Supplementary Online Materials

ADAMTS-7 Constitutes a Positive Feedback Loop with TNF-α in the Pathogenesis of Osteoarthritis

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This PDF file includes:

MATERIALS AND METHODS

1. Generation of ADAMTS-7 transgenic mice

All animal studies were performed in accordance with institutional guidelines and approval by the Institutional Animal Care and Use Committee of New York University. The transgenic construct and genotyping of embryonic founders by eye color selection is described previously[1,2]. To create ADAMTS-7 transgene, a 5.1-kb DNA fragment covering the entire encoding region of human ADAMTS-7 cDNA was prepared by high fidelity PCR. The hADAMTS-7 cDNA was subcloned into the *Swa*I sites of an expression vectors containing chondrocyte-specific Col2a1 promoter and enhancer sequence (as described in **Fig. 2A**) to create Col2a1-ADAMTS7 plasmid. Transgenic mice were produced by microinjecting the plasmid into the pronuclei of fertilized eggs from F1 mice (FVB/n). Transgenic mice were identified by eye color and PCR assays of genomic DNA extracted from tail. Genomic DNA was amplified by transgene-specific PCR using primers derived from the human ADAMTS-7 cDNA 5'- GCCCAGCCACGGCGCCCCCT -3' and from WPRE sequence 5'- GGAGTTGATAGGTGGTGGC -3' to amplify a 350bp product for ADAMTS-7 transgenic mice.

2. Generation of ADTMTS7 knockdown mice

Based on the technique developed previously[3,4], we generated the U6-ploxPneo-ADAMTS7-RNAi knockdown (KD) transgenic mice with C57BL/6 background using a pair of oligos: sense 5' - CGA GGA GAA GGA CTT AAA GTT CAA GAG ACT TTA AGT CCT TCT CCT CGT TTT TT - 3' and anti-sense 5'- AAT TAA AAA ACG AGG AGA AGG ACT TAA AGT CTC TTG AAC TTT AAG TCC TTC TCC TCG GGC C - 3' under the control of the U6 promoter (a ubiquitous promoter). Because a loxP-flanked neomycin cassette is inserted into this promoter to block the promoter activity, there is no expression of RNAi in the absence of Cre. In this study, two independent founders were used for crossing with Sox2 Cre mice in C57BL/6 (Stock Number: 004783 from Jackson Laboratory) to remove the neo cassette in order to activate expression of ADAMTS-7 RNAi. The transgenic lines were genotyped using PCR with the following pair of primers (5'- CGAAGTTATCTAGAGTCGAC -3' and 5'- AAACAAGGCTTTTCTCCAAGG -3'), which amplify 102bp from the U6 promoter and the connecting neo gene. Cre-mediated neo excision was confirmed by genomic PCR using the following pairs of primers: Del-1：5′ -CGCACAGACTTGTGGGAGAA-3′ ; Del-2: 5′ -CACAATTACTTTACAGTTAG-3′ (product size: 267 bp), All animal studies were in accordance with the guidelines and approved by the IACUC committee of New York University.

3. Radiographic analysis

Prior to histologic processing, samples were evaluated via X-ray (Faxitron) and micro-CT using a Scanco μCT 40 scanner (Scanco) as previously reported5. For the BMD analysis of the whole body, Dual Energy X-ray Absorptiometry (DEXA) scanning (PIXImus2 DEXA scanner, Faxitron) was performed.

4. Whole mount staining for the skeleton

Newborn mice were sacrificed and eviscerated, and their skin was peeled off before they were fixed and stained for cartilage for 24 h with a solution of 80% ethyl alcohol and 20% glacial acetic acid that contained 15 mg of Alcian blue 8GX (Sigma-Aldrich)/100 ml. Samples were then stained for mineralized matrix overnight in a solution of 1% KOH that contained 10 mg of alizarin red S (Sigma-Aldrich)/100 ml. Samples were placed in a 1% KOH–20% glycerol clearing solution until the soft tissues became transparent and the skeleton visible. Then they were placed in successive solutions of 0.5% KOH with increasing percentages of glycerol (20, 40, 60, and 80%). Samples were stored in 100% glycerol.

