Sox9 Family Members Negatively Regulate Maturation and Calcification of Chondrocytes through Up-Regulation of Parathyroid Hormone–related Protein

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Sox9 is a transcription factor that plays an essential role in chondrogenesis and has been proposed to inhibit the late stages of endochondral ossification. However, the molecular mechanisms underlying the regulation of chondrocyte maturation and calcification by Sox9 remain unknown. In this study, we attempted to clarify roles of Sox9 in the late stages of chondrocyte differentiation. We found that overexpression of Sox9 alone or Sox9 together with Sox5 and Sox6 (Sox5/6/9) inhibited the maturation and calcification of murine primary chondrocytes and up-regulated parathyroid hormone–related protein (PTHrP) expression in primary chondrocytes and the mesenchymal cell line C3H10T1/2. Sox5/6/9 stimulated the early stages of chondrocyte proliferation and development. In contrast, Sox5/6/9 inhibited maturation and calcification of chondrocytes in organ culture. The inhibitory effects of Sox5/6/9 were rescued by treating with anti-PTHrP antibody. Moreover, Sox5/6/9 bound to the promoter region of the PTHrP gene and up-regulated PTHrP gene promoter activity. Interestingly, we also found that the Sox9 family members functionally collaborated with Ihh/Gli2 signaling to regulate PTHrP expression and chondrocyte differentiation. Our results provide novel evidence that Sox9 family members mediate endochondral ossification by up-regulating PTHrP expression in association with Ihh/Gli2 signaling.

INTRODUCTION

In vertebrae, most of skeleton is formed by endochondral ossification. Endochondral ossification is a unique biological event comprised of multiple processes including mesenchymal cell condensation, differentiation of mesenchymal cells into chondrocytes, maturation of chondrocytes, vascular invasion into cartilage, chondrocyte apoptosis, and the replacement of cartilage with bone tissue (de Crombrughe *et al.*, 2000, 2001; Kronenberg, 2006). These processes are sequentially and harmoniously regulated by several cytokines and hormones that activate downstream signaling pathways and transcription factors (de Crombrughe *et al.*, 2000, 2001; Kronenberg, 2006). Genetic evidence has demonstrated that the HMG-type transcription factor Sox9 plays an essential role in endochondral ossification, and in particular, the regulation of chondrocytic matrix expression (Akiyama *et al.*, 2002, 2004, 2007). Biochemical studies have also demonstrated that Sox9 directly regulates the expression of type 2 collagen (Col2 α 1), aggrecan, type 11 collagen (Col11 α 2), Sox5, and Sox6 initiates the commitment of mesenchymal cells into chondrocytes, and promotes the early stages of chondrogenesis (Lefebvre *et al.*, 1996, 1997, 1998; Bridgewater *et al.*, 1998; Akiyama, 2008).

Sox5 and Sox6 have been shown to regulate the expression of Sox9 target genes and to function as transcriptional partners of Sox9 (Lefebvre *et al.*, 1998; Akiyama *et al.*, 2002). The Sox9 knockin mutant mouse demonstrates dwarfism phenotypes associated with delayed hypertrophy of chondrocytes, suggesting a possible role for Sox9 in the late stages of endochondral ossification (Akiyama *et al.*, 2004). Ikeda and coworkers have also reported that in cooperation with Sox5 and Sox6 cofactors, Sox9 inhibits terminal differentiation of chondrocytic cell lines (Ikeda *et al.*, 2004; Saito *et al.*, 2007). However, the precise functional role of Sox9 in chondrogenesis during the late stages remains unknown to date. In particular, it remains unclear whether Sox9 functions as a negative regulator during the late stages of chondrogenesis. Furthermore, the molecular mechanisms underlying the control of late-stage endochondral ossification by Sox9 remain unclear.

Indian hedgehog (Ihh) is expressed in the prehypertrophic chondrocytes and stimulates the expression of parathyroid hormone–related protein (PTHrP; Lanske *et al.*, 1996; Vortkamp *et al.*, 1996; St-Jacques *et al.*, 1999). PTHrP has been shown to stimulate the proliferation of chondrocytes but to inhibit their maturation and calcification (Karaplis *et al.*, 1994; Kronenberg, 2006). These findings suggest that Ihh and PTHrP function in a negative feedback system during late-stage chondrocyte development. Recently, it has also been shown that Ihh stimulates chondrocyte maturation and calcification in a PTHrP-independent manner (Kobayashi *et al.*, 2005; Amano *et al.*, 2008; Mak *et al.*, 2008). These results indicate that the precise role of the PTHrP/Ihh negative

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Figure 1. Induction of PTHrP by Sox9 family members in C3H10T1/2. (A) C3H10T1/2 was infected with adenoviruses carrying Sox5, Sox6, and HA-tagged Sox9 (HA-Sox9) at 40 moi and cultured for 2 d. Expression of each Sox protein after infection of the adenoviruses was examined by immunoblotting with anti-Sox5 (top), anti-Sox6 (middle), or anti-HA antibody (bottom). (B–D) C3H10T1/2 cells infected with control (Cont) or Sox5/6/9 adenoviruses were cultured for 3 d. Total RNA isolated from the cells was determined by real-time PCR analyses for Col2α1 (B), Aggrecan (C), and PTHrP (D). (E) C3H10T1/2 cells infected with adenovirus carrying Myc-tagged dominant-negative Sox9 (DN-Sox9) at 40 moi were cultured for 2 d. Expression of DN-Sox9 was examined by immunoblotting with anti-Myc antibody. (F) C3H10T1/2 cells infected with control (Cont) or dominant-negative Sox9 (DN-Sox9) adenovirus were cultured for 3 d. Total RNA of the cells was subjected to real-time PCR analysis for PTHrP. Data represent mean $\pm$ SD ($n = 3$). * $p < 0.01$ vs. control; Student's t test.

feedback loop is more complicated than initially proposed and that the regulation of PTHrP expression is complex.

