Smoc1 and Smoc2 regulate bone formation as downstream molecules of Runx2

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Runx2 is an essential transcription factor for bone formation. Although osteocalcin, osteopontin, and bone sialoprotein are well-known Runx2-regulated bone-specific genes, the skeletal phenotypes of knockout (KO) mice for these genes are marginal compared with those of Runx2 KO mice. These inconsistencies suggest that unknown Runx2-regulated genes play important roles in bone formation. To address this, we attempted to identify the Runx2 targets by performing RNA-sequencing and found Smoc1 and Smoc2 upregulation by Runx2. Smoc1 or Smoc2 knockdown inhibited osteoblastogenesis. Smoc1 KO mice displayed no fibula formation, while Smoc2 KO mice had mild craniofacial phenotypes. Surprisingly, Smoc1 and Smoc2 double KO (DKO) mice manifested no skull, shortened tibiae, and no fibulae. Endochondral bone formation was also impaired at the late stage in the DKO mice. Collectively, these results suggest that Smoc1 and Smoc2 function as novel targets for Runx2, and play important roles in intramembranous and endochondral bone formation.
In vertebrates, two different types of bone-formation processes, intramembranous ossification and endochondral ossification, are known to occur during embryonic and postnatal skeletogenesis. In intramembranous ossification, mesenchymal stem cells directly differentiate into osteoblasts that subsequently form bone tissues. For endochondral ossification, chondrocytes differentiate from mesenchymal stem cells to form cartilage tissues that are eventually replaced by bone tissues containing osteoblasts and osteoclasts.

Bone morphogenetic protein (Bmp) family members are powerful cytokines that exhibit bone and cartilage formation activities by inducing osteoblast and chondrocyte differentiation. Among the Bmp family members, Bmp2 regulates the expressions and functions of runt-related transcription factor 2 (Runx2), Sp7 transcription factor 7 (Osterix), and Sex determining region Y-box 9 (Sox9), as critical transcription factors for bone and cartilage development.

In particular, Runx2, a member of the Runt family of transcription factors, plays an indispensable role in bone formation and osteoblastogenesis. Runx2-deficient mice manifest no bone formation. Mutations in the Runx2 gene cause cleidocranial dysplasia, characterized by impaired bone formation in the calvaria and clavicles. Runx2 was also identified as a transcription factor that binds to the osteoblast-specific element 2 present in the osteocalcin (Ocn) gene promoter. In addition, Runx2 was sufficient to promote mesenchymal cell differentiation into osteoblasts. During osteoblast differentiation, Runx2 specifically regulated the expressions of osteoblast-specific and osteogenic genes, including Ocn, osteopontin, and bone sialoprotein.

However, the skeletal phenotypes in knockout (KO) mice for these genes are very marginal or absent compared with those in Runx2 KO mice. Therefore, it is predicted that currently unknown Runx2-target molecules play critical roles in bone formation.

In this study, we aimed to identify critical downstream molecules of Runx2 and investigate their functional roles in skeletal development. RNA-sequence and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses indicated that Smoc1 and Smoc2 were induced by both, Runx2 and Bmp2. Dominant-negative (DN)-Runx2 suppressed Smoc1 and Smoc2 expressions and their expressions were reduced in Runx2 KO mice. Smoc1 and Smoc2 knockdown inhibited mouse osteoblast differentiation and mineralization. Moreover, Smoc1 and Smoc2 double-knockout (DKO) mice manifested no skull formation, dwarfism, and shortened long bones. Histological analyses revealed impaired endochondral ossification at the late stage when Runx2 is required. Therefore, our results demonstrated that Smoc1 and Smoc2 function as transcriptional target modules and play important roles in bone formation.

