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The silencing of NREP aggravates OA cartilage damage through the TGF-β1/Smad2/3 pathway in chondrocytes

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ABSTRACT

Background: Osteoarthritis (OA) is a common chronic degenerative joint disease. Due to the limited understanding of its complex pathological mechanism, there is currently no effective treatment that can alleviate or even reverse cartilage damage associated with OA. With improvement in public databases, researchers have successfully identified the key factors involved in the occurrence and development of OA through bioinformatics analysis. The aim of this study was to screen for the differentially expressed genes (DEGs) between the normal and OA cartilage through bioinformatics, and validate the function of the TGF-β1/Smad2/3 pathway-related neuron regeneration related protein (NREP) in the articular cartilage.

Methods: The DEGs between the cartilage tissues of OA patients and healthy controls were screened by bioinformatics, and functionally annotated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. The expression levels of the DEG in human and murine OA cartilage was verified by reverse transcription-quantitative PCR (RT-qPCR), Western blotting and immunohistochemistry (IHC). RT-qPCR, Western-blotting, Cell Counting Kit-8(CCK8) and EdU assays were used to evaluate the effects of knocking down NREP in normal chondrocytes, and the molecular mechanisms were investigated by RT-qPCR, Western blotting and IHC.

Results: In this study, we identified NREP as a DEG in OA through bioinformatics analysis, and found that NREP was downregulated in the damaged articular cartilage of OA patients and mouse model with surgically-induced OA. In addition, knockdown of NREP in normal chondrocytes reduced their proliferative capacity, which is the pathological basis of OA. At the molecular level, knock-down of NREP inactivated the TGF-β1/Smad2/3 pathway, resulting in the downregulation of the anabolic markers Col2a1 and Sox9, and an increase in the expression of the catabolic markers MMP3 and MMP13.

Conclusion: NREP plays a key role in the progression of OA by regulating the TGF-β1/Smad2/3 pathway in chondrocytes, and warrants further study as a potential therapeutic target.

1. The translational potential of this article

This study provides a biological rationale for the use NREP as a potential target for OA treatment.

2. Introduction

Osteoarthritis (OA) is a chronic disease of the joints, and is characterized by articular cartilage destruction, subchondral bone sclerosis and osteophyte formation [1]. The risk of OA is increased due to mechanical overload, inflammation, metabolic factors, hormonal changes and aging [2]. Currently, over 528 million people worldwide have been diagnosed with OA [3]. Since articular cartilage has no blood vessels, nerves, and lymphatic tissues [4], the damaged tissues cannot regenerate easily. Furthermore, medication, physical therapy and surgical approaches only relieve the symptoms of OA, such as pain and

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inflammation, and cannot reverse cartilage damage [5,6]. When the disease progresses to the terminal stage, total knee arthroplasty (TKA) is the only effective treatment option. Therefore, it is crucial to elucidate the molecular mechanisms underlying the pathogenesis of OA in order to identify novel therapeutic targets, and develop effective therapies to slow down or even reverse articular degeneration.

Neuron regeneration related protein (NREP), also known as P311 and PTZ17, was discovered in 1993 and is mainly expressed in the embryonal mouse brain, and the cerebellum, hippocampus and olfactory bulb in adult mice [7]. NREP is an 8-kDa intracellular protein consisting of 68 amino acids that does not belong to any known protein family and requires interacting partners for its tertiary structure and function [8]. The amino acid sequence of NREP contains a PEST domain, which serves as a binding site for the ubiquitin-proteasome pathway and is therefore involved in regulating the expression of transcription factors, cytokines, and signaling factors [9–11]. Previous studies have identified eukaryotic translation initiation factor 3 subunit B (EIF3B) as a novel NREP binding partner. NREP recruits the transforming growth factor (TGF)β1, β2 and β3 mRNAs to the translation machinery through concomitant binding to EIF3B and the 5′ UTR of transcripts, thereby promoting translation and increasing the expression of TGF-β protein. In fact, NREP is the first documented regulator of the three TGF-β transcripts that stimulates translation of TGF-β [12].

It is reported that NREP is expressed in chondrocytes [13] and regulates TGF-β1/Smad2/3 pathway expression in different tissues [14–16]. However, the expression and potential role of NREP in osteoarthritic chondrocytes have not been studied so far. In this study, we have demonstrated for the first time that NREP is highly expressed in normal cartilage tissue and chondrocyte, and is significantly down-regulated in osteoarthritic cartilage tissues and inflamed chondrocytes. Furthermore, knock-down of NREP in the chondrocytes disrupted the metabolic balance by inhibiting the TGF-β1/Smad2/3 pathway. Our findings highlight the critical role of NREP in the development of OA and its potential as a therapeutic target.

3. Material and methods

3.1. Screening and functional annotation of differentially expressed genes (DEGs)

The microarray dataset GSE169077 including the transcriptomic data of OA and normal human cartilage tissues was downloaded from the Gene Expression Database. The DEGs between the OA and normal groups were screened using the "limma" R package (R4.2.1), with $|\log_2 FC| > 1$ and $P < 0.05$ as the criteria. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on the DEGs using the "cluster Profiler" R software package.

3.2. Collection of human cartilage samples

Knee joint specimens were obtained from 6 patients with osteoarthritis who underwent TKA. The tissues were isolated from the worn cartilage covering the medial tibial plateau condyle (OA) and the complete hyaline cartilage covering the tibial plateau lateral condyle (normal) of the same patient. Part of the cartilage tissue was fixed with 4 % paraformaldehyde for histological staining, and the remaining was frozen at −80 °C for western blotting. The experiments with human specimens were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (No:2022YX167).

3.3. Induction of OA in mice

Traumatic OA was induced in a mouse model by the surgical destabilization of the medial meniscus (DMM). Male C57BL/6 mice (8–10 weeks old) were randomly divided into the sham-operated, 4-week DMM and 8-week DMM groups. To induce DMM, the right knee joint capsule was incised, and the tibial-medial meniscus ligament was severed using micro-tweezers. The medial meniscus was gently pushed to check whether it was completely free, and the wound was closed layer by layer. In the sham-operated group, the right knee joint capsule was incised without disturbing the meniscus. The operation was performed under a stereomicroscope to avoid any damage to the patellar ligament. The animals were housed in a specific pathogen-free (SPF) barrier facility under controlled temperature, humidity, and a 12-h light–dark cycle. The animal experiments were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (No. DW2022063).

3.4. Radiography

The mice were euthanized 4 and 8 weeks post DMM surgery, and the front and side of the right knee joint were imaged by X-ray. The exposure time and radiation dose were set to "automatic". Knee degeneration was graded on the basis of the degree of osteophyte formation as follows: grade 0-normal with no osteophyte formation; grade 1-mild osteophyte formation, grade 2-moderate osteophyte formation, grade 3-severe osteophyte formation. The radiographic scoring was performed independently by two researchers (Yang Liu and Mengrou Liu) under the guidance of an experienced surgeon (Ziquan Yang).