MATERIALS AND METHODS

Cell Culture

C3H10T1/2, Cos7, and BOSC23 cells were purchased from the Riken cell bank (Tsukuba, Japan) and cultured in modified α -minimum Eagle's medium (α -MEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). ATDC5 cells were also purchased from the Riken cell bank and cultured in DMEM/F12 (Sigma) supplemented with 10% FBS.

Primary chondrocytes were isolated from the ribs of 4-wk-old DDY mice by repetitive digestion with 0.2% collagenase at 37°C. The isolated cells were filtered through a 40- μ m nylon mesh (BD Bioscience, San Jose, CA), collected by centrifugation and with the exception of the cells isolated following the first digestion, and then cultured (Kamiya *et al.*, 2002; Gartland *et al.*, 2005). Primary chondrocyte cell cultures were maintained in DMEM (Sigma) supplemented with 10% FBS, 0.1 mg/ml ascorbic acid, and 5 mM β -glycerophos-

phate (Sigma) at 37°C in a humidified 5% CO₂ incubator. Adenoviruses were infected at 40 multiplicity of infection (moi). Recombinant BMP2 was generated as described previously (Ichida *et al.*, 2004). BMP2 (100 ng/ml), PTHrP (5×10^{-8} M, Sigma), and cyclopamine (5 μ M, Biomol International, Tokyo, Japan) were used in the experiments outlined below. All animal experiments were preapproved by the Osaka University Animal Ethics Committee.

Organ Culture

Three central metatarsal rudiments were isolated from each hind limb of 15.5-d-old (E15.5) DDY mouse embryos, placed into 24-well plates, and cultured in organ culture medium as described previously (Haaijman *et al.*, 1997; Yasoda *et al.*, 1998). The metatarsals were cultured at 37°C in a humidified 5% CO₂ incubator for 12 d. Infection of adenoviruses (80 moi) or the administration of anti-PTHrP neutralizing antibody (193 μ g/ml, Chugai Pharmaceutical, Tokyo, Japan) was performed 1 d after dissection (Nifuji *et al.*, 2004). For morphometric analysis, metatarsal explants were photographed under a dissecting microscope (microscope: Stemi 2000-C; camera: AxioCam MRc; acquisition software: AxioVision AC Rel. 4.5, Carl Zeiss, Thornwood, NY). The longitudinal length and dark calcified zone in the diaphysis and epiphysis

Figure 2. Induction of PTHrP by Sox9 in murine primary chondrocytes. (A–C) Primary chondrocytes were infected with control (Cont) or HA-Sox9 adenoviruses at 40 moi and cultured for 3 d. Total RNA isolated from the cells was determined by real-time PCR analyses for Col2α1 (A), Sox5 (B), and Sox6 (C). (D) Primary chondrocytes infected with the adenoviruses (40 moi) as indicated were cultured for 3 d. Total RNA isolated from the cells was determined by real-time PCR analyses for PTHrP. (E) Primary chondrocytes infected with control (Cont) or DN-Sox9 adenovirus at 40 moi were cultured for 3 d. Total RNA of the cells was determined by real-time PCR analysis for PTHrP. Data represent mean $\pm$ SD ($n = 3$). * $p < 0.01$, ** $p = 0.01$, *** $p < 0.05$ vs. control; Student's t test.

were measured as described previously (Yasoda *et al.*, 1998; Amano *et al.*, 2008). For histological analysis, paraffin sections of metatarsals were prepared and stained with hematoxylin and eosin (H&E). Immunohistochemical analysis was performed using the following primary polyclonal rabbit antibodies: α -hemagglutinin (HA) antibody (MBL, Nagoya, Japan) at 1:500 dilution, α -Col2 antibody (LSL Biolafitte, St. Germain en Laye, France) at 1:500 dilution, α -Col10 antibody (LSL Biolafitte) at 1:500 dilution and anti-PTHrP antibody (Yanaihara Institute, Shizuoka, Japan) at 1:1000 dilution. Antigen retrieval was performed using 2.5% hyaluronidase for α -Col2 and α -Col10 and with microwave treatment for 5 min in 0.05 M citrate buffer for anti-PTHrP. Immunoreactivity was visualized with a biotinylated anti-rabbit IgG secondary antibody using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA) and the peroxidase substrate DAB kit (Vector) according to the manufacturer's protocol. For the analysis of alkaline phosphatase activity, metatarsals were fixed in 4% buffered paraformaldehyde, decalcified in 10% EDTA, and immersed in 30% sucrose, and 10- μ m frozen sections were collected using a cryostat. The sections were then washed with phosphate-buffered saline (PBS) and stained with a mixture of 330 μ g/ml nitro blue tetrazolium (Sigma), 165 μ g/ml bromochloroindolyl phosphatase (Sigma), 100 mM NaCl, 5 mM MgCl₂, and 100 mM Tris, pH 9.5, for 10 min at 37°C. For bromodeoxyuridine (BrdU) staining, BrdU-labeling reagent (Roche) was added to the organ culture medium at 1:1000 dilution. After 24-h incubation, the samples were embedded in paraffin and sectioned. Paraffin sections were then incubated with anti-BrdU monoclonal mouse antibody (1:1000, Roche, Indianapolis, IN). Antigen retrieval was performed with trypsin, and the sections were restained using Vectashield (Vector) with DAPI for normalization. Histological sections were photographed using a microscope attached to a digital camera (microscope: Axioskop 2; camera: AxioCam HRC; acquisition software: AxioVision Rel. 4.4, Carl Zeiss). The distal growth plates of the metatarsals were observed histologically because they allowed the clear analysis of hierarchical chondrocyte differentiation.

