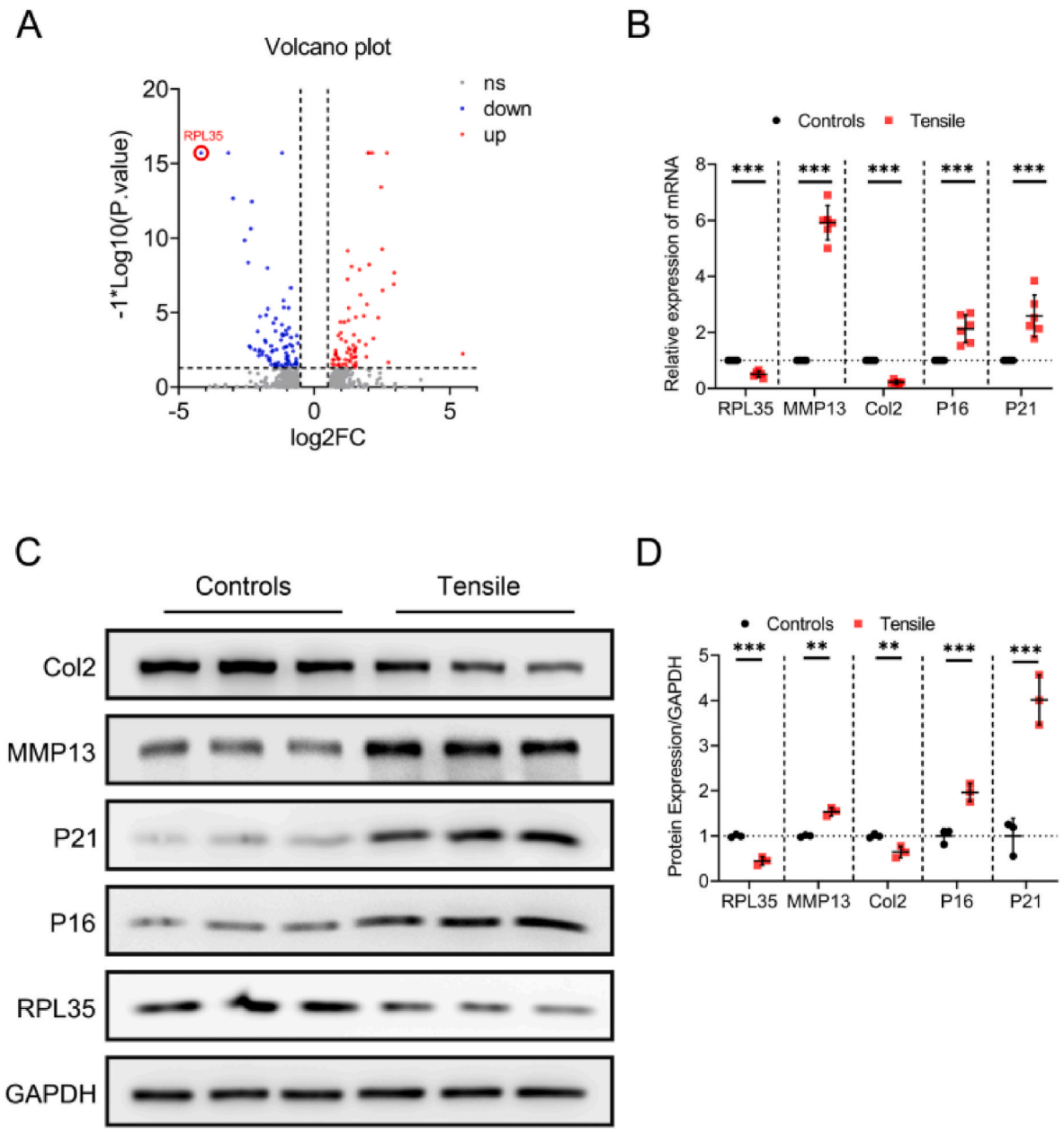

Fig. 1. Ribosomal protein L35 (RPL35) down-regulation in senescent chondrocytes *in vitro* was induced by excessive mechanical loading. (A) Volcano plots of differentially expressed genes. (B) Quantitative PCR analysis of RPL35, Col2, MMP13, and senescence markers (P16, P21) in primary chondrocytes treated with 20 % elongation strain loading for 24 h n = 6 per group. (C, D) Western blotting analysis of RPL35, Col2, MMP13, and senescence markers (P16, P21) in primary chondrocytes treated with 20 % elongation strain loading for 24 h. The GAPDH was used as a loading control; $^{**}P < 0.01$, $^{***}P < 0.001$.

number of cells positive for Senescence-associated β-galactosidase assay staining increased after excessive mechanical loading [Supplemental Fig. 1(A)]. Down-regulation in RPL35 gene expression was noted in chondrocytes treated with excessive mechanical loading compared to normal chondrocytes [Fig. 1(B)]. The corresponding gene expression results obtained from western blot (WB) analysis in three independent experiments confirmed the decreased expression of RPL35 in primary chondrocytes treated with 20 % cyclic tensile strain loading for 24 h [Fig. 1(C) and (D)]. It is known that pro-inflammatory cytokines, such as IL-1β, and TNF-β, play a role in the pathogenesis of OA. Therefore, a model of OA was established using primary mouse chondrocytes stimulated with recombinant IL-1β. WB results showed a decrease in RPL35 in IL-1β-stimulated chondrocytes compared to normal chondrocytes [Supplemental Fig. 1(B)].

