

Matrilin-3 Inhibits Chondrocyte Hypertrophy as a Bone Morphogenetic Protein-2 Antagonist*

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Background: Matrilin-3-deficient mice exhibit increased chondrocyte hypertrophy and early osteoarthritis.

Results: Matrilin-3 binds bone morphogenetic factor-2 (BMP-2) and inhibits downstream BMP-2 signaling.

Conclusion: The biological function of matrilin-3 involves modulating BMP-2 pathway activity.

Significance: This is the first demonstration that matrilin-3 regulates chondrocyte hypertrophy as a BMP-2 antagonist in cartilage.

Increased chondrocyte hypertrophy is often associated with cartilage joint degeneration in human osteoarthritis patients. Matrilin-3 knock-out (*Matn3* KO) mice exhibit these features. However, the underlying mechanism is unknown. In this study, we sought a molecular explanation for increased chondrocyte hypertrophy in the mice prone to cartilage degeneration. We analyzed the effects of *Matn3* on chondrocyte hypertrophy and bone morphogenetic protein (Bmp) signaling by quantifying the hypertrophic marker collagen type X (*Col X*) gene expression and Smad1 activity in *Matn3* KO mice *in vivo* and in *Matn3*-overexpressing chondrocytes *in vitro*. The effect of *Matn3* and its specific domains on BMP activity were quantified by *Col X* promoter activity containing the Bmp-responsive element. Binding of MATN3 with BMP-2 was determined by immunoprecipitation, solid phase binding, and surface plasmon resonance assays. In *Matn3* KO mice, Smad1 activity was increased more in growth plate chondrocytes than in wild-type mice. Conversely, *Matn3* overexpression in hypertrophic chondrocytes led to inhibition of Bmp-2-stimulated, BMP-responsive element-dependent *Col X* expression and Smad1 activity. MATN3 bound BMP-2 in a dose-dependent manner. Multiple epidermal growth factor (EGF)-like domains clustered together by the coiled coil of *Matn3* is required for Smad1 inhibition. Hence, as a novel BMP-2-binding protein and antagonist in the cartilage extracellular matrix, MATN3 may have the inherent ability to inhibit premature chondrocyte hypertrophy by suppressing BMP-2/Smad1 activity.

Matrilins are a family of four noncollagenous extracellular matrix proteins that contain common structural motifs (1, 2).

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During development, matrilin (MATN)-1 and MATN3 are expressed in cartilage, whereas MATN2 and MATN4 have a broader tissue distribution (2, 3). Growth plate chondrocytes undergo well ordered and controlled phases of cell proliferation, maturation, and hypertrophy, which is characterized by synthesis of the hypertrophic marker, collagen type X (*Col X*).³ Hypertrophic cartilage then undergoes mineralization and ultimately is replaced by bone. Interestingly, *Matn3* mRNA is synthesized by epiphyseal and columnar proliferating chondrocytes in mouse femur and tibia but not by hypertrophic chondrocytes (1, 4). However, MATN3 protein is distributed in the hypertrophic cartilage matrix (1). MATN3 exists at low levels in adult articular cartilage, but its expression is induced during osteoarthritis (OA) (5). This pattern of expression suggests a role for MATN3 in the regulation of chondrocyte proliferation and differentiation during endochondral ossification as well as OA pathogenesis, which can be considered an abnormal recapitulation of endochondral ossification (6).

MATN3 is the least complex member of the matrilin family and consists of one von Willebrand factor A (vWFA) domain, four epidermal growth factor (EGF)-like domains, and a C-terminal coiled-coil domain, which mediates oligomerization (3). Like other matrilins, MATN3 plays an important structural role in cartilage and has been shown to bind to cartilage ECM proteins, including collagen types II and IX (7). In addition, MATN3 has recently been shown to play an important regulatory role in the cartilage ECM by promoting chondrogenesis through an interleukin-1 receptor antagonist-dependent mechanism (8, 9). However, the role of MATN3 in regulating chondrocyte differentiation and hypertrophy is unclear.

Mutations in MATN3 result in a variety of skeletal diseases, including multiple epiphyseal dysplasia (characterized by abnormal ossification in the growth plate and early onset OA) (10–12), spondylo-epi-metaphyseal dysplasia (SEMD) (chondrodystrophy occurring during skeletal development in childhood) (11), and hand OA occurring in adults during aging (12).

³ The abbreviations used are: *Col X*, collagen type X; BMP, bone morphogenetic protein; OA, osteoarthritis; vWFA, von Willebrand factor A; BRE, BMP-responsive element; ECM, extracellular matrix; SEMD, spondylo-epi-metaphyseal dysplasia.

Although the MATN3 mutations of multiple epiphyseal dysplasia occur in the vWFA domain, the mutations accounting for hand OA and SEMD reside in the first EGF-like domain (11–13). We have previously shown that *Matn3* knock-out (KO) mice exhibit premature chondrocyte hypertrophy during embryonic development, increased bone mineral density in adulthood, and accelerated joint degeneration during aging (14). These data suggest that MATN3 is an important regulator of chondrocyte proliferation, differentiation, and bone mineralization in the cartilage ECM (35). Elucidating the currently unknown underlying mechanism by which MATN3 modulates cartilage homeostasis and development (as well as bone development) has important implications for better understanding the pathophysiology of OA and other MATN3-associated diseases.

The bone morphogenetic protein (BMP) signaling pathway is known to regulate development and regeneration of bone and cartilage. Several *in vitro* and *in vivo* studies have shown that BMPs promote chondrocyte proliferation and the expansion of all differentiation zones in the developing skeleton (15–17). BMPs were originally identified as molecules that induce ectopic endochondral ossification (18) and are part of the TGF- β superfamily of secreted signaling molecules (19, 20). BMP signaling is essential to early limb development by inducing formation of mesenchymal condensations. Later in life, most long bone expression of BMPs (BMP-2, -3, -4, -5, and -7) is in the perichondrium; BMP-2 and -6 are found in hypertrophic chondrocytes, and BMP-7 is found in proliferative chondrocytes (21, 22). BMPs elicit their function by binding to specific cell surface receptors with serine-threonine kinase activity and then inducing phosphorylation of receptor-regulated Smads (Smad1, -5, and -8) (23). These Smads then complex with the common Smad4 and translocate into the nucleus, where they bind to Smad elements within the promoter regions of target genes. For example, BMP-2 promotes chondrocyte hypertrophy and Col X mRNA expression via Smad signaling and downstream interaction with the BMP-responsive element (BRE) in the *Col X* gene promoter (24, 25).

BMP signaling is controlled by extracellular BMP antagonists (*i.e.* noggin and follistatin) and intracellular molecules such as Smad6, Tob, and Smad ubiquitin regulatory factor 1 (SMURF1) (26). It has been demonstrated that the bioavailability of BMP for interaction with its receptor is modulated by BMP binding to ECM proteins such as heparan sulfate and collagen type IV (27, 28). In this study, we hypothesized that MATN3 modulates chondrocyte hypertrophy by inhibiting BMP-2 signaling via the Smad pathway. Furthermore, we tested whether MATN3 can bind BMP-2 extracellularly thereby preventing BMP-2 from interacting with its receptor.

MATERIALS AND METHODS

Generation of MATN3 KO Mice—*Matn3* KO mice were generated as described previously (14, 29). *Matn3*-targeted embryonic stem cells were injected into C57BL/6J blastocysts for germ line transmission. *Matn3* KO mice were identified by Southern blot analysis and maintained on a mixed 129/C57 background and housed in accordance with Sanger Institute home office regulations (United Kingdom).

