Disruption of Dhcr7 and Insig1/2 in cholesterol metabolism causes defects in bone formation and homeostasis through primary cilium formation

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INTRODUCTION

Cellular cholesterol amounts are regulated by multiple steps of biosynthesis and feedback mechanisms.1 Infants with cholesterol metabolism aberrations have malformations in the craniofacial region.2–5 For example, mutations in genes involved in cholesterol synthesis (DHCR7, SC5D, and DHCR24) have been found in patients with Smith-Lemli-Opitz Syndrome (SLOS) lathosterolosis, and desmosterolosis, who display craniofacial bone abnormalities.1 In addition, high-cholesterol diets during pregnancy are known to be a risk factor for birth defects, including craniofacial bone abnormalities.2,6,7 Despite these findings, it remains unclear how cholesterol metabolism contributes to craniofacial bone formation, particularly intramembranous ossification.

The 7-dehydrocholesterol reductase (DHCR7) catalyzes the final step of cholesterol biosynthesis;8 mutations in DHCR7 cause cholesterol deficiency and an excess of cholesterol precursors, resulting in craniofacial deformities (e.g., microcephaly, cleft palate, craniosynostosis, and micrognathia), intellectual disability, and behavioral problems in humans.9,10 Dhcr7−/− mice show a sucking defect, weight loss, immature lungs, distended bladders, and variable craniofacial abnormalities.11 The molecular mechanism of craniofacial anomalies in these conditions is still elusive. The insulin-induced genes 1 and 2 (INSIG1 and INSIG2) are endoplasmic reticulum (ER) retention proteins that play roles in both the regulation of the activity of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and the translocation of the sterol regulatory element-binding protein (SREBP) to the nucleus for gene regulation.12 Mice deficient for Insig1 and Insig2 (Insig1−/− Insig2−/− mice), which are negative regulators of cholesterol biosynthesis,13 show high-cholesterol levels in craniofacial tissues and display craniofacial deformities such as midfacial cleft, cleft palate, calvarial deformities and micrognathia, while mice deficient for either Insig1 or Insig2 are normal.12,13 These craniofacial deformities are rescued by the normalization of cholesterol levels in Insig1/2 null mice; however, it remains elusive how high-cholesterol levels cause craniofacial deformities and which cells are responsible for the craniofacial anomalies seen in Insig1/2 null mice.

Primary cilia, microtubule-based organelles that function in sensory and signaling pathways, are enriched with cholesterol-rich microdomains (known as lipid rafts) that recruit or retain receptors and ciliary membrane proteins.14 An association between lipid rafts and ciliary membrane proteins has been suggested in other organisms, including vertebrate photoreceptors,15 Chlamydomonas reinhardtii,16 mammalian spermatozoa,17 and Leishmania major.18 Defects of primary cilia cause various deformities, including craniofacial abnormalities (altogether known as ciliopathies, a group of genetic syndromes associated with defects in
primary cilia). The broad-spectrum phenotypes in Dhcr7−/− mice and individuals with SLOS1 (e.g., presenting with craniofacial anomalies such as craniosynostosis, hypertelorism, and cleft palate, as well as immature lungs and enlarged bladders) are similar to those seen in ciliopathies. The phenotypic similarity between ciliopathies and cholesterol synthesis defects suggests that cholesterol metabolism (level and function of mature cholesterol and cholesterol intermediates) can regulate bone metabolism (level and function of mature osteoblasts) and mice with a deletion of Dhcr7 and mice with a deletion of Insig1/2, respectively. To identify the bone abnormalities with either low or high-cholesterol levels, we carried out microCT, skeletal staining, and histological analyses in these mice. Our study aims to elucidate how Dhcr7 and Insig1/2 regulate bone formation.

