Transcriptional Induction of ADAMTS5 Protein by Nuclear Factor-κB (NF-κB) Family Member RelA/p65 in Chondrocytes during Osteoarthritis Development*

Hiroyuki Kobayashi‡, Makoto Hirata§, Taku Saito‡, Shozo Itoh¶, Ung-il Chung‡, and Hiroshi Kawaguchi†‡‌

From the Departments of ‡Sensory and Motor System Medicine and §Bone and Cartilage Regenerative Medicine and ¶Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Background: ADAMTS5 is a crucial proteinase for osteoarthritis development. Exhaustive comparison of the genomic sequences of human, macaque, and mouse ADAMTS5 genes revealed that the proximal 1.4 kb of the 5’-end-flanking regions containing several consensus motifs was highly conserved. Among putative transcription factors for these motifs, NF-κB family member RelA/p65 most strongly stimulated the promoter activity. In the ADAMTS5 gene, there were three NF-κB binding motifs, in which deletion, mutagenesis, and tandem repeat analyses of the luciferase assay identified the core responsive elements of RelA/p65 to be −896/−887 and −424/−415 bp with specific bindings. The endogenous ADAMTS5 expression in ATDC5 cells was increased by Rela/p65 overexpression and decreased by knockdown through its siRNA. The expression was also inhibited by the Rela deletion through Cre transfection in primary articular chondrocytes from Relafl/fl mice. In the ex vivo culture of femoral head cartilage from mesenchymal cell-specific Rela knock-out (Prx1-Cre;Relafl/fl) mice, aggrecan degradation was significantly lower than that in the Rela0/0 cartilage. Finally, in the experimental mouse osteoarthritis model, ADAMTS5 and RelA were co-localized in chondrocytes of degraded articular cartilage. We conclude that Rela/p65 is a potent transcriptional activator of ADAMTS5 in chondrocytes during osteoarthritis development.

**Significance:** The molecular network related to the Rela/p65-ADAMTS5 axis may represent a therapeutic target for osteoarthritis.

Here we sought to identify transcription factors that induce ADAMTS5, a crucial proteinase for osteoarthritis development. Exhaustive comparison of the genomic sequences of human, macaque, and mouse ADAMTS5 genes revealed that the proximal 1.4 kb of the 5’-end-flanking regions containing several consensus motifs were highly conserved. Among putative transcription factors for these motifs, NF-κB family member Rela/p65 most strongly stimulated the promoter activity. In the ADAMTS5 gene, there were three NF-κB binding motifs, in which deletion, mutagenesis, and tandem repeat analyses of the luciferase assay identified the core responsive elements of Rela/p65 to be −896/−887 and −424/−415 bp with specific bindings. The endogenous ADAMTS5 expression in ATDC5 cells was increased by Rela/p65 overexpression and decreased by knockdown through its siRNA. The expression was also inhibited by the Rela deletion through Cre transfection in primary articular chondrocytes from Relafl/fl mice. In the ex vivo culture of femoral head cartilage from mesenchymal cell-specific Rela knock-out (Prx1-Cre;Relafl/fl) mice, aggrecan degradation was significantly lower than that in the Rela0/0 cartilage. Finally, in the experimental mouse osteoarthritis model, ADAMTS5 and RelA were co-localized in chondrocytes of degraded articular cartilage. We conclude that Rela/p65 is a potent transcriptional activator of ADAMTS5 in chondrocytes during osteoarthritis development.

**Results:** NF-κB family member Rela/p65 was identified as a potent transactivator of ADAMTS5 and regulated the expression and aggrecan analysis.

**Conclusion:** Rela/p65 is a potent transcriptional activator of ADAMTS5 in chondrocytes during osteoarthritis development.

**Significance:** The molecular network related to the Rela/p65-ADAMTS5 axis may represent a therapeutic target for osteoarthritis.
nuclear factor κB (NF-κB) family member RelA (NF-κB p65) most strongly activated the human ADAMTS5 promoter activity. The NF-κB family of transcription factors plays a crucial role in a broad range of biological processes, including immune responses, inflammation, proliferation, differentiation, and apoptosis (12–14). The family includes RelA/p65, RelB, Rel, p105/p50, and p100/p52, each of which contains a Rel homology domain that mediates DNA binding and dimerization. Numerous studies have established that IκB proteins are phosphorylated and degraded by a large protein complex IκB kinase (IKK) in response to several signals, thereby allowing free NF-κB complexes to translocate from the cytoplasm into the nucleus, leading to target gene transactivation (15, 16). The NF-κB family genes are expressed in the chick limb cartilage, and blockage of the NF-κB activity causes the arrest of the limb outgrowth (17). The IκB kinase α-deficient mice also exhibit suppression of limb outgrowth (18, 19). RelA/p65 is a key active subunit in NF-κB transcription in several cell types (20, 21), and the knockdown of RelA/p65 with specific siRNA inhibits the expression of cartilage catabolic factors such as matrix metalloproteinases-9 (MMP-9), nitric oxide synthase-2 (NOS-2), and cyclooxygenase-2 (COX-2) in chondrocytes (22). Because these lines of evidence implicate the interaction between RelA/p65 and ADAMTS5 signals in cartilage, the present study investigated the mechanism underlying the transcriptional regulation of the ADAMTS5 promoter by RelA/p65.

