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NOD2 signaling pathway is involved in fibronectin fragment-induced pro-catabolic factor expressions in human articular chondrocytes

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ABSTRACT

The nucleotide-binding and oligomerization domain (NOD) is an innate pattern recognition receptor that recognizes pathogen- and damage-associated molecular patterns. The 29-kDa amino-terminal fibronectin fragment (29-kDa FN-f) is a matrix degradation product found in the synovial fluids of patients with osteoarthritis (OA). We investigated whether NOD2 was involved in 29-kDa FN-f-induced pro-catabolic gene expression in human chondrocytes. The expression of mRNA and protein was measured using quantitative real-time polymerase chain reaction (qrt-PCR) and Western blot analysis. Small interfering RNAs were used for knockdown of NOD2 and toll-like receptor 2 (TLR-2). An immunoprecipitation assay was performed to examine protein interactions. The NOD2 levels in human OA cartilage were much higher than in normal cartilage. NOD1 and NOD2 expression, as well as pro-inflammatory cytokines, including interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), were upregulated by 29-kDa FN-f in human chondrocytes. NOD2 silencing showed that NOD2 was involved in the 29-kDa FN-f-induced expression of TLR-2. Expressions of IL-6, IL-8, matrix metalloproteinase (MMP)-1, -3, and -13 were also suppressed by TLR-2 knockdown. Furthermore, NOD2 and TLR-2 knockdown data demonstrated that both NOD2 and TLR-2 modulated the expressions of their adaptors, receptor-interacting protein 2 (RIP2) and myeloid differentiation 88, in 29-kDa FN-f-treated chondrocytes. 29-kDa FN-f enhanced the interaction of NOD2, RIP2 and transforming growth factor beta-activated kinase 1 (TAK1), an indispensable signaling intermediate in the TLR-2 signaling pathway, and activated nuclear factor-κB (NF-κB), subsequently leading to increased expressions of pro-inflammatory cytokines and cartilage-degrading enzymes. These results demonstrate that 29-kDa FN-f modulated pro-catabolic responses via cross-regulation of NOD2 and TLR-2 signaling pathways.

INTRODUCTION

Osteoarthritis (OA) is characterized by the degeneration of articular cartilage, synovial inflammation, and joint pain (1). Degradation of the extracellular matrix (ECM) is a crucial event leading to joint destruction in OA, rheumatoid arthritis (RA), and septic arthritis (2). Chondrocytes express catabolic mediators in response to proinflammatory cytokines, chemokines, adipokines, and mechanical loading (3). Fibronectin (FN) fragments result from the breakdown of FN, an ECM glycoprotein of cartilage, and in arthritis synovial fluid, up to 50% of the FN is fragmented into molecular sizes of 29 to 200 kDa (4). The most potent fragment, 29-kDa FN-f, stimulates cartilage ECM degradation by increasing the expression of nitric oxide (NO) and of matrix metalloproteinase (MMP)-1, -3, and -13 (5, 6). Innate pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD) receptors, recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (7, 8). In response to various stresses, endogenous ligands, including fibronectin, heparin sulfate, and high mobility group box1 (9), are released by injured tissues or activated cells and activate signals of innate and adaptive immunity (10). Previously, we reported that 29-kDa FN-f induced catabolic responses, including MMPs expression in articular chondrocyte cultures by activation of NF-kappaB (NF-κB) and p38 through an MyD88-dependent TLR-2 signaling pathway (11). Thus, there is a possibility that activation of DAMP signaling forms a vicious cycle in OA pathogenesis by causing matrix damage and generation of matrix degradation products, which result in more DAMP signaling. A recent study provided additional evidence that PPRs are involved in OA development (12). Polymorphisms of the promoter TLR-3 and TLR-9 genes were associated with knee OA in a Chinese population (13, 14). We previously demonstrated that TLR-2, -3, -4, and -5 were significantly upregulated in OA cartilage compared to normal cartilage (11), and

that TLR-2 and TLR-4 ligands strongly induced catabolic responses in chondrocytes (3). In addition to cartilage degeneration, TLR activation has implications in the development of synovitis in OA, since TLRs 1-7 and 9 have been detected in synovial membranes in OA and *in vitro* synovial fibroblasts have been shown to respond to many microbial TLR agonists (15).

NOD1 and NOD2, members of the leucine-rich repeat containing receptor (NLR) family, sense specific bacterial peptidoglycans and modulate expression of inflammatory genes through activation of the NF-kB signaling pathway (16). NOD2 plays an important role in activation of the innate immune response to muramyl dipeptide (MDP) found in all gram-positive and gram-negative bacteria, whereas NOD1 recognizes γ-D-glutamyl-*meso*-diaminopimelic acid which is contained in the proteoglycan of all gram-negative bacteria (17). NOD2 recruits the adaptor protein receptor interacting-protein 2 (RIP2/RICK2) and stimulates a TLR-2 downstream molecule, TNF receptor-associated factor 6, which drives the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways (16, 18, 19). In particular, NOD2 mutation and polymorphism lead to various diseases, including Crohn's disease, Blau syndrome, and early onset sarcoidosis (17). We aimed to reveal the role of NOD2 in 29-kDa FN-f-induced pro-catabolic responses in primary chondrocytes. Tight regulation of innate immune receptor cross-signaling would thus be necessary to evade hyper-activation of pro-inflammatory and pro-catabolic responses.

In this study, we investigated whether 29-kDa FN-f modulated pro-catabolic responses through the NOD2 signaling pathway in articular chondrocytes. In addition, crosstalk between TLR-2 and NOD2 signaling pathways was explored.

