SnoN Suppresses Maturation of Chondrocytes by Mediating Signal Cross-talk between Transforming Growth Factor-β and Bone Morphogenetic Protein Pathways*§

Received for publication, February 2, 2012, and in revised form, June 21, 2012 Published, JBC Papers in Press, July 5, 2012, DOI 10.1074/jbc.M112.349415

Ichiro Kawamura‡§, Shingo Maeda‡1, Katsuyuki Imamura‡§, Takao Setoguchi5, Masahiro Yokouchi3, Yasuhiro Ishidou1, and Setsuro Komiya‡5

From the ‡Department of Medical Joint Materials, §Department of Orthopedic Surgery, and the †Near-Future Locomotor Organ Medicine Creation Course, Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima University, Kagoshima 890-8544, Japan

Background: BMP signaling promotes chondrocyte maturation and, subsequently, endochondral ossification, whereas TGF-β signaling is inhibitory.

Results: TGF-β induced SnoN to suppress BMP signaling and chondrocyte hypertrophy.

Conclusion: SnoN mediates a signal cross-talk between TGF-β and BMP to regulate chondrocyte maturation.

Significance: Our data revealed an effector of TGF-β signaling as a putative therapeutic molecular target for cartilage/bone regeneration or osteoarthritis.

Hypertrophic maturation of chondrocytes is a crucial step in endochondral ossification, whereas abnormally accelerated differentiation of hypertrophic chondrocytes in articular cartilage is linked to pathogenesis of osteoarthritis. This cellular process is promoted or inhibited by bone morphogenetic protein (BMP) or transforming growth factor-β (TGF-β) signaling, respectively, suggesting that these signaling pathways cross-talk during chondrocyte maturation. Here, we demonstrated that expression of Tgfβ1 was increased, followed by phosphorylation of Smad2, during BMP-2-induced hypertrophic maturation of ATDC5 chondrocytes. Application of a TGF-β type I receptor inhibitor compound, SB431542, increased the expression of Id1, without affecting the phosphorylation status of Smad1/5/8, indicating that the activated endogenous TGF-β pathway inhibited BMP signaling downstream of the Smad activation step. We searched for TGF-β-inducible effectors that are able to inhibit BMP signaling in ATDC5 cells and identified SnoN. Overexpression of SnoN suppressed the activity of a BMP-responsive luciferase reporter in COS-7 cells as well as expression of Id1 in ATDC5 cells and, subsequently, the expression of Col10a1, a hallmark of hypertrophic chondrocyte maturation. siRNA-mediated loss of SnoN showed opposite effects in BMP-treated ATDC5 cells. In adult mice, we found the highest level of SnoN expression in articular cartilage. Importantly, SnoN was expressed, in combination with phosphorylated Smad2/3, in prehypertrophic chondrocytes in the growth plate of mouse embryo bones and in chondrocytes around the ectopically existing hypertrophic chondrocytes of human osteoarthritis cartilage. Our results indicate that SnoN mediates a negative feedback mechanism evoked by TGF-β to inhibit BMP signaling and, subsequently, hypertrophic maturation of chondrocytes.

Bone formation is achieved by either intramembranous or endochondral ossification. The former process is characterized by differentiation of mesenchymal cells directly into bone-forming osteoblasts. Endochondral ossification is initiated by condensation of mesenchymal cells expressing a chondrogenic master regulator, Sox9, after which cells further differentiate into chondrocytes that are able to express a specific marker Col2a1, encoding type II collagen (1). Then the committed chondrocytes proliferate and convert into hypertrophic chondrocytes to eventually mineralize the surrounding cartilage matrix to be replaced by bone (2). Hypertrophic chondrocytes are characterized by a round large cell body and the ability to express Col10a1, encoding type X collagen. This hypertrophic conversion process is, at least in part, governed by Runx2 protein (i.e. Runx2 directly activates the promoter of Col10a1 to promote chondrocyte hypertrophy) (3, 4). Mouse models in which dominant-negative Runx2 was overexpressed in chondrocytes showed suppressed chondrocyte hypertrophy, combined with complete loss of endochondral ossification (5). Conversely, forced expression of wild-type Runx2 in mouse chondrocytes resulted in accelerated differentiation of hypertrophic chondrocytes and bone formation (5, 6). Given that in permanent cartilage (e.g. normal articular cartilage on the joint space), chondrocytes do not undergo the late phase hypertrophic maturation, hypertrophic conversion of chondrocytes must be restricted to maintain a normal cartilage phenotype.

In addition to its indispensable role in physiological bone formation, the endochondral ossification process is a promising cellular event in the application of bone/cartilage regenerative medicine, which can be artificially engineered from human mesenchymal stem/stroma cells (7). Interestingly, these mesenchymal stem/stroma cells form bone trabeculae in vivo only
when they have developed hypertrophic structures in vitro before implantation (7), indicating that the efficiency of bone regeneration could be improved by promoting hypertrophic maturation of chondrocytes in vitro. In the case of cartilage regenerative medicine for treatment of cartilage defects, the maturation processes in engineered chondrocytes must be arrested because abnormally matured hypertrophic chondrocytes, expressing type X collagen, cause pathological conditions (e.g. osteoarthritis (OA)) (8–10). In vitro, mesenchymal stem/stroma cells rapidly express COL10A1 in monolayer or pellet culture before the cells demonstrate morphology of hypertrophic chondrocytes, a phenomenon that is a major problem of cartilage tissue engineering (11, 12), suggesting that the mechanism by which mesenchymal stem/stroma cells induce type X collagen is different from that in growth plate chondrocytes. Importantly, it is largely unknown how the expression of type X collagen is induced in degenerating articular chondrocytes.