5. Histological analysis and immunostaining

For the mouse younger than 3 weeks, mouse knee joints were fixed for overnight in 4% PFA, and then dehydrated, embedded in paraffin, and cut with a microtome to generate 5μm thick sections. For adult mice, samples were fixed in 4% PFA for 3 days and decalcified for 2 weeks in 10% w/v EDTA before embedding. Serial sections were stained with Safranin-O/fast green/iron hematoxylin. For immunohistochemistry of ADAMTS-7, sections were pretreated with 0.1% trypsin for 30 min at 37°C, while for the other matrix protein in the cartilage sections were pretreated with chondroitinase ABC (Sigma-Aldrich-Aldrich, 0.25 U/ml) for 60 min at 37°C and then hyaluronidase (Sigma-Aldrich-Aldrich, 1U/ml) for 60 min at 37°C, followed by protein blocking with 20% normal goat serum and 3% BSA for 30 min at room temperature to reduce nonspecific staining. Affinity-purified polyclonal anti-ADAMTS-7 (1:200 dilution), hybridoma cells supernatant containing ColX antibody (no dilution, DSHB), MMP13 antibody (1:200 dilution, ab3208, Abcam), affinity-purified monoclonal anti-COMP (1:200), aggrecan neoepitope 374ARGSV polyclonal antibody (1:100 dilution, AB8135, Millipore) were incubated overnight at 4°C in a method described previously[6]. Detection was performed using the Vectastain Elite ABC kit (Vector, Burlingame, CA), and 0.5 mg/ml 3,3′ -diaminobenzidine (DAB) in 50 mM Tris-Cl substrate (Sigma-Aldrich) was used for visualization, and sections were then counterstained with 1% methyl green. For all of these experiments, at least three slides were analyzed per joint.

6. Double labeling for new bone formation analysis

Dynamic histomorphometric indices were determined by double-fluorescence labeling in vertebral bodies. 2-week-old wild-type and transgenic mice were administered i.p. by 1mg/ml calcein label dissolved in 0.9% NaCl solution containing 2% NaHCO3 (5 mg/kg body weight; Wako Chemicals) followed by a second calcein shot 7 days later. After two days, the mice were killed. Bones were fixed with ethanol and embedded in methylmethacrylate. Sections were cut and viewed using a fluorescence microscope (Eclipse E1000; Nikon).

7. Aging-associated and surgically-induced OA models (DMM)

All animals were provided with water and food ad libitum throughout the duration of theSE studies. For the aging-associated model of OA, WT and ADAMTS-7 transgenic mice were kept up to the age of 8 months and were followed up for spontaneous development of OA. For surgically-induced OA model, we backcrossed the transgenic mice in FVB/n background with C57/BL6 mice for 6 generations to reduce the FVB/n background, and surgery was

performed in 8-week-old ADAMTS-7 transgenic mice, knockdown mice and their corresponding age-matched WT control as described previously[7-9]. In all DMM operations mice were anesthetized with a mixture of xylazine and ketamine (0.15% xylazine and 0.85% ketamine), which was injected intraperitoneally at 1μl/g body weight, and operations were performed. The right knee joint was destabilized by transection of the medial meniscotibial ligament to generate destabilization of the medial meniscus[10]. The left knee joint was sham-operated, in which the joint was prepared using the same approach as that for the right knee joint but without any ligament transection. Six mice were used per time point in each group. These animals were then killed at 4, 8, 12 weeks after surgery. Knee joint tissues were processed for histological evaluation.

8. Histopathologic and quantification evaluation of OA

The proteoglycan content of the articular cartilage was graded on Safranin-O–stained sections using categories of Mankin scores of Safranin-O staining[11,12] (range 0–4), a well accepted scoring system for OA severity[12], in which 0 = normal, 1=slight reduction in staining, 2 = moderate reduction in staining, 3 =severe reduction in staining, and 4 = no dye noted. To determine whether the OA changes in mice were associated with loss of chondrocytes, articular chondrocytes were counted per unit area, and the average diameter of articular chondrocytes was determined following calibration of the microscope at a convenient magnification (100×). The articular cartilage thickness was analyzed by Adobe Photoshop 7.0 (Adobe Systems)[13]. Five random regions of interest were chosen from each joint, and the diameter of all cells within each region of interest was measured. All 3 parameters were determined and averaged in all sections from each mouse, and six mice were analyzed each group. For each experiment, at least three slides were analyzed per joint.