3.2. Chondrocyte RPL35 level was decreased by excessive mechanical loading and reduced in OA articular cartilage

To assess whether the *in vitro* study results can be applied *in vivo*, RPL35 protein levels were first examined in human OA. Immunohistochemical (IHC) staining showed that the level of RPL35 protein was reduced in the medial tibial plateau of OA patients who underwent total knee arthroplasty compared to cartilage samples from the mildly damaged lateral tibial plateau [Fig. 2(A) and (B)]. The same results were observed when using a surgically-induced OA mouse model in which the medial meniscus was destabilized. Specifically, RPL35 protein levels showed a progressive down-regulation in OA mice, accompanied by increased cartilage damage compared to normal cartilage [Fig. 2(C)–(E)]. The effects of mechanical loading on articular cartilage senescence were then further analyzed. In immunofluorescence (IF) staining for

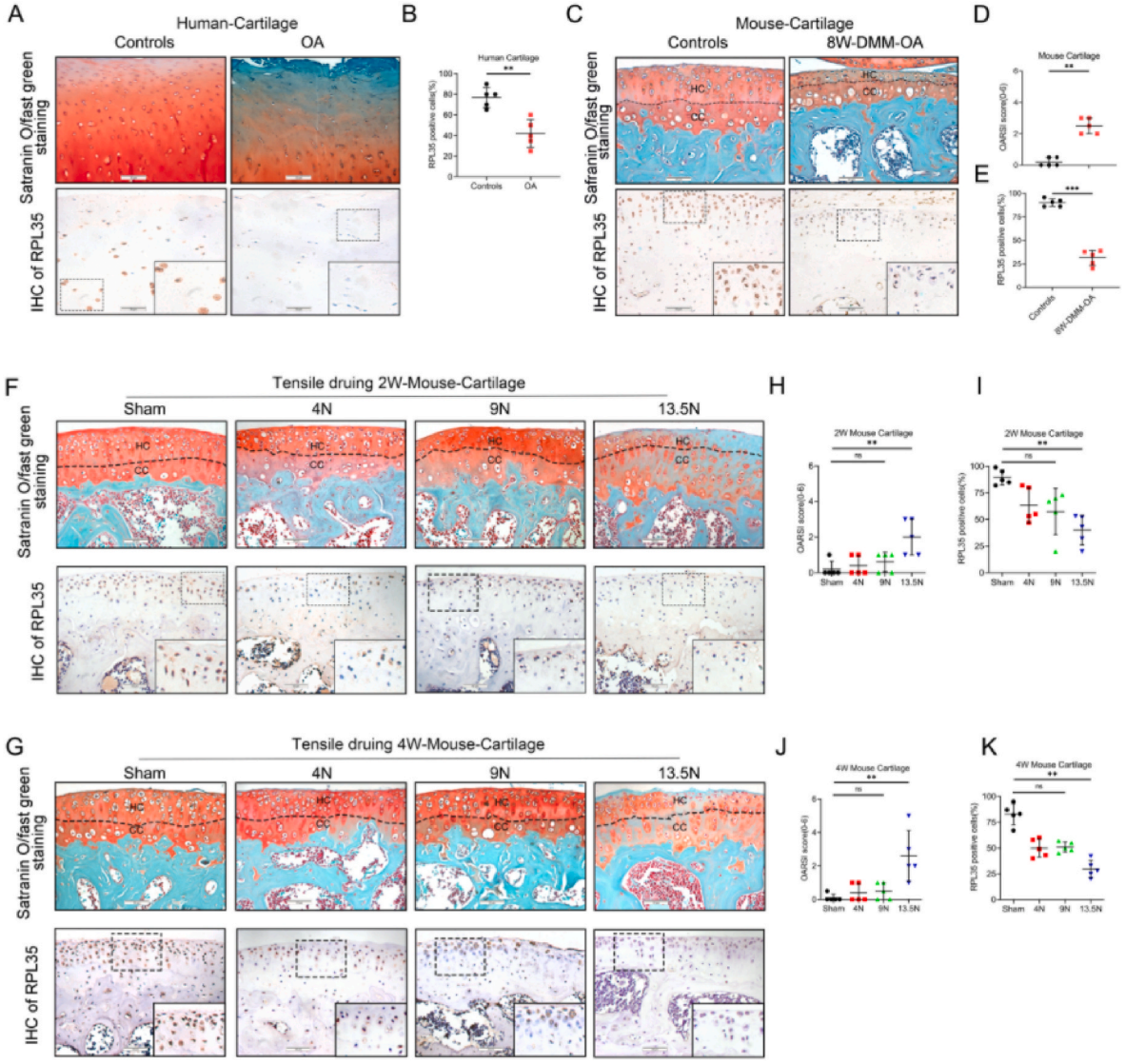

Fig. 2. Chondrocyte RPL35 level was reduced by mechanical overloading and decreased in OA articular cartilage. (A) Safranin O and Fast Green staining (upper) and immunohistochemistry (IHC) of RPL35 (lower) of human articular cartilage from medial (OA) and lateral (NC) tibial plateau of OA patients undergoing total knee arthroplasty. Scale bar: 50 μm. (B) Quantification of RPL35 in human articular cartilage of medial and lateral tibial plateau. n = 5 per group. (C) Safranin O and Fast Green staining (upper) and IHC of RPL35 (lower) of sagittal sections of knees from controls and OA model mice at 8 weeks post destabilization of the medial meniscus (DMM) operations. Scale bar: 50 μm. (D) Osteoarthritis Research Society International (OARSI) grades for the joints described in C. n = 5 per group. (E) Quantification of RPL35 in articular cartilage of controls and DMM mice. n = 5 per group. (F) Safranin O and Fast Green staining (upper) and IHC of RPL35(lower) in articular cartilage of mice treated with multiple loading episodes at peak loads of 13. 5 N for 2 weeks. Scale bar: 50 μm. (G) Safranin O and Fast Green staining (upper) and IHC of RPL35(lower) in articular cartilage of mice treated with multiple loading episodes at peak loads of 13. 5 N for 4 weeks. Scale bar: 50 μm. (H) Osteoarthritis Research Society International (OARSI) grades for the joints described in F. n = 5 per group. (I) Quantitative analysis of RPL35 positive chondrocytes as a proportion of the total chondrocytes in articular cartilage of mice described in F. n = 5 per group. (J) OARSI grades for the joints described in G. n = 5 per group. (K) Quantitative analysis of RPL35 positive chondrocytes as a proportion of the total chondrocytes in articular cartilage of mice described in G. n = 5 per group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant. HC, hyaline cartilage. CC, calcified cartilage.

P16, P21, MMP13, and Col2 in mouse articular chondrocytes, the number of MMP13-, P16-, and P21-positive chondrocytes was increased, whereas the number of Col2-positive chondrocytes was significantly decreased under mechanical stress of 13.5 N for two or four weeks compared to the sham-operated groups [Supplemental Fig. 2(A)]. Collectively, these results showed that mechanical overloading accelerated chondrocyte senescence in articular cartilage. Therefore, the role of RPL35 in the pathogenesis of OA was examined based on the model presented above.