RESULTS

NOD2 expression was elevated in OA cartilage tissue and 29-kDa FN-f-treated chondrocytes

To investigate the role of NOD2 in OA pathogenesis, the differences of NOD2 expression in OA and normal cartilage were compared using quantitative real-time reverse transcription polymerase chain reactions (qRT-PCR) and immunohistochemical (IHC) analyses. NOD2 expression at the mRNA level was significantly increased in OA cartilage compared to normal cartilage (Fig. 1A). IHC analyses also showed that OA cartilages showed strong expression of NOD2 in the chondrocytes in all zones, including the superficial, middle, and deep zones of the articular cartilage, together with the loss of cartilage matrix, compared to normal cartilage (Fig. 1B).

We investigated whether NOD1 and NOD2 expressions could be regulated by 29-kDa FN-f and pro-inflammatory cytokines, including IL-1β and TNF-α, in primary human chondrocytes. Both NOD1 and NOD2 expressions were significantly increased 24 h and 48 h following treatment with 29-kDa FN-f compared to untreated cells (Fig. 1C). Increased expressions of both receptors were also observed in IL-1β- and TNF-α-treated cells (Fig. 1D and E). Taken together, these results demonstrate that 29-kDa FN-f modulated the expression of PRR, including NOD1 and NOD2.

29-kDa FN-f induced pro-catabolic gene expressions via NOD2 in primary human chondrocytes

To examine whether NOD2 and/or TLR-2 signaling pathways were involved in 29-kDa FN-f-induced expression of inflammatory genes, chondrocytes were transfected with control siRNA, si-NOD2, or si-TLR-2, followed by treatment with 29-kDa FN-f. QRT-PCR analysis showed that 29-kDa FN-f highly induced the expression of the IL-6 and IL-8 pro-

inflammatory cytokines, and the proteoglycan degrading enzymes MMP-1, -3, and -13, whereas a single knockdown of NOD2 or TLR-2 significantly suppressed the 29-kDa FN-f-induced expression of IL-6, IL-8, and MMPs (Fig. 2A). In addition, double knockdown of NOD2 and TLR-2 exhibited greater reductions in the expressions of IL-6, IL-8, MMP-1, and MMP-3 than a single knockdown of NOD2 or TLR-2 (Fig. 2A). These data demonstrate that NOD2 and TLR-2 signaling pathways, which are responsible for 29-kDa FN-f-induced expression of pro-catabolic genes, controlled the expressions both independently and synergistically.

Next, we investigated whether NOD2 and TLR-2 affected reciprocal expression of the genes. NOD2 knockdown significantly suppressed 29-kDa FN-f-induced expressions of TLR-2, while TLR-2 knockdown suppressed NOD2 expression induced by 29-kDa FN-f (Fig. 2B). Our data demonstrate that NOD2 and TLR-2 reciprocally regulated 29-kDa FN-f-stimulated expression of each other, and suggested that they may synergistically work together in 29-kDa FN-f-induced pro-catabolic gene expression.

TLR-2 and NOD2 reciprocally modulated their downstream adaptor expression

To explore the mechanism of 29-kDa FN-f-induced expressions of pro-inflammatory factors via NOD2, we analyzed the effect of 29-kDa FN-f on the protein expression of NOD2 and its downstream adaptor RIP2. 29-kDa FN-f increased both NOD2 and RIP2 expressions and elevated TLR-2 expression (Fig. 3A), showing that 29-kDa FN-f positively regulated the expression of the NOD2 receptor and its downstream molecule. We next examined whether the increased levels of NOD2 or TLR-2 influenced the expression of either one's adaptor. Human articular chondrocytes were transfected with si-NOD2 or si-TLR-2, then stimulated with 29-kDa FN-f for 24 h. 29-kDa FN-f greatly increased TLR-2 expression, whereas NOD2

silencing significantly suppressed the expression, as well as the expressions of RIP2 and MyD88 (Fig. 3B). In addition, we observed similar results for TLR-2 knockdown, which significantly suppressed the 29-kDa FN-f-stimulated expressions of NOD2 and the downstream molecule, RIP2 (Fig. 3C). These results demonstrate that TLR-2 and NOD2 reciprocally influenced their signaling pathways through modulation of the expression of their respective receptors and adapters in response to 29-kDa FN-f.

29-kDa FN-f activated the NF-kB signaling pathway through enhanced formation of NOD2, RIP2, and TAK1 complex

29-kDa FN-f-mediated activation of the NF-κB and p38 MAPK signaling pathways was associated with TLR-2 in human chondrocytes (11). To investigate the involvement of NOD2 in the 29-kDa FN-f-mediated signaling pathway, control siRNA or si-NOD2-transfected chondrocytes were stimulated with 29-kDa FN-f for 15 to 60 min. Immunoblot analyses showed that higher phosphorylation of IκBα was observed 15 minutes after 29-kDa FN-f treatment than in untreated cells, while NOD2 knockdown significantly inhibited the phosphorylation (Fig. 4). In contrast, the phosphorylation of JNK, ERK1/2, and p38 MAPK was not altered by NOD2 knockdown (Fig. 4). The results demonstrate that 29-kDa FN-f-induced inflammatory factor expression was dependent on the NOD2-dependent NF-κB signaling pathway.

