Smad7 Inhibits Chondrocyte Differentiation at Multiple Steps during Endochondral Bone Formation and Down-regulates p38 MAPK Pathways

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Bone morphogenetic proteins (BMPs) play critical roles at various stages in endochondral bone formation. In vitro studies have demonstrated that Smad7 regulates transforming growth factor-β and BMP signals by inhibiting Smad pathways in chondrocytes. However, the in vivo roles of Smad7 during cartilage development are unknown. To investigate distinct effects of Smad7 at different stages during chondrocyte differentiation, we generated a series of conditional transgenic mice that overexpress Smad7 in chondrocytes at various stages of differentiation by using the Cre/loxP system. We generated Col11a2-lacZflplox Smad7transgenic mice and mated them with three types of Cre transgenic mice to obtain Smad7, but not Smad6, down-regulated the phosphorylation of Smad7 at different stages during chondrocyte differentiation, we generated a series of conditional transgenic mice that overexpress Smad7 in chondrocytes at various stages of differentiation by using the Cre/loxP system. We generated Col11a2-lacZflplox Smad7transgenic mice and mated them with three types of Cre transgenic mice to obtain Smad7, but not Smad6, down-regulated the phosphorylation of Smad7 at different stages during chondrocyte differentiation, we generated a series of conditional transgenic mice that overexpress Smad7 in chondrocytes at various stages of differentiation by using the Cre/loxP system. We generated Col11a2-lacZflplox Smad7transgenic mice and mated them with three types of Cre transgenic mice to obtain Smad7, but not Smad6, down-regulated the phosphorylation of Smad7 at different stages during chondrocyte differentiation, we generated a series of conditional transgenic mice that overexpress Smad7 in chondrocytes at various stages of differentiation by using the Cre/loxP system. We generated Col11a2-lacZflplox Smad7transgenic mice and mated them with three types of Cre transgenic mice to obtain Smad7, but not Smad6, down-regulated the phosphorylation of Smad7 at different stages during chondrocyte differentiation, we generated a series of conditional transgenic mice that overexpress Smad7 in chondrocytes at various stages of differentiation by using the Cre/loxP system.

The transforming growth factor-β (TGF-β) superfamily regulates cell growth and differentiation in a variety of tissues.

This family includes three major subfamilies: TGFs-β, activins, and bone morphogenetic proteins (BMPs). Signaling by members of the TGF-β superfamily is transduced through type I and II serine/threonine kinase receptors (1). Upon ligand binding, type II receptors phosphorylate type I receptors. Next, type I receptors phosphorylate downstream targets. Receptor-regulated Smads (R-Smads) are phosphorylated by type I receptors (2). Smad1, Smad5, and Smad8 are R-Smads that transduce BMP signals, and Smad2 and Smad3 are R-Smads that transduce TGF-β and activin signals. Phosphorylated R-Smads form heteromers with Smad4, which is a common-partner Smad (referred to as Co-Smad), and translocate into the nucleus. There, they interact with transcription factors and activate gene transcription. Although the Smad pathway exists in most cell types and tissues, additional pathways are activated by BMP/TGF-β in certain cell types (3). BMP and TGF-β activate TAK1 (TGF-β-activated kinase 1), a member of the MAPK kinase kinase family. TAK1 is involved in the activation of several MAPKs, including JNK, p38, and ERK, which ultimately results in the activation of ATF2. TGF-β also activates Rho signaling pathways.

Inhibitory Smads including Smad6 and Smad7 inhibit phosphorylation of R-Smads by competing with R-Smads for binding to phosphorylated type I receptors (2). Smad6 inhibits BMP signaling, whereas Smad7 inhibits both TGF-β and BMP signaling. Smad6 has narrow specificity in its interaction with receptors (4). Smad6 and Smad7 inhibit BMP-activated p38 MAPK pathways in neuronal cells (5). Smad6 and Smad7 interact differently with TAK1 in PC12 cells. Smad6 and Smad7 are differentially expressed during development (6). Mice deficient in exon 1 of Smad7 have abnormal B-cell responses and are small (7). Data on skeletal tissues of these mice are not available. These mice have partial Smad7 function because N-terminally truncated forms of the Smad7 transcript may be produced (7).