9. Sandwich ELISA for COMP

Serum concentration of COMP was analyzed by our new sandwich ELISA[14], using rabbit anti-COMP pAb as a capture antibody, anti-COMP typeIII mAb 2127F5B6 as a detection antibody, both of the antibodies were purified by the Protein A agarose (Invitrogen), and anti-COMP typeIII mAb 2127F5B6 was labeled by HRP using Lightning-Link™ Horseradish Peroxidase Labeling Kit (Innova) as per the manufacturer's protocols. Ninety-six well ELISA plates (Becton, Dickinson and Company) were coated with 50μl /well of purified rabbit anti-COMP pAb diluted with PBS to 2μg/ml, kept on an orbital shaker overnight at 4℃ , then coated wells were completely washed with PBST three times and blocked with 5% BSA(w/v) in PBS for 1.5 h at room temperature. Purified recombinant mouse type III standards (6.25, 12.5, 25, 50, 100ng/ml) and serum samples (1/10 for mouse) diluted with 0.5%BSA in PBST were transferred to blocked wells at 100μl/well, incubated for 2h on a shaker at room temperature. Plates were washed with PBST for three times and 100μl diluted HRP conjugated detection antibody (diluted to 1μg/ml with 0.5%BSA in PBST) were added to each well, incubated for 2h on a shaker at room temperature, washed plates with PBST for six times. Peroxidase substrate TMB solution (eBioscience) was applied to plates at 100μl/well, and development of color was stopped by adding 100μl 2M sulfuric acid to each well, and absorbance at wavelength of 450nm was read. The COMP concentrations in serum were calculated from the linear range of a standard curve. All the samples were assayed in

triplicate and repeated three times.

10. Cartilage explant cultures

Briefly, mouse femoral head cartilage from either WT or ADAMTS-7 transgenic mice was dissected into pieces of diameter of approximately 1 mm of 1- to 2-mm thickness. The cartilage was dispensed into tissue-culture flasks and incubated overnight in control, serum-free medium Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 25mM HEPES, 2mM glutamine, 100µg/ml streptomycin, 100IU/ml penicillin, 2.5µg/ml gentamicin, and 40 units/ml nystatin or with ADAMTS-7 conditioned media collected from HEK-EBNA stable cell lines (in triplicate for statistical analysis). After 3 days of culture, the supernatants were harvested for COMP degradation analysis by Western blotting, and RNA was extracted from the cartilage samples for real-time RT-PCR.

