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Smad1 plays an essential role in bone development and postnatal bone formation

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SUMMARY

Objectives—To determine the role of Smad1 in bone development and postnatal bone formation.

Methods—*Col2a1-Cre* transgenic mice were bred with *Smad1*$^{fx/fx}$ mice to produce chondrocyte-specific *Smad1* conditional knockout (cKO) mice. Embryonic skeletal preparation and staining were performed, alkaline phosphatase activity (ALP) and relative gene expression were examined in isolated primary cells. *Smad1*$^{fx/fx}$ mice were also bred with *Col1a1-Cre* transgenic mice to produce osteoblast-specific *Smad1* cKO mice. Postnatal bone formation was assessed by micro-computed tomography (μCT) and histological analyses in 2-month-old mice. Mineralized bone nodule formation assay, 5-bromo-2′-deoxy-uridine (BrdU) labeling and gene expression analysis were performed.

Results—Mice with chondrocyte- and osteoblast-specific deletion of the *Smad1* gene are viable and fertile. Calvarial bone development was delayed in chondrocyte-specific *Smad1* cKO mice. In osteoblast-specific *Smad1* cKO mice, BMP signaling was partially inhibited and mice developed an osteopenic phenotype. Osteoblast proliferation and differentiation were impaired in osteoblast-specific *Smad1* cKO mice.

Conclusions—Smad1 plays an essential role in bone development and postnatal bone formation.

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Conflict of interest

The authors have no conflict of interest to disclose.

Keywords

Smad1; Conditional knockout; BMP signaling; Chondrocyte; Osteoblast

Introduction

The vertebrate skeletal system is composed of two distinct tissues: cartilage and bone. The variant cartilage types are connective tissues important in numerous and varied roles during prenatal and postnatal skeletal development[1]. Bones are rigid mineralized organs formed in a variety of shapes. The cell type located in cartilage is the chondrocyte and in bone the osteoblast and osteoclast. Chondrocytes and osteoblasts are both originally derived from a common mesenchymal progenitor[2], while osteoclasts have a hematopoietic origin. There are two different mechanisms of bone formation:membranous bone formation and endochondral ossification[3–5] and both of these mechanisms start with condensation of mesenchymal cells. During endochondral ossification most mesenchymal cells will differentiate into chondrocytes and eventually these chondrocytes will be replaced by osteoblasts and most bone tissues in the body are developed through this process. In contrast, bones of the skull vault, or cranium, and the clavicle form directly from mesenchymal progenitors in a process termed membranous ossification.

Prenatal and postnatal bone formation is a complicated process that is regulated by multiple growth factors, signaling molecules, and transcription factors. Sox9, Runx2 and β-catenin are important transcription factors and a signaling protein, respectively, which affect cell fate during early prenatal skeletal development. *Sox9* inactivation in the cranial neural crest causes cranial neural crest-derived cells to deviate from their chondrogenic differentiation into an osteoblastic lineage[6]. *Runx2*-deficient calvarial cells differentiate into chondrocytes instead of osteoblasts when cultured with BMP-2[7]. Conditional inactivation of the β-*catenin* gene in mouse mesenchymal cells results in loss of osteoblasts and ectopic chondrocyte formation in bone tissues through membranous and endochondral bone formation processes[8]. During the postnatal stage, Ihh and β-catenin play an important role in normal cartilage and bone development. Postnatal conditional ablation of *Ihh* leads to condylar disorganization and growth retardation[9], while late stage postnatal conditional activation of the β-*catenin* gene causes articular cartilage degradation[10].

BMPs, especially BMP-2, and their signaling proteins are required in both chondrogenesis and bone formation at embryonic and postnatal stages[11–13]. Smad1 is an immediate downstream transducing molecule of the BMP receptor and plays a central role in mediating BMP signaling[14]. Activation of Smad1 is achieved through phosphorylation by the BMP type I receptor in a ligand-dependent manner. Phosphorylated Smad1 thereafter forms a heteromeric complex with Smad4. This heteromeric Smad complex translocates and accumulates in the nucleus participating in gene transcription in conjunction with other transcription factors. Smad1 is critical in embryonic development. Targeted deletion of the *Smad1* gene results in early embryonic lethality due to failure of the allantois to fuse to the chorion and animals die around embryonic day 10 (E10)[15,16]. Since bone development starts at a later stage of embryonic development and the first ossification center forms around E14.5 in mice[17], it is impossible to study the role of Smad1 in bone development and subsequent bone formation postnatally using *Smad1* knockout (KO) mice.

Although an accumulating body of evidence shows that BMP signaling is critical for chondrogenesis and osteoblast differentiation, the physiological role of bone morphogenetic protein (BMP) signaling in prenatal and postnatal bone formation *in vivo* remains to be defined. Recently, Retting *et al.* found that Smads 1 and 5 are required for chondrogenesis

by analyzing cartilage-specific *Smad1* and *Smad5* double KO mice[18]. However, the precise role of Smad1 in prenatal bone development and postnatal bone formation remains undefined. In this study, we generated chondrocyte- and osteoblast-specific *Smad1* cKO mice using the Cre-loxP system[19–21] and investigated the specific role of Smad1 in bone development and postnatal bone formation. We found that chondrocyte-specific deletion of the *Smad1* gene causes ectopic cartilage formation in calvaria at the embryonic stage, and further leads to delayed calvarial bone ossification. Moreover, osteoblast-specific *Smad1* cKO mice have impaired postnatal bone formation. Our findings provide direct evidence that Smad1 can regulate prenatal bone development and postnatal bone formation.

Materials and methods

Generation of the Smad1 cKO Mice

To generate $Smad1^{fx/fx}$ mice, the Smad1 locus was targeted by pLoxpneo-loxP-Smad1 in which exon 2 was flanked with loxP sites by insertion of pLoxpneo in intron 1 and a third loxP site in intron 2[22]. Viable homozygous $Smad1^{fx/fx}$ mice were obtained, indicating that the insertion of pLoxpneo in intron1 does not interfere with expression of the *Smad1* gene. To generate chondrocyte-specific *Smad1* cKO mice, $Smad1^{fx/fx}$ mice were bred with Col2a1-Cre transgenic mice (Table I). In these *Smad1* cKO mice (hereafter referred to as $Smad1^{Col2a1}$), the *Smad1* gene was deleted by Cre recombinase which was driven by the 3 kb murine type II collagen promoter (*Col2a1-Cre*)[23]. To determine the role of Smad1 in postnatal bone formation, we also generated osteoblast-specific *Smad1* cKO mice. To facilitate the excision activity of Cre, we bred $Smad1^{fx/fx}$ mice with CMV-Cre mice first[24] (Table II). The strain of CMV-Cre mice mediates deletion of loxP-flanked genes in all tissues, including germ cells[25]. In this mouse line every single cell would have only one floxed *Smad1* allele, removal of which would result in compound heterozygosity for the deleted-null allele (*CMV-Cre;* $Smad1^{fx/wt}$). After that, we crossed $Smad1^{del/wt}$ mice with the *Col1a1-Cre* transgenic mice in which Cre recombinase was driven by the 2.3 kb murine osteoblast-specific type I collagen promoter[26–28] (Table II). The 2.3 kb type I collagen promoter is mainly active in mature osteoblasts[29,30]. Mating of these animals with mice homozygous for the floxed allele ($Smad1^{fx/fx}$) generated the progeny with desired genotype, *Col1a1-Cre;*$Smad1^{del/fx}$ mice (osteoblast-specific *Smad1* cKO) (hereafter referred to as $Smad1^{Col1a1}$). The Cre-dependent recombination was detected by polymerase chain reaction (PCR) [Fig. 2(B), Lane 1, 4 and 5].

