Supplementary material 1

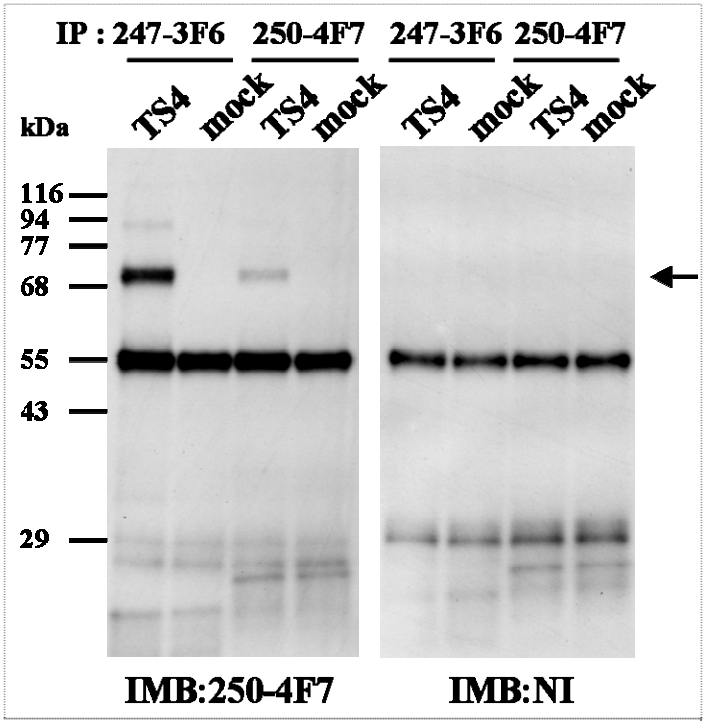
Real-time quantitative PCR: Sequences of the primers and TaqMan probe for ADAMTS1, 4, 5, 8, 9 and 15 were designed using Primer Express software (Applied Biosystems, Foster City, CA): forward primer 5'-CCATCCCAAGAGTATCACATGTCT-3', reverse primer 5'-CACTATGACACAGCAATTCTTTTCAC-3' and TaqMan probe 5'-FAM-CCCACACAAGTCCTGTC-MGB-3 for ADAMTS1; forward primer 5'-TCACTGACTTCCTGGACAATGG-3', reverse primer 5'-ACTGGCGGTCAGCATCATAGT-3' and TaqMan probe 5'-FAM-CATCTGCCTGTGACTTTCCCTG-TAMRA-3' for ADAMTS4; forward primer 5'-CACTGTGGCTCACGAAATCG-3', reverse primer 5'-GGAACCAAAGGTCTCTTCACAGA-3' and TaqMan probe 5-FAM-CTTGGCCTCTCCCATGACGATTCCA-TAMRA-3' for ADAMTS5; forward primer 5'-GCAGAACCACATCCTGACGTTAA-3', reverse primer 5'-ACGATCAGCACTTTTACCACCAT-3' and TaqMan probe 5'-FAM-CTACAAGCACCCCAGCATCAAGAATTCCAT-TAMRA-3 for ADAMTS8; forward primer 5'-TCCGAGACTGCCGTAGAAAGA-3', reverse primer CCGACAAAACCTGAAGCAAAA-3' and TaqMan probe 5'-FAM-TTAACTCAACAGATCGCATTGAGCAA-TAMRA-3' for ADAMTS9; forward primer 5'-GGTGAAATACCGATCCTGCCA-3', reverse primer 5'-GTAGCCGTTGAAAGCCTCACA-3' and TaqMan probe 5'-FAM-TCAGCCTCCGGAAAGAGCTTCCG-TAMRA-3' for ADAMTS15. The total gene specificity of nucleotide sequences chosen for the primers and probes, and the absence of DNA polymorphisms were ascertained by BLASTN and Entrez on an NIH web sites (http://www.ncbi.nlm.nih.gov/).