Both BMP and TGF-β signaling promote early chondrogenesis. Members of the TGF-β family, including BMPs, transduce signals through type II and type I receptors to activate receptor-regulated Smads (R-Smads). Upon ligand binding, TGF-β type I receptors activate Smad2/3, whereas BMP type I receptors phosphorylate Smad1/5/8 in the cytoplasm. After forming a trimeric complex with Smad4 (co-Smad), R-Smads translocate into the nucleus to directly or indirectly regulate the transcription of target genes (13). Forced expression of an extracellular BMP-antagonist, Noggin (Nog), in chondrocytes in vivo showed no cartilage formation in transgenic mice (14). Similarly, cartilage-specific combined loss of BMP type I receptors (Bmpr1a and Bmpr1b) or double deletions of BMP-type R-Smads (Smad1 and Smad5) showed severely impaired chondrogenesis in mice (15, 16). These mouse models clearly demonstrated the critical roles of the BMP-Smad pathway in early chondrogenesis. TGF-β signaling promotes the early stage of chondrogenesis by enabling Smad3 to form an active transcriptional complex with CEBP/p300 and Sox9 (17). However, at the late maturation stage, BMP and TGF-β signaling show mutually opposite roles in chondrocyte hypertrophy. BMP signaling directly accelerates the expression of Col10a1 in concert with Runx2 (18–20), whereas the chondrocyte-specific expression of constitutively active Bmpr1a promoted the maturation and hypertrophy of chondrocytes in transgenic mice (21). An intraarticular injection of BMP-2 in mice accelerated cartilage chondrocyte hypertrophy and endochondral ossification to form osteophytes, a phenotype of OA (22). In contrast, loss of TGF-β signaling in the cartilage of mice, achieved by targeted ablation of the Smad3 gene or forced expression of the dominant negative TGF-β type II receptor, resulted in accelerated differentiation of chondrocytes into hypertrophy coupled with an OA-like destruction of cartilage (23, 24). Similarly, chondrocyte-specific transgenic mice of Smurf2, an E3 ubiquitin ligase of the negative regulator for TGF-β signaling, also showed an OA-like change in articular cartilage with ectopic hypertrophic chondrocytes (25). These mouse models indicated an indispensable role of TGF-β signaling in preventing chondrocyte hypertrophy in articular cartilage, although the underlying molecular mechanisms are unclear. Interestingly, Smad3-deficient chondrocytes showed enhanced BMP signaling and accelerated hypertrophic differentiation in vitro, suggesting a role of endogenous TGF-β signaling in suppressing BMP signaling during chondrocyte maturation (26). Taken together, promoting TGF-β signaling in maturing chondrocytes is a promising candidate approach to manipulate the differentiation to fine tune chondrocyte hypertrophy. However, because TGF-β signaling regulates a wide range of pivotal biological functions (e.g. cell growth, differentiation, motility, extracellular matrix production, and apoptosis) in various target cells, blocking of the entire TGF-β signaling pathway might result in undesirable side effects. Therefore, to eliminate these problems, the direct mediator(s) induced by TGF-β signaling to inhibit BMP signaling in maturing chondrocytes should be an appropriate molecular target. Here, we show that SnoN is as a spatial and temporal effector of TGF-β signaling to inhibit the BMP-Smad pathway and, subsequently, chondrocyte hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The chondrogenic cell line ATDC5 was obtained from RIKEN BioResource Center. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (1:1) (Invitrogen) containing 5% fetal bovine serum (FBS) and 100 units/ml penicillin G and 100 μg/ml streptomycin. Differentiation of ATDC5 cells was induced in serum-free medium containing insulin/transferrin/selenium supplement (Sigma) on collagen type I-coated culture plates (Iwaki). For expression analysis of differentiation markers in ATDC5 cells, micromass culture was performed as previously described (27), to accelerate maturation of chondrocyte differentiation. A monolayer culture system was employed for analysis of phosphorylation of Smads by immunoblotting because the cell lysis of micromass culture for cytoplasmic/nuclear protein was less efficient, and in addition, these experiments were not intended to evaluate the chondrogenic differentiation of early time points but rather the intracellular signaling. Monolayer culture was also performed for immunocytochemistry because of the difficulty in identifying individual cells in the micromass culture. Primary chondrocytes were harvested from 4-day-old mice of C57BL/6J background as described (28). Primary chondrocytes were induced to differentiate in a monolayer culture. COS-7 cells were purchased from the RIKEN BioResource Center and maintained in DMEM supplemented with 10% FBS and antibiotics.

**Ligands and Inhibitors**—BMP-2 (Peprotech) was applied at a concentration of 300 ng/ml, whereas TGF-β1 (Peprotech) was used at 5 ng/ml. The day of the first ligand application was referred to as day 0. SB431542 (Sigma) was applied at 1 μM for 30 min prior to ligand stimulation, and DMSO was used as a mock control. When indicated, cells were treated with a proteasome inhibitor, MG132 (Merck), at 10 μM 12 h prior to cell lysis.

**Bone Organ Culture**—Metatarsal bone rudiments were harvested from C57BL/6J embryos at 17.5 days postcoitum (E17.5) and cultured in DMEM supplemented with 10% FBS, 100
units/ml penicillin G, and 100 μg/ml streptomycin, as described (29). Cultured bones were stained with Alcian blue and alizarin red dyes according to a standard protocol for skeletal preparation. Briefly, bones fixed in 96% ethanol were stained with 0.015% Alcian blue 8G (Sigma) in a mixture solution of 96% ethanol, acetic acid (4:1) for 1 day, followed by a dehydration step in 100% ethanol. Dehydrated bones were immersed briefly in 1% potassium hydroxide (KOH), followed by staining in 0.001% alizarin red S (Sigma) in 1% KOH for 1 day. Images were analyzed using ImageJ software (National Institutes of Health). Animal experiments were approved by the Institutional Animal Care and Use Committee of Kagoshima University (MD11019).

RNA Interference—Dharmacon siRNA ON-TARGETplus SMARTpool, a mixture of four independent siRNAs of mouse SnoN (Skil), and the control reagent were purchased from Thermo Scientific. siRNA was transfected into cells using Lipofectamine RNAiMax (Invitrogen). Ligands and SB431542 were added to the culture simultaneously after an overnight transfection of siRNA.

Plasmids—9xCAGA luc, BRE luc, ALK3TD, ALK3QD, and FLAG-tagged human SnoN in pcDEF3 were kind gifts from Dr. Kohei Miyazono (University of Tokyo). pGL4.75hRlucCMV was purchased from Promega. cDNA of mouse SnoN was cloned from ATDC5 by employing a reverse transcription-polymerase chain reaction (RT-PCR)-based technique, subcloned into the entry vector, pENTR, and further transferred into pEFDEST51 by attL-attR (LR) recombination (Invitrogen). Flag ligands and SB431542 were added to the culture simultaneously after an overnight transfection of siRNA.

Lentiviruses—pENTR-SnoN and pENTR-5’EF1αP were subjected to LR recombination with pLent6.4/R4R2/V5-DEST (Invitrogen) to generate a lentiviral vector, which expresses C-terminal V5-tagged SnoN from the EF1α promoter. The lentiviral expression vector or pLent6/V5/GW-lacZ control vector was transfected into 293FT cells to generate lentivirus. The viral expression vector or pLenti6/V5/GW-lacZ control vector was further transferred into pEFDEST51 by attL-attR (LR) recombination (Invitrogen).