The sequences of PCR primers for genotyping and detecting Cre transgene are Cre-5′, 5′-CCT ggA AAA TgC TTC TgT CCg TTT gCC-3′; Cre-3′, 5′-gAg TTg ATA gCT ggC Tgg Tgg Cag ATg-3′, and the size of the PCR product is 600-bp. The sequences of PCR primers for genotyping $Smad1^{fx/fx}$ mice are SIF400, 5′-gTT CCC ATT Tgg TTC CAA gC-3′; LoxP2, 5′-gAg CTC TgC TCC gCC ACT CA-3′. The 360-bp PCR product was detected in wild-type mice and the 410-bp PCR product was detected in $Smad1^{fx/fx}$ mice [Fig. 1(A)]. The sequences of PCR primers for genotyping *Smad1* cKO mice are S1F400, 5′-gTT CCC ATT Tgg TTC CAA gC-3′; LoxP2, 5′-gAg CTC TgC TCC gCC ACT CA-3′; Rec-Smad1, 5′-CAC CTg TgC CCC CTC CAA gT-3′. The 360-bp PCR product was detected in wild-type mice, the 410-bp PCR product was detected in $Smad1^{fx/fx}$ mice, and the 300-bp product was detected after Smad1 recombination [Fig. 2(A)].

The *Smad1-loxP*, *Col1a1-Cre* and *Col2a1-Cre* mice were produced with mixed backgrounds[22,23,26] and were then converted into C57 background in our lab after breeding with C57 mice for over 15 generations.

Isolation of calvarial mRNA from E16.5 embryos

E16.5 embryos were sacrificed and tail tissues were obtained for genotyping. Calvarial tissues of E16.5 *Smad1*^{Col2a1} or Cre-negative control embryos were harvested to obtain total mRNA. After washing with sterile phosphate buffered saline (PBS) twice, the calvariae were collected in 1.5 ml micro-centrifuge tubes. 1 ml Trizol reagent (Qiagen) and one 5 mm stainless steal bead (Qiagen) were added into each tube. This was followed by vibrating the tube at 4°C for 1 min using Tissue Lyser (Qiagen) to breakdown the tissue. Total mRNA was harvested following Trizol manufacture's instructions. Expression of *Col2a1*, *Cre* and *Smad1* mRNA was analyzed by PCR or real-time PCR.

Embryonic skeletal alizarin red/alcian blue staining

Embryos were sacrificed at E16.5 and E18.5 and tail tissues were obtained for genotyping. Embryo skin was removed before placing them in 95% ethanol for 1–2 days to dehydrate. Embryos were then transferred in acetone (J. T. Baker) for 1 day to remove fat. The alcian blue/alizarin red staining was performed using a solution containing 5 ml 0.3% alcian blue 8GS (Sigma) dissolved in 70% ethanol, 5 ml 0.1% Alizerin red S (Sigma) dissolved in 95% ethanol, 5 ml glacial acetic acid (J. T. Baker), and 85 ml 70% ethanol in 37°C incubator for 3 days. The embryos were then transferred in 2% KOH (Sigma) for 1–2 days until skeletons are clearly visible through the surrounding skin. The embryos were transferred in 20% glycerin (Sigma) containing 1% KOH, and finally store in 100% glycerin.

Assessment of three-dimensional (3D) trabecular microstructure

The calvariae of chondrocyte-specific *Smad1* cKO embryos (E16.5) and proximal tibiae of osteoblast-specific *Smad1*^{Col1a1} mice (2-month-old) were scanned using a micro-computed tomography (μCT) scanner (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) and Cre-negative littermates were used as controls. A small X-ray tube with a μ focal spot is used as a source. The detector consists of a linear charged coupled device (CCD)-array. A scout view scan was obtained first for selection of the examination volume of the specimens, followed by automatic positioning, measurement, and offline reconstruction[32]. The field of view was 30 mm×30 mm, and matrix size was 2048 × 2048. Images with isotropic resolution of 15 μm^3 were obtained.

Alkaline phosphatase staining

Twenty-four hours after isolated from 3-day-old Cre-negative and *Smad1*^{Col2a1} mice, primary calvarial osteoblasts were treated with vehicle or BMP-2 (100 ng/ml) for 48 h. Prior to staining, cells were washed with PBS and fixed with 10% Neutral buffered formalin (NBF) at 4°C for 15 min. Cells were washed twice with milliQ H_2O after removal of NBF. Alkaline phosphatase (ALP) staining was performed using a solution containing 66.7 μl 25 mg/ml naphthol AS-MS-PO4 (Sigma) dissolved in N-N-Dimethyl formamide (Sigma), 8.33 ml 0.2 M Tris–HCl (pH= 8.3), 8.33 ml milliQ H_2O, and 10 mg Red Violet LB Salt in 37°C incubator for 45 min.

Isolation of primary sternal chondrocytes

Three-day-old neonatal pups were sacrificed and tail tissues were obtained for genotyping. The anterior rib cage and sternum of *Smad1*^{Col2a1} and Cre-negative control mice were harvested to obtain sternal chondrocytes. This was followed by washing with sterile PBS twice, and digesting with 2mg/ml pronase (Roche) dissolved in PBS in a 37°C water bath with continuous shaking for 60 min. The rib cage and sternum were then incubated in 3 mg/ml collagenase D (Roche) solution dissolved in serum-free Dulbecco's modified Eagle's medium (DMEM)(GIBCO) at 37°C for 90 min. The soft tissue debris was carefully removed. This was followed by further digestion in fresh 3 mg/ml collagenase D solution in

petri dishes at 37°C for 5 h with intermittent shaking. The digestion solution was filtered using a 40 μm cell strainer to collect the sternal chondrocytes. Cells were counted and plated in complete DMEM with 50 μg/ml ascorbic acid (Sigma).

Isolation of primary calvarial osteoblasts

Three-day-old neonatal pups were sacrificed and tail tissues were obtained for genotyping. Calvariae of 3-day-old Cre-negative and *Smad1*^{Col2a1} mice were harvested to obtain calvariae. Calvariae were washed with PBS twice, serum-free α-Minimum Essential Medium (α-MEM) (GIBCO)twice, and then digested with 1 mg/ml Collagenase A (Roche) dissolved in serum-free α MEM in a 37°C water bath with continuous shaking. Keep changing fresh 1 mg/ml Collagenase A every 15–20 minutes for four times. On the 4th time digestion, a 40 μm cell strainer was used to filter the digestion solution. Cells were centrifuged and resuspended in complete α MEM (GIBCO) with 50 μg/ml Ascorbic Acid (Sigma). The remaining calvariae were further digested twice with Collagenase A for 15–20 minutes each time to collect digestion solution. Cells were centrifuged and resuspended under the same condition.

