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Fibronectin fragment inhibits xylosyltransferase-1 expression by regulating Sp1/Sp3- dependent transcription in articular chondrocytes

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SUMMARY

Objective: We investigated the effects of 29-kDa amino-terminal fibronectin fragment (29-kDa FN-f) on xylosyltransferase-1 (XT-1), an essential anabolic enzyme that catalyzes the initial and rate-determining step in glycosaminoglycan chain synthesis, in human primary chondrocytes.
Methods: Proteoglycan and XT-1 expression in cartilage tissue was analyzed using safranin O staining and immunohistochemistry. The effects of 29-kDa FN-f on XT-1 expression and its relevant signaling pathway were analyzed by quantitative real-time-PCR, immunoblotting, chromatin immunoprecipitation, and immunoprecipitation assays. The receptors for 29-kDa FN-f were investigated using small interference RNA and blocking antibodies.
Results: The expression of XT-1 was significantly lower in human osteoarthritis cartilage than in normal cartilage. Intra-articular injection of 29-kDa FN-f reduced proteoglycan levels and XT-1 expression in murine cartilage. In addition, in 29-kDa FN-f-treated cells, XT-1 expression was significantly suppressed at both the mRNA and protein levels, modulated by the transcription factors specificity protein 1 (Sp1), Sp3, and activator protein 1 (AP-1). The 29-kDa FN-f suppressed the binding of Sp1 to the promoter region of XT-1 and enhanced the binding of Sp3 and AP-1. Inhibition of mitogen-activated protein kinase and nuclear factor kappa B signaling pathways restored the 29-kDa FN-f-inhibited XT-1 expression, along with the altered expression of Sp1 and Sp3. Blockading toll-like receptor 2 (TLR-2) and integrin α5β1 via siRNA and blocking antibodies revealed that the effects of 29-kDa FN-f on XT-1 expression were mediated through the TLR-2 and integrin α5β1 signaling pathways.
Conclusion: These results demonstrate that 29-kDa FN-f negatively affects cartilage anabolism by regulating glycosaminoglycan formation through XT-1.

Introduction

Articular cartilage damage is a key event leading to joint deformity in various arthritides, including rheumatoid arthritis (RA), osteoarthritis (OA), and septic arthritis[1]. The dynamic equilibrium between the anabolic and catabolic activities of chondrocytes is of utmost importance for the maintenance of the cartilage extracellular matrix (ECM). Although the role of cartilage matrix catabolism in the pathogenesis of OA has been widely investigated, the role of matrix anabolism in OA is not well understood.

Proteoglycans (PG) are major constituents of cartilage ECM, which, by covalently attaching highly charged glycosaminoglycan (GAG) chain such as chondroitin sulfate (CS), heparan sulfate (HS), and keratan sulfate, confer compressive integrity to cartilage[2,3]. The biosynthesis of GAGs is accomplished through multiple steps, beginning with the formation of a core protein linkage tetrasaccharide that functions as a primer for GAG chain elongation. Xylosyltransferase-1 (XYLT1; XT-1; EC 2.4.2.26) catalyzes the first and rate-limiting step in the biosynthesis of GAGs by transferring xylose to specific serine residues in PG core proteins[4].

Previous reports have demonstrated that XT-1 functions as a key regulator of the cartilage repair process[5]. XT-1 expression was lower in damaged regions of OA cartilage than in unaffected regions, and modulation of XT-1 expression using RNA interference

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and overexpression vectors revealed that XT-1 was closely associated with GAG synthesis[6]. *In vivo* animal studies have shown that XT-1 expression was significantly reduced in rat cartilage in antigen-induced arthritis, and XT-1 knockdown prevented cartilage repair by decreasing PG synthesis[5]. In addition, XT-1 was identified as a regulator of chondrocyte maturation and endochondral ossification in a recessive dwarf mouse mutant[7]. XT-1 expression was downregulated by the catabolic cytokine interleukin-1 beta (IL-1β) in human primary chondrocytes and cartilage explants, and upregulated by the anabolic cytokine transforming growth factor beta 1 (TGF-β1) in cartilage explants[3,6].

Chondrocytes express degradative enzymes and catabolic mediators in response to matrix degradation products as well as proinflammatory cytokines and mechanical loading[8]. Fibronectin fragments (FN-fs) are generated through the proteolysis of fibronectin (FN), a component of articular cartilage matrix, and are detected at high levels in OA cartilage and synovial fluids[9,10]. FN-fs, including 29-kDa, 50-kDa, and 140-kDa FN-fs, induce cartilage catabolism by increasing the expression of matrix metalloproteinases (MMPs), and intra-articular injection of 29-kDa FN-f into rabbit knee joints caused cartilage PG loss[10–12]. Previously, we reported that the 29-kDa amino-terminal FN-f (29-kDa FN-f) stimulated catabolic responses through the MyD88-dependent toll-like receptor 2 (TLR-2) signaling pathway in primary human articular chondrocytes, suggesting that it may function as a mediator of damage-associated molecular pattern (DAMP) and induce a vicious cycle accelerating cartilage matrix damage[1]. It is unknown whether FN-fs also regulate anabolic signaling in chondrocytes.

In this study, we investigated the influence of 29-kDa FN-f on cartilage matrix anabolic response by examining the transcriptional regulation of XT-1 and GAG synthesis in human articular chondrocytes.

Materials and methods

Materials, cartilage collection, primary chondrocyte culture, cartilage explants culture, and surgically induced OA and intra-articular injection of 29-kDa FN-f in mice are described in the supplementary information.

Histology and immunohistochemistry (IHC) analysis

Safranin O staining and immunohistochemistry analysis of normal and OA human cartilage and mouse joint cartilage tissues were performed as previously described[13].

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from chondrocytes and cartilage tissues using TRIzol reagent. XT-1, Sp1, and Sp3 gene expression were quantified as previously described[13]. The primer sequences are shown in Table S1.

Transfection with siRNA and overexpression vectors

Transfections with siRNA and plasmids were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as previously described[1]. The siRNA sequences are shown in Table S2.

Overexpression plasmids for Sp1 and Sp3 were prepared. Briefly, PCR products amplified using Sp1-and Sp3-specific primers containing HindIII (AAGCTT) and BamHI (GGATCC) restriction sites were ligated into a pGEM-T Easy vector and subcloned into a pcDNA3.1-myc-His vector. Purified Sp1 and Sp3 overexpression vectors were transfected into chondrocytes using Lipofectamine 2000. The expression of Sp1 and Sp3 was detected using western blot analysis.

Western blot analysis

Cell lysates were prepared with radioimmunoprecipitation assay (RIPA) lysis buffer and immunoblotting was performed as described previously[1].

Chromatin immunoprecipitation (ChIP) and immunoprecipitation (IP) assays

ChIP was performed according to the manufacturer's instructions (EpiQuik, Farmingdale, NY, USA). Briefly, chondrocytes were cross-linked with 1% formaldehyde for 10 min and the cross-linked protein–DNA complexes were extracted from cells. The extracted genomic DNA was sheared into smaller pieces by sonication and immunoprecipitated with specific antibodies against immunoglobulin G (IgG), Sp1, Sp3, and c-jun. Cross-linked DNA was released from the antibody-target protein-DNA complex and purified using Fast-Spin columns. The levels of DNA bound to each protein were determined using PCR and agarose gel electrophoresis.

