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Research Article

Regulation of chondrocyte differentiation by ADAMTS-12 metalloproteinase depends on its enzymatic activity

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Abstract. ADAMTS-12, a metalloproteinase that belongs to ADAMTS family, is strongly upregulated during chondrogenesis and demonstrates prominent expression in the growth plate chondrocytes. ADAMTS-12 potently inhibits chondrocyte differentiation, as revealed by altered expression of both early and later genes critical for chondrogenesis. In addition, ADAMTS-12-mediated inhibition of chondrogenesis depends on its enzymatic activity, since its point mutant lacking enzymatic activity completely loses this activity. Furthermore, the C-terminal four thrombospondin motifs known to bind COMP substrate is necessary for its full proteolytic activity and inhibition of chondrocyte differentiation. Mechanism studies demonstrate that ADAMTS-12 induces PTHrP, whereas it inhibits IHH during chondrogenesis. Furthermore, PTHrP induces ADAMTS-12 and ADAMTS-12 is hardly detectable in PTHrP-/-growth plate chondrocytes. Importantly, knocking down ADAMTS-12 mRNA levels or blocking ADAMTS-12 activity almost abolishes the PTHrP-mediated inhibition of type X collagen expression. Collectively, these findings demonstrate that ADAMTS-12, a downstream molecule of PTHrP signaling, is a novel regulator of chondrogenesis.

Keywords. Chondrocyte, differentiation, ADAMTS-12, metalloproteinase, PTHrP signaling.

Introduction

The ADAMTS (a disintegrin and metalloproteinase with thrombospondin type 1 motifs) family consists of secreted zinc metalloproteinases with a precisely ordered modular organization that includes at least one thrombospondin type I repeat [1, 2]. Important functions have been established for several members of the ADAMTS family. ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, ADAMTS-9, ADAMTS-16 and ADAMTS-18 degrade aggrecan [3–8] and ADAMTS-5 plays a primary role in aggrecan loss in murine arthritis [4, 6]. ADAMTS-2, ADAMTS-3, and ADAMTS-14 are procollagen N-propeptidases [9, 10]. ADAMTS-2 mutations cause dermatosparaxis, an inherited disorder characterized by severe skin fragility [11]. ADAMTS-13 is a von Willebrand factor–cleaving protease, and its mutations lead to heritable life-threatening thrombocytopenic purpura

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[12]. ADAMTS-7 and ADAMTS-12 share the same domain organization and form a subgroup with unique properties within ADAMTS family [2, 13, 14]. Our previous reports demonstrate that ADAMTS-7 and ADAMTS-12 directly associate with and degrade cartilage oligomeric matrix protein (COMP), a prominent noncollagenous component of cartilage. In addition, Alpha-2-Macroglobulin inhibits their degradation of COMP [15]. Recent report revealed that ADAMTS-12 also degraded aggrecan [16].

COMP is a 524-kDa pentameric, disulfide-bonded, multidomain glycoprotein composed of approximately equal subunits (~110 kDa each) [17, 18]. Although the function of COMP is not completely understood, it appears to mediate chondrocyte attachment by an integrin receptor [19, 20], and accumulating evidence suggests that COMP may function to stabilize the extracellular matrix of articular cartilage by specific cation-dependent interactions with matrix components, including collagen type II (Col II) and collagen type IX, aggrecan and fibronectin [21–24]. In addition, mutations in the human COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia [25–31], autosomal-dominant forms of short-limb dwarfism [32, 33]. We recently found that COMP associates with granulin-epithelin precursor (GEP), a novel chondrogenic growth factor, and potentiates GEP-stimulated chondrocyte proliferation[34]. Fragments of COMP have been detected in diseased cartilage, synovial fluid, and serum of patients with knee injuries, posttraumatic, primary osteoarthritis (OA) and rheumatoid arthritis (RA) [35–37]. Monitoring of COMP levels in either joint fluid or serum can be used to assess the presence and progression of arthritis [22, 38–42].

In addition to their cartilage-degrading role in arthritis, several metalloproteinases have been shown to play important functions in regulating chondrogenesis [43–45]. Well-orchestrated chondrogenesis is controlled exquisitely by cellular interactions with the growth factors and surrounding matrix proteins, including metalloproteinases, that mediate cellular signaling pathways and transcription of specific genes in a temporal-spatial manner [46–48]. In this study we report that ADAMTS-12 is a downstream molecule of parathyroid hormone related peptide (PTHrP) and adversely mediates chondrocyte differentiation based on the following observations: (1) ADAMTS-12 demonstrates prominent expression in proliferating and prehypertrophic chondrocytes in the wildtype embryonic growth plate, whereas its expression is hardly detectable in PTHrP-/-embryos; (2) ectopic expression of ADAMTS-12 dramatically inhibits the expression of marker genes for chondrocyte differentiation, including type II collagen (Col II), Sox9, type X collagen (Col X), Core-binding factor α1 (Cbfa1) and Indian hedgehog (IHH), while it enhances PTHrP expression; (3) the enzymatic activity and the C-terminal four thrombospondin motifs of ADAMTS-12 are important for ADAMTS-12-mediated inhibition of chondrogenesis, and (4) PTHrP signaling is almost abolished when ADAMTS-12 is repressed, and PTHrP action is largely restored when ADAMTS-12 is re-expressed.

Materials and methods

Transfection of the cells and generation of stable cell lines. HEK 293-EBNA, C3H10T1/2, rat chondrosarcoma (RCS) cells were seeded at 5×10^6 cells/well of a six-well plate in media containing 10% fetal calf serum. The cells were cultured overnight and transfected the following day with appropriate plasmid DNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The stable lines were selected with 1 mg/ml of G418 and maintained with 200 μg/ml of G418.

Preparation and purification of an antiserum to ADAMTS-12. Rabbit polyclonal antibodies against ADAMTS12 were generated using a C-terminal 14-amino acids peptide (RQRRQRLLQKSKEL) as antigen by Zemed Laboratories Inc. via pay-for-service. To affinity-purify anti-ADAMTS-12 antibody, the rabbit serum was incubated with Affi-Gel-10 beads (Bio-Rad) to which peptide antigen was covalently linked. The bound antibodies were eluted from the beads with 0.15M glycine buffer (pH 2.5) and immediately neutralized with 1.5M Tris-HCl buffer (pH 8.0) [49].

Construction of human ADAMTS-12 and its series of C-terminal domain deletion mutants. Full-length human pcDNA3-ADAM-TS12-HA and its point mutant plasmid were kindly provided by Dr. Cal S. [14]. Full-length ADAMTS-12 and its domain deletion mutants were created using the PCR method. The PCR was performed with pcDNA3-ADAM-TS12-HA as a template and amplified by Pfu turbo DNA polymerase using the following primers: Forward primer, 5'-ATGCAGCGGCC-GCCATCTGAATCATGCCATGTGCCC-AGAGGAGCTG (ADAMTS-12 FW), containing a Not I restriction site (underlined), and the reverse primer, 5'-ATGCATCTAGAGAG-TTCTTTTGACTTTTGGAG-3' (ADAMTS-12 RV), 5'-ATGCATCTAGAGTGTGCAGAGCC-GTGGCCGT-3' (ADAMTS-12-M1 RV), 5'-ATG-CATCTAGAGCATTGCTGGAGGCCAC-

ACA-3' (ADAMTS-12-M2 RV), 5'-ATG-CATCTAGAGAAGTACATCTGCTCAAC-3' (ADAMTS-12-M3 RV), 5'-ATGCATCTAGA-GGAAGAGCCATCTCCCAGGC-3' (ADAMTS-12-M4 RV), 5'-ATGCATCTAGAACAGGGGTG-GACGTTGCACA-3' (ADAMTS-12-M5 RV), or 5'-ATGCATCTAGACTTGGACTTCAAGCCTTTC-3' (ADAMTS-12-M6 RV) containing a XbaI restriction enzyme site (underlined). The PCR was carried out for 35 cycles of denaturation (60 s at 94 °C), annealing (60 s at 60 °C), and extension (7 min at 72 °C). The PCR products were ligated into the pcDNA3.1/myc-His (A) vector, using the NotI and XbaI restriction enzyme sites. A diagram of the ADAMTS-12 constructs (TS12-M1 – TS12-M6) created is shown in Figure 3A. All constructs were verified by nucleic acid sequencing; Subsequent analysis was performed using Curatools (Curagen, New Haven, CT) and BLAST software (available at http://www.ncbi.nlm.nih.gov/BLAST/).