Western blot analysis

The cells were lysed using Golden Lysis Buffer supplemented with protease inhibitor cocktail tablets (Sigma). The Bio-Rad Protein Assay (Bio-Rad) kit was used to detect protein concentration. The protein was detected with mouse monoclonal antibody against Smad1 (Santa Cruz) or phosphorylated Smad1/5/8. A monoclonal antibody against β-actin (Sigma) at a dilution of 1:8000 was used as loading control. The immune complexes were detected using West Pico chemiluminescent agents (Pierce).

Histological and histomorphometric analyses

Histomorphometric analyses were performed on proximal tibial metaphyses in 2-month-old osteoblast-specific *Smad1*^{Col1a1} mice and control littermates that had been injected intraperitoneally with calcein 7 and 2 days before animals were sacrificed[28,31]. Half of the bone samples from each group were decalcified in 14% ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, and sections stained with H&E or for tartrate-resistant acid phosphatase (TRAP). Histological changes in trabecular bones were analyzed and changes in osteoblast and osteoclast numbers were quantified as previously described[28,31]. The other half of the bone samples were embedded in methylmethacrylate and sections used for measurements of bone formation rates (BFR) or stained with von Kossa to determine changes in the trabecular bone mineralization and changes in bone volume (BV). Trabecular BV was quantified in the bone marrow cavity 0.5–2.5 mm from the growth plates and expressed as a percentage of total tissue volume (TV). Values were expressed as the mean ± standard error calculated from three non-consecutive sections per mouse from each of 10 *Smad1*^{Col1a1} mice and littermate control mice. The calcein labeled bone surface or mineralizing surface (MS) and the distance between two fluorescent labels was measured and BFR/bone surface (BS), ($\mu m^3/\mu m^2$/day), were calculated.

5-bromo-2′-deoxy-uridine (BrdU) labeling

BrdU labeling was performed using Zymed BrdU labeling reagent and BrdU staining kit according to the manufacture's protocols (Zymed). Briefly, BrdU was injected intraperitoneally into mice 24 h before sacrifice. Bone tissues were fixed in 10% NBF and embedded in paraffin. 3–4 μm thick sections were cut and placed on polylysine coated slides. Slides were dried in a 60°C oven overnight, deparaffinized in two changes of xylene for 5 min each, and then rehydrated in a graded series of alcohol. Slides were stained for BrdU according to manufacture's protocols. Streptavidin-peroxidase was used as a signal

generator for the BrdU system and deaminobenzidine in the presence of hydrogen peroxide was used as a chromogen. BrdU-incorporated nuclei were stained in a dark brown color[34].

Mineralized bone nodule formation assay

Primary osteoblasts isolated from the calvariae of neonatal *Smad1*^{Col1a1} mice and littermate control mice were used for mineralized bone nodule formation assays as described previously[28,31,33]. Cells were plated in 24-well culture plates at a density of 2 ×10^4 cells/well and cultured with αMEM supplemented with 10% fetal calf serum (FCS). When cells reached confluence (day 0), the medium was changed to αMEM containing 5% FCS, 100 μg/ml ascorbic acid and 5 mM β-glycerol phosphate with or without addition of 100 ng/ml of BMP-2. The medium was changed every other day and fresh reagents were added. After 10-day incubation, von Kossa staining was performed to analyze the formation of mineralized bone nodules.

Luciferase assay

Primary osteoblasts isolated from *Smad1*^{Col1a1} and Cre-negative control mice were transiently transfected with BMP signaling reporter construct 12× SBE-OC-Luc followed by treatment with 4, 20 and 100 ng/ml of BMP-2 using Superfect (Qiagen, CA, USA) according to the manufacturer's instruction. The DNA to transfection reagent ratio was 1:3 (w/w) in all experiments. β-gal plasmid was cotransfected as an internal control. Cells were lysed using passive lysis buffer (Promega, WI, USA) and extracts were prepared to detect the luciferase activity using the Dual Luciferase Assay System (Promega, WI, USA) following manufacturer's directions.

Statistical analysis

The data were presented as mean ± 95% confidence limit (CL). For comparison of two groups of data, unpaired student's *t*-test was performed. For comparison of multiple groups of data, one-way analysis of variance (ANOVA) was performed followed by Dunnett's test. **P*< 0.05 was considered as significant difference between two experimental groups.

Results

To determine the role of Smad1 in skeletal development, E16.5 and E18.5 chondrocyte-specific *Smad1*^{Col2a1} embryos were analyzed. We first extracted RNA from calvarial tissues of E16.5 Cre-negative and *Smad1*^{Col2a1} embryos to determine if *Col2a1* gene is expressed and Cre-recombination occurs in calvarial tissues of *Smad1*^{Col2a1} embryos. We detected *Col2a1* and *Cre* mRNA expression in calvariae of Cre-negative and *Smad1*^{Col2a1}embryos [Fig. 1(B and C)]. We then analyzed *Smad1* expression and found that *Smad1* expression was significantly reduced (about 70% reduction) in the calvaria of *Smad1*^{Col2a1} mice [Fig. 1(D and E)]. These findings indicate that mouse calvarial tissues indeed express the *Col2a1* gene at early developmental stage. Embryos were placed in 95% ethanol for 2 days for dehydration and then placed in acetone for 1 day to remove fat. Alizarin red/Alcian blue staining was performed. *Smad1*^{Col2a1} embryos and their littermate Cre-negative embryos are similar in size [Fig. 1(F)]. Delayed ossification of frontal, parietal and supraoccipital bones was observed in chondrocyte-specific *Smad1*^{Col2a1} embryos at E16.5 stage [Fig. 1(F and G)]. In addition, formation of the ossification center of proximal phalangeal bones was also delayed in E16.5 *Smad1*^{Col2a1} embryos [Fig. 1(H)]. We also analyzed calvarial bone formation and mineralization of E16.5 *Smad1*^{Col2a1} embryos by μ-CT and found that calvarial bone formation and mineralization were reduced in *Smad1*^{Col2a1} embryos [Fig. 1(I and J)]. However, no obvious difference was observed in E18.5 *Smad1*^{Col2a1} embryos compared to same aged Cre-negative embryos [Fig. 1(K)], indicating that the delayed

calvarial mineralization and ectopic cartilage formation observed at E16.5 *Smad1*^{Col2a1} embryos are stage-specific phenomenon.

