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Loss-of-function of ACVR1 in osteoblasts increases bone mass and activates canonical Wnt signaling through suppression of Wnt inhibitors SOST and DKK1

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Abstract

BMPs (Bone morphogenetic proteins) such as BMP2 and BMP7 have been used about one decade as bone anabolic agents in orthopaedics. The BMP receptor ACVR1, which is a key receptor of BMP7, is expressed in bone. The pathological role of ACVR1 in humans has been reported: a point mutation in ACVR1 can cause fibrodysplasia ossificans progressiva (FOP) in which ectopic ossification occurs in skeletal muscles and deep connective tissues. The physiological function of ACVR1 in bone, however, is totally unknown. The purpose of this study is to investigate the endogenous role of ACVR1 in osteoblasts, one of the most dominant cell-types in bone. We generated *Acvr1*-null mice in an osteoblast-specific manner using an inducible Cre-loxP system. Surprisingly, we found that bone mass was increased in the *Acvr1*-null mice. Interestingly, canonical Wnt signaling was increased and expression levels of Wnt inhibitors *Sost* and *Dkk1* were both suppressed in the null bones during the developmental stages. In addition, we confirmed that expression levels of both *Sost* and *Dkk1* were upregulated by BMP7 dose-dependently *in vitro*. These results suggest that the *Acvr1*-deficiency can increase bone mass by activating Wnt signaling in which both *Sost* and *Dkk1* expression levels are diminished. This study leads to a new concept of the BMP7-ACVR1-SOST/DKK1 axis in osteoblasts, in which BMP7 signaling through ACVR1 can reduce Wnt signaling via SOST/DKK1 and then inhibits osteogenesis. Although this concept is beyond the current known function of BMP7, it can explain the varied outcomes of BMP7 treatment. We believe BMP signaling can exhibit multifaceted effects by context and cell type.

Keywords

BMP; ACVR1; Wnt; SOST; DKK1; Osteoblast

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Introduction

ACVR1 is a key receptor of BMP (bone morphogenetic protein) 7 [1], which is also called OP1 (osteogenic protein 1). BMP7, approved by the FDA (Food and Drug Administration) in 2001, has been clinically applied as a bone anabolic agent for lumbar spinal fusion and treatment of long bone non-union fracture [2] because of its ability to induce osteoblast differentiation *in vitro* and ectopic bone formation *in vivo* [3]. The advantage of BMP7 therapy has been highlighted commercially, but its effectiveness reviewed during one decade is not robust because the outcomes are varied [4].

Marshall Urist made the key discovery that demineralized bone matrix induced bone formation in 1965 [5]. BMPs are members of the transforming growth factor-β (TGF-β) superfamily [6]. BMP signals, like those of other TGF-β family members, are mediated through a heteromeric receptor complex of type I and type II transmembrane Ser/Thr kinase receptors [7]. Upon ligand binding, type II receptors, which are constitutively active kinases, phosphorylate and activate type I receptors (also called ALKs). There are three BMP type I receptors, type IA (BMPR1A or ALK3), type IB (BMPR1B or ALK6), and ACVRI (or ALK2). ACVR1 is expressed in bone [8]; however, the physiological role of ACVR1 in osteoblasts has not been studied yet [9].

By contrast, a pathological role of ACVR1 in humans has been reported. A single point mutation of ACVR1 has been linked as the causative mutation in patients with fibrodysplasia ossificans progressiva (FOP; OMIM ID: 135100) [10] that display congenital malformations of the progressive heterotopic ossification in skeletal muscles and other connective tissues [11; 12]. It is recently reported that cells mediating heterotopic ossification in FOP may be of endothelial origin [13] because exceeded BMP signaling through ACVR1 can convert vascular endothelial cells into multipotent stem-like cells. Because endogenous bone in FOP patients is not affected in general, it is important to identify distinct molecular mechanisms of ACVR1 in endogenous ossification versus ectopic (i.e. heterotopic) ossification.

To elucidate the endogenous role of ACVR1 in bone development, it is necessary to study loss-of-function of ACVR1 using animal models. The conventional *Acvr1*-null mice are not suitable for this purpose because they die before bone development [14]. In this study, we generated conditional *Acvr1*-null mice using a tamoxifen-inducible Cre-loxP system under the control of a 3.2 kb type I collagen promoter. In the conditional null mice, we unexpectedly found increased bone mass. Intriguingly, canonical Wnt signaling was upregulated in the null bones in conjunction with a reduction of Wnt inhibitors *Sost* and *Dkk1*. Based on our findings, we conclude that ACVR1 negatively regulates bone mass by suppressing Wnt signaling through SOST and DKK1.