11. Real-time RT-PCR

Total RNA was extracted from the articular cartilage using RNeasy kit (Qiagen), and first-strand cDNA was generated with ImProm-II reverse transcription system (Promega). Real-time PCR was performed with SYBR Green I dye used to monitor DNA synthesis. Data from each sample were normalized to GAPDH. Primers used for real-time RT-PCR were designed to generate products between 100bp and 200bp in length. Oligonucleotides used as the specific primers to amplify mouse genes are as follows: The following primer sequences were used: for ADAMTS-7 (for both mouse and human), 5'-TCACCAGGTTCCTTGACCGTG-3' and 5'-CCAGCTTGGAGTGACAGGTGGT-3'; for ColX, 5'-TTTCTGCTGCTAATGTTCTTGACC-3' and 5'-TTACTCTTTATGCGTATGGGATG-3'; for alkaline phosphatase, 5'-TCCTGACCAAAAACCTCAAAGG-3'and 5'-ATCTCCACTGCTTCATGCAGAG-3'; for osteocalcin, 5'-GGACCATCTTTCTGCTCACTCTG-3' and 5'-ACCTTATTGCCCTCCTGCTT-3'; for MMP-3, 5'- TCCCACAGCATCCCCTGATGTCC -3' and 5'-TTGCGCCAAAAGTGCCTGTCT-3'; for MMP-9, 5'-ACCACATCGAACTTCGA-3' and 5'-CGACCATACAGATACTG-3'; for MMP 13, 5'-CGGGGAAGACCCTCTTCTTC-3' and 5'-CTTTGTTGCCAATTCCAGGG-3'; for MMP-14, 5'- GGCCGCTCGGATGGTTACCG -3' and 5'-TACGGTCGCGTCCACTCGGG-3'; for ADAMTS-1, 5'-CTGGGCAAGAAATCTGATGA-3' and 5'-AAGCACAGCCACAGTTTATCA-3'; for ADAMTS-4, 5'-CATCCGAAACCCTGTCAACTTG-3' and 5'-GCCCATCATCTTCCACAATAG C-3'; for ADAMTS-5, 5'-GTCACATGAATGATGCCCAC-3' and 5'- TCAGTCCCATCCGTAACCTTTG -3'; for TNF-α, 5'- GCCCACGTCGTAGCAAACCAC-3' and 5'- TCGGGGCAGCCTTGTCCCTT -3'; for GAPDH:5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3'. Reactions were performed in a 50µl SYBR green PCR volume in a 96-well optical reaction plate formatted in the 7300 sequence detection system (Applied Biosystems) under the following PCR conditions: 40 cycles at 95°C for 15 s and at 60°C for 1 min. For each gene, three independent PCRs from the same reverse transcription sample were performed.

12. Western blotting analysis

Total protein from WT and ADAMTS-7 transgenic mice articular cartilage culture media was precipitated by adding 1 volume of 100% TCA to 4 volumes of serum, mixed well and incubated on ice for 20 min, spin out the supernatant, washed the pellet with ice-cold acetone twice, dried the pellet for 5 min to drive off the residual acetone, finally dissolved the pellet with 1×reducing SDS-PAGE loading buffer and boiled for 5 minutes. Samples were subject to 10% SDS- PAGE and electrotransferred to nitrocellulose membrane, then the membrane was blocked with 5% non-fat milk in TBST for 1 hour at room temperature, and incubated with rabbit anti-COMP pAb overnight at 4°C, anti-rabbit IgG conjugated horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution was used as secondary antibody, signals were detected by using the enhanced chemiluminescence (ECL) system (Pelkin Elmer Life Sciences).

13. Chromatin Imunoprecipitation (ChIP)

The ChIP assay was performed as described previously[15]. Briefly, C28/I2 cells were cultured in DMEM media supplemented with 10% fetal bovine serum and 20ng/ml TNF-α for 48 hours, then cells were treated with formaldehyde, and chromatin was prepared and sheared to a size range of 300 to 600kbp by sonication. Chromatin was immunoprecipitated overnight using antibodies for NF-κB p65, and nonspecific rabbit IgG. The immune complexes were allowed to react with protein A-agarose beads (Santa Cruz); after extensive washing, the precipitates were removed from the beads using elution buffer (50mM NaHCO3 and 1% SDS) and mild vortexing. The cross-linking was reversed and the samples were sequentially digested with RNase A and proteinase K. Immunoprecipitated chromatin was purified and subjected to PCR using primers specific for human ADAMTS-7 promoter region (-418 to -97) , sense 5'-GAGGCCTCCCGCGTCCCCT-3', antisense 5'-CCCCTCGGACTCCTTCC-3'.