To further characterize these mice, we isolated primary sternal chondrocytes and primary calvarial cells from 3-day-old chondrocyte-specific *Smad1*^{Col2a1} mice and Cre-negative control mice. We analyzed the expression of genes related to calvarial bone formation and mineralization and ALP activity. We found that several of chondrocyte and osteoblast differentiation-related genes were down-regulated in *Smad1*^{Col2a1} mice [Fig. 2(A and B)] and ALP activity in calvarial cells was reduced [Fig. 2(C)]. It seems that transition from cartilage to bone phase at the specific developmental stage is delayed in *Smad1*^{Col2a1} mice and Smad1-mediated BMP signaling plays an important role in this process.

To determine the role of Smad1 in postnatal bone formation, we have generated osteoblast-specific *Smad1*^{Col1a1} mice. The *Smad1*^{Col1a1} mice are viable, fertile, and survive into adulthood. To determine the expression of *Smad1* mRNA and protein, we isolated bone marrow cells from 2-month-old *Smad1*^{Col1a1} mice and control littermates. Expression of *Smad1* mRNA was reduced about 80% in *Smad1*^{Col1a1} cells [Fig. 3(C)]. Smad1 protein expression was detected by Western blotting using an anti-Smad1 mouse monoclonal antibody (Santa Cruz, CA, USA) in osteoblasts derived from control mice. In *Smad1*-^{Col1a1} cells, expression of Smad1 protein was significantly reduced [Fig. 3(D)]. These results demonstrate that expression of the *Smad1* gene is disrupted in bone marrow cells in osteoblast-specific *Smad1*^{Col1a1}mice. To determine changes intrabecular bone formation between *Smad1*^{Col1a1} mice and littermate controls, 2-month-old mice were analyzed using histology and μCT methods on proximal tibial metaphyses[32]. BV was measured on von Kossa-stained tibia sections. Compared to the Cre-negative control mice, the trabecular BV of 2-month-old osteoblast-specific *Smad1*^{Col1a1} mice was reduced 30% [Fig. 3(E and F)]. To investigate the dynamic changes in bone formation in *Smad1*^{Col1a1} mice, we measured BFR by performing calcein/calcein labeling experiments. Fluorescent labels were administered by intraperitoneal injection of calcein 7 and 2 days before mice were sacrificed[28,31]. Un decalcified bone tissues were processed by methylmethacrylate embedding. A significant reduction (36%) in BFR was found in osteoblast-specific *Smad1*^{Col1a1} mice [Fig. 3(G and H)]. Since trabecular BV and BFR represent the percentage of bone in a well-defined area of bone marrow cavities these results suggest that *Smad1*^{Col1a1} mice developed osteopenia. We also analyzed bone mass in the heterozygous *Smad1*^{Col1a1} mice and found that there is no significant reduction in bone mass in those heterozygous *Smad1*^{Col1a1} mice (data not shown).

μCT analysis confirmed trabecular BV per unit of metaphyses to be significantly decreased in osteoblast-specific *Smad1*^{Col1a1} mice [Fig. 4(A)]. Similarly, trabecular number (Th.N), trabecular thickness (Tb.Th), and connectivity density (CD) were significantly decreased in *Smad1* cKO mice compared with Cre-negative controls [Fig. 4(B, C and E)]. Trabecular separation (Tb.Sp) and structure model index (SMI) were significantly higher in *Smad1*^{Col1a1} mice [Fig. 4(D and F)]. These results suggest that trabecular BV and mechanical property of the bone are reduced in *Smad1*^{Col1a1} mice.

To determine if there were changes in osteoblast number, we quantified osteoblast number in the trabecular bone area of mouse tibiae and found a 19% decrease in *Smad1* cKO mice compared with Cre-negative control mice [Fig. 3(I)]. No obvious changes in osteoclast numbers were observed in *Smad1*^{Col1a1} mice. To determine whether osteoblast proliferation was affected, BrdU labeling experiments were performed[28]. BrdU was injected intraperitoneally into mice 24 h before sacrifice. Bone tissues were then processed and stained. A significant decrease (22%) in BrdU-positive osteoblasts was found on calvarial bone surfaces in *Smad1*^{Col1a1} mice [Fig. 5(A and B)]. These results demonstrate that defects

in bone formation in osteoblast-specific *Smad1*^{Col1a1} mice may be partially due to the impairment of osteoblast proliferation.

To determine changes in osteoblast differentiation, primary osteoblasts were isolated from the calvariae of neonatal *Smad1*^{Col1a1} mice and Cre-negative control mice and were used to examine mineralized bone nodule formation[8,31,33]. The mineralized bone nodules were formed at day 10 and treatment with BMP-2 (100 ng/ml) stimulated the nodule formation in osteoblast cells derived from control mice [Fig. 5(C, left panels)]. However, either at basal levels or with BMP-2 treatment, nodule formation was inhibited in osteoblasts derived from *Smad1*^{Col1a1} mice [Fig. 5(C, right panels)]. To determine if BMP signaling is blocked in osteoblasts derived from *Smad1*^{Col1a1} mice, we transfected the BMP signaling reporter construct 12×SBE-OC-Luc[8] into primary osteoblasts of *Smad1*^{Col1a1} mice and Cre-negative control mice followed by BMP-2 treatment. In osteoblasts derived from *Smad1*^{Col1a1} mice, BMP signaling was significantly inhibited compared with that in osteoblasts from Cre-negative control mice [Fig. 5(D)]. We also analyzed osteoblast maker gene expression[34] and found that expression of osteoblast marker genes such as *Runx2*, *osterix*, *bone sialoprotein* (*BSP*) and *osteocalcin* was significantly reduced in osteoblasts derived from *Smad1*^{Col1a1} mice compared with those from Cre-negative control mice [Fig. 6(A–D)]. These results demonstrate that BMP signaling and osteoblast differentiation are impaired in osteoblast-specific *Smad1*^{Col1a1} mice. To determine if Smad5/8 phosphorylation is impaired in *Smad1*^{Col1a1} mice, we performed Smad5/8 phosphorylation assay. We found that BMP-2-induced Smad5/8 phosphorylation remains intact in *Smad1-*^{Col1a1} cells although the levels were reduced compared to control cells [Fig. 6(E)]. Our previous study demonstrates that β-catenin could act upstream of BMP signaling[35]. To determine if deletion of the *Smad1* gene could inhibit β-catenin signaling in osteoblasts, we treated osteoblasts derived Cre-negative and *Smad1*^{Col1a1} mice with BIO, a GSK-3β inhibitor, which mimics the activation of β-catenin signaling. We found that BIO significantly up-regulated ALP activity in control cells. In contrast, its stimulatory effect was significantly reduced in *Smad1*^{Col1a1} cells [Fig. 6(F)]. These results suggest that BMP-Smad1 could serve as downstream signaling molecules of β-catenin signaling in osteoblasts. Since BMP signaling was not completely blocked in osteoblasts derived from *Smad1*^{Col1a1} mice, the results suggest that BMP signaling through Smad5 or Smad8 remains intact in these osteoblasts and Smad5/8 may partially compensate the loss of Smad1 function. Generation of *Smad1/5* double KO mice or *Smad1/5/8* triple KO mice would be necessary to examine this possibility and to clarify the individual contributions of Smad1, 5 and 8 to BMP signaling in postnatal bone formation and adult bone remodeling.

