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TCF12 regulates the TGF-β/Smad2/3 signaling pathway to accelerate the progression of osteoarthritis by targeting CXCR4

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ABSTRACT

Objective: Osteoarthritis (OA), which involves total joint damage and dysfunction, is a leading cause of disability worldwide. However, its exact pathogenesis remains unclear. Here, we identified TCF12 as an important regulator of the progression of OA.

Methods: qRT-PCR, immunoblotting and immunohistochemistry (IHC) were used to detect the expression level of TCF12. The interaction of TCF12 with its downstream factor CXCR4 was assessed by Western blotting, immunofluorescence, qRT-PCR and luciferase assays. A mouse model was generated to examine the functions and mechanism of TCF12 in vivo.

Result: TCF12 expression was upregulated in chondrocytes stimulated with IL-1β and osteoarthritic chondrocytes. TCF12 upregulates the expression of CXCR4 and leads to dysfunction of the TGF-β signaling pathway. Furthermore, knockdown of TCF12 alleviated cartilage damage in a mouse model generated by destabilization of the medial meniscus (DMM).

Conclusion: TCF12 aggravates the progression of OA by targeting CXCR4 and then activating the TGF-β signaling pathway, suggesting that TCF12 may be a new target for the treatment of OA.

The translational potential of this article: Transcription Factor 12(TCF12), is known to regulate cell development and differentiation, It has been widely studied in various organs and diseases, but its role in OA remains unclear. Here, we identified Transcription Factor 12(TCF12) as an important regulator mediating chondrocyte senescence and cartilage extracellular matrix degradation indicating its role in OA. We found that TCF12 expression was upregulated both locally and systemically as OA advanced in patients with OA, and in mice after DMM surgery to induce OA. TCF12 expression caused striking progressive articular cartilage damage, synovial hyperplasia in OA mice, and remarkably, it was relieved by intra-articular administration of mutant mouse TCF12 lentiviral vector (shTCF12). Furthermore, TCF12 upregulated the expression of CXCR4, leading to exacerbation of experimental OA partially through activation of TGF-β signaling in chondrocytes. TCF12 expression was upregulated in chondrocytes treated with IL-1β and osteoarthritic chondrocytes. Our findings established an essential role of

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TCF12 in chondrocyte senescence and cartilage extracellular matrix degradation during OA, and identified intra-articular injection of TCF12 as a potential therapeutic strategy for OA prevention and treatment.

1. Introduction

Osteoarthritis (OA) is a chronic and highly prevalent joint disease, leading to chronic pain, joint stiffness, and physical disability in aging population. It begins with destructive changes in the cartilage and progresses to joint space narrowing, synovial inflammation, subchondral bone sclerosis and osteophyte formation, with the manifestations of joint pain, deformity and dysfunction [1–3]. Present OA management is broadly divided into nonpharmacological, pharmacological, and surgical treatments [4–6]. Nonpharmacological treatments, such as exercise, weight loss, and physical therapy, are recommended for early-stage OA patients. Pharmacological treatments are mainly focused on pain management with analgesics and anti-inflammatory medication. Surgical treatment is most widely used for patients in the late phase of the disease process [7,8]. However, the specific mechanisms leading to OA have not been fully elucidated, Current OA treatments are limited and insufficient to prevent the initiation and progression of the disease. Thus, further study on pathogenesis of the disease and exploring new therapeutic strategies is of great clinical significance.

The underlying mechanism of OA has not yet been fully clarified, and It is generally accepted that an imbalance in ECM homeostasis triggers osteoarthritic cartilage destruction. Intact ECM is essential for the normal weight-bearing functions of articular cartilage. In addition, the ECM regulates most cellular behaviors and is required for various developmental processes [9]. However, in OA, many matrix-degrading enzymes, including matrix metalloproteinases (MMPs) and metalloproteinases containing thrombospondin motifs (ADAMTS), show significantly upregulated expression, thus accelerating remodeling and loss of flexibility of the ECM even before the onset of cartilage destruction [10]. Another cellular process related to ECM degradation and chondrocyte abnormalities is senescence. Cellular senescence is characterized by cell cycle arrest, metabolic changes, and loss of proliferative capacity. Senescent chondrocytes exhibit an impaired regenerative capacity and loss of the normal phenotype. Moreover, aging chondrocytes manifest a senescence-associated secretory phenotype (SASP) characterized by enhanced secretion of proinflammatory factors such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α), increased production of ECM-degrading enzymes, and accumulated oxidative stress, which exacerbate cartilage damage and promote the development of OA. Senescent chondrocytes exhibit a suppressed regenerative capacity and a loss of original phenotype. Moreover, aging chondrocytes manifest a SASP characterized by enhanced secretion of proinflammatory factors such as IL-1β, IL-6, and TNF-α, increased production of ECM degrading enzymes, and accumulated oxidative stress, which exacerbate cartilage damage and promote OA development.

The basic helix-loop-helix (bHLH) family of transcription factors contains two distinct motifs, the HLH domain and adjacent basic regions, and is involved in cell growth and differentiation processes, including myogenesis, neurogenesis, lymphocyte and osteoblast differentiation [11–13]. The bHLH protein family can be divided into nontissue-specific expressed class A proteins and tissue-specific expressed class B proteins [14]. Class A proteins, also known as E proteins because of their ability to bind directly to DNA (E-box, CANTG), are composed of TCF3, TCF4, and Transcription Factor 12(TCF12). TCF12 can form homodimers or heterodimers with other family members to regulate cell development and differentiation in various tissues [15–18]. TCF12 has been shown to be involved in osteogenic differentiation. In our previous study, it was found that TCF12 was up-regulated in rat OA patients [19]. However, the role of TCF12 in OA and its molecular mechanism have not been reported. The transforming growth factor beta (TGF-β) family includes not only TGF-βs but also activins and bone morphogenetic proteins (BMPs), which play important roles in tissue development and stability of the internal environment, regulate cell proliferation, differentiation, apoptosis, and metastasis and control the synthesis and decomposition of the ECM. Increasing evidence has shown that TGF-β plays a crucial role in maintaining the homeostasis of articular cartilage and subchondral bone [20–22].

In the present study, significant upregulation of TCF12 expression was detected under osteoarthritic conditions. Therefore, we hypothesized that TCF12 may play an important role in the development of OA. To validate this hypothesis, we determined the destructive effects of TCF12 in primary murine chondrocytes and in surgically induced OA mice. Upregulation of TCF12 expression aggravated OA, whereas inhibition of TCF12 had a therapeutic effect. In addition, we found that TCF12 regulates TGF-β activation, while upregulated TCF12 expression in OA significantly promotes CXCR4 expression. The TCF12/CXCR4/TGF-β regulatory axis was therefore established in an experimental mouse model of OA.