**EXPERIMENTAL PROCEDURES**

**Similarity Search of the Proximal Promoter Sequences among Species**—We compared the sequences of the 5’-end-flanking regions relative to the transcription start site (TSS) among 2-kb human, macaque, and mouse ADAMTS5 genes, using BLASTN search (23). TSS and exon1 were determined from the ADAMTS5 gene information of the GenBank™ database. The detected sequences were aligned by the Vector-NTI software (Invitrogen), and the transcription factor-binding motifs were predicted using the TFSEARCH web site (Computational Biology Research Center).

**Cell Cultures**—Mouse chondrogenic ATDC5 cells (RIKEN Cell Bank) were grown and maintained in DMEM/F12 (1:1) with 5% FBS. Human chondrogenic OUMS-27 cells (Japan Health Sciences Foundation) were grown and maintained in DMEM with 10% FBS. Primary articular chondrocytes were isolated from femoral heads, femoral condyles, and tibial plateau of 5–6-day-old mice and cultured in DMEM/F12 (1:1) with 10% FBS (24).

**Construction of Expression Vectors**—Full-length human cDNA sequences of the transcription factors were PCR-amplified and cloned into pCMV-HA vector (Clontech). The GenBank accession numbers are as follows: HIF1A NM_001530.3, HIF2A NM_001430.4, CEBPA NM_004364.3, CEBPB NM_005194.3, CEBPD NM_005195.3, CEBPE NM_001806.3, DDIT3 NM_00195053.1, RUNX1 NM_001754.4, RUNX2 NM_001024630.4, RUNX3 NM_001031680.2, NFKB1 NM_003998.3, NFKB2 NM_001077494.2, RELA NM_029711.1, RELB NM_006509.3, REL NM_002908.2, STAT1 NM_007315.3, STAT3 NM_138276.2, STAT5A NM_003152.3, STAT5B NM_012448.3, POU2F1 NM_002697.3, POU2F2 NM_002698.4, POU5F1NM_002701.4, POU3F3 NM_005604, SOX5 NM_006940.4, SOX6 NM_0175082.2, SOX9 NM_000346.3, SP1 NM_138473.2, and FOS NM_005252.3. The primer sequences are available upon request. We prepared siRNA vectors for the murine RelA (NM_009045.4: nucleotides 1,490–1,514) in piGENEmU6 vectors (iGENE Therapeutics). We generated adenovirus vectors by the AdenoX expression system (Clontech) and verified all vectors by DNA sequencing.

**Luciferase Assay**—We prepared the ADAMTS5 promoter region (from −1,400 to +1 bp relative to the TSS) by PCR using human genomic DNA as the template and cloned it into the pGL4.10 (luc2) vector (Promega). We created deletion and mutation constructs by PCR. Furthermore, tandem repeat constructs containing −896/−887 and −424/−415 bp were created by ligating the double-strand oligonucleotides of −903/−869 and −442/−410 bp, respectively, into the EcoRI site of the modified pGL3 vector. Transfection of ATDC5 cells was performed in triplicate in 48-well plates using FuGENE 6 transfection reagent (Roche Diagnostics): FuGENE 6 with a total amount of 150 ng of plasmid DNA, 100 ng of pGL3 or pGL4 reporter vector, 50 ng of effector vector, and 4 ng of pRL-TK vector (Promega) for internal control per well. For the dose-response experiment of RelA/p65, we used 25, 50, and 75 ng of the expression vector. We performed luciferase assays with the PicaGene dual sea pansy luminescence kit (Toyo Ink) using a GloMax 96 microplate luminometer (Promega) and showed the data as the ratio of the firefly activities to the Renilla activities.

**Electrophoretic Mobility Shift Assay (EMSA)**—We prepared nuclear extracts from COS-7 cells transfected with expression vector of RelA/p65 using FuGENE 6 transfection reagent and performed the EMSA with the digoxigenin gel shift kit (Roche Diagnostics). Regions of the oligonucleotide probes were about 20–30 bp containing −896/−887 and −424/−415 bp. Binding reactions were incubated for 30 min at room temperature. For competition analysis, we used 50-fold excess of unlabeled competitor probe containing the binding reaction. For the supershift experiment, we added 1 μl of an antibody to RelA/p65 (C-20; Santa Cruz Biotechnology) after 30 min of the binding reaction, and the reaction was incubated for an additional 30 min at room temperature. Samples were loaded onto Novex 6% Tris borate-EDTA gels (Invitrogen) and electrophoresed at 100 V for 60 min.

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP assay was performed with a OneDay ChIP kit (Diagenode, Liège, Belgium) according to the manufacturer’s instructions. In vivo cross-linking was performed after 1 day with and without IL-1β (10 ng/ml) treatment in the culture of human chondrogenic OUMS-27 cells, and then the cell lysates were sonicated to shear genomic DNA. For immunoprecipitation, an antibody to RelA/p65 and the control normal rabbit immunoglobulin G (IgG) were used. Primer sets that amplify the area including the NF-κB motif (−951/−793 or −494/−326 for −896/−887 or −424/−415, respectively) or that do not include the motif (+17/+213) were employed. PCR was performed using KOD FX Neo (TOYOBO).