Immunocytostaining localization of ADAMTS-12 and its domain deletion mutants in RCS chondrocytes. The RCS cells were transiently transfected with an expression plasmid encoding ADAMTS-12 or its mutants described above and cultured in serum-free DMEM with or without heparin (100 μg/ml) for 48 h. To localize each recombinant ADAMTS-12 protein, the cells were washed, fixed with 100 % methanol in the freezer compartment for 5 min, washed twice in 4 °C phosphate-buffered saline for 5 min, and then incubated with 20 % goat serum in phosphate-buffered saline for 30 min, the cells were incubated with primary antibodies (i. e. mouse monoclonal anti-C-Myc antibodies and rabbit polyclonal anti-COMP antibodies) at room temperature for 1 h. After being washed with phosphate-buffered saline, the cover slips were incubated with secondary antibodies (i. e. goat anti-mouse IgG conjugated with rhodamine (Santa Cruz Biotechnology; diluted 1:100) and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Santa Cruz Biotechnology; diluted 1:100)) for 1 h. The specimens were observed under a fluorescence microscope with appropriate optical filters. Microscopic images were captured using the Image Pro program (Media Cybernetics) and an Olympus microscope. Images were arranged using the Adobe Photoshop program.

For revealing the induction of ADAMTS-12 by PTHrP, C3H10T1/2 cells were treated with or without 10^{-7} M of PTHrP for three days, and the expression of ADAMTS-12 was visualized with affinity-purified anti-ADAMTS-12 antibodies.

Generation of ADAMTS-12 siRNA expression constructs. For silencing the expression of ADAMTS-12, three regions of mouse ADAMTS-12 were targeted for small interfering RNA (siRNA) using mammalian expression pSUPER vector (OligoEngine) according to the manufacturer's instructions. To generate siRNAs, equimolar amounts of complementary sense and antisense strands were separately mixed, annealed, and slowly cooled to 10 °C in a 50 μl reaction buffer (100 mM NaCl and 50 mM HEPES, pH 7.4). The annealed oligonucleotides were inserted into the BglII/HindIII sites of pSUPER vector. The resultant plasmids and control vector pSUPER were transfected into C3H10T1/2 cells using Lipofectamine 2000 reagent (Invitrogen), and the expression of ADAMTS-12 was determined using real-time PCR. The data demonstrated that all siRNA corresponding to the different coding sequence of ADAMTS-12 gene (5'-TGAGCACAAGTACTCCCAC-3', 5'-CA-CAAGTACTCCCACAATC-3' and 5'-ACTCCCA-CAATCAACAGCC-3') were able to efficiently reduce the expression of mouse ADAMTS-12.

Western blotting. Total cell extracts, conditioned medium, or *in vitro* digested products were subjected to SDS-PAGE and examined by Western blotting with mouse monoclonal C-Myc antibodies (Santa Cruz Biotechnology), rabbit polyclonal COMP antibodies [49] or rabbit polyclonal ADAMTS-12 antibodies, followed by anti-mouse IgG-conjugated horseradish peroxidase (HRP) or anti-rabbit IgG-conjugated horseradish peroxidase (HRP) at 1:1000 dilution. The signals were detected using the ECL chemiluminescent system.

In order to examine the ADAMTS-12 expression in the course of chondrogenesis, micromass cultures of C3H10T1/2 cells treated with 300 ng/ml bone morphogenetic protein-2 (BMP-2) for various time points were detected by rabbit polyclonal ADAMTS-12 antibodies or anti-tubulin antibodies (internal control), and the signal was visualized as described above.

RNA preparation and real time PCR. Total RNA was extracted from micromass cultures of C3H10T1/2 cells stably transfected with either control, ADAMTS-12 (TS12), its point mutant (TS12-PM), or its C-terminal deletion mutants in the presence of 300 ng/ml recombinant BMP-2 for various time points using RNAeasy kit (Qiagen). One microgram of total RNA per sample was reverse-transcribed using the ImProm-II Reverse Transcription system (Promega). The following sequence-specific primers were synthesized: 5'-TGGTGGAGCAGCAAGAGCAA-3' and 5'-CAGTGGACAGTAGACGGAGGAAA-3' for

mouse Col II, 5'-CTGCTGCTAATGTTCTTGAC-3' and 5'-ACTGGAATCCCT TTACTCTTT-3' for mouse Col X, 5'-TGATGACACTGCCACCTGTG-3' and 5'-ACTCTGGCTTTGGGAAGAGC-3' for mouse Cbfa1, 5'-CGCTCGCAATACGA-CTACGC-3' and 5'-TAGAGCCCTGAG-CCCTGTCC-3' for mouse Sox9, 5'-CAGTG-GAGTGTCCTGGTATT-3' and 5'-GATCTCCGC-GATCAGATGGT-3' for mouse PTHrP, 5'-GCTCGTGCCTCTTGCCTACA-3' and 5'-CGTGTTCTCCTCGTCCTTGA-3' for mouse IHH; 5'-CTCGATCGTGGCTCAGCTTA-3' and 5'-TGCTGGTAGTGACAGGGTGA-3' for mouse ADAMTS-12; 5'-GTGAGCCAT-GATTCGCCTCGG-3' and 5'-CACCAGGTT-CACCAGGATTGCC-3' for human Col II; 5'-CCCTTTTTGCTGCTAGTATCC-3' and 5'-CTGTTGTCCAGGTTTTCCTGGCAC-3' for human Col X. The following pair of oligonucleotides was used as internal controls: 5'-AGGTCGGTGTGAACGGATTTG-3'and 5'-TGTAGACCATGTAGTTGAGGTCA-3' for mouse GAPDH; 5'-ATGACATCAAGA-AGGTGGTG-3'and 5'-CATACCAGGAAATG-AGCTTG-3' for human GAPDH. Reactions were performed in a 50 µl SYBR GREEN PCR volume in a 96-well optical reaction plate formatted in the 7300 Sequence Detection System (ABI PRISM, Applied Biosystems) using the following PCR conditions: 40 cycles, 95 °C for 15 s, 60 °C for 1 min. The transcript of GAPDH mRNA was employed as an internal control for RNA quality. For each gene, three independent PCRs from the same reverse transcription sample were performed. The presence of a single specific PCR product was verified by melting curve analysis and confirmed on an agarose gel and further sequenced by the Applied Biosystems sequencing system (Foster City).

Aggregate culture of human mesenchymal stem cells. Human mesenchymal stem cells (hMSC) were cultured until approximately 80 % confluent and collected. After washing with a defined medium consisting of DMEM-high glucose with ITStPremix (Collaborative Biomedical Products, Bedford), with pyruvate (1 mM; Gibco), ascorbate 2-phosphate (100 mM; Wako), and dexamethasone (10^{-7} M), hMSCs were centrifuged at 500 g for 5 min and resuspended in the defined medium at a final concentration of 2×10^5 cells/ml. Aliquots (1 ml) were added to 15 ml conical polypropylene centrifuge tubes and the cells pelleted by centrifugation at 500 g for 5 min. The medium in each tube was replaced with 0.5 ml defined medium containing 300 ng/ml BMP-2 in the presence of conditioned medium obtained from HEK 293-EBNA cells stably transfected with pcDNA3.1, ADAMTS-12 or its mutants. The aggregates were then incubated at 37 °C in 95 % humidified air and 5 % CO_2. Culture medium was changed every other day. Total RNA from aggregate cultures were isolated at day 14 and real-time PCR for chondorcyte marker genes, including Col II and Col X, was performed as described above.