2. Results

2.1. TCF12 expression is upregulated in osteoarthritic cartilage

To determine the expression level of TCF12, we extracted protein and RNA from normal articular cartilage of patients who underwent post-traumatic osteotomy (NC) and from damaged areas of articular cartilage from patients with OA (OA) and detected the expression levels of TCF12. TCF12 expression was significantly upregulated in the cartilage of the OA patients (Fig. 1A). In addition, the expression levels in the damaged areas of articular cartilage and normal cartilage in the patients with post-traumatic osteotomy were detected by IHC. The damaged osteoarthritic cartilage exhibited significant proteoglycan loss compared to the normal cartilage, as indicated by reduced safranin O staining and significantly increased TCF12 expression in the injured areas (Fig. 1C). We incubated mouse chondrocytes with IL-1β. We found that IL-1β stimulate of mouse chondrocytes increased TCF12 expression in a dose-dependent manner (Fig. 1B). In addition, after we stimulated mouse cartilage explants with IL-1β, we examined the expression of TCF12 by IHC and found that it was upregulated in the IL-1β-stimulated group (Fig. 1D). To determine whether the upregulation of TCF12 expression and increased cartilage degeneration remained consistent in a DMM surgery-induced experimental mouse model of OA, we performed IHC analysis and found that the number of TCF12-positive chondrocytes was strongly increased in the osteoarthritic cartilage compared with the sham-treated cartilage (Fig. 1E). These results indicate that TCF12 expression is upregulated in inflammatory chondrocytes and knee osteoarthritic cartilage.

2.2. TCF12 promotes IL-1β-induced ECM degradation

The expression of TCF12 is elevated in OA, suggesting that TCF12 may be involved in the pathogenesis of OA. To test this hypothesis, we transfected TCF12-overexpressing or TCF12-silencing viruses into chondrocytes and assessed the effect of TCF12 on IL-1β-induced ECM degradation. As expected, transfection of chondrocytes with the TCF12-interfering virus disrupted the stability of TCF12 and resulted in decreased TCF12 levels, whereas after transfection with the TCF12-overexpressing virus, TCF12 expression was substantially elevated compared with that of the cells transfected with the control (Fig. 3B). To determine whether TCF12 in chondrocytes contributes to ECM

degradation and cellular senescence, we treated primary chondrocytes with or without IL-1β in the presence of TCF12 overexpression or interfering virus. In the TCF12-silenced group, Col2a1 expression was significantly increased, while Mmp13 expression was suppressed (Fig. 2A). In contrast, TCF12 overexpression led to downregulation of Col2a1 expression and upregulation of Mmp13 expression (Fig. 2B). WB further confirmed the role of TCF12 in ECM metabolism (Fig. 2C and D). To investigate the role of TCF12 in IL-1β-induced chondrocyte senescence, we examined senescence-associated proteins, including P16, P21, and P53. The results showed that TCF12 overexpression increased the expression of senescence-associated proteins, while TCF12 knockdown decreased P16, P21, and P53 expression (Fig. 2E and F). The role of TCF12 in chondrocyte senescence was further confirmed by β-galactosidase staining (Fig. 2G). In addition, the results of confocal microscopy suggested that TCF12 could reverse the nuclear translocation of P16 caused by IL-1β treatment (Fig. 2H). Together, these results indicate that the interfering with the expression of TCF12 can partially reverse IL-1β-induced ECM degradation and senescence.

2.3. TCF12 directly targets CXCR4

To elucidate the key molecules involved in the promotion of OA by TCF12, we predicted the target genes of TCF12 by the public databases TRANSFAC, MotifMaph and TFtarget and then plotted the Venn graphic intersection of the list of target genes found in the above databases (Fig. 3A). Subsequently, we screened target genes associated with OA and further examined them with RT-qPCR and Western blots. The results confirmed that TCF12 overexpression significantly increased CXCR4 expression (Fig. 3B and C). Given the critical role of CXCR4 in OA progression [23,24], we investigated the role and underlying mechanism of CXCR4 in promoting the osteoarthritic effect of TCF12 in OA, which had a high correlation with CXCR4 according to the results of GEPIA database analysis (Fig. 3E). Next, the IF results showed that CXCR4 expression was substantially increased in the cartilage of the mice exposed to DMM (Fig. 3D). To further determine whether CXCR4 is a direct target of TCF12, we used the JASPAR database (http://jaspar.net/) to identify potential TCF12 binding sites on the CXCR4 promoter. According to JASPAR target prediction, there were multiple binding sites between mouse CXCR4 and TCF12, and the four with the highest scores were selected for dual-luciferase experiments (Fig. 3F). Dual-luciferase reporter gene expression analysis showed that TCF12 significantly decreased the reporter fluorescence of CXCR4 vectors, indicating that this interaction had a constraining effect; the reporter fluorescence in mutant vectors 1 and 4 did not change significantly after mutation of the corresponding binding site. In this experimental model, the sites of mutant vectors 1 and 4 were nonregulatory sites, and the degree of fluorescence inhibition of mutant vectors 2 and 3 was significantly reduced, indicating that these two sites are binding sites for TCF12 and CXCR4. chip qPCR further proved the conclusion of double luciferase. In order to ensure the reliability of chip experiment, we designed two pairs of detection primers, both of which could amplify the target group Because we set up the negative and positive controls of IgG,