**Real-time RT-PCR**—Total RNA from cells was isolated with an RNeasy mini kit (Qiagen) according to the manufacturer’s
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instructions, and an aliquot (1 µg) was reverse-transcribed with QuantiTect reverse transcription (Qiagen) to make single-stranded cDNA. Real-time RT-PCR was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems) using QuantiTect SYBR Green PCR master mix (Qiagen) according to the manufacturer's instructions. Standard plasmids were synthesized with a ZERO Blunt TOPO TA Cloning Kit (Invitrogen), according to the manufacturer's instructions. All reactions were run in triplicate. Copy numbers of target gene messenger RNA (mRNA) in each total RNA were calculated by reference to standard curves and were adjusted to the human or mouse standard total RNA (Applied Biosystems) with the human GAPDH or rodent GAPDH as an internal control. The primer sequences are available upon request.

Western Blotting—Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific). The cell lysates were fractionated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were incubated with primary antibodies to RelA/p65 (C-20; Santa Cruz Biotechnology) and actin (Sigma). Membranes were incubated with HRP-conjugated antibody (Promega), and immunoreactive proteins were visualized with ECL Plus (Amersham Biosciences).

Gene Silencing—For siRNA experiments, ATDC5 cells were transfected with siRNA using FuGENE 6 transfection reagent according to the manufacturer's protocol. After 48 h of culture, cells were washed and placed in DMEM supplemented with or without IL-1β (10 ng/ml). After 24 h of culture, RNA was harvested with TRIzol reagent (Invitrogen) for gene expression analysis.

Mice—All experiments were performed according to protocols approved by the Animal Care and Use Committee of the University of Tokyo. In each experiment, we compared genotypes of littermates that were maintained in a C57BL/6 background. We purchased Prx1-Cre mice from The Jackson Laboratory (25). To generate Prx1-Cre;Relafl/fl mice, Relafl/fl mice were mated with Prx1-Cre mice to obtain Prx1-Cre;Relafl/+ mice and then mated with Relafl/+ mice. To harvest articular chondrocytes and femoral heads, we used two mice/group/genotype.

Proteoglycan Release Assay—Proteoglycan release was assessed as reported previously (26). Briefly, femoral heads were harvested from 3-week-old Relafl/+ and Prx1-Cre;Relafl/+ mice. The explants were cultured for 3 days with or without IL-1β (10 ng/ml) in DMEM containing 1% penicillin/streptomycin. The proteoglycan content in the medium was measured as sulfated glycosaminoglycan by a colorimetric assay using dimethylmethylene blue.

Histological Analysis—Tissue was dissected and fixed in 4% paraformaldehyde buffered with PBS (pH 7.4) for 1 day at 4 °C. The specimens were decalcified for 2 weeks with 10% EDTA (pH 7.4) at 4 °C. After dehydration with an increasing concentration of ethanol and embedding in paraffin, 4-µm sagittal sections were cut from the specimens. Safranin-O staining was performed according to standard protocols. For immunohistochemistry, we incubated the sections with antibodies to RelA/p65 (C-20) (1:200; Santa Cruz Biotechnology) and ADAMTS5 (H-200) (1:500; Santa Cruz Biotechnology) or the control non-immune IgG diluted in blocking reagent without antigen retrieval. For immunofluorescence, we used a secondary antibody conjugated with Alexa Fluor 568 (Invitrogen) for RelA/p65 and a CSA II biotin-free catalyzed amplification system (DAKO) for ADAMTS5. For immunoperoxidase methods in ADAMTS5 detection, we also used the CSA II system and applied methyl green for counterstaining. These histological analyses were performed at least three times using 2–3 mice/group/genotype for the confirmation of the results.

OA Experiment—We performed the surgical procedure to create an experimental OA model with removal of the medial collateral ligament and transection of the medial meniscus in knee joints of 8-week-old male mice, as reported previously (27, 28). Briefly, the knee joint was exposed following a medial capsular incision and gentle lateral displacement of the extensor mechanism without transection of the patellar ligament. The medial collateral ligament was transected, and the medial meniscus was removed without anterior cruciate ligament transection. We analyzed various regions of the joint cartilage 8 weeks after surgery. To confirm the reproducibility, the analyses were performed at least three times using three mice.

Statistical Analyses—We performed statistical analyses of experimental data with the unpaired two-tailed Student's t test. p values less than 0.05 were considered significant.

RESULTS

Identification of Transactivators of the ADAMTS5 Promoter by Comparison of Mouse, Macaque, and Human Genes—To identify transcription factors that activate the ADAMTS5 promoter, we initially performed exhaustive comparison of the sequences of about 2 kb of the 5′-end-flanking regions among mouse, macaque, and human genes and found that the 1.4 kb upstream from the TSS was about 80% conserved among all the species (Fig. 1A). The sequence search identified the binding motifs of HIF, CCAAT/enhancer binding protein (C/EBP), RUNX, NF-κB, STAT, OCT, SOX, and SP-1 in the highly conserved region (Fig. 1B).