14. Construction of ADAMTS-7 specific reporter constructs

For reporter gene assay, genomic DNA was extracted from cultured C28/I2cells using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendations. PCR products were obtained using 50ng of genomic DNA template, 0.5μM of each primer (full length -2.7 kb/+61bp, forward:5'-CCCCTCGAGATGCCTGGCTCACATCTTCTC -3', -2105/+61, forward: 5'-CCCCTCGAGTGAGACAGGGCCTCACTTTG-3'; -1078/+61, forward: 5'-CCCCTCGAGTCCTGAGTATCACTGGGACTA-3' -1078/+61, forward: 5'-CCCCTCGAGTGGCTGAGGTGGAGTGGA-3';-735/+61, forward: 5'-CCCCTCGAGGGCTGGGGCCACGAA-3'; -416/+61, forward: 5'-CCCCTCGAGGCCTCCCGCGTCCCCT-3'; -99/+61, forward: 5'-CCCCTCGAGGGAAGGAGTCCGAGG-3' and reverse: 5' -TCCCAAGCTTGTCCGCGGGCAACAAAG -3', underlined sequences represent *Xho*I and *Hind*III recognitaion sites, respectively), 200μM dNTPs and 0.5 U of Phusion® High-Fidelity DNA Polymerase (Finnzymes) in the manufacturer's buffer. The amplified fragments were subcloned into the *Xho*I and *Hind*III sites of the pGL3 basic for generation of −1702TS7luc, −735TS7luc, −416TS7luc and -99TS7luc reporter vectors.

15. Site-directed mutagenesis

Mutations in the ADAMTS-7 promoter were created in putative NF-κB p65 binding sites at nucleotides −785 to −7767 (site 1), −645 to −636 (site 2), -318 to -309 using the Quickchange XL site-directed mutagenesis kit (Stratagene). For construction of site-directed mutants of the ADAMTS-7 promoter, the reporter vector −1702TS7luc was used as a template. To generate a site 1 mutant, primer A, 5'- TGTGCTCCCAGGAGCTGAATTTCTCCCTTCTGCCG-3'; and primer B, 5'- CGGCAGAAGGGAGAAATTCAGCTCCTGGGAGCACA -3' were used. The mutant nucleotides are underlined. The same protocol was used to create mutations in the site 2 and 3 region, for site 2 mutation, we used primer C, 5'-GACTTTCAGCAGGGGCTCCCGGGGAGCCTGGAGGC -3'; and primer D, 5'-GCCTCCAGGCTCCCCGGGAGCCCCTGCTGAAAGTC-3', for site 3 mutant, primers 5'-CCCGGAAAGAATCTCTGGAATTGAGAGAATTAATGTGTTG-3' and 5'-CAACACATTAATTCTCTCAATTCCAGATTCTTTCCGGG-3' were used. The authenticity of mutation of all constructs was confirmed by nucleotide sequencing analysis.

16. Luciferase assay

C28/I2 cells were plated into 24-well plates at 20,000 cells/ well. 24h later, cells were co-transfected with each pGL3 construct and p65 expression plasmid pCMV-p65 or its empty control in combination with renilla luciferase plasmid pRLSV40 (Promega) as an internal control in triplicate using 200ng DNA in total and Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendation. Besides, the same amount of cells transfected with each pGL3-ADAMTS7-luc construct in combination with pRLSV40 were cultured in DMEM with or without 20ng/ml TNF-α. 48h later, cells were lysed and assayed using Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol by GloMax®-96 Microplate Luminometer (Promega). For the NF-κB inhibition assay, different doses of Bay 11-7082 were added to the culture media and incubated for 4 hours after the treatment of TNF-α.

17. Statistical analysis

Results were expressed as mean values±S.E.M. Statistics were conducted as Student's t-test using SPSS software (SPSS Inc, Chicago, IL). $P<0.05$ was considered statistically significant.

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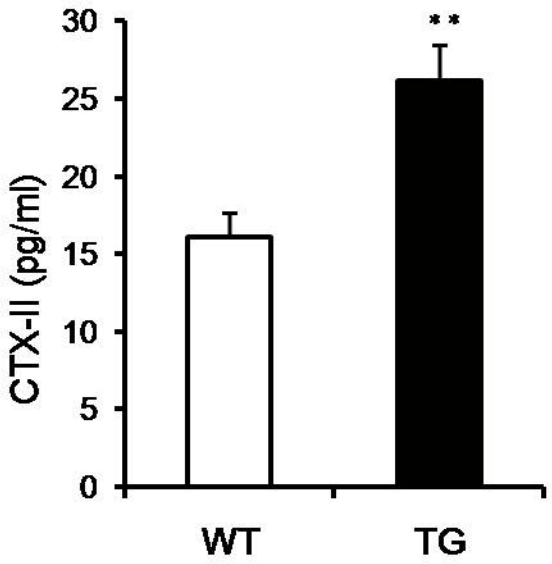