The application of multiple loading episodes at a peak load of 13.5 N for two or four weeks induced proteoglycan loss in mouse knee joints, as evidenced by Safranin O staining. However, no significant changes in chondrocytes were present under stimuli of 4 N and 9 N [Fig. 2(F) and (G)]. As expected, IHC cartilage staining confirmed a marked decrease in chondrocyte RPL35 protein level after the application of excessive mechanical stress of 13.5 N [Fig. 2(F)–(K)]. Overall, these findings revealed that RPL35 protein levels decreased as OA progressed, suggesting an association between OA severity and RPL35 expression.

3.3. Loss of RPL35 accelerates OA progression

To explore the roles of RPL35 in OA progression, RPL35 was first silenced using RPL35 siRNA. Efficient knockdown was confirmed on protein levels [Supplemental Fig. 3(A)]. Next, whether siRNA depletion of RPL35 drives the degeneration of primary mouse chondrocytes was evaluated. WB and qPCR results revealed that RPL35 knockdown

reduced Col2 levels and increased the levels of MMP13, P16, and P21 [Supplemental Figs. 4(A) and (B)]. Furthermore, to investigate the role of RPL35 *in vivo*, we administered RPL35 siRNA, designed for animal use, via intra-articular injection in mice. We observed a notable reduction in the expression of RPL35 in chondrocytes after knocking down RPL35 *in vivo*. This reduction was accompanied by degenerative changes in the cartilage. Similar phenomena were also observed in mice subjected to excessive mechanical stress on their articular cartilage. However, the intra-articular injection of RPL35 siRNA in mice with osteoarthritis induced by mechanical stress did not exacerbate the progression of the osteoarthritis [Supplemental Figs. 4(C)–(E)]. Further assessments are required to evaluate the extent of cartilage damage.

3.4. *RPL35 restored chondrocyte homeostasis and deferred chondrocyte senescence in OA*

Previous experiments validated the effect of RPL35 knockdown on chondrocytes. Next, the effects of RPL35 on chondrocyte homeostasis were examined. Adenovirus-mediated overexpression of RPL35 (Ad-RPL35) and negative control (Ad-NC) vectors were constructed. Ad-RPL35 transfection and overexpression efficiency were verified by IF staining and WB [Supplemental Figs. 3(B) and (C)]. Then, male C57 mice aged 10 weeks were treated by intraarticular injection of Ad-NC or Ad-RPL35 once a week after mechanical loading for the following four or six weeks. In mechanical loading mice with intraarticular injections of Ad-RPL35, OA severity was significantly lower compared to that in mice treated with mechanical loading and Ad-NC. No cartilage erosion was observed, as confirmed by the Osteoarthritis Research Society International scores system. Interestingly, IHC showed that changes in the expression of RPL35 were consistent with the trend of the above-analyzed degree of articular cartilage damage [Fig. 3(A) and Supplemental Fig. 5(A)]. In addition, the number of MMP13-, P16-, and P21-positive chondrocytes was increased, whereas the number of Col2-positive chondrocytes was significantly decreased in mice treated with