RESULTS

Dhcr7 deficiency increases osteogenesis

Dhcr7−/− knockout (KO) mice presented microcephaly, accelerated bone formation, and thicker calvaria bones at birth with complete penetrance, and died within 1 day after birth (Fig. 1a–c and Supplementary Fig. S1). The accelerated bone formation resulted in immature suture fusion after culturing calvaria explants for 3 days (Supplementary Fig. S1d). To examine the cellular mechanism of how cholesterol metabolic aberrations cause accelerated bone formation in Dhcr7−/− mice, we carried out biological analyses, namely BrdU incorporation assays and Ki67 immunohistochemistry for cell proliferation, TUNEL assays for cell apoptosis, and von Kossa staining for mineralization, and immunoblotting for type I collagen for osteogenic differentiation. We found that osteogenic differentiation, but not cell proliferation and apoptosis, was increased in Dhcr7−/− frontal bones (Fig. 1d, e and Supplementary Fig. S2). Next, to determine the regulatory mechanism of osteogenic differentiation, we performed quantitative RT-PCR (qRT-PCR) analyses for osteogenic factors (Runx2, Alp, Col1a1, Col1a2, Bglap, Sparc, Sp7, and Spp1) using frontal bones from Dhcr7−/− mice at embryonic day (E) E14.5, E15.5, E16.5, and postnatal day (P) P0 (Fig. 1f and Supplementary Fig. S3). Col1a1 gene expression was significantly and consistently upregulated in Dhcr7−/− frontal bones compared to controls at E14.5-P0. Expression of RUNX2, COL1A1 and SP7 (aka Osterix) was increased, and the area with these positive signals was expanded compared to controls and correlated with increased expression of these genes in Dhcr7−/− frontal bones (Fig. 1f, g). Next, we evaluated the effect of loss of Dhcr7 on osteogenic differentiation using cultured osteoblasts from P0 frontal bones. The Dhcr7−/− osteoblast characteristics (no proliferation defect but accelerated osteogenic differentiation through upregulated Col1a1 expression) were well conserved in cultured primary osteoblasts (Fig. 1h and Supplementary Fig. S3). Taken together, our results indicate that a failure in cholesterol synthesis causes accelerated osteogenesis through upregulated Col1a1 expression.

Insig1/2 deficiency suppresses osteogenesis

To determine the tissue-specific contribution of high-cholesterol status to craniofacial deformities, we employed Insig1/2 conditional knockout (cKO) mice in cranial neural crest (CNC) cells which give origin to the majority of craniofacial bones. Insig1/2 cKO mice were viable, but they exhibited very thin frontal bones at birth and later in life (Fig. 2a, b), while the other craniofacial structures derived from CNC cells in these mice were intact. To examine the cellular mechanism of how high-cholesterol amount results in decreased bone formation in Insig1/2 cKO mice, we carried out biological analyses, as performed in Dhcr7−/− mice. We found that osteogenic differentiation, but not cell proliferation and apoptosis, was decreased in Insig1/2 cKO frontal bones (Supplementary Fig. S4). We also found that Col1a1 gene expression was significantly and consistently downregulated in frontal bones of Insig1/2 cKO mice at E14.5, E16.5 and P0 (Fig. 2c), with protein expression correlating with gene expression (Fig. 2d). Expression of COL1A1 and SP7, but not RUNX2, was decreased, which was correlated with the expressions of these genes in Insig1/2 cKO frontal bones (Fig. 2e). We further evaluated the effect of loss of Insig1/2 on osteogenic differentiation using cultured osteoblasts from Insig1/2 cKO mice (Fig. 2f and Supplementary Fig. S4). The Insig1/2 cKO osteoblast characteristics (no proliferation defect but reduced osteogenic differentiation through decreased Col1a1 expression) were well conserved in cultured primary osteoblasts. Since COL1 expression was decreased in Insig1/2 cKO mice, the abnormalities may resemble osteogenesis imperfecta, which is a congenital bone disorder characterized by thinner and fragile bones that affects 6–7 in 100,000 individuals worldwide. Altogether, our results indicate that either too much or too little cholesterol causes calvarial bone abnormalities through dysregulation of Col1a1 expression.