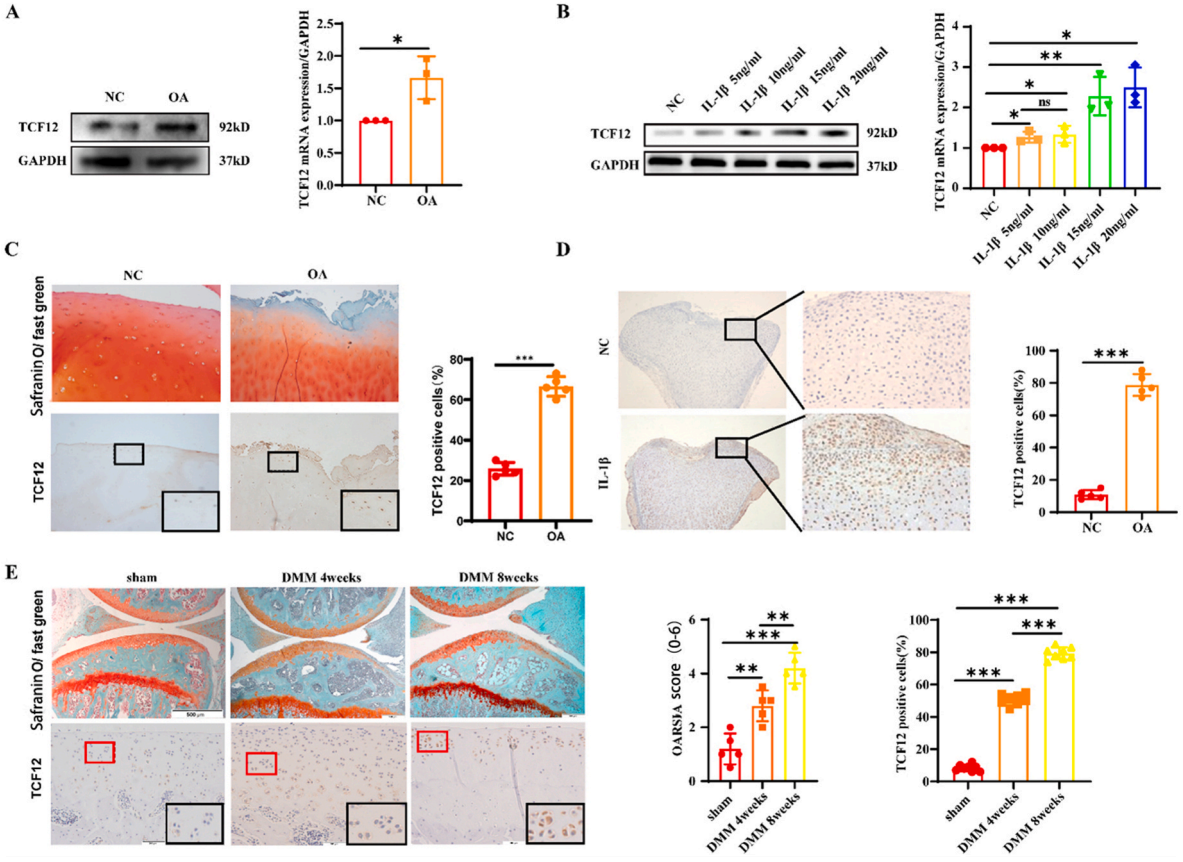

Fig. 1. Upregulation of TCF12 expression in IL-1β-stimulated mouse chondrocytes and osteoarthritic cartilage(A) WB and qRT-PCR analysis of protein and RNA samples extracted from human cartilage (B) WB and qRT-PCR analysis of TCF12 expression in osteoarthritic patient cartilage stimulated with 0, 5, 10, 15 or 20 ng/mL IL-1β for 24 h (C) Safranin O/fast green staining (upper panel) and IHC (lower panel). Scale: 200 μm (D) IL-1β at 50 ng/mL stimulated mouse cartilage explants for 14 days, TCF12 expression was verified by IHC (left panel), and quantification of TCF12 in the IL-1β-stimulated cartilage explants was performed (right panel) (E) Safranin O and fast green staining (upper) and immunostaining of TCF12 (lower) in sagittal sections of knees from the controls and mice with OA at 4 weeks and 8 weeks after DMM. Scale bar: 200 μm. ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are shown as means ± SEM of three independent experiments in (A)and (E). Student's t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

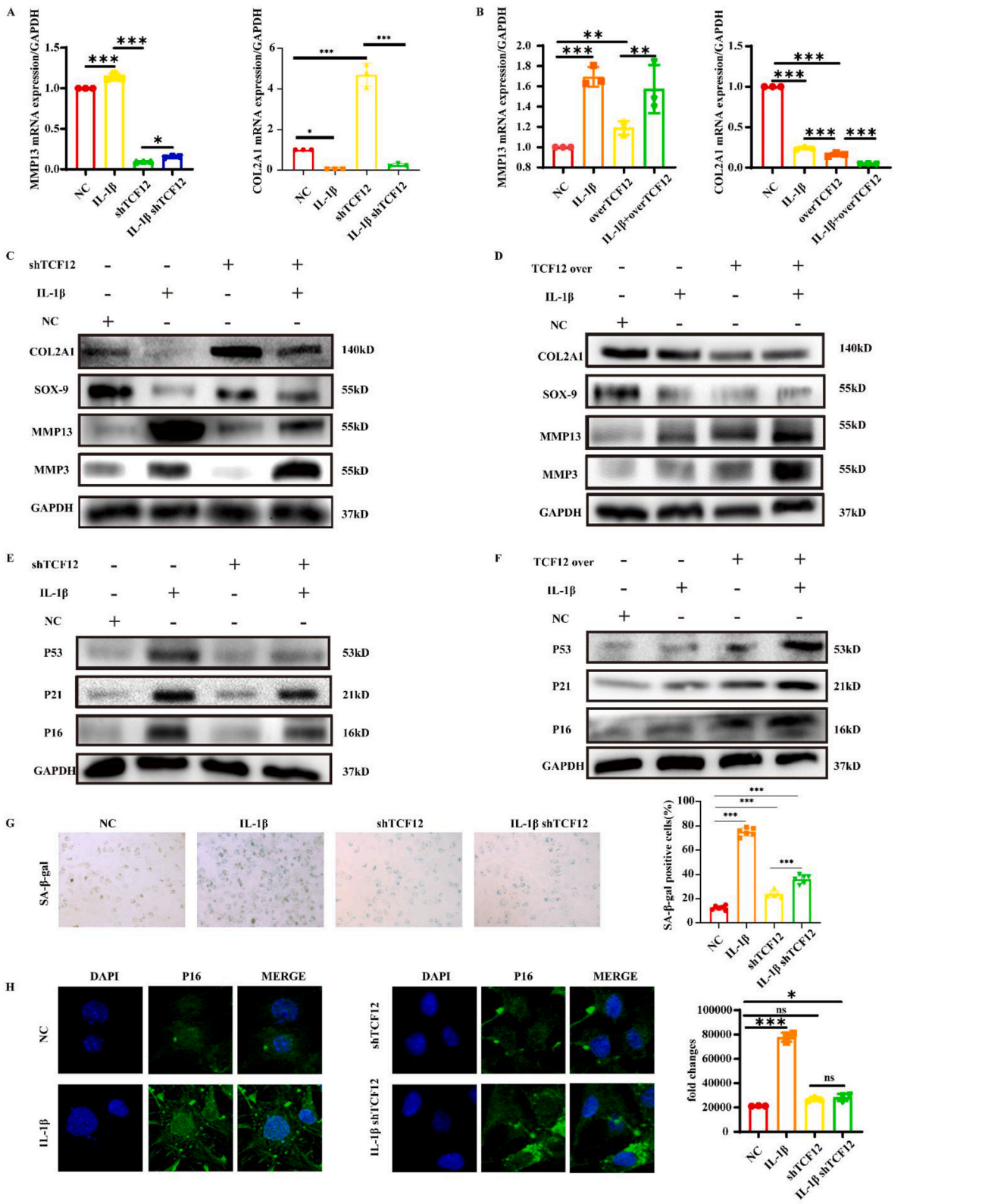

Fig. 2. TCF12 promotes IL-1β-induced cartilage ECM degradation and senescence (A, B, C, D) Mouse chondrocytes were transfected with TCF12-interfering virus or overexpressing virus. The effect of TCF12 levels in mouse chondrocytes on COL2A1, SOX9, and MMP3 expression was assessed by WB and qRT-PCR in the presence or absence of IL-1β stimulation (E, F) The effect of TCF12 levels in mouse chondrocytes on P16, P21, and P53 expression was assessed by WB in the presence or absence of IL-1β stimulation (G) IL-1β and TCF12 interfered with β-galactosidase staining in virus-treated primary mouse chondrocytes (H) IL-1β and TCF12 interfered with virus-treated primary mouse chondrocytes, and the effect of TCF12 levels on P16 nuclear translocation in mouse chondrocytes was assessed by confocal microscopy (left) and a P16 fluorescence quantification map (right). ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are shown as means ± SEM of three independent experiments in (A) (B) (G) and (H). Student's t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