**Results**

**Smoc1 and Smoc2 regulation by Runx2 and Bmp2.** To identify important molecules involved in osteoblastogenesis and bone formation, we performed RNA-sequence analyses using limb bud cells infected with control or Runx2 adenoviruses, or Bmp2 adenovirus, which regulates Runx2 function and expression, and induces bone formation. Searching for genes that displayed a log2 fold-change value of >1 expression level in cells infected with Runx2 or Bmp2, we determined the upregulation of 653 genes by Bmp2 and 497 genes by Runx2 (Supplementary Fig. 1 and Supplementary Data 2 and 3). From the 180 common genes that were upregulated by both, Bmp2 and Runx2 (Supplementary Fig. 1d), we focused on the matrix proteins Smoc1 and Smoc2, which contain an extracellular calcium-binding domain, and are members of the secreted acidic cysteine-rich glycoprotein (SPARC)-related family genes. We confirmed the induction of Smoc1 and Smoc2 expressions in limb bud cells by both, Runx2 and Bmp2 by RT-qPCR analyses (Figs. 1a, b). To understand the importance of Runx2 for Smoc1 and Smoc2 expressions, we determined the effects of DN-Runx2 on their expressions in primary osteoblasts isolated from mouse calvariae. As expected, DN-Runx2 overexpression markedly inhibited Osterix and Osteocalcin expressions, both of which are well-known Runx2 transcriptional targets in primary osteoblasts (Fig. 1c, d). Smoc1 and Smoc2 expressions were significantly inhibited by DN-Runx2 overexpression (Fig. 1e, f). To further assess the role of Runx2 in Smoc1 and Smoc2 regulation, we examined the effects of Bmp2 on limb bud cells prepared from Runx2-deficient and wild-type (WT) littermate mice. Sustained exposure of limb bud cells to Bmp2 induced Runx2 expression (Supplementary Fig. 2), and Bmp2 induced Osterix and osteocalcin expressions in WT limb bud cells, but not in Runx2 KO limb bud cells (Fig. 1g, h). DN-Runx2 treatment suppressed Bmp2-dependent Smoc1 and Smoc2 induction in WT limb bud cells (Fig. 1i). In addition, Bmp2-dependent Smoc1 induction was absent in Runx2 KO limb bud cells (Fig. 1i). By contrast, Bmp2 still induced Smoc2 expression in Runx2 KO limb bud cells. This result suggests that a Runx2-independent Bmp2 signal is also involved in Smoc2 upregulation. Indeed, Bmp2 and Runx2 synergistically induced Smoc1 and Smoc2 expressions as an early response to infection (Supplementary Fig. 3a, b). Furthermore, we confirmed Runx2 binding on the Smoc1 and Smoc2 genes promoters as determined by chromatin immunoprecipitation assays (Supplementary Fig. 4), referred to published ChIP-seq datasets. These results suggest that Smoc1 and Smoc2 are downstream molecules of Runx2 and Bmp2 signaling.

**Smoc1 and Smoc2 expressions in skeletal tissues.** To clarify whether Smoc1 and Smoc2 expressions are regulated by Runx2 in vivo, we examined their expressions in Runx2 KO and WT littermate mice at the E12.5 stage as determined by whole-mount in situ hybridization. The analyses revealed that Smoc1 was strongly expressed in the skull (Fig. 2a), forelimbs, hindlimbs (Fig. 2b), and vertebrae (Fig. 2c). The Smoc1 signals in the skull and vertebrae of Runx2 KO mice were moderately less than those of WT mice (Fig. 2a, c). Strong Smoc2 expression was observed in the skull of WT mice (Fig. 2d), whereas only very weak signals were detected in other tissues (Fig. 2e, f). Notably, a drastic decrease of Smoc2 expression in the skull of Runx2 KO mice was seen compared with that in the skull of WT mice (Fig. 2d). In addition, the localization of Runx2 expression was similar to Smoc2 expression in the skull (Supplementary Fig. 5a, b). These results suggest that Smoc1 and Smoc2 are downstream molecules of Runx2 in vivo.