For IP, cells were lysed with RIPA lysis buffer containing protease inhibitors. Cell lysates were centrifuged at 13,000×*g* for 10 min at 4°C and the supernatants were collected. The supernatant samples containing equal amounts of proteins were pre-cleared with protein A agarose bead slurry for 4 h at 4°C on a rotating shaker. The pre-cleared samples (1 μg) were incubated with specific antibodies against c-jun, Sp1, or IgG in the presence of protein A agarose beads at 4°C overnight with gentle rotation. Proteins were eluted by boiling in 2x SDS sample buffer. A portion of the lysates was used as an input control. Eluted protein samples were subjected to western blot analysis as described above.

Direct enzyme-linked immunosorbent assay (ELISA) to detect the binding of 29-kDa FN-f to TLR-2

Round-bottom microtiter plates were coated with 100 μL recombinant human TLR-2 (1 μg/mL) overnight at 4°C and blocked with 300 μL BSA (2% w/v) for 2 h at room temperature. 29-kDa FN-f (1, 5, 10, and 30 μg/mL) or IgG was added to TLR-2-coated wells for 2 h at room temperature. Anti-FN antibody (100 μL/well, 1:1000 dilution) was added to each well for 1 h at room temperature, followed by incubation with HRP-conjugated anti-mouse IgG. To detect HRP activity, the wells were incubated with 1-step Turbo TMB Direct ELISA reagent (Thermo Fisher Scientific, Rockford, IL, USA) for 30 min, and then stop solution was added to each well. The absorbance was measured at 450 nm.

Neutralization assay using neutralizing antibodies against TLR-2 and integrin α5β1

Chondrocytes were pre-incubated for 2 h with antibodies against TLR-2 or integrin α5β1 (Merck Millipore, Billerica, MA, USA) and treated with 29-kDa FN-f for 14 h. The expression levels of XT-1, Sp1, and Sp3 in anti-TLR-2- and anti-integrin α5β1-treated cells were measured by qRT-PCR.

Statistical analysis

For *in vivo* studies, 4–6 mice in each group were used. For *in vitro* studies, individual experiments were performed using chondrocytes from more than three different donors with duplicate

or triplicate per donor. Multi-level mixed effects models were used for statistical analyses by using Statistical Analysis System 9.4 (Statistical Analysis System, Cary, NC, USA). Data are expressed as the mean ± standard deviation (SD). A value of $P < 0.05$ was considered statistically significant.

Results

XT-1 expression is significantly downregulated in OA cartilage

To investigate whether XT-1 and ACAN expression changes in OA, we compared XT-1 and ACAN expression in normal and OA cartilage. The mRNA expression of XT-1 and ACAN were significantly downregulated in OA cartilage compared with normal cartilage [Fig. 1(A)]. To further investigate XT-1 protein levels in cartilage tissues, we analyzed XT-1 levels using IHC. ECM, as assessed by safranin O staining, and XT-1 expression levels were lower in human OA cartilage than in normal cartilage [Fig. 1(B)]. In addition, XT-1 expression and ECM levels were reduced in DMM-induced OA murine knee-joint cartilage relative to those of sham mice [Fig. 1(C)], suggesting that reduced XT-1 protein expression may be closely related to OA pathogenesis.

29-kDa FN-f suppresses the expression of aggrecan (ACAN) and XT-1 in primary chondrocytes and cartilage explant culture

We examined the basal expression of ACAN, a representative PG, and XT-1 in chondrocyte monolayer cultures incubated in the presence or absence of FBS for 72 h. The mRNA expression of XT-1 and ACAN oscillated during the 24 h of culture, peaking after 14 h of culture (Fig. S1). Interestingly, at 72 h, XT-1 and ACAN expression tended to increase again compared with baseline levels (Fig. S1). To determine whether 29-kDa FN-f modulates XT-1 and ACAN expression in primary chondrocytes, cells were stimulated with 29-kDa FN-f for 24 h. PCR analysis demonstrated that 29-kDa FN-f suppressed XT-1 expression at 6 h and at 14 h, when XT-1 expression was maximally induced, but had no effect on XT-1 expression at 24 h [Fig. 2(A)]. Unlike its mRNA expression, XT-1 protein expression did not decrease at 24 h, and treatment with 29-kDa FN-f suppressed the protein expression of XT-1 at 14 and 24 h [Fig. 2(B)]. In the case of ACAN, 29-kDa FN-f suppressed mRNA expression of ACAN throughout the 24-h period [Fig. 2(A)]. We examined whether ACAN has influence on XT-1 expression, and found out that regardless of the presence of 29-kDa FN-f, ACAN knockdown suppressed XT-1 expression (Fig. S2). Conversely, XT-1