Immunoblotting—Cells were lysed in either M-PER lysis buffer (Thermo Scientific) supplemented with aprotinin, sodium orthovanadate, and phenylmethylsulfonyl fluoride (PMSE) or directly with 1× SDS sample buffer. SDS-PAGE, membrane transfer, and chemiluminescence were performed by using a standard protocol. Blots were incubated with anti-SnoN (1:200, H-317, sc-9142, Santa Cruz Biotechnology, Inc.), anti-phospho-Smad1/5/8 (1:1000, catalog no. 9511), anti-phospho-Smad2 (1:1000, catalog no. 3108), anti-Smad1 (1:1000, catalog no. 9743), horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody and anti-mouse secondary antibody (1:10,000) (Cell Signaling), anti-Smad2/3 (1:1000, catalog no. 610842, BD Biosciences), anti-tubulin (1:1000, DM1A, T9026, Sigma), and anti-Mmp13 (1:1000, ab58836, Abcam). Signals were detected using the LAS 4000 mini image analyzer (Fujifilm).

Luciferase Assay—COS-7 cells were seeded in triplicate in 24-well plates and transiently transfected with firefly reporter constructs, pGL4.75hRlucCMV Renilla vector, constitutively active type I receptor constructs, and the SnoN vector. Dual luciferase assays were performed as described (30) using the GloMax 96 microplate luminometer (Promega).

Statistical Analysis—Data in this study are expressed as the mean ± S.D. of at least three independent experiments. A p value of <0.05, which was determined by Student’s t test, was accepted as statistically significant.

RESULTS

Endogenous TGF-β Signaling Suppresses Terminal Chondrocyte Maturation in Bone—To identify physiologically active target genes of TGF-β signaling in chondrocytes, we employed


TGF-β-SnoN Axis Prevents Maturation of Chondrocytes

A

B

C

D

E

FIGURE 1. Endogenous TGF-β signaling prevents maturation of chondrocytes in bone organ culture. Metatarsal bones from E17.5 mouse embryo were cultured with BMP-2 (B2; 300 ng/ml) or TGF-β1 (Tβ1; 5 ng/ml) in combination with SB431542 (SB; 1 μM) for 4 days. A, cartilage matrix was stained with Alcian blue, whereas chondrocyte matrix calcified by mature hypertrophic chondrocytes was stained by alizarin red. The clear zone represented uncalcified hypertrophic chondrocytes (Fig. 1A). Upon treatment with BMP-2, matrix calcification was induced, whereas coapplication of TGF-β/H9252 confirmed the inhibitory action of endogenous TGF-β signaling in chondrocyte hypertrophy and maturation in bone rudiments.

Next, to investigate cell-autonomous actions of endogenous TGF-β signaling in BMP-induced maturation of chondrocytes, we applied SB431542 in a chondrocyte micromass culture, which is a system widely employed to study the multiple steps of cartilage differentiation (31). Upon treatment with BMP-2 in the presence of insulin/transferrin/selenium supplements, expression of Col2a1 and Col10a1 was induced in ATDC5 cells, as reported previously (32), whereas combined application of SB431542 mildly suppressed the level of Col2a1 mRNA, suggesting the promotive role of TGF-β signaling in early chondrogenesis (Fig. 2A). In contrast, BMP-induced expression of Col10a1 was further enhanced by SB431542 in ATDC5 cells as well as dramatically enhanced in mouse primary chondrocytes (Fig. 2A). Whereas treatment with BMP-2 accelerated the production of the type X collagen protein (encoded by Col10a1) in ATDC5 cells, SB431542 further strengthened the signal (Fig. 2B). The fluorescent signal of immunocytochemistry against type X collagen was significantly increased by SB431542 treatment (Fig. 2B). Error bars, S.D.
ment (Fig. 2C). Moreover, the expression of Mmp13, encoding the collagenase matrix metalloproteinase 13 specifically expressed by the terminal hypertrophic chondrocytes, was dramatically up-regulated by blockade of the TGF-β signaling pathway at both mRNA and protein levels (Fig. 2, D and E). These data demonstrate the cell-autonomous inhibitory action of endogenous TGF-β signaling in the late stage of chondrocyte differentiation.

Endogenous TGF-β Signaling Is Up-regulated during Maturation of Chondrocytes—Because SB431542 affected the late stage of chondrocyte differentiation, we assessed if the expression of any of the three TGF-β ligands was increased during BMP-induced maturation of ATDC5 chondrocytes. Although the mRNA levels of Tgfb2 and Tgfb3 were marginally changed by application of BMP-2 or SB431542, Tgfb1 (encoding TGF-β1) was dramatically increased from day 4 and further remained up-regulated by BMP-2 treatment, whereas it was not affected by SB431542 (Fig. 3A). Because TGF-β ligands transduce the signal through TGF-β type I receptor ALK5, encoded by Tgfr1, we asked if expression of Tgfr1 was accelerated in maturing ATDC5 cells. However, the expression level of Tgfr1 was unchanged by BMP-2 application (Fig. 3A). Therefore, we
evaluated if increased Tgfβ1 expression reflected intracellular signal transduction (Fig. 3B). As a positive control, exogenously applied TGF-β1 potently induced the C-terminal phosphorylation of Smad2, which was weakened by SB431542. Importantly, in BMP-2-treated ATDC5 chondrocytes, Smad2 was phosphorylated at a comparatively weak but detectable level, which was suppressed by SB431542 (Fig. 3B). In addition, an immunocytochemistry for phosphorylated Smad2 in ATDC5 cells showed that endogenous TGF-β signaling was activated in BMP-2-treated chondrocytes, which was blocked by SB431542 (Fig. 3C). These data indicated that the activation of Smad2 by BMP-2 was achieved through the TGF-β type I receptor, probably by the up-regulated production of TGF-β1 in ATDC5 cells. In developing humerus growth plate of E17.5 mouse embryo, moderate expression of TGF-β1 was detected in proliferative chondrocytes, suggesting its role in promoting early chondrogenesis (Fig. 3D). Importantly, the expression of TGF-β1 was further accentuated in the prehypertrophic zone and the matrix around the hypertrophic chondrocytes (Fig. 3D). Similarly, Smad2 was phosphorylated in some of the proliferative chondrocytes, whereas phospho-Smad2 was detected in most of the prehypertrophic chondrocytes (Fig. 3D). Interestingly, Smad2 was not phosphorylated in hypertrophic chondrocytes. These results from immunohistochemistry suggested that expression of TGF-β1 was elevated to up-regulate TGF-β signaling in prehypertrophic chondrocytes, whereas the signaling was inhibited with in hypertrophic chondrocytes, in the endochondral ossification process.