Supplementary material 2

Immunoprecipitation of ADAMTS4 with the antibodies against human ADAMTS4: To study whether the mouse monoclonal antibodies (247-3F6 and 250-4F7) [1] can immunoprecipitate ADAMTS4, cell lysates of ADAMTS4 stable transfectants that synthesize active ADAMTS4 of 73 kDa and mock transfectants [2] were prepared in a lysis buffer of 50 mM Tris-HCl buffer, pH 7.5 containing 1% Triton X-100, 150 mM NaCl, 10 mM $CaCl_2$, and subjected to immunoprecipitation with anti-ADAMTS4 antibody (0.5 μg/ml; 247-3F6 or 250-4F7), followed by incubation with Protein G Sepharose 4 Fast Flow beads (GE Healthcare, UK Ltd., Buckinghamshire, UK). Beads carrying immune complexes were washed five times in the lysis buffer and boiled for 10 minutes in SDS reduction buffer. The immunoprecipitates were separated on 10% SDS-PAGE and the resolved proteins on gels were transferred onto polyvinylidene difluoride membranes. The membranes were blotted with anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) or non-immune mouse IgG (0.5 μg/ml; Daiichi Fine Chemicals, Ltd., Takaoka, Japan), followed by the reaction with horseradish peroxidase-labeled anti-mouse IgG, and the immunoreactive bands were detected with enhanced chemiluminescence Western

blotting reagents according to the instructions of the manufacturer (GE Healthcare, UK Ltd.). As shown in supplementary Figure, both antibodies immunoprecipitated the 73-kDa ADAMTS4 species from ADAMTS4 stable transfectants but not mock transfectants, whereas no such immunoprecipitation was observed with non-immune IgG.

Supplementary Figure: Cell lysates (2 x 10^6 cells) were subjected to immunoprecipitation (IP) with anti-ADAMTS4 antibodies (247-3F6 or 250-4F7), and then immunoblotting (IMB) with anti-ADAMTS4 antibody (250-4F7) or non-immune IgG (NI). Arrow indicates 73-kDa ADAMTS4. Bands of 55 kDa and ~25 kDa are IgG, which appeared because secondary antibody against mouse IgG was used for immunoblotting.



Supplementary material 3

Transfection of ADAMTS4 siRNA to osteoarthritic chondrocytes and cartilage explants: Transfection of siRNA for ADAMTS4 and non-silencing oligonucleotide to osteoarthritic chondrocytes and cartilage explants was performed principally according to our previous methods [3, 4]. In our preliminary study, the optimal condition for silencing the ADAMTS4 expression in osteoarthritic chondrocytes was determined by trying various amounts of the siRNAs (2, 3 and 5 μg) and different cell numbers (0.5 x 10^6 and 1.0 x 10^6 cells) in 100 μl of Human Chondrocyte Nucleofector solution (Amaxa Inc., Gaithersburg, MD) and two different Nucleofector programs (U-24 and U-28). Based on the data, chondrocytes (1 x 10^6 cells) suspended in 100 μl of the solution were transfected with siRNA (3 μg) using the program U24 according to the manufacturer's instructions. The cells were cultured in the presence of IL-1α (1 ng/ml) for 72 hours and then the ADAMTS 4 expression and aggrecanase activity were examined by RT-PCR and immunoblotting using anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) and anti-aggrecan neoepitope-specific antibody (2 μg/ml). Cartilage tissue from osteoarthritic joint was cut into