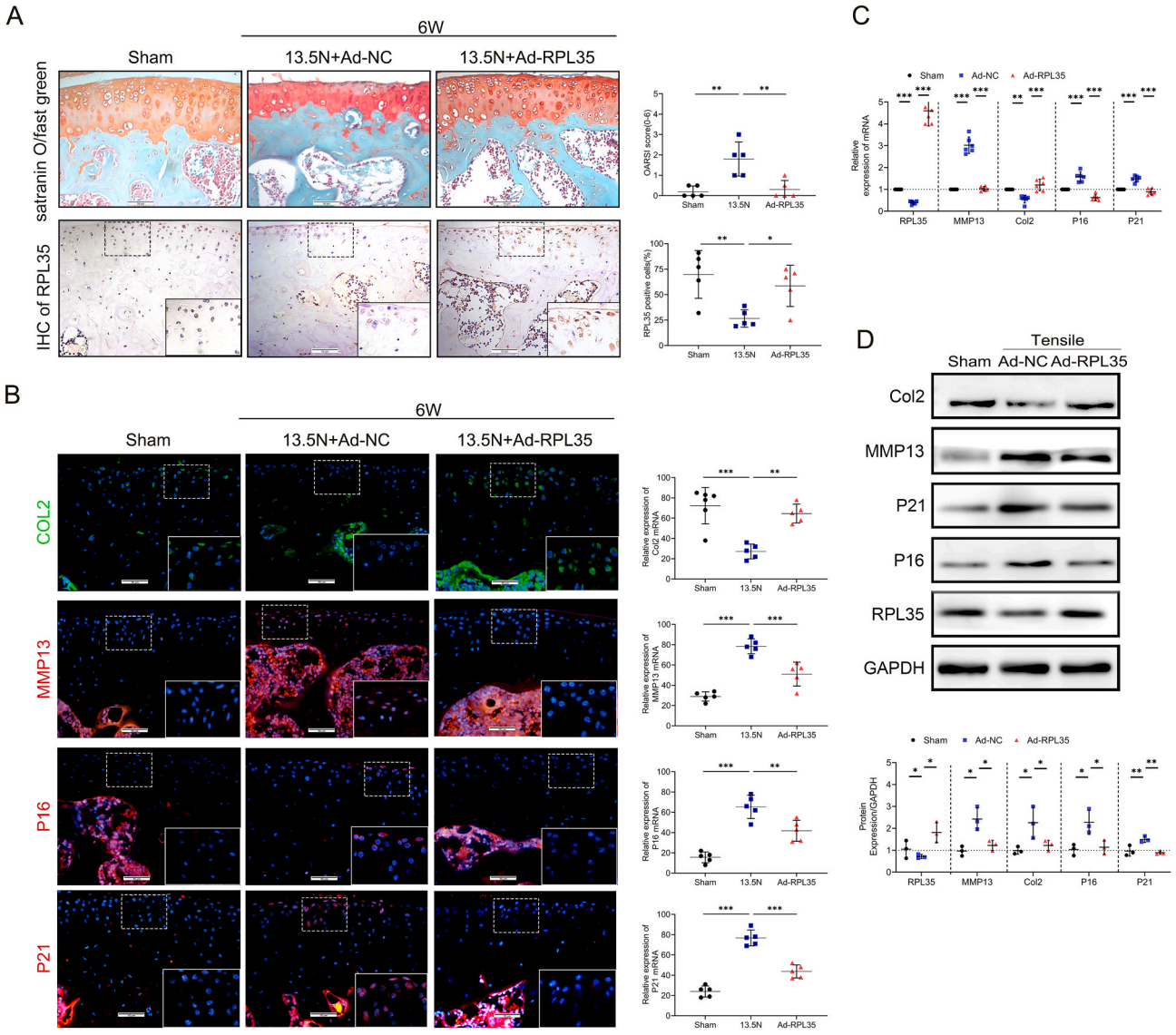

Fig. 3. RPL35 overexpression in chondrocytes protects against mechanically induced chondrocyte senescence and OA development. (A) Safranin O and Fast Green staining (upper) and immunostaining of RPL35 (lower) in articular cartilage of mice intra-articularly injected with or without Adenovirus-mediated overexpression of RPL35 (Ad-RPL35) for 6 weeks under mechanical loading. Scale bar: 50 μm. n = 5 per group. (B) Immunofluorescent staining and quantification of Col2, MMP13, P16, P21 in articular cartilage described in A. n = 5 per group. Scale bar: 50 μm. (C, D) Western blot (n = 3 per group) and quantitative PCR (n = 6 per group) analysis of RPL35, Col2, MMP13, P16, and P21 in mouse primary Chondrocytes treated with or without Ad-RPL35 for 48 h under 20 % elongation strain loading for 24 h $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mechanical loading for four or six weeks compared to the control mice. The RPL35 supplement relieved the degenerative pathology [Fig. 3(B) and Supplemental Fig. 5(B)]. Additionally, excessive mechanical loading stimulation promoted the expression of these senescence factors, while the RPL35 supplement reversed these effects *in vitro* [Fig. 3 (C) and (D)], indicating its protective effect against chondrocyte senescence.