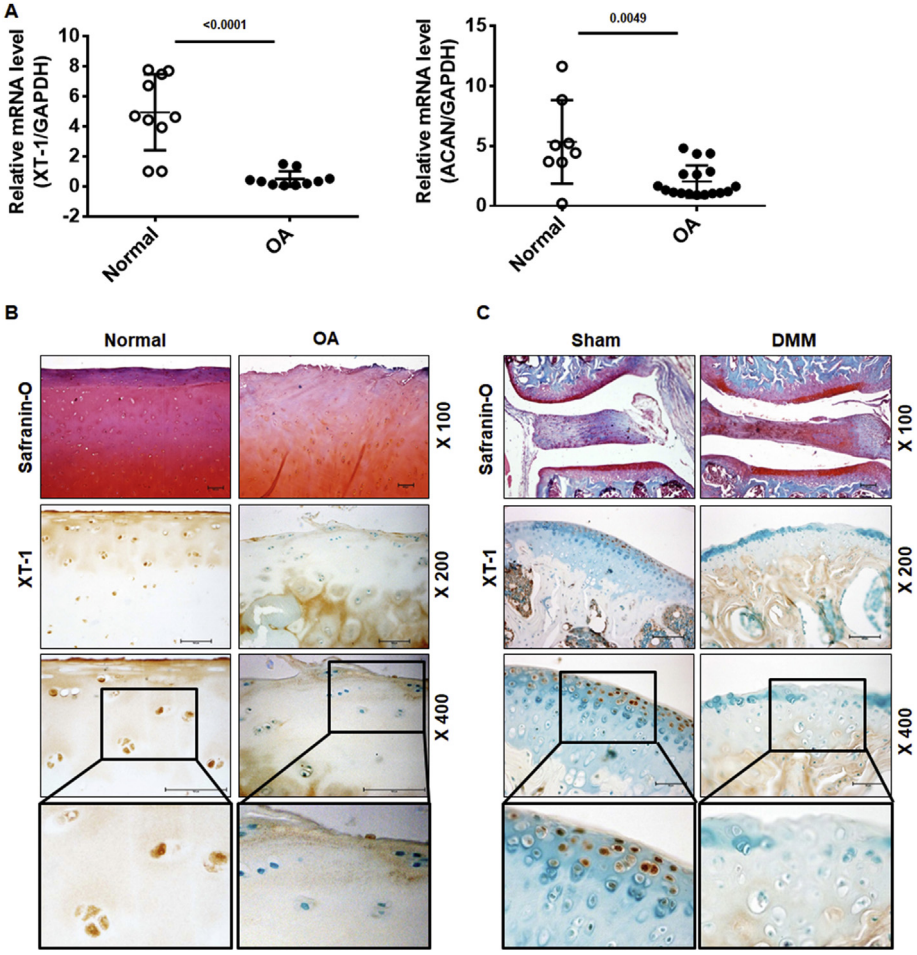

Fig. 1. Reduced xylosyltransferase-1 (XT-1) expression in human osteoarthritis (OA) articular cartilage and destabilization of medial meniscus (DMM)-induced OA murine joint cartilage. (A) The relative expression of XT-1 and ACAN in normal and OA cartilage was determined using Quanti Fast SYBR Green-based real-time PCR (RT-PCR). The expression ratios of XT-1 relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the internal control, are shown. Data represent the mean ± standard deviation (SD) for duplicate data from 10 separate donors for XT-1 and from 8 normal and 17 OA donors for ACAN. *P* value was presented between two groups. Dot indicates the representative value obtained from each donor. Expression of XT-1 in normal and OA cartilage (B) and in joint cartilage of sham and DMM mice (C) was determined by immunohistochemistry (IHC) using anti-XT-1 antibodies. Data represent the results from three normal and OA cartilage samples (B) and four sham and six DMM 10-week-old mice (C). Scale bars = 100 μm for 100× and 200× magnifications and 50 μm for ×400 magnification.

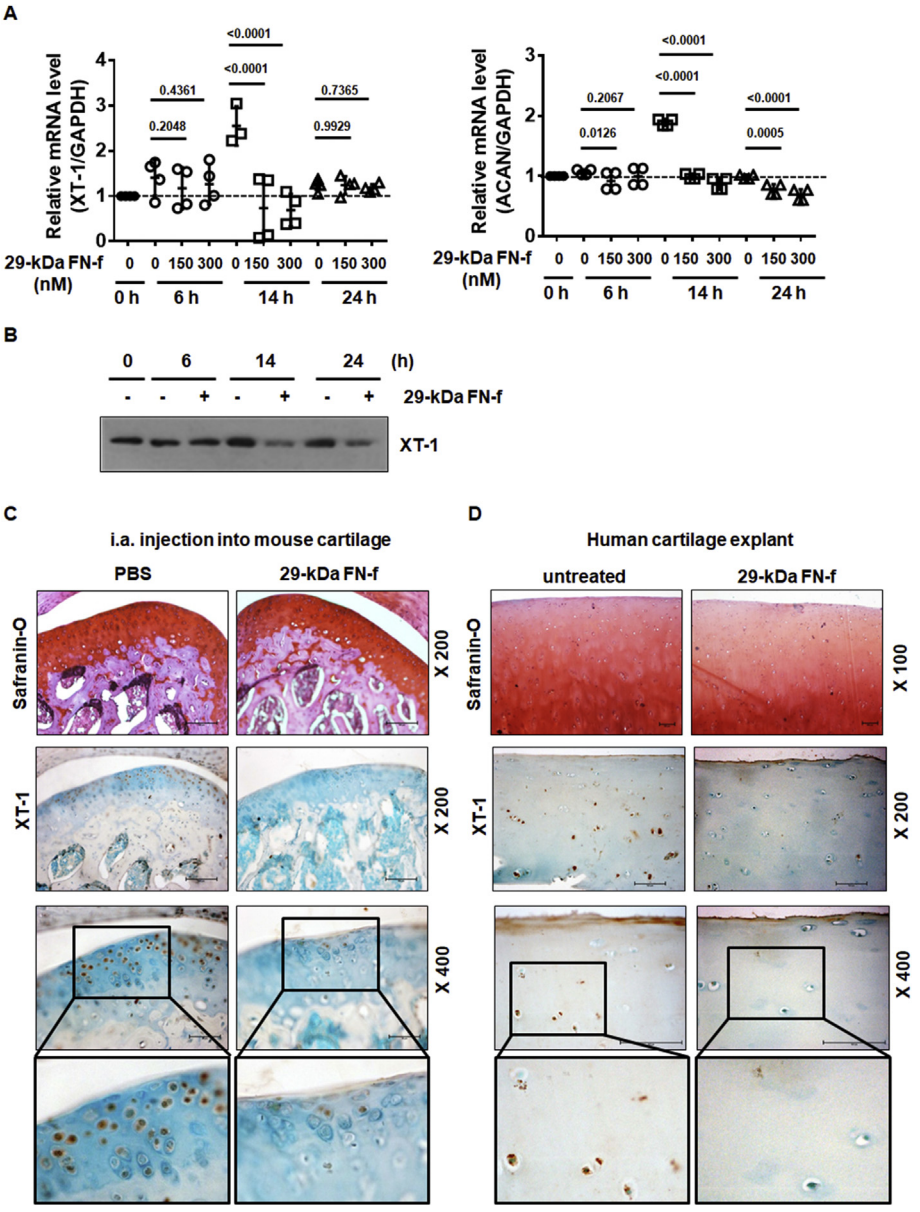

Fig. 2. The 29-kDa fibronectin fragment (FN-f) suppresses XT-1 expression in primary chondrocytes and joint cartilage. (A) mRNA expression of XT-1 and aggrecan (ACAN) decreased following treatment with 29-kDa FN-f. Chondrocytes were stimulated with 29-kDa FN-f (150 and 300 nM) for 6, 14, and 24 h. The mRNA levels of XT-1 and ACAN were measured using RT-PCR. Data represent the mean ± SD of duplicate data from more than three different donors. *P* value was presented between two groups. Dot indicates the representative value obtained from each donor. (B) XT-1 protein expression was significantly suppressed following 24 h incubation with 29-kDa FN-f. Chondrocytes were treated with 29-kDa FN-f (300 nM) for 6, 14, and 24 h. Protein levels of XT-1 were measured using western blot analysis. The blot shown is representative of blots from six independent donors. (C, D) IHC and safranin O staining in (C) murine joint cartilage injected with 29-kDa FN-f (3 μM/10 μL; $n = 6$) or PBS (10 μL; $n = 6$) and (D) explant culture of human cartilage in the presence of 29-kDa FN-f (300 nM) for 7 days. XT-1 expression was determined by IHC using anti-XT-1 antibody and cartilage degeneration was examined by safranin O staining. i.a., intra-articular. Scale bars = 100 μm for 100× and 200× magnifications and 50 μm for ×400 magnification.

knockdown led to ACAN suppression, suggesting that ACAN and XT-1 reciprocally affect the expression of the other (Fig. S2). To further investigate the effects of 29-kDa FN-f on the *in vivo* expression of XT-1 protein, 29-kDa FN-f (3 μM/10 μL) was intra-articularly injected into the left knee joints of mice. The animals were sacrificed 7 days after injection. IHC analysis revealed that administration of 29-kDa FN-f decreased XT-1 expression in murine joint cartilage compared with PBS injection [Fig. 2(C)]. Human cartilage explants exposed to 29-kDa FN-f (300 nM) for 7 days showed ECM depletion and decreased XT-1 expression [Fig. 2(D)] similar to the observations in 29-kDa FN-f-injected murine joint cartilage [Fig. 2(C)]. The results demonstrate that 29-kDa FN-f negatively regulated XT-1 and PG expression in primary human chondrocytes and cartilage tissue.