Activated NOD2 binds to its adaptor, RIP2, and recruits transforming growth factor β-activated kinase 1 (TAK1) to the complex, subsequently leading to NF-κB activation (20). We investigated whether 29-kDa FN-f influenced formation of the NOD2/RIP2/TAK1 complex. Primary chondrocytes were transfected with si-NOD and treated with 29-kDa FN-f for 24 h. Cell lysates were immunoprecipitated (IP) with an antibody against RIP2. 29-kDa

FN-f induced the expressions of NOD2, RIP2, and TAK1, but NOD2 silencing lowered their expressions (Fig. 4B). IP data revealed that 29-kDa FN-f increased formation of the NOD2/RIP2/TAK1 complex, whereas NOD2 silencing inhibited it due to decreased expression of NOD2, RIP2, and TAK1, indicating that 29-kDa FN-f activated the NOD2/RIP2/TAK1 pathway (Fig. 4B). Therefore, these results suggest that 29-kDa FN-f activated the pro-catabolic NF-κB signaling pathway via the NOD2 signaling pathway.

DISCUSSION

In this study, we examined whether NOD2 expression was elevated in OA cartilage compared to normal cartilage. Consistent with previous reports, 29-kDa FN-f significantly induced pro-inflammatory gene expressions, including IL-6 and IL-8, and proteoglycan degrading enzymes, including MMP-1, -3, and -13, through synergistic action of the NOD2 and TLR-2 signaling pathways. In addition, NOD2 and TLR-2 reciprocally modulated the 29-kDa FN-f-stimulated expression of their respective adaptors, RIP2 and MyD88. Further, 29-kDa FN-f activated NF-κB through NOD2 signaling and promoted the formation of NOD2/RIP2/TAK1, subsequently increasing expressions of pro-catabolic factors.

Synovial fluids contain various molecules released from damaged joint tissue. In particular, molecules derived from the cartilage extracellular matrix, including fragments of aggrecan, hyaluronan, collagen, and fibronectin (FN), have been found in the cartilage and synovial fluid from patients with OA and RA. For example, FN-fs in OA synovial fluids were present at concentrations of more than 1 μM, together with increased proteolytic enzymes (21). Aggrecan fragments (150-250 kDa high-molecular and 32-amino acids) were also identified in the synovial fluid from OA patients (22). A recent report showed that the naturally-occurring

aggrecan 32-mer fragment triggered catabolic factor expression via TLR-2 and activation of NF-κB (23). Another report showed that the aggrecan 32-mer excited dorsal root ganglion nociceptive neurons, both in culture and in intact explants, which was mediated through TLR-2 (24), suggesting that DAMPs induce the cartilage catabolic process, as well as pain, in OA. In addition, promoter polymorphism of the TLR-9 gene was associated with end-stage knee OA, while that of TLR-3 was associated with susceptibility to knee OA in a Chinese Han population (13, 14). Thus, the elucidation of signaling mechanisms controlling the DAMP response is gaining importance in understating the pathogenesis of OA.

Cell surface TLRs sense intact bacterial peptidoglycans (PGN), whereas intracellular NOD2 recognizes degradation products resulting after the uptake of PGN and its release into the cytosol (25). NOD1 and NOD2 mRNA expressions have been demonstrated in the synovial tissues from RA and OA patients, and NOD2-deficient mice showed decreases in joint inflammation and cytokine production in a streptococcal cell wall (SCW)-induced arthritis model (26). A previous report showed that Ag-induced arthritis developed in NOD2 and RIPK2-deficient mice, and impaired NOD2/RIP2 signaling pathways in joints led to reduced production of proinflammatory cytokines and chemokines, including TNF, IL-1β, and CXCL1 (27). TLR-2 and NOD2 receptor expressions were enhanced by IL-32γ and Pam3Cys/MDP stimulation, and AdIL-32γ transduction, followed by the injection of SCW, displayed aggravated joint inflammation and cartilage destruction in mice (28). TLR-2/NOD2 activation significantly induced cartilage-degrading mediators, including MMP-1, -3, inducible nitric oxide synthase, TLR-2, and NOD2, in fibroblast-like synoviocytes (28). Several studies have reported cross-regulation between NOD2 and TLR-2 signaling. IL-12 expression was increased by a variety of TLR ligands in NOD2-deficient splenic macrophages compared to wild type cells, indicating that NOD2 is a negative regulator of the TLR-2 response (29). On the other hand, NOD2 and

TLR-2 were required for PGN-induced cytokine production and had a synergistic influence on murine peritoneal macrophages (29, 30). In our *in vitro* study, we demonstrated that 29-kDa FN-f modulated IL-6 and IL-8 expressions, as well as MMPs, through the coordinated actions of NOD2 and TLR-2, indicating a close synergistic relationship between the NOD2 and TLR-2 signaling pathways in the 29-kDa FN-f-induced catabolic responses.

IL-1β induces expression of proteoglycan-degrading enzymes and functions synergistically with other cytokines and chemokines, such as IL-6 and IL-8, subsequently worsening inflammation (31, 32). In addition, cartilage proteoglycan loss was inhibited in the knee joints of IL-6-deficient mice compared to wild type, suggesting that IL-6 aggravates cartilage destruction (33). NOD2 activation produced IL-6 and IL-8 in RA synovial fibroblasts by synergistic action with TLRs, indicating that up-regulation of proinflammatory cytokines was produced by the increased expression of NOD2 from TLR-2 activation (34). A previous study using human B lymphocytes showed that the NOD2 ligands enhanced TLR-induced B cell activation (35). Thus, the intracellular cytoplasmic sensor, NOD2, and membrane-bound TLR-2 may recognize a variety of pathogens and stimuli due to different localization, whereas upon recognition of PAMPs, the two receptors converge into common pathways, i.e. NF-κB and MAPK, leading to inflammatory immune responses and host defense (36). Furthermore, NOD2 may respond to PAMPs that are delivered into the cytoplasm without detection by extracellular TLRs. Therefore, these receptors may function synergistically through tight regulation of the crosstalk between their signaling pathways.