Immunohistochemistry—Hind limbs were dissected from *Matn3* null and wild-type embryos (E16.5). Limbs were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, dehydrated in ethanol, cleared in xylene, and embedded in paraffin, and 6- μ m sections were cut. Tissue sections were deparaffinized, rehydrated, and digested with bovine testicular hyaluronidase (100 units/ml in PBS Sigma) for 30 min at 37 °C. Serum block was applied for 30 min at room temperature before incubation of the primary antibody of phosphorylated Smad1 (p-Smad1) (Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C. For detection, biotinylated secondary antibody and horseradish peroxidase (HRP)-streptavidin complex (Zymed Laboratories Inc.) were used. HRP substrate was used for visualization, and sections were then counterstained with Mayer's hematoxylin. Sections were then dehydrated and mounted. Photography was performed with a Nikon microscope.

Cell Culture—Primary chicken chondrocytes were cultured as described previously (30). Hypertrophic chondrocytes were isolated from the cephalic part of embryonic (day 17) chick sternal cartilage and then seeded in 6-well plates at a density of 1.5×10^6 cells/well. Each well contained Ham's F-12 medium with 10% fetal bovine serum (FBS). MCT is a chondrogenic murine cell line (derived from newborn mouse rib chondrocytes) that has been immortalized via temperature-sensitive large T antigen. MCT cells proliferate at 33 °C but will stop proliferating and begin differentiating at 37 °C. In our experiments, MCT cells were first allowed to proliferate at 33 °C until 80% confluent and then plated in 12-well plates at 2.0×10^4 cells/well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. After transient transfection, cells were incubated at 37 °C. Primary mouse rib chondrocytes were isolated from rib cage of newborn wild-type mice. Rib cages were digested using 3.0 mg/ml collagenase D (Roche Applied Science) for 2 h followed by a second digest for 6 h at 37 °C. Cells were strained to remove clumps, washed three times with DMEM, and plated in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen) and maintained at 37 °C in the presence of 5% CO₂. For treatment experiments, BMP-2 (R&D Systems, Minneapolis, MN) was added to the medium for the designated concentrations and times indicated in the figures prior to cell harvesting for luciferase assay or RNA extraction.

Retrovirus-mediated Cell Infection—The coding sequence of the mouse MATN3 cDNA was cloned into replication competent retroviral vectors RCASBP(A). Chicken embryo fibroblasts were transfected with the retroviral plasmid DNA. Viral supernatants were collected, and aliquots were rapidly frozen at -80 °C. After primary chicken chondrocytes were attached to the plates, they were incubated for 48 h with viral supernatant mixed in a 1:9 ratio with plating medium. RCASBP(A) retrovirus lacking the MATN3 cDNA insert was used as a control.

Transient Transfection and Reporter Assay—Transient transfection experiments were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. For *Matn3* overexpression, the *Matn3* transgene was overexpressed by transfection using pcDNA3.1-*Matn3*, which contained a C-terminal V5 tag for immunodetection. For *Matn3*

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TABLE 1

Mouse (m) and chicken (c) oligonucleotide primer sequences used for RT-PCR

| Gene | Sense | Antisense |
|--------------------|-------------------------------|---------------------------------|
| (m) <i>Col X</i> | 5'-CCAGGTGTCCAGGATTCCTCC-3' | 5'-CAAGCGGCATCCCAGAAAGC-3' |
| (m) <i>Matn3</i> | 5'-TCCCGCATCATCGACACTCTG-3' | 5'-CCCAGCCTCCACAGTGAAG-3' |
| (c) <i>Col X</i> | 5'-AGTGCTGTTCATTGTCTCATGGA-3' | 5'-TCAGAGGAATAGAGACCATTGGATT-3' |
| (m, c) <i>18 S</i> | 5'-CGGCTACCACATCCAAGGAA-3' | 5'-GCTGGAATTACCGCGGCT-3' |

knockdown experiments, cells were transiently transfected with 40 pmol of a murine *Matn3* siRNA (Dharmacon, Lafayette, CO). A scrambled siRNA was used as a negative control. Protein and mRNA were collected 48 h after transfection for subsequent analysis. For reporter assay transfection, chondrocytes were seeded in 12-well plates (2.0×10^4 MCT cells/well; 3.0×10^5 primary chicken chondrocytes/well). The BMP-responsive chicken *Col X* promoter/luciferase constructs, prepared as described previously (12), and the BRE-luc reporter (generously provided by Dr. Peter ten Dijke) (34) were transfected into chondrocytes 1 day after plating in monolayer culture. Cotransfection with pRL-null *Renilla* luciferase control vector or pRL-SV40 Firefly luciferase control vector was used for controlling transfection efficiency. Sixteen hours before lysis, cells transfected with the BRE-luc reporter were exposed to BMP-2 (50 ng/ml). Luciferase activity was measured with the Dual-Luciferase[®] assay system (Promega, Fitchburg, WI). Activity assays for the *Renilla* luciferase/Firefly luciferase or Firefly luciferase/*Renilla* luciferase were performed sequentially with a manual TD-20/20 luminometer. Statistical analysis was performed with an unpaired *t* test; values of *p* < 0.05 were considered significant.

RNA Extraction and Quantitative Real Time (RT)-PCR—Total RNA was extracted using the RNAqueous kit (Ambion, Austin, TX) following the manufacturer's recommendations. 1 μ g of total RNA was used for each reverse transcriptase reaction in a reaction buffer containing 1 μ l of oligo(dT) and 1 μ l of 10 mM dNTP mix (Bio-Rad). RT-PCR amplification was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) with the DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research, Waltham, MA). Primers used in amplification of target genes' mRNA are shown in Table 1. Target gene mRNA levels were normalized to the level of the housekeeping gene, ribosomal RNA 18 S. Calculation of mRNA values was performed as described previously (13). The housekeeping gene 18 S was amplified at the same time and used as an internal control. The cycle threshold (C_t) values for 18 S and target genes were measured and calculated by computer software (PerkinElmer Life Sciences). Relative transcript levels were calculated as $x = 2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t = \Delta E - \Delta C$, and $\Delta E = C_{t, \text{exp}} - C_{t, 18 S}$; $\Delta C = C_{t, \text{ctl}} - C_{t, 18 S}$.

Western Blot and Immunoprecipitation—MCT cells or mouse chondrocytes were transfected with pcDNA3.1-V5-MATN3 by the method described above. For SDS-PAGE/Western blotting, conditioned media were collected at the designated time points and concentrated with Microcon columns (Millipore, Bedford, MA). Cells transfected with empty vector were used as a negative control. Cell lysates and protein pellets from the concentrated media were resuspended in 4% SDS, 0.2 M Tris, pH 6.8, and 40% glycerol, and the protein was deter-