3.5. Chondrocyte culture and IL-1β induction

The ATDC5 chondrocyte cell line was cultured in DMEM-F12 (Gibco, USA) supplemented with 10 % fetal bovine serum (CellMax, China) and 1 % penicillin-streptomycin liquid (Solarbio, China) at 37 °C under 5 % CO_2. The chondrocytes were seeded in 6-well plates and cultured till 60–70 % confluent. After adding 10 ng/ml IL-1β (Peprotech, USA) to each well, the cells were induced for 16 h.

3.6. RT-qPCR

Total RNA was extracted from the cells using the TRIzol™ reagent (TAKARA, Japan) and cDNA was obtained by reverse transcription using the PrimeScript ™ RT Master Mix reagent (TAKARA, Japan) according to the manufacturer's instructions. The cDNA samples were amplified by qPCR using 2xS6 Universal SYBR qPCR Mix (EnzyArtisan, China) in Biosystems™ QuantStusio™ 6 Flex Real-Time Fluorescent PCR System (Thermo Fisher Scientific, USA). The 18 S rRNA was used as the internal reference and the relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Supplementary Table S1.

3.7. Western blotting

Proteins were extracted from the cultured cells or human cartilage tissues using the Lysis Buffer (KeyGEN BioTECH, China), separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, USA). The membranes were incubated overnight with primary antibodies at 4 °C, followed by incubation in a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The positive bands were visualized using the Bio-Rad Chemi Doc™ XRS $^{+}$ System, and quantified using Image J software. The following antibodies were used: rabbit anti-NREP (Bioss, 1:500), rabbit anti-MMP13 (Abcam, 1:1500), rabbit anti-MMP3 (Abcam, 1:1000), rabbit anti-Col2a1 (Abcam, 1:1500), rabbit anti-Sox9 (Abclonal, 1:1000), rabbit anti-TGF-β1 (Abclonal, 1:1500), rabbit anti-p-Smad2/3 (Abclonal, 1:1500), rabbit anti-PCNA(Abclonal, 1:1000), rabbit anti-β-actin (Abclonal, 1:2500), goat anti-rabbit IgG (Boster, 1:5000).

3.8. Histology and IHC

Cartilage tissue specimens were serially sectioned (5 μm) after routine fixation, decalcification, dehydration, and paraffin embedding. The tissue sections were stained with 0.5 % Safranin O (Sigma, USA),

0.2 % Fast green (Sigma, USA) and 1 % Alcian blue (Sigma, USA) to detect cartilage. In addition, the sections were immuno-stained using the following antibodies: rabbit anti-NREP (Bioss, 1:50), rabbit anti-MMP13 (Abcam, 1:100), rabbit anti-MMP3 (Bioss, 1:100), rabbit anti-Col2a1 (Abcam, 1:100), rabbit anti-TGF-β1 (Abclonal, 1:100), rabbit anti-p-Smad2/3 (Abclonal, 1:100), rabbit anti-ColX (Abcam, 1:50). Images were acquired using a digital section scanner (3DHISTECH, Hungary). The Osteoarthritis Research Society International (OARSI) grading system was used to analyze the results of Safranin-O/Fast green staining. ImageJ was used to determine the number of NREP-positive cells and cartilage area.

3.9. SiRNA transfection

ATDC5 cells were seeded in 6-well plates, cultured to reach 60–70 % confluency, and transfected with 20 nM siRNA using Lipo8000™ Transfection Reagent (Beyotime, China) in complete DMEM-F12. The cells were harvested 48 h later and analyzed as appropriate. All siRNAs used in this study were purchased from Ribo Bio (China) and the sequences are listed in Supplementary Table S2.

3.10. CCK8 assay

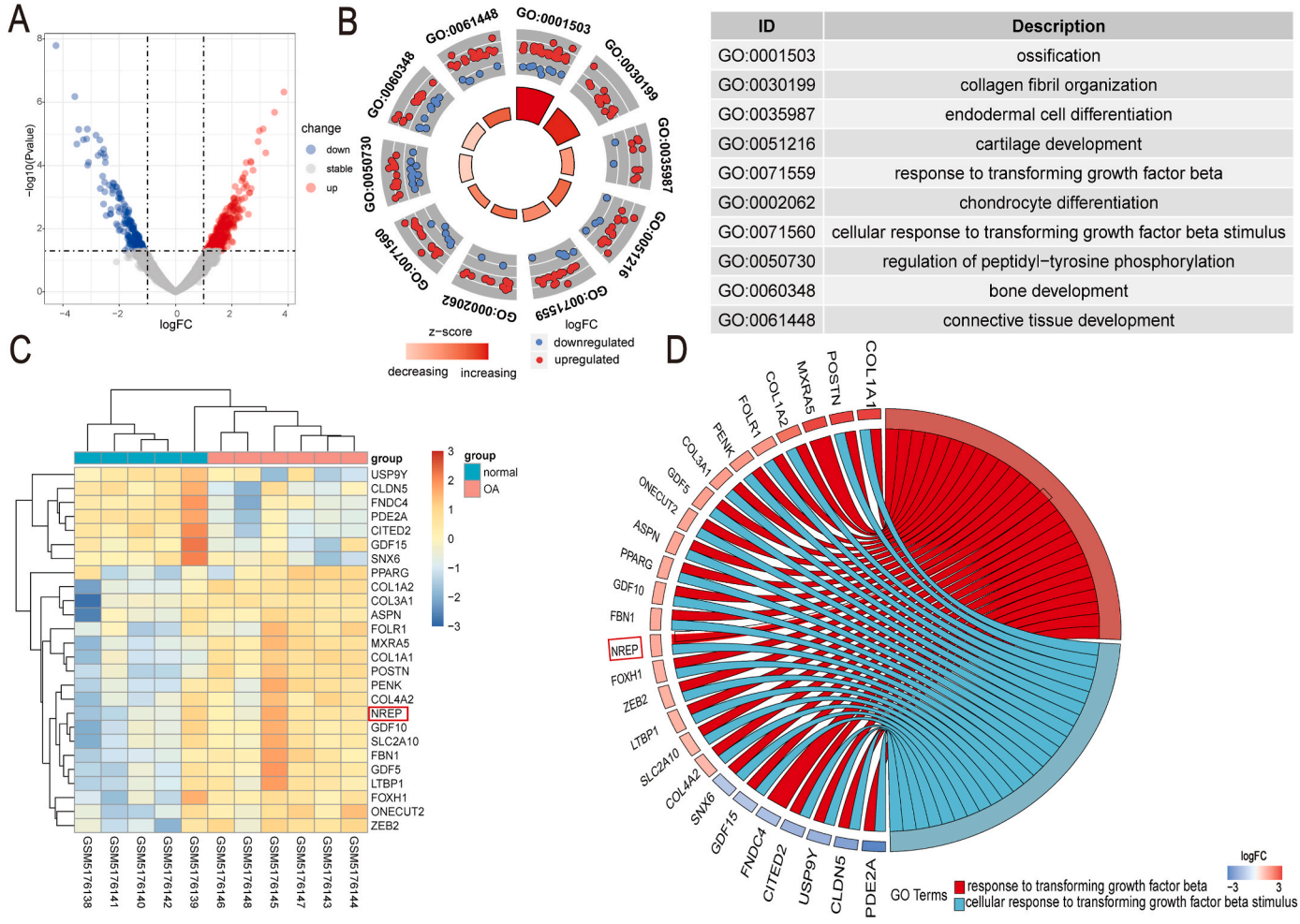
Cell Counting Kit-8 (CCK8) (Beyotime, China) was used to assess chondrocyte viability. Briefly, chondrocytes transfected with si-NC or si-NREP were seeded in 96-well plates at the density of 3000 cells per well and cultured for 24, 48 and 72 h. At the stipulated time-points, 100 μl basal medium and 10 μl CCK8 reagent were added, and the cells were incubated for 1 h. The optical absorbance of the corresponding wells was measured at 450 nm.