Transcription factors Sp1 and Sp3 transcriptionally regulate XT-1 expression

We analyzed the 5′-flanking region of the human XT-1 gene to identify transcription factors that regulate XT-1 expression. Genome sequence analysis revealed three activator protein 1 (AP-1) and two Sp1 binding sites in the XT-1 promoter region[3]. In addition, Sp3 is a transcription factor that competes for the same DNA region bound by Sp1[3]. PCR analysis demonstrated that Sp1 and Sp3

expression increased in the absence of 29-kDa FN-f after 14 h of incubation, whereas 29-kDa FN-f treatment up-regulated Sp1 and Sp3 transcripts at 6 h, followed by reduced Sp1 expression and increased Sp3 expression after 14 h of treatment [Fig. 3(A)]. Western blot results showed that 29-kDa FN-f treatment reduced Sp1 expression and increased Sp3 expression at 14 h, and down-regulated XT-1 expression [Fig. 3(B)]. 29-kDa FN-f exposure (at 6 h) did not affect XT-1 protein expression compared with control levels, whereas its mRNA expression decreased [Fig. 2(A) and (B)]. The results suggest that the reduction in XT-1 expression was likely due to altered cellular levels of Sp1 and Sp3 in 29-kDa FN-f-treated chondrocytes.

Next, to clarify whether the alterations of Sp1 and Sp3 expression by 29-kDa FN-f affect XT-1 expression, knockdown or overexpression of Sp1 and Sp3 was performed using Sp1 and Sp3 siRNA or Sp1 and Sp3 overexpression vectors. Whereas Sp1 knockdown