mined with the Bradford protein assay (Bio-Rad). Aliquots of 30 μ g of protein from each sample were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose. Anti-type X collagen (Abcam, Cambridge, MA), P-Smad1, Smad1, and actin antibodies (Cell Signaling Technology, Beverly, MA) were used in Western blotting (31). Concentrated supernatants were resuspended with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, and 1% Triton X-100) in the presence of protease and phosphatase inhibitors (Roche Applied Science). The samples were pre-cleared using 40 μ l of protein A/G-Sepharose beads (Pierce) and 10 μ l of mouse normal IgG for 1 h at 4 °C. The supernatant was then incubated with 10 μ l of monoclonal anti-V5 antibody (Invitrogen) and 40 μ l of protein A/G-Sepharose beads overnight at 4 °C. Following incubation, the supernatant and beads were collected. The beads were then washed three times with lysis buffer. The bound proteins were released by boiling in Laemmli loading buffer for 5 min. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and blotted with anti-BMP-2 antibody (Sigma) or V5 antibody. The supernatant after immunoprecipitation with V5 antibody was further concentrated and resuspended with 2 \times protein loading buffer and boiled followed by Western blot with BMP2 antibody V5 antibody. Membranes were washed and probed with IRDye-conjugated secondary antibody, and the proteins were visualized using fluorescence detection on the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Solid Phase Binding Assay—Ninety six-well plates were coated with 20 nM recombinant human MATN3 (R&D Systems, Minneapolis, MN) or Noggin (Humanzyme, Chicago, IL) in Tris-buffered saline (TBS) (0.9% NaCl, 20 mM Tris, pH 7.4) overnight at 4 °C. Nonspecific binding sites were blocked with TBS containing 3% (w/v) bovine serum albumin for 2 h. Following four rinses in wash buffer (1 \times TBST), recombinant human BMP-2 was added for 1 h at a range of concentrations (0.78 to 3.125 nM). Recombinant BMP-2 was added to wells that were coated with recombinant MATN3, Noggin, or negative controls containing no coated protein (experiments were conducted in triplicates). Wells were then washed and incubated with 1 μ g/ml mouse monoclonal anti-BMP-2 primary antibody for 2 h. Wells were then washed and incubated with goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:5000) (Bio-Rad) for 1 h. Wells were then washed, and color was developed with SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and absorbance was determined using a plate reader (Packard Fusion Universal Microplate Reader) at 450 nm. Nonspecific binding of BMP-2 (*i.e.* binding in wells without coated protein) was subtracted from corresponding absorbance readings.

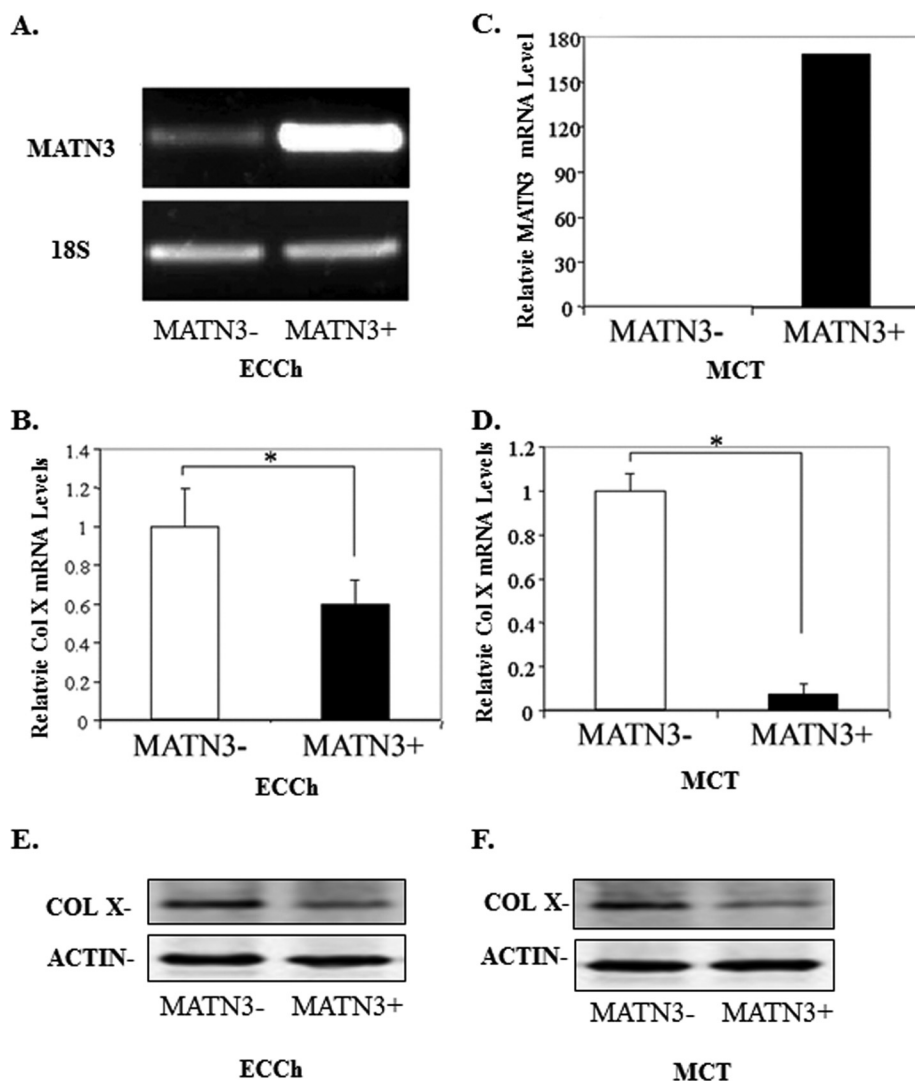


FIGURE 1. *Matn3* overexpression in hypertrophic chondrocytes inhibits *Col X* mRNA expression. RT-PCR demonstrates *Matn3* overexpression in embryonic chicken chondrocytes (ECCh) infected with a viral construct containing *Matn3* cDNA (A) or in MCT cells transfected with MATN3 cDNA (C) (MATN3+) compared with the empty vector (MATN3-). MATN3 overexpression was associated with reduced expression of *Col X* mRNA in hypertrophic chicken chondrocytes (B) and MCT cells (D). (Values are the mean \pm S.D. * indicates significant difference ($p < 0.05$) compared with control values. Data from one representative experiment are presented ($n = 3$, $p < 0.05$.) Western blot analysis of *Col X* protein levels in embryonic chicken chondrocytes (E) and in MCT (F) is shown. An equal amount of protein was loaded in each lane. Data from one representative experiment are presented ($n = 3$).

Surface Plasmon Resonance—MATN3 (30 μ g/ml in 10 mM sodium acetate, pH 4.4) was covalently immobilized on a CM5 sensor chip at 10 μ l/min for 2 min via amine coupling, according to the manufacturer's instructions (final response units = 7500). Binding studies were performed in HBS-P₊ running buffer. BMP-2 (6.25–100 nM; diluted in running buffer) was injected over immobilized MATN3 at 20 μ l/min for 3 min. Chip surface was regenerated with 10 mM glycine-HCl, pH 2.5, at 10 μ l/min for 30 s. Studies were conducted using BIAcore T100 machine and software. All assay reagents used were manufactured by BIAcore (BIAcore/GE Healthcare).

RESULTS

***Matn3* Inhibits *Col X* Expression in Hypertrophic Chondrocytes**—To determine whether MATN3 inhibits chondrocyte hypertrophy, *Matn3* was overexpressed in hypertrophic chondrocytes using two *in vitro* models. First, pcDNA3.1

containing the full-length coding region of *Matn3* (MATN3+) or the empty vector (MATN3-) were transfected into MCT chondrogenic cells proliferating at 33 $^{\circ}$ C (temperature at which large T antigen is activated). Following transfection, temperature was shifted to 37 $^{\circ}$ C, which inactivated the large T antigen and caused MCT cells to stop proliferating and begin expressing markers of hypertrophic chondrocytes, such as *Col X*. Second, embryonic chicken hypertrophic chondrocytes were infected by virus containing *Matn3* cDNA. *Matn3* overexpression was confirmed by RT-PCR 72 h after infection (Fig. 1A) or transfection (Fig. 1C) and was associated with significantly reduced expression of *Col X* mRNA expression in hypertrophic chicken chondrocytes (Fig. 1B) and MCT cells (Fig. 1D). Analysis of *Col X* protein levels using Western blot also confirmed the reduction of *Col X* expression in *Matn3*-overexpressing embryonic chicken chondrocytes (Fig. 1E) and MCT cells (Fig. 1F).