Results

ADAMTS-12 is a potent negative regulator of chondrocyte differentiation. Given that several metalloproteinases play important roles in chondrogenesis and cartilage development [43–45], and ADAMTS-12 is highly expressed in the musculoskeletal tissues, including cartilage and bone [50], we sought to determine the role of ADAMTS-12 in chondrogenesis. The mouse embryonic mesenchymal stem cell line C3H10T1/2 is a pluripotent cell line. It differentiates specifically to the cartilage lineage at high yields when incubated under high-cell-density micromass cultures as well as when exposed to osteoinductive-proteins such as BMP-2 [51]. Therefore, C3H10T1/2 cells have the potential to become chondrocytes, making them a valuable *in vitro* correlate for studying the mechanisms of chondrogenesis. Micromass cultures of both control (CTR) and ADAMTS-12 (TS12) stably transfected C3H10T1/2 cell lines (Fig. 1A) were stimulated with 300 ng/ml BMP-2 protein for seven days and real-time PCRs were performed using specific primers for mouse Col II, Sox9, Col X, Cbfa1 and PTHrP. The data in Figures 1B–1D showed that ADAMTS-12 was a potent inhibitor of chondrocyte differentiation, since it suppressed the expression of both early (Col II and Sox9) and late (Col X) marker genes for chondrogenesis. Specially, the Col X expression was completely inhibited by ectopic expression of ADAMTS-12, indicating that ADAMTS-12 may be a critical mediator for chondrocyte hypertrophy.

The transcription factor Cbfa1, which is needed for osteoblast differentiation, is also required for hypertrophic chondrocyte differentiation [52]. Signaling molecules IHH and PTHrP establish a negative feedback loop that regulates the pace of chondrocyte differentiation. PTHrP action keeps chondrocytes proliferating and delays their further differentiation. IHH is expressed at the prehypertrophic–hypertrophic boundary so that cells that escape the inhibitory action of PTHrP signaling in the growth plate express IHH, which in turn will stimulate PTHrP expression [53, 54]. We examined whether ADAMTS-12 mediated the expressions of these critical molecules in

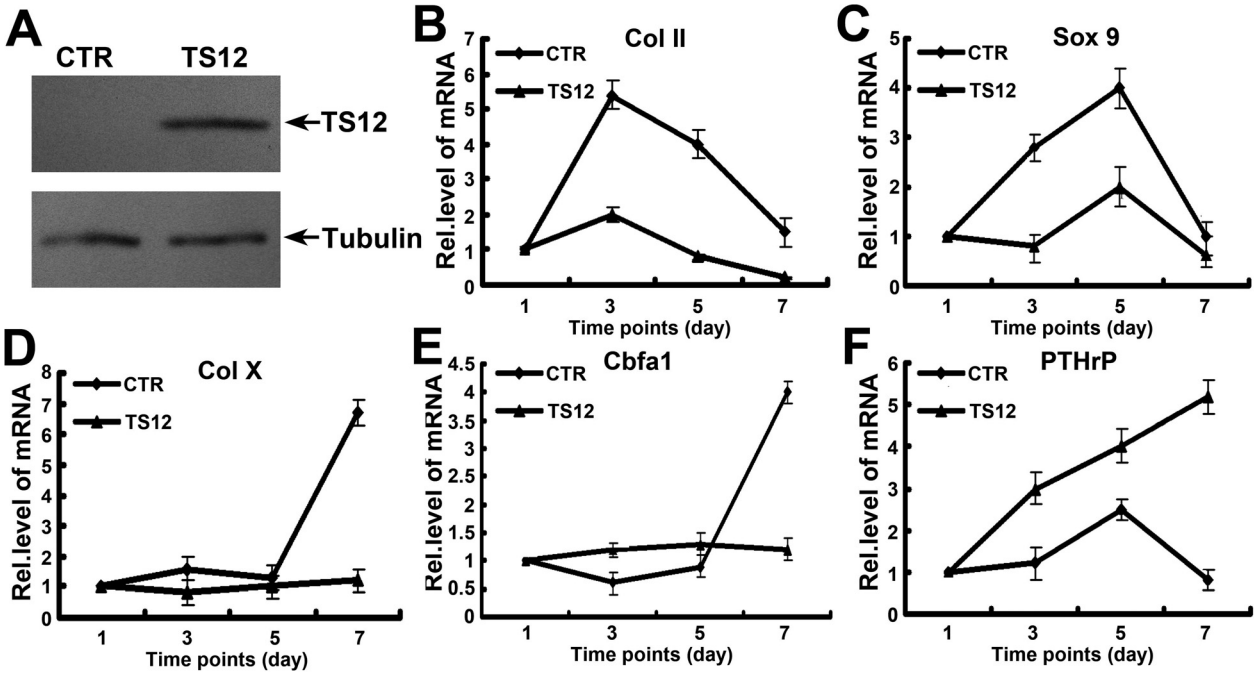

Figure 1. ADAMTS-12 inhibits chondrocyte differentiation. (*A*) C3H10T1/2 were stably transfected with pcDNA3.1 (CTR) or ADAMTS-12 (TS12), as indicated, and the level of ADAMTS-12 in the cell lysates was visualized by Western blotting with anti-ADAMTS-12 antibody; (*B-F*) Micromass cultures of both pcDNA3.1 (CTR) and ADAMTS-12 (TS12) stably transfected C3H10T1/2 cells were stimulated with 300 ng/ml BMP-2 protein for various time points, as indicated, and the levels of mRNAs for Col II (*B*), Sox9 (*C*), Col X (*D*), Cbfa1 (*E*), and PTHrP (*F*) were determined using real-time PCR.

chondrocyte differentiation. Compared to the control (CTR), ADAMTS-12 (TS12) dramatically inhibits the expression of Cbfa1 (Fig. 1E) whereas it enhances the expression of PTHrP (Fig. 1F).

Enzymatic activity of ADAMTS-12 is required for the ADAMTS-12-mediated inhibition of chondrocyte differentiation. ADAMTS-12 has been found to digest COMP and aggrecan [16, 50], we next determined whether the proteolytic activity of ADAMTS-12 is needed for its regulation of chondrocyte differentiation. For this purpose, we employed a point mutant of ADAMTS-12 (TS12-PM) that lacks enzymatic activity [16]. We first examined the specificity of the affinity-purified polyclonal antibodies against ADAMTS-12 using Western blots with conditioned medium collected from HEK 293-EBNA cells stably transfected by either pcDNA3.1 vector (CTR) or C-Myc-tagged wildtype ADAMTS-12 (TS12, shown in Fig. 2A, panel a): a band with an apparent molecular mass of approximately 200 kDa was resolved in the medium bearing C-Myc-tagged ADAMTS-12 but not in the control medium (Fig. 2A, panel a). In addition, this band was also specifically recognized by anti-C-Myc monoclonal antibody (Fig. 2A, panel b). However, the point mutant of ADAMTS-12 did not produce any detectable COMP-degrading activity, although wildtype ADAMTS-12 efficiently cleaved COMP (Fig. 2A, panel c). As expected, the mutation construct expressed comparable amount of point mutant of ADAMTS-12 (TS12-PM) [14] compared to the construct encoding wildtype ADAMTS-12 (TS12) in the stably transfected C3H10T1/2 cells using Western blotting analysis by above-mentioned anti-TS12 polyclonal antibody (Fig. 2A, panel d).