Ciliogenesis is altered in either Dhcr7- or Insig1/2-deficient mice. As cholesterol is abundant in cellular membranes, we carefully investigated cellular membranous structures and found that there were fewer and shorter primary cilia in Dhcr7−/− osteoblasts compared to controls (Fig. 3a–c). Ciliogenesis starts with the interaction of the basal body (mother centriole) with primary ciliary vesicles (CVs), which can be labeled with RAB11, and then the axoneme grows within the ciliary membrane while fusing with secondary CVs, which can be labeled with RAB8. The elongated primary cilium eventually fuses with the plasma membrane, allowing the distal part of the cilium to interact with the extracellular milieu. To track ciliogenesis in Dhcr7−/− osteoblasts, CVs were immunostained with anti-RAB11 and anti-RAB8 antibodies. We found that RAB11-positive primary CVs accumulated in the cells and that RAB8-positive secondary CVs failed to gather at sites of cilium formation in Dhcr7−/− osteoblasts (Fig. 3d, e).

On the other hand, Insig1/2 cKO osteoblasts displayed multiple and longer primary cilia compared to controls (Fig. 4a–c), and RAB8-positive CVs were abnormally fused with each other (Fig. 4d). The osteogenic and primary cilium phenotypes in Insig1/2 cKO osteoblasts were recapitulated with transfection of an adenovirus-Cre system (Supplementary Fig. S5). In addition, multiple basal bodies were detected, consistent with the presence of multiple primary cilia in Insig1/2 cKO osteoblasts (Fig. 4e and Supplementary Figs. S5 and S6). We then hypothesized that gene expression of molecules involved in the formation of the pericentriolar material (PCM) complex (i.e., Atf5, Aurka, Bbs4, Cdk5rap2, Cep152, Cep192, Copap, Lck, Nin, Pcm1, Pcnt, Plk1, Plk4, Sass6, Stil, Tube1, Tubg1, Tunkt, and Tunkt2), which is important for proper cilium formation, was altered in Insig1/2 cKO osteoblasts. Previous studies indicate that overexpression of Plk1, Plk4, Stil and Sass6 induces the formation of multiple centrioles. Among them, by conducting a bioinformatics promoter analysis we found that the Plk4 promoter contained two potential sterol regulatory elements (SREs) for the SRE-binding protein (SREBP) binding. To access the binding experimentally, we carried out chromatin immunoprecipitation (ChIP) assays for the sites [site 1; GTGGA-GAGT (~244 bp to ~252 bp) and site 2; TACTCCAG (~1295 bp to ~1303 bp)] in Insig1/2 cKO and control osteoblasts and found increased binding of SREBP1 and SREBP2 to the SREs in Insig1/2 cKO osteoblasts (Fig. 4f). Indeed, expression of the Plk4 gene was significantly and specifically upregulated in Insig1/2 cKO osteoblasts.
osteoblasts compared to controls (Fig. 4g), while the expression of other PCM-related genes was not altered (Supplementary Fig. S6b). We further confirmed that PLK4 protein expression was increased in the mutant osteoblasts compared to controls (Fig. 4h). Taken together, our results indicate that Dhcr7 and Insig1/2 play an important role in primary cilium formation.