3.5. Knockdown of RPL35 accelerated OA progression through activation of the hedgehog (Hh) pathway

Next, the present study sought to determine which signaling pathway was responsible for the induction of chondrocyte senescence after RPL35 knockdown. Primary mouse chondrocytes were transfected with RPL35 siRNA or treated with mechanical loading. Next, 4989 transcripts were identified, the levels of which were increased in mouse chondrocytes transfected with RPL35 siRNA compared to normal chondrocytes. The levels of 5122 transcripts were increased in mouse chondrocytes treated with mechanical loading compared to normal chondrocytes [Fig. 4(A)]. Importantly, 2100 of these upregulated transcripts overlapped, most of which were enriched in the Hh pathway [Fig. 4(B)]. The qPCR results revealed that RPL35 knockdown or excessive mechanical loading resulted in increased chondrocyte expression of GLI-1 and SMO [Fig. 4 (C)], which are markers of Hh pathway activation.

Excitingly, IHC and IF illustrated increased SMO, Gli-1, and PTCH1 Hh signaling in cartilage under excessive mechanical loading, while

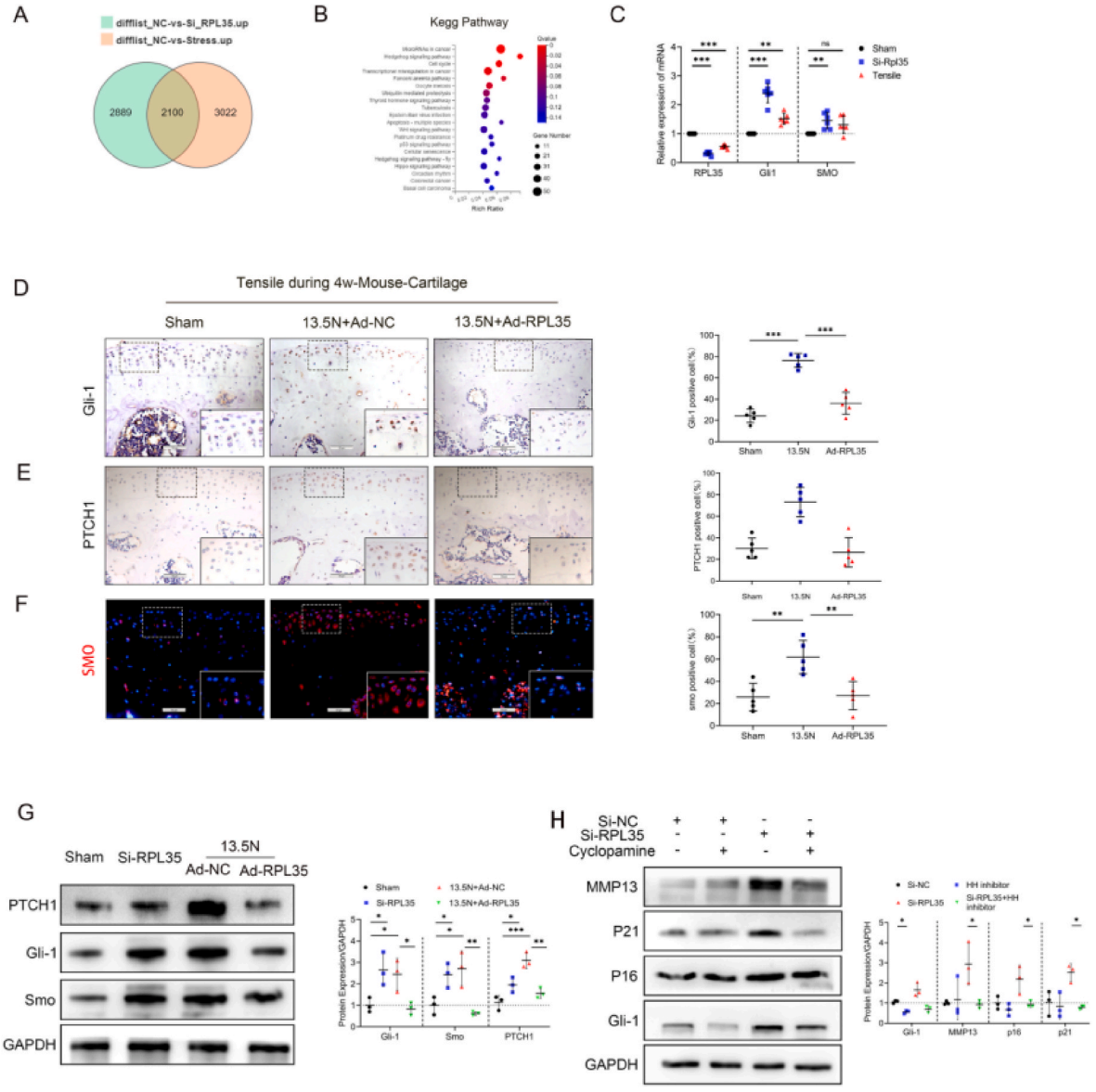

Fig. 4. RPL35 regulates the chondrocyte phenotype in osteoarthritis via the hedgehog (Hh) pathway. (A) Venn diagram analysis of mouse primary chondrocytes treated with 20 % elongation strain loading for 24 h or RPL35 siRNA (Si-RPL35) for 48 h. (B) The bubble plots showing KEGG pathway enrichment data for genes that were up-regulated. (C) Quantitative PCR analysis of RPL35, Gli-1, SMO in chondrocytes treated with 20 % elongation strain loading for 24 h or RPL35 siRNA for 48 h n = 6 per group. (D, E) Immunostaining and quantification of Gli-1, PTCH1 in articular cartilage of mice intra-articularly injected with or without Ad-RPL35 for 4 weeks under mechanical loading. n = 5 per group, Scale bar: 50 μm. (F) Immunofluorescent staining and quantification of SMO in articular cartilage of mice intra-articularly injected with or without Ad-RPL35 for 4 weeks under mechanical loading. n = 5 per group, Scale bar: 50 μm. (G) Western blotting analysis of Gli-1, SMO, PTCH1 in normal primary chondrocytes, RPL35 siRNA-transfected chondrocytes, and mechanical loading chondrocytes treated with or without Ad-RPL35 for 48 h; The GAPDH was used as a loading control. n = 3 per group. (H) Western blotting analysis of Gli-1, MMP13, P16, and P21 in mouse primary chondrocytes treated with RPL35 siRNA for 48 h with or without cyclopamine (10 μM) for 24 h n = 3 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant.

RPL35 supplementation rescued the expression of SMO, Gli-1, and PTCH1 [Fig. 4(D)–(F)]. The above results were further corroborated via WB *in vitro* [Fig. 4(G)]. Overall, these results illustrate the suppressive effect of RPL35 on the Hh pathway. Furthermore, whether Hh activation contributed to RPL35 deficiency-induced chondrocyte senescence was also determined. In primary mouse chondrocytes, the Hh pathway inhibitor cyclopamine partially reduced the protein expression levels of P21, P16, and MMP13 induced by RPL35 loss [Fig. 4(H)]. In summary, the above results suggested that elevated Hh pathway activation was at least in part responsible for articular cartilage degeneration caused by the loss of RPL35.