3.11. EdU incorporation assay

Cell light Apollo488 staining kit was used to evaluate chondrocyte proliferation according to the manufacturer's instructions. The EdU-labelled cells were photographed under an inverted fluorescence microscope, and the number of positive cells was counted in three random fields. The fluorescence intensity was quantified using Image J. The proliferation index was calculated as the percentage of EdU-positive cells relative to the number of Hoechst 33,342-stained cells.

3.12. Statistical analysis

GraphPad Prism 8.0.2 was used for all statistical analyses. The data were presented as mean ± standard deviation. Unpaired two-tailed Student's t test was used to compare two groups, and multiple groups were compared by one-way ANOVA and two-way ANOVA, followed by Tukey's test. All experiments were repeated more than three times. $P < 0.05$ was considered statistically significant.



ID	Description
GO:0001503	ossification
GO:0030199	collagen fibril organization
GO:0035987	endodermal cell differentiation
GO:0051216	cartilage development
GO:0071559	response to transforming growth factor beta
GO:0002062	chondrocyte differentiation
GO:0071560	cellular response to transforming growth factor beta stimulus
GO:0050730	regulation of peptidyl−tyrosine phosphorylation
GO:0060348	bone development
GO:0061448	connective tissue development

Figure 1. Screening and identification of differentially expressed genes (DEGs) using bioinformatics. (A) Volcano plot showing the significant DEGs between human OA and normal groups. (B) GO-enriched loop plot of DEGs. (C) Heat map of DEGs associated with TGF-β. (D) Chord plot of TGF-β-related pathways with their associated DEGs. Statistical indicators: $Log_2FC > 1$, $P < 0.05$.

4. Results

4.1. Screening and functional annotation of DEGs between OA and healthy cartilage

The transcriptomic data of OA and normal human cartilage tissues was retrieved from the Gene Expression Omnibus (GEO) database, and the DEGs were screened using the "limma" generalized linear model, with $|\log_2 FC| > 1$ and $P < 0.05$ as the criteria. As shown in Fig. 1A, there were 685 DEGs between the two groups, of which 470 were upregulated and 215 were downregulated in the OA group. The DEGs were subjected to GO and KEGG pathway analyses. The enrichment analysis based on the GO database annotated the DEGs into three categories of biological processes (BP), cellular components (CC), and molecular functions (MF). There were 277 significantly enriched pathways or related functions with q value < 0.05, including TGF-β pathway and chondrocyte growth (Fig. 1B). TGF-β is essential for the anabolic processes in the cartilage and stimulates Col2a1 expression and proteoglycan synthesis [17]. A total of 26 DEGs showed significant association with the TGF-β pathway, and might play a key role in OA development (Fig. 1C and D). To our knowledge, NREP has a regulatory role in the TGF-β1 pathway in non-articular cartilage. Injection of NREP-overexpressing MSCs into mouse skin tissues significantly increased the production of the anti-inflammatory cytokine IL-10, and decreased that of the pro-inflammatory cytokines TNF-α and IFN-g relative to the control MSCs [18]. In addition, a recent study revealed the expression of IL-10 was significantly reduced in the wound-healed granulation tissue of NREP$^{-/-}$ mice compared to WT mice [19]. Moreover, NREP has not been investigated in OA chondrocytes. For the above reasons, we included NREP as a predicted target gene of OA and designed the following basic experiments to investigate it.

4.2. NREP was downregulated in the damaged articular cartilage of OA patients

Cartilage specimens were obtained from OA patients who underwent TKA. As expected, the cartilage surface of the medial condyle was significantly worn and rough compared to the relatively intact cartilage of the lateral condyle. In addition, the intensity of Safranin O staining was relatively light in the median condyle cartilage, suggesting a