**Roles of Smoc1 and Smoc2 in osteoblastogenesis.** To examine the functional roles of Smoc1 and Smoc2 in osteoblast differentiation, we performed knockdown experiments by employing short hairpin RNA (shRNA) approaches in primary osteoblasts isolated from mouse calvariae. shRNA retrovirus infection against Smoc1 (shSmoc1) and Smoc2 (shSmoc2) efficiently inhibited Smoc1 and Smoc2 expressions in osteoblasts, respectively (Fig. 3a, b). Runx2 and Osterix expressions were significantly suppressed in osteoblasts by shSmoc1 and/or shSmoc2 retrovirus infection (Fig. 3c, d). Furthermore, shSmoc1 and/or shSmoc2 retrovirus infection inhibited osteoblast alkaline phosphatase activity (Fig. 3e, f) and mineralization (Fig. 3g). These data suggest that both, Smoc1 and Smoc2, play a role in osteoblast differentiation and maturation.

**Roles of Smoc1 and Smoc2 in bone and cartilage development.** To evaluate the functional roles of Smoc1 and Smoc2 in bone formation in vivo, we generated Smoc1 and Smoc2 KO mice (Supplementary Figs. 6 and 7). To generate Smoc1 KO mice,
we first generated Smoc1 flox mice and mated these with CAG-Cre transgenic mice. Following the generation of Smoc1 heterozygous deficient mice, we established Smoc1 KO mice by mating the heterozygous mice (Supplementary Fig. 6). Okada et al. previously reported that Smoc1 homozygous mutant mice displayed a phenotype of ocular dysplasia, limb malformation, and fibula loss24. These Smoc1 KO mice were viable at postnatal day 0 (P0) but died within 3 weeks. Rainger et al. also showed that Smoc1 mutant mice have defects of eye development, mispositioned femur and fibula, and hindlimb malformation25. Our Smoc1 KO mice had a similar skeletal phenotype to these, however, they died shortly after birth, not surviving for more than a day (Supplementary Fig. 8); although we do not know the reason why our Smoc1 KO mice are lethal. A recent study by Marchant et al. reported that canine brachycephaly
is associated with the SMOC2 gene function and that the dogs in which the SMOC2 gene was mutated displayed dysmorphic skulls. Our Smoc2 KO mice showed almost normal growth and fertility (Supplementary Fig. 9a–f). However, a moderate craniofacial phenotype, with shorter nasal to eye and nasal to parietal bone distances, was observed in Smoc2 KO mice (Supplementary Fig. 9g–i). Because Smoc1 and Smoc2 have been proposed to functionally compensate for one another based on their highly conserved homology, we generated DKO mice deficient in both Smoc1 and Smoc2 and assessed their phenotypes. Based on our
observation that Smoc2−/− mice had only marginal phenotypes in skeletal tissues compared with their WT littermates (Supplementary Fig. 9), we first mated Smoc1+/−;Smoc2−/− mice with each other and used the Smoc2−/− littermate mice as control mice for the Smoc1 and Smoc2 DKO mice. Deletion of the Smoc1 or Smoc2 gene did not affect the expression level of the other gene in vivo (Supplementary Fig. 5b, c). A severe craniofacial phenotype, which resulted in neonatal lethality of the mice, and moderate dwarfism were observed in Smoc1−/−;Smoc2−/− mice (Fig. 4a–c), whereas clavicle formation appeared normal in Smoc1−/−;Smoc2−/− mice and Smoc2−/− mice at E17.5 (Fig. 4d). Shortened scapula, humerus, tibia, and forelimb were observed in Smoc1−/−;Smoc2−/− mice compared with Smoc2−/− mice (Fig. 4e–h). The tibia in Smoc1−/−;Smoc2−/− mice was also bent (Fig. 4g). Cranial hypoplasia was also confirmed in the earlier developmental stages of E12.5 and E13.5 (Supplementary Fig. 10). To further analyze the skeletal phenotype of Smoc1 and Smoc2 DKO mice, we next mated Smoc1+/−;Smoc2+/− mice. As a result
of counting all the genotyping patterns of the E18.5 offspring, the number of individuals obtained was approximately according to Mendel’s laws (Supplementary Data 4). Consistent with the results shown in Fig. 4, Smoc1 and Smoc2 DKO mice displayed dwarfism, complete loss of calvariae, skull hypoplasia, and shortened limbs (Supplementary Fig. 11). Interestingly, Smoc1−/−;Smoc2+/− mice also showed severely deficient calvariae morphogenesis and exhibited craniofacial anomalies (Supplementary Fig. 11 and Supplementary Data 4). These results indicate that Smoc1 and Smoc2 play important roles in bone formation and craniofacial development.