Because Smad3 signaling had been suggested to suppress BMP pathway in maturing chondrocytes (26), we examined immunoblotting for the phosphorylated Smad1/5/8 to investigate the role of endogenous TGF-β signaling against the BMP-Smad signaling system in chondrocytes. BMP-2 induced potent C-terminal phosphorylation of Smad1/5/8 in ATDC5 cells, even at day 4 of induction, whereas combined treatment with SB431542 showed no effect (Fig. 3E). However, interestingly, the expression of Id1, a specific direct target of the canonical BMP-Smad pathway (33), was significantly elevated by SB431542 treatment, whereas it was suppressed by exogenously applied TGF-β1 (Fig. 3F). These data suggest that BMP-induced activation of endogenous TGF-β signaling inhibited BMP signaling downstream of R-Smad activation as a negative feedback mechanism. These data also confirmed that SB431542 is an appropriate tool to screen for putative molecular mediator(s) downstream of endogenous TGF-β signaling to inhibit BMP signaling in chondrocytes.

SnoN Is Induced by Endogenous TGF-β Signaling in Maturing Chondrocytes—The BMP signaling system is negatively regulated at multiple steps (e.g. by extracellular antagonists (e.g. Noggin, Chordin, Dan, and Cerberus), inhibitory Smads (I-Smads; Smad6 and Smad7), E3 ubiquitin ligases (e.g. Smurf1 and Smurf2), and the Ski/SnoN family of transcriptional corepressors) (34, 35). We asked if any of these BMP signaling inhibitors were induced downstream of endogenous TGF-β signaling in maturing chondrocytes. In micromass cultures of ATDC5 chondrocytes, treatment with TGF-β1, as positive control, significantly elevated only the expression of Smad7 and SnoN, both of which are the direct target genes of the TGF-β-Smad pathway (supplemental Fig. 1). In BMP-2-induced maturing cells, Smad6, Smad7, and SnoN were up-regulated, whereas combined treatment with SB431542 completely inhibited only the elevation of SnoN (supplemental Fig. 1). In mouse primary chondrocytes at day 14 of BMP treatment, SnoN was also significantly elevated; this effect was not observed by combined application with SB431542 (Fig. 4A). These data indicate that, among the examined BMP inhibitors, SnoN is exclusively induced by the enhanced endogenous TGF-β signaling in maturing chondrocytes. During the maturation phase of ATDC5 chondrocytes, the level of SnoN mRNA was increased from days 4 to 10 of BMP application and mildly decreased thereafter, whereas this effect was completely prevented by SB431542 (Fig. 4B). Because SnoN is an unstable protein, which has a half-life of 30 min in the presence of TGF-β signaling (36, 37), we asked if the protein level of SnoN reflects the pattern of its mRNA expression and performed an immunoblot for SnoN protein (Fig. 4C). As control to identify specific bands for endogenous SnoN protein, we performed a knockdown assay against SnoN in monolayer cultures of ATDC5 cells (Fig. 4C, lanes 5–7). At day 3, the signals of two major bands of around 80 kDa were increased in BMP-2-treated cells, both of which were efficiently abolished by siSnoN, indicating that these bands represented the two isoforms, SnoN and SnoN2 (38). In micromass cultures of ATDC5 chondrocytes at day 7 of BMP-2 application, SnoN protein expression was also induced and further weakened by SB431542 (Fig. 4C, lanes 1–4), indicating that the expression of the SnoN protein was indeed induced by endogenous TGF-β signaling in maturing chondrocytes. We also confirmed the induction of SnoN protein by immunocytochemistry. Treatment with TGF-β1 for 16 h potently increased the signal of SnoN protein, which was only faintly detected in cells transfected with SnoN siRNA (Fig. 4D). At day 4 of BMP-2 stimulation, the level of SnoN protein was up-regulated, which was weakened by siSnoN, in maturing ATDC5 chondrocytes of monolayer cultures (Fig. 4E). The knockdown efficiency of SnoN in BMP-2-treated cells was relatively weak, probably because 5 days had passed since the transfection of siRNA. Next, to confirm that the inhibitory effect of SB431542 against the expression level of SnoN was exclusive for the late maturation phase, we stimulated ATDC5 cells with BMP-2 in combination with SB431542 and harvested the cells at the early time points within 36 h of induction (Fig. 4F). To our surprise, SnoN was rapidly induced by BMP-2 after 1 h and was decreased to reach the basal level thereafter, an expression pattern that was similar to that of Id1 (Fig. 4F). However, this transient induction of SnoN by BMP-2 was not affected by SB431542, suggesting that endogenous TGF-β signaling was not responsible for this up-regulation of SnoN.

To investigate the possibility that SnoN plays a role in chondrogenesis in vivo, its expression in cartilage tissue was examined. We extracted RNA from various tissues of 3-month-old adult mice to prepare tissue panels of cDNA to analyze the tissue distribution. Consistent with the report that heterozygous SnoN knock-out mice developed T lymphomas in the spleen, which indicates an important role of SnoN in the spleen (39), we detected the highest level of SnoN expression in the spleen (Fig. 4G). Interestingly, the comparatively highest level
of SnoN expression was found in articular cartilage (Fig. 4G). Immunohistochemistry for the growth plate of developing bone of mouse embryos at E17.5, in which endochondral ossification was in progress, detected the signal of type X collagen protein from the lower half of prehypertrophic chondrocytes to the entire area of hypertrophic chondrocytes (Fig. 4H). Importantly, although SnoN protein was detected weakly in proliferating chondrocytes, its potent signal was present in prehypertrophic chondrocytes, whereas it was weakened in hypertrophic chondrocytes (Fig. 4H). These data suggest that SnoN is highly expressed in premature chondrocytes before hypertrophic conversion, in vitro and in vivo.
SnoN suppresses the BMP-Smad Signaling Pathway to Inhibit Hypertrophic Maturation of Chondrocytes—SnoN interacts with Smad2, Smad3, and Smad4 in the cytoplasm to prevent their nuclear translocation (40, 41). In the nucleus, it represses their transcriptional activity by disrupting the active trimeric Smad complex and recruiting transcriptional corepressors (36), thereby negatively regulating TGF-β signaling. It is not known whether SnoN suppresses canonical BMP-Smad signaling in the same manner as the related family molecule c-Ski (42); however, SnoN may inhibit the pathway because of its ability to bind Smad4, although it cannot bind Smad1/5/8 (43). To test this hypothesis, SnoN was investigated using a BRE luciferase reporter specifically responding to the BMP-Smad pathway (33) in COS-7 cells. As a positive control for the SnoN function, SnoN was applied to the 9xCAGA TGF-β signaling reporter (44), activated by constitutively active TGF-β type I receptor ALK5, to show a dose-dependent potent inhibition (Fig. 5A, left). Interestingly, SnoN did suppress the activity of the BMP-Smad signaling reporter (Fig. 5A, right).}