Taken together, our findings underline the important role of NOD2 signaling in the 29-kDa FN-f-induced catabolic responses in articular chondrocytes. NOD2 and TLR-2 pathways contribute to the independent triggering of 29-kDa FN-f-induced catabolic gene expressions and, at least in part, cooperate through managing the complex formation of NOD2/RIP2 and TAK1, a

downstream effector of TLR-2, suggesting that TLR-2 and NOD2 cross-regulation pathways may be useful targets to prevent the development and progression of arthritis.

MATERIALS AND METHODS

Materials, IHC analysis, transfection with siRNA, qRT-PCR, immunoblot analysis, and statistical analyses are described in the Supplementary Materials.

Immunoprecipitation (IP) assays

Proteins were extracted from chondrocytes with RIPA lysis buffer and supernatants were obtained by centrifugation at 13,000 × *g* for 10 min at 4°C. Supernatants containing equal amounts of proteins were pre-incubated with a protein A agarose bead slurry for 4 h at 4°C on a rotating shaker, then incubated with specific antibodies against RIP2 or IgG in the presence of protein A agarose beads at 4°C overnight with gentle rotation. Proteins eluted in 2x SDS sample buffer were separated on SDS-PAGE and subjected to immunoblot analyses as described above. A portion of the protein extracts was used as an input control.

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Disclosure statement

The authors declare no conflicts of interest.

FIGURE LEGENDS

Figure 1. NOD2 expression increased in osteoarthritis (OA) cartilage and 29-kDa FN-f-treated chondrocytes. (A) Relative expression of NOD2 in human normal and OA cartilage. Relative expression of NOD2 in normal and OA cartilage was measured using SYBR Green-based real-time polymerase chain reaction (qPCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. *P < 0.05 vs. normal cartilage. Data are presented as the mean ± standard deviation (SD) of data from duplicate experiments using cartilages from different donors (normal cartilage, n = 7 and OA cartilage, n= 25). (B) IHC staining for NOD2 in normal and OA cartilage. NOD2 expression was determined by IHC using an antibody against NOD2. Sections were counterstained with methyl green. Data are representative of results from three normal and OA cartilages. Scale bars = 100 μm for 100× and 200× magnifications. The arrows indicate NOD2-positive chondrocytes. (C)-(E). (C) 29-kDa FN-f and inflammatory cytokines, including (D) IL-1β and (E) TNF-α, positively increased NOD1 and NOD2 expression. Chondrocytes were exposed to 29-kDa FN-f, IL-1β, or TNF-α for 6, 24, and 48 h and mRNA levels of NOD1 and NOD2 were measured by qPCR. Data are presented as the mean ± SD of duplicate data from more than five different donors. *P < 0.05, ***P < 0.005, and ****P < 0.001 vs. untreated control. ns, not significant.

Figure 2. 29-kDa FN-f increased pro-catabolic factor expressions through synergistic activation of NOD2 and TLR-2. (A) NOD2 and TLR-2 silencing inhibited 29-kDa FN-f-induced IL-6, IL-8, MMP-1, -3, and -13 expressions. Chondrocytes were transfected with control small interfering (si) RNA (si-con), si-NOD2 RNA (si-NOD2), or TLR-2 RNA (si-TLR-2), and 48 h later chondrocytes were treated with 29-kDa FN-f for 24 h. mRNA levels

were measured using qPCR. Data represent the mean ± SD of duplicate data from more than five different donors. GAPDH served as an endogenous control. *P < 0.05 and ****P < 0.001 vs. si-con-transfected cells. #P < 0.05, ## P < 0.01, ### P < 0.005, and ####P < 0.001 vs. 29-kDa FN-f+si-NOD2 and siTLR-2-transfected cells. ns, not significant. (B) The reciprocal regulation of NOD2 and TLR-2 on their mRNA expressions. mRNA expression of NOD2 and TLR-2 was measured in 29-kDa FN-f+si-NOD2- or 29-kDa FN-f+si-TLR-2-transfected chondrocytes using SYBR Green-based real-time PCR assay. GAPDH was used as an endogenous control. Data represent the mean ± SD of duplicate data from more than five different donors. *** P < 0.005 and ****P < 0.001 vs. si-NOD2- or si-TLR-2-transfected cells.

Figure 3. NOD2 and TLR-2 cross-regulated the expression of their respective adaptor molecules. (A) 29-kDa FN-f increased the protein levels of TLR-2 and NOD2 in a time-dependent manner. Chondrocytes were incubated with 29-kDa FN-f for 4, 8, 16, and 24 h. (B), (C) NOD2 and TLR-2 receptors were involved in expression of the adaptor molecules of NOD2 and TLR-2. Chondrocytes were transfected with control siRNA and (B) si-NOD2 or (C) si-TLR-2. Forty-eight hours later, chondrocytes were stimulated with 29-kDa FN-f for 24 h. The protein levels were measured by immunoblot analyses. β-actin served as a loading control.

Figure 4. 29-kDa FN-f activated the NF-κB signaling pathway through the NOD2/RIP2/TAK1 signaling pathway. (A) NOD2 silencing inhibited the 29-kDa FN-f-induced NF-kB activation. Human chondrocytes were transfected with control small interfering RNA (si-con) or si-NOD2, then stimulated with 29-kDa FN-f (300 nM) for 15, 30, and 60 min. The levels of p-ERK/ERK, p-JNK/JNK, p-p38/p38, and p-IκBα/IκBα were determined by immunoblot analyses.