Material and Methods

Mice and tamoxifen administration

A transgenic mouse line expressing the tamoxifen (TM)-inducible Cre fusion protein *CreERT* under the control of a 3.2 kb mouse pro-collagen *a1(I)* promoter (*Col1-CreERT*) was generated by a pronuclear injection [15; 16; 17] and crossed with floxed *Acvr1* mice [18], which become functional null after Cre recombination [19]. Tamoxifen (TM, Sigma) was dissolved in a small volume of ethanol, diluted with corn oil at a concentration of 10 mg/ml, and stored at −20°C until use. To generate cKO mice in embryonic stages, we set up a breeding pair (i.e. male; *Col1-CreERT*+:*Acvr1*fx/fx, female; *Col1-CreERT*−:*Acvr1*fx/fx), injected TM (75mg/kg) intraperitoneally into pregnant females from E13.5 to E17.5, and collected fetuses at E18.5 [16]. After the administration of TM, 50% of fetuses were

expected to be cKO (Col1*CreERT*+:*Acvr1fx/fx*) and the rest were control (Col1*CreERT* −:*Acvr1fx/fx*). To generate cKO mice in weanling stages, we injected TM intraperitoneally into nursing females every three days from P2 (Postnatal day 2) until euthanasia at P21 [17]. Under this condition, TM was delivered to progeny through milk. For adult stages, TM was injected intraperitoneally twice a week for 10 weeks. Cre activity of *Col1-CreERT* mice was detected specifically in immature osteoblasts, mature osteoblasts, and osteocytes as we reported previously [16; 17]. *TOPGAL* [20] mice were obtained from the Jackson Laboratory. Wildtype tissues and osteoblasts were harvested from C57BL/6 mice [21]. The animal protocol was approved by the Institutional Animal Care and Use Committee.

X-ray and histological analyses

For X-ray analysis, rib bones from *Acvr1* cKO and control mice at P21 were harvested. Images were taken using a Faxitron X-ray system (Faxitron). For H&E staining, bones (i.e. humerus, calvariae, and tibiae) were fixed in 4% paraformaldehyde, decalcified with 10% EDTA, and embedded in paraffin. Paraffin sections were cut at 8 μm and stained using a standard protocol. For β-galactosidase (β-gal) staining on sections, decalcified calvariae and tibiae from P21 mice were soaked in up to 30% sucrose before frozen sectioning. Sections were stained with X-gal for β-gal activity and counterstained with eosin. For β-gal staining on whole tissues, bones (i.e. tibiae and calvariae) were stained with X-gal as described previously [16; 17].

Quantitative real-time RT-PCR (qRT-PCR)

RNA was isolated from P21 calvariae and newborn tissues (i.e. heart, skeletal muscles, and skull bones) using Trizol (Invitrogen) and from primary osteoblasts using Picopure (Arcturus). cDNA was synthesized using the SuperScript™ Preamplification System (Invitrogen). PCR reactions, data quantification, and analysis were performed according to the manufacturer's standard protocol for TaqMan gene expression assays (Applied Biosystems). Values were normalized to *Gapdh* using TaqMan Rodent GAPDH Control Reagents. All measurements were performed in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method [22].

Primary osteoblast culture

Newborn calvariae from C57BL/6 wild type mice were digested with type I collagenase and dispase II to isolate osteoblasts as previously described [16]. Primary osteoblasts were maintained in α-MEM containing 10% FBS and ascorbic acid (50 mg/ml, Sigma) and then treated with BMP7 (R & D) for 3 hr at increasing concentrations (10, 50, and 100 ng/ml).

Statistical analysis

All statistical analyses were performed using a two-tailed Student's *t*-test. All results were expressed as mean ± SD and compared between control and *Acvr1* cKO. A p value of <0.05 indicates statistical significance.