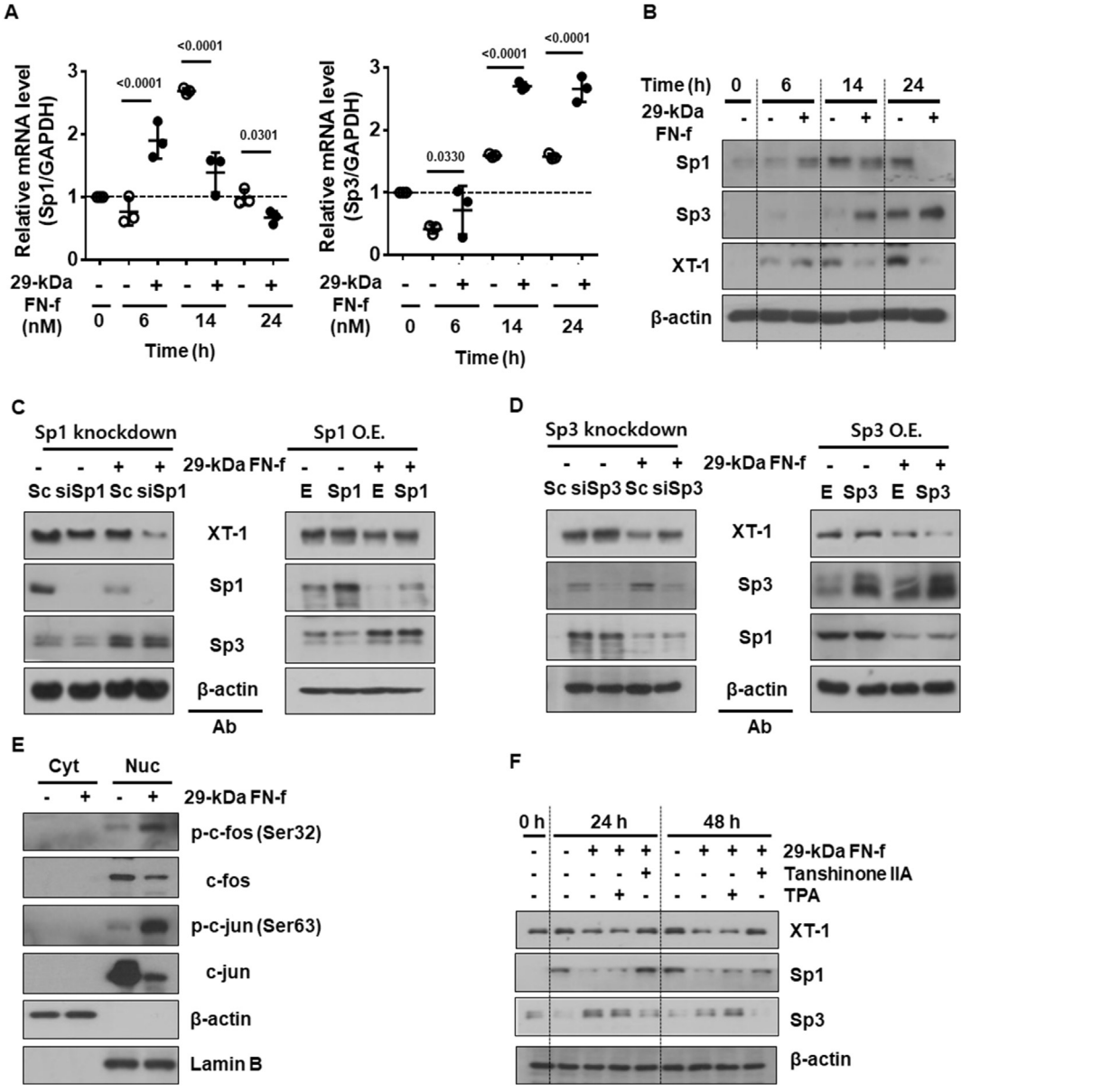

Fig. 3. Specificity protein 1 (Sp1), Sp3, and activator protein 1 (AP-1) are transcriptional regulators of XT-1. (A, B) 29-kDa FN-f regulates the expression of Sp1 and Sp3 at both the (A) mRNA and (B) protein levels. Chondrocytes were treated with 29-kDa FN-f (300 nM) for 6, 14, and 24 h. mRNA and protein levels of Sp1 and Sp3 were measured using RT-PCR and western blot analysis, respectively. RT-PCR data is expressed as the mean ± SD of duplicate data from three independent experiments. *P* value was presented between two groups. Dot indicates the representative value obtained from each donor. (C, D) Knockdown and overexpression of Sp1 and Sp3 affect XT-1 expression in primary human chondrocytes. (C) Chondrocytes were transfected with control siRNA (Sc), small interfering Sp1 RNA (siSp1), and Sp1 overexpression (O.E.) vector. (D) Chondrocytes were transfected with small interfering Sp3 RNA (siSp3), empty vector (E), and Sp3 O.E. vector. After 48 h, chondrocytes were stimulated with 29-kDa FN-f (300 nM) for 24 h. (B–D) The protein levels of XT-1, Sp1, and Sp3 were measured using western blot analysis. β-actin served as the loading control. The blots shown are representative of three or more independent experiments using chondrocytes from three different donors. E, empty vector. (E) Treatment with 29-kDa FN-f activates transcription factor AP-1. Chondrocytes were treated with 29-kDa FN-f (300 nM) for 24 h. The levels of c-jun/phospho-c-jun (Ser63) and c-fos/phospho-c-fos (Ser32) in the cytosol and nuclear fractions of 29-kDa FN-f-treated chondrocytes were measured by western blot analysis. β-actin and lamin B served as loading controls for the cytoplasmic and nuclear fractions, respectively. Cyt, cytosolic; Nuc, nuclear. (F) AP-1 inhibition recovers XT-1 expression suppressed by 29-kDa FN-f treatment. Chondrocytes were exposed to Tanshinone IIA, an AP-1 inhibitor, for 6 h, and to 12-O-tetradecanoylphorbol acetate (TPA), an AP-1 activator, for 24 h, followed by treatment with 29-kDa FN-f for 24 and 48 h. The levels of XT-1, Sp1, and Sp3 were determined using western blot analysis. The blots shown are representative of three or more independent experiments using chondrocytes from three different donors.

further downregulated XT-1, Sp1 overexpression slightly increased XT-1 expression in 29-kDa FN-f-treated chondrocytes [Fig. 3(C)]. On the other hand, Sp3 knockdown enhanced XT-1 expression, whereas its overexpression downregulated XT-1 in 29-kDa FN-f-treated cells [Fig. 3(D)]. Collectively, these data demonstrate that 29-kDa FN-f regulates cellular levels of Sp1, a transcriptional activator, and Sp3, a transcriptional repressor, which are involved in XT-1 expression, suggesting that Sp1 and Sp3 may be transcriptional regulators of XT-1.

AP-1 transcription factor modulates the expression of Sp1 and Sp3

The XT-1 promoter contains three sites that interact with the transcription factor AP-1 (c-jun/c-fos)[3]. We examined whether 29-kDa FN-f affects AP-1 activity by examining the phosphorylation of c-jun and c-fos. Western blot analysis of nuclear extract demonstrated that 29-kDa FN-f enhanced the nuclear levels of phospho-c-jun and phospho-c-fos [Fig. 3(E)], indicating that 29-kDa FN-f activates the transcription factor AP-1. We also assessed whether AP-1 is implicated in 29-kDa FN-regulated XT expression using 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an AP-1 activator, and Tanshinone IIA, an AP-1 inhibitor. AP-1 inhibition by Tanshinone IIA recovered 29-kDa FN-f-suppressed XT-1 expression to the levels observed in untreated cells, accompanied by increased Sp1 and decreased Sp3 expression [Fig. 3(F)]. By contrast, activation of AP-1 by TPA did not further decrease 29-kDa FN-f suppression of XT-1. These results demonstrate that 29-kDa FN-f activates AP-1, modulating Sp1 and Sp3 expression and ultimately downregulating XT-1.

29-kDa FN-f modulates the binding of Sp1, Sp3, and AP-1 to the XT-1 promoter

We next investigated whether 29-kDa FN-f directly regulates the binding of Sp1, Sp3, and AP-1 to the promoter region of XT-1 using a ChIP assay. The putative Sp1 and AP-1 binding sites are shown in Fig. 4(A). Our ChIP data indicate that 29-kDa FN-f promoted the recruitment of Sp3 and c-Jun, transcriptional repressors of the XT-1 gene, to the XT-1 promoter. By contrast, 29-kDa FN-f suppressed the binding of Sp1, a transcription activator, to the XT-1 promoter [Fig. 4(A)]. Therefore, the results indicate that 29-kDa FN-f modulated the recruitment of transcriptional activators and repressors of the XT-1 gene to their individual binding sites.

c-Jun regulates a variety of genes through its associations with transcription factors such as Sp1, ATF, and Smad[14]. To investigate whether the interaction between Sp1 and c-Jun is mediated by 29-kDa FN-f, we performed IP using anti-c-Jun or -Sp1 antibodies. Binding between c-Jun and Sp1 was observed in untreated control cells, whereas 29-kDa FN-f treatment decreased the interaction between c-Jun and Sp1 [Fig. 4(B)], suggesting that 29-kDa FN-f inhibits the formation of the c-Jun/Sp1 complex. Taken together, these results suggest that 29-kDa FN-f regulates the binding of the transcription factors Sp1, Sp3, and AP-1 to the XT-1 promoter, as well as the c-Jun/Sp1 complex.

MAPK and NF-κB signaling pathways are responsible for 29-kDa FN-f-modulated XT-1 expression

To determine whether the MAPK and NF-κB signaling pathways are involved in XT-1 and ACAN expression, chondrocytes were exposed to MAPK and NF-κB inhibitors 2 h prior to 29-kDa FN-f treatment. Pretreatment with JNK inhibitor SP203580, p38 inhibitor SB600125, ERK1/2 inhibitor PD98059, and IκBα inhibitor Bay11-7082 recovered XT-1 expression suppressed by 29-kDa FN-f [Fig. 5(A)]. In addition, ACAN downregulation by 29-kDa FN-f was reversed by pretreatment with MAPK and NF-κB inhibitors [Fig. 5(A)]. MAPK and NF-κB inhibitors also restored the expression levels of Sp1 and Sp3 altered by 29-kDa FN-f to those of untreated cells [Fig. 5(B)]. These results demonstrate that the MAPK and NF-κB signaling pathways play an important role in the regulation of XT-1 transcription factors Sp1 and Sp3, as well as in XT-1 expression.

TLR-2 and integrin α5β1 are receptors for 29-kDa FN-f in human chondrocytes

We previously reported that TLR-2 knockdown suppressed 29-kDa FN-f-mediated catabolic factor expression and signaling pathways in human primary chondrocytes[1]. Previous reports have shown that FN and 120-kDa FN-f interact with and stimulate members of the integrin family[15–17]. To verify the direct binding of 29-kDa FN-f to TLR-2, the interaction of TLR-2 with various concentrations of 29-kDa FN-f was assessed using a modified ELISA method. TLR-2 was incubated with 29-kDa FN-f to induce complex formation, then 29-kDa FN-f in the complex was probed using ELISA with an anti-FN antibody. Our ELISA data showed that the interaction of 29-kDa FN-f with TLR-2 increased in a dose-dependent manner, suggesting direct binding [Fig. 6(A)]. A slightly increased interaction in the IgG-treated sample used as a negative control was considered non-specific binding [Fig. 6(A)]. However, we could not confirm that 29-kDa FN-f directly binds integrin α5β1 with ELISA because purified integrin α5β1 is unavailable.