During development, the limb skeleton is formed through endochondral bone formation, which consists of multiple steps of cellular differentiation (8, 9). Mesenchymal cells initially undergo condensation, which is followed by the differentiation of prechondrogenic cells within these condensations into round chondrocytes to form cartilage. Round chondrocytes in cartilage proliferate and produce cartilage extracellular matrix composed of collagen fibrils and proteoglycans. Proliferating chondrocytes in the central region of the cartilage then exit the cell cycle and differentiate into hypertrophic chondrocytes. The proliferating chondrocytes closest to the hypertrophic

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1 The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; dpc, day(s) postcoitus; rhBMP2, recombinant human BMP2; RT, reverse transcription; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
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chondrocytes flatten out and form orderly columns of still proliferating flat chondrocytes. The zone of the hypertrophic chondrocytes is invaded by blood vessels along with osteoblasts, osteoclasts, and hematopoietic cells to form primary ossification centers. BMPs play critical roles at various stages in endochondral bone formation (10). Cartilage formation is severely disturbed in mice lacking BMP receptors (11) and in mice overexpressing Noggin, a BMP antagonist (12), in prechondrogenic cells; these results suggest that BMP signaling is necessary for cartilage formation.

Smad7 is expressed in growth plate cartilage (13) and osteoarthritic cartilage (14, 15). In vitro studies using cell culture systems or organ culture of mandibular explants have shown that Smad7 inhibits chondrocyte differentiation and/or proliferation induced by TGF-β (16, 17) or BMP (18, 19). These in vitro studies have demonstrated down-regulation of R-Smad activation by Smad7 in chondrocytes. On the other hand, recent mouse genetic studies have revealed mild abnormalities in cartilage in mice lacking Smad4 (20) and in mice overexpressing Smad6 (21) in chondrocytes, raising the possibilities that non-Smad pathways may also mediate BMP-induced cartilage formation. In addition, conditional inactivation of the TGF-β type II receptor gene (Tgbr2) in prechondrogenic cells and chondrocytes results in mice without any long bone defects, leading to the conclusion that TGF-β signaling is not needed in the limb endochondral process (22). In vivo effects of Smad7 on cartilage development are unknown and thus are worth examining.

To examine the effects of Smad7 during cartilage development, we generated a series of conditional transgenic mice that overexpress Smad7 in chondrocytes at various steps of differentiation by using the Cre/loxP system. Smad7 overexpression on cartilage development are unknown and thus are needed in the limb endochondral process (22). I

EXPERIMENTAL PROCEDURES

Construction of the Transgene—The α2(XI) collagen gene (Col11a2)-based expression vector, p742lacZInt, contains the Col11a2 promoter (−742 to +380), an SV40 RNA splice site, the β-galactosidase reporter gene (lacZ), the SV40 polyadenylation signal, and 2.3 kb of the first intron sequence of Col11a2 as an enhancer (23). To create a Col11a2-lacZfloxed-Smad7 transgene plasmid, a loxp sequence was inserted into the 5′-untranslated region of lacZ p742lacZInt, and the NotI sites at both ends of the lacZ segment were mutated and abolished. Next, a sequence consisting of an SV40 RNA splice site, a loxp sequence, a NotI site, and the SV40 polyadenylation signal was inserted into the 3′-end of the SV40 polyadenylation signal of the plasmid. Finally, mouse Smad7 cDNA tagged with NotI sites at both ends was inserted into the NotI sites. To create the Col11a2prom-Cre-ehn plasmid, the lacZ sequence in p742lacZInt was replaced with a NotI-tagged Cre sequence at the NotI sites. To create the Col11a2prom-Cre plasmid, the lacZ sequence in p742lacZInt was replaced with a NotI-tagged Cre sequence at NotI sites.

Staining of the Skeleton—Embryos were dissected, fixed in 100% ethanol overnight, and then stained with Alcian blue followed by alizarin red S solution according to standard protocols (25).

Histological Analysis—Embryos were dissected with a stereomicroscope, fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Serial sections were stained with hematoxylin and eosin or with safranin O/fast green/iron hematoxylin. Immunohistochemistry was performed with a rabbit polyclonal antibody against Smad7 (1:100 dilution; Santa Cruz Biotechnology, Inc.). Immune complexes were detected using streptavidin-peroxidase staining and Histofine SAB-PO kits (Nichirei, Tokyo, Japan). RNA in situ hybridization was performed using 35S-labeled antisense riboprobes as described previously (45). To detect proliferating cells in tissue sections, digoxigenin-11-UTP-labeled Hist2 (histone cluster 2) RNA probes were prepared (26).