Our Smoc1 KO mice displayed loss of the fibulae and consistent phenotypes with Smoc1tm1a/tm1a mutant mice (Supplementary Fig. 8). Because it has been reported that fibula loss is caused by Homeobox C11 (Hoxc11) gene overexpression27, we examined the involvement of Hoxc11 in the phenotype observed in Smoc1 KO mice. Interestingly, Hoxc11 expression was significantly upregulated by Bmp2 (Supplementary Fig. 12a). We also found an association of Hoxc11 with Runx2 by performing co-immunoprecipitation experiments (Supplementary Fig. 12b). However, Hoxc11 overexpression did not affect Smoc1 or Smoc2 expressions (Supplementary Fig. 12c, d). Runx2, Osterix, and osteocalcin expressions were unaltered by Hoxc11 overexpression (Supplementary Fig. 12e–g). Taken together, it is unlikely that Hoxc11 plays a role in fibula formation through Smoc1 or Smoc2, and other molecules would be involved in Bmp2-induced Smoc1 and Smoc2 expressions.

Fig. 4 Skeletal abnormalities in Smoc1/2 DKO mice. Smoc1+/−;Smoc2−/− mice were mated, and Smoc1+/++;Smoc2+/− and Smoc1−/−;Smoc2−/− littermate were analyzed at E17.5 macroscopically under a stereoscopic microscope (a) and by skeletal preparations stained with alizarin red and alcan blue (b–h). a Growth retardation in Smoc1−/−;Smoc2−/− mice. Staining in the whole body (b), skull (c), clavicle (d), scapula (e), humerus (f), tibia and fibula (g), and forelimb (h) of Smoc1+/++;Smoc2−/− and Smoc1−/−;Smoc2−/− littermate mice. a, b Scale bars: 5 mm. c–h Scale bars: 1 mm.
Because we observed dwarfism and shortened long bones in Smoc1 and Smoc2 DKO mice (Fig. 4 and Supplementary Figs. 10 and 11), we investigated the roles of Smoc1 and Smoc2 in endochondral bone formation. To achieve this, we histologically examined the tibia in Smoc1 and Smoc2 DKO mice at E15.5. Endochondral ossification appeared delayed in Smoc1 and Smoc2 DKO mice compared with Smoc2 KO mice (Fig. 5a, b). Collagen, type I, alpha 1 (Col1a1), Collagen, type II, alpha 1 (Col2a1), and Parathyroid hormone 1 receptor expressions appeared normal in Smoc1 and Smoc2 DKO mice (Fig. 5c).

Col10a1 expression was clearly separated from diaphysis in Smoc2 KO mice, however, it was not still separated in the diaphysis of Smoc1 and Smoc2 DKO mice, supporting the notion that the late stage of endochondral bone formation was delayed in the DKO mice (Fig. 5c, d). Similarly, Matrix metallopeptidase 13 (Mmp13), Osterix, and Runx2 expressions were reduced in Smoc1 and Smoc2 DKO mice (Fig. 5c, d). These data demonstrate that Smoc1 and Smoc2 are required for the late stage of endochondral bone formation, during which step Runx2 plays an important role (Fig. 6).