We next determined whether enzymatic activity of ADAMTS-12 was involved in ADAMTS-12-mediated chondrogenesis. As shown in Figure 2B, panels a–f, ADAMTS-12 potently inhibited expressions of Col II, Col X, Cbfa1 and IHH, whereas it increased the expression of PTHrP, during *in vitro* chondrogenesis of a micromass culture of C3H10T1/2 cells; however, its point mutant of ADAMTS-12 (TS12-PM) totally lost these regulatory activities, clearly indicating that ADAMTS-12-mediated chondrogenesis depends on its enzymatic activity. These observations were also repeated with aggregate culture system of human mesenchymal stem cells (MSCs) (Fig. 2C, panel a and b): ADAMTS-12 potently inhibited expressions of Col II and Col X during *in vitro* chondrogenesis of human MSCs pellet cultures, whereas its point mutant failed to do so.

Effects of ADAMTS-12 non-catalytic ancillary domains on its biochemical activities and regulation of

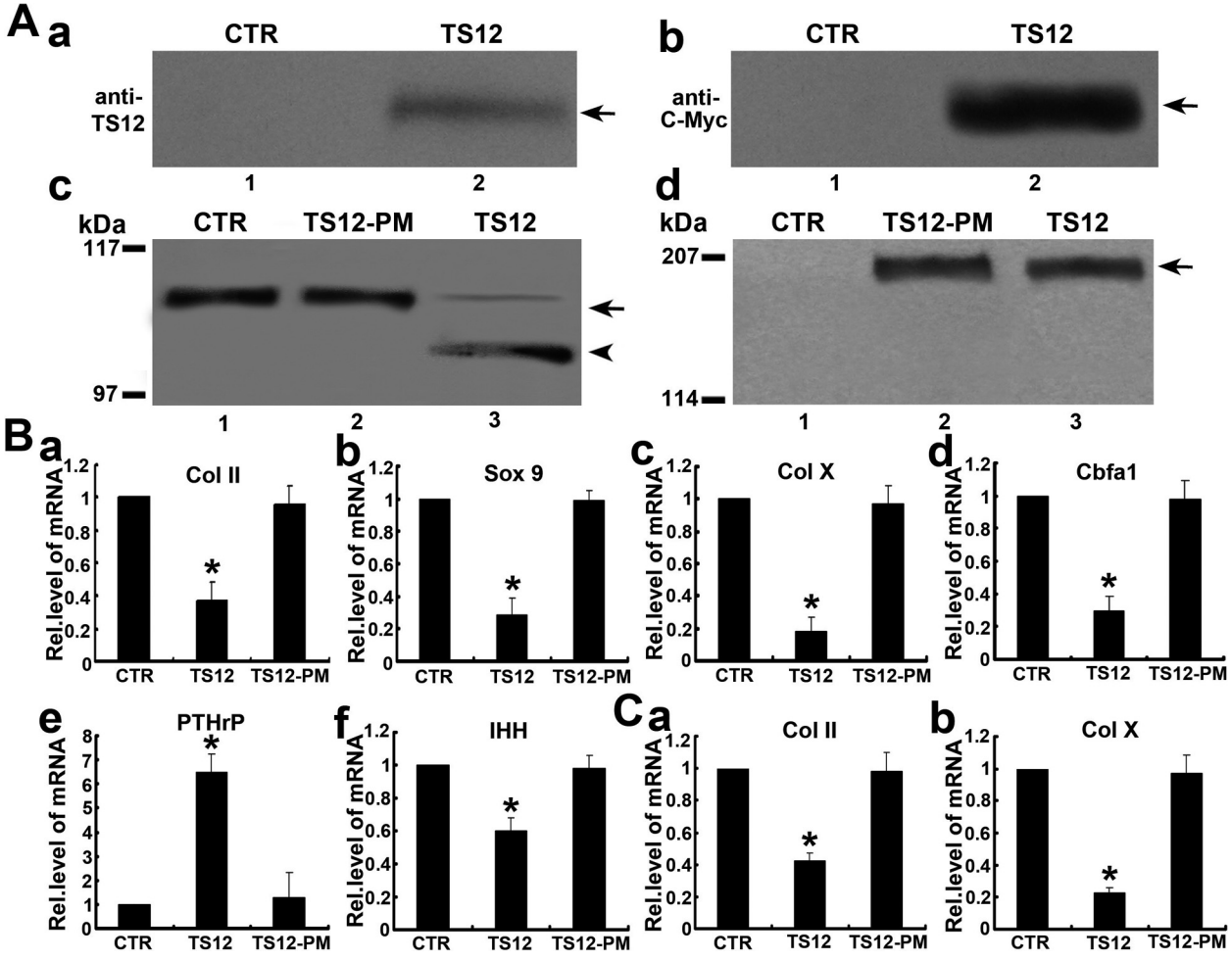

Figure 2. The proteolytic activity of ADAMTS-12 is required for its regulation of chondrogenesis. (*A*) a and b. Western blotting analysis of ADAMTS-12 with affinity-purified anti-ADAMTS-12 or anti-C-Myc antibody. Conditioned medium collected from HEK 293-EBNA cells stably transfected by either pcDNA3.1 (CTR) or C-Myc-tagged full length ADAMTS-12 (TS12) was examined by Western blotting analysis with anti-TS12 polyclonal antibody (a) or anti-C-Myc monoclonal antibodies (b). (*A*) c. *In vitro* digestion assay of COMP by ADAMTS-12 and its point mutant. COMP was incubated with the conditioned medium collected from HEK 293-EBNA cells stably transfected by pcDNA3.1 vector (CTR, lane 1), or expression constructs encoding wild-type ADAMTS-12 (TS12, lane 3), or ADAMTS-12 point mutant (TS12-PM, lane 2), as indicated. The cleaved products were subjected to reduced 8% SDS-PAGE and detected with anti-COMP polyclonal antibodies. The intact COMP and its digested fragments are indicated with arrow and arrowhead, respectively. (*A*) d. Expression of ADAMTS-12 and its point mutant (TS12-PM) in stably transfected C3H10T1/2 cells. Cell lysates prepared from C3H10T1/2 cells stably transfected with a control (CTR), ADAMTS-12 (TS12) and its point mutant (TS12-PM) expression plasmid were examined by Western blotting analysis with purified anti-TS12 polyclonal antibodies. (*B*) ADAMTS-12 inhibits chondrocyte differentiation, whereas the point mutant of ADAMTS-12 lacking enzymatic activity loses this inhibition. Micromass cultures of C3H10T1/2 cells stably transfected with a control (CTR), ADAMTS-12 (TS12) and its point mutant (TS12-PM) expression plasmid were incubated with 300 ng/ml of BMP-2 and the mRNA levels of Col II (a), Sox9 (b), Col X (c), Cbfa1 (d), PTHrP (e) and IHH (f) were determined using real-time PCR. The units are arbitrary, and the normalized values were calibrated against control (CTR), here given the value of 1. The asterisk (*) indicates significant increase or decrease from control (*, $P<0.05$). (*C*) Effect of ADAMTS-12 and its point mutant on chondrocyte differentiation using human mesenchymal stem cells (MSCs). MSCs pellets were cultured for 14 days in the presence of the conditioned medium obtained from HEK 293-EBNA cell lines stably transfected with either control vector (CTR), an expression plasmid encoding ADAMTS-12 (TS12) or its point mutant (TS12-PM), and Col II (a) and Col X (b) expression were analyzed by Real-time PCR.

chondrocyte differentiation. ADAMTS-12 contains a zinc catalytic domain followed by non-catalytic ancillary domains, including a disintegrin domain, a thrombospondin domain, a cysteine-rich domain, a spacer-1 domain, three thrombospondin motifs, a spacer-2 domain, and a C-terminal four thrombospondin motifs (Fig. 3A). Non-catalytic ancillary domains have been shown to play important roles in regulating the subcellular localizations and enzymatic activities of ADAMTS [55, 56]; we thus generated various C-terminal domain deletion mutants of ADAMTS-12 in order to dissect their effects on ADAMTS biochemical activities and regulation of chondrogenesis. Constructs encoding C-Myc-tagged either full-length of ADAMTS-12 or its C-terminal domain deletion mutants were transfected into HEK 293-EBNA cells