We further investigated whether TLR-2 and integrin α5β1 are implicated in 29-kDa FN-f-mediated XT-1 and ACAN expression using siRNA knockdown and blocking antibodies. First, human chondrocytes were transfected with siTLR-2, si-integrin α5β1, and control siRNA, followed by incubation with 29-kDa FN-f. We found that TLR-2 and integrin α5β1 knockdown recovered XT-1 and ACAN expression suppressed by 29-kDa FN-f [Fig. 6(B)]. This suggests that both TLR-2 and integrin α5β1 are primarily, or at least partially, responsible for 29-kDa FN-f-mediated pathways in the regulation of XT-1 and ACAN expression. Next, TLR-2 and integrin α5β1 receptors on chondrocytes were blocked using antibodies, followed by treatment with 29-kDa FN-f. Although XT-1 and ACAN expression were suppressed in response to 29-kDa FN-f, their expression was partially recovered by treatment with TLR-2 or integrin α5β1 antibodies [Fig. 6(C)]. Furthermore, a combination of two antibodies synergistically restored XT-1 and ACAN expression to the levels observed in untreated cells [Fig. 6(C)]. Blockading these receptors with siRNA and blocking antibodies also reversed the effects of 29-kDa FN-f on Sp1 and Sp3 expression [Fig. 6(B) and (C)]. Taken together, these results demonstrate that both TLR-2 and integrin α5β1 are responsible for 29-kDa FN-f modulation of the transcription factors Sp1 and Sp3 as well as XT-1 expression.

Discussion

XT-1, a crucial enzyme involved in GAG synthesis, was downregulated in human OA cartilage and surgically induced OA mouse cartilage compared with normal cartilage and sham surgery mouse cartilage, respectively. In addition, 29-kDa FN-f suppressed the expression of both XT-1 and ACAN in human articular chondrocytes. This study focused on the mechanism by which 29-kDa FN-f regulates the expression of XT-1, the enzyme regulating the rate-limiting step of GAG synthesis in cartilage matrix in primary human chondrocytes. 29-kDa FN-f downregulated XT-1 expression via TLR-2 and integrin α5β1 receptor-mediated signaling pathways. The transcription factors Sp1, Sp3, and AP-1 played particularly important roles in 29-kDa FN-f-suppression of XT-1.

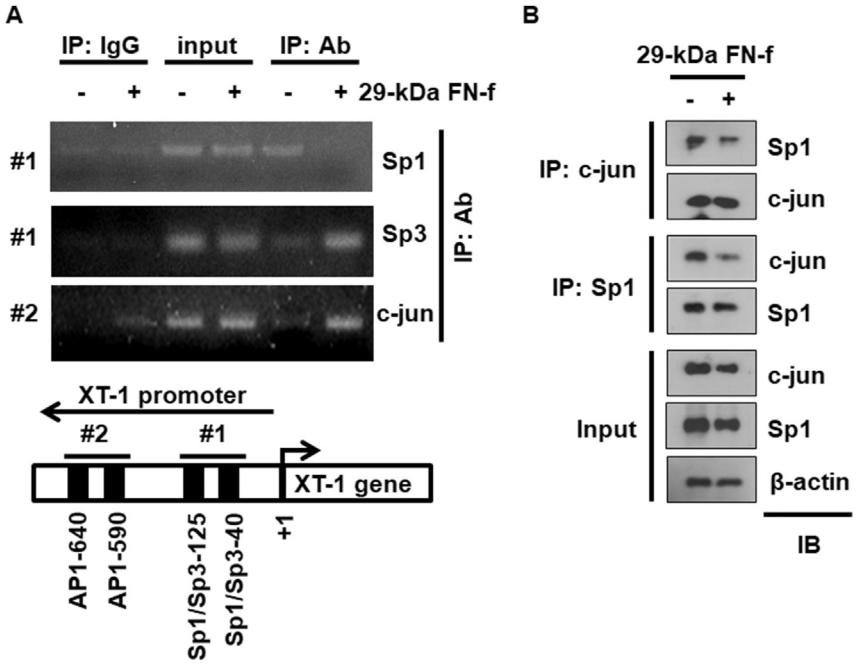

Fig. 4. 29-kDa FN-f alters the binding of transcription factors Sp1, Sp3, and AP-1 to the XT-1 promoter. (A) Chromatin immunoprecipitation (ChIP) assay for the binding of Sp1, Sp3, and AP-1 to the XT-1 promoter region in 29-kDa FN-f-treated chondrocytes. ChIP assays were performed using 29-kDa FN-f-treated chondrocytes. Chromatin was sheared by sonication and protein-DNA complexes were immunoprecipitated with antibodies against Sp1, Sp3, and c-jun. DNA retrieved from the precipitates was amplified with primers for specific sequences encompassing the Sp1 (#1), Sp3 (#1), and AP-1 (#2) binding regions in the XT-1 promoter. The PCR products were resolved on agarose gels and stained with GelRed. IgG was used as a negative control. Input represents 5% of the total cross-linked chromatin before immunoprecipitation. #1, Sp1/Sp3-40 and Sp1/Sp3-125; #2, AP1-590 and AP1-640 regions of the XT-1 promoter. Data are representative of three independent experiments using chondrocytes from three different donors. (B) 29-kDa FN-f inhibited the association between Sp1 and c-jun. 29-kDa FN-f-stimulated chondrocytes were lysed with RIPA buffer and the nuclear extracts were immunoprecipitated with antibodies against Sp1 or c-jun. The proteins were analyzed by western blot analysis with antibodies against Sp1, c-jun, and β-actin. Input, nuclear extracts. Data are representative of three independent experiments using chondrocytes from three different donors.