We subsequently attempted to examine whether Smoc1 and Smoc2 play a role in the differentiation of mesenchymal cells to osteoblasts and/or chondrocytes. To address this, we examined the effect of Smoc1 and Smoc2 on the expressions of osteoblast and chondrocyte marker genes in Bmp2-stimulated limb bud cells isolated from Smoc1 or Smoc2 KO and WT littermate mice. The early chondrocyte markers, Col2a1 and Sox9, were decreased in the Smoc1-deficient limb bud cells (Supplementary Fig. 13a), but not in the Smoc2-deficient limb bud cells (Supplementary Fig. 14a, b). Similar expression patterns were observed for Mmp13 (Supplementary Figs. 13a and 14f). In addition, Smoc1 deficiency decreased Osterix and Runx2 expressions (Supplementary Fig. 13a, both of which are essential transcription factors for mesenchymal cell differentiation to osteoblasts, whereas Smoc2 deficiency did not affect Runx2 or Osterix expressions (Supplementary Fig. 14d, e). These results suggest that Smoc1 might be
involved in mesenchymal cell differentiation to osteoblasts and chondrocytes, but not Smoc2. The findings were supported by the results that chondrocyte and osteoblast differentiation were suppressed in Smoc1-deficient cells as determined by alcian blue staining and alkaline phosphatase (ALP) activity assays, respectively (Supplementary Fig. 13b, c). These results suggest that Smoc1 and Smoc2 have different effects on osteoblast and chondrocyte differentiation from mesenchymal cells.

**Discussion**

In this study, we attempted to identify Runx2-target molecules involved in bone formation and isolated Smoc1 and Smoc2 as such molecules. Smoc1 and Smoc2, are members of the SPARC/osteonectin family, are matricellular proteins associated with basement membranes, and are characterized by the presence of an extracellular domain and a follistatin-like domain. Osteonectin is predominantly expressed in osteoblasts, and participates in the regulation of cell–matrix interactions, consequently influencing bone mineralization and angiogenesis. However, osteonectin KO mice appeared normal and fertile until 6 months of age and had no obvious phenotypes in their skeletal tissues. Although osteonectin was not increased by Bmp2 treatment, Smoc1 and Smoc2 were upregulated by both, Runx2 and Bmp2 (Fig. 1). Consistently, DN-Runx2 overexpression significantly inhibited Bmp2-induced Smoc1 and Smoc2 expressions. Moreover, Bmp2 failed to upregulate Smoc1 expression in Runx2-deficient limb bud cells. Importantly, Bmp2-induced osteoblast differentiation of primary osteoblasts was clearly suppressed by Smoc1 or Smoc2 knockdown. In addition, Smoc1 and Smoc2 were highly expressed in mouse embryo calvariae and limbs (Fig. 2).

Taken together, these results indicate that the SPARC family matrix proteins, Smoc1 and Smoc2, are expressed in skeletal tissues, particularly the skull, function as important downstream molecules of Runx2, and play roles in osteoblastogenesis (Fig. 6).

Mutations in the SMOC1 gene were shown to result in ophthalmo-acromelic syndrome (OAS), also known as Waardenburg anophthalmia syndrome, which manifests a distinctive pattern of distal limb anomalies. Genetically constructed Smoc1 mutant mice by gene-trapped technology, Smoc1<sup>tm1a/tm1a</sup>, with disruption of Smoc1 gene expression to ~10% of the normal level, displayed similar malformation of the hindlimbs to that observed in the limbs of human OAS patients. Consistently, our Smoc1 KO mice showed similar skeletal phenotypes to Smoc1<sup>tm1a/tm1a</sup> mice (Supplementary Fig. 8).

Because the homology between Smoc1 and Smoc2 is very high and their expressions coincide in skeletal tissues, we hypothesized that Smoc1 and Smoc2 functionally compensate one another during bone formation. Interestingly, Smoc1 and Smoc2 DKO mice displayed severe phenotypes in skeletogenesis, including the complete loss of calvariae, ocular dysplasia, and shortened limbs. In addition, Smoc1<sup>+/−</sup>;Smoc2<sup>−/−</sup> mice showed no skull formation. Considering that Smoc2<sup>−/−</sup> mice appeared normal skull formation, it would be possible that Smoc2 deficiency might not be essential for skull formation. However, consistent with the previous literature, we observed mild skull dysplasia in Smoc2 KO mice (Supplementary Fig. 9). Therefore, dosages and different tissue expressions of Smoc1 and Smoc2 are closely associated with the different bone phenotypes observed in the Smoc1 and Smoc2 mutant mice. In addition, histological analyses of Smoc1 and Smoc2 DKO mice revealed delayed endochondral bone formation.
oscillation at the late stage, when Runx2 is functional. Taken together, these results indicate that Smoc1 and Smoc2 compensate each other and play important roles in both intramembranous and endochondral bone formation. By contrast, clavicle formation appeared normal in Smoc1 and Smoc2 DKO mice compared with control mice. We speculate that the normal clavicle formation in Smoc1 and Smoc2 DKO mice was due to low Smoc1 and Smoc2 expression levels in the clavicle and/or the involvement of other molecules.