Results

Generation of Acvr1 cKO mice

ACVR1 is known to be expressed in many tissues [8; 23], but its quantity by tissue is unknown. We quantitatively measured the expression levels of endogenous *Acvr1* in the heart, skeletal muscles, and bones during postnatal development of wildtype mice. Interestingly, *Acvr1* is highly expressed in skull bones compared with skeletal muscles at new bone stage (Fig. 1A). To investigate the physiological role of ACVR1 in bone, we next generated conditional knockout (cKO) mice for *Acvr1* in an osteoblast-dependent manner

using a tamoxifen-inducible Cre-loxP system. In cKO bones (i.e. skull bones), expression levels of *Acvr1* was significantly reduced compared with control bones as assessed by qRT-PCR at P21 (Fig. 1B), suggesting a sucessful Cre recombination in the cKO mice. Consistent with this data, expression levels of *Id1*, a known downstream target of BMP signaling [24], was also significantly reduced. Although cKO mice appeared normal in skeletal size and length, X-ray analysis demonstrated a dramatic increase in radiodensity of adult cKO bones including the sternum and ribs (Fig. 1C). Bone mineral density assessed by DEXA was significantly increased in adult cKO bones (Control rib bones; 0.0193g/cm^2, cKO rib bones; 0.0277g/cm^2, $p = 0.0006$, $n = 5$). These results indicate that loss of ACVR1 in osteoblasts increases bone density.

Increased bone mass in Acvr1 cKO bones

Although *Acvr1* is highly expressed in bone during developmental stages (Fig. 1A), physiological roles of ACVR1 in bone development is totally unknown. To investigate the *Acvr1*-deficiency in bone development, we generated *Acvr1* cKO during embryonic stages and postnatal stages. At E18.5 (i.e. embryonic day 18.5), trabecular bone mass in cKO humerus appeared increased compared with controls as assessed by H&E staining (Fig. 2A), which is consistent with the increase in radiodensity during adult stages (Fig. 1C). In addition, the thickness of skull bones (i.e. calvariae) was increased. Similarly, we observed increased trabecular bones in cKO tibiae as well as thickened lamellar bones in cKO calvariae compared with controls at P21 (i.e. postnatal day 21) (Fig. 2B). These results demonstrate that *Acvr1* cKO mice exhibited an increase in bone mass during both embryonic and postnatal bone developmental stages. It is also suggested that *Acvr1*-deficiency affects regulation of bone mass both in the endochondral ossification process (i.e. humerus and tibiae) as well as the intramembranous ossification process (i.e. calvariae).

Upregulation of canonical Wnt signaling in Acvr1 cKO bones

Activation of Wnt signaling in osteoblasts also increases bone mass [25; 26]. It is, however, largely unknown how BMP and Wnt signaling pathways affect each other *in vivo*. To assess canonical Wnt signaling in *Acvr1* cKO mice, we used *TOPGAL* Wnt reporter mice that express a β-galactosidase transgene driven by a T cell factor (TCF) β-catenin responsive promoter [20]. When assessed by a whole mount via β-gal staining, *Acvr1* cKO:*TOPGAL* mice demonstrated increased Wnt activity in the tibiae and calvariae at P21 compared to controls (Fig. 3A). Upregulation of canonical Wnt signaling was further confirmed by a sectioned specimen with β-gal staining (Fig. 3B). It is noted that the upregulation of Wnt signaling was observed in conjunction with increased bone thickness compared to controls. These facts suggest that enhanced canonical Wnt signaling resulting from a loss of BMP signaling through ACVR1 may be a cause of increased bone mass found in the cKO bones.

We further sought to determine the molecular mechanisms responsible for the increased Wnt signaling. In the P21 cKO calvariae, expression levels of *Dkk1* and *Sost* mRNAs as assessed by qRT-PCR were significantly reduced while *Dkk2* and *Lrp5* were unchanged (Fig. 4A). We next investigated a potential link between the expression of Wnt inhibitors *Sost* and *Dkk1* and BMP signaling using wildtype primary osteoblasts. In primary osteoblasts treated with BMP7, a potent ACVRI ligand [1], levels of *Sost* and *Dkk1* increased up to 4.5- and 19-fold, respectively, after 3 hr as assessed by qRT-PCR (Fig. 4B, C). These results suggest that canonical Wnt signaling was upregulated in *Acvr1* cKO bones in conjunction with downregulation of Wnt inhibitors *Sost* and *Dkk1*. In turn, ACVRI-mediated BMP signaling can negatively regulate canonical Wnt signaling in osteoblasts under physiological conditions.