Discussion

To determine the specific contribution of Smad1 in bone development and postnatal bone formation, we have generated chondrocyte- and osteoblast-specific *Smad1* cKO mice using *Col2a1-Cre* (the Cre transgene is driven by the 3 kb *Col2a1* transgene) and *Col1a1-Cre* (the Cre transgene is driven by the 2.3 kb *Col1a1* promoter) transgenic mice. Our findings demonstrate a delay in calvarial bone mineralization and reduction of postnatal bone formation in these *Smad1* cKO mice. Abzhanov *et al.* showed that type II collagen is expressed in mouse calvaria during the embryonic stage and large population of calvarial cells are derived from Col2a1-positive chondrocyte-like cells[36]. In the paper published by Ovchinnikov *et al.*[23], the authors analyzed β-gal activity at E15 embryos of *Col2a1-Cre* transgenic mice and they found *Col2a1* promoter activity is active in the axial and appendicular skeleton, calvarial bones (temporal and basioccipital bones), and the other elements of the skull. Our results show that chondrocyte-specific deletion of the *Smad1* gene causes a transient ectopic cartilage phase and delayed ossification of the calvarial bones during the prenatal stage. Our previous studies demonstrate that hypertrophic marker genes

such as *ColX* and *Alp* are upregulated by Wnt3a in primary chondrocytes. Activation of Wnt/β-catenin signaling up-regulated *Bmp-2* and *Bmp4* expression and addition of noggin, a BMP antagonist, inhibited Wnt3a-induced chondrocyte differentiation[37]. In the present study, we further demonstrated that BIO-induced ALP activity was inhibited in *Smad1*-deficient cells. These findings suggest that BMP/Smad1 signaling is downstream of Wnt/β-catenin signaling in osteoblasts. Interestingly, ectopic cartilage formation in calvariae was also observed when the β-*catenin* gene was inactivated[8], and these results suggest that the disruption of the β-catenin-BMP/Smad1 signaling pathway may be responsible for the inhibition of osteoblast differentiation in calvariae.

Cumulative evidence shows that intact BMP signaling is necessary for postnatal bone formation. Our previous studies demonstrate that disruption of normal BMP signaling by expressing dominant-negative type I BMP receptor causes osteopenic phenotype[28]. Tob is a member of anti-proliferative protein family and inhibits BMP-induced and Smad-dependent transcription in osteoblasts by direct inhibition of Smad1 or 5[38]. *Tob* KO mice show increased BMP signaling and enhanced responsiveness to BMP-2-induced osteoblast proliferation and differentiation. Bone mass and BMP-2-induced local bone formation are increased in *Tob* KO mice[38,39]. These observations demonstrate that Smad1 and other BMP-activated Smad proteins play an important role in postnatal bone formation. The importance of BMP signaling in postnatal bone formation is reinforced by studies using transgenic mice in which noggin and sclerostin transgenes were overexpressed in mature osteoblasts. In these mice, osteopenic and osteoporotic phenotypes were observed[40–42]. These findings provide evidence that activation of endogenous BMP signaling can enhance bone formation and that regulation of the amount of BMP signaling in postnatal life is required for normal bone formation. Our current findings are consistent with previous observations in *Bmpr1a* cKO mice. Deletion of the *Bmpr1a* gene in mature osteoblasts targeted by the *Og2-Cre* transgenic mice showed reduced BV and BFR in 3-month-old cKO mice[43]. However, when the *Bmpr1a* gene is deleted in early progenitor cells using the *3.2Col1-CreER* transgenic mice, bone mass was increased in the cKO mice[44]. The different phenotypes of these mice may be due to targeting different populations of cells at different stages. In our study, we used *2.3 Col1-Cre* transgenic mice which target mature osteoblasts population similar to *Og2-Cre* transgenic mice and found similar osteopenic phenotype. Although both Smad1 and Smad5 are expressed in primary osteoblasts and multiple osteoblast-like cell lines, such as 2T3 and MC3T3 cells (data not shown), our present findings suggest that Smad1 is one of the critical molecules mediating BMP signaling and regulating postnatal bone formation. To fully understand the BMP signaling in postnatal bone formation, osteoblast-specific deletion of the *Smad1/5* genes or *Smad1/5/8* genes will be required.

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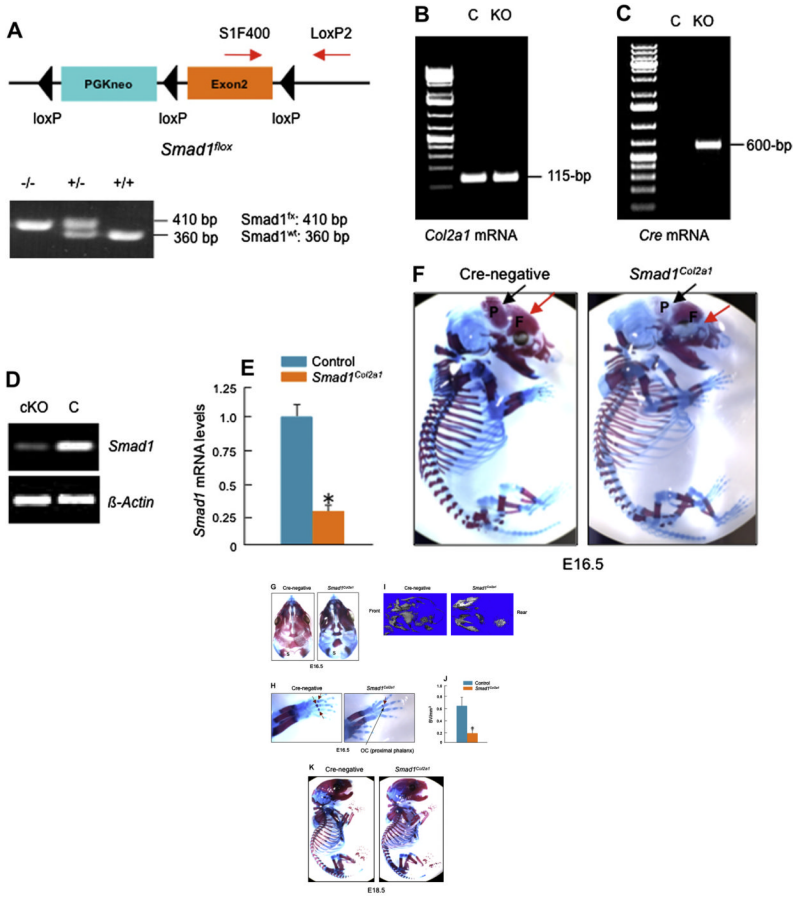

Fig. 1.