Vector Construction

HA-tagged Sox9, Sox5, Sox6, Myc-tagged Gli1, Flag-tagged Gli2, and Flag-tagged Gli3 expression vectors were used as described previously (Shimoyama *et al.*, 2007; Hata *et al.*, 2008). Myc-tagged dominant-negative Sox9 (aa2-234) and Myc-tagged dominant-negative Gli2 (aa2-968) that lacked the corresponding transcriptional activation domains were also used as described previously (Shimoyama *et al.*, 2007; Hata *et al.*, 2008). The human PTHrP gene promoter including the 1120-base pair 5'-flanking region (Gallwitz *et al.*, 2002) and the deletion mutants were ligated into the pGL4.10 vector (Promega, Madison, WI). Four deletion mutants were constructed with or without the P2 midregion GC-rich promoter (between -1120 and -611) or the P3 TATA promoter (between -513 and -505) as described previously (Vasavada *et al.*, 1993). His-tagged Sox9 and His-tagged Venus were generated by subcloning the corresponding PCR products into pCold vector (Takara, Tokyo, Japan).

Generation of Adenoviruses

Recombinant adenoviruses carrying Sox5, Sox6, HA-tagged Sox9, a dominant-negative Myc-tagged Sox9, Ihh, Myc-tagged Gli2, or a dominant-negative Gli2 were constructed by homologous recombination between the expression cosmid cassette (pAxCawt) and the parental virus genome in 293T cells (Riken Cell Bank) using the adenovirus construction kit (Takara) as described previously (Shimoyama *et al.*, 2007; Hata *et al.*, 2008). Mock adenovirus was used as a control adenovirus. The viruses showed no proliferative activity because of the lack of E1A-E1B. Virus titers were determined using a modified point assay (Shimoyama *et al.*, 2007; Hata *et al.*, 2008).

Western Blotting

The cells were rinsed twice with PBS and solubilized in lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM AEBSF, and 0.2 mM sodium orthovanadate. The lysates were then centrifuged for 10 min at 15,000 $\times$ g at 4°C and boiled in SDS sample buffer containing 0.5 M β -mercaptoethanol for 5 min. The supernatants were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies, and immunoreactivity was visualized with horseradish peroxidase-coupled anti-mouse, -rabbit or -goat IgG antibody using the ECL detection kit. Anti-Sox5, -Sox6, -HA, and α -Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Real-Time PCR

Total RNA was isolated using the total RNA isolation kit according to the manufacturer's protocol (Macherey-Nagel, Bethlehem, PA). After denaturation at 70°C for 10 min, cDNA was synthesized using oligo dT primer and reverse transcriptase (Takara). Real-time PCR was performed using the Taqman PCR protocol and an ABI 7300 real-time PCR system (Applied Biosystems, Tokyo, Japan). Taqman primers and probes used for the amplification were as follows: mouse Col2a1 (sense primer, 5'-CCTCCGCTACTGTCCACTGA-3'; anti-sense primer, 5'-ATTGGAGCCCTGGATGAGCA-3'; probe, 5'-CTTGAGGTTC-CAGCCGCTTCGTCC-3'), mouse Ihh (sense primer, 5'-GACTCATTGCCTC-

CCAGAACTG-3'; anti-sense primer, 5'-CCAGGTAGTAGGGTACATTGC-3'; probe, 5'-CCACAGCCAGCTGGACATCCCA-3'), mouse alkaline phosphatase (sense primer, 5'-ATCTTTGGTCTGGCTCCCATG-3'; anti-sense primer, 5'-TTTCCCGTTCACCGTCCAC-3'; probe, 5'-TGAGCGACACGGACAAGAAGC-CCTT-3'), mouse β -actin (sense primer, 5'-TTAATTTCGAATGGCCAG-GTCT-3'; anti-sense primer, 5'-ATTGGTCTCAAGTCAGTGTACAGG-3'; probe, 5'-CCTGGCTGCCTCAACACCTCAACCC-3'), mouse PTHrP (sense primer, 5'-GAACATCAGCTACTGCATGACAAG-3'; anti-sense primer, 5'-TCTGATT-TCGGCTGTGTGGATC-3'; probe, 5'-CCATCCAAGACTTGGCCGCCGCTT-3'), mouse aggrecan (sense primer, 5'-TCACTGTTACCGCCACTTTC-3'; anti-sense primer, 5'-TGCTGCTCAGATGTGACTGC-3'; probe, 5'-ACCGTCTCTC-CGCATCCACCCAGG-3'), mouse Sox5 (sense primer, 5'-AGGCAGGAAATGC-GACAGTAC-3'; anti-sense primer, 5'-CTCGGAGGGCAGGTGAGG-3'; probe, 5'-ACGTGGGGCAACAAGCACAGATCCCC-3'), mouse Sox6 (sense primer, 5'-TACCCACAGCTCCCTGAAG-3'; anti-sense primer, 5'-CTCACCTTCAGTG-GCAAGAGC-3'; probe, 5'-TCAGCAGCAGCGTTCACGAGCAGC-3'), mouse Patched (sense primer, 5'-CTCCAAAAGAAGAGCGGCTAATG-3'; anti-sense primer, 5'-GCACAAATGTTCACCTTCCATTG-3'; probe, 5'-ACCACAGAG-GCTCTCTGCAACACCT-3'). mRNA expression levels were normalized to that of β -actin.