Discussion

The key finding of this study is an increase in endogenous bone mass in *Acvr1* cKO mice. This observation is in striking contrast to the current understanding of BMPs as osteogenic growth factors, which has been extensively documented with numerous *in vitro* studies [27; 28]. Similar to our current results, negative regulation of bone mass by BMPs has been genetically demonstrated in mice: 1) overexpression of *Bmp4* in osteoblasts reduced bone mass in embryos, 2) overexpression of *Noggin*, an antagonist of BMP2 and BMP4, in osteoblasts increased bone mass in embryos and weanlings [29], and 3) loss-of-function of BMPR1A, a potent receptor of BMP2 and BMP4, in osteoblasts demonstrated an increased bone phenotype in embryos, weanlings, and adults [16; 17; 21]. Collectively, these facts suggest that osteoblasts have ability to restrain bone mass by responding to BMPs. In fact, recent preclinical studies demonstrated that BMPs can reduce bone mass and density when applied to bone defect models [30; 31].

In addition to BMP signaling, Wnt signaling in osteoblasts has been examined for a decade because of its role in bone formation and bone mass [25; 26]. Both BMPs and Wnt are understood as bone mass inducers; however, the physiologic relationship between BMP and Wnt is largely unknown. This study, which focused on prenatal and postnatal developmental stages, demonstrates two key findings: 1) Wnt signaling was increased in the *Acvr1* cKO bones where bone mass was also increased and 2) Wnt inhibitors SOST and DKK1 were both suppressed in the *Acvr1* cKO bones. The physiological function of SOST and DKK1 is well documented *in vivo*: Conventional knockouts of *Sost* (*Sost*$^{-/-}$ mice) exhibit increased bone mass [32]. In humans, loss-of-function and hypomorphic mutations in *SOST* cause sclerosteosis [33] and Van Buchem disease [34], respectively, with a high bone mass (HBM) phenotype. Similarly, mice heterozygous for *Dkk1* (*Dkk1*$^{+/-}$ mice) exhibit a HBM phenotype [35], while overexpression of *Dkk1* in osteoblasts causes osteopenia [36]. These two Wnt inhibitors are upregulated by BMP7, a potent ligand of ACVRI (Fig. 4). We propose a working model of the BMP7-ACVR1-SOST/DKK1 axis, in which both SOST and DKK1 are downstream targets of ACVR1 and act as a Wnt inhibitor. As a new strategy of bone anabolic drugs, a clinical trial for treatment of osteoporosis using neutralizing antibodies for SOST (AMG785) and DKK1 (BHQ 880) has recently started [37]. Our study presented here may bring a new direction to develop ACVR1 inhibitors that can be used to increase bone mass as potential bone anabolic drugs for osteoporosis and bone fracture. For this direction, further characterization of bone anabolic parameters (i.e. bone formation rate, mineral apposition rate) in the cKO adult bones will be desired.

In conclusion, this study demonstrates that loss of BMP signaling via ACVR1 directs osteoblasts to increase endogenous bone mass. Signaling by BMPs via ACVR1 in osteoblasts negatively regulates endogenous bone mass and downregulates Wnt signaling possibly through Wnt inhibitor SOST and DKK1. This study, which proposes a new concept of BMP7-ACVR1-SOST/DKK1 functional axis, can help to understand complex roles of BMP signaling and varied outcomes of BMP treatments at molecular levels *in vivo*.

Highlights

- We investigate physiological roles of the BMP receptor ACVR1, a potent receptor for BMP7.
- Loss of ACVR1 in osteoblasts increases bone mass and activates Wnt signaling in mice.
- Wnt inhibitors SOST and DKK1 are both downregulated in the mutant bones.

> ACVR1 can repress osteogenesis and Wnt signaling via SOST/DKK1.

> This study proposes a new concept of the BMP7-ACVR1-SOST/DKK1 axis in osteoblasts.

Abbreviations

ACVR1	Activin A Receptor Type I
ALK	Activin Receptor-like Kinase
BMP	Bone Morphogenetic Protein
BMPR	BMP Receptor
DEXA	Dual-emission X-ray Absorptiometry
E	Embryonic day
FOP	Fibrodysplasia Ossificans Progressiva
HBM	High Bone Mass
P	Postnatal day
TGF-β	transforming growth factor-β
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction

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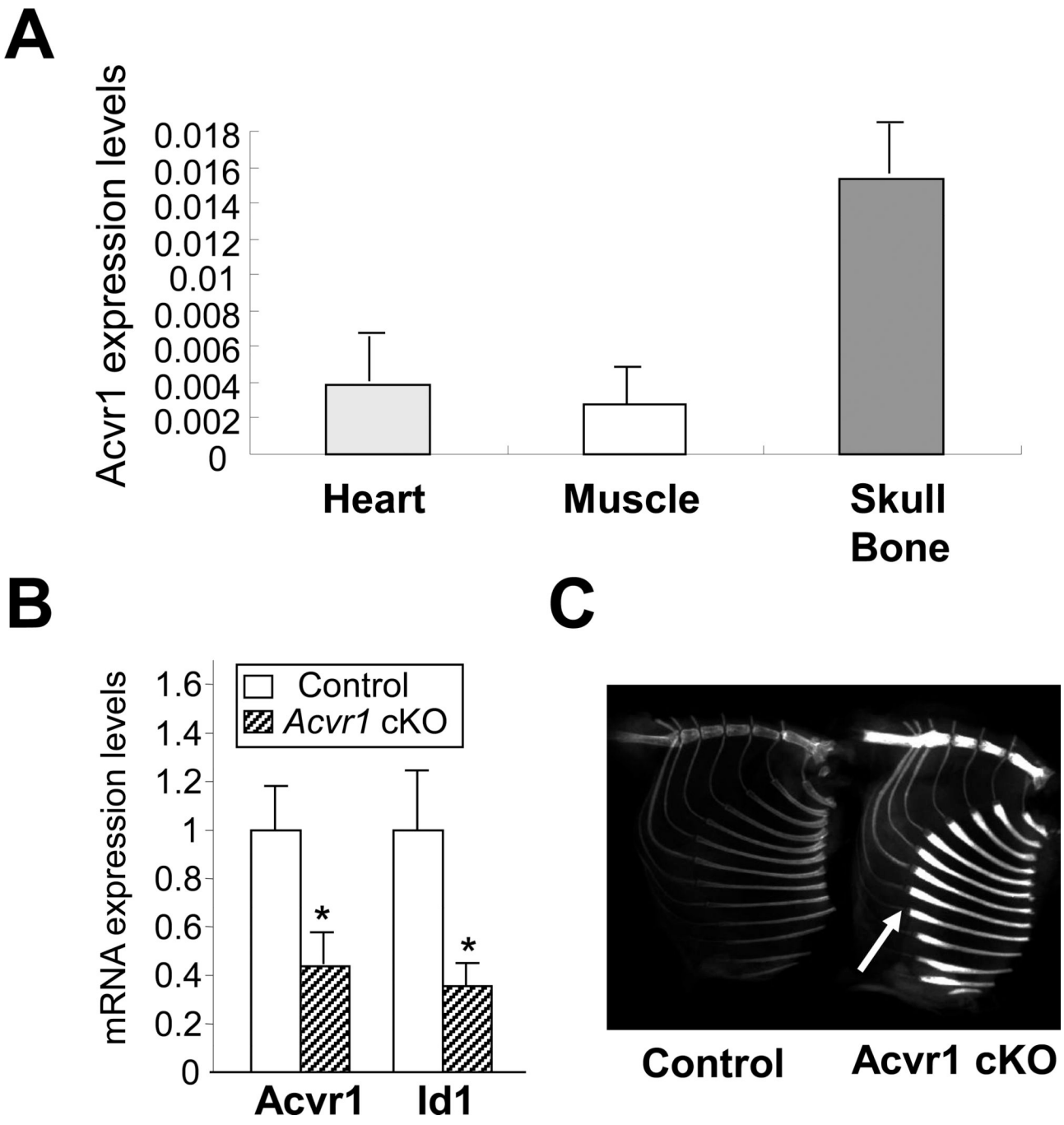

Figure 1.

Acvr1 conditional knockout (cKO) mice. (A) *Acvr1* expression levels in normal tissues. RNA was isolated from newborn wildtype mice. Endogenous expression levels of *Acvr1* were assessed by qRT-PCR. Absolute values were expressed as mean ± SD (n = 3). (B) qRT-PCR analysis for *Acvr1* and *Id1* using P21 calvariae. Expression levels of *Acvr1* and *Id1* were significantly reduced in cKO bones. Values in cKO bones (striped bar) are expressed relative to controls (open bar). mean ± SD; *, $p < 0.05$. (C) X-ray image of *Acvr1* cKO adult mice. The radiodensity of cKO rib bones was notably increased compared with controls. White arrows indicate the rib flaring in cKO mice.