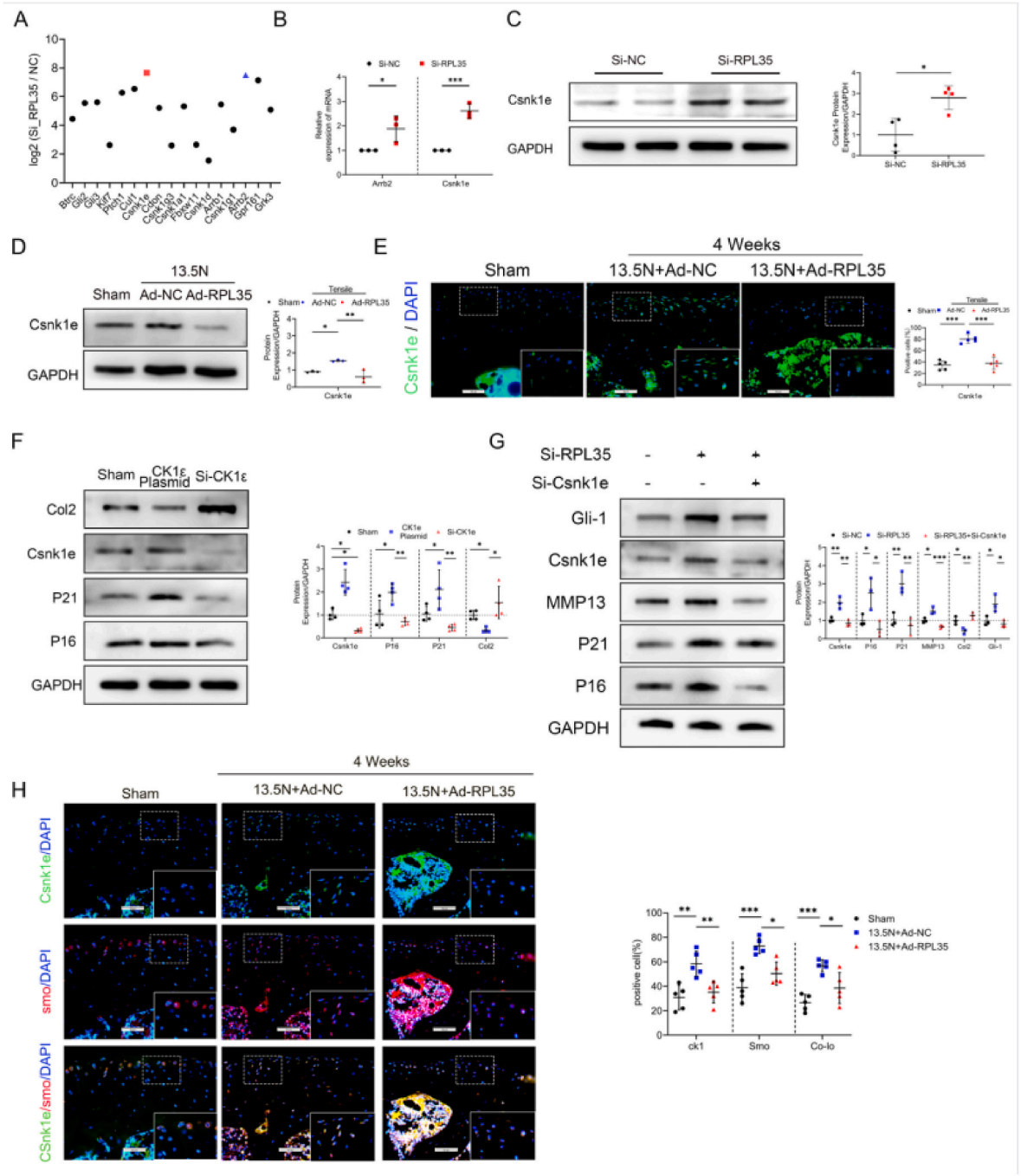

Fig. 5. RPL35 regulated Hh signaling activity through Csnk1e. (A) Genes that were up-regulated in pathway enrichment. (B) Quantitative PCR analysis of Arrb1, Csnk1e in mouse primary chondrocytes treated with or without RPL35 siRNA for 48 h, n = 6 per group. (C) Western blot analysis of Csnk1e in mouse primary chondrocytes treated with or without RPL35 siRNA for 48 h, n = 4 per group. (D) Western blot analysis of Csnk1e (CK1ε) in mouse primary Chondrocytes treated with or without Ad-RPL35 for 48 h under 20 % elongation strain loading for 24 h, n = 3 per group. (E) Immunofluorescent staining and quantification of CK1ε in articular cartilage of mice intra-articularly injected with or without Ad-RPL35 for 4 weeks under mechanical loading. n = 5 per group. Scale bar: 50 μm. (F) Western blotting analysis of Col2, P16, P21, CK1ε in mouse primary chondrocytes treated with Csnk1e siRNA (Si– CK1ε) or Csnk1e overexpression plasmid (CK1ε plasmid) for 48 h n = 4 per group. (G) Western blotting analysis of Gli-1, Col2, MMP13, P16, P21, Csnk1e in mouse primary chondrocytes treated with Csnk1e siRNA or RPL35 siRNA for 48 h n = 3 per group. (H) Immunofluorescent staining and quantification of Csnk1e and SMO in articular cartilage of mice intra-articularly injected with or without Ad-RPL35 for 4 weeks under mechanical loading. n = 5 per group. Scale bar: 50 μm *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant.

3.6. RPL35 regulated Hh signaling through Csnk1e (CK1ε)