**FIGURE 5.** SnoN suppresses BMP signaling and subsequent chondrocyte maturation. A, SnoN expression vector was transfected with plasmids of constitutively active type I receptors and a luciferase reporter of 9xCAGA luc (for TGF-β signaling) or BRE luc (for BMP signaling) into COS-7 cells. Expression of transfected SnoN was confirmed by anti-SnoN immunoblot. Tubulin served as loading control. B, FLAG-tagged SnoN expression vector was stably transfected into ATDC5 chondrocytes, and its expression was confirmed by anti-SnoN immunoblotting. C, SnoN suppressed expression of Id1 as well as Col10a1 at day 7 of BMP-2 (300 ng/ml) treatment in a stable transfectant of ATDC5 chondrocytes. D and E, metatarsal bones of E17.5 mouse embryo were infected with indicated lentivirus (LV) for 16 h. Immunostaining using FITC-linked anti-V5 antibody on bone coronal sections was performed at day 2 of culture to evaluate the efficiency of lentiviral infection. Nuclei were stained with Hoechst dye. Merged images are presented. LV-LacZ-V5 served as a lentiviral infection control (D). Scale bars, 200 μm. Alcian blue/alizarin red staining was performed at day 2 of BMP-2 treatment (E). The arrowheads indicate the calcified cartilage matrix in bone rudiments of triplicate culture. *, p < 0.05. Error bars, S.D.
the BRE reporter induced by the constitutively active type I BMP receptor ALK3 (encoded by Bmpr1a) (Fig. 5A, right) in a dose-dependent manner, although more than 3 times the amount of SnoN plasmid DNA was required for the inhibition compared with the reporter assay using 9xCAGA. Next, we stably transfected a FLAG-tagged SnoN expression vector into ATDC5 cells and confirmed the transgene expression by anti-SnoN immunoblotting (Fig. 5B). In ATDC5 chondrocytes, gain of SnoN suppressed the elevated expression of Id1 at day 7 of BMP-2 induction (Fig. 5C, left). The increased expression of the hypertrophic marker Col10a1 was also down-regulated by SnoN in BMP-treated ATDC5 cells (Fig. 5C, right). Thus, forced expression of SnoN in ATDC5 chondrocytes inhibited BMP signaling and, subsequently, chondrocyte maturation. To investigate the role of SnoN in cartilage maturation, we infected lentivirus carrying V5-tagged SnoN expression cassette into E17.5 metatarsal bone rudiments. We confirmed the infection efficiency of the lentivirus by performing immunohistochemistry on the coronal sections of the bones, using anti-V5-FITC antibody to detect the transgene product (Fig. 5D). In lentivirus-infected bones, we found the certain expression of V5-tagged transgenes in the cells of perichondrium and the chondrocytes in zones of reserve, proliferative, and prehypertrophic but not hypertrophic chondrocytes. Treatment of BMP-2 induced calcification of the hypertrophic zone of bone rudiments (Fig. 5E, arrowheads), whereas overexpression of SnoN by lentivirus completely prevented the cartilage mineralization (Fig. 5E). These data demonstrate that gain of SnoN inhibits the maturation of the hypertrophic chondrocytes.

Next, we investigated the physiological role of endogenous SnoN in ATDC5 chondrocytes by performing an siRNA-mediated knockdown assay. We confirmed that SnoN protein expression was induced upon BMP-2 induction, even at the early time points, and that the two bands (representing SnoN and SnoN2) could be efficiently eliminated by siSnoN (Fig. 6A). BMP-2 induced a potent phosphorylation of Smad1/5/8, whereas siSnoN showed no effect on the phosphorylation level, even after the phosphorylation declined at 8 h (Fig. 6A). However, knockdown of SnoN mildly, but significantly, enhanced the BMP-2-induced increase of the Id1 gene at day 4 in ATDC5 cells (Fig. 6B). siSnoN further significantly enhanced the BMP-induced up-regulation of Col10a1 as well as alkaline phosphatase (Alp), another marker of matured hypertrophic chondrocytes (Fig. 6C). These data for the SB431542-mimicking effects of siSnoN (Figs. 2 and 3) indicated that SnoN mediated a major part of the roles of endogenous TGF-β signaling in chondrocytes to physiologically suppress BMP signaling and subsequent chondrocyte hypertrophic maturation without affecting the activating step of Smad1/5/8.

TGF-β Signaling and Expression of SnoN Are Accentuated in “Prehypertrophic” Chondrocytes Adjacent to Pathologically Hypertrophic Chondrocyte in Moderately Affected OA Cartilage—Given the role of SnoN in the prevention of hypertrophic conversion of chondrocytes, SnoN protein may be present before pathologic chondrocytes gained hypertrophic phenotype in OA cartilages (8, 9). We performed immunohistochemistry assays for SnoN in human OA cartilages of various severities. In normal adult human femoral head cartilage, expression of type X collagen was entirely absent, whereas SnoN protein was weakly detected in the superficial zone (Fig. 7A). In moderate OA cartilage, in which the severity was graded as 6 according to the modified Mankin score (45, 46), type X collagen-positive pathologically hypertrophic chondrocytes were located in the upper layer of degenerated cartilage, which was poorly stained by Safranin O. Strikingly, we detected potent signals for SnoN protein in chondrocytes in which the cell body was mildly enlarged, resembling “prehypertrophic” chondrocytes in the developing bone. These SnoN-positive cells formed colonies located in close proximity of the colonies of hypertrophic chondrocytes (Fig. 7A). In severe OA cartilage of a grading score of 10, the cartilage surface of which was entirely destroyed, a few colonies of pathologically hypertrophic chondrocytes expressing type X collagen were present; however, we could no longer detect SnoN-positive cells (Fig. 7A). These data are consistent with our hypothesis that SnoN prevents the progression of hypertrophic conversion of articular chondrocytes. Finally, we asked if this enhanced expression of SnoN protein was associated with TGF-β signaling in human OA cartilage, similarly as observed in the growth plate of mouse embryo (Figs. 3D and 4H). In moderate OA cartilage, an immunofluorescence assay revealed the abundant accumulation of TGF-β1 protein around the “prehypertrophic” chondrocytes (Fig. 7B). Moreover, phosphorylated active Smad3 was detected in these pathologic chondrocytes. Again, SnoN protein was also expressed in chon-
TGF-β-SnoN Axis Prevents Maturation of Chondrocytes