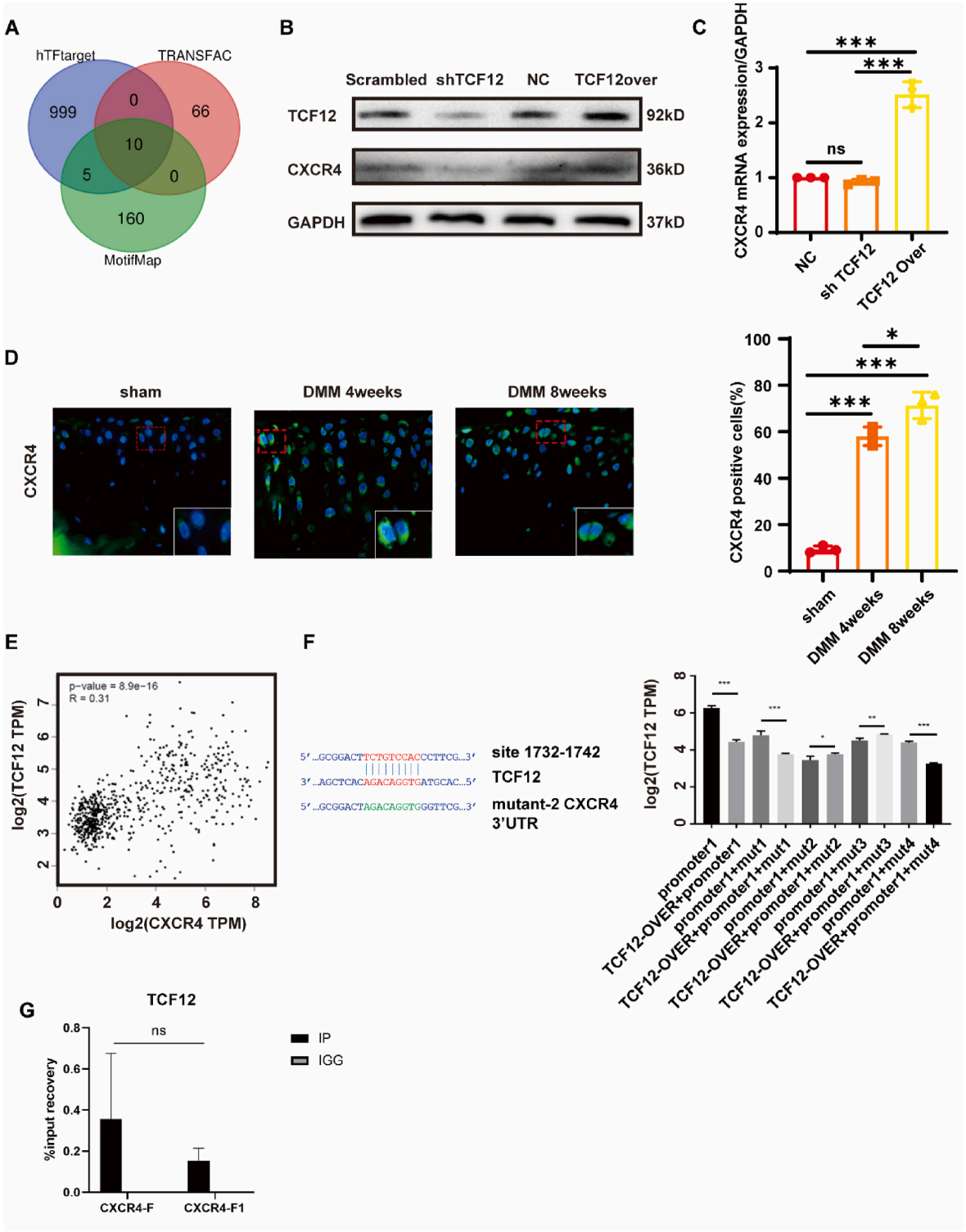

Fig. 3. CXCR4 was identified as a direct target of TCF12 in chondrocytes (A)Venn diagram showing the overlap of TCF12 target genes (B) Western blot analysis of CXCR4 protein levels in chondrocytes after TCF12 inhibition or overexpression, and the data were normalized with GAPDH (C) qRT-PCR analysis of CXCR4 RNA levels in chondrocytes after TCF12 inhibition or overexpression (D) IF analysis (left) and quantitative data (right) of cartilage CXCR4 in the mouse model of OA (E) Correlation analysis between TCF12 and CXCR4 via the GEPIA database (F) The binding site sequence of TCF12 to CXCR4. TCF12 overexpression vectors with 3′ UTR wt or 3′ UTR mut were cotransfected into HEK-293T cells. Luciferase activity was measured after transfection. Luciferase reporter assays showed that TCF12 decreased the luciferase activity of the wild-type reporter plasmid (G)The binding of TCF12 to CXCR4 promoter was detected by chip-qPCR. ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are shown as means ± SEM of three independent experiments in (C) (D) and (F). Student's t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

it can be seen that the negative control of IgG did not amplify the target gene Fragment. According to the amplification curve, it can be seen that both Input and TCF12 IP products can amplify the target gene, indicating good specificity of the experiment (Fig. 3G) .Together, our data show that TCF12 binds to CXCR4 promoter region.

2.4. TCF12 activates the TGF-β signaling pathway

We next sought to identify the TCF12-derived pathways responsible for chondrocyte senescence and macrophage reprogramming during OA. The TGF-β signaling pathway is a well explored inflammatory pathway. TCF12 functions with SMAD2/3 at distal enhancer elements and also directly associates with PRC2 at a subset of developmental promoters that are important for mesoderm and endoderm (ME) formation [1] Therefore, we hypothesize that TCF12 may play an important role in OA development through activation of TGF-β. To examine activation of the TGF-β pathway, we next measured phosphorylation of Smad2 in mouse primary chondrocytes after transfection with the TCF12-interfering virus or overexpression virus. The results showed that IL-1β substantially enhanced the expression of P-Smad2, whereas TCF12 interference significantly inhibited P-Smad2 phosphorylation (Fig. 4A). TCF12 overexpression also significantly increased P-Smad2 expression (Fig. 4B). In addition, IF results confirmed the role of TCF12 in activating the TGF-β signaling pathway (Fig. 4C). We confirmed that TCF12 activates the TGF-β signaling pathway by enhancing the phosphorylation of Smad2 and then promotes OA with the TGF-β signaling pathway-specific inhibitor ITD-1 , It does not inhibit the kinase activity of TGFBR1 or TGFBR, but effectively inhibits TGFβ2-induced phosphorylation of the effector SMAD2/3. ITD-1 was added after overexpression of TCF12, and the chondrocyte degeneration caused by overexpression of TCF12was ameliorated by ITD-1 (Fig. 4D and E). Together, these results indicate that TCF12 significantly increases TGF-β pathway activation in chondrocytes stimulated by IL-1β.

2.5. The biological functions of TCF12 are mediated by targeting CXCR4 in chondrocytes

Next, we performed rescue experiments to determine whether the effect of TCF12 expression on the OA phenotype was achieved via CXCR4. Chondrocytes were transfected with siRNA (siCXCR4-1 and siCXCR4-2) to successfully inhibit CXCR4 expression (Fig. 5A). Cotransfection of the TCF12-overexpressing virus and siCXCR4 into chondrocytes was performed, and the qRT-PCR results suggested that siCXCR4 reversed the effects of TCF12 on downregulation of COL2A1 and SOX9 expression and increased MMP3 and MMP13 expression, and changes in the protein levels of these ECM anabolic and catabolic markers also supported this reversal, as shown by WB analysis (Fig. 5B and C). In addition, β-galactosidase staining showed increased β-galactosidase-positive cells in the IL-1β and TCF12 overexpression groups compared with the control group, while β-galactosidase-positive cells were significantly decreased in the siCXCR4 cotransfection group (Fig. 5D). SiCXCR4 reversed TGF-β activation by TCF12 after cotransfection of the TCF12-overexpressing virus and siCXCR4 into chondrocytes (Fig. 5E). Thus, our data suggest that TCF12 promotes ECM catabolism and chondrocyte senescence by targeting CXCR4, which in turn activates the TGF-β signaling pathway.