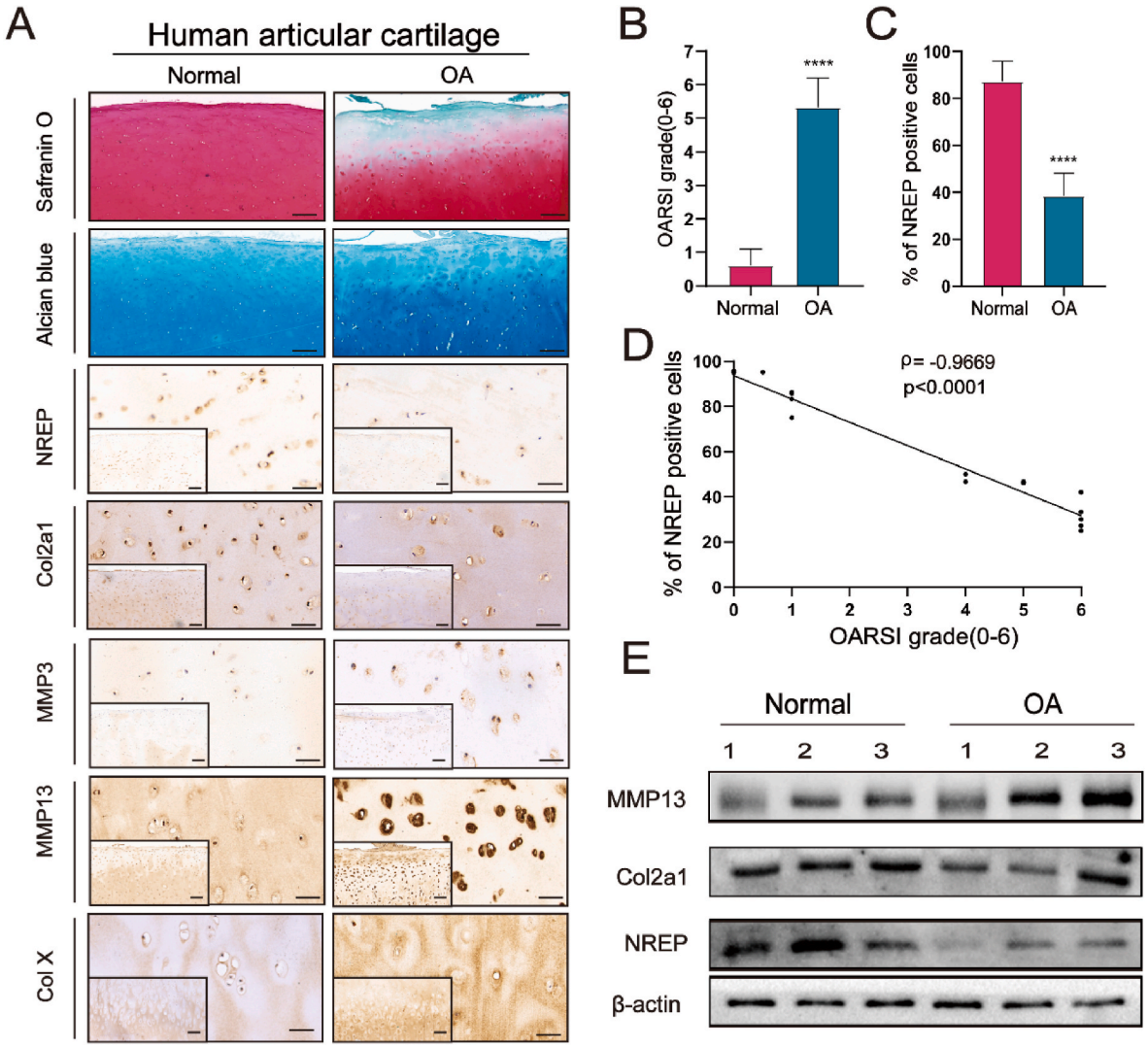


Figure 2. NREP was downregulated in the degenerated articular cartilage of patients with OA. (A) Representative images of Safranin O/Fast green, Alcian blue and immunohistochemical (IHC) staining of sections from intact and damaged knee cartilage from OA patients. Scale bars: Safranin O and Alcian blue staining - 200 μm, IHC - 50 μm (bottom of the panel) and 200um (top of the panel). (B) OARSI grading of human cartilage tissues. (C) Number of NREP-positive chondrocytes in the OA and control groups. (D) Scatter plot showing the correlation between NREP expression and OARSI grade in articular cartilage from OA patients. (E) Immunoblots showing NREP protein levels in the intact and damaged articular cartilage from OA patients. Data are expressed as mean ± SD (B and C). P values are from two-tailed unpaired Student's t-test (B, C) and two-tailed Spearman's rank correlation test (D). ****$P < 0.0001$.

significant loss of proteoglycan. Likewise, Alcian blue staining also suggested a reduction in the amount of cartilage and aggregated proteoglycans in the OA group. Consistent with the pathophysiological process of OA, the cartilage anabolic factor Col2a1 showed reduced expression in the affected tissues, whereas the expression of the catabolic factors MMP3 and MMP13, and the hypertrophic factor Col X were higher compared to that in the control tissues (Fig. 2A). The OA group also had a higher OARSI score compared to the control group (Fig. 2B). In addition, there were markedly fewer NREP positive chondrocytes in the damaged cartilage compared to the intact cartilage (Fig. 2C), and these cells were predominantly distributed in the transitional zone of the cartilage. NREP expression was localized to the cytoplasm of the chondrocytes. There was also a significant negative correlation between the OARSI score and the number of NREP-positive chondrocytes (Fig. 2D). The results of IHC were confirmed by western blotting (Fig. 2E and Supplementary Fig. S1A). Taken together, NREP expression is reduced in the degenerated cartilage from OA patients.

4.3. *NREP was downregulated in the in vitro and in vivo mouse models of OA*

To further validate the role of NREP in the pathogenesis of OA, we established a mouse model of OA through surgical DMM, and simulated chondrocyte inflammation *in vitro* using IL-1β.

DMM was performed on the right knee of the mice and samples of the articular cartilage were taken 4 and 8 weeks post-surgery. X-ray imaging revealed numerous osteophytes in the tibial plateau and tibial intercondylar ridge 4 and 8 weeks post-surgery, and the knee joint was significantly enlarged 8 weeks after the surgery. The sham-operated joints had fewer osteophytes (Fig. 3A). As shown in Fig. 3D, the osteophytosis score was significantly different among the three groups. At 4 weeks post DMM surgery, the cartilage surface was depressed and the Safranin O-stained regions were relatively pale and sparse compared to that in the sham-operated group. In addition, Alcian blue staining also showed degeneration of the articular cartilage. Surgical DMM significantly increased the OARSI score, and gradually decreased the cartilage area (Fig. 3B and C) and Col2a1 expression, and enhanced MMP13 expression (Fig. 3A) with the progression of OA. These pathological changes were indicative of the successful establishment of the OA model. Consistent with our findings in human cartilage specimens, the number of NREP-positive articular chondrocytes in the knee joint decreased significantly 4 and 8 weeks after surgical DMM, and NREP was primarily expressed in the cartilage transitional zone (Fig. 3E). Furthermore, the OARSI score was negatively correlated with the