Alizarin Red Staining

Cultured murine primary chondrocytes were rinsed twice with PBS, fixed in 4% buffered paraformaldehyde and then 95% ethanol, and stained with 1% alizarin red solution (Wako, Osaka, Japan) for 10 min. Stained samples were

Figure 3. Inhibition of calcification by Sox9 family members in primary chondrocytes. (A and B) Primary chondrocytes cultured with or without BMP2 (100 ng/ml) were infected with control (Cont) or Sox5/6/9 adenoviruses, cultured for 7 d, and stained with alizarin red (A). Alizarin red-positive area of the cells was measured by Image Proplus (B). Data represent mean $\pm$ SD (n = 3). *p < 0.01 vs. BMP2; one-way ANOVA. (C) Primary chondrocytes infected with control (Cont) or HA-Sox9 adenovirus at 40 moi were cultured for 3 d. Total RNA of the cells was determined by real-time PCR analyses for ALP. Data represent mean $\pm$ SD (n = 3). *p < 0.01 vs. control; Student's *t* test. (D) Primary chondrocytes were placed at a density of 10×10^4 /well of 24-multiwell plates. The cells were infected with control (Cont) or HA-Sox9 adenovirus and cultured for 3 d. MTT assay was performed to evaluate proliferation of the cells. Data represent mean $\pm$ SD (n = 4). *p < 0.05 vs. control; Student's *t* test. (E and F) Primary chondrocytes cultured with or without BMP2 (100 ng/ml) were treated with or without PTHrP (5×10^{-8} M) for 7 d and then stained by alizarin red (E). The alizarin red-positive area of the cells was measured with Image Proplus (F). Data represent mean $\pm$ SD (n = 3). *p < 0.01 vs. BMP2; one-way ANOVA.

scanned using an Epson GT-9500 (Long Beach, CA), and the alizarin red-positive area of the cells measured using Image ProPlus (Media Cybernetics, Bethesda, MD).

MTT Assay

Cell proliferation was examined using reagent WST-1 according to the manufacturer's protocol (Roche). The WST-1 reagent was added to cultured cells at a final dilution of 1:10. The samples were then incubated at 37°C for 2 h, and the absorbance of the samples was measured against a background control at 450 nm using a microplate reader (model 550, Bio-Rad).

Luciferase Assay

The luciferase reporter construct for the human PTHrP gene promoter and the TK-renilla luciferase construct (Promega) were cotransfected with expression vectors into Cos7 or ATDC5 cells using Fugene6 reagent (Roche). After 48-h incubation, cells were lysed, and the luciferase and renilla activity was measured in the substrates on a luminometer (Promega) according to the manufacturer's instructions. Renilla was used to normalize the transfection efficiency.

Oligonucleotide Pulldown Assay

Protein lysates were prepared from BOSC23 transfected with HA-Sox9 or C3H10T1/2 cells infected with Sox5, Sox6, and Sox9 adenoviruses. Lysates were then incubated with the biotinylated double-stranded DNA oligonucleotide 5'-GCTCGCCCCGCGCGCTTCCTAGGCGCCA-3' for 3 h, and incubated with streptavidin beads (Vector) for 1 h. After five washes in lysis

buffer, the precipitated samples and the beads were boiled in 20 μ l sample buffer and subjected to Western blotting. Competition was performed in the presence of a 40-fold amount of nonbiotinylated oligonucleotide.

In Vitro Binding Assay

His-tagged Sox9 and His-tagged Venus proteins were generated in the BL21 *Escherichia coli* strain using the pCold system (Takara). Cell lysates were then incubated with His-tagged Sox9 or Venus protein for 4 h, and the His-tagged Sox9 or Venus protein was precipitated with TALON beads (Clontech, Palo Alto, CA). The proteins associated with His-tagged Sox9 or Venus were determined using immunoblotting.

Statistical Analysis

Data were analyzed using the Student's *t* test, multiple comparisons (Tukey procedure) of one- or two-way ANOVA. Data are presented as the mean $\pm$ SD.

RESULTS

Sox9 Family Induces PTHrP Expression in Chondrocytes

To understand the role of the Sox9 family members in chondrocyte differentiation, we first examined the effects of Sox9 family protein overexpression on the commitment of undifferentiated mesenchymal cells to chondrocytes in the

Figure 4. Suppression of calcification of chondrocytes by Sox9 family members in organ culture. (A) Mouse metatarsals isolated from 15.5-d-old embryo (E15.5), 1 d after birth (P1), and 14-d-old mice (P14) were observed under a dissecting microscope (top). Metatarsals were histologically analyzed by H&E staining (bottom, magnification, $\times 100$). Bars (top) show calcified region. R, resting zone; P, proliferating zone; H, hypertrophic zone; C, calcified zone; S, secondary ossified area. (B) Mouse metatarsal explants isolated from E15.5 embryo were cultured for 6 and 12 d and then macroscopically (top) and histologically (bottom; magnification, $\times 100$) examined. Bars (top) show calcification region. R, resting zone; P, proliferating zone; H, hypertrophic zone; C, calcified zone; S, secondary ossified area. (C) Mouse metatarsal explants isolated from E15.5 embryo were infected with control (Cont) or HA-Sox9, Sox5, and Sox6 adenoviruses at 80 moi and cultured for 12 d. Organ cultured metatarsals were immunostained with anti-HA antibody. (D) Mouse metatarsal explants isolated from E15.5 embryo were infected with control (Cont) or Sox5/6/9 adenoviruses at 80 moi and cultured for 12 d. Left, red bars, calcified area; black arrows, secondary ossification. Right, longitudinal length and calcified zone were measured under a microscope. Data represent mean $\pm$ SD ($n = 12$). * $p < 0.01$ vs. control; Student's *t* test.