WNT/β-catenin and hedgehog signaling pathways are downstream signaling cascades responsible for the bone phenotype of Dhcr7- and Insig1/2-deficient mice

We examined how the ciliary phenotypes resulted in osteogenic abnormalities. The primary cilia coordinate with multiple signaling pathways such as hedgehog (HH) and WNT during

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**Fig. 1** Loss of Dhcr7 accelerates osteogenesis. a MicroCT images of the calvaria—top view (upper panels) and side view (lower panels)—of newborn wild-type (WT) control and Dhcr7−/− knockout (KO) mice. Red arrows indicate early closure of the metopic, coronal, and sagittal sutures. Yellow dotted lines in the lower panels indicate the tips of premaxillae. b Skeletal staining of skulls from newborn WT control and KO mice. Yellow boxed areas are enlarged in the lower images. Black arrows in KO images indicate overlapping of frontal and parietal bones at the coronal suture and left and right parietal bones at the sagittal suture. c Hematoxylin and Eosin (H&E) staining of the sagittal sutures of newborn WT and KO mice. Arrows indicate the osteogenic front. Scale bar, 50 µm. d von Kossa staining of the sagittal sutures of newborn WT and KO mice. Arrows indicate the osteogenic front. Scale bar, 100 µm. e Immunoblotting for type I collagen (COL1) in P0 calvaria of WT and KO mice. GADPH was used as loading control. f Quantitative RT-PCR of the indicated osteogenic genes at E14.5 (left) and E16.5 (right) in WT (blue bars) and KO (red bars) mice. n = 6 per genotype per stage. **P < 0.01; ***P < 0.001. g Immunohistochemistry analysis for RUNX2, COL1A1 and SP7 (Osterix) in newborn WT and KO mice. Nuclei were counterstained with 0.04% methylene blue. Scale bar, 50 µm. h Alkaline phosphatase (left) and Alizarin Red (right) staining of osteoblasts isolated from newborn WT and KO calvaria after induction of osteogenic differentiation at Day 0, 7, and 14.
To investigate HH signaling in Dhcr7−/− calvaria, we analyzed Gli1 and Ptch1 levels by qRT-PCR, which are readout gene expressions for HH signaling activity.39–43 We found that expression of these genes was significantly downregulated in Dhcr7−/− calvaria (Fig. 5a). Furthermore, we employed Gli1-LacZ reporter mice to evaluate the HH signaling activity in vivo. We found that HH signaling was compromised in the calvaria of Dhcr7−/− mice compared to controls (Fig. 5b). To further confirm the reduced HH signaling activity in the calvaria of Dhcr7−/− mice, we performed cell fractionation and the consequent immunoblotting analyses for GLI1. Previous studies indicate that in the absence of HH ligands, GLI1 is truncated and degraded by the proteasome in the cytoplasm, while GLI1 translocates into the nucleus in the presence of HH ligands by escaping from degradation.44,45 As expected, full-length GLI1 was decreased in the nuclear fraction from Dhcr7−/− osteoblasts (Fig. 5c).

Next, we investigated the HH signaling activity in Insig1/2 cKO mice. We carried out qRT-PCR for Gli1 and Ptch1 in Insig1/2 cKO calvaria and found that expression of these genes was significantly upregulated in Insig1/2 cKO mice (Fig. 5d). Furthermore, we performed LacZ staining for Gli1 expression in Insig1/2 cKO/Gli1LacZ and control mice. As expected, Gli1-LacZ expression was increased in the calvaria of Insig1/2 cKO/Gli1-LacZ mice compared to that of control mice (Fig. 5e). Next, we examined whether Col1a1 expression was regulated through HH signaling, we conducted promoter analyses for GLI binding on the Col1a1 promoter and found that the Col1a1 promoter contained a putative GLI-binding site (GGCCACGCA; −68 bp to −60 bp) (Supplementary Fig. S7a).

ChIP assays validated that GLI binding to the Col1a1 promoter region was correlated with the activity of HH signaling in osteoblasts from Dhcr7−/− and Insig1/2 cKO mice (Fig. 5f, g).