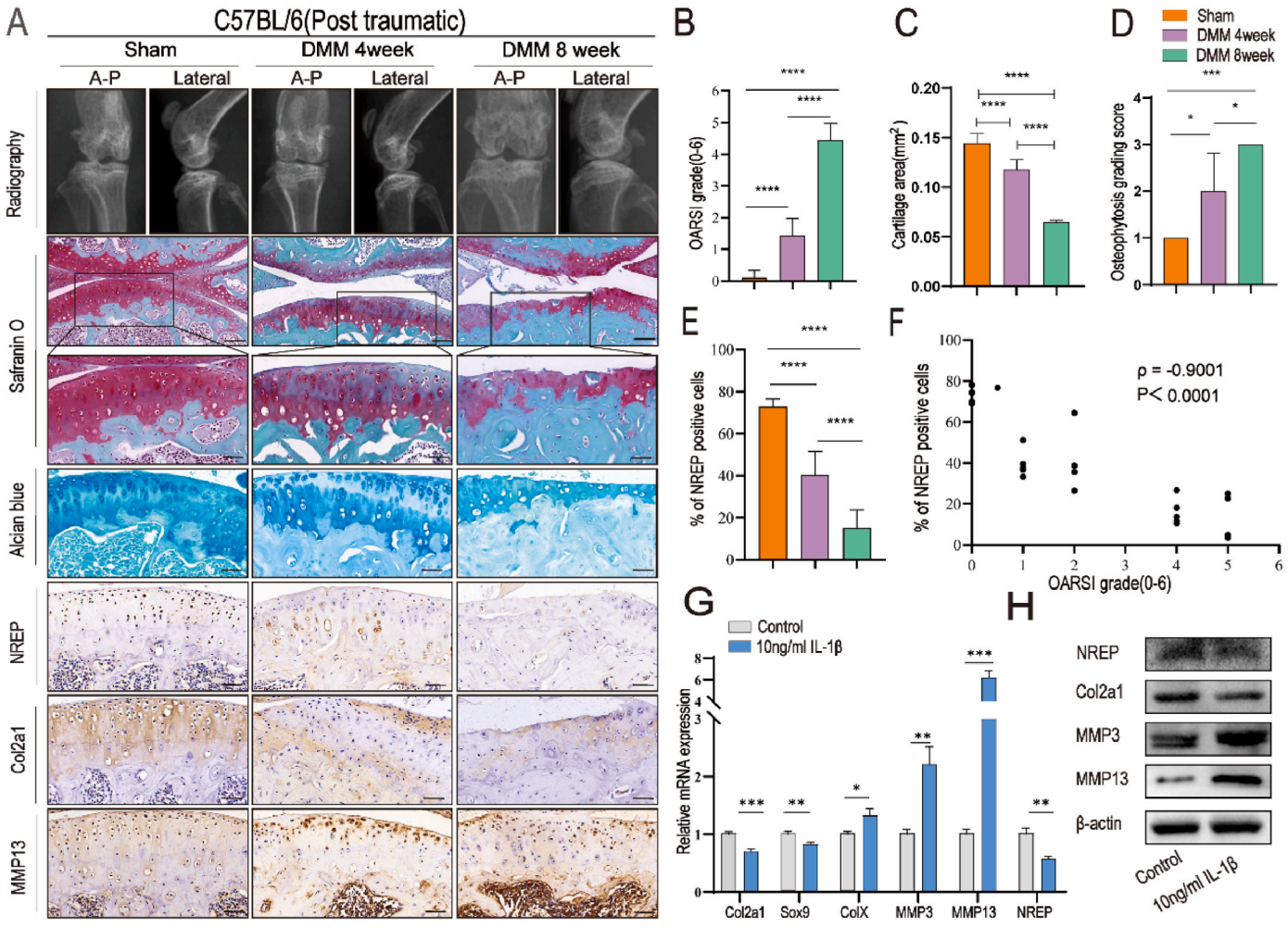


Figure 3. NREP expression was downregulated in the *in vivo* and *in vitro* OA models. (A) Representative X-ray images of the knee joints of control (sham-operated) and OA (4 weeks, 8 weeks after DMM surgery) mice, and representative images of cartilage sections with Safranin O/Fast green, Alcian blue and IHC staining. Scale bar: Safranin O - 100 μm (top) and 50 μm (bottom), Alcian blue and IHC - 50 μm. (B) OARSI scores of the cartilage, (C) cartilage area, (D) osteophytosis scores and (E) number of NREP positive cells in the indicated groups. (F) Scatter plot showing Spearman correlation between NREP expression and OARSI grade in knee cartilage. (G) RT-qPCR results and (H) immunoblots showing NREP mRNA and protein levels in normal and IL-1β-induced chondrocytes. Data are expressed as mean ± SD (B, C, D, E, G). *P* values are from one-way ANOVA with Tukey's multiple comparisons (B, C, D, E), two-tailed Spearman's rank correlation test (F), two-tailed unpaired Student's t-test (G). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

number of NREP-positive cells (Fig. 3F).

To simulate the inflammatory milieu of OA *in vitro*, ATDC5 chondrocytes were induced with 10 ng/ml IL-1β. The stimulated cells showed decreased expression of Col2a1 and Sox9, and increased expression of MMP3, MMP13 and Col X (Fig. 3G and H), which was consistent with the in-situ observations. Furthermore, NREP mRNA and protein levels were also significantly reduced following IL-1β induction (Fig. 3G and H and Supplementary Fig. S1B). The above findings suggested that the downregulation of NREP is related to the pathological changes in the cartilage during OA progression.

4.4. Knockdown of NREP disrupted the homeostasis in the chondrocyte matrix

To further assess the role of NREP in the pathogenesis of OA, we silenced the gene in chondrocytes using three siRNAs. As shown in Fig. 4A, si-NREP#2 and si-NREP#3 effectively decreased the expression of NREP compared to the control siRNA (si-NC), and si-NREP#3 led to the strongest knock-down. Therefore, si-NREP#3 (hereafter referred to as si-NREP) was used for the subsequent knock-down experiments. The mRNA expression levels of chondrocyte anabolic biomarkers (Sox9, Acan, Col2a1), catabolic biomarkers (MMP3), osteogenic markers (Runx2) and hypertrophic markers (Col X) indicated that knock-down of NREP promoted catabolism and hypertrophic differentiation of chondrocytes, and inhibited chondrocyte anabolism (Fig. 4B). Similar trends were observed with the cartilage anabolic markers at the protein level (Fig. 4C and D). Taken together, NREP maintains the balance between chondrocyte anabolism and catabolism, and its loss can induce articular cartilage damage by disrupting this balance.

4.5. Knockdown of NREP inhibited chondrocyte proliferation

Chondrocyte proliferation is closely related to articular cartilage destruction and matrix degradation during OA progression. Therefore, we also analyzed the role of NREP in chondrocyte proliferation. As shown in Fig. 5A, the proliferative marker proliferating cell nuclear antigen (PCNA) was significantly downregulated in the si-NREP group. Consistent with this, knock-down of NREP also decreased the viability of the cultured chondrocytes in the CCK8 assay, and significantly inhibited EdU incorporation (Fig. 5B–D), which are indicative of reduced proliferative capacity. Taken together, knock-down of NREP in chondrocytes can inhibit proliferation, thereby inducing OA.

4.6. NREP maintained cartilage homeostasis via the TGF-β1/Smad2/3 pathway

The TGF-β pathway is critical to maintaining articular cartilage homeostasis [20] and has been identified as a key signaling pathway in OA [21]. In this study as well, TGF-β pathway was significantly enriched among the DEGs between the OA and normal cartilage.