Pathway analysis showed that 17 upregulated transcripts mediate the Hh signaling pathway in primary chondrocytes treated with RPL35 siRNA compared to normal chondrocytes. CK1ε was the most prominent among them [Fig. 5(A) and (B)]. The qPCR and WB analyses revealed that RPL35 knockdown in chondrocytes increased the endogenous CK1ε level compared to that in normal chondrocytes [Fig. 5(C)]. Moreover, elevated CK1ε levels were noted in chondrocytes treated with excessive mechanical loading, while the RPL35 supplement rescued the expression of CK1ε [Fig. 5(D)]. The same tendency was observed through IF staining analysis [Fig. 5(E)]. Previous studies have shown that blocking CK1 activity in the Drosophila wing disc attenuated pathway activity and that activation of Indian hedgehog (IHH) increased the expression of MMP13 in chondrocytes. The role of CK1ε in OA development was therefore explored. First, western blot results verified the knockdown efficiency of siRNA targeting Csnk1e [Supplemental Fig. 6(A)]. CK1ε-overexpressing chondrocytes exhibited elevated MMP13 levels, decreased Col2 levels, and heightened senescence-related marker P16 and P21 levels compared to normal chondrocytes, whereas Csnk1e knockdown was protective [Fig. 5(F)]. To investigate whether siRPL35 regulates the OA process by targeting CK1ε/SMO signaling, WB results showed that the effects of RPL35 knockdown were markedly diminished by the CK1ε knockdown [Fig. 5(G)]. In addition, immune co-localization showed increased levels of CK1ε and increased co-localization with SMO after the RPL35 knockdown [Fig. 5(H)], further suggesting the possibility of CK1ε activation of SMO. In conclusion, RPL35 inhibition led to OA, likely by elevating CK1ε protein levels, and thus contact activation with SMO, thereby activating the Hh pathway.