Thus, HH signaling activity was correlated with the primary cilium phenotype of Dhcr7−/− and Insig1/2 cKO mice. However, osteogenesis.36–38 To investigate HH signaling in Dhcr7−/− calvaria, we analyzed Gli1 and Ptc1 levels by qRT-PCR, which are readout gene expressions for HH signaling activity.39–43 We found that expression of these genes was significantly downregulated in Dhcr7−/− calvaria (Fig. 5a). Furthermore, we employed Gli1-LacZ reporter mice to evaluate the HH signaling activity in vivo. We found that HH signaling was compromised in the calvaria of Dhcr7−/− mice compared to controls (Fig. 5b). To further confirm the reduced HH signaling activity in the calvaria of Dhcr7−/− mice, we performed cell fractionation and the consequent immunoblotting analyses for GLI1. Previous studies indicate that in the absence of HH ligands, GLI1 is truncated and degraded by the proteasome in the cytoplasm, while GLI1 translocates into the nucleus in the presence of HH ligands by escaping from degradation.44,45 As expected, full-length GLI1 was decreased in the nuclear fraction from Dhcr7−/− osteoblasts (Fig. 5c).

Next, we investigated the HH signaling activity in Insig1/2 cKO mice. We carried out qRT-PCR for Gli1 and Ptc1 in Insig1/2 cKO calvaria and found that expression of these genes was significantly upregulated in Insig1/2 cKO mice (Fig. 5d). Furthermore, we performed LacZ staining for Gli1 expression in Insig1/2 cKO/Gli1LacZ and control mice. As expected, Gli1-LacZ expression was increased in the calvaria of Insig1/2 cKO/Gli1-LacZ mice compared to that of control mice (Fig. 5e). Next, we examined whether Col1a1 expression was regulated through HH signaling, we conducted promoter analyses for GLI binding on the Col1a1 promoter and found that the Col1a1 promoter contained a putative GLI-binding site (GGCCACGCA; −68 bp to −60 bp) (Supplementary Fig. S7a).

ChIP assays validated that GLI binding to the Col1a1 promoter region was correlated with the activity of HH signaling in osteoblasts from Dhcr7−/− and Insig1/2 cKO mice (Fig. 5f, g).

Thus, HH signaling activity was correlated with the primary cilium phenotype of Dhcr7−/− and Insig1/2 cKO mice. However,
previous studies show that upregulated and downregulated HH signaling cause defective and normal craniofacial bone formation, respectively.46–48 This suggests that altered HH signaling was not responsible for the bone phenotypes in Dhcr7−/− and Insig1/2 cKO mice.

We, therefore, investigated another candidate cilium-mediated signaling that regulates intramembranous ossification, WNT/β-catenin signaling.49,50 To investigate the activity of WNT/β-catenin signaling pathway, we conducted quantitative RT-PCR for Axin2, a readout of WNT/β-catenin signaling activity, and found that its expression was upregulated and downregulated in Dhcr7−/− and Insig1/2 cKO osteoblasts, respectively (Fig. 6a, b and Supplementary Fig. S8). To validate the relationship between WNT/β-catenin signaling and cholesterol metabolism, we treated primary osteoblasts from Dhcr7−/− and Insig1/2 cKO mice with either WNT3A or lithium chloride (LiCl), a known WNT/β-catenin signaling activator, While both WNT3A and LiCl induced Axin2 expression five-fold in wild-type controls, Axin2 expression was upregulated and downregulated in Dhcr7−/− and Insig1/2 cKO osteoblasts, respectively (Fig. 6c, d). We confirmed that in mice with Toppal reporter for the WNT/β-catenin signaling activity, its activity was increased at osteogenic front of the calvaria in Dhcr7−/− mice compared to control mice (Fig. 6e). Next, to examine whether Col1a1 expression was regulated through WNT/β-catenin signaling, we conducted promoter analyses for β-catenin binding on the Col1a1 promoter and found four putative β-catenin binding sites (Supplementary Fig. S7b). To evaluate these predicted binding sites, we conducted ChIP assays for β-catenin binding to the Col1a1 promoter region, and found that β-catenin bound at binding sites 1 and 4, and the binding was correlated with the activity of WNT/β-catenin signaling in osteoblasts from Dhcr7−/− and Insig1/2 cKO mice (Supplementary Fig. S9).