2.6. TCF12 promotes progression of OA

For further determination of the role of TCF12 during OA progression, 10-week-old male C57 mice were intra-articularly injected with TCF12-interfering virus or TCF12-overexpressing virus once a week after DMM surgery (Fig. 6A). of note, the overexpression virus resulted in significantly higher OA scores than that of the sham group and resulted in fewer chondrocytes, more severe cartilage erosion, and loss of proteoglycan at 4 and 8 weeks after DMM surgery, as demonstrated by the OARSI scale and HC/CC (Fig. 6B and Fig. S1B). Strikingly, compared to those of the controls, high levels of synovial hyperplasia and abundant cell infiltration were observed in the synovial tissue of the mice with OA treated with the TCF12-overexpressing virus, combined with significantly higher synovitis scores at 8 weeks after surgery (Fig. S1C). Silencing with TCF12 expression prevented the loss of ECM in osteoarthritic cartilage (Fig. 6C, D and Fig. S1A). These findings suggest that TCF12 plays an important role in OA pathology. In general, TCF12 overexpression aggravates OA, whereas interfering with TCF12 expression reverses the OA phenotype (Fig. 6E).

3. Discussion

In this study we demonstrated for the first time that TCF12 is a key factor mediating chondrocyte senescence and cartilage extracellular matrix degradation during the pathogenesis and progression of OA. We showed that TCF12 expression markedly upregulated CXCR4, which in turn exacerbated cartilage degeneration, synovial inflammation during OA partly through TGF-β signaling. Our findings demonstrated a functional pathway important for OA development and identified intra-articular injection of TCF12 as a potential therapy for OA prevention and treatment.

Aging has long been recognized as a major risk factor for arthritis [23,25]. Evidence showed that senescent chondrocytes (SnCs) aggregate with age and are markedly increased in human OA cartilage compared with healthy controls. In addition, excessive SnC accumulation can lead to severe destruction of articular cartilage, while removal of SnCs reduces surrounding cell and tissue damage, thereby delaying the progression of OA [24]. In addition, SnCs release large amounts of SASP molecules that can digest the ECM and trigger further inflammation, which is thought to be a major driver of aging-induced OA progression [26]. These findings suggest that aging is closely linked to the severity of OA, but the regulatory mechanism of SnCs in OA is unknown. In our study, we found that TCF12 significantly increased SA-β-gal-positive cells. Remarkably, inhibition of TCF12 effectively reversed the enhanced SnC markers, including p16, p53, and p21, in articular cartilage of mice subjected to DMM. These results indicate that TCF12 regulates chondrocyte senescence, which may be related to the pathogenesis of OA.

In addition, the chemokine receptor (CXCR) plays a crucial role in regulating the development and progression of OA. Several mechanisms by which CXCR regulates OA pathology have been well studied [27–29]. We hypothesize that TCF12-induced chondrocyte senescence in OA is due to aberrant expression of CXCR4. In the present study, we validated the role of CXCR4 in OA pathogenesis. Higher levels of CXCR4 were detected in osteoarthritic cartilage, and knockdown of CXCR4 expression reversed the effects of overexpression of TCF12, leading to cartilage degradation and chondrocyte senescence. Dual-luciferase reporter assays and CHIP qPCR are demonstrated that TCF12 directly binds the promoter of CXCR4, suggesting that TCF12 plays a crucial role in OA by binding and promoting CXCR4.

In previous studies, intra-articular gene delivery systems mainly included lentiviruses, adenovirus vectors, nanoparticles, and enucleated collagen [30–33]. These biological drug delivery approaches have good safety and target effects. In this study, shTCF12, a specially modified short hairpin RNA, was administered intra-articularly in a mouse model of OA induced by DMM. Our results indicate that interfering with TCF12 expression can ameliorate surgically induced OA in mice. Thus, our study suggests that TCF12 vectors may be a promising strategy for the treatment of OA. Further studies will be conducted to confirm its therapeutic potential in humans.

In conclusion, our findings broaden the potential clinical application of TCF12. In chondrocytes, TCF12 increases the expression of CXCR4, leading to cartilage degeneration by targeting chondrocyte senescence, which initiates and promotes OA development. Intra-articular interference with TCF12 expression is a strategy to delay OA progression.

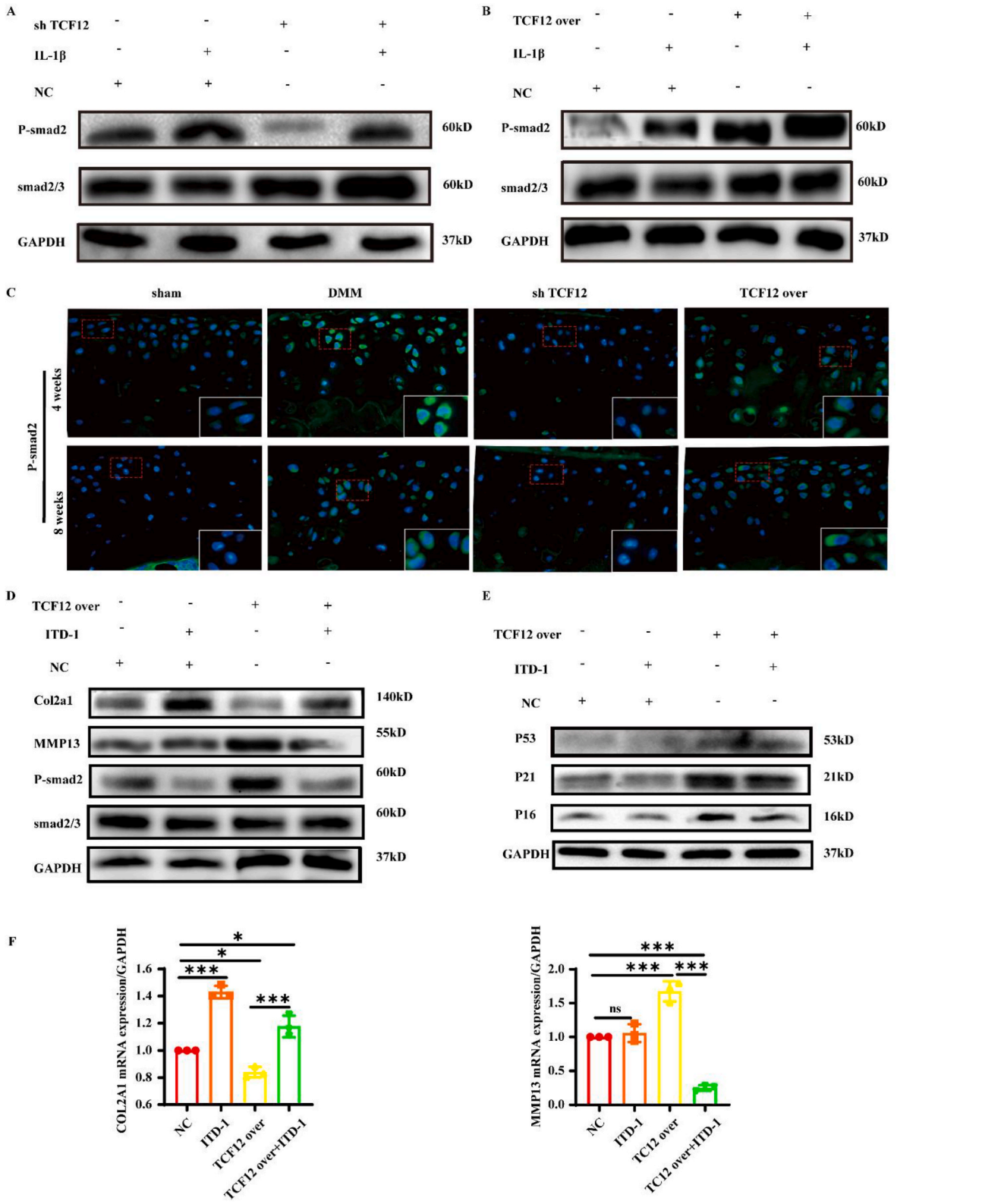

Fig. 4. TCF12 promotes the activation of the TGF-β signaling pathway(A) Western blot analysis and quantitative data of P-Smad2 in chondrocytes after TCF12 interference. Data were normalized with GAPDH (B) Western blot analysis of P-Smad2 in chondrocytes after TCF12 overexpression. Data were normalized with GAPDH (C) Representative images of P-Smad2 were examined in the TCF12-silenced or TCF12-overexpressing mouse chondrocytes by inverted fluorescence microscopy. DAPI, 4,6-diamino-2-phenylindole. Scale bar, 400 μm (D, E,F) The effect of TCF12 levels in mouse chondrocytes on P-Smad2 expression in mouse chondrocytes was assessed by WB and qRT-PCR in the presence or absence of ITD-1, and the data were normalized with GAPDH. ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are shown as means ± SEM of three independent experiments in (F). Student's t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