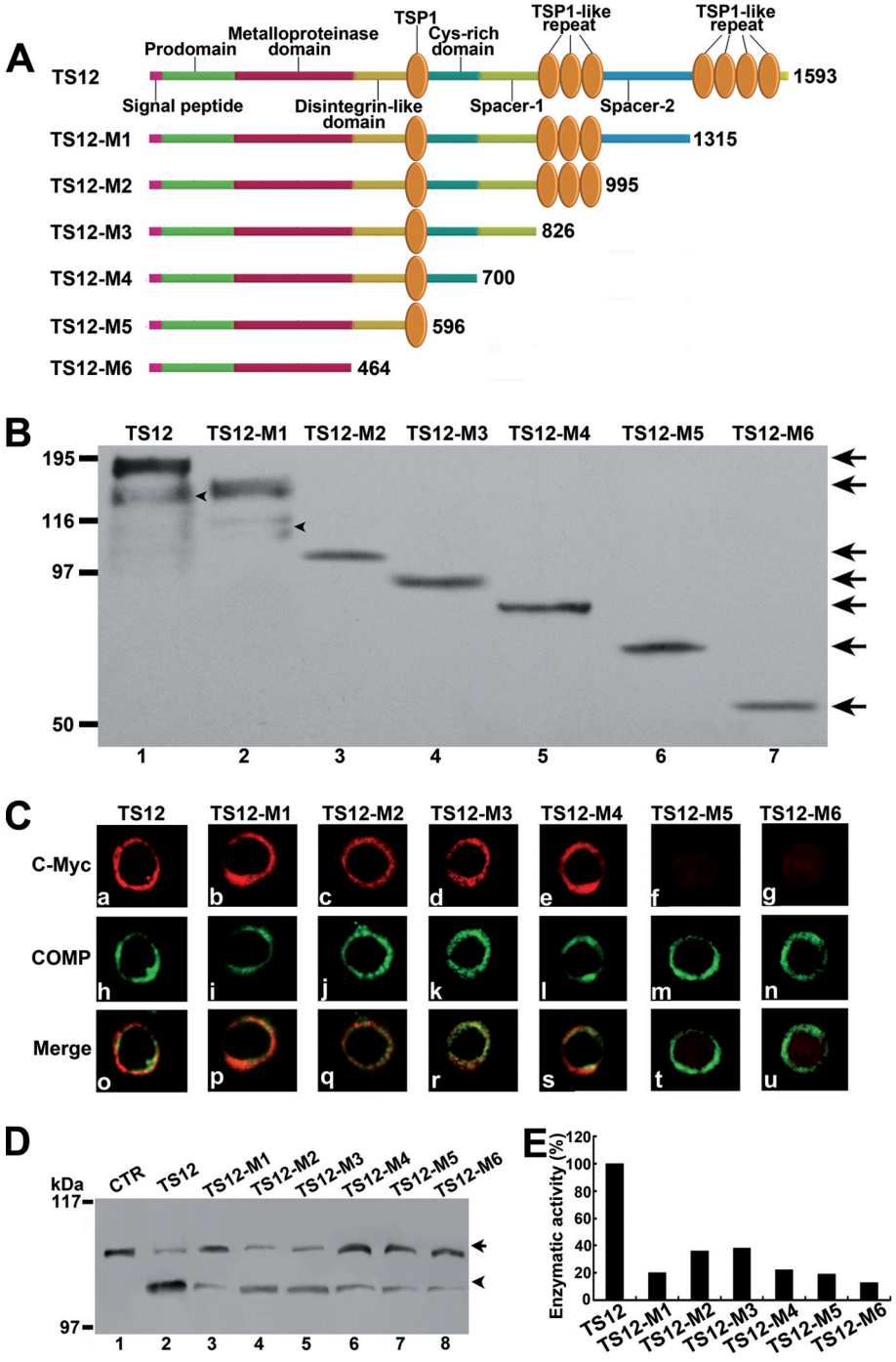

Figure 3. Effects of ADAMTS-12 functional domains on its cell surface location and enzymatic activity. (*A*) Schematic structure of the ADAMTS-12 and its C-terminal deletion mutants. Numbers refer to amino acid residues in ADAMTS-12. The functional domains are indicated. (*B*) Western blotting analysis of ADAMTS-12 and its C-terminal deletion mutants. The conditioned medium collected from the HEK 293-ENBA cells stably transfected by either ADAMTS-12 or its C-terminal deletion mutants were detected by Western blotting analysis with anti-C-Myc antibodies. The arrows indicate the full length of the ADAMTS-12 and its series of mutants, and the arrowheads indicate the fragments resulted from several ADAMTS-12 deletion mutants. (*C*) The subcellular localization of the ADAMTS-12 and its domain deletion mutants in RCS cells. RCS chondrocytes were transiently transfected with expression constructs encoding the ADAMTS-12 and its C-terminal deletion mutants. The expression of ADAMTS-12 or its C-terminal deletion mutants was visualized with anti-C-Myc antibodies. COMP was visualized by cell staining with anti-COMP polyclonal antibodies. Overlapping signals are indicated with "merge". (*D*) *In vitro* digestion assays of COMP mediated by ADAMTS-12 and its C-terminal domain deletion mutants. COMP was incubated with the conditioned medium collected from HEK 293-EBNA cells stably transfected by pcDNA3.1 (CTR), ADAMTS-12 (TS12) or its C-terminal domain deletion mutants (TS12-M1 to TS12-M6), as indicated. The cleaved products were subjected to reduced 8 % SDS-PAGE and detected with anti-COMP polyclonal antibodies. The intact COMP and its digested fragments are indicated with arrow and arrowhead, respectively. (*E*) Qualitative analysis of enzymatic activity of ADAMTS-12 and its C-terminal domain deletion mutants. The values were calibrated against ADAMTS-12 (TS12), here given the value of 100 %.

and the expressions of ADAMTS-12 and its mutants were examined by Western blotting analysis with anti-C-Myc antibody (Fig. 3B). The molecular masses of the recombinant enzymes estimated by SDS-PAGE were slightly higher than that predicted from their amino acid composition, probably due to the glycosylation of the protein that contains potential *N*-glycosylation sites and potential mucin-type *O*-glycosylation site [14]. Note that some deletion mutants exhibit the smaller fragments in addition to the predicted protein bands, including wildtype TS12 and mutant TS12-M1. These smaller fragments are likely the result of the intracellular processing of the protein.

Immunolocalization of TS12 in RCS chondrocytes with the anti-C-Myc monoclonal antibody indicated that wildtype TS12 was associated with extracellular matrix (ECM) and co-localized with its binding partner COMP (Fig. 3C, TS12). Removal of a C-terminal four thrombospondin motifs (TS12-M1) known to bind COMP, further removal of a spacer-2 domain (TS12-M2), further deletion of three thrombospondin motifs (TS12-M3) and further deletion of a spacer-1 domain (TS12-M4), did not change the cell surface localization of the protein; however, further deletion of the cysteine-rich domain (TS12-M5) released the enzyme from cell surface into the medium. These data indicate that the cysteine-rich domain of ADAMTS-12 is required for its binding to ECM and cell surface appearance.

In order to define the effects of functional domains of ADAMTS-12 on its enzymatic activities, the conditioned medium obtained from HEK 293-EBNA cells stably transfected with pcDNA3.1 (CTR), ADAMTS-12 (TS12) and its series of C-terminal deletion mutants, which contains comparable amounts of proteins (Fig. 3B), was incubated with recombinant COMP. In accordance with previous report of COMP cleavage by ADAMTS-12 [50], full-length ADAMTS-12 (TS12) efficiently digested COMP, with the release of an approximately 100 kDa fragment; deletion of C-terminal four thrombospondin motifs (TS12-M1) required for binding COMP substrate dramatically reduced enzymatic activity; surprisingly, further removal of a spacer-2 domain (TS12-M2) actually enhanced the COMP degradation, indicating that this domain is likely an inhibitory domain for its enzymatic activity; further deletion of three thrombospondin motifs (TS12-M3) did not affect enzymatic activity; but further deletion of a spacer-1 domain (TS12-M4) diminished the COMP cleavage and further deletion of cysteine-rich domain (TS12-M5) did not affect enzymatic activity. In addition, the catalytic domain of ADAMTS-12 alone (TS12-M6) poorly demonstrated COMP degradation activity. Collectively, this set of experiments indicates that non-catalytic ancillary domains of ADAMTS-12 are involved in regulation of its enzymatic activity. Qualitative analysis of enzymatic activity of ADAMTS-12 (TS12) and various mutants (TS12-M1 to TS12-M6) on the cleavage of COMP is summarized in Figure 3E.