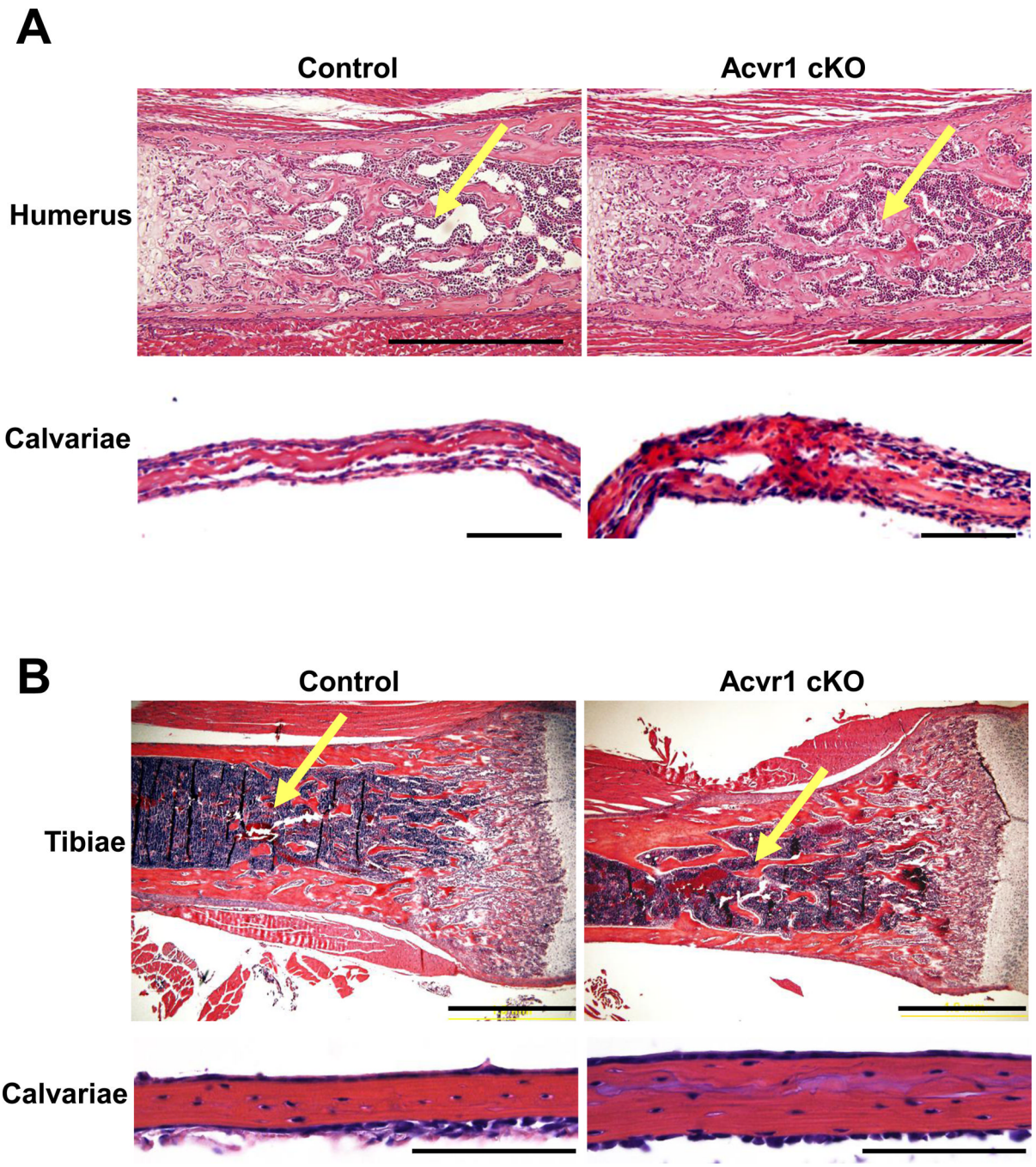

Figure 2.

Increased bone mass in *Acvr1* cKO mice during embryonic and weanling stages. (A) H&E staining of humerus and calvariae was compared between control and cKO bones at E18.5. Bars: 500 μm (humerus), 100 μm (calvariae). (B) H&E staining of tibiae and calvariae was compared between control and cKO bones at P21. Bars: 1 mm (tibiae), and 500 μm (calvariae). Yellow arrows indicate the trabecular bone area where the bone mass is more increased in cKO bones compared with controls.