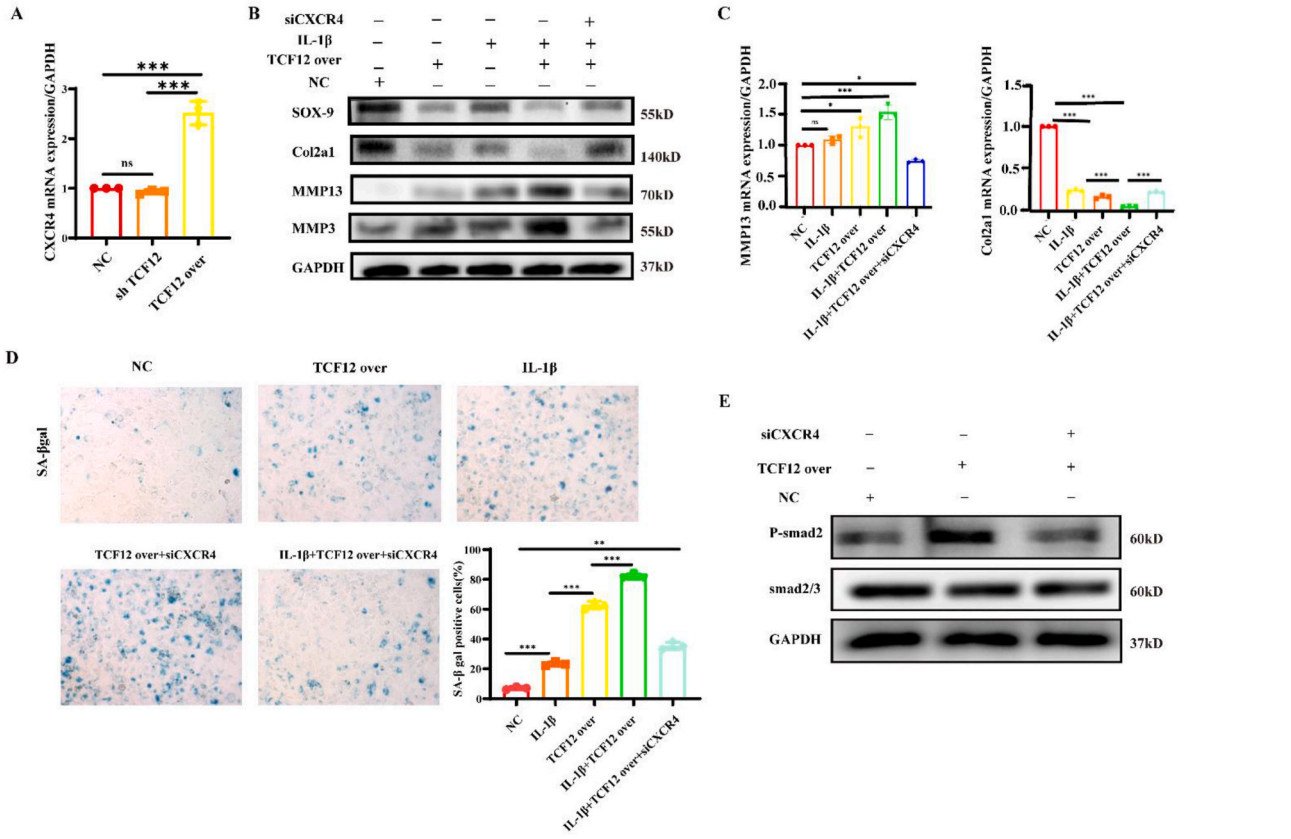

Fig. 5. TCF12 exerts biological functions by targeting CXCR4 (A) Mouse primary chondrocytes were transfected with two siRNAs (siCXCR4-1 and siCXCR4-2) at a final concentration of 30 nM. Twenty-four hours after transfection, the knockdown efficiency of CXCR4 was confirmed by qRT-PCR (B) WB analysis of COL2A1, SOX9, MMP3 and MMP13 in human chondrocytes with a combination of TCF12 inhibition or TCF12 overexpression and CXCR4 inhibition (with or without IL-1b stimulation) was performed, and data were normalized with GAPDH (C) qRT-PCR analysis of COL2A1 and MMP13 RNA levels in chondrocytes after TCF12 inhibition or combined TCF12 overexpression with CXCR4 inhibition (D) β-galactosidase staining in chondrocytes after TCF12 inhibition or combined TCF12 overexpression and CXCR4 inhibition (left); quantitative data (right) (E) WB analysis of P-Smad2 protein levels in chondrocytes following TCF12 inhibition or combined TCF12 overexpression and CXCR4 inhibition. ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are shown as means ± SEM of three independent experiments in (A), (c) and (D). Student's t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

3.1. Limitations of the study

The limitations of this study include the lack of information on the role of TCF12 in relieving OA pain and restoring joint function, as well as subchondral bone, Experimental studies relating to this should be explored in future studies.

4. Materials and methods

4.1. Human samples

After the study was approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University, samples were obtained from OA patients who underwent total knee replacement surgery and provided informed consent (n = 20), Normal human cartilage was obtained from victims of road traffic accidents with no history of arthritic diseases (n = 7, aged 34.14 ± 4.98 years, four males and three females). The clinical characteristics of the patients are shown in the Supplementary Tables 1–2.

4.2. Experimental OA model and histomorphometry

Animal handling and experimental procedures were performed with the approval of the Ethics Committee of Southern Medical University. Importation, transportation and housing of the mice were all conducted according to the recommendations of "The use of non-human primates in research." All mice were maintained in accordance with institutional animal care and use guidelines. Surgery to destabilize the medial meniscus (DMM) was performed on the right knee of 14-week-old mice to surgically create a mouse model of induced OA. Briefly, the right knee joint of the mice was exposed under a stereomicroscope through an internal capsule incision after anesthesia with intraperitoneal tribromoethanol (0.7 mg/mL). The meniscotibial ligament of the medial meniscus was released. Finally, the incision was sutured, and the skin was closed. Sham surgery was performed by opening and exposing the tissues of the left knee and then suturing the incision without intervention of the meniscus in age-matched mice. Mice in the OA groups underwent weekly intra-articular injections of The mutant mouse TCF12 lentiviral vector (shTCF12 group) and TCF12-overexpressing lentiviral vector (TCF12 over group) and empty lentiviral vector (vector group) lentiviruses. A total of 10 μL of solution was slowly injected into the right knee joint cavity. The right legs were harvested 4 or 8 weeks post-surgery (n = 7 in each group). Then, the samples were fixed, decalcified, dehydrated and sectioned.