To further explore the possible regulatory mechanisms of NREP expression in OA, we analyzed the expression of TGF-β1 pathway

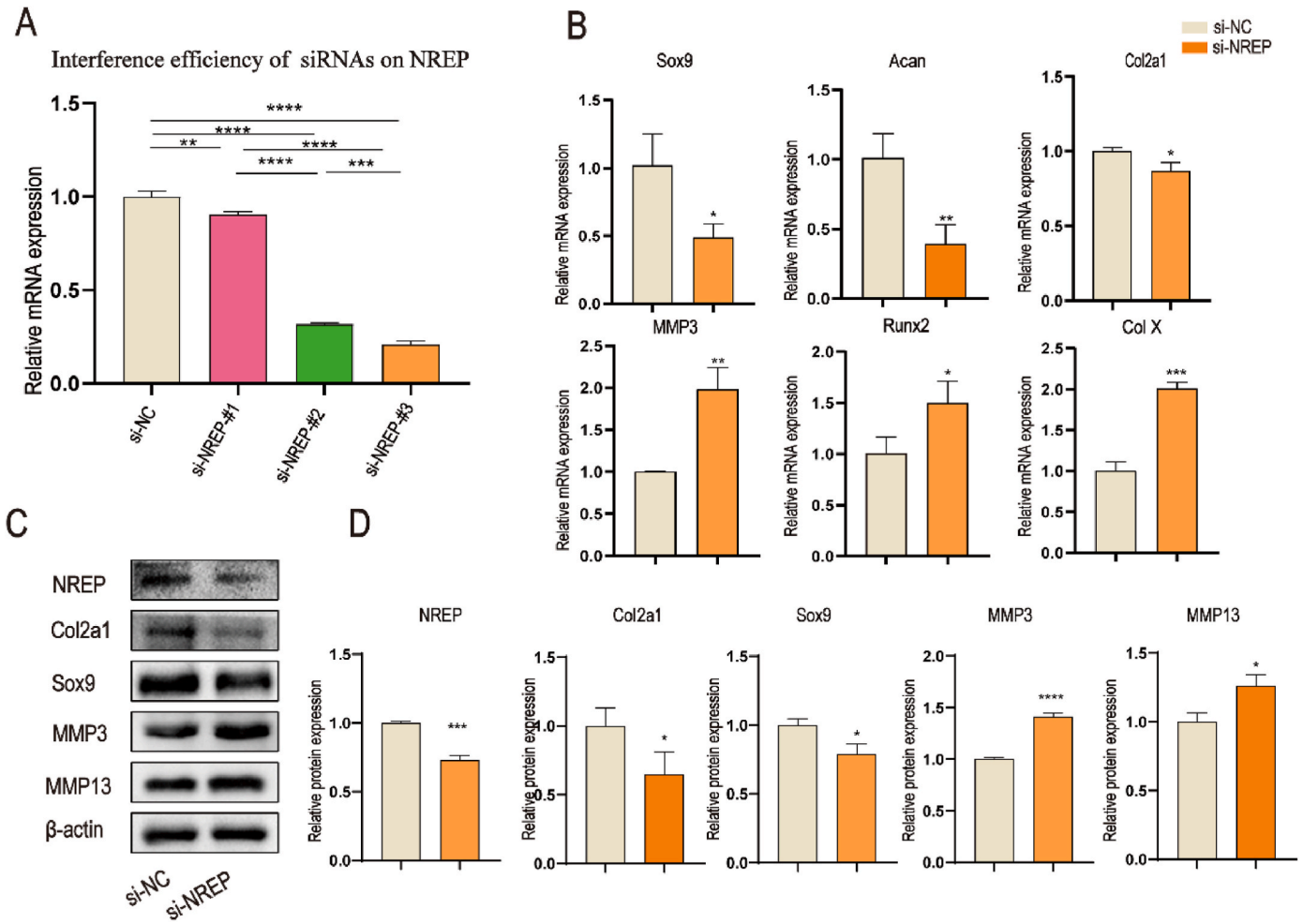


Figure 4. Knock-down of NREP impaired chondrocyte matrix homeostasis. (A) NREP mRNA levels in chondrocytes transfected with si-NC, si-NREP#1, si-NREP#2 and si-NREP#3. (B, C, D) Expression of cartilage-specific markers in the si-NREP and si-NC groups. Data are expressed as mean ± SD (A, B, D), n = 3. *P* values are from one-way ANOVA with Tukey's multiple comparisons (A), two-tailed unpaired t-test (B, D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