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MATN3 Inhibition of Col X Expression Is Dependent on BMP-2—We next studied whether *Matn3* inhibition of *Col X* mRNA expression in hypertrophic chondrocytes was dependent on BMP-2 signaling. The activity of chicken *Col X* promoter constructs with or without BRE was quantified by luciferase assay 2 days after cotransfection into chondrocytes with either pcDNA3.1 *Matn3* (MATN+) or empty vector (MATN-). Three *Col X* promoter constructs were utilized as follows: full-length (Luc-1), deletion of 5' distal region of *Col X* promoter with intact BRE (Luc-2), and deletion of BRE (Luc-3) (Fig. 2A). *Matn3* overexpression in MCT cells inhibited the *Col X* promoter activity of Luc-1 and Luc-2 (Fig. 2B). However, deletion of the BRE within the *Col X* promoter in Luc-3 decreased baseline *Col X* promoter activity and eliminated the inhibitory effect of *Matn3* (Fig. 2B). Furthermore, RT-PCR showed that *Matn3* overexpression inhibited *Col X* mRNA expression induced by BMP-2 (50 and 100 ng/ml) in chicken chondrocytes (Fig. 2C). *Matn3* continued to inhibit *Col X* mRNA expression induced by 250 ng/ml BMP-2, but the observed difference was not statistically significant. However, the highest dose of BMP-2 (500 ng/ml) rescued down-regulated *Col X* mRNA expression, and there was no difference between expression in the experimental and control groups (Fig. 2C). Western blot analysis was used to measure p-Smad1 and total Smad1 levels in *Matn3*-overexpressing chondrocytes. As expected, treatment with 50–500 ng/ml BMP-2 activated the phosphorylation of Smad1. Overexpression of *Matn3* inhibited Smad1 activation but failed to do so in the presence of 500 ng/ml BMP-2 (Fig. 2D).

Matn3 Decreases p-Smad1 Expression—To further demonstrate the role of BMP-2 signaling in mediating *Matn3*'s inhibition of *Col X* mRNA expression, we examined p-Smad1 expression by Western blot and in paraffin sections of WT and *Matn3* KO mice by immunohistochemistry. Western blot was performed 2 days after transfection and demonstrated reduced levels of p-Smad1 in *Matn3*-overexpressing chondrocytes (MATN3+) compared with empty vector control (MATN3-) cells (Fig. 3A). To confirm the effect of *Matn3* on Smad1 phosphorylation in the mouse, we overexpressed the *Matn3* gene in primary mouse chondrocytes. As shown in Fig. 3B, the p-Smad1 level was increased when treated with BMP. However, *Matn3* overexpression resulted in the inhibition of Smad1 phosphorylation relative to control cells (empty vector). BMP-2 treatment activated the p-Smad1 level to a lesser degree in *Matn3*-overexpressing chondrocytes compared with the control cells (Fig. 3B). Furthermore, when the *Matn3* gene was knocked down using a siRNA in mouse chondrocytes, Smad1 phosphorylation was increased relative to chondrocytes that were transfected with a scrambled control siRNA (Fig. 3C). Gene expression analysis was conducted via RT-PCR to measure the efficiency of *Matn3* gene knockdown (Fig. 3D). We further performed immunohistochemistry analysis to detect p-Smad1 expression through the entirety of the growth plate in both *Matn3* KO and WT mice at embryonic day 18.5 (Fig. 3E). p-Smad1 expression was up-regulated in both epiphyseal and columnar, but not hypertrophic, cartilage of *Matn3* KO compared with WT mice. No reactivity was detected when sections were incubated without the primary antibody.

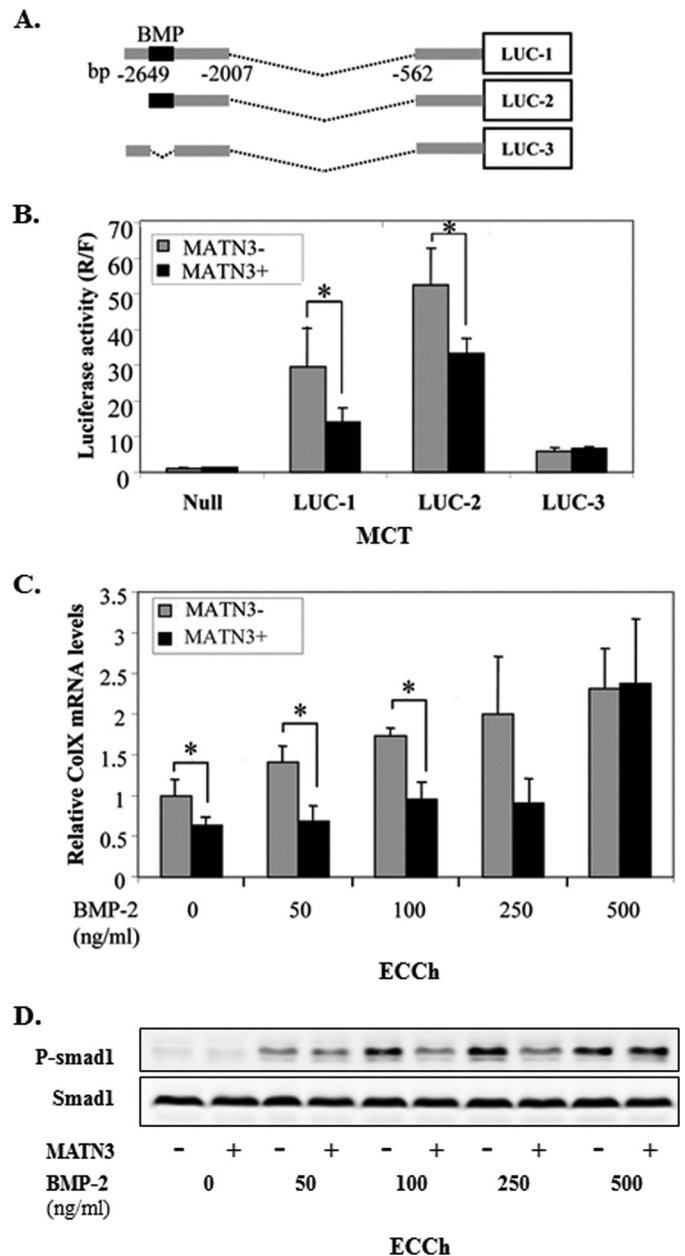


FIGURE 2. MATN3 inhibition of *Col X* expression is dependent on BMP-2 signaling. A, schematic representations of chicken *Col X* promoter constructs are as follows: full-length (LUC-1), deletion of 5' distal region of *Col X* promoter with intact BMP-responsive element (BRE) (LUC-2), and deletion of BRE (LUC-3). B, promoter luciferase activity quantified 2 days after construct cotransfection into MCT cells with either *Matn3* pcDNA3.1 (MATN+) or empty vector (MATN-). *Matn3* overexpression inhibited the *Col X* promoter activity of Luc-1 and Luc-2; however, the BRE in Luc-3 decreased baseline activity and eliminated the inhibitory effect of *Matn3*. C, *Col X* mRNA expression in chicken chondrocytes infected with MATN3 (MATN3+) or empty vector (MATN3-) was quantified by RT-PCR. BMP-2 induced *Col X* mRNA expression in dose-dependent manner. MATN3 overexpression inhibited *Col X* mRNA expression induced by 50 and 100 ng/ml BMP-2. Highest dose of BMP-2 (500 ng/ml) overcame MATN3-mediated inhibition of *Col X* mRNA expression. (Values are the mean ± S.D. of three cultures. * indicates significant difference ($p < 0.05$) from control values. Data from one representative experiment are presented.) D, Western blot analysis of phospho-Smad1 levels in response to MATN3 and BMP-2. Equal amount of protein was loaded in each lane. Data from one representative experiment are presented ($n = 3$). ECCh, embryonic chicken chondrocytes.