Generation and analysis of *Smad1*^{Col2a1} mice. (A) Heterozygous Smad1 floxed mice (*Smad1*$^{fx/wt}$) were bred with each other (Table I) and the production of homozygous *Smad1* floxed mice (*Smad1*$^{fx/fx}$) were identified by PCR using primers SIF400/LoxP2 (lower panel, Lane 1, 410-bp band). *Smad1*$^{fx/fx}$ mice were then bred with *Co21a1-Cre* mice (Table I, step a and b) and *Smad1*^{Col2a1} mice were identified by PCR using Cre-5′/Cre-3′ primers (600-bp PCR product) and SIF400/LoxP2 primers (410-bp PCR product). (B–E) Total RNAs were extracted from calvarial tissues of E16.5 Cre-negative and *Smad1*^{Col2a1} embryos. Expression of *Col2a1* mRNA was detected in both Cre-negative and *Smad1*^{Col2a1} embryos and expression of *Cre* mRNA was only detected in Cre-positive *Smad1*^{Col2a1} embryos. Expression of Smad1 mRNA was significantly reduced in E16.5 *Smad1*^{Col2a1} embryos. (F) Chondrocyte-specific *Smad1*^{Col2a1} embryos and littermate control embryos are indistinguishable in size at embryonic day 16.5 (E16.5). Alizarin red/Alcian blue staining was performed on E16.5 embryos. E16.5 *Smad1*^{Col2a1} embryos had delayed mineralization and ectopic cartilage formation of frontal [F] and parietal [P] bones compared to the same aged control embryos. (G) Calvarial Alizarin red/Alcian blue staining showed delayed mineralization of supraoccipital [S] bone in E16.5 *Smad1*^{Col2a1} embryo. (H) The formation of ossification center of proximal phalangeal bone was also delayed in E16.5 *Smad1*^{Col2a1} embryos. (I and J) μ-CT analysis showed reduced calvarial BV and mineralization in E16.5 *Smad1*^{Col2a1} embryos. Unpaired Student's *t*-test, $P = 0.045$ ($n = 3$). (K) E18.5 *Smad1*^{Col2a1} embryos and littermate control embryos are indistinguishable in size. No delayed mineralization and ectopic cartilage formation were observed in E18.5 *Smad1*^{Col2a1} embryos.

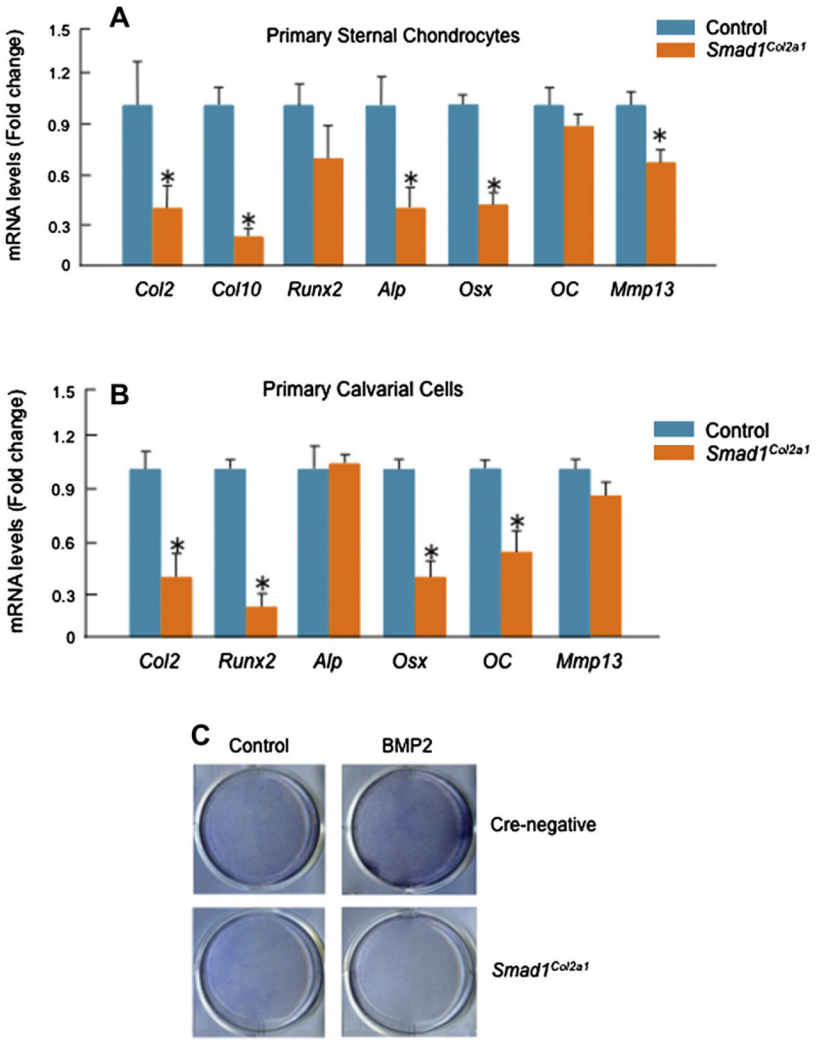

Fig. 2.

Reduction in marker gene expression in sternal chondrocytes and calvarial cells derived from *Smad1*^{Col2a1} mice. (A) Primary sternal chondrocytes were isolated from 3-day-old Cre-negative and *Smad1*^{Col2a1} mice and total RNAs were extracted from these cells. Expression of genes related to chondrocyte differentiation and was measured by real-time PCR. Significant reduction in expression of these genes was observed in *Smad1*-deficient cells. Unpaired Student's *t*-test, *Col2a1*, $P = 0.033$; *Col10a1*, $P = 0.005$; Runx2, $P = 0.012$; ALP, $P = 0.048$; *Osterix*, $P = 0.004$; *OC*, $P = 0.080$; *Mmp13*, $P = 0.022$ (Cre-negative, $n = 6$; *Smad1*^{Col2a1}, $n = 4$). (B) Primary calvarial cells were isolated from 3-day-old Cre-negative and *Smad1*^{Col2a1} mice and total RNAs were extracted from these cells. Expression of genes related to osteoblast differentiation and mineralization was measured by real-time PCR. Unpaired Student's *t*-test, *Col2a1*, $P = 0.005$; Runx2, $P = 0.001$; *Alp*, $P = 0.452$; *Osterix*, $P < 0.001$; *OC*, $P = 0.002$; *Mmp13*, $P = 0.032$ (Cre-negative, $n = 6$; *Smad1*^{Col2a1}, $n = 4$). (C) Primary calvarial cells were isolated from 3-day-old *Smad1*^{Col2a1} and their littermate control mice. Cells were treated with vehicle or BMP-2 (100 ng/ml) for 24 h, followed by ALP staining. *Smad1*-deficient cells had decreased basal ALP activity. BMP-2 stimulated ALP activity in control cells but had no effect on *Smad1*-deficient cells.