There were differences in phenotypic severity, particularly in the extremity bones during endochondral bone formation. We observed that fibular hypoplasia and tibial bending were present in Smoc1 and Smoc2 DKO mice, whereas there was little morphological difference in the humerus, scapula, and forelimb between Smoc1 and Smoc2 DKO mice and their control littermate mice (Fig. 4). Furthermore, toe fusion was observed in the hindlimb discs and to inhibit BMP signaling40. Meanwhile, a recent report that a better understanding of the molecular mechanisms could further detailed analyses are required in future studies. We believe that a better understanding of the molecular mechanisms could be achieved through the identification of the cell-surface receptors for Smoc1 and Smoc2. Thus, our findings reveal that the Bmp2–Runx2–Smoc1/Smoc2 axis plays an important role in bone formation; it may offer novel and effective therapeutic strategies associated with various bone and cartilage diseases.

Methods
Cells and reagents. LentiX-293T cells were purchased from Takara (Shiga, Japan). Plat-E cells were a generous gift from Dr. Kitamura (The University of Tokyo, Tokyo, Japan). Osteoblasts and limb bud cells were cultured in alpha modification of Eagle’s minimum essential media (α-MEM; Thermo Fisher, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO2 incubator. Plat-E cells and LentiX-293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher) containing 10% FBS. Recombinant Bmp2 was obtained from a conditioned medium of LentiX-293T cells transfected with a Bmp2 expression vector as described previously42. Bmp2 activity was determined by comparison with human recombinant Bmp2 (Peprotech, Rocky Hill, NJ, USA). Mouse osteoblasts were isolated from calvariae of 3–5-day-old neonatal mice by a sequential enzymatic digestion method as described previously.43 Briefly, mouse calvariae were gently incubated with 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 1 h at 37 °C, followed by three 20-min digests with 0.25% collagenase in DMEM for 20 min at 37 °C. Cells obtained during the last two digestion processes were collected together in α-MEM containing 10% FBS. Throughout the subsequent experiments, α-MEM containing 10% FBS, 50 μg/mL ascorbic acid, and 5 mM sodium β-glycerophosphate were used to induce osteoblastic differentiation. Limb bud cells were isolated from mouse embryos at E12–E13 and digested with 0.05% trypsin/0.53 mM EDTA in PBS for 10 min at 37 °C. Cells obtained at 37 °C were collected in α-MEM containing 10% FBS. For monolayer culture, limb bud cells were seeded at 1.6 × 10⁵ cells/cm². All other chemicals used were of the highest purity commercially available.

RNA-sequence and data analysis. Limb bud cells were infected with Venus, or Bmp2 adenovirus. After 4 days of incubation, total RNA was extracted from cells using the RNaseasy Mini Kit (Qiagen). Sequencing was done at Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) in a 75-base single-end mode. Sequenced reads without trimming were mapped to the mouse reference genome sequences (mm10) using TopHat version 2.1.1 in combination with Bowtie 2 version 2.4.2 and SAMtools version 1.11. The fragments per kilobase of gene per million mapped fragments were calculated using Cufflinks version 2.2.1. Adjusted P values from RNA-sequence data and principal component analysis (PCA) were analyzed by IDEP 9.144. Upregulated genes were defined using a threshold of false discovery rate (FDR) < 0.1 and log2 fold change >1, following analysis with the DESeq2 package. Raw reads from these samples were submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (accession number: GSE166982).