4.3. Cartilage explants

Three-week-old male C57 mice were euthanized to isolate tibial plateau cartilage explants. Microforceps were used for blunt dissection of cartilage from the underlying bone.

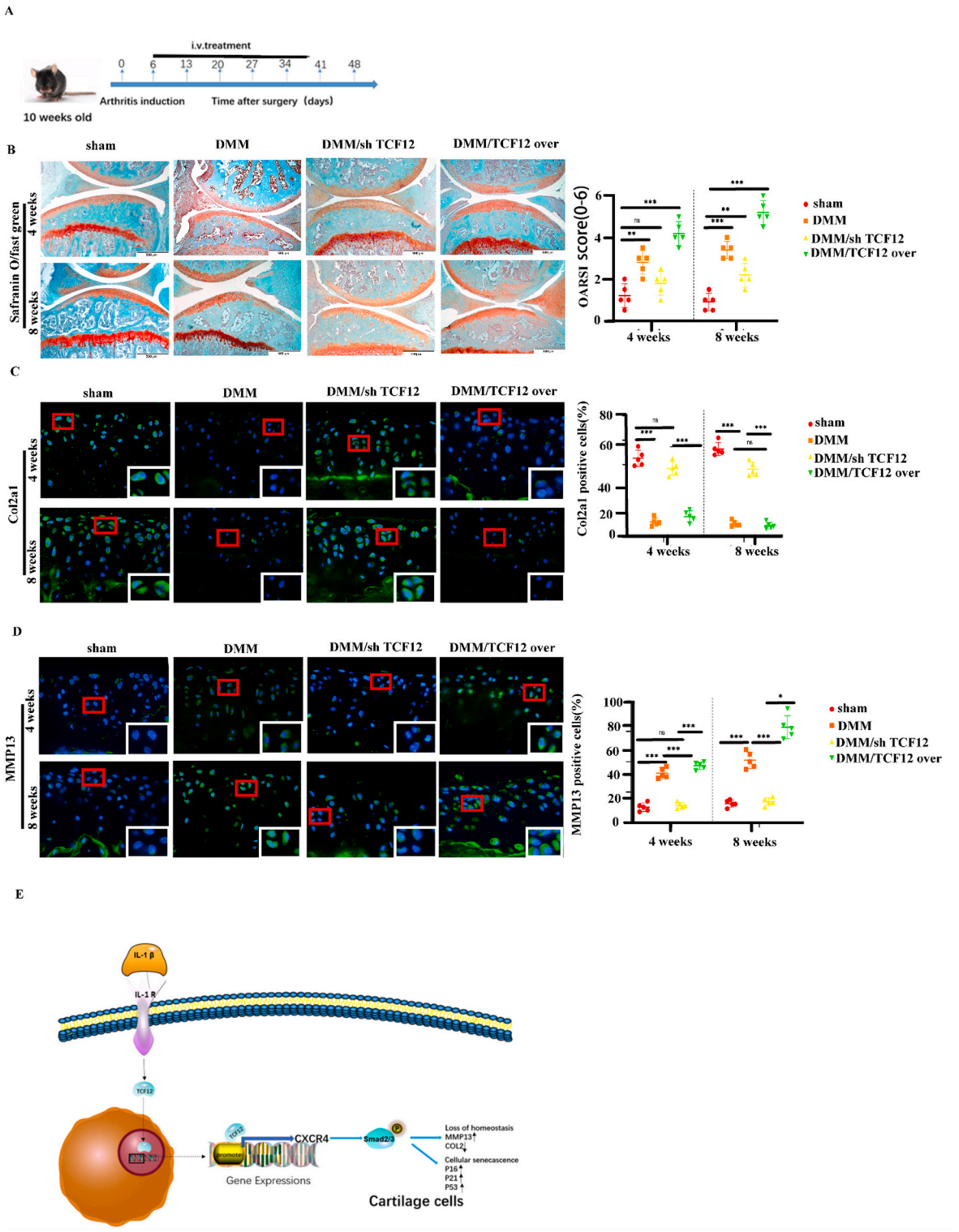

Fig. 6. TCF12 alleviates OA in vivo(A) Schematic of the experimental design for OA treatment targeting TCF12 (B) The mouse model of OA generated by DMM was injected with TCF12-overexpressing lentivirus or TCF12-interfering virus. Articular cartilage was stained with safranin O/fast green (SOFG). Scale bar, 200μm (left). The OARSI score was measured based on the SOFG staining results (right) (C, D) The expression of COL2A1 and MMP13 in articular cartilage was detected by IF. Scale bar, 400 μm. OARSI, Osteoarthritis Research Society International (E) Schematic of the working hypothesis. The upregulation of TCF12 expression in inflammatory cytokine-stimulated human chondrocytes and osteoarthritic cartilage tissues is directly associated with ECM catabolism and cell apoptosis. TCF12 activates the TGF-β pathway by directly targeting CXCR4. ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are shown as means ± SEM of three independent experiments in (B) (C) and (D). Student's t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

Explants were cultured for 3 days in DMEM/F12 containing 10 % fetal bovine serum (FBS) in 96-well plates before further processing.

4.4. Cell culture

Primary chondrocytes were isolated using femoral heads and tibial plateaus from C57BL/6 neonatal mice [34]. Briefly, femoral heads, femoral condyles, and tibial plateaus were dissected from mice 4–6 days old and rinsed with phosphate-buffered saline (PBS), and the pellet was collected by centrifugation after 30 min of 0.25 % trypsin digestion at 37 °C and digested overnight in Dulbecco's modified Eagle's medium (DMEM) freshly prepared with 0.1 mg/mL collagenase II (Thermo Fisher Scientific, Waltham, MA, USA). After digestion, the pellet was collected by centrifugation and resuspended in complete medium. Then, the cells were transferred to a 60-mm culture dish, mixed evenly in DMEM (Gibco, Thermo Fisher Scientific) with 10 % FBS (Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin sulfate, and added to the culture dish. The cells were placed in a 37 °C, 5 % CO_2 cell incubator for culture, and the medium was changed every other day. Primary murine chondrocytes were treated with 30 ng/mL IL-1β (R&D Systems) for 24 h to create an in vitro osteoarthritic chondrocyte model [23].

4.5. Real-time quantitative PCR

Total RNA was isolated from primary murine chondrocytes and ground cartilage from human tibial plateaus using TRIzol reagent (TaKaRa Bio, Inc., Shiga, Japan). For mRNA quantification, 1 mg of total RNA was purified with a genomic DNA (gDNA) remover and reverse transcribed using 5 × HiScript II qRT SuperMix II (Vazyme Biotech, Nanjing, China). Each PCR consisted of 10 μL of 2 × ChamQ SYBR qPCR Master Mix (Vazyme), 10 μM forward and reverse primers, and 500 ng of cDNA. For miRNA quantification, 1 mg total RNA was purified with gDNA wiper mix and then reverse transcribed using HiScript II Enzyme Mix, 10 × RT Mix, and specific stem-loop primers. Template DNA was mixed with 2 × miRNA Universal SYBR qPCR Master Mix, specific primers and mQ primer R (Vazyme). All reactions were run in triplicate. Primers are shown in the Supplementary Table 3.