C3H10T1/2 mesenchymal cell line. Overexpression of Sox5, Sox6, and Sox9 (Figure 1A) markedly induced Col2a1 and aggrecan expression in the C3H10T1/2 cells (Figure 1, B and C). Interestingly, Sox9 family proteins also induced the expression of PTHrP in the cells (Figure 1D). In contrast, overexpression of a dominant-negative Sox9 inhibited PTHrP expression (Figure 1, E and F). These results suggested that Sox9 family members are involved in not only the promotion of chondrocyte differentiation, but also the regulation of PTHrP expression. To confirm this finding, we next examined the role of Sox9 on PTHrP expression in primary chondrocytes isolated from mouse ribs. We found that overexpression of Sox9 increased PTHrP expression in the primary chondrocytes along with up-regulation of

Col2a1, Sox5, and Sox6 expression (Figure 2, A–D). In addition, overexpression of Sox5 or Sox6 alone also significantly increased PTHrP expression in the cells (Figure 2D). Furthermore, overexpression of a dominant-negative Sox9 suppressed PTHrP expression in primary murine chondrocytes (Figure 2E). These results suggested that Sox9 controls chondrocyte differentiation via PTHrP. PTHrP is known to play an important role in the regulation of proliferation, maturation, and apoptosis processes in chondrocytes (Weir *et al.*, 1996; Amling *et al.*, 1997; Zerega *et al.*, 1999; Beier *et al.*, 2001; Yamanaka *et al.*, 2003). Furthermore, previous genetic studies have suggested an inhibitory role for Sox9 in the maturation of chondrocytes (Akiyama *et al.*, 2004). However, it remains unknown as to whether Sox9 directly regulates

Figure 5. Stimulation of PTHrP expression and suppression of chondrocyte maturation of by Sox9 family members in organ culture. (A) Organ-cultured metatarsals were histologically analyzed by H&E staining and immunostained with anti-Col2 and anti-Col10 antibodies. S, secondary ossified region; H+C, hypertrophic and calcified areas. Red bars, immunostained areas. Magnification, $\times 100$. (B) Metatarsals infected with green fluorescent protein (GFP) adenovirus or Sox5/6/9 adenoviruses were cultured for 12 d. The cultured metatarsals were frozen with dry ice for a moment and ground with lysis buffer to isolate mRNA. Total RNA of the cells was subjected to real-time PCR analysis for PTHrP (left), Col2a1 (middle), and Col10a1 (right) expression. $p < 0.01$ vs. GFP control; Student's t test. (C) Organ cultured metatarsals infected with control (Cont) or Sox5/6/9 adenoviruses were histologically analyzed by immunostaining with anti-PTHrP antibodies. No Ab, not incubated with PTHrP primary antibody. Magnification, $\times 100$. (D) Organ cultured metatarsals infected with control (Cont) or Sox5/6/9 adenoviruses were subjected to ALP staining. Magnification, $\times 100$. (E and F) Organ-cultured metatarsals were subjected to BrdU and DAPI staining (E), and BrdU-positive chondrocytes were counted and normalized by DAPI-stained chondrocytes present at the resting and proliferating zone (F). Magnification, $\times 100$. Bars, immunostained areas. Data represent mean $\pm$ SD ($n = 3$). * $p < 0.05$ vs. control; Student's t test.

chondrocyte maturation and calcification. Thus, we next examined the effects of Sox9 protein on the calcification of chondrocytes. As shown in Figure 3, A and B, overexpression of Sox9 proteins abolished BMP2-induced calcification of primary chondrocytes. In addition, overexpression of Sox9 markedly suppressed the expression of ALP (alkaline phosphatase) in primary chondrocytes (Figure 3C). In contrast, Sox9 overexpression was found to significantly stimulate proliferation of primary chondrocytes (Figure 3D). As expected, treatment with PTHrP also inhibited the BMP2-induced calcification of primary chondrocytes (Figure 3, E and F). In combination, these results suggested that the Sox9 family members may negatively control chondrocyte calcification via the up-regulation of PTHrP.

Sox9 Family Inhibits Late-Stage Chondrogenesis

To further examine the functional relationship between the Sox9 family and PTHrP in endochondral ossification, we used an ex vivo organ culture system using metatarsal explants isolated from E15.5 mouse embryos. As shown in Figure 4, A and B, the metatarsal explants cultured ex vivo for either 6 or 12 d showed a pattern of development similar to bone isolated from 1- or 14-d-old mice (P1 or P14), respectively. We also confirmed that the Sox9 adenovirus was efficiently introduced into the metatarsals (Figure 4C). This data suggested that our organ culture system combined with the Sox9 adenovirus may be useful for the study of chondrocyte development in vitro (Haaijman *et al.*, 1997; Yasoda *et al.*, 1998; Nifuji *et al.*, 2004).