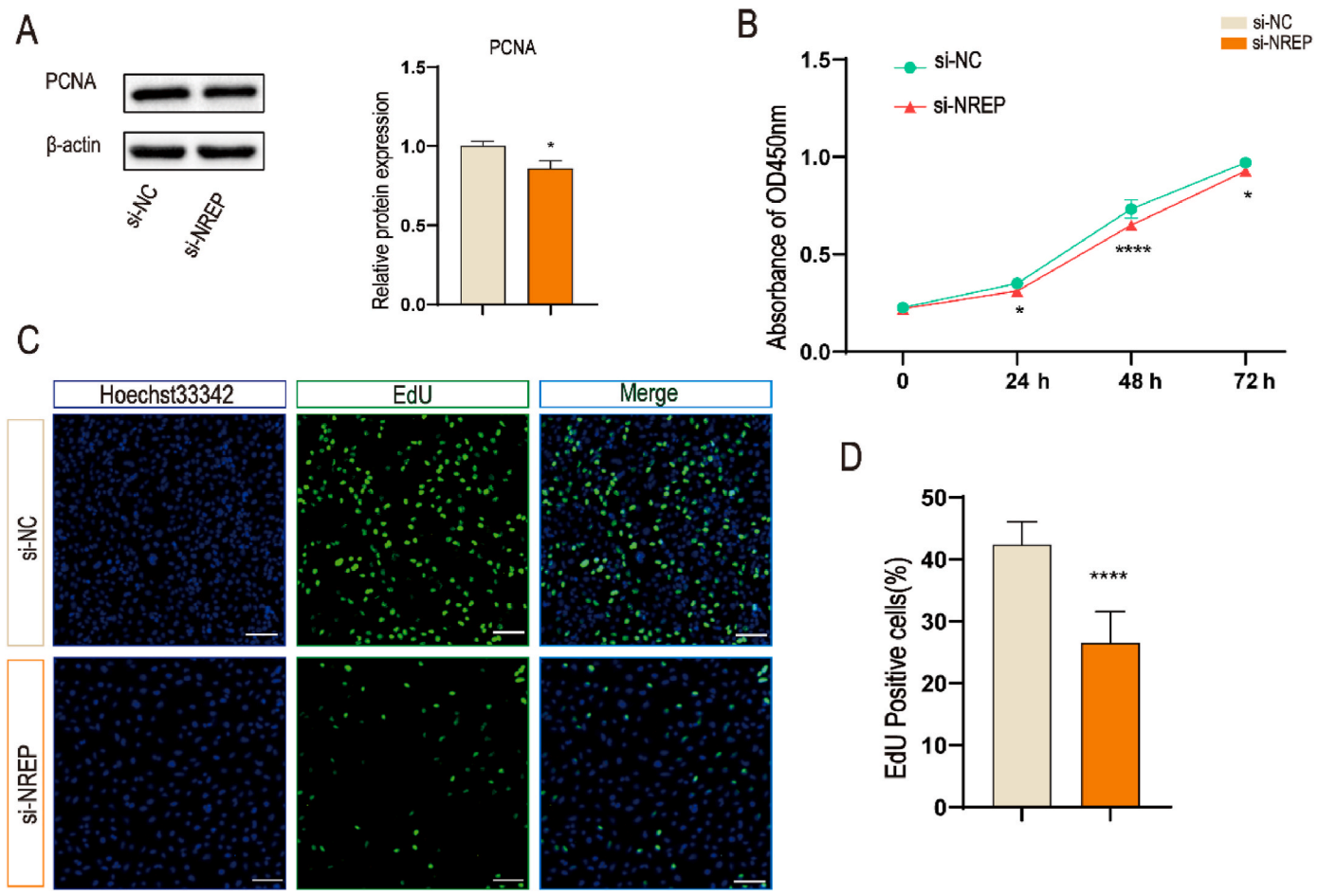


Figure 5. Knock-down of NREP inhibited chondrocyte proliferation. (A) Immunoblots showing PCNA expression in the si-NREP and si-NC groups (n = 3). (B) Viability of si-NC and si-NREP chondrocytes. (C) EdU incorporation in the si-NC and si-NREP cells. Cells stained with Hoechst 33,342 and EdU are actively proliferating. Scale bar: 20 μm. (D) Percentage of EdU-positive cells in the indicated groups. Data are expressed as mean ± SD (A, D). *P* values are from two-way ANOVA with Tukey's multiple comparisons (B), two-tailed unpaired t-test (D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

mediators in the normal and damaged cartilage from human and murine specimens. The in-situ expression of p-Smad2/3 was decreased in the worn cartilage from OA patients (Fig. 6A), and from mice that underwent surgical DMM (Fig. 6B) compared to the respective controls. Similar results were obtained for the IL-1β-induced chondrocytes (Fig. 6C and D) and the cartilage specimens from OA patients (Fig. 6E) through western blotting. Furthermore, TGF-β1 mRNA was significantly downregulated, whereas Smad2/3 mRNA levels were unaffected in the cells stimulated with IL-1β compared to the untreated controls (Fig. 6F). Interestingly, the expression levels of the TGF-β1 and Smad2/3 mRNAs were not significantly altered after knock-down of NREP (Fig. 6G), whereas TGF-β1 and p-Smad3 proteins showed a marked reduction (Fig. 6H). Taken together, knock-down of NREP impairs cartilage homeostasis by inhibiting the TGF-β1/Smad2/3 signaling pathway, thereby promoting OA.

5. Discussion

Osteoarthritis is a major public health concern worldwide [22] and imposes a considerable financial burden on individuals and society [23]. Sandell et al. showed that the progression of OA is associated with aberrant proliferation and death of chondrocytes, along with an imbalance between cartilage anabolism and catabolism, which lead to cartilage degeneration and bone redundancy [24]. In this study, we found that NREP expression is reduced in OA cartilage, which disturbs the homeostasis in the articular cartilage through inactivation of the TGF-β1/Smad2/3 pathway.

TGF-β inhibits the terminal differentiation of chondrocytes, which blocks cartilage matrix calcification and angiogenesis, thus maintaining the integrity of the extracellular matrix (ECM) [25]. Mice with chondrocyte-specific knockout of the TGF-β type II receptor (TGFβRII) gene show a significant reduction in articular cartilage area at 3 months of age, which progresses to the loss of the entire articular cartilage, significant bone redundancy and an increase in subchondral bone mass by 6 months of age [26]. In addition, mice lacking Smad3, the downstream target of TGF-β, exhibit progressive joint degeneration and osteophyte formation [27]. However, the target genes located upstream of TGF-β have not been elucidated so far. In the present study, we identified NREP as an upstream factor of the TGF-β pathway by bioinformatics analysis. NREP expression was significantly downregulated in the damaged cartilage from OA patients and the mouse model compared to the intact cartilage. Knock-down of NREP in a chondrocyte line significantly decreased the expression of Col2a1 and Sox9, and increased that of MMP3 and MMP13, which degrade collagen and A disintegrin [28]. Chondrocyte hypertrophy is an important pathological process in OA [29], and ColX, one of the specific markers of chondrocyte hypertrophy, increased expression after NREP knockdown. The above is the molecular basis of human OA pathogenesis.

Given the low regenerative capacity of the articular cartilage, improving its proliferative potential may slow the development of OA [30]. Knocking down NREP significantly inhibited chondrocyte proliferation, as indicated by the decreased expression of the PCNA, a DNA