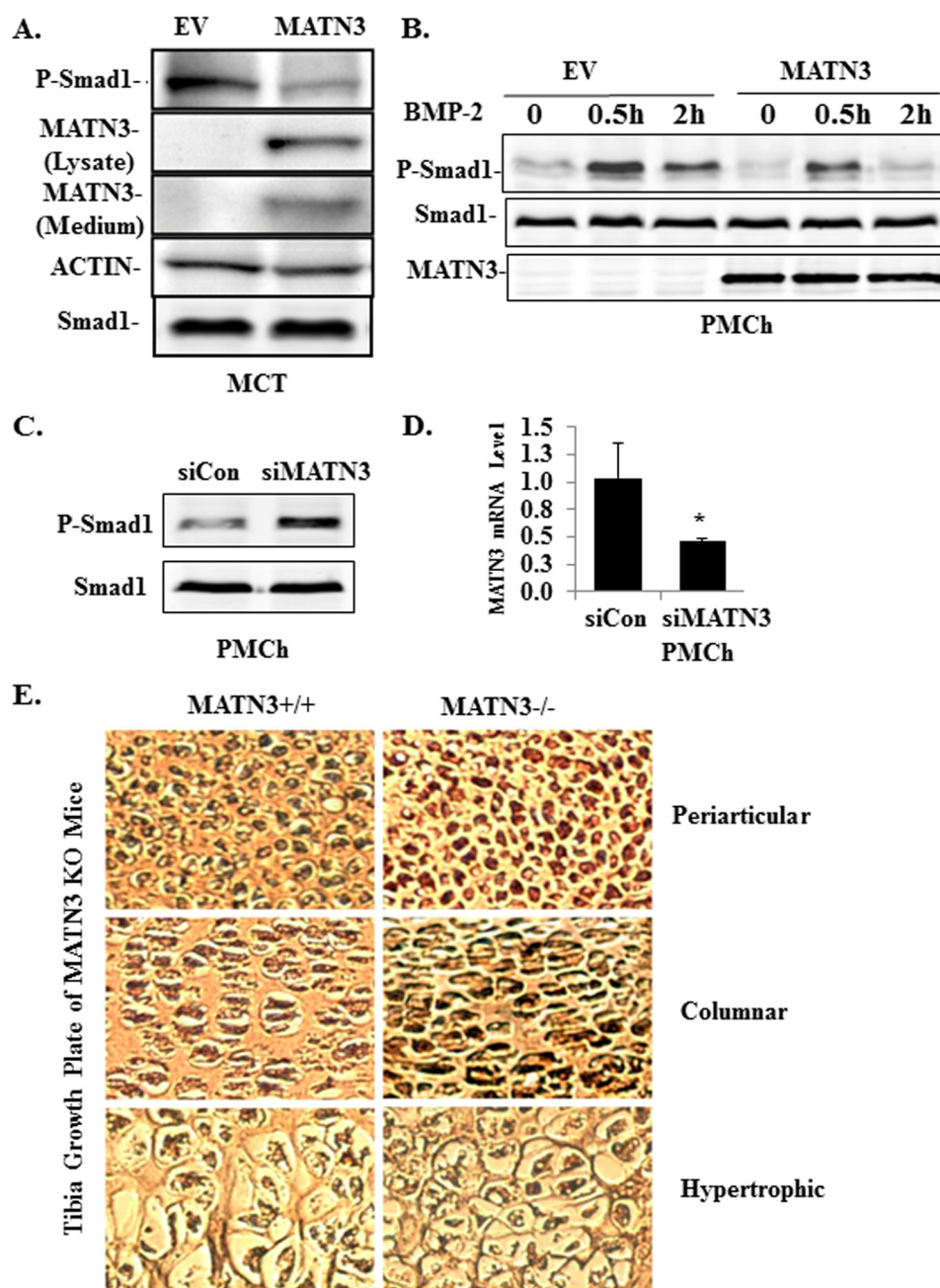


FIGURE 3. MATN3 decreases p-Smad1 expression. *A*, MCT cells were transfected with either *Matn3* pcDNA3.1 (*MATN*⁺) or empty vector (*MATN*⁻). MATN3 overexpression was associated with decreased p-Smad1 protein as analyzed by Western blot. Equal amounts of protein were loaded into lanes, and β -actin serves as a loading control. *EV*, empty vector. *B*, Western blot analysis of p-Smad1 levels in primary mouse chondrocytes transfected with either an empty vector construct or a pcDNA3.1-V5-*Matn3* construct and treated with 100 mg/ml recombinant BMP-2 for 0.5 and 2 h. Total Smad1 protein levels were used as a loading control. Data from one representative experiment are presented ($n = 3$). *C*, Western blot analysis demonstrates that primary mouse chondrocytes transfected with a *Matn3* siRNA exhibit increased phosphorylation of Smad1 protein relative to cells transfected with a scrambled siRNA control. Data from one representative experiment are presented ($n = 3$). *D*, real time RT-PCR data confirms that the *Matn3* siRNA successfully reduced *Matn3* mRNA levels in primary mouse chondrocytes (PMCh). * indicates significant difference ($p < 0.05$) from empty vector control. *E*, immunohistochemistry performed on paraffin sections from *Matn3* knock-out (KO) and wild-type (WT) mice at embryonic day 18.5. p-Smad1 expression was up-regulated in epiphyseal and columnar, but not hypertrophic, cartilage in *Matn3* KO compared with WT mice. No reactivity was detected when sections were incubated without the primary antibody.

Matn3 Inhibits *Smad1/5* Transcription Activity, Which Requires Multiple EGF-like Repeats and the Coiled-coil Domain—Among members of the matrilin family, *Matn1* distribution in the developing growth plate overlaps with *Matn3* (1). However, no expanded zone of chondrocyte hypertrophy is observed in *Matn1* KO mice (data not shown). Compared with MATN1, MATN3 lacks the second vWFA domain and pos-

sesses three additional EGF-like domains. Thus, we hypothesized that the multiple EGF-like domains of MATN3 are involved in modulation of chondrocyte hypertrophy and BMP signaling. To further characterize this structure-function relationship, we utilized full-length *Matn3* (MATN3), *Matn3* containing vWFA domain and the first EGF-like domain (T1-MATN1), *Matn3* containing only vWFA domain but no EGF-