We next examined whether the individual domains of ADAMTS-12 affect its activity in regulating chondrocyte differentiation. As shown in Figure 4A, ADAMTS-12-mediated regulation of chondrogenesis was completely lost with deletion of C-terminal four thrombospondin motifs (TS12-M1); further removal of a spacer-2 domain (TS12-M2), further deletion of three thrombospondin motifs (TS12-M3) slightly affected the expressions of late marker genes for chondrogenesis, including Col X (Fig. 4A, panel c) and PTHrP (Fig. 4A, panel d), whereas there was no effect on the expression of early markers of chondrocytes, assayed by Col II (Fig. 4A, panel a) and Sox9 (Fig. 4A, panel b); Similar to deletion of C-terminal four thrombospondin motifs (TS12-M1), further deletion of a spacer-1 domain (TS12-M4), the cysteine-rich domain domain (TS12-M5), and catalytic domain of ADAMTS-12 alone (TS12-M6) did not exhibit any regulatory activity of chondrogenesis. These findings were also observed with human MSCs pellet cultures (Fig. 4B, panels a and b). Taken together, C-terminal four thrombospondin motifs of ADAMTS-12, the substrate-binding domain, is important for its inhibition of chondrocyte differentiation.

PTHrP induces ADAMTS-12 expressions *in vitro*. Because ADAMTS-12 is highly expressed in proliferating and prehypertrophic chondrocytes (not shown) and negatively regulates chondrocyte differentiation (Fig. 1), we next used C3H10T1/2 cells and chondroprogenitor ATDC5 cells to examine whether ADAMTS-12 is the downstream target of PTHrP, a critical negative signal in controlling chondrocyte differentiation. Micromass cultures of both C3H10T1/2 and ATDC5 cells pretreated with 300 ng/ml of BMP-2 for one week were cultured with or without 10^{-7} M of PTHrP for various time points and the level of ADAMTS-12 mRNA was measured using real-time PCR (Fig. 5A). ADAMTS-12 mRNA was increased to 2.4-fold at day 1 and to 5.1-fold by day 3 in the PTHrP-untreated control C3H10T1/2 cells. PTHrP enhanced the level of ADAMTS-12 mRNA to 3.6-fold at day 1 and to 7.2-fold by day 3. In the case of ATDC5 cells, ADAMTS-12 mRNA was very slightly increased by day 1 but dramatically reduced by day 3 in the PTHrP-untreated cells; PTHrP significantly induced ADAMTS-12 to 2.2-fold by day 1. Although ADAMTS-12 mRNA nearly returned to baseline by

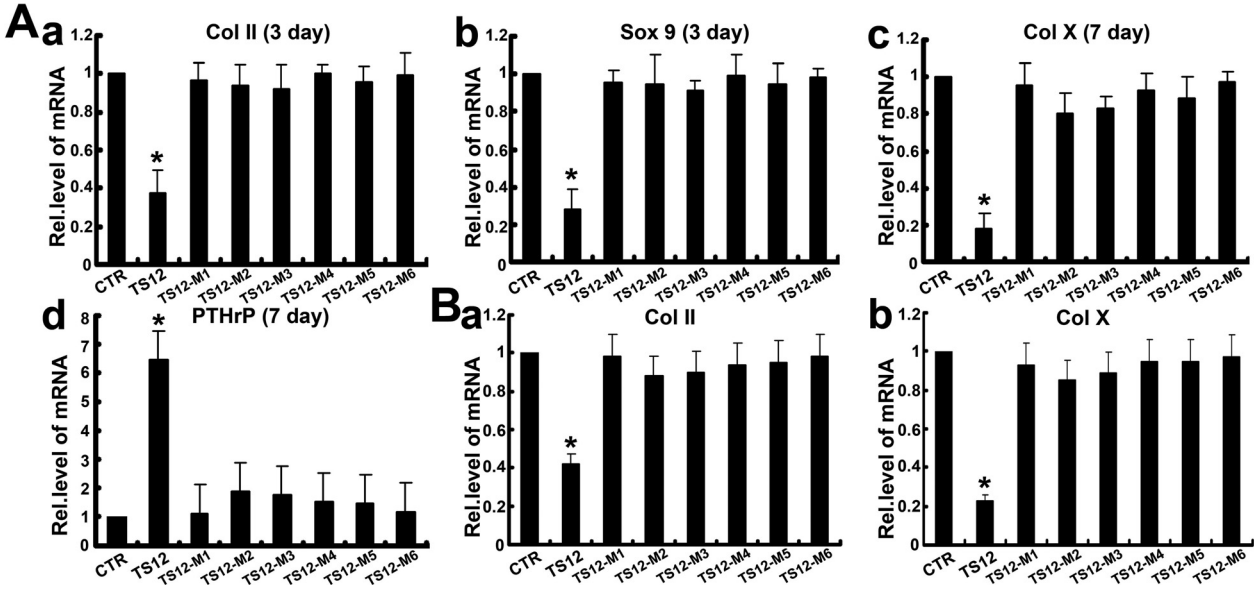

Figure 4. Substrate-binding domain of ADAMTS-12 is important for its regulation of chondrogenesis. (*A*) Effect of ADAMTS-12 and its C-terminal deletion mutants on chondrocyte differentiation of murine C3H10T1/2 cells. Micromass cultures of either control (CTR), wildtype ADAMTS-12 (TS12), or its C-terminal mutants (TS12-M1 to TS12-M2) C3H10T1/2 stable cell lines were stimulated with 300 ng/ml BMP-2 protein for 3 or 7 days, and the mRNA levels of Col II (a), Sox9 (b), Col X (c) and PTHrP (d) were determined using real-time PCR. The units are arbitrary, and the normalized values were calibrated against control (CTR), here given the value of 1. The asterisk (*) indicates significant increase or decrease from control (*, $P<0.05$). (*B*) Effect of ADAMTS-12 and its C-terminal deletion mutants on the expressions of Col II and Col X during chondrogenesis of human mesenchymal stem cells (MSCs). MSCs pellets were cultured for 14 days in the presence of the conditioned medium obtained from HEK 293-EBNA cell lines stably transfected with either control vector (CTR), an expression plasmid encoding ADAMTS-12 (TS12) or its C-terminal deletion mutants (TS12-M1 to TS12-M2), and Col II (a) and Col X (b) expression was analyzed by Real-time PCR, as described in (*A*).

day 3, it was still approximately 5-fold higher than the mRNA in the control at the same time point. Collectively, these findings demonstrate that ADAMTS-12 is a PTHrP-inducible gene in the late stage of chondrogenesis. Furthermore, induction of ADAMTS-12 by PTHrP was also visualized via immunofluencent cell staining (Fig. 5B).

ADAMTS-12 is dramatically reduced in PTHrP-/- embryonic growth plate chondrocytes. We next determined whether ADAMTS-12 expression depends on PTHrP signaling *in vivo* via performing immunohistochemistry with the sections of long bone from 18.5-day-old wildtype (PTHrP +/+) and PTHrP null (PTHrP-/-) mouse embryos. As expected, ADAMTS-12 demonstrates a prominent expression in the proliferating and prehypertrophic chondrocytes in the embryonic growth plate of control mice (Fig. 5C, left). Since PTHrP inhibits the hypertrophic chondrocyte differentiation, PTHrP (-/-) mice display reduced zones of proliferating chondrocytes, which indicates accelerated onset of hypertrophic differentiation. In the disordered growth plate of PTHrP knock-out embryonic growth plates, ADAMTS-12 is markedly reduced (Fig. 5C, right), indicating that *in vivo* expression of ADAMTS-12 is tightly controlled by PTHrP signaling.