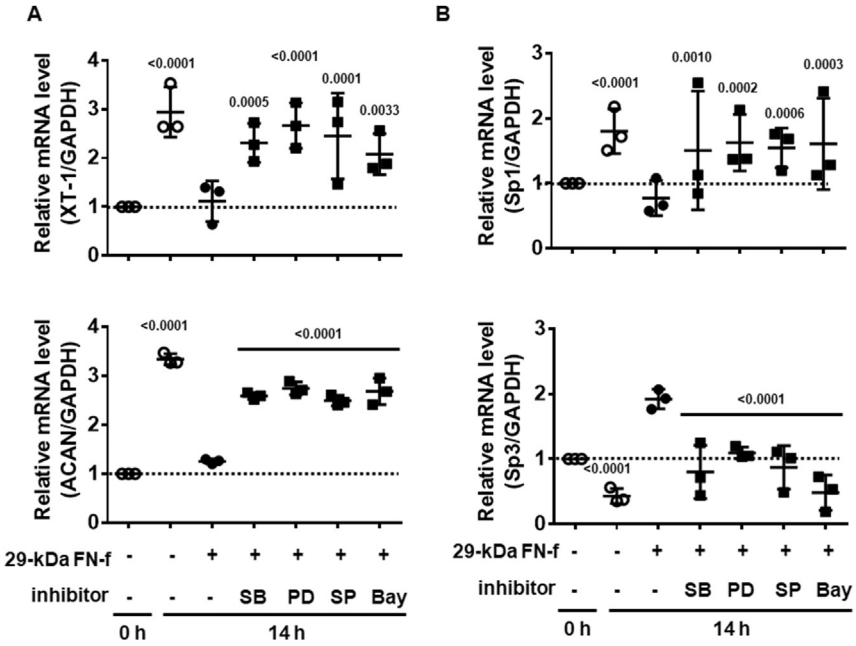

Fig. 5. 29-kDa FN-f downregulates XT-1 expression by activating nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. The NF-κB and MAPK signaling pathways are implicated in the expression of (A) XT-1 and ACAN and (B) Sp1 and Sp3. Chondrocytes were pretreated for 2 h with various inhibitors and stimulated with 29-kDa FN-f for 14 h. mRNA levels of XT-1 were measured using Quanti Fast SYBR Green-based RT-PCR. SP (SP6001250), JNK inhibitor; SB (SB203580), p38 inhibitor; PD (PD98059), MEK1/2 inhibitor; Bay (Bay 11-7082), NF-κB inhibitor. Data are expressed as the mean ± SD of duplicate data from three independent donors. *P* value was presented between two groups (vs 29-kDa FN-f-treated cells). Dot indicates the representative value obtained from each donor.

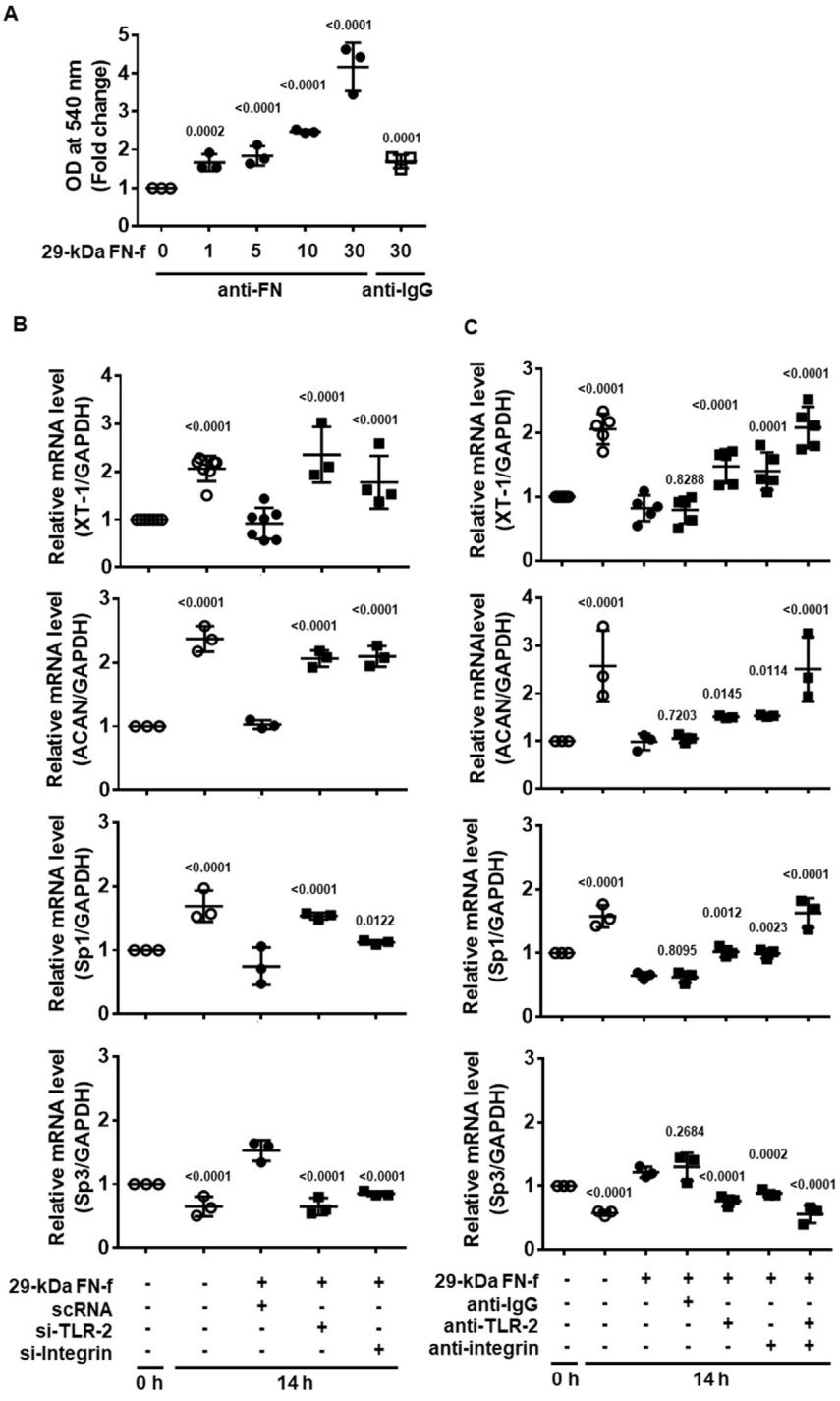

Fig. 6. Toll-like receptor 2 (TLR-2) and integrin α5β1 function as receptors for 29-kDa FN-f in human primary chondrocytes. (A) 29-kDa FN-f significantly binds to TLR-2. 29-kDa FN-f (1, 5, 10, and 30 μg/mL) was added to TLR-2-coated microplates for 2 h, and antibodies specific to FN or IgG were added to each well for 1 h. Data are expressed as the mean ± SD of triplicate data from three independent donors. *P* value was presented between two groups (vs untreated controls). (B) Effect of TLR-2 or integrin α5β1 knockdown on 29-kDa FN-f-suppressed XT-1, ACAN, Sp1, and Sp3 expression. 29-kDa FN-f-inhibited XT-1 expression was recovered by knockdown of endogenous TLR-2 and integrin expression. Chondrocytes were transfected with siTLR-2, si-integrin α5β1, and control siRNA. After 48 h, cells were stimulated with 29-kDa FN-f for 14 h. XT-1, ACAN, Sp1, and Sp3 expression were measured using Quanti Fast SYBR Green-based RT-PCR. Data are expressed as the mean ± SD of duplicate data from more than three independent donors. *P* value was presented between two groups (vs scRNA- and 29-kDa FN-f-treated cells). (C) Effects of TLR-2 and integrin α5β1 antibodies on 29-kDa FN-f-suppressed XT-1 expression in primary chondrocytes. To neutralize TLR-2 and integrin α5β1, chondrocytes were incubated with antibodies specific to TLR-2, integrin α5β1, and IgG for 24 h followed by treatment with 29-kDa FN-f for 14 h. XT-1, ACAN, Sp1, and Sp3 expression were measured by Quanti Fast SYBR Green-based RT-PCR. Data are expressed as the mean ± SD of duplicate data from three independent donors. *P* value was presented between two groups (vs 29-kDa FN-f-treated cells). Dot indicates the representative value obtained from each donor.