Immunoblot data are representative of three independent experiments from different donors. (B) 29-kDa FN-f increased the formation of the NOD2/RIP2/TAK1 complex. 29-kDa FN-f-stimulated chondrocytes were lysed with RIPA buffer and the cell extracts were immunoprecipitated with an antibody against RIP2. The proteins were analyzed by immunoblot analyses using antibodies against NOD2, RIP2, and TAK1. Immunoprecipitation with IgG served as a negative control.

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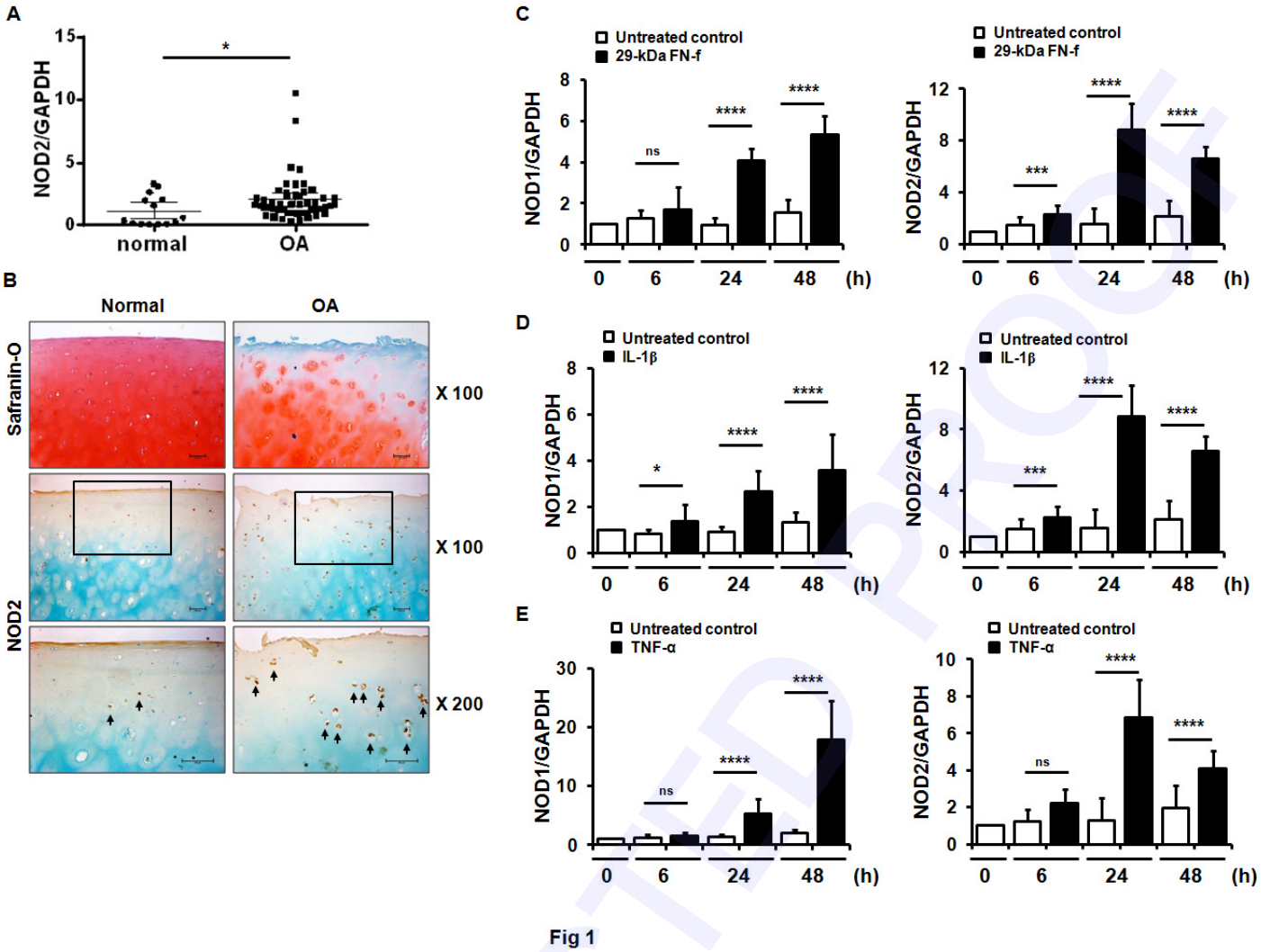