Micromass Culture of Mesenchymal Cells—Micromass culture was performed according to previously described methods (27). The distal quarters of limb buds from 12.0-day postcoitus (dpc) wild-type and transgenic mouse embryos were dissected and digested with 0.1% collagenase (Sigma) and 0.1% trypsin (Sigma) for 45 min in 5% CO2 at 37°C. The dissociated cells were filtered through nylon mesh (40-μm pore size; Tokyo Screen, Tokyo) to generate a single cell suspension and were then adjusted to 2 × 105 cells/ml in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum. Cell suspensions (20-ml drops) were placed in the center of each well of 12-well plates. After the cells were allowed to attach for 90 min in 10% CO2 at 37°C, they were overlaid with 2 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The medium was replaced by fresh medium every other day.
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FIGURE 1. Generation of Col11a2-lacZ$^{\text{floxed}}$-Smad7 transgenic mice. A, the structure of the Col11a2-lacZ$^{\text{floxed}}$-Smad7 transgene is shown. In the presence of Cre recombinase, the lacZ sequence would be excised, and Smad7 would be expressed instead of LacZ under the control of the Col11a2 promoter/enhancer sequences. B–G, two Col11a2-lacZ$^{\text{floxed}}$-Smad7 transgenic mouse lines were established. Both lines showed similar cartilage-specific patterns of X-gal staining and developed normally. One line (B–F) showed stronger X-gal staining than the other line (G). B, at 12.5 dpc, mesenchymal condensation in limbs was strongly stained. C, at 14.5 dpc, primordial cartilage in limbs and ribs showed X-gal staining. D, a histological section showed X-gal-stained mesenchymal condensation in the forelimb at 12.5 dpc. E, shown is a histological section of the femur at 14.5 dpc. F, a higher magnification of the boxed region in E is shown. G, the other transgenic line at 14.5 dpc showed weak X-gal staining compared with the first line (C). Scale bars = 2.5 mm (B and C), 100 μm (D and E), 50 μm (F), and 2.5 mm (G).

To induce cartilaginous nodule formation, medium containing 50 or 100 ng/ml recombinant human BMP2 (rhBMP2) (Astellas Pharma, Tokyo) in 10% fetal bovine serum was used unless specifically described otherwise. For infection with adenoviral vectors for Smad6 and Smad7 (18), cells were infected 1 day after plating. For Alcian blue staining, micromass cultures 4 days after plating were fixed with 4% paraformaldehyde for 10 min and stained overnight with 1% Alcian blue in 3% acetic acid at 37 °C. The cultures were then digitally photographed with a Nikon SMZ-U microscope. The size and number of nodules were measured using WinROOF software (Mitani Shoji, Fukui, Japan). For Western blot analysis, adenovirus-infected cells were starved for 16 h before replacement with Dulbecco’s modified Eagle’s medium in the presence or absence of rhBMP2. One hour after treatment with rhBMP2, the cells were lysed and subjected to Western blotting. For activation of MAPK pathways, 2 ng/ml anisomycin was added to the culture medium and used as a positive control.

Western Blotting—Cells were cultured, lysed, subjected to SDS-PAGE, electrophoresed, and immunnostained. The antibodies used were anti-Smad7 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.); anti-Smad6 antibody (1:200 dilution; Zymed Laboratories Inc.); anti-Smad1 antibody (1:1000 dilution; Calbiochem); anti-phospho-Smad1/5/8 antibody (1:1000 dilution), anti-phospho-ATF2 antibody (1:1000 dilution), anti-ATF2 antibody (1:1000 dilution), anti-phospho-p38 MAPK antibody (1:1000 dilution), anti-p38 MAPK antibody (1:1000 dilution), anti-phospho-Smad2 antibody (1:1000 dilution), and anti-Smad2/3 antibody (1:1000 dilution) (Cell Signaling Technology). Proteins in the blots were visualized using an ECL Plus kit (Amersham Biosciences).

RESULTS

Generation of Floxed lacZ-Smad7 Transgenic Mice—Because Smad7 inhibits various signaling pathways, we anticipated that its overexpression in chondrocytes might cause severe cartilage abnormalities in transgenic mice. To avoid lethality, we employed a conditional transgenic mouse system to express the Smad7 transgene. We first established floxed lacZ-Smad7 transgenic mouse lines bearing the Col11a2-lacZ$^{\text{floxed}}$-Smad7 transgene (Fig. 1A). Col11a2 promoter/enhancer sequences direct expression to condensed mesenchymal cells and chondrocytes (23). We expected that the mice bearing this construct would express lacZ but not Smad7 due to the poly(A) signal sequence that immediately follows the lacZ sequence. In the presence of Cre recombinase, the lacZ sequence would be deleted, and Smad7 would be expressed instead of lacZ under the control of the Col11a2 promoter/enhancer sequences. We obtained and analyzed two independent lines of Col11a2-lacZ$^{\text{floxed}}$-Smad7 transgenic mice. Staining of transgenic embryos from one line with X-gal showed that lacZ was expressed in condensed mesenchyme at 12.5 dpc (Fig. 1B) and in primordial cartilage in limbs and ribs at 14.5 dpc (Fig. 1C). Histological analysis confirmed lacZ activities specifically in mesenchymal condensation at 12.5 dpc (Fig. 1D) and in chondrocytes at 14.5 dpc (Fig. 1E). lacZ activities were not recognized in cells in the perichondrium (Fig. 1F). Transgenic embryos from the other line showed a similar but weaker (Fig. 1G) pattern of lacZ activities than the first line (Fig. 1C) at 14.5 dpc. Both lines of mice developed normally.