In the pathogenesis of OA, a variety of proteins found in OA synovial fluids activate inflammatory and catabolic signaling pathways and act as DAMPs[18–21]. For example, S100A8/S100A9 in synovial fluid in OA and RA modulates the expression of catabolic factors, including MMPs and IL-6, and anabolic factors, including ACAN and type II collagen[22]. Exposure of human OA chondrocytes to high-mobility group box-1 (HMGB1) induces the expression of nitric oxide (NO), IL-1β, TNF, and MMP-13[23]. We previously reported that 29-kDa FN-f, which was found in the synovial fluid of OA joints, triggered TLR-2/MAPK/NF-kB signaling pathways and was implicated in the induction of catabolic enzymes, such as MMP-1, -3, and -13[1]. A contradictory report found that low-concentration 29-kDa FN-f (1 nM) increased the synthesis of PG and the release of anabolic factors, such as insulin-like growth factor-1 and TGF-β1, indicating that it possesses both catabolic and anabolic activities[24]. In addition to stimulating catabolic signaling, DAMPs, such as soluble biglycan, S100A8/S100A9, and ACAN 32-mer fragment, negatively regulated the expression of anabolic factors, including ACAN and type II collagen in chondrocytes by modulating the TLR-2- or TLR-4-NF-κB pathways[22,25,26]. TLR-4 activation by lipopolysaccharide (LPS) was found to decrease the expression of ACAN and type II collagen, and blocking p38 inhibited the negative effects of LPS on matrix biosynthesis[27]. These observations suggest that the DAMP/TLR pathway may be important in both the catabolic and anabolic processes of ECM. Compared with the regulation of catabolic responses by DAMPs in OA cartilage, relatively few studies have reported its role in anabolic signaling pathways in the pathogenesis of OA.

XTs, including XT-1 and XT-2, initiate GAG biosynthesis by transferring a sugar to an acceptor protein or peptide[28]. They show different tissue expression patterns; for example, XT-1 expression is predominant in mouse testis, kidney, and brain, whereas XT-2 is dominant in the liver, indicating the different physiological functions of the two enzymes[29,30]. A previous study in dwarf mutant mice showed that XT-1, but not XT-2, was associated with skeletal development, suggesting a lack of redundancy in XT expression in cartilage tissue[7]. Mutational analysis of the XT gene in OA patients suggested that although single nucleotide polymorphisms of XT-1 and XT-2 were not correlated with OA duration, joint involvement, XT activity, and hip or knee phenotype, elevated serum levels of XT activity served as a marker for the progression of articular cartilage damage[31]. Furthermore, XT activity was reported to decrease depending on cartilage aging in rats[32]. Consistent with previous reports, our data showed that XT-1 was significantly downregulated in human OA and DMM-induced mouse OA cartilage relative to normal cartilage, and 29-kDa FN-f, a DAMP, significantly suppressed the expression of XT-1 in monolayer chondrocyte culture, explant culture of human cartilage tissue, and *in vivo* mouse joint cartilage, indicating that 29-kDa FN-f may be involved in the pathogenesis of OA through the regulation of XT-1 expression. In a previous study, injection of FN-fs into rabbit knee joints resulted in up to 70% depletion of proteoglycan within 7 days[11], while our study showed that such injection had little influence on ECM of mouse joint cartilage. This discrepancy may result from the difference in experimental animal (species and age) and the source of FN-f. For example, a mixture of FN-f (29- & 50-kDa FN-f) purified from rabbit plasma was used in the previous study whereas we used 29-kDa FN-f isolated from human plasma. XT-1 was found to be co-regulated with ACAN and XT-1 inhibition was shown before loss of safranin O (Fig. S2 and Fig. 2(C)). This suggests that XT-1 might serve as a surrogate marker for ACAN or cartilage loss.

Sp1 and Sp3 are members of the specificity protein (Sp)/Krüppel-like transcription factor family[33]. Sp1 has the capacity to form tetramers owing to its C-terminal D domain, which is involved in synergistic transactivation by regulating chromatin looping between enhancer and promoter regions of target genes[34]. By contrast, Sp3 binds as a monomer to Sp binding sites in promoters without forming multimers, and shows no synergistic transactivation[35]. Sp1 and Sp3 participate in the expression of a variety of genes by competitively binding to the same region in the promoters of target genes, and the Sp3/Sp1 ratio consequently influences promoter activity[36]. In chondrocytes, IL-1β increases Sp3 protein expression and inhibits Sp1 protein biosynthesis while decreasing the binding activity of both Sp1 and Sp3 to the COL2A1 promoter[37]. The siRNA-mediated knockdown of Sp3 led to a highly significant reduction of XT-1 mRNA, and the c-Jun/AP-1 transcription factor was essential for full XT-1 promoter activity in SW1353 cells[38]. On the other hand, Sp1 knockdown partially inhibited ADAMTS-4 induction by IL-1 in chondrocytes[38]. Consistent with these reports, our results from Sp1 and Sp3 silencing and overexpression vector transfections showed that 29-kDa FN-f altered the expression of Sp1 and Sp3 and thus the Sp3/Sp1 ratio, leading to reduced XT-1 expression in human articular chondrocytes. Sp1 and Sp3 interact directly and indirectly with transcription factors, transcriptional regulators, and chromatin remodeling factors, and the activities of Sp1 and Sp3 are modulated by binding proteins as well[36]. The interactions of Sp1 and c-Jun are implicated in the regulation of claudin-4, damage-induced neuronal endopeptidase, and integrin-linked kinase[39–41]. Our IP analysis revealed that 29-kDa FN-f suppressed the association of Sp1 and c-jun in the XT-1 promoter, reducing XT-1 expression.

We previously reported that chondrocytes expressed TLRs and that 29-kDa FN-f induced catabolic responses by binding to TLR-2 and increasing TLR-2 expression[8]. Previous reports demonstrated that integrin α5β1 also functioned as the receptor for FN and the FN-derived proteolytic peptides 29-, 50-, and 140-kDa FN-fs[16,42]. Here, by using antibodies against TLR-2 and α5β1 integrin and siRNA tools, we demonstrated that both receptors were involved in the regulation of XT-1 expression by 29-kDa FN-f, and blocking both using antibodies had synergistic effects on reversing the effects of 29-kDa FN-f. The significant overlap of signaling pathway of FN-f and IL-1β may be due to signaling through TLR-2 by FN-f which utilizes MyD88-IRAK-MAPK-AP-1 pathway.

In conclusion, our results demonstrate that 29-kDa FN-f suppresses anabolic responses by activating TLR-2- and integrin α5β1-dependent signaling pathways in human articular chondrocytes. Specifically, 29-kDa FN-f affects the pathogenesis of OA by reducing the expression of cartilage matrix synthesis enzyme XT-1, suggesting that a relevant pathway may be a useful therapeutic target in OA.

Author contributions

HSH and HAK contributed to study conception and design. MHL contributed to acquisition of data. HSH contributed to analysis and interpretation of data. HSH, MHL and HAK drafted the article or revised it critically for important intellectual content. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflicts of interest.

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Supplementary data

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