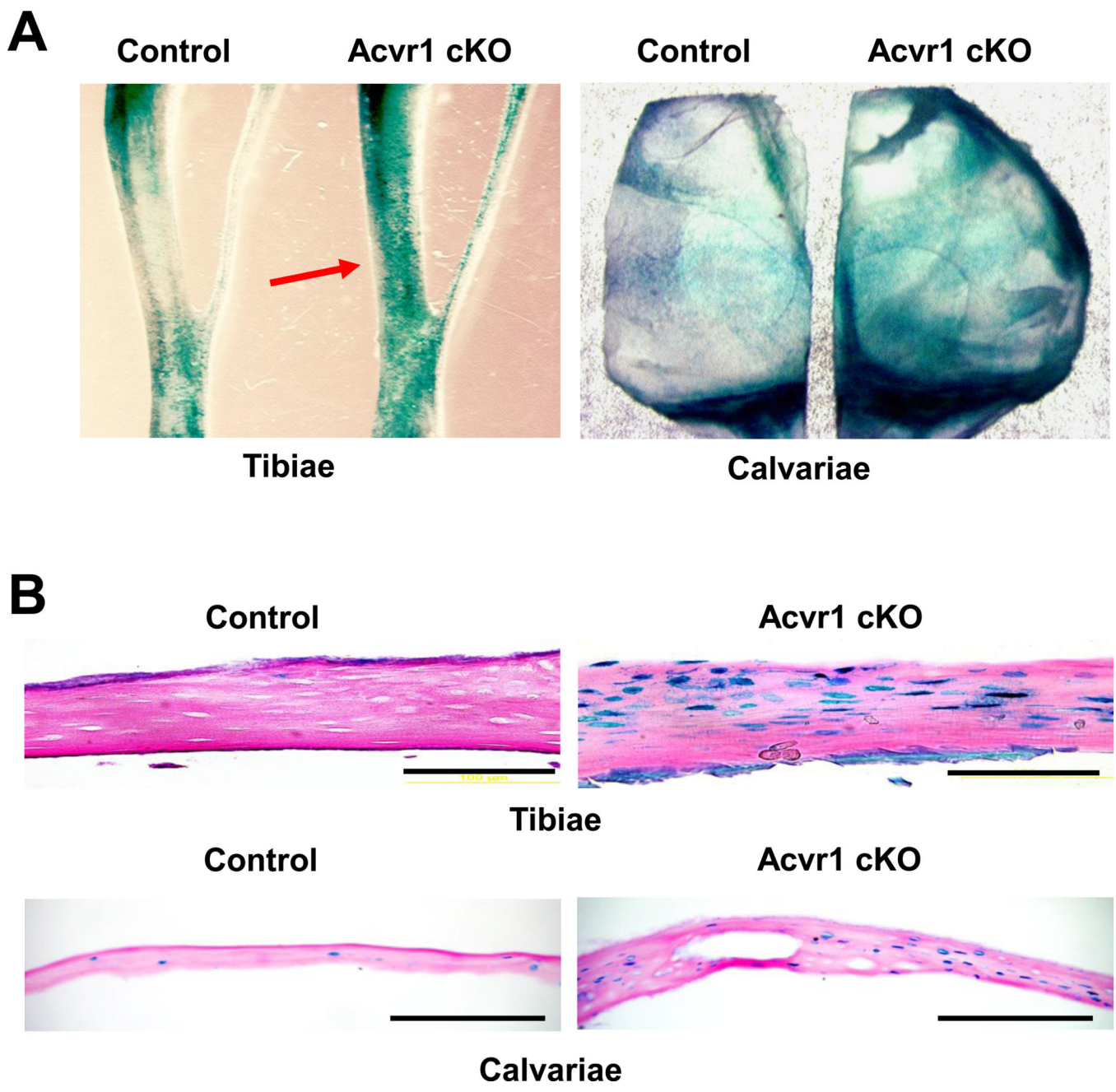

Figure 3.

Upregulation of canonical Wnt signaling in *Acvr1* cKO bones. (A) Using *TOPGAL* mice, canonical Wnt signaling was assessed by whole mount β-gal staining at P21. Wnt activity was increased in both tibiae (red arrow) and calvariae. (B) Histological analysis of Wnt signaling by β-gal staining. Note that the number of β-gal positive osteoblasts was dramatically increased in both cKO tibiae and calvariae compared with controls at P21. Bars: 200 μm.