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**Generation of Cre Transgenic Mice**—Next, we prepared three types of Cre transgenic mice. First, Prx1-Cre transgenic mice were obtained from Dr. Malcolm Logan (24). Second, we linked the Cre sequence to the Col11a2 promoter plus intron enhancer to prepare the transgene construct 11Enh-Cre (Fig. 2A). Third, we prepared Cre linked to the Col11a2 promoter without an intron enhancer to prepare the transgene construct 11Prom-Cre (Fig. 2A). We injected 11Enh-Cre and 11Prom-Cre into fertilized ova and established transgenic lines.

We examined the expression patterns of Cre by mating CAG-CATfloxed-lacZ reporter transgenic mice (29) with these Cre transgenic mice. As reported previously (24), Prx1-Cre mice showed Cre recombinase activity throughout the early limb bud mesenchyme at 12.5 dpc. (Fig. 2B). 11Enh-Cre transgenic mice started to show very weak Cre activities in mesenchymal condensation in forelimb buds at 12.5 dpc (Fig. 2C). 11Prom-Cre transgenic mice showed no lacZ activities in limb buds at 12.5 dpc (Fig. 2D). aberrant Cre activities were recognized in forebrains. At 14.5 dpc, 11Enh-Cre transgenic mice showed Cre-mediated recombination in all primordial cartilage of limbs and ribs (Fig. 2E). In contrast, 11Prom-Cre transgenic mice showed recombination in a limited part of each primordial cartilage (Fig. 2F). X-gal staining was absent in the epiphysseal part of each primordial cartilage. Histological analysis of the distal ulnas of 11Enh-Cre transgenic mice at 14.5 dpc showed recombinase activities in all chondrocytes, including round proliferative chondrocytes, flat proliferative chondrocytes, prehypertrophic chondrocytes, hypertrophic chondrocytes, and perichondral cells (Fig. 2G). Histological analysis of the distal ulnas of 11Prom-Cre transgenic mice at 14.5 dpc showed recombinase activities in flat chondrocytes, prehypertrophic chondrocytes, and hypertrophic chondrocytes, but not in round chondrocytes located at the ends of each primordial cartilage (Fig. 2H). These results suggest that 11Enh-Cre induces recombination from the step of round proliferative chondrocytes and that 11Prom-Cre induces recombination from the step of flat proliferative chondrocytes. The recombination pattern of our 11Enh-Cre transgenic mice was consistent with that of previously reported Cre transgenic mice containing identical promoter/enhancer sequences (30). None of the Cre transgenic mice showed detectable abnormalities.

**Skeletal Abnormalities in Smad7 Conditional Transgenic Mice**—We mated Col11a2-lacZfloxed-Smad7 transgenic mice with Prx1-Cre, 11Enh-Cre, and 11Prom-Cre transgenic mice, respectively, and generated double transgenic pups as follows: pups bearing Col11a2-lacZfloxed-Smad7 and Prx1-Cre transgenes (Smad7Prx1), those bearing Col11a2-lacZfloxed-Smad7 and 11Enh-Cre transgenes (Smad711Enh), and those bearing Col11a2-lacZfloxed-Smad7 and 11Prom-Cre transgenes (Smad711Prom). We examined the skeletons of Smad7 conditional transgenic mice. Staining with Alcian blue and alizarin red S revealed that the Smad7Prx1 double transgenic mice had very hypoplastic limb skeletons (Fig. 3B) compared with Col11a2-lacZfloxed-Smad7 control mice (Fig. 3A) at 16.5 dpc. The Smad7Prx1 axial skeleton was relatively preserved because the Prx1-Cre transgene directs Cre expression mainly in limbs. Hind limb skeletons were less affected than forelimb skeletons. Smad711Enh mice had moderately hypoplastic skeletons (Fig. 3C) compared with Col11a2-lacZfloxed-Smad7 control mice (Fig. 3A). Mineralization indicated by alizarin red staining was reduced in Smad711Enh mice. The cartilaginous components of Smad711Prom mice were slightly small (Fig. 3D) compared with those of Col11a2-lacZfloxed-Smad7 control mice (Fig. 3A). Mineralization was reduced slightly in Smad711Prom mice. We also mated the other line of Col11a2-lacZfloxed-Smad7 mice with Prx1-Cre mice, and the resultant Smad7Prx1 transgenic pups showed similar but milder abnormalities in limb skeleton (Fig. 3E) than the Smad7Prx1 mice from the first line of Col11a2-lacZfloxed-Smad7 mice (Fig. 3B). Overall, the skeletal lengths in each type of double transgenic pups varied with regard to cartilage size and mineralization depending on the types of Cre transgenic mice used for mating (Fig. 3F).