Endogenous TGF-β Signaling Is Activated in Maturing Chondrocytes to Induce SnoN—Although the importance of TGF-β signaling in preventing hypertrophic conversion of chondrocytes has been demonstrated (23, 24, 47), the mode of TGF-β signaling during chondrocyte maturation and its direct target gene(s) responsible for inhibiting the differentiation remain largely unknown. While exogenously applied TGF-β has been reported to have a positive role in early chondrogenesis in vitro, SB431542 showed no effect on bone growth, whereas it dramatically enhanced the matrix calcification by the mature hypertrophic chondrocytes in a bone organ culture system (Fig. 1). This result suggested that endogenous TGF-β signaling was more active in the late stage of maturing chondrocytes than in the early stage of chondrocyte differentiation. Indeed, expression of Tgfb1 was up-regulated in the late phase of differentiation of ATDC5 chondrocytes (Fig. 3A), coupled with an increase in phosphorylation of Smad2, which was inhibited by SB431542 (Fig. 3, B and C). This was completely linked to the expression pattern of SnoN (Fig. 4, A–C). SnoN expression was probably directly induced by endogenous TGF-β signaling because SnoN had been shown to be directly induced by TGF-β-activated Smad2 through its binding to the Smad-binding element in the promoter of the SnoN gene (48). Interestingly, in Smad3-deficient chondrocytes, the expression of TGF-β1, TGF-β type 1 receptor ALK5 (Tgfr1), and SnoN was suppressed, whereas the expression of Smad1, Smad5, Smad6, BMP-2, and BMP-6 was elevated (26), suggesting SnoN to be one of the downstream targets of TGF-β signaling in chondrocytes. For the first time, we demonstrated the rapid and transient induction of SnoN by exogenously applied BMP ligand, whereas it was not suppressed by SB431542 (Fig. 4F). The SnoN expression pattern in the early phase, which resembled that of Id1 (Fig. 4F), led us to hypothesize that the BMP-Smad pathway, in addition to TGF-β-Smad signaling, can directly induce SnoN in a context-dependent manner. Indeed, the sequences of GGCACC or GGCGCG, both of which contain a 1-base mismatch with the BMP-Smad-responsive motif GGCGCC (33), can be found adjacent to three TGF-β-responsive CAGA Smad-binding elements in the SnoN promoter (48). Further experiments are required to resolve this hypothesis and to evaluate the roles of BMP-Smad-induced SnoN.

SnoN Interferes with BMP Signaling to Suppress Hypertrophic Differentiation of Chondrocytes—The Sno gene (whose name derived from “Ski-related novel gene”) was initially discovered on the basis of its close homology to v-Ski and c-Ski. Ski proteins can suppress TGF-β signaling, as well as BMP signaling, by binding to R-Smads and Smad4. Interestingly, although c-Ski can interact weakly with Smad1/5, the strong interaction with Smad4 is indispensable for suppression of BMP signaling by c-Ski (42). Although SnoN interacts with Smad4, the role of SnoN in the context of BMP signaling has not been well investigated. We showed here that SnoN suppressed BMP signaling in a comparative but slightly weaker manner compared with TGF-β signaling (Fig. 5A). This weaker suppression of BMP signaling is probably due to the difference in the accessibility to Smads (i.e. SnoN could bind to Smad2/3 and Smad4 but not to Smad1/5, unlike c-Ski) (43). Loss of SnoN mildly enhanced the expression of Id1, whereas it did not affect the phosphorylation level of Smad1/5/8 (Fig. 6, A and B), supporting the notion that the functional molecular target of SnoN was not the phosphor-
ylation step of Smad1/5/8 but rather Smad4. Because siSnoN and SB431542 showed an essentially similar effect with regard to phospho-Smad1/5/8 and Id1 expression (compare Fig. 3, E and F, and Fig. 6, A and B), SnoN seems to be one of the major mediators of TGF-β signaling to inhibit BMP signaling in chondrocytes. This is interesting because we previously reported a similar negative feedback mechanism regulated by signal cross-talk between TGF-β and BMP signaling in osteoblast differentiation, in which I-Smads were induced by endogenous TGF-β signaling to inhibit BMP signaling in the maturation phase (49). However, the expression of I-Smads was not dramatically affected by SB431542 in ATDC5 chondrocytes (supplemental Fig. 1), suggesting context-dependent mechanisms in the selection of TGF-β target genes in these mesenchymal cells. The effect of forced expression, or knockdown, of SnoN showed more dramatic changes in the Col10a1 expression than in that of Id1 (Figs. 5C and 6C). The persistently mild suppression of BMP signaling by SnoN during the maturing phase may account for the major inhibition of hypertrophic maturation of chondrocytes.

Expression of SnoN Is Associated with the Enhanced TGF-β Signaling in Prehypertrophic Chondrocytes in Mouse Developing Bone and Human OA Cartilage—Maintenance of the articular cartilage depends on the function of articular chondrocytes, which produce cartilage matrix and are constrained from undergoing the maturation program seen in growth plate chondrocytes of developing bone. Genetic association of single nucleotide polymorphisms (SNPs) in the SMAD3 or ASPN gene (encoding asporin) with human OA has been reported (50, 51). Asporin was shown to bind TGF-β ligands on the cell surface to block their signal transduction (52). In articular cartilage of old mice, decreased expression of TGF-β ligands and receptors, coupled with strongly dropped phosphorylation of Smad2, was observed (53). Moreover, as mentioned in the Introduction, mouse models of loss of TGF-β signaling showed an OA phenotype with accelerated hypertrophic conversion of chondrocytes. These lines of evidence clearly demonstrate the importance of TGF-β signaling in preventing OA change of articular chondrocytes. To date, the mechanism by which TGF-β signaling regulates this process is largely unknown. One candidate for targeting TGF-β signaling is Smurf2, a protein whose expression was up-regulated in human OA cartilage, whereas forced expression of Smurf2 in chondrocytes developed OA change in joints of the transgenic mice (25). Because Smurf2 is a TGF-β-inducible molecule to inhibit TGF-β signaling, it may mediate the effect of TGF-β signaling in chondrocyte hypertrophy. However, in Smad3-deficient chondrocytes, which showed enhanced BMP signaling and accelerated maturation, expression of Smurf2 was not affected (26). Similarly, in our ATDC5 micromass culture system, Smurf2 was not elevated in maturing ATDC5 chondrocytes stimulated by BMP-2 (supplemental Fig. 1). In contrast, we detected an increase of SnoN expression, not only in both maturing ATDC5 cells and primary chondrocytes in vitro but also in prehypertrophic chondrocytes in mouse developing bone and in human OA cartilage (Figs. 4 and 7). Although we demonstrated the inhibitory role of SnoN in chondrocyte hypertrophy in vitro (Figs. 5 and 6), hypertrophy of chondrocytes was present in both developing normal mouse cartilage and degenerating human OA cartilage, despite the accentuated expression of SnoN in contiguous prehypertrophic chondrocytes (Figs. 4H and 7). In mouse E17.5 bone, phosphorylation of Smad2 and expression of SnoN were diminished in hypertrophic chondrocytes despite the abundant accumulation of TGF-β1 protein in the extracellular matrix (Figs. 3D and 4H), suggesting a disorder of the signal transduction by unknown mechanisms to be resolved. Similarly, in human OA cartilage, expression of SnoN was weakened in colonies of COL X-positive hypertrophic cells (Fig. 7A). Taken together, it is likely that the hypertrophic conversion was induced after the decline of SnoN expression in these chondrocytes. In this regard, further investigation is required to understand why the transduction of TGF-β signaling and subsequent expression of SnoN are diminished in hypertrophic chondrocytes. Although our results do not establish an etiologic role for SnoN in the progression of OA, they at least establish a strong association that explains a possible causal relationship between SnoN regulation and hypertrophic conversion of chondrocytes in OA cartilage. Loss of SnoN in mice in vivo should reveal its putative roles in cartilage hypertrophy or endochondral ossification; however, SnoN knock-out mice showed embryonic lethality at E3.5 (39). Therefore, a conditional knock-out mouse line of chondrocyte-specific ablation of the SnoN gene would be suitable to address these questions. In conclusion, our data revealed a novel role of SnoN in regulating BMP signaling and subsequent chondrocyte maturation as a downstream mediator of TGF-β signaling. SnoN may be targeted in chondrocytes to inhibit its function in order to accelerate endochondral ossification or to enhance its activity in the case of treating OA or cartilage regeneration for repair.