pieces (2 x 2 x 2 mm) for explant culture. They were cultured for 12 hours in 24-well plates (4 pieces/well) with 500 μl of DMEM/F-12 containing 0.2% lactalbumin hydrolysate, and then transfected with ADAMTS4 siRNA (4 μg/ml) or non-silencing siRNA (4 μg/ml) for 24 hours in cationic cardiolipin analogue (CCLA)-based liposome (NeoPharm, Inc., Waukegan, IL) with 3:2 charge ratio of CCLA-liposome to siRNA. The condition was determined based on our previous study [3] and preliminary experiments by trying different charge ratios (3:2, 3:1 and 6:1) of CCLA-liposome to siRNA. After the transfection was repeated for 24 hours by replacing with the same transfection medium, IL-1α (1 ng/ml) was added to the media and incubation was continued for 72 hours. Cartilage tissues collected from each well were freeze-milled under liquid nitrogen into a fine powder. The cartilage powder obtained was extracted for 48 hours at 4℃ with 10 volumes of 4 M guanidine hydrochloride, 10 mM EDTA, 1 M amino caproic acid, 50 mM sodium acetate, pH 6.8. The extracts were centrifuged at 3,000 g for 15 minutes at 4℃ and the supernatants were dialyzed against 50 mM Tris-acetate, pH 7.3 for 48 hours at 4℃. The supernatants in the buffer containing 15 mM EDTA were deglycosylated with 0.4 unit/ml chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan) and 0.4 unit/ml keratanase (Seikagaku Corporation) for 4 hours at 37℃ and were subjected to immunoblotting for aggrecanase activity with anti-aggrecan neoepitope-specific antibody (2 μg/ml).

Supplementary material 4

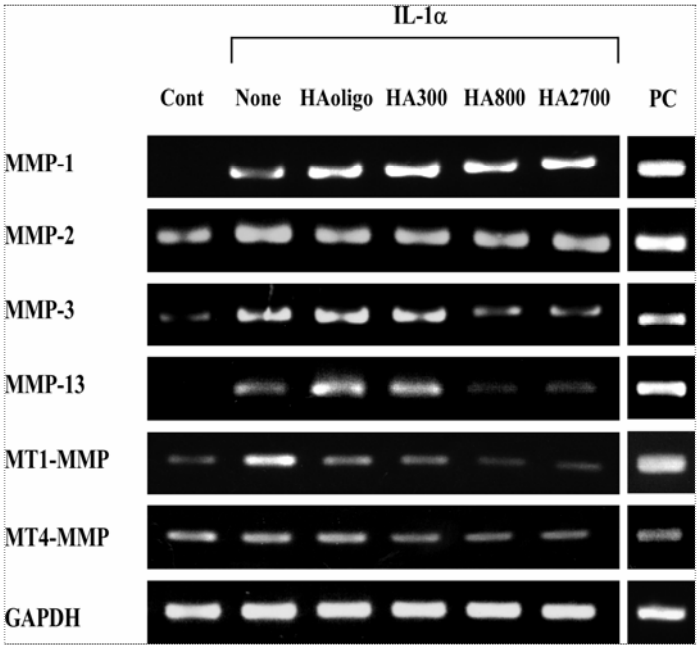
Blocking of HA2700-binding to chondrocytes with anti-HA receptor antibodies and analyses of cell signaling: Chondrocytes were plated in 6 cm dishes and cultured under ~80% confluency in DMEM/F-12 containing 10% FBS. After culturing for 48 hours in serum-free DMEM/F-12 containing 0.2% lactalbumin hydrolysate, they were treated with buffer alone, non-immune IgG (20 μg/ml; BD Biosciences, San Diego, CA), anti-CD44 antibody (20 μg/ml; IM7, BD Biosciences), anti-ICAM-1 antibody (20 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a mixture of anti-CD44 antibody (20 μg/ml) and anti-ICAM-1 antibody (20 μg/ml) for 30 minutes and cultured for 24 hours with 2.5 mg/ml HA2700. Then, IL-1α (1 ng/ml) was added to the media and cultured for 24 hours for real-time PCR and for 48 hours for immunoblotting analysis. At the end of the experiment, cells were lysed by sonication twice for 30 seconds using a sonicator (Handy Sonic; Tomy Seiko Co., Ltd, Tokyo, Japan) in SDS reduction buffer and boiled for 10 minutes. Then, they were subjected to immunoblotting with anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) or anti-GAPDH antibody (0.2 μg/ml; Abcam plc, Cambridge, UK). For the experiments of other signaling molecules, cell lysates were harvested 30 minutes after IL-1α (1 ng/ml) stimulation, and then immunoblotted with antibodies against IRAK-1 (0.2 μg/ml), p-IRAK-1 (0.2 μg/ml), ERK1/2 (0.2 μg/ml), p-ERK1/2 (0.2 μg/ml) or anti-IRAK-M antibody (0.2 μg/ml; Cell Signaling Technology Co., Beverly, MA). To examine the effects of MEK inhibitor (PD98059) on IL-1α-induced ADAMTS4 expression and ERK1/2 phosphorylation, chondrocytes were treated for 30 minutes without or with PD98059 (50 μM; Calbiochem, San Diego, CA), and then cultured in the absence or presence of IL-1α (1 ng/ml) for 48 hours to detect ADAMTS4 and GAPDH by immunoblotting with anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) and