Plasmin. Venus, Runx2, DN-Runx2, or Bmp2 cDNAs were ligated to the pAX-cawt adenovirus vector (Takara) as described previously45. Flag-tagged-DN-Runx2 used in this study contains amino acids 2–247 of Runx2. This construct lacks the transcriptional activation domain at the C-terminal region. The generation of these adenoviruses was performed using an adenovirus generation kit (Takara). A Venus adenovirus was used as the control adenovirus46. For shRNA vector construction for Smoc1 and Smoc2, the following oligo DNAs were used: shSmoc1 forward, 5′-GATCCGCAAGACTCAGAATTACCAAGATATTACTTCTTGAGCTTCTGGTAGTTTTG-3′ and reverse, 5′-AATTCAGAAAAACAGCTGGAGTACAACTACTCTC-3′; shSmoc2 forward, 5′-GATCCGCCAAGAATGACAATGTAGTGATCTCTTGAATCACTA-3′ and reverse, 5′-AATTCAGAAAAACAGCTGGAGTACAACTACTCTC-3′. Oligo DNAs were annealed at a concentration of 25 μM, and incubated at 95 °C for 5 min. The annealed oligo DNAs were individually inserted into the pSIREN-retroQ shRNA expression vector (Takara) at the BamHI/EcoRI site.

Retrovirus infection. PLAT-E cells were seeded at 8 × 10⁵ cells/cm² 1 day before transfection. Polyethylenimine (PEI) was used for all transfections. The pSIREN-retroQ shRNA expression vectors for shSmoc1 and shSmoc2 were mixed with PEI, and the plasmid-PEI complexes were incubated in Opti-MEM (Thermo Fisher) for 15 min at room temperature and added to PLAT-E cells. The virus supernatant was collected at 48 h after transfection and used to infect osteoblasts for 48 h in the presence of 4 μg/mL polybrene.

Determination of ALP activity. ALP activity was determined as described previously43,47,48. In brief, cells were washed with PBS and solubilized with 0.1% Triton X-100, followed by determination of the ALP activity in lysates using p-nitrophenol phosphate as a substrate. Protein contents of the lysates were determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA). For cytochemical analysis, cells were washed with PBS and fixed with 4% parafomaldehyde in PBS. Subsequently, cells were stained with a mixture of 330 μg/mL nitroblue tetrazolium, 175 μg/mL bromochloroindolyl phosphate, 100 mM NaCl, 50 mM MgCl2, and 100 mM Tris (pH 9.5).

Alizarin red staining. Following induction of differentiation, cultured osteoblasts were washed with PBS twice, fixed in 70% ethanol, and stained with 0.4% alizarin red solution for 10 min.

Skeletal preparation of mice. Following removal of the skin and viscera, mice were fixed in 96% ethanol for 24 h. Cartilage was stained for 24 h with alcian blue solution containing 0.015% alcian blue 8GX, 20% acetic acid, and 80% ethanol. Following dehydration with 100% ethanol for 3 days, the whole bodies were digested with 1% KOH at room temperature until the skeleton became clearly visible. The specimens were subsequently stained with 0.05% alizarin red in H₂O for 24 h. Finally, the specimens were maintained in 100% glycerol and observed and photographed under an S-APO microscope (Leica, Wetzlar, Germany).
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Cultured cells were washed twice with PBS and subjected to total RNA extraction with a NucleoSpin RNA Plus Kit (Takara). cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). The individual cDNAs were amplified with THUNDERBIRD® SYBR qPCR Mix (TOYOBO) using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression levels of the target genes were determined by the delta-delta Ct method using transcripts of Actb as the internal reference for each mouse RNA sample. The primer pairs and probes used for amplification were:

- Smoc1 forward, 5′-TGCCCTGGTTGGTACGAAAG-3′, reverse, 5′-GGGTCCGTCGGATGATGAAACG-3′, and probe, 5′-TGGGCTTTGGGCACAGAGAG-3′;
- Smoc2 forward, 5′-GGCTTGGGTCACACCAGAG-3′, reverse, 5′-CTGGGCTGTCTTATTAGAAGAAAC-3′, and probe, 5′-AACGCAACACCCAGAGGACGCCA-3′;
- Osterix forward, 5′-AGGGCCACCTTGACGACAACT-3′, reverse, 5′-GGCGCTTGGATGCTCTCTCTC-3′, and probe, 5′-CCGGACGGCGATGGCCTCTCC-3′;
- Runx2 forward, 5′-CCTCCTGCCGAGGTTGCTTCC-3′, reverse, 5′-CTGGTGTCGTCACCGTCCT-3′, and probe, 5′-TGGAGCTCGCTCAACACCC-3′;
- Actb forward, 5′-TAAATCTTTCAGAAGGACAGG-3′, and probe, 5′-GTTACGTCGACCTTGACTT-3′.