**Cre-mediated Recombination in Each Type of Conditional Transgenic Mouse**—The scheme in Fig. 4A shows the expected recombination events in this experiment. In Smad7Prx1 mice, recombination occurs in mesenchymal cells in limb buds before the onset of mesenchymal condensation when Col11a2 promoter/enhancer sequences start to direct expression. Thus, the
expression of the Smad7 transgene in Smad7^Prox1 mouse limbs may be controlled by Col11a2 promoter/enhancer sequences in the Col11a2-lacZfloxed-Smad7 transgene, and the Smad7^transgene may be gradually expressed from the step of condensed mesenchyme. The expression pattern of the Smad7 transgene in Smad7^Prox1 conditional transgenic mouse limbs is considered to be similar to the expression pattern of the transgene expression in conventional transgenic mouse bearing the Col11a2-lacZfloxed-Smad7 transgene. In Smad7^Prox1 mice, recombination occurs at the step of round proliferative chondrocytes when the amount of Cre protein may be increased enough to catalyze recombination. In Smad7^Prom mice, recombination occurs at the step of flat proliferative chondrocytes.

We examined Cre-catalyzed recombination by monitoring the disappearance of lacZ activities. Col11a2-lacZfloxed-Smad7 mice showed LacZ expression in condensed mesenchyme at 12.5 dpc (Fig. 4B). Smad7^Prox1 double transgenic mice did not exhibit LacZ expression (Fig. 4C), indicating that Prx1-Cre transgene products induced recombination of the Col11a2-lacZfloxed-Smad7 transgene in limb buds. Both Smad7^1Enh mice and Smad7^1Prom mice showed LacZ expression in condensed mesenchyme (Fig. 4D and E), as did Col11a2-lacZfloxed-Smad7 control mice (Fig. 4B), indicating that recombination did not occur at this stage.

At 14.5 dpc, Col11a2-lacZfloxed-Smad7 control mice showed LacZ expression in all primordial cartilage (Fig. 4F). Smad7^Prox1 mice showed LacZ expression in primordial cartilage in the ribs and spine, but not in the limbs (Fig. 4G). LacZ expression in Smad7^1Enh mice disappeared in primordial cartilage in trunk and proximal segments in the limbs. LacZ expression remained in paws, which developed later than proximal segments in the limbs (Fig. 4H). Smad7^1Prom mice showed LacZ expression in the most of the primordial cartilage at this stage except for weak LacZ expression in the humerus in femur, which developed earlier than the distal segments (Fig. 4I).

Histological Analysis of Various Smad7 Conditional Transgenic Mice—To investigate the function of Smad7 at various steps during endochondral bone formation, we performed histological analysis of skeletons of Smad7 conditional transgenic mice bearing various Cre transgenes. At 16.5 dpc, wild-type mice formed ossification centers in the humerus (Fig. 5A). Smad7^Prox1 mice showed very small cartilage. The Smad7^1Enh transgenic cartilage of the humerus contained proliferative and hypertrophic chondrocytes, but not ossification centers. The humeri of Smad7^1Prom mice had an almost normal sized proliferative cartilage and exhibited incomplete formation of ossification centers.

At 14.5 dpc, proliferative chondrocytes started hypertrophy at the center of the humeri of wild-type mice (Fig. 5B). The Smad7^1Prom transgenic cartilage from the humerus contained proliferative (but not hypertrophic) chondrocytes. These results suggest that Smad7 expressed in flat proliferative chondrocytes delays chondrocyte hypertrophy.