We therefore created expression vectors of the putative transcription factors whose binding motifs were identified: HIF1α, HIF2α, C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPδ, RUNX1, RUNX2, RUNX3, NFκB1, NFκB2, RelA/p65, RelB, C-Rel, STAT1, STAT3, STAT5A, STAT5B, OCT1, OCT2, OCT3/4, OCT7, SOX5, SOX6, SOX9, and SP1; and transfected them in ATDC5 cells with a luciferase reporter construct containing the 5′-end-flanking region (−1,400/+1) of the human ADAMTS5 gene (Fig. 1C). Among the transcription factors, the luciferase reporter assay revealed that NF-κB family member RelA/p65 most strongly activated the ADAMTS5 promoter activity in ATDC5 cells, causing us to speculate that RelA/p65 is a potent transcriptional activator for ADAMTS5 induction. Because NF-κB family members are known to often function as a heterodimer with a binding partner such as Stat3 (29), we looked at the ADAMTS5 promoter activity by the co-transfection of RelA/p65 with several candidates of the partners and found that NFκB1 most strongly enhanced the ADAMTS5 transactivation by RelA/p65 (Fig. 1D).

Identification of the Core Region Responsive to RelA/p65 in the ADAMTS5 Proximal Promoter—We initially confirmed that RelA/p65 activated the ADAMTS5 promoter activity in a
FIGURE 1. Identification of transcription factors of the ADAMTS5 promoter. A, the sequence similarity of the proximal promoter (2 kb) sequences among the mouse, macaque, and human ADAMTS5 genes analyzed by BLASTN search and Vector-NTI software (Invitrogen). B, conserved nucleotides in the two or three species are denoted by white letters shaded in gray or black, respectively. An arrow shows the TSS of the human gene. Putative binding motifs of transcription factors identified in highly conserved regions are indicated below. C, luciferase assay for the human ADAMTS5 promoter activity by the putative transcription factors whose binding motifs were identified above. ATDC5 cells were co-transfected with a luciferase reporter construct containing the 5′-end-flanking region (−1,400/−1) of the human ADAMTS5 gene and the effector vectors or the control empty vector (EV). *, p < 0.05, **, p < 0.01 versus EV. D, luciferase assay for the human ADAMTS5 promoter activity by the co-transfection of putative binding partners with RelA/p65 in the reporter construct containing ATDC5 cells as above. For C and D, data are shown as means (bars) ± S.E. (error bars) of relative luciferase activity (the ratio of the firefly activities to the Renilla activities) for three wells/group.
dose-dependent manner of the expression vector (Fig. 2A). There were three NF-kB motifs in the 1.4-kb region of the human ADAMTS5 proximal promoter: −1,196/−1,187 bp, −896/−887 bp, and −424/−415 bp (Fig. 1B). Hence, to identify the region responsive to RelA/p65 in the human proximal promoter, we performed the deletion analysis of the luciferase assay in ATDC5 cells co-transfected with RelA/p65. A series of 5′-deletions of the −1,400/+1 fragment identified the decreases of the transcriptional activity moderately between −1,400 and −1,022 bp, and significantly between −1,022 and −811 bp and between −506 and −405 bp, all of which contained the three motifs (Fig. 2B). Hence, we next compared the luciferase activity by a single and double mutagenesis of the respective motifs (M1, M2, and M3 in one motif) and found that the promoter activities were significantly decreased by the mutagenesis in the −896/−887 and in the −424/−415 motif, although not in the −1,196/−1,187 motif (Fig. 2C). We further compared the luciferase activity of the tandem repeats of the
−896/−887 and the −424/−415 motif elements and found that both responded to RelA/p65 in a repeat number-dependent manner, indicating that the two proximal motifs are responsive regions to RelA/p65 (Fig. 2D).

EMSA revealed the complex formation of the COS-7 nuclear extracts overexpressing RelA with the oligonucleotide probes including the −896/−887- or −424/−415-bp region (Fig. 2E, lane 2, arrowheads). The complex formation was abolished by mutations inside the NF-κB motif (M1) (Fig. 2E, lane 3). Cold competition with excess amount of the unlabeled wild-type probe suppressed the complex formation (Fig. 2E, lane 4), whereas that with the unlabeled mutation probes did not affect it (Fig. 2E, lane 5). Both complexes by the −896/−887 and the −424/−415 oligonucleotide probes underwent supershifts with the addition of an antibody to RelA/p65 (Fig. 2E, lane 6), although not by the nonimmune IgG (Fig. 2E, lane 7). These results demonstrate the specific binding between RelA/p65 and the two NF-κB motifs in the ADAMTS5 promoter.

ChIP assay showed in vivo binding of RelA/p65 with the ADAMTS5 promoter including the −424/−415-bp region in cell lysates of human chondrogenic OUMS-27 cells stimulated by IL-1β, a putative inducer of the NF-κB signal as well as OA development, although it was not detected with that including the −896/−887-bp region, indicating that the −424/−415-bp region may be more physiologically important for the RelA binding than the −896/−887-bp region (Fig. 2F). The specificity of the binding was confirmed because it was not immunoprecipitated by the nonimmune IgG, and no amplification was seen with a primer set that does not span the NF-κB motif.