Acknowledgment—We gratefully acknowledge the technical assistance of Hui Gao.

REFERENCES
1. Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedd, A., and de Crombrugghe, B. (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 16, 2813–2828
2. Provot, S., and Schipani, E. (2005) Molecular mechanisms of endochondral bone development. Biochem. Biophys. Res. Commun. 328, 658–665
3. Enomoto, H., Enomoto-Iwamoto, M., Iwamoto, M., Nomura, S., Himeno, M., Kitamura, Y., Kishimoto, T., and Komori, T. (2000) Cbfa1 is a positive regulatory factor in chondrocyte maturation. J. Biol. Chem. 275, 8695–8702
4. Zheng, Q., Zhou, G., Morello, R., Chen, Y., Garcia-Rojas, X., and Lee, B. (2003) Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. J. Cell Biol. 162, 833–842
5. Ueta, C., Iwamoto, M., Kanatani, N., Yoshida, C., Liu, Y., Enomoto-Iwamoto, M., Ohmori, T., Enomoto, H., Nakata, K., Takada, K., Kurisu, K., and Komori, T. (2001) Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J. Cell Biol. 153, 87–100
6. Takeda, S., Bonnamy, J. P., Owen, M. J., Ducy, P., and Karsenty, G. (2001) Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev. 15, 467–481
7. Scotti, C., Tonnarelli, B., Papadimitropoulos, A., Scherberich, A., Schae ren, S., Schauerte, A., Lopez-Rios, J., Zeller, R., Barbero, A., and Martin, I.
TGF-β-SnoN Axis Prevents Maturation of Chondrocytes

(2010) Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proc. Natl. Acad. Sci. U.S.A. 107, 7251–7256

8. Pullig, O., Weseloh, G., Ronneberger, D., Käkönen, S., and Swoboda, B. (2000) Chondrocyte differentiation in human osteoarthritis. Expression of osteocalcin in normal and osteoarthritic cartilage and bone. Calcif. Tissue Int. 67, 230–240

9. Sandell, L. J., and Aigner, T. (2001) Articular cartilage and changes in arthritis. An introduction. Cell biology of osteoarthritis. Arthritis Res. 3, 107–113

10. Dreier, R. (2010) Hypertrophic differentiation of chondrocytes in osteoarthritis. The developmental aspect of degenerative joint disorders. Arthritis Res. Ther. 12, 216

11. Nelea, V., Luo, L., Demers, C. N., Antoniou, J., Petit, A., Leroure, S., R Werthermeier, M., and Mwale, F. (2005) Selective inhibition of type X collagen expression in human mesenchymal stem cell differentiation on polymer substrates surface-modified by glow discharge plasma. J. Biomed. Mater. Res. A 75, 216–223

12. Sekiya, I., Vouriotou, J. T., Larson, B. L., and Prockop, D. J. (2002) In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc. Natl. Acad. Sci. U.S.A. 99, 4397–4402

13. Miyazono, K., Kamiya, Y., and Morikawa, M. (2010) Bone morphogenic protein receptors and signal transduction. J. Biochem. 147, 35–51

14. Tsumaki, N., Nakase, T., Miyaji, T., Kakiuchi, M., Kimura, T., Ochi, T., and Yoshikawa, H. (2002) Bone morphogenic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis. J. Bone Miner. Res. 17, 898–906

15. Yoon, B. S., Ovchinnikov, D. A., Yoshi, I., Mishina, Y., Behringer, R. R., and Lyons, K. M. (2005) Bmp1r1a and Bmp1r1b have overlapping functions and are essential for chondrogenesis in vivo. Proc. Natl. Acad. Sci. U.S.A. 102, 5062–5067

16. Retting, K. N., Song, B., Yoon, B. S., and Lyons, K. M. (2009) BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. Development 136, 1093–1104

17. Furumatsu, T., Tsuda, M., Taniguchi, N., Tajima, Y., and Ashara, H. (2005) Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. J. Biol. Chem. 280, 8343–8350

18. Volk, S. W., Luvabelle, P., Leask, T., and Lebov, P. S. (1998) A BMP-responsive transcriptional region in the chicken type X collagen gene. J. Bone Miner. Res. 13, 1521–1529

19. Leboy, P., Grasso-Knight, G., D’Angelo, M., Volk, S. W., Lian, J. V., Drissi, H., Stein, G. S., and Adams, S. L. (2001) Smad-Runx interactions during chondrocyte maturation. J. Bone Joint Surg. Am. 83-A, Suppl. 1, S15–S22

20. Kempf, H., Ionescu, A., Udager, A. M., and Lassar, A. B. (2007) Bone morphogenetic signaling pathways regulate cartilage formation and differentiation. J. Bone Miner. Res. 22, 2280–2291