ADAMTS-12 is a crucial downstream target for PTHrP action. We next investigated whether endogenous ADAMTS-12 is required for PTHrP-mediated inhibition of Col X expression during chondrocyte differentiation using the siRNA approach. Three siRNAs that specifically recognize three distant regions of murine ADAMTS-12 were selected (see Materials and methods for details). As shown in Figure 6A, these siRNAs led to approximately 65–75% inhibition of endogenous ADAMTS-12 in C3H10T1/2 cells. Micromass cultures of pSuper vector (CTR) and pSuper-TS12 (siTS12(1), siTS12(2) or siTS12(3)) stably transfected C3H10T1/2 cells were cultured in the presence of BMP-2 (300 ng/ml) for five days and then treated with 10^{-7} M PTHrP for additional three days. The level of Col X mRNA was determined by real-time PCR. As shown in Fig. 6B, PTHrP strongly inhibited Col X expression in the control cells, but this PTHrP-mediated inhibition was nearly lost when ADAMTS-12 was knocked down by siRNA (compare bar 2 and 3, 4 or 5). In addition, the inhibition was largely restored when ADAMTS-12 was re-expressed via co-transfecting an ADAMTS-12 expression plasmid (compare bar 2 and 6, 7 or 8). The requirement of ADAMTS-12 for PTHrP-mediated inhibition on Col X expression was verified using antibody blocking assay (Fig. 6C). Taken together,

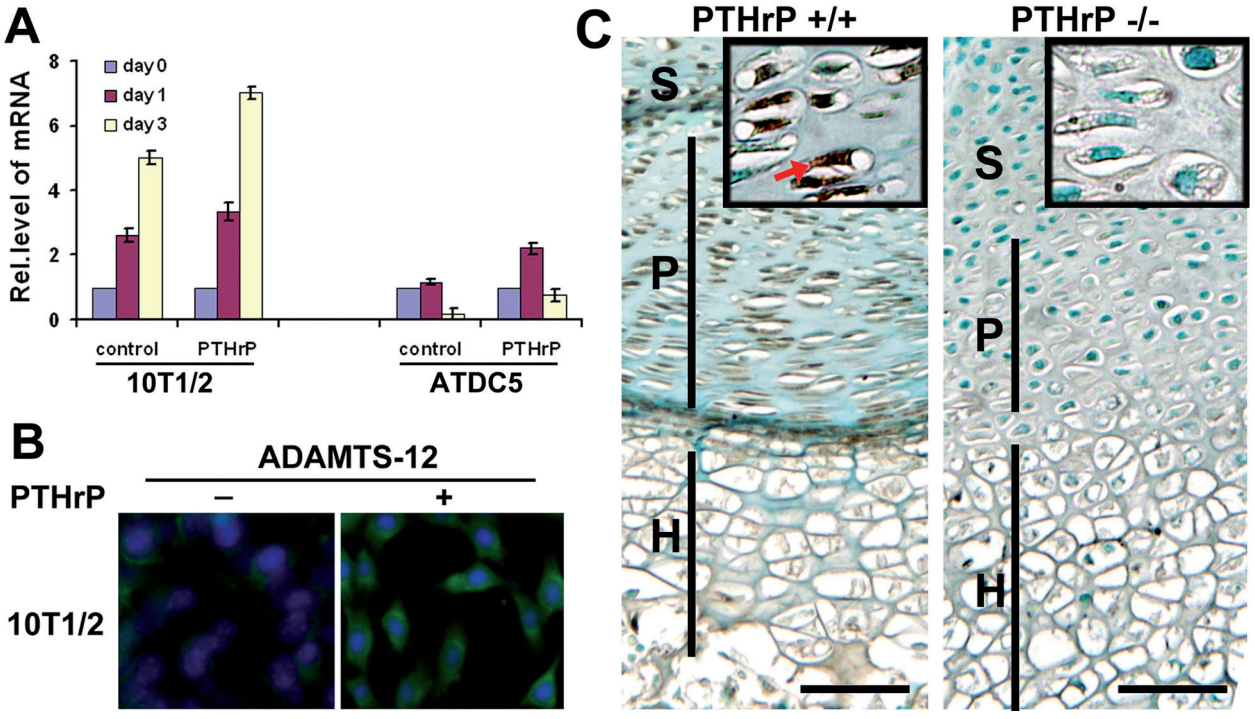

Figure 5. ADAMTS-12 expression depends on PTHrP signaling. (*A*) PTHrP induces the expression of ADAMTS-12 mRNA, assayed by Real-time PCR. C3H10T1/2 and ATDC5 cells pretreated with recombinant 300 ng/ml of BMP-2 for one week were cultured without (control) or with PTHrP (10^{-7} M) for various time periods, as indicated. The normalized values against GAPDH mRNA were calibrated against controls (day 0), given the value of 1. (*B*) PTHrP increase the level of ADAMTS-12 protein, assayed by Immunofluencent cell staining. Micromass cultures of C3H10T1/2 cells treated with or without PTHrP (10^{-7} M) for three days were stained with ADAMTS-7 antibody (green). The nuclei were stained with DAPI. (C) ADAMTS-12 is markedly reduced in the growth plate chondrocytes of PTHrP null embryos, revealed by Immunohistochemistry. Sections of long bone from 18.5-day-old PTHrP null (PTHrP-/-) and wildtype (PTHrP +/+) mouse embryos were stained with anti-ADAMTS-12 antibody (brown, indicated with arrows) and counterstained with Methyl green (green). S, resting chondrocytes; P, proliferating chondrocytes; H, hypertrophic chondrocytes. Bar = 100 μm.

these results indicated that PTHrP-mediated chondrocyte differentiation depends, at least in part, on ADAMTS-12 metalloproteinase.

Discussion

We previously reported the importance of ADAMTS-12 in cartilage degradation and arthritis[15, 50], the current study focused on the role of ADAMTS-12 in chondrogenesis as well as the molecular events involved. ADAMTS-12 protein was highly induced in the course of chondrogenesis *in vitro* and also demonstrated prominent expression in the growth plate chondrocytes *in vivo* (not shown). Real-time PCR for measurements of ADAMTS-12 mRNA showed that the level of ADAMTS-12 mRNA was relatively low until day 5, at day 7 it tripled and thereafter remained at high levels during the late differential stage. The discrepancy between protein and mRNA of ADAMTS-12 during chondrogenesis suggests that post-transcriptional regulation, such as translation, mRNA stability and protein degradation, might be also important in the control of ADAMTS-12 expression during chondrogenesis.