Effects of Gain and Loss of Functions of RelA/p65 on ADAMTS5 Expression and Aggrecanolysis—We then looked at the endogenous ADAMTS5 expression by gain and loss of functions of RelA/p65 in chondrocytes. Adenoviral overexpression of RelA/p65 increased the endogenous ADAMTS5 expression in ATDC5 cells (Fig. 3A). IL-1β enhanced Adams5 and Rela expressions in ATDC5 cells. The Adams5 induction by IL-1β was suppressed by the knockdown of Rela through its specific siRNA transfection (Fig. 3B). Furthermore, Adams5 expression was inhibited in a dose-dependent manner by the Rela deletion through adenoviral transfection with Cre in primary articular chondrocytes derived from mice homozygous for a floxed Rela allele (Relafl/fl mice) (Fig. 3C).

Moreover, we used mesenchymal cell-specific Rela knockout (Prx1-Cre;Relafl/fl) mice. Primary chondrocytes derived from articular cartilage of the Prx1-Cre;Relafl/fl mice were confirmed to express Rela in small amounts as compared with the control Relafl/fl chondrocytes (Fig. 4A, upper panel). ADAMTS5 expression was stimulated by IL-1β treatment in the Relafl/fl chondrocyte culture; however, the basal level was much lower in the Prx1-Cre;Relafl/fl chondrocyte culture and did not respond to IL-1β treatment (Fig. 4A, lower panel). In the ex vivo culture of femoral head cartilage of the Relafl/fl mice, the release of proteoglycan into the medium was stimulated by IL-1β treatment; however, this stimulation was abrogated in the Prx1-Cre;Relafl/fl cartilage (Fig. 4B). Immunohistochemical analysis revealed that ADAMTS5 expression was lower in the Prx1-Cre;Relafl/fl chondrocytes than in the Relafl/fl chondrocytes in the presence and absence of IL-1β (Fig. 4C). These indicate that loss of function of RelA reduces ADAMTS5 expression and subsequent aggrecanolysis of the articular cartilage.

Co-localization of RelA and ADAMTS5 in Chondrocytes of the Degraded Articular Cartilage—To know the possible interaction between RelA and ADAMTS5 and in vivo, we next created an experimental mouse OA model by surgical induction of

**FIGURE 2. Identification of the core region responsive to RelA/p65 in the ADAMTS5 proximal promoter.** A, dose response of the ADAMTS5 promoter activity with different amounts (25, 50, and 75 μg) of the RelA/p65 expression vector in the ATDC5 cells co-transfected with a luciferase reporter construct containing the 5′-end-flanking region (−1,400/+1) of the human ADAMTS5 gene. *, p < 0.05 versus EV.B, deletion analysis of the luciferase assay in ATDC5 cells co-transfected with a series of deletion fragments of the human ADAMTS5 promoter including three NF-κB motifs (M1, M2, and M3 in one motif), and Rela or EV. *, p < 0.05; **, p < 0.01 versus EV.WT + Rela.D, tandem repeat analysis of the luciferase assay in ATDC5 cells co-transfected with a luciferase reporter construct ligated to the −896/−887 motif (upper) or to the −424/−415 motif (lower). C, mutagenesis analysis of the luciferase assay in ATDC5 cells co-transfected with a luciferase reporter construct containing wild-type (WT) or a single and double mutations in the NF-κB motifs (M1, M2, and M3 in one motif), and Rela or EV. *, p < 0.05; **, p < 0.01 versus WT + Rela. D, EMSA for binding of nuclear extracts (NE) of COS-7 cells transfected with Rela or the control empty vector (−) and the WT or the mutated (M1) probe above of the −896/−887 (top) and the −424/−415 (bottom) oligonucleotide probes. The arrowheads indicate the protein-probe binding. Cold competition with 50-fold excess of unlabelled WT or M1 probe is presented. A supershift of the binding by an antibody to RelA or nonimmune IgG is also shown. F, ChIP assay for specific binding of Rela to the NF-κB motif using cell lysates of human chondrogenic OUMS-27 cells with or without IL-1β treatment. Primer sets that amplify the area including the NF-κB motif (−896/−887 or −424/−415) or that do not include the motif (+171/+213) were employed before (input) and after immunoprecipitation with an antibody to RelA or the control nonimmune IgG.
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![Graph showing the effects of IL-1β on RelA and ADAMTS5 mRNA levels in primary chondrocytes of articular cartilage derived from Prx1-Cre;Relafl/fl mice and the control Relafl/fl littermates. The graph illustrates the concentration of total glycosaminoglycan released into the medium in the presence and absence of IL-1β.](image)

**FIGURE 4.** Effects of loss of functions of RelA on aggrecanolyis. A, Rela and Adami55 mRNA levels in primary chondrocytes of articular cartilage derived from Prx1-Cre;Relafl/fl mice and the control Relafl/fl littermates in the presence and absence of IL-1β for 24 h. *p < 0.05, **p < 0.01 versus Relafl/fl + control. # *p < 0.05, ## *p < 0.01 versus Relafl/fl - IL-1β. B, the concentration of total glycosaminoglycan released into the medium in the ex vivo cultures of femoral head cartilage derived from the two genotype mice above with and without IL-1β for 72 h. *p < 0.05 versus Relafl/fl + control. # *p < 0.05 versus Relafl/fl - IL-1β. For A and B, data are shown as means (bars) ± S.E. (error bars) of three cultures/group using two mice/ genotype. C, representative images of immunohistochemical staining by an antibody to ADAMTS5 or the control nonimmune IgG in the cultured femoral head cartilages above (images with high magnification are inserted). Positive stainings are brown by diaminobenzidine with methyl green counterstaining. Scale bars, 20 μm. Immunohistochemistry was performed at least three times using two mice/group/genotype and obtained reproducible results.