Whole-mount in situ hybridization. Digoxigenin (DIG)-labeled single-stranded RNA probes were prepared using a DIG RNA Labelling Kit (Roche, Basel, Switzerland). The Smoc1 probe was an 847-bp fragment of the coding sequence (position 386–1232 in the NM_001146217.1 cDNA sequence). The Smoc2 probe was a 1041-bp fragment of the coding sequence (position 460–1500 in the NM_022315.2 cDNA sequence). The Runx2 probe was a 639-bp fragment of the coding sequence (position 3173–3807 in the NM_001146308.2 cDNA sequence).

C57BL/6J mouse embryos (E12.5) were fixed with 4% paraformaldehyde in PBS containing 0.1% Tween-20 overnight at 4°C. Samples were hybridized with gene-specific DIG-labeled RNA probes overnight at 70°C, washed, and incubated with 12500 diluted anti-DIG-AP Fab fragments (Roche) for 3 h at room temperature. For optimum signal detection, samples were treated with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate without interactions with Smad1 and Smad5. Therefore, we decided to generate mutant mice, in which exon 1 of the Smoc1 locus was floxed (Supplementary Fig. 6a). Similarly, three splice variants are known to exist for Smoc2 at the protein level. All splice variants contain exon 1 (chromosome 17: 14279506–14279799), exon 2 (chromosome 17: 14325535–14325706), and exon 3 (chromosome 17: 14336547–14336653). Exon 1 includes the ATG start codon of Smoc2. Therefore, we decided to generate mutant mice, in which exon 1 of the Smoc2 locus was floxed by the PGK Neo cassette (Supplementary Fig. 7a). These targeting vectors were electroporated into T22 embryonic stem cells46 and their homologous recombination was examined by Southern blotting analysis. Germline transmission of the mutant allele of Smoc1 flox or Smoc2 mutant was achieved by mating with C57BL/6J mice and confirmed by Southern blotting, genomic PCR, and RT-qPCR (Supplementary Materials and Methods, Supplementary Figs. 6b–d and 7b–d). The accession numbers for the Smoc1 floxed and Smoc2 heterozygous deficient mice are CDB0719K and CDB0802K, respectively (http://www2.cstr.riken.jp/~argus/mouse%20list.html). CAG-Cre transgenic mice were provided by the RIKEN Bioresource Center (Tsukuba, Japan; RBR01828). For genotyping, the product sizes were: Smoc1 WT allele, 202 bp (forward primer, 5′-TCTCCTCCATTGGCTTCCC-3′; reverse primer, 5′-GAGTGGCGGACCTTGGCTCT-3′); Smoc1 deletion allele, 112 bp (forward primer, 5′-AAGCGCCCTCCCTACCTCT-3′; reverse primer, 5′-GGTCAGGACGACAACCTTTAT-3′; Smoc2 WT allele, 216 bp (forward primer, 5′-GTTCGACAGCAGCAGTCTC-3′; reverse primer, 5′-GTTGCACAGCAGCAGTCTC-3′; Smoc2 KO allele, 205 bp (forward primer, 5′-GTTCGACAGCAGCAGTCTC-3′; reverse primer, 5′-GGTTTCAGTGGAGGAGCAACAG-3′).

All protocols for animal use and experiments were approved by the Osaka University Institute Animal Experiment Committee and the Institutional Animal Care and Use Committee of the RIKEN Kobe Branch.

Data availability

The source data for the graphs and charts in the main figures are available as Supplementary Data 1, and any remaining information can be obtained from the corresponding author upon reasonable request.

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