21. Kobayashi, T., Lyons, K. M., McMahon, A. P., and Kronenberg, H. M. (2005) BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. Proc. Natl. Acad. Sci. U.S.A. 102, 18023–18027

22. van Beuningen, H. M., Glansbeek, H. L., van der Kraan, P. M., and van den Berg, W. B. (1998) Differential effects of local application of BMP-2 or TGF-β1 on both articular cartilage composition and osteophyte formation. Osteoarthr. Cartil. 6, 306–317

23. Yang, X., Chen, L., Xu, X., Li, C., Huang, C., and Deng, C. X. (2001) TGF-β1/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J. Cell Biol. 153, 35–46

24. Serra, R., Johnson, M., Filvaroff, E. H., LaBorde, J., Sheehan, D. M., Derynck, R., and Moses, H. L. (1997) Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J. Cell Biol. 139, 541–552

25. Wu, Q., Kim, K. O., Sampson, E. R., Chen, D., Awad, H., O’Brien, T., Puzas, J. E., Drissi, H., Schwarz, E. M., O’Keefe, R. J., Zusick, M. J., and Rosier, R. N. (2008) Induction of an osteoarthritis-like phenotype and degradation of phosphorylated Smad3 by Smurf2 in transgenic mice. Arthritis Rheum. 58, 3132–3144

26. Li, T. F., Darowish, M., Zusick, M. J., Chen, D., Schwarz, E. M., Rosier, R. N., Drissi, H., and O’Keefe, R. J. (2006) Smad3-deficient chondrocytes have enhanced BMP signaling and accelerated differentiation. J. Bone Miner. Res. 21, 4–16

27. Bobick, B. E., and Kulyk, W. M. (2004) The MEK-ERK signaling pathway is a negative regulator of cartilage-specific gene expression in embryonic limb mesenchyme. J. Biol. Chem. 279, 4588–4595

28. Gosset, M., Berenbaum, F., Thirion, S., and Jacques, C. (2008) Primary culture and phenotyping of murine chondrocytes. Nat. Protoc. 3, 1253–1260

29. Alvarez, J., Sohn, P., Zeng, X., Doetschman, T., Robbins, D. J., and Serra, R. (2002) TGFβ2 mediates the effects of hedgehog on hypertrophic differentiation and PTHrP expression. Development 129, 1913–1924

30. Tominaga, H., Maeda, S., Hayashi, M., Takeda, S., Akira, S., Komiy, S., Nakamura, T., Akiyama, H., and Imamura, T. (2008) CCAAT/enhancer-binding protein β promotes osteoblast differentiation by enhancing Runx2 activity with ATF4. Mol. Biol. Cell 19, 5373–5386

31. Ahrens, P. B., Solursh, M., and Reiter, R. S. (1977) Stage-related capacity for bone formation in vitro during cartilage development and osteoarthritis. The developmental aspect of degenerative joint disorders. Endocr. Rev. 24, 218–235

32. Miyazono, K., Maeda, S., and Imamura, T. (2005) BMP receptor signaling. Transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev. 16, 251–263

33. Stroschein, S. L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999) Negative feedback regulation of TGF-β signaling by the SnoN oncoprotein. Science 286, 711–714

34. Serra, R., Johnson, M., Filvaroff, E. H., LaBorde, J., Sheehan, D. M., Derynck, R., and Moses, H. L. (1997) Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J. Cell Biol. 139, 541–552

35. Wang, W., Mariani, F. V., Harland, R. M., and Luo, K. (2000) Ski represses bone morphogenetic protein signaling in Xenopus and mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 97, 14394–14399

36. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (2004) Interaction with Smad4 is indispensable for suppression of BMP signaling by c-Ski. Mol. Biol. Cell 15, 963–972

37. Mankin, H. J., Dorfman, H., Lippiello, L., and Zarin, A. (1971) Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic
human hips. II. Correlation of morphology with biochemical and metabolic data. J. Bone Joint Surg. Am. 53, 523–537

46. Kuroki, H., Nakagawa, Y., Mori, K., Ohba, M., Suzuki, T., Mizuno, Y., Ando, K., Takenaka, M., Ikeuchi, K., and Nakamura, T. (2004) Acoustic stiffness and change in plug cartilage over time after autologous osteochondral grafting. Correlation between ultrasound signal intensity and histological score in a rabbit model. Arthritis Res. Ther. 6, R492–R504

47. Li, T. F., Gao, L., Sheu, T. J., Sampson, E. R., Flick, L. M., Konttinen, Y. T., Chen, D., Schwarz, E. M., Zuscik, M. J., Jonason, J. H., and O’Keefe, R. J. (2010) Aberrant hypertrophy in Smad3-deficient murine chondrocytes is rescued by restoring transforming growth factor β-activated kinase 1/activating transcription factor 2 signaling. A potential clinical implication for osteoarthritis. Arthritis Rheum. 62, 2359–2369

48. Zhu, Q., Pearson-White, S., and Luo, K. (2005) Requirement for the SnoN oncoprotein in transforming growth factor β-induced oncogenic transformation of fibroblast cells. Mol. Cell Biol. 25, 10731–10744

49. Maeda, S., Hayashi, M., Komiya, S., Imamura, T., and Miyazono, K. (2004) Endogenous TGF-β signaling suppresses maturation of osteoblastic mesenchymal cells. EMBO J. 23, 552–563

50. Valdes, A. M., Spector, T. D., Tamm, A., Kisand, K., Doherty, S. A., Denison, E. M., Mangino, M., Tamm, A., Kerna, I., Hart, D. J., Wheeler, M., Cooper, C., Lories, R. J., Arden, N. K., and Doherty, M. (2010) Genetic variation in the SMAD3 gene is associated with hip and knee osteoarthritis. Arthritis Rheum. 62, 2347–2352

51. Kizawa, H., Kou, I., Iida, A., Sudo, A., Miyamoto, Y., Fukuda, A., Mabuchi, A., Kotani, A., Kawakami, A., Yamamoto, S., Uchida, A., Nakamura, K., Notoya, K., Nakamura, Y., and Ikegawa, S. (2005) An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. Nat. Genet. 37, 138–144

52. Nakajima, M., Kizawa, H., Saitoh, M., Kou, I., Miyazono, K., and Ikegawa, S. (2007) Mechanisms for asporin function and regulation in articular cartilage. J. Biol. Chem. 282, 32185–32192

53. Blaney Davidson, E. N., Scharstuhl, A., Vitters, E. L., van der Kraan, P. M., and van den Berg, W. B. (2005) Reduced transforming growth factor-β signaling in cartilage of old mice. Role in impaired repair capacity. Arthritis Res. Ther. 7, R1338–R1347