4.6. Western blot analysis

Cells cultured in 6-well dishes were lysed with 200 μL of radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing protease inhibitor and phosphatase inhibitor. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Beyotime). After incubation with 5 % skim milk in 50 mM Tris-buffered saline (TBS) (pH 7.4) containing 0.1 % Tween-20 (TBST) for 1 h at room temperature, the membranes were further incubated overnight at 4 °C with primary antibodies diluted with TBST with 5 % BSA. Following three washes with TBST (5 min each), the membranes were incubated with secondary antibodies (diluted at 1:3000 in TBST) for 1 h at room temperature. Target protein bands were visualized by FDbio-Dura ECL (FDbio science, Hangzhou, China). Antibodies used for Western blotting (WB) were as follows: GAPDH (RayBiotech, 1:5000, Cat# RM2002, RRID: AB_2756459), TCF12 (1:000, Proteintech, Cat# 14419-1-AP, RRID: AB_2198838), MMP13 (1:1000, Proteintech, 18165-1-AP, RRID), MMP3 (1:1000, Abclonal, Cat#A11418, RRID: AB_2861563), COL2A1 (1:1000, Abclonal, Cat#A1560, RRID: AB_2763005), SOX9 (1:1000, Affinity, AF6330, RRID: AB_2835186), CXCR4 (1:1000, Immunoway, YT1800, RRID: AB_2814754), rabbit anti-P16 (Abcam, 1:1,000, ab51243, RRID: AB_2757075), rabbit anti-P21 (Abcam, 1:1,000, ab188224), rabbit anti-P53 (Proteintech, 1:1,000, 10442-1-AP, RRID: AB_2206609), Smad2/3 (1:2000, GeneTex, Cat# GTX111123, RRID: AB_2038001), P-Smad2 (1:2000, GeneTex Cat# GTX133614, RRID: AB_2887051) and species-matched horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

4.7. Immunohistochemical and immunofluorescence (IF) analyses

Knee joint tissues were fixed in 4 % paraformaldehyde for 48 h, decalcified for 21 days, dehydrated and embedded in paraffin. Serial midsagittal sections (3 μm thick) were cut and stained with safranin O-fast green/hematoxylin and eosin (H&E)/toluidine blue for morphological analysis. Immunohistochemistry (IHC) and IF staining were performed on 4-μm thick tissue sections. Slides were deparaffinized, rehydrated, and washed three times in PBS for 5 min each. Antigen retrieval was performed by soaking slides in citric acid in a 60 °C water bath overnight. After three washes in PBS, the slides were quenched in 3 % hydrogen peroxide for 10 min at room temperature and washed with PBS three times. Then, the slides were blocked with 10 % normal bovine serum (Solarbio, Beijing, China) for 1 h at room temperature. The slides were then incubated with primary antibodies at 4 °C overnight. The secondary antibody for IHC or fluorescent secondary antibody for IF was applied for 1 h at room temperature, and then, the IHC-stained slides were stained with DAB and hematoxylin, dehydrated and mounted. IF slides were processed with 4,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA, USA) staining solution and mounted. Photomicrographs of the sections were obtained, histomorphometric measurements of the entire area of the tibial cartilage were made with a Zeiss mirror (Zeiss, Heidelberg, Germany), and quantitative histomorphometric analysis was blindly performed with OsteoMeasureXP software (OsteoMetrics, Inc., Atlanta, USA). The number of positively stained cells in the entire tibial cartilage region was calculated in three consecutive sections for each mouse in each group. Antibodies used for IHC/IF staining were as follows: TCF12 (1:000, Proteintech, Cat# 14419-1-AP, RRID: AB_2198838), CXCR4 (1:1000, Immunoway, YT1800, RRID: AB_2814754), MMP13 (1:1000, Abclonal, Cat#A11418, RRID: AB_2861563), COL2A1 (1:1000, Abclonal, Cat#A1560, RRID: AB_2763005), P-Smad2 (1:2000, GeneTex Cat# GTX133614, RRID: AB_2887051) and P16 (Abcam, 1:1,000, ab51243, RRID: AB_2757075).

4.8. Grading of cartilage structure

Histological sections of the knee joints were graded by two blinded observers based on the Osteoarthritis Research Society International (OARSI) scoring system developed by Glasson et al. [34]. Generally, sections were assigned a grade of 0–6: 0, normal cartilage; 0.5, slight loss of safranin O staining without structural changes; 1 small fibrillations without loss of cartilage; 2, vertical clefts down to the layer below the superficial layer; 3–6, vertical clefts or erosion to the calcified cartilage affecting <25 % (grade 3), 25–50 % (grade 4), 50–75 % (grade 5) and >75 % (grade 6) of the articular surface. Toluidine blue staining of the cartilage explants was graded by two blinded observers based on the area of staining loss by using a 6-point scale.

4.9. Luciferase assay

First, 293T cells were seeded in 96-well plates 24 h before transfection. Then, 100 ng pmirGLO plasmids with the CXCR4 wild-type 3′ untranslated region (UTR) (3′UTR wt) or mutant CXCR4-3′ UTR (3′UTR mut), 100 ng TCF12 overexpression vector and NC were cotransfected with Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h of incubation, firefly and Renilla luciferase activities were calculated by PerkinElmer EnSpire according to the manufacturer's instructions. Firefly Luciferase Assay Reagent (Beyotime, RG005) was used to measure the firefly luciferase activities, while Renilla Luciferase Assay Reagent (Beyotime, RG016) was used to measure the Renilla luciferase activities. Firefly and Renilla luciferase activities were measured to evaluate the relative luciferase

activity.

4.10. Senescence-associated β-galactosidase (SA-βGal) staining

SA-βGal activity was measured using a staining kit (Solarbio). Chondrocytes were seeded in wells of 6-well plates, fixed for 10 min at room temperature, washed, and incubated with staining solution overnight at 37 °C.

4.11. Lentivirus packaging and cell infections

The mutant mouse TCF12 lentiviral vector (shTCF12 group), overexpression mouse TCF12 lentiviral vector (TCF12over group) and empty lentiviral vector (vector group) were constructed by Hanbio Biotechnology Co. Ltd (Shanghai, China). Chondrocytes were cultured in DMEM (Gibco, Thermo Fisher Scientific) with 10 % FBS (Gibco), 100 U · mL-1 penicillin and 100 mg/mL streptomycin sulfate Chondrocytes were transfected with lentivirus medium at a multiplicity of infection (MOI = the quantity of lentiviruses/the quantity of cells) of 1–20, and 2 μg/mL polybrene was added to promote efficiency. Chondrocytes at concentrations of 50–70 % were exposed to lentiviruses (vector, TCF12over groups and shTCF12 groups) for 24 h, and then, the lentivirus medium was replaced with normal medium. After the transfected chondrocytes were cultured for 48–72 h, green fluorescent protein (GFP) expression, representing the success of transfection, was observed under an inverted fluorescence microscope.

4.12. Statistical analysis

All results are expressed as the mean ± standard deviation using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, CA, USA) and were analyzed using Student's t test or analysis of variance (ANOVA). Pearson's linear correlation coefficient was used to measure the dependence of two variables. The significance level was set at $P < 0.05$.

Authors contribution

Conceptualization, Z.H.,L.Y.H,.F.J.L,.and L.W; Formal Analysis,C.S.X.,H.G.X, and Z.Y.M.; H.S:.Investigation,Z.H.,L.W.; Writing, Z.H. and L.W.; Visualization,Z.R.K.; Supervision,Z.R.K. Z.Y.X:and F.H.; Funding Acquisition,Z.R.K.

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Availability of data

All reagents used in this work are available upon request and a brief statement describing the purpose for their use.

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.2023.11.006.

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