During OA development, it is noteworthy that the activity of ADAMTS5 is regulated not only at the transcriptional level, but also by post-translational modifications starting with the processing of the proprotein form by proprotein convertases or proteases, such as furin, proprotein convertase 7 (PC7), paired basic amino acid cleaving enzyme 4 (PACE4), and syndecan-4 (35–37). ADAMTS5 is synthesized as latent, inactive zymogens and requires removal of the prodomain before the catalytic activity is obtained. Furin and a furin-like enzyme PC7 have major roles in activating ADAMTS enzymes by recognizing the furin-like recognition sequence. PACE4, which is known to bind extracellular matrix, also contributes to the processing/ activation of proADAMTS5. Syndecan-4 modulates the activa-
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The present study showed that RelA/p65 is a potent transcriptional activator of a catabolic factor ADAMTS5 in chondrocytes. Similarly, we previously reported that RelA/p65 is a transactivator of early stage or anabolic factors for chondrocyte differentiation: SOX9, SOX6, and type II collagen α1 chain (COL2A1) (44, 45). We also previously identified RelA/p65 as the most potent transcriptional factor of HIF-2α that controls later stages of chondrocyte differentiation and cartilage degradation (46). Furthermore, a recent study demonstrated that Nkx3.2 supports chondrocyte survival by activating RelA/p65 via a ligand-independent mechanism (47). Hence, RelA/p65 may extensively control anabolic and catabolic phases of cartilage during skeletal growth, cartilage homeostasis, and OA development with distinct upstream and downstream signals depending on the stages. Taken together, RelA/p65 and ADAMTS5 do not function in a one-to-one correspondence fashion. In fact, the global knock-out mice of Rela are embryonically lethal due to liver dysfunction (48), whereas those of Adamts5 exhibit minor abnormality with digit fusion and valve anomalies under physiological conditions (49, 50).

Proinflammatory cytokines such as IL-1 and TNF-α are known to be representative ligands for the NF-κB signal (51–54). In fact, IL-1β enhanced Adamts5 and Rela expressions in ATDC5 cells (Figs. 3B and 4). Regarding therapeutic intervention, however, ADAMTS5 expression at least by human synoviocytes is not affected by neutralization of either IL-1 or TNF-α alone or in combination, whereas ADAMTS4 expression by the same cells is severely inhibited by the TNF-α blocker etanercept and an anti-IL-1 neutralizing antibody (9). Although it may not be simple to apply the present finding to its therapeutic application, the molecular network related to the Rela/p65-ADAMTS5 axis may represent a therapeutic target for OA.

FIGURE 5. Immunohistochemistry using antibodies to RelA and ADAMTS5 and the merged staining, as well as the control nonimmune IgG in various regions of the joint cartilage (the superficial layer with high degeneration, the superficial layer with low degeneration, and the deep layer) of the knee joint cartilage of the experimental mouse OA model. The model was created by surgical induction of instability in the knee joints of 8-week-old mice, and the staining was performed 8 weeks after surgery. The boxed areas in the Safranin-O staining indicate regions shown in the enlarged images of lower panels. Scale bars, 100 μm. These analyses were performed at least three times using three mice and obtained reproducible results.
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REFERENCES

1. Verma, P., and Dalal, K. (2011) ADAMTS-4 and ADAMTS-5: key enzymes in osteoarthritis. J. Cell. Biochem. 112, 3507–3514

2. Brophy, R. H., Rai, M. F., Zhang, Z., Torgomyan, A., and Sandell, L. J. (2012) Molecular analysis of age and sex-related gene expression in mesimal tissue with and without a concomitant anterior cruciate ligament tear. J. Bone Joint Surg. Am. 94, 385–393

3. Glasson, S. S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H. L., Flannery, C. R., Peluso, D., Kanki, K., Yang, Z., Majumdar, M. K., and Morris, E. A. (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 434, 646–648

4. Stanton, H., Rogerson, F. M., East, C. J., Golub, S. B., Lawlor, K. E., Meeker, C. T., Little, C. B., Last, K., Farmer, F. J., Campbell, I. K., Fourie, A. M., and Fossang, A. J. (2005) ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. Nature 434, 648–652

5. Glasson, S. S., Askew, R., Sheppard, B., Carito, B. A., Blanchet, T., Ma, H. L., Flannery, C. R., Kanki, K., Wang, E., Peluso, D., Yang, Z., Majumdar, M. K., and Morris, E. A. (2004) Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. Arthritis Rheum. 50, 2547–2558

6. Gendron, C., Kashiwagi, M., Lim, N. H., Enghild, J. J., Thøgersen, I. B., Lim, N. H., Abe, T., Lees, D. C., and Firestein, G. S. (2002) Expression and regulation of aggrecanase-2/ADAM-TS5 protein and activity in osteoarthritis. J. Biol. Chem. 277, 1072–1076