4. Discussion

The earliest and most significant pathological changes in osteoarthritis (OA) occur in the articular cartilage. Senescent cells have been found in chondrocytes extracted from mice with OA and patients who underwent total knee replacement surgery, suggesting a correlation between OA and senescent degeneration of chondrocytes [29,30]. The interaction of mechanical and biological factors is currently thought to contribute to OA [27,31]. The present study found that excessive mechanical stress promoted chondrocyte senescence and degeneration, leading to OA both *in vivo* and *in vitro*, and explored the role of the RPL35/CK1/Hh pathway axis in the excessive mechanical loading-mediated effects.

Ribosomal protein L35 (RPL35) is a component of the ribosomal large subunit, working in conjunction with other ribosomal proteins and rRNA to form the ribosomal structure. Ribosomes play a pivotal role in protein synthesis, and RPL35, being a part of the ribosome, indirectly contributes to this process [20]. Current studies indicate that an increased expression of RPS29 in chondrocytes correlates with the progression of OA [32], while RPL38 knockdown inhibits chondrocyte inflammation and apoptosis [33]. However, specific protein alterations in OA induced by various pathological stressors, such as inflammation, aging, and excessive stress, are not solely determined by the quantity of ribosomal proteins. Cells can shift their translation mode favoring Internal Ribosome Entry Site-dependent translation and prioritizing the synthesis of specific proteins [34,35]. Additionally, ribosomal proteins possess extra-ribosomal functions, which are potentially related to cellular signaling, DNA repair, and gene expression regulation [36]. This adds complexity to the study of the relationship between ribosomal proteins and specific OA-associated proteins. In the sequencing results, down-regulation of the RPL35 gene was most pronounced in chondrocytes treated with excessive mechanical loading. However, its involvement in chondrocyte senescence and OA development remains unknown. Therefore, the present study verified the expression of RPL35 in a mechanically induced OA model using sequencing results and found that RPL35 knockdown led to chondrocyte senescence and degeneration. *In vitro* and *in vivo* experiments demonstrated that RPL35 overexpression attenuated chondrocyte senescence and extracellular matrix degradation induced by excessive stress loading. Co-sequencing RPL35 knockdown and overstressed chondrocytes helped to identify the downstream hedgehog (Hh) pathway after RPL35 knockdown, which was previously shown to be activated in OA. Inhibition of the Hh pathway in both mouse and human OA cartilage grafts reduced the severity of OA [24,37–39]. Our experimental results illustrated the activation of the Hh pathway after RPL35 knockdown, while inhibition of the Hh pathway effectively alleviated the senescence and degeneration of chondrocytes caused by RPL35 knockdown.

Hh signaling shares many components with the Wnt/β-catenin pathway, including Csnk1e (CK1α) and GSK-3β [40,41]. The function of CK1α in the Wnt pathway remains unclear. Previous studies have shown that CK1α can phosphorylate LRP6 to activate the Wnt pathway [42]. However, target genes in Wnt signaling, such as cyclin D1, c-myc, and CD44, have been induced at both the mRNA and protein levels in CK1α knockout mice [43]. Similarly, the specific function of CK1ε in Hh remains to be verified [44]. In Drosophila, Ck1α, both inhibit the Hh signal in the absence of the ligand and are required during Hh response through phosphorylation of SMO [45,46]. The siRNA screening also identified Csnka1a1 as a positive regulator of mammalian Hh signaling [47]. However, the specific function of CK1ε in articular cartilage remains to be verified. In the sequencing results, CK1ε was the most significantly elevated after the knockdown of RPL35 in mouse primary chondrocytes. As a tyrosine kinase family member, CK1ε may be involved in the relationship between RPL35 and Hh pathways in chondrocytes. Thus, the role of CK1ε in the pathogenesis of OA was explored. The results showed that high levels of CK1ε were detected in OA cartilage, while CK1ε overexpression led to cartilage anabolism inhibition. Furthermore, inhibition of CK1ε alleviated the Hh activation caused by the RPL35 knockdown, thus alleviating OA. This suggested that CK1ε may exacerbate the OA process by activating the Hh pathway. The limitations of the present study included the use of adenovirus overexpression and siRNA to verify the function of RPL35 instead of using transgenic mice. In addition, the mechanism for the activation of the Hh pathway by CK1ε needed to be further explained. The present study showed tight spatial proximity between CK1ε and SMO under mechanical stress, suggesting that CK1ε may activate the Hh pathway by interacting with SMO. In conclusion, the present study identified the role and connection of RPL35 and CK1ε in OA, while the experimental results verified that CK1ε overexpression in articular cartilage activated the Hh pathway, demonstrating that RPL35 deletion and activation of the CK1ε-Hh signaling pathway played a key role in biomechanically-induced chondrocyte senescence and degeneration. Therefore, targeting intra-articular supplementation of RPL35 is a way to slow down the development of OA.

Author contributions

JJZ, LLL, RGL, and XTG performed experiments, analyzed data, drafted and and revised the manuscript; JBY, HYX, YHL, and ZCZ analyzed data and prepared figures; HBZ, ZHY, and HYZ performed bioinformatics and statistical analyses; CZ, XJW, and DZC designed the experimental study, analyzed data, and edited the manuscript. The final manuscript was read and approved by all writers.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2024.01.003.

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