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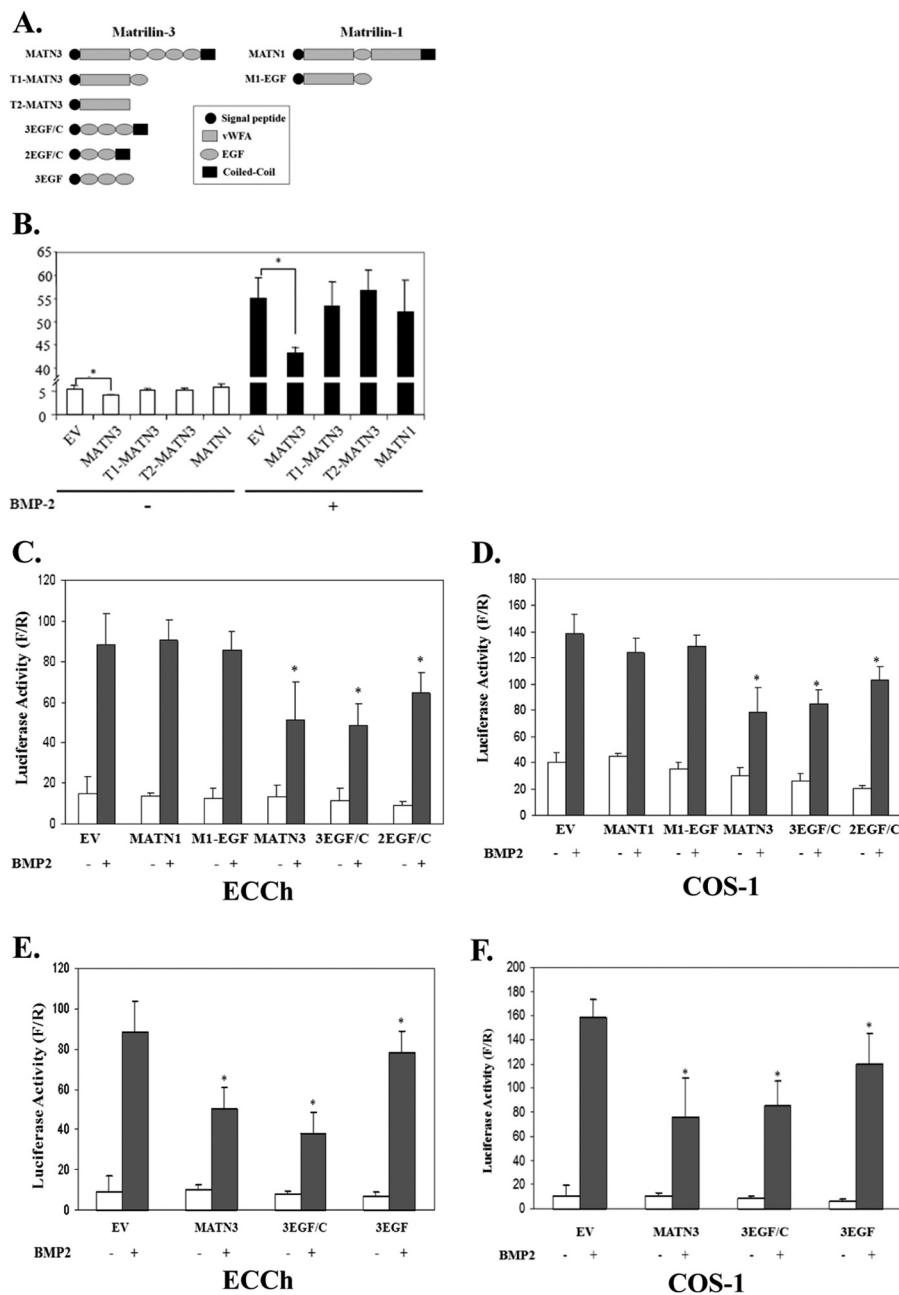


FIGURE 4. Matn3 inhibition of *Smad1/5* promoter activity is dependent on presence of EGF-like structural and coiled-coil domains. *A*, schematic representations of plasmids are as follows: full-length *Matn3*; *Matn3* containing three EGF and coiled-coil domains (*3EGF/C*); *Matn3* containing only vWFA domain (*T2-Matn3*); *Matn3* containing one EGF and coiled-coil domains (*3EGF/C*); *Matn3* containing two EGF and coiled-coil domains (*2EGF/C*); *Matn3* containing three EGF domains (*3EGF*); full-length *Matn1* (*MATN1*); *Matn1* with terminal coiled-coil and vWFA domains deleted (*M1-EGF*). *B*, plasmids and empty vector (*EV*) transfected into MCT cells with BRE-luc reporter specific for *Smad1/5* transcriptional activity. BMP-2 stimulated *Smad1/5* promoter activity. Overexpression of *Matn3*, but not *T1-Matn3*, *T2-Matn3*, or *Matn1*, was associated with decreased *Smad1/5* promoter activity. *C*, plasmids and empty vector transfected into chicken primary chondrocyte cells with BRE-luc reporter specific for *Smad1/5* transcriptional activity. BMP-2 stimulated *Smad1/5* promoter activity. *Matn3*, but not *Matn1*, significantly reduced induction of BRE promoter activity by BMP-2. The peptide containing only the third and fourth EGF repeats (*2EGF/C*) had a smaller inhibitory effect. *D*, *Matn3* modulates BMP signaling in COS-1 cells. Plasmids and empty vector transfected into COS-1 cells with BRE-luc reporter specific for *Smad1/5* transcriptional activity. *E*, the peptide without the coiled-coil domain (*3EGF*) did not have a significant inhibitory effect on BMP-2-induced BRE promoter activity, unlike the *3EGF* peptide with the coiled-coil domain (*3EGF/C*) or full-length *Matn3*. *E*, *Ev*, the peptide without the coiled-coil domain (*3EGF*) did not have a significant inhibitory effect on BMP-2-induced BRE promoter activity, unlike the *3EGF* peptide with the coiled-coil domain (*3EGF/C*) or full-length *Matn3*. *F*, *3EGF* peptide had a small inhibitory effect on BMP-2-induced BRE promoter activity than *3EGF/C* or full-length *MATN3*. *Error bars* represent standard deviation of the mean of three experiments. * indicates significant difference ($p < 0.05$) from empty vector control. *F*, *3EGF* peptide had a small inhibitory effect on BMP-2-induced BRE promoter activity than *3EGF/C* or full-length *MATN3*. *Error bars* represent standard deviation of the mean of three experiments. * indicates significant difference ($p < 0.05$) from empty vector control. *ECCh*, embryonic chicken chondrocytes.

like domain (*T2-MATN3*), and full-length *Matn1* (*MATN1*) (Fig. 4A). These constructs or an empty vector (–) were cotransfected into MCT cells with BRE-luc reporter specific for *Smad1/5* transcriptional activity. Following transfection, luciferase activity was quantified in cells treated in the presence or

absence of BMP-2 (50 ng/ml). Although BMP-2 increased luciferase activity (Fig. 4B), *Matn3* overexpression decreased BMP-2-induced *Smad1/5* promoter activity. No significant difference in *Col X* promoter activity was observed with overexpression of *T1-MATN3*, *T2-MATN3*, or *MATN1*. This

finding suggests that C-terminal EGF repeats and/or the coiled-coil domains are required for MATN3 inhibition of Smad1 activity.

To determine which domain is sufficient for inhibiting BMP signaling, we generated a series of cDNA constructs derived from the cartilage-specific *Matn1* and *Matn3* (Fig. 4A). These cDNAs were co-transfected into primary chicken chondrocytes or nonchondrocyte COS-1 cells with a BRE promoter/luciferase reporter. In primary chondrocyte culture, addition of 100 ng/ml BMP-2 increased the BRE promoter activity by more than 4-fold (Fig. 4C, EV). Although transfection of *Matn1* did not affect the induction of BRE promoter activity by BMP-2, transfection of *Matn3* significantly reduced the induction (Fig. 4C, MATN3). Elimination of the vWFA and the first EGF domain from MATN3 (3EGF/C) did not affect the inhibition of BRE activity induction by BMP-2. The peptide containing the third and fourth EGF repeats (2EGF/C) had a smaller inhibitory effect compared with that seen with three EGF repeats (3EGF/C) (Fig. 4C). This finding in primary chicken chondrocytes were consistent with that of COS-1 cells (Fig. 4D) suggesting that multiple EGF repeats play a role in Matn3 inhibition of BMP-2 activity. To determine whether the coiled-coil domain in *Matn3* is involved in modulating BMP-2 activity, we deleted the coiled coil from *Matn3* (Fig. 4A, 3EGF). The peptide without the coiled-coil domain (3EGF) had a smaller inhibitory effect on BRE activity than the 3EGF peptide with the coiled-coil domain (3EGF/C) (Fig. 4E), indicating that the presence of the coiled-coil domain may intensify the inhibitory effect of BMP signaling. Again, our findings in primary chicken chondrocytes were consistent with our findings in COS-1 cells (Fig. 4F).