anti-GAPDH antibody (0.2 μg/ml), respectively. For the detection of p-ERK1/2 and ERK1/2, the chondrocytes treated with IL-1α for 30 minutes were subjected to immunoblotting with anti-p-ERK1/2 antibody (0.2 μg/ml; Cell Signaling Technology Co) and anti-ERK1/2 antibody (0.2 μg/ml; Cell Signaling Technology Co). All experiments were performed in triplicate.

Supplementary material 5

Effect of HA on mRNA expression of MMPs: Total RNA extracted from cultured chondrocytes was reverse-transcribed to cDNA using a random oligonucleotide hexamer (Takara Bio, Otsu, Japan) and Molony murine leukemia virus reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan). PCR amplification by EX Taq DNA polymerase (Takara Bio) was performed on a thermal cycler using primers specific to MMP-1, MMP-2, MMP-3, MMP-13, MT1-MMP, MT4-MMP and GAPDH according to the methods described previously [5, 6]. As for positive controls, total RNA was extracted from synovial fibroblasts from patients with rheumatoid arthritis for these MMPs and GAPDH. As shown in supplementary Figure below, expression of MMP-1 and MMP-13 was induced by IL-1α treatment, and the expression of MMP-2, MMP-3 and MT1-MMP appeared to increase with IL-1α. On the other hand, MT4-MMP was constitutively expressed. When IL-1α-stimulated chondrocytes were treated with HA species, the expression of MMP-3, MMP-13, MT1-MMP and MT4-MMP appeared to decrease in a manner directly related to molecular weight of the HA used. No definite decrease in the expression of MMP-1 and MMP-2 was obtained by the treatment.

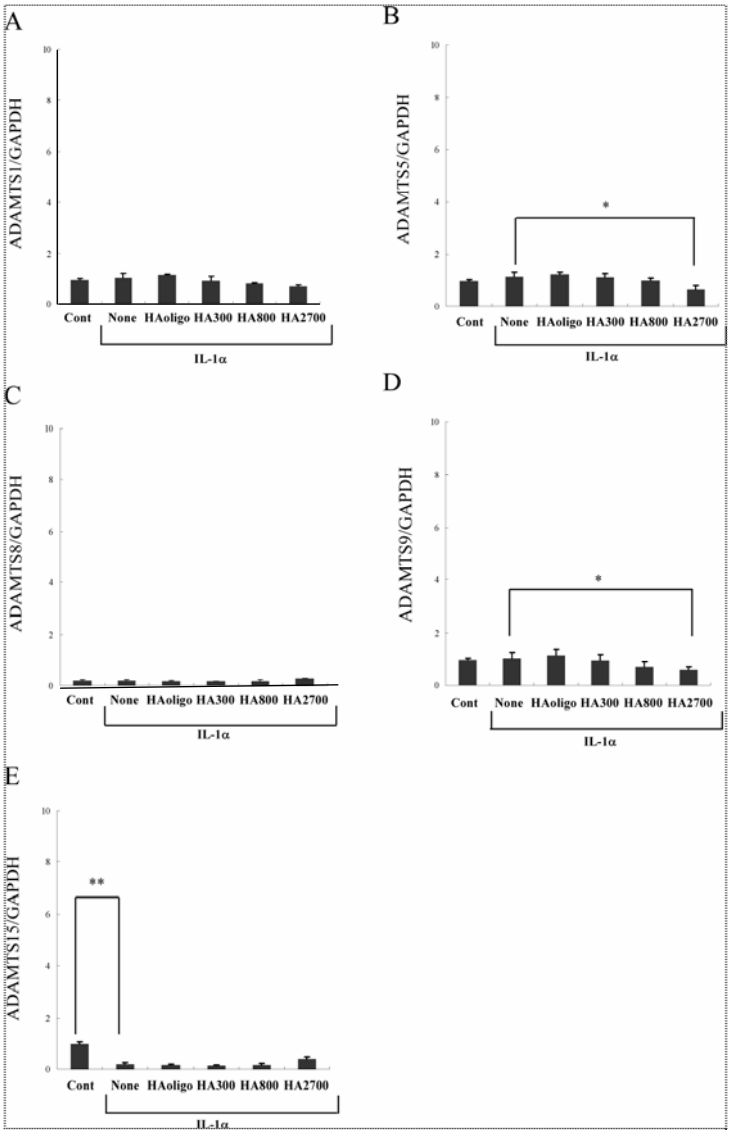
Supplementary Figure: mRNA expression of MMP species in osteoarthritic chondrocytes. Effects of HA species on the expression were examined by RT-PCR in osteoarthritic chondrocytes, which were treated for 24 hours without (Cont) or with IL-1α (1 ng/ml) and HA species (250 μg/ml for HAoligo and 2.5 mg/ml for other HA species). The experiments were carried out in triplicate and representative data are shown. PC, positive controls.