ADAMTS-12 appears to be a potent negative regulator of chondrocyte differentiation and its inhibition of chondrogenesis strictly depend on its enzymatic activities, since its point mutant lacking enzymatic activity completely lost this inhibition (Fig. 2B). ADAMTS-12 is composed of multiple functional domains, including a prodomain, a catalytic domain, a disintegrin domain, a thrombospondin motif, a cysteine-rich domain, a spacer-1 domain, three thrombospondin motifs, a spacer-2 domain, and a C-terminal four thrombospondin motifs (Fig. 3A). In addition to its C-terminal four thrombospondin motifs known to bind to COMP [50] and GEP (Zhang and Liu, unpublished data), the role of individual domains in regulating the biochemical properties of ADAMTS-12 remains unknown. To address this issue, we generated a series of C-terminal domain deletion mutants of ADAMTS-12 and found that the cysteine-rich domain is required for ADAMTS-12 binding to the cell surface and ECM in chondrocytes (Fig. 3C). The addition of heparin to the culture medium released the majority of TS12–1 from cell surface

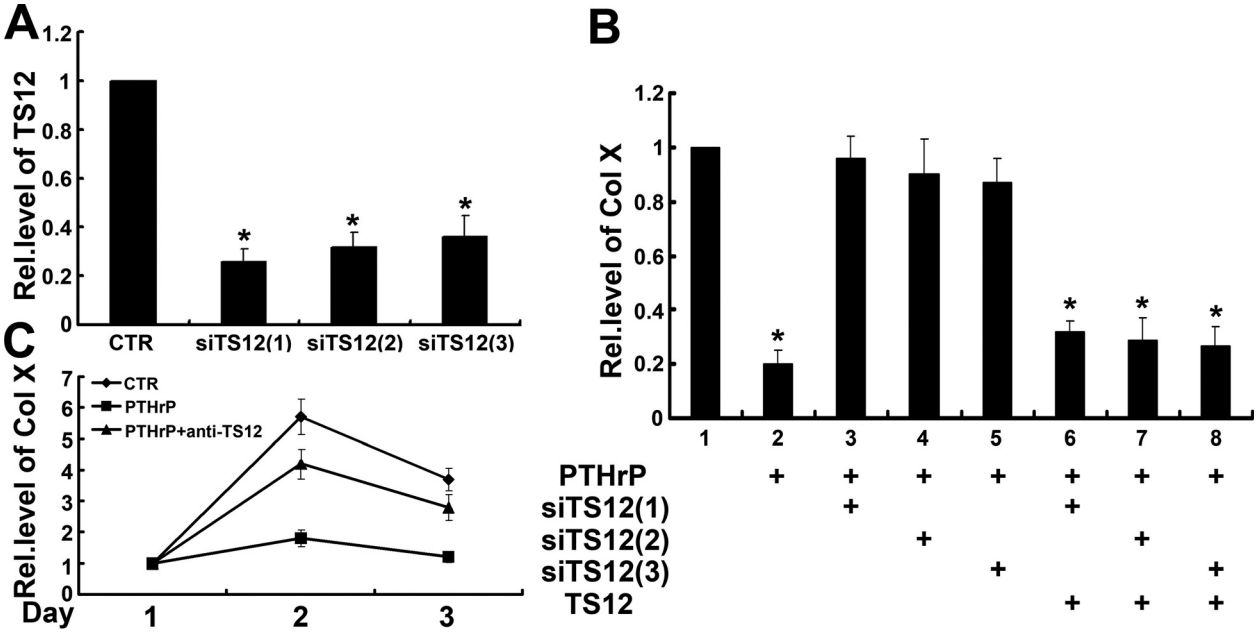

Figure 6. ADAMTS-12 is required for the PTHrP-mediated inhibition on chondrocyte differentiation. (*A*) ADAMTS-12 siRNAs efficiently represses the expression of endogenous ADAMTS-12 in C3H10T1/2 cells. C3H10T1/2 cells were transfected with either pSUPER vector (CTR) or one of three ADAMTS-12 siRNAs cloned into pSUPER, as indicated, and the level of ADAMTS-12 was measured using real-time PCR. (*B*) Knockdown of ADAMTS-12 almost abolished PTHrP-mediated inhibition of Col X expression, whereas its re-expression largely restored PTHrP action. Micromass cultures of C3H10T1/2 cells transfected with either pSuper, siTS12 (siTS12(1), siTS12(2), siTS12(3)), ADAMTS12 (TS12) expression plasmid, or various combinations, as indicated, were pretreated with BMP-2 and then cultured in the presence or absence of PTHrP (10^{-7} M) and the level of Col X was determined by real-time PCR. The units are arbitrary, and the normalized values were calibrated against control, here given the value of 1. (*C*) ADAMTS-12 antibody dramatically attenuated PTHrP-mediated inhibition of Col X expression. ATDC5 cells pretreated with BMP-2 for three days were cultured in the absence (CTR) or presence of PTHrP (10^{-7} M) without (PTHrP) or with anti-ADAMTS12 (1μg/ml) antibody (PTHrP + anti-TS12) for various time points, as indicated, and Col X was measured and analyzed as described in (*B*).

(not shown). This suggests that TS12 binds to negatively charged glycosaminoglycans on the cell surface and in the ECM. Interestingly, this cysteine-rich domain-dependent localization appears to be cell type-specific, since ADAMTS-12 and its deletion mutants are predominately localized in the cytoplasm of COS7 cells (not shown). In addition, the enzymatic activities of ADAMTS-12 are also precisely regulated by its non-catalytic domains, specially its substrate-capturing C-terminal four thrombospondin motifs and an inhibitory spacer-2 domain (Fig. 3D). Furthermore, C-terminal four thrombospondin motifs is also important for its mediation of chondrogenesis (Fig. 4). Multiple signaling pathways are involved in endochondral ossification in epiphyseal growth plate [57]. Among them, PTHrP and IHH coordinately regulate the rate of chondrocyte differentiation through a negative feedback loop [58–60]. Several lines of evidence indicate that PTHrP negatively regulated the endochondral bone formation. PTHrP prevents chondrocyte hypertrophy in the growth plate and maintains a pool of cells above the hypertrophic zone in a proliferative condition [59]. In embryonic mice lacking PTHrP, chondrocytes stop proliferating prematurely, with accelerated differentiation [61]. Mice lacking either PTHrP [62, 63] or the parathyroid hormone (PTH)/PTHrP receptor [64, 65] also have accelerated chondrocyte differentiation and impaired skeletal growth, exhibiting shortened zones of proliferative chondrocytes and premature hypertrophic differentiation, while animals overexpressing PTHrP in chondrocytes show delayed hypertrophic differentiation [66]. Chondrocyte-specific expression of a constitutively active PTH/PTHrP receptor also delays the conversion of proliferative to hypertrophic chondrocytes [67]. In mouse models both PTHrP and PTH/PTHrP receptor knockout mice display advanced endochondral bone formation [63–65]. Our studies demonstrate that 1) overexpressing ADAMTS-12 enhanced the expression of PTHrP whereas it inhibited IHH (Figs. 1, 2), 2) PTHrP induced ADAMTS-12 expression in the course of chondrogenesis *in vitro* (Figs. 5A, B), and 3) ADAMTS-12 expression strictly depends on PTHrP in the growth plate chondrocytes in mice (Fig. 5C), suggesting that there exists a positive feedback regulatory loop between ADAMTS-12 and PTHrP signaling in the course of chondrogenesis. In addition, ADAMTS-12 appears to be an important downstream target of PTHrP based on the facts that 1) repression of ADAMTS-12 via

siRNA approach or blocking ADAMTS-12 activity using its blocking antibodies almost abolished PTHrP-mediated inhibition of Col X expression, but reexpression of ADAMTS-12 largely restored PTHrP action (Fig. 6). Similar to PTHrP, which is known to stimulate chondrocyte proliferation [68], ADAMTS-12 was also found to increase chondrocyte proliferation and its stimulation on cell proliferation also depends on its substrate-binding C-terminal four thrombospondin motif (not shown). Since ADAMTS-12 negatively regulates chondrocyte differentiation from mesenchymal stem cells whereas it positively mediates chondrocyte proliferation, it remains to be determined how ADAMTS-12 affects cartilage development and endochondral bone formation *in vivo.* ADAMTS-12 was found to associate with COMP [50] and Granulin Epithelin Precusor (GEP, Zhang, et al, unpublished data), and both COMP and GEP have been shown to be important for chondrocyte differentiation and proliferation [34], suggesting that there may exist a function- and protein-interaction network among ADAMTS-12 metalloproteinase, GEP growth factor, and COMP extracellular matrix molecule in chondrocytes. In summary, this study extends our understanding of the actions of metalloproteinases in cartilage biology and provides an excellent example of how metalloproteinases acts as a direct effecter(s) of PTHrP signaling in the course of chondrogenesis.

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