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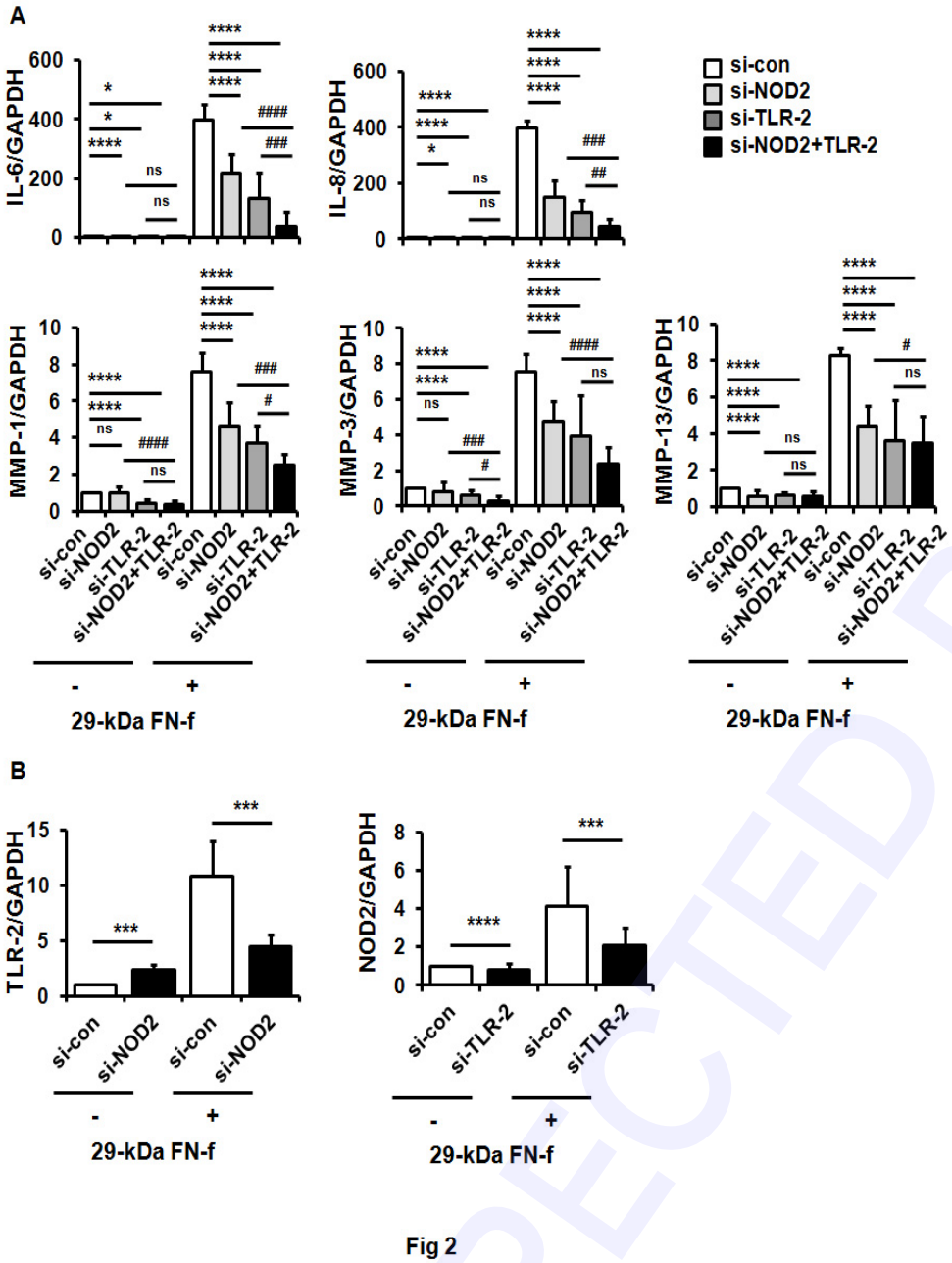

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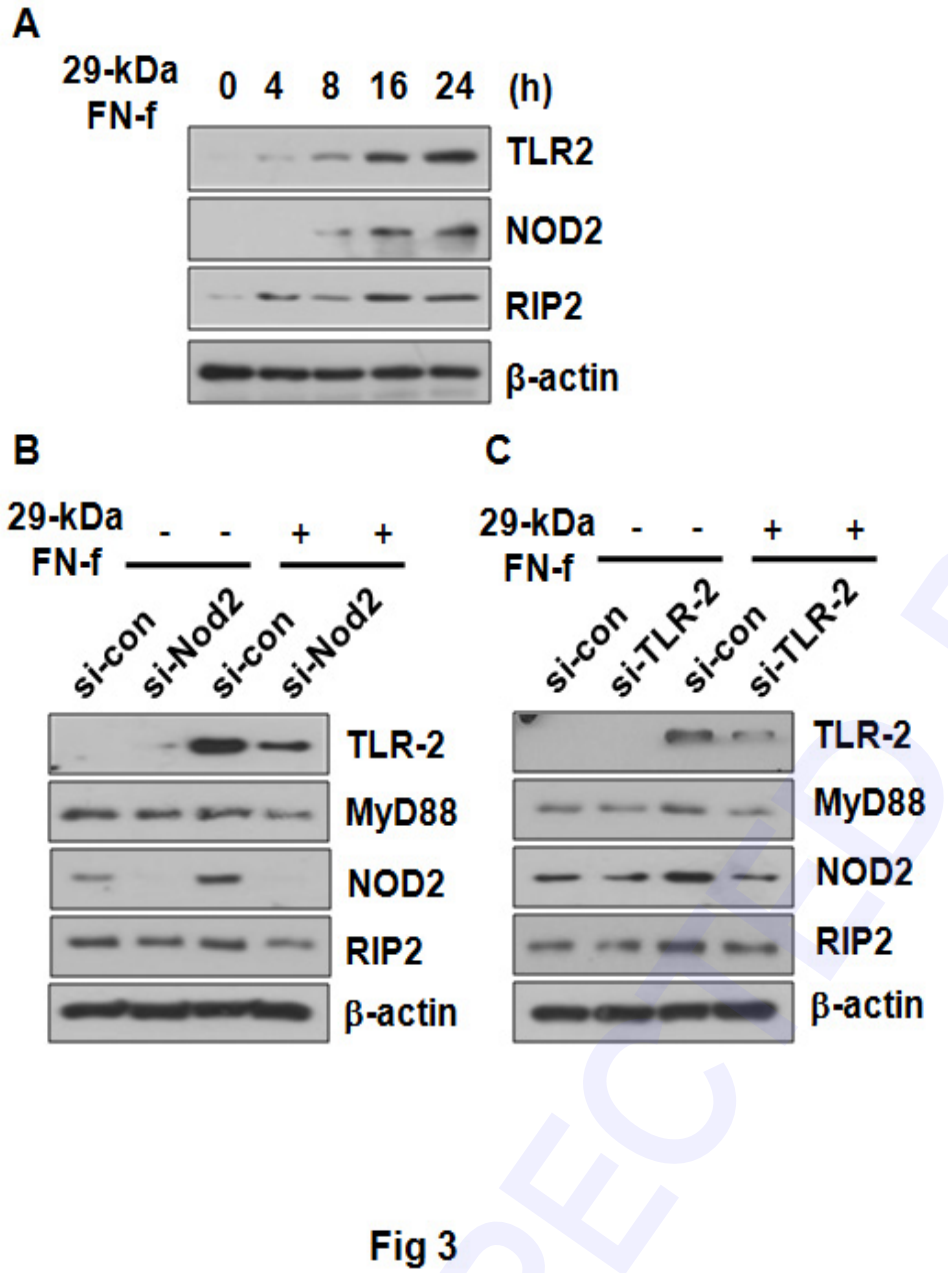