Supplementary material 6

Effect of HA on mRNA expression of ADAMTS1, 5, 8, 9 and 15: The mRNA expression levels of ADAMTS1, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15 were analyzed by real-time PCR. Consistent with the data of RT-PCR, the levels of ADAMTS5 and ADAMTS9 in the IL-1α-treated samples were significantly decreased by treatment with HA2700 (supplementary Figure). The expression level of ADAMTS15 was significantly decreased after treatment with IL-1α, while no effects of HA species on ADAM15 expression in IL-1α-treated chondrocytes were seen (supplementary Figure).

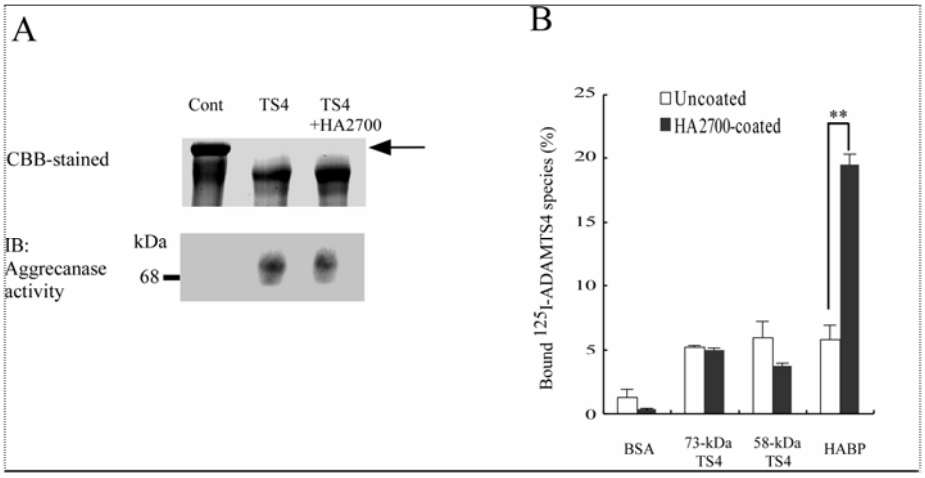
Supplementary Figure: Relative levels of mRNA expression of ADAMTS1, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15 were examined by real-time quantitative PCR in osteoarthritic chondrocytes treated without (Cont) or with IL-1α (1 ng/ml) and HA species (250 μg/ml for HAoligo and 2.5 mg/ml for other HA species). Relative expression levels of the ADAMTS species were normalized to an endogenous control GAPDH. Bars, mean ± SD (n=5). *, $p<0.05$; **, $p<0.01$.