Fig. S1 Collgen II cleavage was significantly increased in 8-month old ADAMTS-7 transgenic mice. The serum level of C-terminal fragment of type II collagen degradation (CTX-II) was measured with the commercially available Serum Pre-clinical Cartilaps enzyme-linked Immunosorbent assay (ELISA) (Nordic Bioscience, Herlev, Denmark). **, $P<0.01$ versus WT mice.

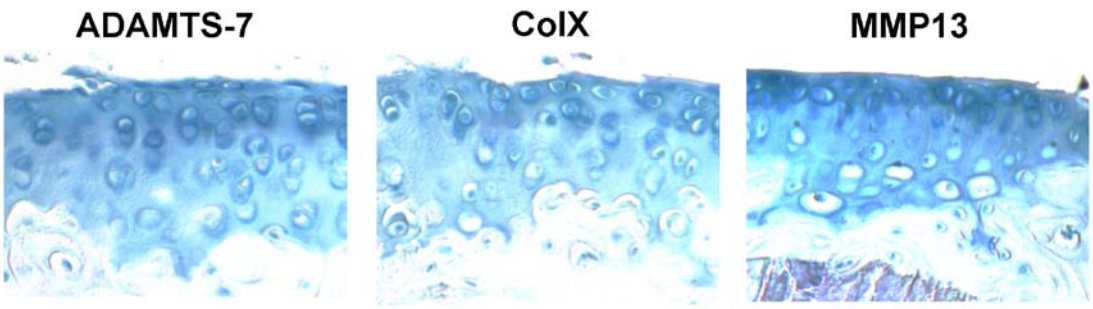

Fig. S2 Negative controls for immunohistochemistry involved in this study. Negative Immunostaining for ADAMTS-7 (left), Collagen X (ColX, middle), and MMP13 (right) was performed on mice articular cartilage using corresponding preimmune serum (ADAAMTS-7 and control IgGs (ColX and MMP13), respectively.