Proliferation of Proliferative Chondrocytes Is Inhibited by Smad7 Overexpression—We examined the effect of Smad7 overexpression on the proliferation of proliferative chondrocytes by in situ hybridization using Hist2 cRNA probes on histological sections of distal femur at 16.5 dpc and performed statistical analysis (Fig. 5, C and D). In situ hybridization for
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Smad7 (but Not Smad6) Overexpression Inhibits Cartilaginous Nodule Formation Induced by BMP and Inhibits BMP-activated MAPK Pathways—Mouse genetic experiments have shown that BMP signal inactivation dramatically inhibits cartilage formation (11), but TGF-β signal inactivation does not affect cartilage formation in the limbs (22). To further examine how Smad7 overexpression inhibited cartilage formation, we analyzed BMP signals during cartilaginous nodule formation using micromass cultures of limb bud mesenchymal cells prepared from the first line of Smad7 mice and the other line of Smad7 mice. The other line of Smad7 mice (Fig. 3E) showed less severe skeletal abnormalities than the first line of Smad7 mice (Fig. 3B). We also prepared mesenchymal cells from Col11a2-Smad6 transgenic mice, which express Smad6 in condensed mesenchymal cells and chondrocytes (21). The addition of rhBMP2 to the medium stimulated the formation of cartilaginous nodules, which were visualized by Alcian blue staining (Fig. 7A, top row). The cartilaginous nodule formation induced by rhBMP2 was decreased in mesenchymal cells prepared from the first line of Smad7 mice (Fig. 7A, second row) and in mesenchymal cells prepared from the other line of Smad7 mice (third row), but not in mesenchymal cells from Col11a2-Smad6 transgenic mice (fourth row). The mean number of cartilaginous nodules was more in the mesenchymal cell cultures of the other line of Smad7 mice (Fig. 7A, third row) than that in the mesenchymal cell cultures of the first line of Smad7 mice (second row).

We next examined marker gene expression in micromass cultures of transgenic mesenchymal cells in the presence of rhBMP2 by real-time RT-PCR (Fig. 7B). The relative expression levels of Smad7 were increased in mesenchymal cells from the

histone mRNA is as an appropriate technique for assessment of cell proliferation as bromodeoxyuridine immunohistochemistry (26). Compared with the control mice, Smad7 mice had a reduced number of Hist2-positive cells. In contrast, Smad7 mice had a normal number of Hist2-expressing cells.

We analyzed the transgene expression in distal femur by in situ hybridization (Fig. 5E). In wild-type mice, the expression levels of Smad7 and Smad6 were relatively low in cartilage compared with those in the surrounding tissues. Smad7 mice showed increased Smad7 expression in both epiphyseal and metaphyseal chondrocytes, suggesting that Cre-mediated deletion of the lacZ sequence had occurred and that the Smad7 transgene was transcribed in round and flat chondrocytes. Smad7 mice showed increased Smad7 expression in the metaphyseal chondrocytes and lacZ expression in the epiphyseal chondrocytes, suggesting that Cre-mediated recombination had occurred and that the Smad7 transgene was transcribed mainly in flat chondrocytes. Immunohistochemical analysis showed that anti-Smad7 immunoreactivity was increased in the femoral chondrocytes of Smad7 mice compared with those of wild-type mice, suggesting that Smad7 transgene mRNA was translated (Fig. 5F).

Mesenchymal Condensation Is Inhibited and Sox9 Expression Is Decreased in Smad7 mice—In situ hybridization analysis of axial sections of 12.5-dpc wild-type limb buds showed that Sox9 and Col11a2 were expressed in condensing mesenchyme that was recognized in sections stained with safranin O (Fig. 6A). In Smad7 mice, mesenchymal condensation was not apparent; Sox9 expression was weak; and Col11a2 expression was not detected at this stage. In situ hybridization analysis of mice at 16.5 dpc showed that the expression levels of Smad6 and Smad7 mRNAs in chondrocytes relatively low compared with the levels in the surrounding tissues in the wild-type mice. The relative expression levels of Smad7 in femoral chondrocytes were similar to those of Smad7 in surrounding tissues in Smad7 mice (Fig. 6B). These results suggest that the Smad7 transgene was transcribed mainly in chondrocytes.

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We next examined marker gene expression in micromass cultures of transgenic mesenchymal cells in the presence of rhBMP2 by real-time RT-PCR (Fig. 7B). The relative expression levels of Smad7 were increased in mesenchymal cells from the
first line of Smad7Prx1 mice, indicating transgene expression. The expression levels of Smad6 were increased in mesenchymal cells from Smad7Prx1 mice, probably because the culture was composed mainly of non-cartilaginous cells. Non-chondrocytic cells expressed endogenous Smad6 at higher levels compared with chondrocytes (Fig. 6B). The relative expression levels of Col2a1, Col11a2, and Sox9 were decreased, and the expression level of Aldh1a2, which is the target gene of BMP signals and down-regulates Sox9 gene expression (31), was increased. In contrast, the relative expression levels of the marker genes were not significantly changed in mesenchymal cells from Col11a2-Smad6 mice. The relative expression levels of Smad6 were elevated in Col11a2-Smad6 mice. These results revealed different effects on cartilaginous nodule formation and marker gene expression between Smad7 and Smad6 overexpression, although we could not deny the possibility that the expression level of the Smad6 transgene was not high enough compared with that of the Smad7 transgene.