Fig 3

Fig. 3. Figure 3

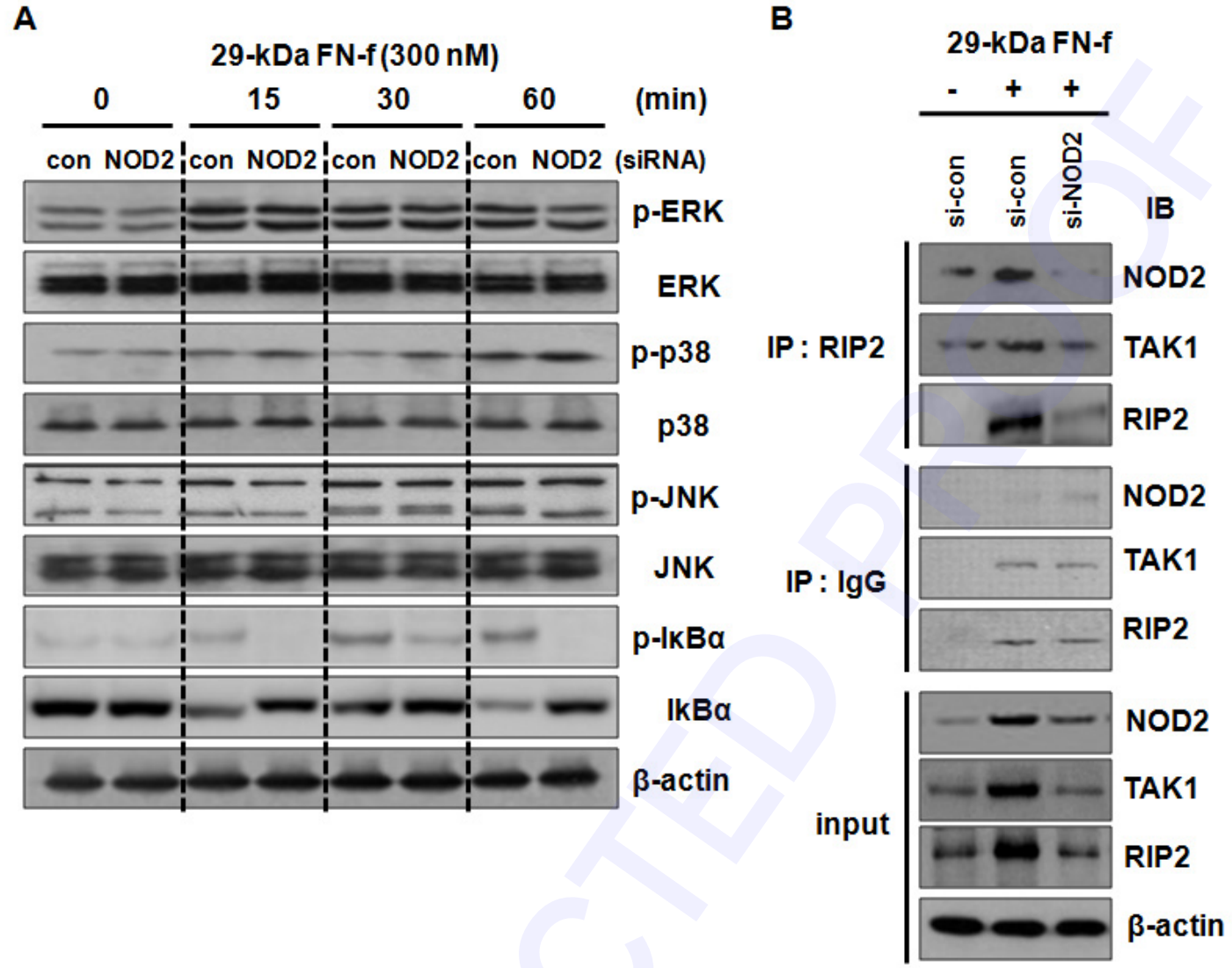

Fig 4

Fig. 4. Figure 4

Materials

Recombinant human 29-kDa FN-f and an antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were from Cell signaling Technology (Danvers, MA, USA): Myd88, NOD2, RIP2, SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p38 MAPK (p38), phospho-p38 (Thr180/Tyr182), p44/42 MAPK (Erk1/2), phospho-ERK1/2 (Thr202/Tyr204), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) and phosphor-IκBα (Ser32). An antibody against TLR-2 was obtained from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Small interfering RNAs (siRNAs) against TLR-2 and NOD2 were purchased from Bioneer (Daejeon, South Korea). Primers for NOD1, NOD2, IL-6, IL-8, TLR-2, MMP-1, -3, and -13, and GAPDH were obtained from Cosmo Genetech Co. (Seoul, South Korea). Recombinant human TNF-α and IL-1β were obtained from R&D Systems (Minneapolis, MN, USA).