7. Zhang, Z., Xing, X., Hensley, G., Chang, L. W., Liao, W., Abu-Amer, Y., Xu, J., and Verma, I. M. (1999) IKKI-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev. 13, 1322–1328

8. De Martin, R., Hoeth, M., Hofer-Warbinek, R., and Schmid, I. A. (2000) The transcription factor NF-κB and the regulation of vascular cell function. Arterioscler. Thromb. Vasc. Biol. 20, E83–E88

9. Lin, K., and Lin, A. (2002) NF-κB at the crossroads of life and death. Nat Immunol. 3, 221–227

10. Lianxu, C., Hongti, J., and Changlong, Y. (2006) NF-κBp65-specific siRNA inhibits expression of genes of COX-2, NOS-2, and MMP-9 in rat IL-1β-induced and TNF-α-induced chondrocytes. Osteoarthritis Cartilage 14, 367–376

11. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410

12. Ggosset, M., Berenbaum, F., Thirion, S., and Jacques, C. (2008) Primary culture and phenotype of chondrocytes. Nat Protoc. 3, 1253–1260

13. Logan, M., Martin, J. F., Nagy, A., Lobe, C., Olson, E. N., and Tabin, C. J. (2002) Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis 33, 77–80

14. Bostrom, K., and Liu, X. (2008) A transcriptional enhancer from the coding region of ADAMTS5(aggrecanase-2) gene promoter. Mol. Biol. Rep. 34, 225–231

15. Lin, A. C., Seeto, B. L., Bartoszek, J. M., Khoury, M. A., Whetstone, H., Ho, L., Hsu, C., Ali, S. A., and Alman, B. A. (2009) Modulating hedgehog signaling can attenuate the severity of osteoarthritis. Nat. Med. 15, 1421–1425

16. Tsushima, H., Okazaki, K., Hayashida, M., Ushijima, T., and Iwamoto, Y. (2012) CCAAT/enhancer binding protein β regulates expression of matrix metalloproteinase-3 in arthritis. Ann Rheum Dis 71, 99–107

17. Mikihira, S., Yan, W., Murakami, H., Furukawa, M., Kawai, T., Nikaia, H., Yoshida, E., Hamada, T., Okada, Y., and Kato, Y. (2003) Thyroid hormone enhances aggrecanase-2/ADAM-TS5 expression and proteoglycan degradation in growth plate cartilage. Endocrinology 144, 2480–2488

18. Barthel, K. K., and Liu, X. (2008) A transcriptional enhancer from the coding region of ADAMTS5. PLoS One 3, e2184

19. Longpré, J. M., McCulloch, D. R., Koo, B. H., Alexander, J. P., Apte, S. S., and Leduc, R. (2009) Characterization of proADAMTS5 processing by proprotein convertases. Int. J. Biochem. Cell Biol. 41, 1116–1126

20. Malfait, A. M., Arner, E. C., Song, R. H., Alston, J. T., Markosyan, S., Slaton, N., Yang, Z., Griggs, D. W., and Tortorella, M. D. (2008) Proprotein convertase activation of aggrecanases in cartilage in situ. Arch. Biochem. Biophys. 478, 43–51

21. Echtmeyer, F., Bertrand, J., Dreier, R., Meinecke, I., Neugebauer, K., Fuerst, M., Lee, Y. I., Song, Y. W., Herzog, C., Thelmeier, G., and Pap, T. (2009) Syndecan-4 regulates ADAMTS5 activation and cartilage breakdown in osteoarthritis. Nat. Med. 15, 1072–1076

22. Han, Z., Boyle, D. L., Manning, A. M., and Firestein, G. S. (1998) AP-1 and NF-κB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 28, 197–208

23. Sosulova, V., Townsend, P. A., Mann, J., van der Loos, C. M., Motterle, A., D’Acquisto, F., Mann, D. A., and Ye, S. (2010) Allele-specific regulation of matrix metalloproteinase-3 gene by transcription factor NFκB. PLoS One 5, e13132

24. Li, Q., Lu, Q., Hwang, J. Y., Büsscher, D., Lee, K. F., Izpisua-Belmonte, J. C., and Verma, I. M. (1999) IKKI-deficient mice exhibit normal development of skin and skeleton. Genes Dev. 13, 1322–1328

25. Logan, M., Martin, J. F., Nagy, A., Lobe, C., Olson, E. N., and Tabin, C. J. (2002) Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis 33, 77–80

26. Stanton, H., Golub, S. B., Rogerson, F. M., Last, K., Little, C. B., and Fossang, A. J. (2011) Investigating ADAMTS-mediated aggrecanase in mouse cartilage. Nat Protoc. 6, 388–404

27. Kamekura, S., Kawasaki, Y., Hoshi, K., Shimoaka, T., Chikuda, H., Maruyama, Z., Komori, T., Sato, S., Takeda, K., Karsenty, G., Nakamura, K., Chung, U. I., and Kawaguchi, H. (2006) Contribution of runt-related transcription factor 2 to the pathogenesis of osteoarthritis in mice after induction of knee joint instability. Arthritis Rheum. 54, 2462–2470