We next infected adenoviruses carrying Sox5, Sox6, and Sox9 into the metatarsals and examined their effects on

chondrogenesis. As shown in Figure 4D, infection of these adenoviruses significantly reduced longitudinal development of the metatarsals. Consistent with the results shown in Figure 3, A and B, the Sox9 family also markedly inhibited chondrocyte calcification at the diaphysis and secondary ossification (Figure 4D). Histologically analysis also demonstrated inhibition of chondrocyte hypertrophy at the diaphysis and secondary ossification at the epiphysis after infection with the Sox5/6/9 adenoviruses (Figure 5A). We also found that Sox5/6/9 infection increased the Col2-positive area and Col2a1 expression, but decreased the Col10-positive area and Col10a1 expression (Figure 5, A and B). In addition, infection of the Sox5/6/9 adenoviruses increased PTHrP expression and PTHrP-positive area (Figure 5, B and C), but decreased the ALP active area (Figure 5D). The Sox9 family also stimulated the proliferation of chondrocytes in metatarsal explants (Figure 5, E and F). Collectively, these results supported the hypothesis that the Sox9 family induces PTHrP expression, thereby stimulating proliferation of chondrocytes and negatively regulating late-stage chondrocyte differentiation.

To investigate the involvement of PTHrP in the regulation of chondrogenesis by the Sox9 family, we next examined the effects of anti-PTHrP neutralizing antibody on the metatarsal explants. Treatment with anti-PTHrP antibody was found to recover the suppression of chondrocyte calcification caused by the Sox9 family (Figure 6A). Moreover, histological analysis indicated that treatment with the anti-PTHrP antibody also recovered the chondrocyte hypertrophy observed in the metatarsals (Figure 6, B

Figure 6. Rescue of chondrocyte maturation and calcification by anti-PTHrP antibody. (A) Metatarsals infected with control (Cont) or Sox5/6/9 adenovirus were cultured for 12 d with or without anti-PTHrP-antibody (193 μg/ml). Organ-cultured metatarsals were photographed (left; blue bars, calcified area; black arrows, secondary ossification) and measured (right). Data represent mean ± SD (n = 5). N.S., no significant difference between Sox5/6/9 and Sox5/6/9+Anti-PTHrP. *p < 0.01 versus Sox5/6/9 **p < 0.05 versus control; one-way ANOVA. (B and C) The organ-cultured metatarsals shown in A were subjected to H&E staining (B) and immunostained with anti-Col10 antibody (C). S, secondary ossification; H+C, hypertrophic and calcified areas. Red bars, immunoreactive area. Magnification, ×100.

Figure 7. Regulation of the PTHrP gene promoter by Sox9 family members. (A) Cos7 cells were transfected with a PTHrP gene promoter luciferase (1120 bp) and TK-renilla luciferase constructs together with empty vector (Cont) or Sox5, Sox6, or Sox9 expression vectors, as indicated. After 48 h, luciferase activity of the cell lysates was measured. Transfection efficiency was normalized by renilla luciferase activity. Data represent mean $\pm$ SD (n = 4). *p < 0.01 (vs. control), **p < 0.05 (vs. Sox5 or Sox6), ***p < 0.01 (vs. Sox6 or Sox9), ****p < 0.01 (vs. Sox5+Sox6, or Sox6+Sox9); one-way ANOVA. (B) Cos7 cells were transfected with PTHrP gene promoter luciferase (1120 bp) and TK-renilla luciferase constructs with empty vector (Cont) or a combination of Sox5/6/9 or dominant-negative (DN)-Sox9 expression vector as indicated. After 48 h, luciferase activity of the cell lysates was measured. Transfection efficiency was normalized by renilla luciferase activity. Data represent mean $\pm$ SD (n = 4). *p < 0.01 vs. Sox5/6/9; one-way ANOVA. (C) Schematic diagram of a series of human PTHrP gene promoter luciferase constructs. (D) Cos7 cells were transfected with a series of PTHrP gene promoter luciferase and TK-renilla luciferase constructs together with empty vector (Cont) or combination of Sox5/6/9 as indicated. After 48 h, luciferase activity of the cell lysates was measured. Transfection efficiency was normalized by renilla luciferase activity. Data represent mean $\pm$ SD (n = 4). *p < 0.01 vs. control, -440, -611 and -803; two-way ANOVA. (E) Lysates of BOSC23 transfected with empty or HA-Sox9 vector were incubated with biotinylated oligonucleotide (Bio-oligo) containing a putative Sox9 binding element of the PTHrP gene promoter. After precipitation with streptavidin beads, associated proteins with the oligonucleotide were determined by immunoblotting with anti-HA antibody. Comp, competition with 40-fold of nonbiotinylated oligonucleotide. (F) Lysates of C3H10T1/2 infected with control or Sox5/6/9 adenoviruses were incubated with biotinylated oligonucleotide (Bio-oligo) containing a putative Sox9-binding element of the PTHrP gene promoter. After precipitation with streptavidin beads, associated proteins with the oligonucleotide were determined by immunoblotting with anti-Sox5 antibody. Comp, competition with 40-fold nonbiotinylated oligonucleotide.

and C). Taken together, these findings indicated that the Sox9 family members regulate late-stage endochondral ossification via the up-regulation of PTHrP expression.

Sox9 Family Members Directly Control PTHrP Expression

To further understand the molecular mechanisms underlying the regulation of PTHrP expression by the Sox9 family, we next examined the effects of the Sox9 proteins on the PTHrP gene promoter using a luciferase-reporter gene construct containing a 1120-base pair fragment of the 5' end flanking region of the human PTHrP gene (Vasavada *et al.*, 1993; Gallwitz *et al.*, 2002). Sox5 or Sox6 were found to significantly stimulate PTHrP gene promoter activity (Figure 7A), whereas Sox9 did not. Overexpression of Sox5 and Sox6 together with Sox9 was found to stimulate PTHrP gene promoter activity more efficiently than each of the Sox9 family member genes alone (Figure 7A). Up-regulation of PTHrP gene promoter activity by the Sox9 family was inhibited by overexpression of a dominant-negative Sox9 (Figure 7B).