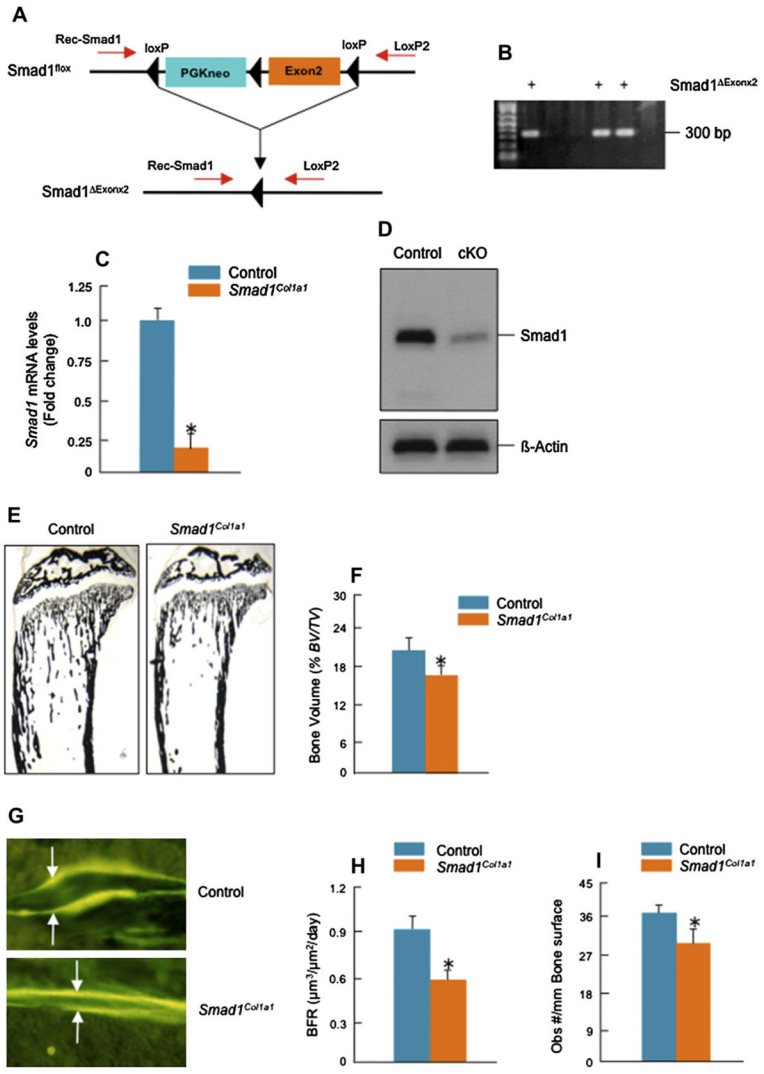

Fig. 3.

Generation and analysis of *Smad1*^{Col1a1} mice. (A and B) *Smad1*$^{del/wt}$ mice were bred with *Col1a1-Cre* mice and *Col1a1-Cre;Smad1*$^{del/wt}$ mice were identified by PCR using Cre-5′/Cre-3′ primers (lower panel, Lane 1, 4, 5, 600-bp PCR product) and Rec-Smad1/LoxP2 primers (300-bp PCR product). Heterozygous Smad1 null allele mediated by CMV-Cre was detected by Rec-Smad1/LoxP2 primers. To generate osteoblast-specific *Smad1*^{Col1a1} mice, the *Col1a1-Cre;Smad1*$^{del/wt}$ mice were bred with *Smad1*$^{fx/fx}$ mice. The desired progeny, *Col1a1-Cre;Smad1*$^{del/fx}$ mice (*Smad1*^{Col1a1} mice), were identified by PCR using SIF400/LoxP2 primers (tail tissue: 410-bp PCR product), Cre-5′/Cre-3′ primers (600-bp PCR product) and Rec-Smad1/LoxP2 primers (300-bp PCR product). (C and D) Expression of *Smad1* mRNA and protein was measured by real-time PCR and Western blotting in bone marrow cells. Expression of *Smad1* mRNA was reduced about 80%. Unpaired Student's *t*-test, $P < 0.001$ ($n = 3$). Expression of Smad1 protein was detected by Western blotting using an anti-Smad1 monoclonal antibody in bone marrow cells derived from control mice but significantly reduced in those derived from *Smad1*^{Col1a1} mice. (E and F) BV was analyzed in a defined area 0.5–2.5 mm from the growth plate in proximal tibiae of 2-month-old *Smad1*^{Col1a1} mice and littermate control mice. The BV was normalized to TV. A 30% decrease in BV was observed in *Smad1*^{Col1a1} mice compared with littermate controls.

Unpaired Student's *t*-test, $P = 0.006$ ($n = 6$). (G and H) The animals were labeled with calcein/calcein. In *Smad1*^{Col1a1} mice, the distance between the two labels was significantly reduced as indicated by white arrows. The BFR/BS were measured in the area of the marrow cavity 0.5–2.5 mm from the growth plates of proximal tibiae and expressed as $mm^3/mm^2/day$ (F). Unpaired Student's *t*-test, $P = 0.004$ ($n = 6$). (I) Osteoblast numbers in trabecular bone in the same area were quantitated and a 19% decrease in osteoblast numbers was found in *Smad1*^{Col1a1} mice. Unpaired Student's *t*-test, $P < 0.001$ ($n = 6$).

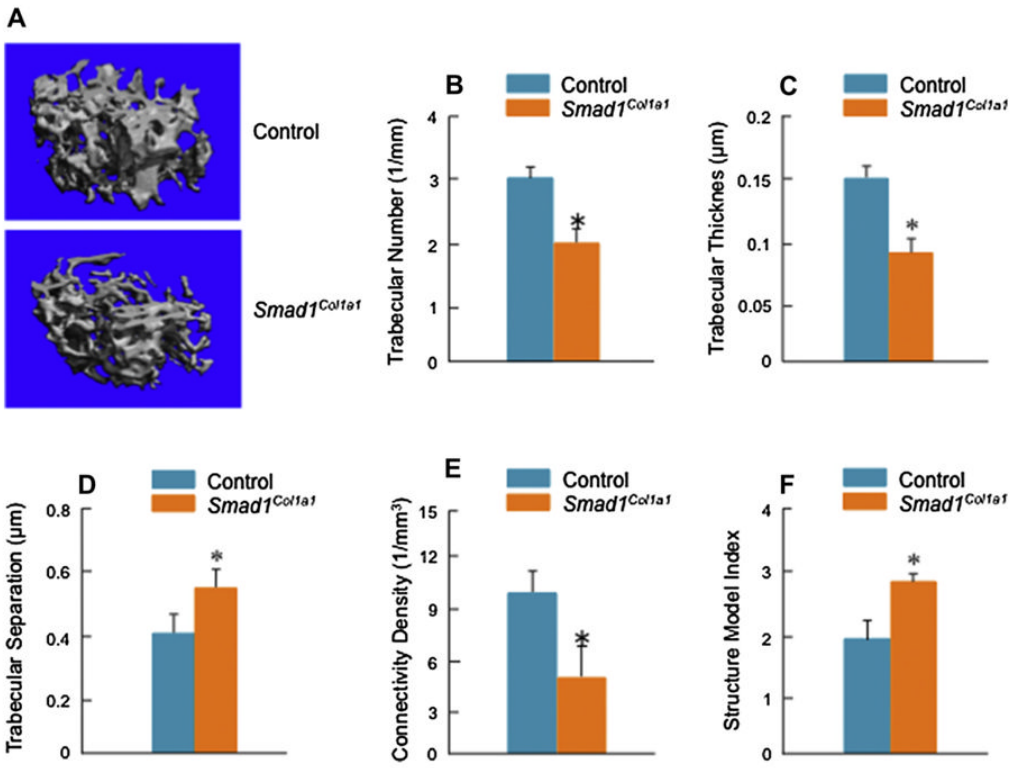

Fig. 4.