Because Smad6 overexpression did not disturb cartilaginous nodule formation or marker gene expression, we speculated that non-Smad pathways might mediate BMP-induced nodule formation. It has been reported that BMPs activate MAPK pathways (3). MAPKs control chondrogenesis (32). JNK phosphorylation is not affected during chondrogenesis, suggesting that JNKs play minor roles (33). During chondrogenesis of chick mesenchymal cells, p38 phosphorylation is increased, and ERK phosphorylation is decreased. The inhibitor of p38 MAPK pathways blocks chondrogenesis, whereas the inhibitor of MEK/ERK activity enhances chondrogenesis (34). This line of findings suggests that p38 MAPK pathways may be involved in BMP-induced nodule formation.

Next, we examined whether our results from the transgenic overexpression experiments were consistent with results from the adenoviral overexpression experiments. Consistently, cartilaginous nodule formation in wild-type mouse mesenchymal cells was inhibited by Smad7 adenoviral infection (Fig. 7C, second row), but not by Smad6 adenoviral infection (third row). Real-time RT-PCR analysis showed that the relative mRNA expression levels for
Smad7 and Smad6 were elevated in micromass cultures infected with Smad7 and Smad6 adenoviral vectors, respectively (Fig. 7D). Both Smad6 and Smad7 adenoviral infection decreased the expression levels of Id1, the target gene of Smad1/5/8 (35, 36), suggesting that Smad1/5/8 pathways were affected. We used immunoblot analysis to examine the effects of BMP signals on the phosphorylation of signaling molecules in micromass cultures of wild-type mouse mesenchymal cells (Fig. 7E). The addition of rhBMP2 induced the phosphorylation of Smad1/5/8 but only slightly induced the phosphorylation of Smad2. The addition of rhBMP2 also induced the phosphorylation of p38 and ATF2, a downstream target molecule of the MAPK pathways, like anisomycin, a positive control for MAPK pathways. Infection with Smad7 and Smad6 adenoviral vectors inhibited the phosphorylation of Smad1/5/8 to similar extents. Smad7 adenoviral infection inhibited the phosphorylation of p38 and ATF2 to a greater extent than did Smad6 adenoviral infection. These results indicate the following. 1) Cartilaginous nodule formation induced by rhBMP2 was associated with activation of p38 MAPK pathways, and 2) Smad7 overexpression inhibited cartilaginous nodule formation induced by rhBMP2 and down-regulated BMP-activated p38 MAPK pathways.

We also examined the effects of BMP signals using an undifferentiated ATDC5 cell line. The addition of rhBMP2 to ATDC5 cells increased the phosphorylation of p38 and ATF2 (Fig. 8A). The increased phosphorylation of p38 and ATF2 in the presence of rhBMP2 was decreased by Smad7 adenoviral infection to a greater extent compared with Smad6 adenoviral infection. Infection with an adenoviral construct of a constitutively active form of MKK3 (a gift from Dr. Riko Nishimura), a MAPK kinase that activates p38 (37), increased the phosphorylation of p38 and ATF2 in ATDC5 cells (Fig. 8B). Infection with both the constitutively active form of MKK3 and the Smad7 adenoviral construct showed increased phosphorylation of p38 and ATF2, suggesting that Smad7 affects MAPK pathways upstream of MKK3 in ATDC5 cells.

DISCUSSION

Smad7 Overexpression Affects Cartilage Development at Multiple Steps—To examine the effects of Smad7 on cartilage at various stages of chondrocyte differentiation in vivo, we generated mice that conditionally overexpress Smad7 in chondrocytes. To avoid potential lethality, we employed a conditional transgenic mouse system. Conditional transgenic mouse systems that use Cre/loxP in other tissues have been developed previously (38, 39). We established Col11a2-lacZfloxed-Smad7 transgenic lines and transgenic lines expressing Cre under the control of the Col11a2 regulatory sequences. By using these mice, we controlled the start of Smad7 overexpression at three steps of chondrocyte differentiation. We found that Smad7 overexpression exerts specific functions at multiple stages of chondrocyte differentiation. Smad7 overexpression in condensing mesenchymal cells inhibited their chondrocyte differentiation; Smad7 overexpression in round chondrocytes decreased proliferation; and Smad7 overexpression in flat chondrocytes inhibited maturation toward hypertrophy. In situ hybridization showed that Smad7 expression in chondrocytes was relatively low compared with that of the surrounding tissues during normal development.
 Roles of Smad7 in Chondrocyte Differentiation

![Image](50x298 to 407x733)