We next investigated the effects of the Sox9 family on the PTHrP gene promoter using a series of PTHrP gene promoter deletion mutants (Figure 7C). We found that there are Sox9-responsive element(s) between -1120 and -803 of the promoter region, a region that also contained a potential Sox9-binding element (Figure 7D). Thus, we next examined whether the Sox9 family members are able to bind to this region by performing oligonucleotide binding assays. As shown in Figure 7, E and F, Sox9 and Sox5 bound to the element defined in the PTHrP gene promoter. These results indicated that Sox9 proteins directly regulate PTHrP gene transcription.

Collaboration between Sox9 Family and Ihh/Gli2 Signaling

Given that Ihh regulates hypertrophic conversion by induction of PTHrP (Lanske *et al.*, 1996; Vortkamp *et al.*, 1996), we next examined whether Ihh signaling is involved in PTHrP induction by Sox9. Consistent with previous reports, Ihh was found to increase the expression of PTHrP in primary

for 3 d. Total RNA of the cells was determined by real-time PCR analysis for Patched1. Data represent mean $\pm$ SD (n = 3). N.S., no significant difference as determined; one-way ANOVA.

chondrocytes in a manner similar to that of Patched1, one of the direct target genes of Ihh (Figure 8A). These effects of Ihh were inhibited after treatment with a hedgehog inhibitor cyclopamine (Figure 8A). We also found that cyclopamine significantly inhibited PTHrP expression induced by Sox9 (Figure 8B). In contrast, Sox9 did not show any effects on the expression of Ihh and Patched1 in the primary chondrocytes (Figure 8, C and D). These results suggested that Sox9 proteins regulate PTHrP expression in association with Ihh signaling, but not through the up-regulation of Ihh and Patched1 expression.

Because the Gli family members Gli1, Gli2, and Gli3 are known to play a central role in Ihh signaling (Hilton *et al.*, 2005; Sterling *et al.*, 2006), we next tested the effects of Gli family members on PTHrP gene promoter activity. We found that only Gli2 stimulated PTHrP gene promoter activity (Figure 9A). We also found that Gli2 stimulated PTHrP gene promoter activity in the region spanning -1120 and -803 , a region that is also known to contain a Sox9-responsive element (Figure 9B). In addition, the overexpression of Gli2 also increased PTHrP expression in the primary chondrocytes (Figure 9C). These results suggested that Gli2 serves as an important regulator of PTHrP expression in chondrocytes. We next examined the functional relationships between the Sox9 family members and Gli2 in the regulation of PTHrP gene transcription. Sox9 family members were found to increase PTHrP gene promoter activity in the presence of Gli2 (Figure 9D). Consistent with these findings, overexpression of a dominant-negative Gli2 was found to suppress the PTHrP expression up-regulated by Sox9 (Figure 9E). Furthermore, we found that Gli2 was able to directly bind to Sox9 (Figure 9F). However, we did not observe any direct associations between Gli2 and the PTHrP gene (data not shown). This data indicated that the Sox9 family members collaborate with Gli2 to regulate PTHrP expression during chondrogenesis.

DISCUSSION

A large body of evidence indicates that the Sox9 gene family plays a critical role in endochondral ossification, especially

in the commitment of undifferentiated mesenchymal cells into chondrocytes and early chondrocyte differentiation (Bi *et al.*, 1999; Akiyama *et al.*, 2002). The molecular mechanisms underlying the regulation of early stage chondrogenesis by Sox9 have recently been discussed (Akiyama, 2008). In addition, several studies have also shown that Sox9 is involved in the regulation of late-stage chondrogenesis (Ikeda *et al.*, 2004). Although endochondral ossification is thought to be a sequential event, it remains unclear as to how Sox9 controls the balance between early- and late-stage chondrogenesis. In the present study, we first demonstrated that Sox9 family members up-regulate PTHrP expression in collaboration with Ihh/Gli2 signaling. We next revealed that the inhibition of chondrocyte maturation and calcification by Sox9 family members was rescued by treatment with an anti-PTHrP antibody. These results demonstrated that Sox9 functions as a negative regulator of chondrocyte maturation and calcification by utilizing the Ihh/PTHrP loop.

In the present study, we identified PTHrP as a direct transcriptional target of Sox9. This result is supported by the reported finding that PTHrP is down-regulated in Sox9 conditional knockout mice driven off the Col2a1-Cre transgene (Akiyama *et al.*, 2002). These mice exhibited severe chondrodysplasia, suggesting that Sox9 controls chondrogenesis, at least in part, through PTHrP. Given that PTHrP stimulates phosphorylation of Sox9 via protein kinase A (PKA), a reaction that subsequently results in the up-regulation of the DNA binding affinity and transcriptional activity of Sox9 (Huang *et al.*, 2000; Huang *et al.*, 2001), we hypothesized that Sox9 and PTHrP may generate a positive loop required for their function. In addition, we also suggest that Sox5 and Sox6 function as cofactors with Sox9 on the PTHrP gene promoter, as is the case for Col2a1 (Lefebvre *et al.*, 1998). Overexpression of either Sox5 or Sox6 in primary chondrocytes also marginally increased PTHrP expression, although overexpression of Sox9, which could induce both Sox5 and Sox6 and subsequently form protein complexes on specific chondrogenic genes, dramatically increased PTHrP expression. Using the described luciferase assay, we found that the most efficient effects of Sox9 were observed in the presence of Sox5 and Sox6. These results are supported by the finding