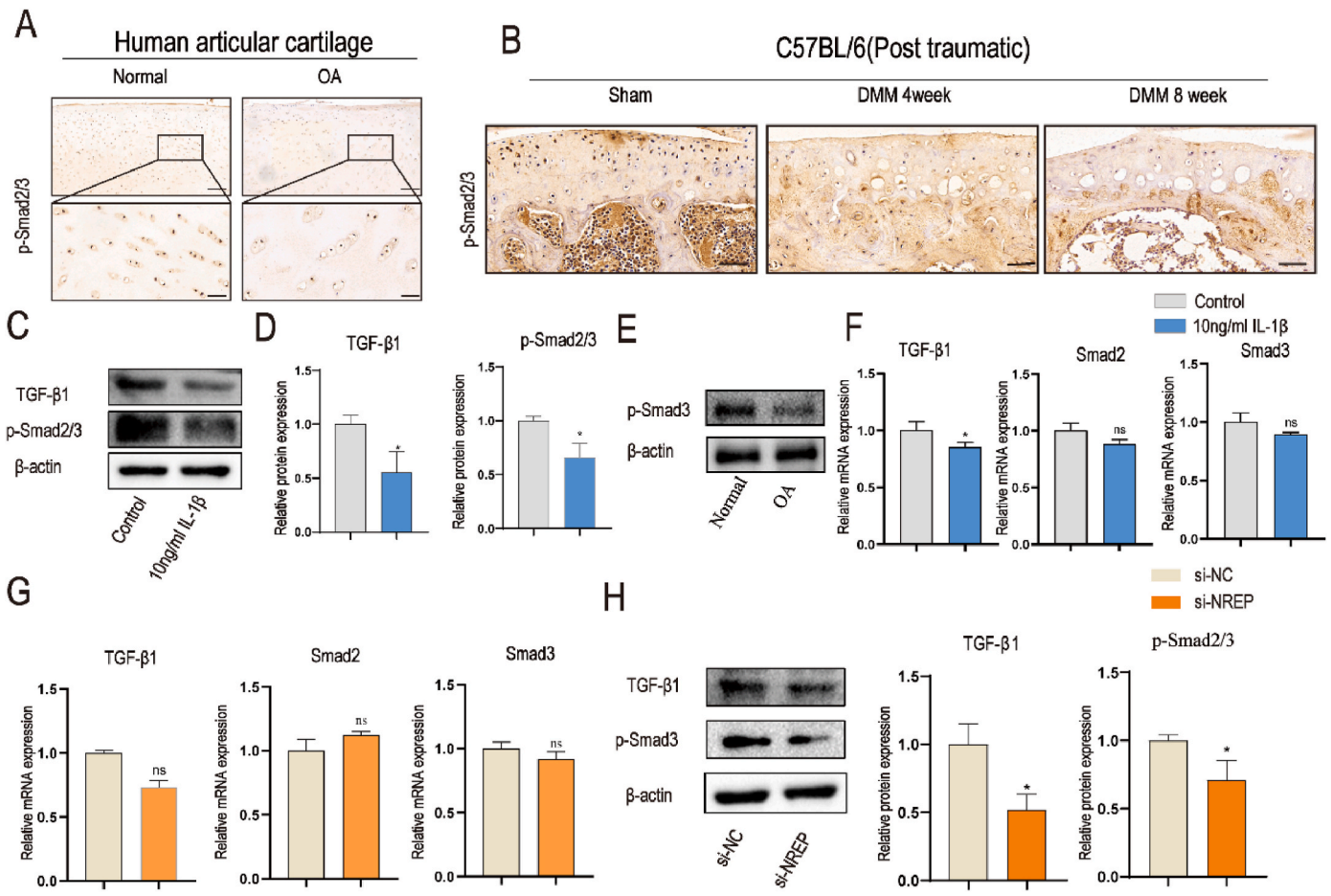


Figure 6. Knock-down of NREP disrupted articular cartilage homeostasis via the TGF-β1/Smad2/3 pathway. (A) Representative images of intact and damaged cartilage tissues from OA patients immuno-stained for p-Smad2/3. Scale bars: 200 μm (top) and 50 μm (bottom). (B) Representative images of knee cartilage tissues from sham-operated and OA (4 weeks, 8 weeks after DMM surgery) groups immuno-stained for p-Smad2/3. Scale bar:50 μm. (C) Immunoblots showing p-Smad2/3 levels in control and IL-1β-induced chondrocytes and (D) quantification of protein levels. (E) Immunoblots showing p-Smad3 levels in intact and damaged knee cartilage from OA patients. (F) TGF-β1, Smad2 and Smad3 mRNA levels in control and IL-1β-induced chondrocytes. (G) TGF-β1, Smad2 and Smad3 mRNA levels in the si-NREP and si-NC groups. (H) TGF-β1, p-Smad3 protein expression in the si-NREP and si-NC groups. Representative blots (n = 3). Data are expressed as mean ± SD (D, F, G, H), *P* values from two-tailed unpaired t-test (D, F, G, H). NS, not significant, $^{*}P < 0.05$, $^{****}P < 0.0001$.

polymerase δ co-protein involved in the coordination of DNA replication, cell division and proliferation [31]. Thus, targeting NREP can be of therapeutic value in OA since it regulates the proliferation of chondrocytes.

TGF-β1 is the predominant and most extensively studied isoform of TGF-β in animals [32], and the TGF-β1 pathway is differentially regulated in various tissues of the knee joint. SolRII can inhibit the generation of osteophytes by blocking the activity of TGF-β1 and β3 [33]. Furthermore, subchondral bone injection of TGF-βRI inhibitor (SB505124) can significantly reduce subchondral bone angiogenesis, proteoglycan loss and articular cartilage ossification [34]. TGF-β induces synovial tissue fibrosis, which is characterized by fibroblast proliferation and accumulation of type I and type III collagen [35]. Several studies have shown that the TGF-β1/Smad2/3 pathway plays an important role in the development of OA by regulating chondrocyte function and maintaining articular cartilage homeostasis [36,37]. We detected lower expression of p-Smad2/3 in the damaged articular cartilage of OA patients and mice, as well as in the IL-1β-stimulated chondrocytes, suggesting an important role of this pathway in maintaining joint homeostasis. Consistent with our results, Wang et al. found that p-Smad2/3 expression was decreased in severely degenerated cartilage tissue [38]. Furthermore, knock down of NREP decreased the expression of the TGF-β1 and p-Smad3 proteins in the cultured chondrocytes.

However, the TGF-β1/Smad2/3 transcripts were not significantly affected by knock-down of NREP. Likewise, knock-down of NREP did not affect TGF-β1 gene levels in the $NREP^{-/-}$ mice with unilateral ureteral obstruction (UUO)-induced renal fibrosis compared to that in the $NREP^{+/+}$ counterparts, whereas the TGF-β1/Smad2/3 proteins were significantly downregulated [15]. However, Tan et al. found that NREP overexpression in human fibroblasts upregulated TGF-β1 mRNA and its knock-down had the opposite effect [39]. NREP promoted TGF-β1 translation in the lung tissues without affecting the regulation at the transcriptional level [40]. In the vascular smooth muscle cells however, NREP inhibited TGF-β1 transcription but promoted translation of TGF-β1, p-Smad2 and p-Smad3 by interacting with the TGF-β 5′-UTR [41]. These conflicting results suggest that NREP may promote TGF-β1 expression at the post-translational level depending on the tissue and cell type.

A major limitation of this study is that our results are based on *in vitro* experiments, and will have to be validated in NREP knock-out or knock-down mice. Our future studies will focus on the regulatory role of NREP in OA using these experimental approaches.

6. Conclusion

NREP maintains articular cartilage homeostasis via the TGF-β1/Smad2/3 signaling pathway, and is a potential therapeutic target for

OA.

Ethics statement

All animal procedures were performed in accordance with the Guidelines for care and Use of Laboratory Animals of the National Institutes of Health and approved by the Animal Ethics Committee of the Second Hospital of Shanxi Medical University. All human samples were extracted in accordance with the approved guidelines which meet the standard of the Declaration of Helsinki and approved by the Ethics Committee of the Second Hospital of Shanxi Medical University.

Credit author statement

Conceptualization and design of study: Yang Liu, Ziquan Yang; Data curation: Yang Liu, Mengrou Liu; Methodology: Chengming zhang; software: Xiaoke Li, Peidong Liu, Siyu Zheng, Le Wen; funding acquisition: Ziquan Yang, Pengcui Li.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2023.11.004.

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