MATN3 Binds to BMP-2 in Vitro—To test our hypothesis that Matn3 and Bmp-2 bound each other, we performed an immunoprecipitation assay on concentrated supernatants from MCT cells transfected with either pcDNA3.1-*Matn3* or empty vector (negative control). When V5-*Matn3* was pulled down, Bmp-2 was detected in complex with Matn3 in the conditioned medium of *Matn3*-expressing MCT cells (MATN3+) but not in that of empty vector controls (MATN3-) (Fig. 5A). Additionally, we performed Western blot analysis to quantify unbound Bmp-2 that may have remained in the flow-through fraction after immunoprecipitation with V5 antibody (Fig. 5B). Western blot results showed that the level of unbound Bmp-2 after immunoprecipitation was significantly reduced in the Matn3 sample, suggesting their interaction (Fig. 5B). To quantitatively assess the MATN3/BMP-2 binding interaction, a solid phase binding assay was performed. BMP-2 binding to MATN3 was dose-dependent, similar to BMP-2 binding with Noggin, a known BMP-2 antagonist (Fig. 5C). Surface plasmon resonance demonstrated a dose-dependent specific binding interaction approaching saturation with a calculated $K_d = 2.17 \times 10^{-7}$ M (217 nM) (Fig. 5D).

DISCUSSION

Matrilins have been shown to play an integral structural role in the cartilage ECM (13, 31). Past studies have inferred functional redundancy among matrilins and postulated that MATN3 plays no significant role in mouse skeletal development (32). However, recent studies have demonstrated that

MATN3, in addition to having an important structural function, also has a regulatory function in cartilage (8, 9). Our laboratory has demonstrated that *Matn3* KO mice exhibit an expanded zone of chondrocyte hypertrophy during development, increased bone mineral density in adulthood, and accelerated joint degeneration during aging (14). In this study, we further characterize the *Matn3*KO mouse phenotype and investigate the mechanism by which the loss of Matn3 affects cartilage.

Matn3 KO mice have also been shown to have an expanded zone of chondrocyte hypertrophy and increased *Col X* mRNA expression during embryonic development (14). It is important to note that this increase in chondrocyte hypertrophy was only observed prenatally, but it was comparable with WT at birth. As a result, it has been suggested that Matn3 modulates chondrocyte differentiation in the growth plate during the prenatal period. In this study, *Matn3* overexpression in both primary chicken chondrocytes and MCT cells was associated with decreased mRNA expression of *Col X*, a marker of chondrocyte hypertrophy. This finding supports that Matn3 negatively regulates chondrocyte hypertrophy. We hypothesized that this regulatory effect of Matn3 was accomplished by inhibition of Bmp-2 signaling.

BMPs are important regulators of bone and cartilage development, which act via phosphorylation of Smads and eventual activation of transcription factors. BMP-2 has been shown to stimulate chondrocyte proliferation and differentiation, as well as induce *Col X* mRNA expression via the BRE in the *Col X* gene promoter (25, 36). One of the early downstream signaling molecules of BMP-2 (following binding to its extracellular receptor) is Smad1. In this study, we demonstrate that Matn3 inhibition of the *Col X* promoter activity is dependent on the presence of the BRE within the *Col X* promoter. Furthermore, *Matn3* overexpression inhibits the dose-dependent induction of *Col X* mRNA expression by exogenous Bmp-2. Taken together, these data indicate that Matn3 regulation of *Col X* expression, a marker of chondrocyte hypertrophy, is via inhibition of BMP-2 signaling. In addition, it is important to note that the highest dose of BMP-2 tested (500 ng/ml) rescued *Col X* mRNA expression from MATN3 inhibition. This finding raised the possibility of a direct, saturable interaction between BMP-2 and MATN3.

BMP-2 has been shown to interact with other ECM proteins, such as heparin sulfate, heparin sulfate proteoglycan, and type IV collagen. These interactions regulate BMP-2 signaling by limiting the availability of BMP-2 to bind to its receptor on the chondrocyte membrane (27). In this study, we isolated Matn3 complexed with Bmp-2 by immunoprecipitation in the medium of *Matn3*-overexpressing chondrocytes. By solid phase binding assay and surface plasmon resonance, we demonstrate that MATN3 binds to BMP-2 with a $K_d = 217$ nM. Taken together, these findings suggest that MATN3 directly interacts with BMP-2 in the cartilage ECM, likely limiting BMP-2 availability to its receptor and thus inhibiting downstream signaling. To our knowledge, this is the first study to demonstrate binding between MATN3 and a growth factor present in the cartilage ECM, suggesting a novel mechanism by which MATN3 executes its regulatory role.