Figure 9. Functional cooperation of Sox5/6/9 and Ihh/Gli2 signaling in regulation of PTHrP expression. (A) Cos7 (left) or ATDC5 cells (right) were transfected with a PTHrP gene promoter luciferase (1120 bp) and TK-renilla luciferase constructs with empty vector (Cont), Gli1, Gli2, or Gli3 vectors. After 48 h, luciferase activity of the cell lysates was measured. Transfection efficiency was normalized by renilla luciferase activity. Data represent mean $\pm$ SD (n = 4). * p < 0.01 or ** p < 0.05; one-way ANOVA (vs. control, Gli1, and Gli3). (B) Cos7 cells were transfected with a series of PTHrP gene promoter luciferase and TK-renilla luciferase constructs together with empty (Cont) or Gli2 vector. After 48 h, luciferase activity of the cell lysates was measured. Transfection efficiency was normalized by renilla luciferase activity. Data represent mean $\pm$ SD (n = 4). * p < 0.01; two-way ANOVA (vs. control, -440, -611 and -803). (C) Primary chondrocytes infected with control (Cont) or Gli2 adenovirus were cultured for 3 d. Total RNA of the cells was determined by real-time PCR analysis for PTHrP. Data represent mean $\pm$ SD (n = 3). * p < 0.01 vs. control; Student's t test. (D) Cos7 (left) or ATDC5 cells (right) were transfected with a PTHrP gene promoter luciferase (1120 bp) and TK-renilla luciferase constructs with empty vector (Cont), Gli2, or Sox5/6/9 as indicated. After 48 h, luciferase activity of the cell lysates was measured. Transfection efficiency was normalized by renilla luciferase activity. Data represent mean $\pm$ SD (n = 3). * p < 0.01; one-way ANOVA (vs. control, Sox5/6/9, or Gli2). (E) Primary chondrocytes infected with control (Cont), HA-Sox9, or dominant-negative (DN)-Gli2 adenoviruses as indicated were cultured for 3 d. Total RNA of the cells was determined by real-time PCR analysis for PTHrP. Data represent mean $\pm$ SD (n = 3). * p < 0.01 vs. Sox9; one-way ANOVA. (F) Lysates of 293FT cells transfected with or without Myc-Gli2 were precipitated by TALON beads with His-tagged Sox9 or Venus protein, and then the precipitates were determined by immunoblotting with anti-Myc antibody (top). Inputs of Myc-Gli2 (middle) and His-tagged Sox9 protein (bottom) were determined by immunoblotting with anti-Myc and Sox9 antibodies, respectively.

that Sox9 null mutants exhibit severe chondrodysplasia, whereas Sox5 and Sox6 single null mice are born with mild skeletal abnormalities only (Smits *et al.*, 2001; Smits *et al.*, 2004).

Our *ex vivo* experiments using isolated mouse metatarsals indicated that the Sox9 family inhibited secondary ossification. Therefore, it appears likely that the Sox9 family not only inhibits terminal differentiation of chondrocytes at the growth plate, but also prevents the hypertrophy of resting and periarticular chondrocytes during secondary ossification processes via the up-regulation of PTHrP. Consistent with this finding, LacZ transgenic mice driven off the PTHrP gene promoter demonstrated PTHrP expression in the resting or periarticular chondrocytes, as well as in the perichondrium (Chen *et al.*, 2006). Indeed, secondary ossification appears to be initiated at the PTHrP-rich periarticular region (Chen *et al.*, 2006). Moreover, PTHrP-positive chondrocytes not only participated in the proximal proliferation stage in the growth plate, but were also separated into distal and differentiated subarticular chondrocytes (Chen *et al.*, 2006). As secondary ossification is thought to be a physiologically important process that results in the organization of articular cartilage and spongiosa in the epiphysis, we suggest that Sox9 family proteins may be involved in the development of secondary ossification by up-regulating PTHrP expression.

There appears to be a discrepancy in the literature regarding the expression pattern of PTHrP and Sox9 in cartilage tissues. As described by Chen *et al.* (2006), PTHrP is expressed in the perichondrium, an area where Sox9 does not appear to be expressed. In contrast, the proliferating columnar chondrocytes express Sox9, but not PTHrP (Akiyama *et al.*, 2004). A potential explanation for this discrepancy is that Sox9 may not be essential for the induction of PTHrP. It is also possible that the Sox9 and Ihh dose gradient is involved in the regulation of PTHrP. Further investigations using appropriate *in vivo* systems may be required to further our understanding of the roles of Sox9 in PTHrP regulation.

We also observed that Gli2 may serve as an effective positive regulator of PTHrP gene promoter activity. Gli3 has also been demonstrated to be a negative regulator of Ihh-dependent PTHrP expression (Hilton *et al.*, 2005). Distinct roles for Gli2 and Gli3 have also been described previously in other systems (Motoyama *et al.*, 1998; Ruiz i Altaba, 1998; Shimoyama *et al.*, 2007). In combination, these findings suggest that PTHrP expression may be regulated by Gli2 and Gli3 in a dose-dependent manner.

In summary, we demonstrate that the Sox9 gene family regulates terminal differentiation of chondrocytes in association with a Ihh/PTHrP loop. These findings advance our

understanding of the molecular mechanisms underlying the spatial and temporal regulation of endochondral ossification by the Sox9 gene family.

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