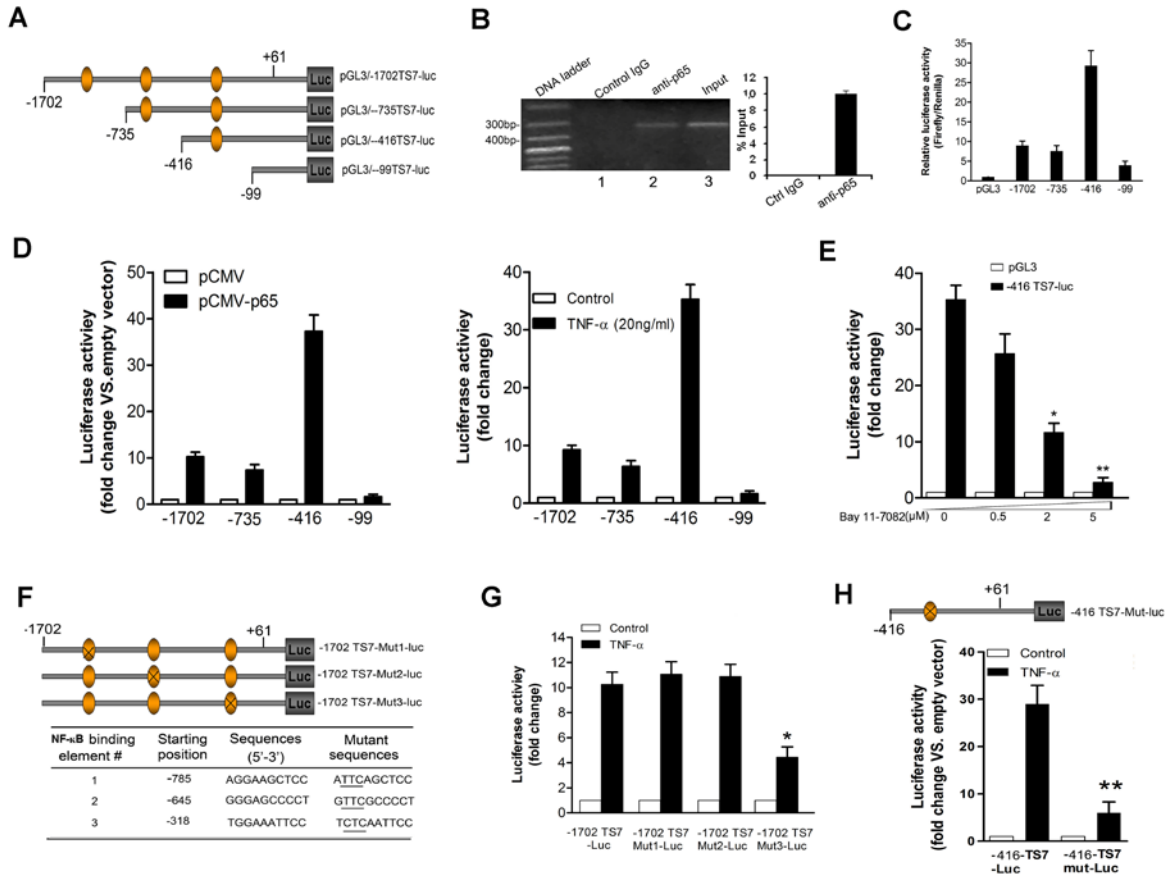

Fig. S3 TNF-α upregulates ADAMTS-7 in an NF-κB-dependent manner. (**A**) Schematic representation of four ADAMTS-7-specific reporter constructs. The potential NF-κB binding sites are indicated by ovals. (**B**) Interaction between NF-κB p65 and ADAMTS-7 5'-flanking regulatory region, assayed by ChIP. C28/I2 cells were cultured with 20ng/ml TNF-α for 48h. Following crosslinking with formaldehyde, cell lysates were subjected to immunoprecipitation with anti-p65 and control IgG, Purified DNA from the cell lysate as input DNA, and DNA recovered from immunoprecipitation were amplified by PCR using specific primers. (**C**) C28/I2 cells were transfected with four reporter constructs containing different length fragments from 5'-flanking region of ADAMTS-7 along with pRLSV40 as an internal control. Relative luciferase activity was measured, the value from pGL3-basic was set as 1, values are plotted as mean ± SEM. (**D**) C28/I2 cells transfected with various ADAMTS-7 reporter constructs, as indicated, were cultured in the presence of 20ng/ml TNF-α (right panel) or cotransfected with an plasmid encoding NF-κB p65 (left panel). Luciferase activity was analyzed as described above. (**E**) C28/I2 cells transfected with -416-TS7-luc were cultured with 20ng/ml TNF-α for 48h, then treated with various dosages of Bay 11-7082 for 4 hours, and the luciferase activity was analyzed as described above. pGL3-basic transfection group was used as control. * $p<0.05$, ** $p<0.01$ compared with the group treated with TNF-α only. (**F**) Schematic of three mutants of -1702 TS7-luc reporter construct. The NF-κB-specific sequence in the -1072 TS7-luc reporter construct was altered, mutant nucleotides are indicated by underlines. (**G**) C28/I2 cells transfected with various -1702 TS7-luc mutants were cultured with 20ng/ml TNF-α for 48 hours, and the luciferase activity was measured. Data were presented as fold of changes over the non-treatment group after being normalized with renilla activity.* $p<0.05$, compared with the -1702 TS7-luc WT construct transfection group. (**H**) Alteration of the NF-κB-specific sequence almost abolished TNF-α transactivation of ADAMTS-7 reporter gene. Schematic of mutated NF-κB binding site in -416GEP-luc reporter construct. The NF-κB-specific sequence in the -416 TS7-luc reporter construct was altered, as indicated in (F). C28/I2 cells were transfected, processed, and the reporter gene activities analyzed as described. * $p<0.05$, compared with the -416 TS7-luc WT construct transfection group.