Cartilage collection, primary chondrocyte culture, and cartilage explant culture

Cartilage samples were obtained from the knee joints of OA patients at the time of total knee replacement surgery. Patient diagnoses were determined using the criteria developed by the American College of Rheumatology. The collection and use of human tissue samples was reviewed and approved by the Institutional Review Board of Hallym University Sacred Heart Hospital, Anyang, South Korea (approval number 2013-I022). All patients provided written informed consent for the use of their discarded cartilage samples.

Chondrocytes were isolated by carefully dissecting articular cartilage from a relatively lesion-free area, followed by sequential digestion with a protease from *Streptomyces griseus*, collagenase from *Clostridium histolyticum*, and hyaluronidase from bovine testes (Sigma-Aldrich). Chondrocytes were maintained in monolayer culture in Dulbecco's modified Eagle's

medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. First-passage primary chondrocytes were used for all experiments within 1 week after seeding.

IHC analysis

Freshly dissected normal and OA human cartilage tissues were fixed with 4% paraformaldehyde, decalcified, and embedded in paraffin. Five-μm-thick sections were blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h, followed by incubation with an antibody against NOD2 (1:100 dilution) for 16 h at 4°C. Sections were incubated with biotinylated secondary antibody for 30 min, treated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min, and visualized with 3,3′-diaminobenzidine. The tissue sections were mounted and observed under a microscopy (Nikon, Tokyo, Japan).

Transfection with siRNA

Transfection of chondrocytes with siRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Human chondrocytes were transfected with control (sense, 5′-CCU-ACG-CCA-CCA-AUU-UCG-U-3′; antisense, 5′-ACG-AAA-UUG-GUG-GCG-UAG-G-3′), NOD2 (sense, 5′-UAU-UGU-UAU-CGC-GCA-AAU-ACA-GAG-C-3′; antisense, 5′-GCU-CUG-UAU-UUG-CGC-GAU-AAC-AAU-A-3′), and TLR-2 (sense, 5′-GGC-UUC-UCU-GUC-UUG-UGA-C-3′; antisense, 5′-GUC-ACA-AGA-CAG-AGA-AGC-C-3′) at a concentration of 50 nM for 48 h using Lipofectamine 2000. Cells were then exposed to 29-kDa FN-f (300 nM) for 6 or 24 h and used in all subsequent experiments.

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

Total RNA was extracted from chondrocytes or cartilage tissues using TRIzol reagent as previously described (37). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed using a StepOnePlus real-time PCR system with the following primers: IL-6 forward, 5'-TAC-CCC-CAG- GAG-AAG-ATT-CC-3'; IL-6 reverse, 5'-TTT-TCT-GCC-AGT-GCC-TCT-TT-3'; IL-8 forward, 5'-CTG-GCC-GTG-GCT-CTC-TTG-3'; IL-8 reverse, 5'-CTT-GGC-AAA-ACT-GCA-CCT-TCA-3'; TLR-2 forward, 5'-TTG-TGA-CCG-CAA-TGG-TAT-CTG-3'; TLR-2 reverse, 5'-GCC-CTG-AGG-GAA-TGG-AGT-TT-3'; NOD1 forward, 5'-TTC-CGT-GCT-GCC-TTT-GAA-G-3'; NOD1 reverse, 5'-GAC-ATC-TGT-CAG-GGT-CAT-CGT-3'; NOD2 forward, 5'-CTG-TCC-AGA-CCC-TGC-TCT-TC-3'; NOD2 reverse, 5'-CAG-AGA-AGC-CCT-TGA-GGT-TG-3'; MMP-1 forward, 5'-CCT-CGC-TGG-GAG-CAA-ACA-3'; MMP-1 reverse, 5'-TTG-GCA-AAT-CTG-GCG-TGT-AA-3'; MMP-3 forward, 5'-AGG-CAT-CCA-CAC-CCT-AGG-TTT-3'; MMP-3 reverse, 5'-ATC-AGA-AAT-GGC-TGC-ATC-GAT-3'; MMP-13 forward, 5'-CCT-TCA-AAG-TTT-GGT-CCG-ATG-T-3'; MMP-13 reverse; 5'CAG-CAA-TGC-CAT-CGT-GAA-GT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-ATG-GAA-ATC-CCA-TCA-CCA-TCT-T-3'; GAPDH reverse, 5′-CGC-CCC-ACT-TGA-TTT-TGG-3′. GAPDH was used as an internal control.

Immunoblot analysis

Proteins from chondrocytes were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer (Biosesang, Kyunggi, South Korea) and protein concentrations were quantified using bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a polyvinylidene difluoride membrane (Bio-Rad

Laboratories, Hercules, CA, USA). The membrane was blocked with 5% (w/v) nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with primary and secondary antibodies at the appropriate dilutions. The membrane was developed using an enhanced chemiluminescence kit (GE Healthcare Life Science, Buckinghamshire, UK).

Immunoprecipitation (IP) assays

Proteins were extracted from chondrocytes with RIPA lysis buffer and the supernatants were obtained by centrifugation at 13,000 × *g* for 10 min at 4°C. The supernatant containing equal amounts of proteins were pre-incubated with protein A agarose bead slurry for 4 h at 4°C on a rotating shaker and then incubated with specific antibodies against RIP2 or IgG in the presence of protein A agarose beads at 4°C overnight with gentle rotation. Proteins eluted in 2x SDS sample buffer were separated with SDS-PAGE and subjected to immunoblot analysis as described above. A portion of the protein extracts was used as an input control.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Mann–Whitney *U* test or two-way analysis of variance (ANOVA) in GraphPad Prism 6 (GraphPad software). A value of $P < 0.05$ was considered statistically significant.