28. Yamada, T., Kawanou, H., Koshizuka, Y., Fukuda, T., Yoshimura, K., Kamekura, S., Saito, T., Ikeda, T., Kawasaki, Y., Azuma, Y., Ikegawa S., Hoshi K., Chung, U. I., Nakamura, K., Kato, S., and Kawaguchi, H. (2006) Carminerin contributes to chondrocyte calcification during endochondral ossification. Nat. Med. 12, 665–670

29. Grivennikov, S. I., and Karin, M. (2010) Dangerous liaisons: STAT3 and NF-κB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev. 21, 11–19

30. Thirunavukkarasu, K., Pei, Y., and Wei, T. (2007) Characterization of the human ADAMTS5(aggrecanase-2) gene promoter. Mol. Biol. Rep. 34, 644–648

31. Takeda, K., Takeuchi, O., Tsujiura, T., Iimori, S., Adachi, O., and Kawai, T. (1999) Limb and skin abnormalities in mice lacking IKKα. Science 284, 313–316
5, e9902

40. Miyaki, S., Sato, T., Inoue, A., Otsuki, S., Ito, Y., Yokoyama, S., Kato, Y., Takemoto, F., Nakasa, T., Yamashita, S., Takada, S., Lotz, M. K., Ueno-Kudo, H., and Asahara, H. (2010) MicroRNA-140 plays dual roles in both cartilage development and homeostasis. *Genes Dev.* **24**, 1173–1185

41. Miyaki, S., Nakasa, T., Otsuki, S., Grogan, S. P., Higashiyama, R., Inoue, A., Kato, Y., Sato, T., Lotz, M. K., and Asahara, H. (2009) MicroRNA-140 is expressed in differentiated human articular chondrocyte and modulates interleukin-1 response. *Arthritis Rheum.* **60**, 2723–2730

42. Young, D. A., Lakey, R. L., Pennington, C. J., Jones, D., Kevorkian, L., Edwards, D. R., Cawston, T. E., and Clark, I. M. (2005) Histone deacetylase inhibitors modulate metalloproteinase gene expression in chondrocytes and block cartilage resorption. *Arthritis Res. Ther.* **7**, R503-R512

43. Kashiwagi, M., Tortorella, M., Nagase, H., and Brew, K. (2001) TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). *J. Biol. Chem.* **276**, 12501–12504

44. Ushita, M., Saito, T., Ikeda, T., Yano, F., Higashikawa, A., Ogata, N., Chung, U., Nakamura, K., and Kawaguchi, H. (2009) Transcriptional induction of SOX9 by NF-κB family member RelA in chondrogenic cells. *Osteoarthritis Cartilage* **17**, 1065–1075

45. Itoh, S., Saito, T., Hirata, M., Ushita, M., Ikeda, T., Woodgett, J. R., Algül, H., Schmid, R. M., Chung, U. I., and Kawaguchi, H. (2012) GSK-3α and GSK-3β proteins are involved in early stages of chondrocyte differentiation with functional redundancy through RelA protein phosphorylation. *J. Biol. Chem.* **287**, 29227–29236

46. Saito, T., Fukai, A., Mabuchi, A., Ikeda, T., Yano, F., Ohba, S., Nishida, N., Akune, T., Yoshimura, N., Nakagawa, T., Nakamura, K., Tokunaga, K., Chung, U. I., and Kawaguchi, H. (2010) Transcriptional regulation of endochondral ossification by HIF-2α during skeletal growth and osteoarthritis development. *Nat. Med.* **16**, 678–686

47. Park, M., Yong, Y., Choi, S. W., Kim, J. H., Lee, J. E., and Kim, D. W. (2007) Constitutive RelA activation mediated by Nkx3.2 controls chondrocyte viability. *Nat. Cell Biol.* **9**, 287–298

48. Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. *Nature* **376**, 167–170

49. McCulloch, D. R., Nelson, C. M., Dixon, L. J., Silver, D. L., Wylie, I. D., Lindner, V., Sasaki, T., Cooley, M. A., Argraves, W. S., and Apte, S. S. (2009) ADAMTS metalloproteases generate active versican fragments that regulate interdigital web regression. *Dev. Cell* **17**, 687–698

50. Dupuis, L. E., McCulloch, D. R., McGarity, J. D., Bahan, A., Wessels, A., Weber, D., Diminich, A. M., Nelson, C. M., Apte, S. S., and Kern, C. B. (2011) Altered versican cleavage in ADAMTS5 deficient mice; a novel etiology of myxomatous valve disease. *Dev. Biol.* **357**, 152–164

51. Sitcheran, R., Cogswell, P. C., and Baldwin, A. S., Jr. (2003) NF-κB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. *Genes Dev.* **17**, 2368–2373

52. Silverman, N., and Maniatis, T. (2001) NF-κB signaling pathways in mammalian and insect innate immunity. *Genes Dev.* **15**, 2321–2342

53. Ghosh, S., and Karin, M. (2002) Missing pieces in the NF-κB puzzle. *Cell* **109**, S81–96

54. Li, Q., and Verma, I. M. (2002) NF-κB regulation in the immune system. *Nat. Rev. Immunol.* **2**, 725–734