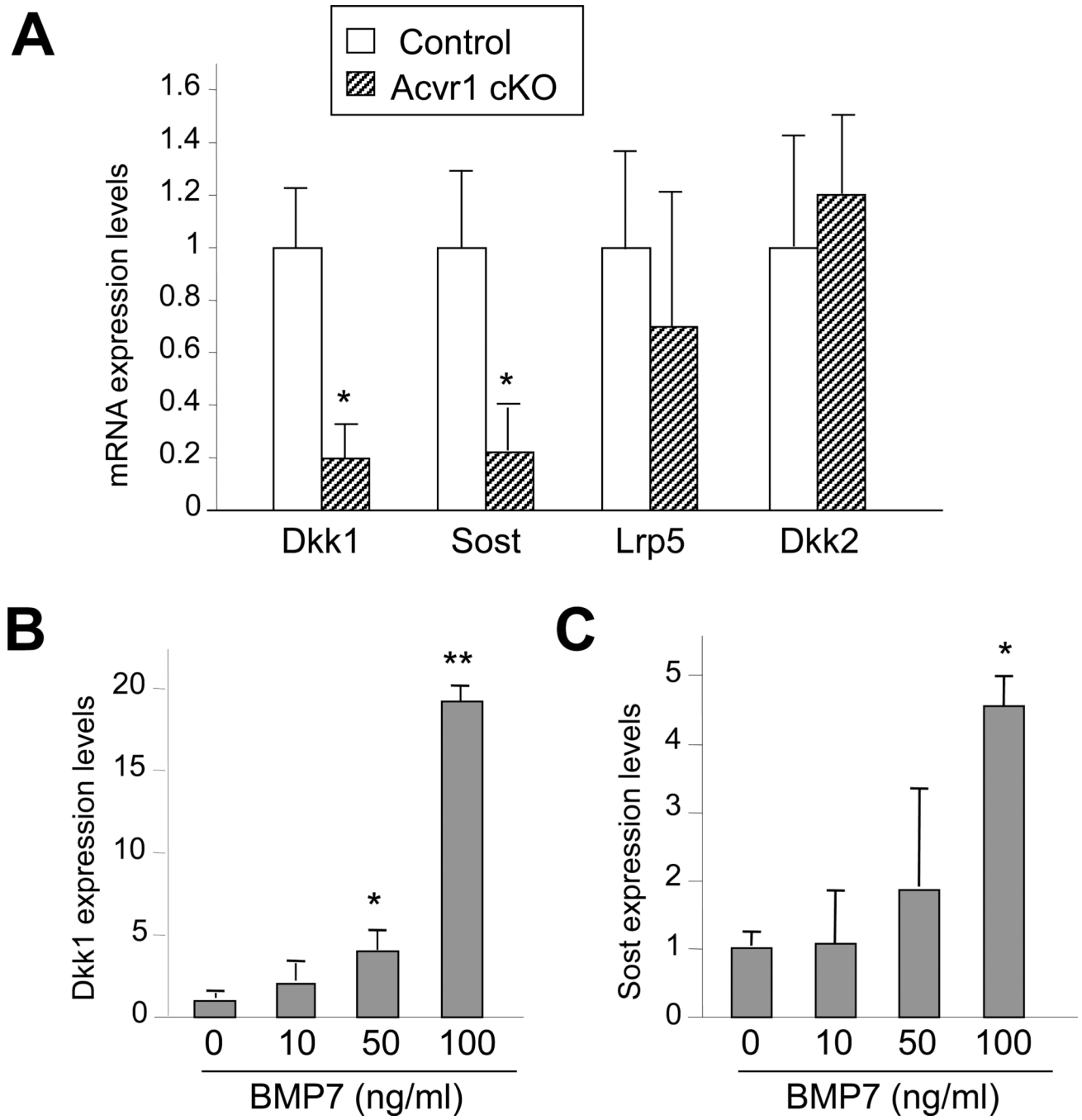

Figure 4.

Reduction of *Sost* and *Dkk1* expression in cKO bones. (A) qRT-PCR analysis for *Dkk1*, *Dkk2*, *Lrp5*, and *Sost* using P21 calvariae. Expression levels of Wnt inhibitors *Dkk1* and *Sost* were significantly reduced in cKO calvariae, while expression of co-receptor *Lrp5* was unchanged. Values in cKO bones (striped bar) are expressed relative to controls (open bar). mean ± SD; *, $p < 0.05$. (B, C) Positive regulation of *Dkk1* and *Sost* expression by BMP7. Primary osteoblasts were isolated from wildtype newborn calvariae. mRNA was extracted from osteoblasts treated with BMP7 at indicated concentrations for 3 hr. Dose-dependent effects of BMPs on *Dkk1* and *Sost* expression as assessed by qRT-PCR. Values are expressed relative to untreated osteoblasts. mean ± SD; *t*-test, *, $p < 0.05$; **, $p < 0.01$.