Supplemental material 7

No direct action of HA2700 upon ADAMTS4: To study whether HA2700 directly inhibits the aggrecanase activity of ADAMTS4, recombinant full-length ADAMTS4 of 73 kDa and its deletion mutant of 58 kDa were reacted with or without HA2700 for 2 hours at 37˚C and incubated with aggrecan in an enzyme to substrate ratio of 1:100 for 16 hours at 37˚C. Then, the aggrecanase activity was monitored by immunoblotting. As shown in supplementary Figure A, no inhibition of ADAMTS4 activity was observed with HA2700. We also examined the binding activity of HA2700 to ADAMTS4. Microtiter plates with 96 wells were coated with 0.1 mg/ml HA2700. After washing the plates with 50 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$, 0,05% Brij 35, 0.02% NaN_3 and blocking with 3% Block-Ace (Dainippon-Sumitomo Pharmaceutical, Tokyo, Japan), the plates were incubated with ^{125}I-labeled full-length recombinant ADAMTS4, its deletion mutant, bovine serum albumin or hyaluronan binding protein (Seikagaku Corporation) (5 x 10^5 cpm/well; ~20 ng/well) for 24 hours at 4ºC. The bound proteins were dissociated by treatment with 1 N NaOH, and the radioactivity of the bound fractions was counted using a γ-counter (ARC-600, Aloka, Tokyo, Japan). As shown in supplementary Figure B, there was no definite binding between ADAMTS4 species and HA2700, although the hyaluronan binding protein could bind to HA2700.

Supplementary Figure: No direct inhibition or interaction of ADAMTS4 with HA2700. **A**, Recombinant ADAMTS4 was incubated with buffer alone (TS4) or HA2700 (2.5 mg/ml) (TS4 + HA2700) and incubated with aggrecan. Then, aggrecan digestion was monitored by gels stained with Coomassie brilliant blue (CBB) and immunoblotting with anti-aggrecan neoepitope antibody. The arrow indicates the intact aggrecan core protein. No inhibition of ADAMTS4 aggrecanase activity is observed. **B**, Microtiter plates were coated or uncoated with HA2700 and then incubated with ^{125}I-labeled bovine serum albumin (BSA), 73-kDa and 58-kDa forms of ADAMTS4 (TS4) [2] or hyaluronan binding protein (HABP). Bound radioactivity was measured by a γ-counter. Note that no binding of ADAMTS4 species to HA2700 is seen, while HABP binds to HA2700. Bars, mean ± SD (n=3). **, $p<0.01$.



References

[1] Naito S, Shiomi T, Okada A, Kimura T, Chijiiwa M, Fujita Y, et al. Expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic cartilage. Pathol Int. 2007;57:703-11.

[2] Hashimoto G, Shimoda M, Okada Y. ADAMTS4 (aggrecanase-1) interaction with the C-terminal domain of fibronectin inhibits proteolysis of aggrecan. J Biol Chem. 2004;279:32483-91.

[3] Mitsui Y, Mochizuki S, Kodama T, Shimoda M, Ohtsuka T, Shiomi T, et al. ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. Cancer Res. 2006;66:9913-20.

[4] Okada A, Mochizuki S, Yatabe T, Kimura T, Shiomi T, Fujita Y, et al. ADAM-12 (meltrin alpha) is involved in chondrocyte proliferation via cleavage of insulin-like growth factor binding protein 5 in osteoarthritic cartilage. Arthritis Rheum. 2008;58:778-89.

[5] Konttinen YT, Ainola M, Valleala H, Ma J, Ida H, Mandelin J, et al. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis. 1999;58:691-7.

[6] Yokoyama T, Nakamura H, Otani Y, Kubota T, Fujimoto N, Seiki M, et al. Differences between scirrhous and non-scirrhous human gastric carcinomas from the aspect of proMMP-2 activation regulated by TIMP-3. Clin Exp Metastasis. 2004;21:223-33.