Smad7 in Prechondrogenic Cells Inhibits Chondrocyte Differentiation and Down-Regulates BMP-activated p38 MAPK Pathways—Overexpression of Smad7 in condensing mesenchymal cells inhibited condensation and subsequent cartilage formation in Smad7Prx1 mice. Because mouse limb cartilage develops almost normally without TGF-β receptor type II (22), we speculated that Smad7 overexpression inhibited cartilage formation by inhibiting BMP signals in Smad7Prx1 mice. Cartilage does not form in mice that lack BMP signals in chondrocytes (11, 12). However, cartilage forms in mice lacking Smad4 (20) and in mice overexpressing Smad6 (21) in chondrocytes, suggesting that non-Smad pathways may mediate BMP-induced cartilage formation. During chondrogenesis of chick mesenchymal cells, p38 phosphorylation is increased. The inhibitor of p38 MAPK pathways blocks chondrogenesis (34). BMP signals increase Sox9 expression in mesenchymal cells (31). Sox9 is likely a downstream target of the p38 MAPK pathway in chondrocytes (40). Sox9 promotes cartilage formation (41). Based on these findings, it is reasonable to assume that MAPK pathways mediate BMP-induced cartilage formation through increased expression of Sox9. Sox9 expression was decreased in condensing mesenchyme in Smad7Prx1 mice. Together with these findings, the present result that Smad7 overexpression down-regulated the phosphorylation of p38 and ATF2 suggested that Smad7 inhibits cartilage formation, possibly by down-regulating BMP-activated p38 MAPK pathways. Accordingly, Smad7 has been reported to inhibit activation of BMP-activated p38 MAPK pathways in neuronal cells (5).

A high level of overexpression of Smad6 or Smad7 could nonspecifically affect signaling pathways (2). In the present study, the expression levels of the Smad7 transgene were as much as those of endogenous Smad7 in the tissues surrounding cartilage as examined by in situ hybridization analysis (Fig. 6B). Although we con-

These results collectively suggest that controlled expression of Smad7 is crucial for normal cartilage development and homeostasis to sustain anabolic BMP/TGF-β signals.
 Roles of Smad7 in Chondrocyte Differentiation

The decreased expression of Hist2 in Smad7/11E10 mice proved that Smad7 overexpression inhibits cartilage formation because Smad7 transgene expression begins at the stage of round proliferative chondrocytes, allowing a normal transition from condensed mesenchyme to round chondrocytes in Smad7/11E10 mice. It was reported that the chondrocyte proliferation rate is decreased in Bmpr1b<sup>+</sup>/Bmpr1a<sup>CKO</sup>;Col2a1<sup>Cre</sup> mice (42). In contrast, the chondrocyte proliferation rate is normal in mice lacking TGF-β receptor type II in chondrocytes (22). The chondrocyte proliferation rate is normal in transgenic mice overexpressing Smad6 in chondrocytes, although the phosphorylation of Smad1/5/8 is down-regulated (21). These findings suggest that chondrocyte proliferation is controlled by BMP signals and Smad7, but not by TGF-β signals or Smad6. Because the activation of the p38 MAPK pathways decreases the chondrocyte proliferation rate (40), another pathway may mediate BMP-induced chondrocyte proliferation. This other pathway remains to be elucidated.

Smad7 in Flat Chondrocytes Delays Maturation toward Hypertrophy—Smad7/11Prom mice showed delayed chondrocyte hypertrophy. We previously reported that Smad6 overexpression inhibits BMP-induced chondrocyte hypertrophy associated with the inhibition of Smad1/5/8 phosphorylation (21). BMP signals generally stimulate chondrocyte hypertrophy (10, 43). However, the chondrocyte-specific deletion of Tgfr2 does not affect chondrocyte hypertrophy (22). p38 MAPK activity is required for chondrocyte hypertrophy (44). Combined with these findings, the present results raise possibilities that Smad7 overexpression may delay hypertrophy of flat chondrocytes by inhibiting the activation of Smad1/5/8 and p38 MAPK pathways. It remains to be determined whether Smad7 affects p38 MAPK pathways in differentiated chondrocytes.

In summary, we established a Smad7 conditional transgenic mouse system and found that Smad7 overexpression exerts functions at multiple stages of chondrocyte differentiation. Our results suggest that Smad7 inhibits cartilage formation, possibly by down-regulating BMP-activated p38 MAPK pathways. Smad7 is overexpressed in various pathological states, including cancers and inflammation. In joint diseases such as osteoarthritis, cartilage expresses Smad7 (15). In cultured articular chondrocytes, stimulation with the inflammatory cytokine interleukin-1β up-regulates Smad7 (14, 15). Although we should be cautious in interpreting our data, the findings of this Smad7 overexpression study should contribute to an understanding of the mechanism that interferes with the recovery of damaged cartilage despite treatment in the course of joint diseases.

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