Matrilin-3 Modulates BMP-2 Pathway

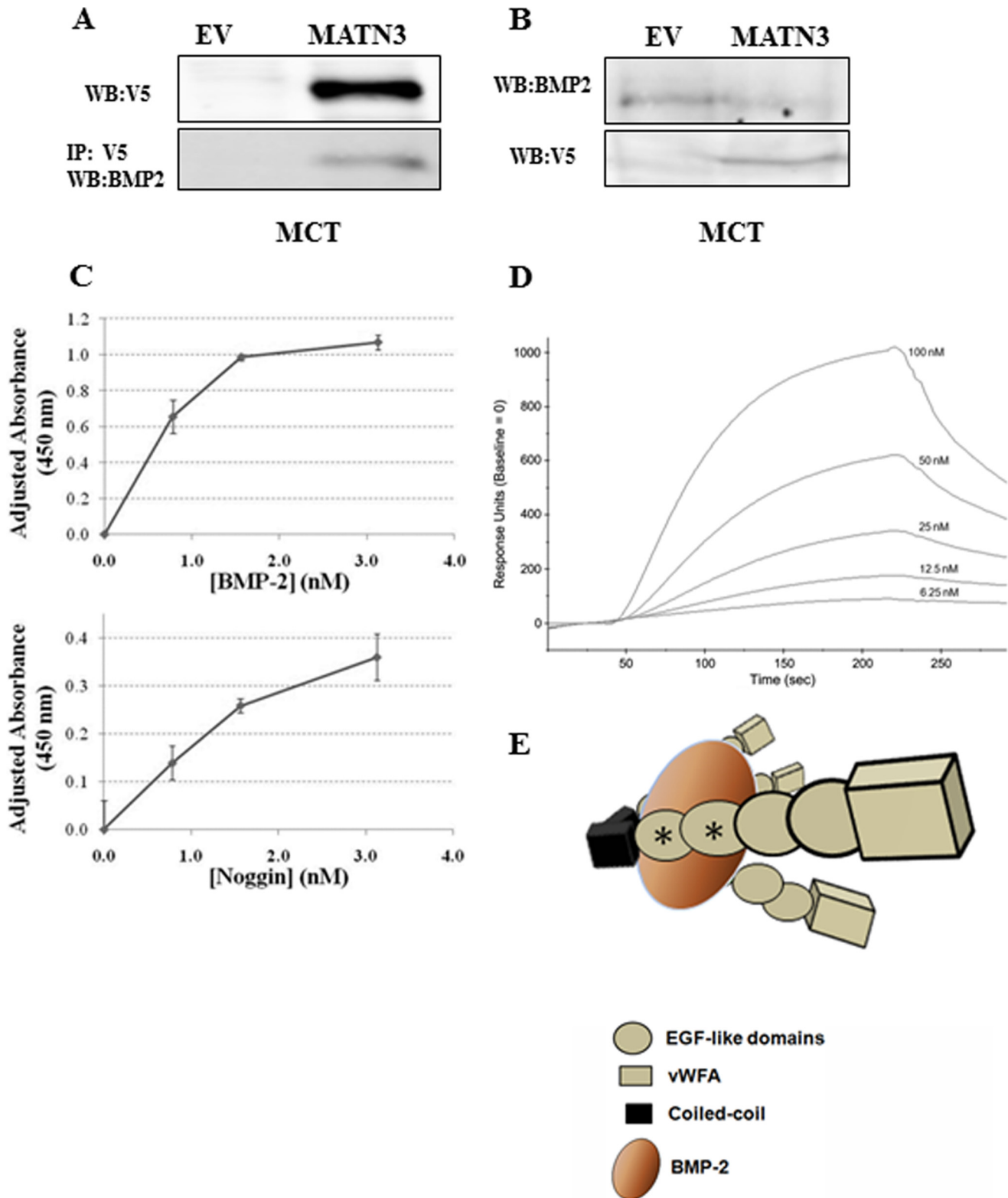


FIGURE 5. MATN3 binds BMP-2. *A*, immunoprecipitation (IP) pulldown (bottom row) detected BMP-2 in complex with MATN3. Western blot (WB) (top row) with anti-V5 antibody detected high concentration of Matn3 in conditioned medium of *Matn3*-overexpressing MCT cells (MATN3) but not empty vector controls (EV). *B*, supernatant after immunoprecipitation with V5 antibody from *A* were further concentrated and followed by Western blot analysis with BMP2 antibody or Matn3/V5 antibody. BMP-2 protein level was significantly reduced in the unbound fraction of *Matn3*-overexpressing cells. *C*, solid phase binding assay demonstrated dose-dependent binding interaction between BMP-2 and MATN3, in parallel to BMP-2 interaction with NOGGIN (known BMP-2 antagonist). *D*, surface plasmon resonance also demonstrated dose-dependent binding interaction approaching saturation with $K_d = 217$ nM. (Values are the mean \pm S.D. of conditions performed in triplicate. Data from one representative experiment are presented.) *E*, diagram schematic depicting the proposed interaction between MATN3 and BMP-2. As demonstrated in this study, only the coiled-coil domain and the two outer most EGF-like domains of MATN3 are required to inhibit BMP-2 signaling. This diagram illustrates the proposed concept that multimeric MATN3 binds to BMP-2 via two EGF-like domains (*) closest to the coiled-coil domain.

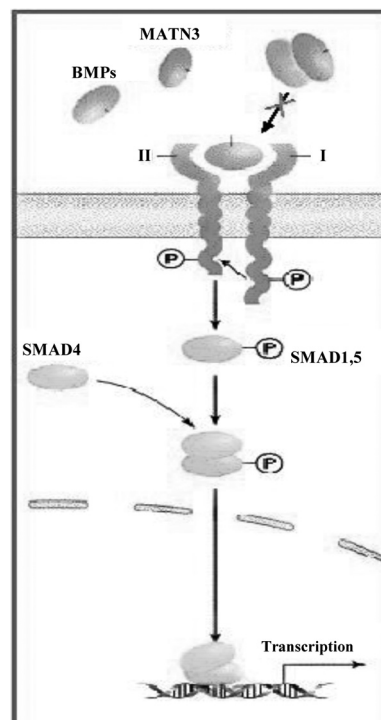


FIGURE 6. Model of matrilin-3 regulating chondrocyte hypertrophy through binding bone morphogenetic protein. MATN3 binds to BMP-2 through the EGF domains clustered together by the coiled-coil domain. This binding sequesters BMP from interacting with the BMP receptors on the cell membrane, thereby inhibiting activation of intracellular Smad1, its translocation into the nucleus, and binding to the promoter region of the hypertrophic genes such as type X collagen with co-transcriptional factor Runx2. Thus, MATN3 inhibits chondrocyte hypertrophy by acting as an antagonist of BMP in cartilage matrix.

To evaluate downstream BMP-2 signaling, the effect of MATN3 on Smad1 activation (by phosphorylation) was analyzed. We found that MATN3 overexpression decreases p-Smad1 in chondrocytes. Conversely, p-Smad1, which was present throughout the growth plate in both WT and *Matn3* KO mice, was significantly up-regulated in the epiphyseal and columnar (but not hypertrophic) chondrocytes in *Matn3* KO mice during development. This distribution corresponds to *Matn3* expression and thus supports our hypothesis that MATN3 inhibits BMP-2 signaling.

MATN3 consists of one vWFA domain, four EGF-like domains, and a C-terminal coiled-coil domain. Missense mutations in the vWFA domain have been associated with multiple epiphyseal dysplasia (37), although the EGF-like domains of MATN3 have been associated with Sox9 regulation (38). In this study, we show that *Matn3*, but not *Matn1* (which only contains one EGF-like domain), inhibits Smad1/5 promoter activity induced by BMP-2. Furthermore, *Matn3*'s ability to inhibit BRE activity was abolished in *Matn3* constructs that included one or no EGF-like domain repeats. This suggests that multiple EGF domains are required for inhibiting BRE promoter activity. Indeed, the presence of EGF2, -3, and -4 is necessary and sufficient for *Matn3* to inhibit Bmp-2 activity. Furthermore, the presence of the coiled-coil domain, which oligomerizes *Matn3* into tetramers, enhances MATN3 inhibition of Bmp-2 activity. Thus, although the N-terminal vWFA domain is essential for matrilin binding to ECM molecules and integrins through the MIDAS motif (7, 39), the C-terminal EGF repeats and the coiled-coil domain are essential for *Matn3* to bind Bmp-2 and subsequently inhibit its activity.

Interestingly, previous studies have shown that follistatin (Fs12) binds and inhibits TGF- β family members, including activin, myostatin, and BMP-2, -4, -6, and -7 (40, 41). Fs12 binds to activin A with a K_d of 430 nM, which is of the same magnitude as the K_d value for the MATN3/BMP-2 binding interaction (42). The crystal structure of activin A complex with Fs12 shows that Fs12 molecules wrap around the back of the "wings" of activin, blocking the type II receptor-binding site on activin A, thereby inhibiting activin A activity (42). Based on the sequence homology between MATN3 and Fs12 (specifically the EGF repeats contained in both MATN3 and Fs12) and between BMP-2 and activin A, we propose a model in which the multiple EGF repeats of MATN3, clustered together by the coiled-coil domain, form a pocket to bind BMP-2, thereby inhibiting BMP-2 activity through blocking the BMP type II receptor-binding site (Fig. 5D).

In conclusion, we show that MATN3 plays an important regulatory role in maintaining the cartilage ECM microenvironment. It inhibits BMP-2 signaling via a direct extracellular binding interaction between MATN3 and BMP-2, which prevents BMP-2 from interacting with its receptor (Fig. 6). This study is the first to demonstrate that MATN3 binds with a growth factor present in the cartilage ECM. As a novel antagonist of BMP-2 signaling, MATN3 prevents premature chondrocyte hypertrophy, which is a key feature of osteoarthritis (33). Furthermore, MATN3's inhibitory effect on BMP-2 signaling is localized to its EGF repeat and coiled-coil domains. Thus, we provide evidence of a novel function of MATN3 and potential

structural basis for this function. There are several important physiological implications of these findings. First, MATN3 mutations responsible for SEMD chondrodysplasia and OA, which occur in the EGF domain of MATN3, may be the result of a defect in inhibition of Smad1 activity and chondrocyte hypertrophy. Indeed, it has been shown recently that the MATN3 SEMD chondrodysplasia mutant resulted in premature chondrocyte hypertrophy (47). Second, MATN3 peptides containing the Smad1 inhibitory activity, as identified in this study, may be used to inhibit excessive chondrocyte hypertrophy during SEMD chondrodysplasia and OA.

Although BMP-2 was generally thought to play a positive role in cartilage repair, recent data have shown its pleiotropic effects on cartilage hypertrophy and OA (43–45). BMP-2 has recently been shown to directly bind another cartilage ECM protein, cartilage oligomeric matrix protein (COMP) (46). These findings, taken with our findings, suggest that BMP-2 activity in the cartilage ECM is regulated by cartilage ECM proteins, including MATN3.

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