µ-CT analysis of *Smad1*^{Col1a1} mice. µ-CT images showed a significant reduction in bone mass in *Smad1*^{Col1a1} mice. (A) Trabecular structural parameters in the secondary spongiosa of the proximal tibia were measured using µCT analysis. Samples were examined with an isotropic resolution of 5-µm voxel size. Tb.N (B), Tb.Th (C) and trabecular connectivity density (Conn D) (E) were significantly decreased in *Smad1*^{Col1a1} mice compared with littermate controls. Trabecular separation (Tb.Sp) (D) and SMI (F) were significantly increased in *Smad1*^{Col1a1} mice. Unpaired Student's *t*-test; Tb.N., $P = 0.047$; Tb.Th., $P = 0.001$; Tb.Sp., $P = 0.007$; Conn D, $P = 0.012$; SMI, $P < 0.001$ ($n = 6$).

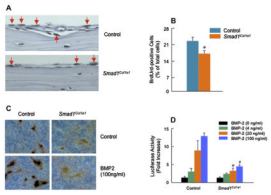

Fig. 5.

Inhibition of osteoblast proliferation and differentiation in *Smad1*^{Col1a1} mice. (A and B) Paraffin-embedded sections of calvariae were stained with BrdU. The BrdU-positive osteoblasts were indicated by red arrows and a 22% reduction of BrdU-positive osteoblasts was found in *Smad1*^{Col1a1} mice. Unpaired Student's *t*-test, $P = 0.002$ ($n = 4$). (C) Primary osteoblasts isolated from *Smad1*^{Col1a1} mice and littermate control mice were cultured for 10 days in the absence or presence of BMP-2 (100 ng/ml). von Kossa staining was performed after the cell culture was ended and the formation of mineralized bone nodules was analyzed. In osteoblasts derived from *Smad1*^{Col1a1} mice, the formation of mineralized bone nodules was inhibited. (D) Primary osteoblasts isolated from *Smad1*^{Col1a1} mice and littermate control mice were transfected with BMP signaling reporter construct 12× SBE-OC-Luc and treated with 4, 20 and 100 ng/ml of BMP-2. The luciferase activity was measured and normalized to β-gal activity. In osteoblasts derived from *Smad1* cKO mice, BMP signaling was significantly inhibited compared with cells isolated from littermate control mice. One-way ANOVA followed by Dunnett's test, BMP-2 (4 ng/ml), $P = 0.003$; BMP-2 (20 ng/ml), $P = 0.014$; BMP-2 (100 ng/ml), $P < 0.001$ ($n = 3$) between control and *Smad1*^{Col1a1} cells.

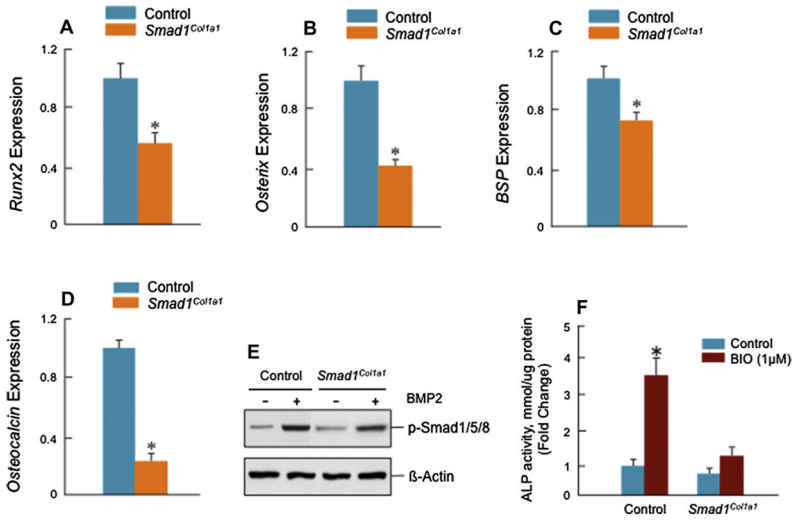

Fig. 6.

Reduction in expression of osteoblast marker genes in *Smad1*^{Col1a1} mice. (A–D) mRNA expression of osteoblast marker genes was examined by real-time PCR. The expression of *Runx2*, *Osterix* (*Osx*), *BSP* and osteocalcin (D) genes was significantly decreased in osteoblasts derived from *Smad1*^{Col1a1} mice. Unpaired Student's *t*-test; *Runx2*, $P = 0.002$; *Osterix*, $P = 0.022$; *BSP*, $P = 0.050$; *OC*, $P = 0.002$ ($n = 6$). (E) Primary osteoblasts were starved for 12 h and then treated with BMP-2 (100 ng/ml) for 6 h. Smad1/5/8 phosphorylation was measured by Western blotting using an anti-p-Smad1/5/8 antibody. BMP-2-induced Smad1/5/8 phosphorylation was reduced in *Smad1*-deficient cells. (F) Primary osteoblasts were treated with BIO (1 μM) for 48 h and ALP activity in cell lysates was measured. Significant inhibition of ALP activity was found in *Smad1*-deficient cells. One-way ANOVA followed by Dunnett's test, basal ALP, $P = 0.127$; BIO, $P < 0.001$ ($n = 3$), between control and *Smad1*-deficient cells.

Table I

Breeding and production of *Smad1*^{Col2a1} mice

Breeding	Desired progeny
a) Col2a1-Cre$^{+/-}$ ×Smad1$^{fx/fx}$	a) Col2a1-Cre$^{+/-}$; Smad1$^{fx/wt}$
b) Col2a1-Cre$^{+/-}$; Smad1$^{fx/wt}$× Smad1$^{fx/fx}$	b) Col2a1-Cre$^{+/-}$; Smad1$^{fx/fx}$

Table II

Breeding and production of *Smad1*^{Col1a1} mice

Breeding	Desired progeny
a) CMV-Cre$^{+/-}$× Smad1$^{fx/fx}$	a) Smad1$^{del/wt}$
b) Col1a1-Cre$^{+/-}$×Smad1$^{del/wt}$	b) Col1a1-Cre$^{+/-}$; Smad1$^{del/wt}$
c) Col1a1-Cre$^{+/-}$; Smad1$^{del/wt}$ ×Smad1$^{fx/fx}$	c) Col1a1-Cre$^{+/-}$; Smad1$^{del/fx}$