Lastly, to test the functional significance of WNT/β-catenin signaling, we generated and investigated compound mutant mice with a haploinsufficiency of Axin2 in the Dhcr7 mutant background and found that normalized WNT/β-catenin signaling restored the accelerated bone formation in Dhcr7−/− mice (Fig. 6f–h). Altogether, these findings are well supported by the fact that WNT/β-catenin signaling positively regulates osteogenesis.52

The bone phenotype in Dhcr7−/− and Insig1/2-deficient mice is restored by the normalization of aberrant cholesterol metabolism with simvastatin.

To test whether abnormalities in primary cilium and bone formation can be restored by the normalization of aberrant cholesterol metabolism, we treated Dhcr7−/− and Insig1/2 cKO osteoblasts with simvastatin, which can normalize cholesterol metabolic aberrations by inhibiting the activity of the HMG-CoA reductase. Simvastatin treatment restored primary cilium formation in cultured osteoblasts from Dhcr7−/− mice (Fig. 7a–c). In addition, the in vivo administration of simvastatin to Dhcr7−/− mice (10 mg·kg−1 body weight per day, intraperitoneal injection to a pregnant mouse, E12.5–E18.5) could normalize the accelerated bone formation in newborn mice (Fig. 7d, e). We confirmed that expression of genes related to bone formation and WNT signaling was restored in Dhcr7−/− mice treated with simvastatin (Fig. 7f). These results indicate that cholesterol intermediates caused the bone defects in Dhcr7−/− mice. In Insig1/2 cKO mice, simvastatin treatment restored both the increased number and length of primary cilium (Fig. 8a, b) and the reduced bone formation in Insig1/2 cKO osteoblasts (Fig. 8c). Importantly, simvastatin treatment (10 mg·kg−1 body weight per day from P7 to P42)
improved the decreased bone formation in these mice (Fig. 8d). As expected, the expression of genes related to osteogenesis, cilia and WNT signaling was normalized in Insig1/2 cKO mice treated with simvastatin (Fig. 8e). Taken together, our results indicate that proper cholesterol metabolic status is crucial for normal primary cilium formation, which is responsible for osteogenesis in osteoblasts (Fig. 9).

**DISCUSSION**

Previous studies show that cholesterol metabolism is important for the formation and homeostasis of bone and cartilage during endochondral ossification. For example, mice with a deletion of Scap, a regulator for cholesterol biosynthesis, in either postcranial somatic lateral plate mesodermal cells (ScapF/F;Prx1-Cre mice) or chondrocytes (ScapF/F;Col2a1-Cre mice), display compromised chondrogenesis and disorganized growth plates, resulting in short limbs. By contrast, increased intracellular cholesterol in mice deficient for Insig1/2 in postcranial somatic lateral plate mesodermal cells (Insig1F/F;Insig2−/−;Prx1-Cre mice) or chondrocytes (Insig1F/F;Insig2−/−;Col2a1-Cre mice) show defects in growth plate organization and chondrogenesis, also resulting in short limbs. Thus, either too much and too low of cholesterol results in short limbs through defective endochondral ossification. In this study, we aimed to study the role of cholesterol in intramembranous ossification using both gain-of-function (Insig1/2 cKO mice) and loss-of-function (Dhcr7−/− mice) mouse models, and found that cholesterol metabolism plays a role in intramembranous ossification in a dose-dependent manner. These findings suggest that effects of abnormal
cholesterol metabolism differ between intramembranous and endochondral ossification.

7-DHC is a cholesterol intermediate as well as a precursor of vitamin D; 7-DHC is converted to pre-vitamin D3 by ultraviolet light on the skin and further converted to active vitamin D3 in the liver and kidney. Previous studies indicate that suppression of DHCR7 activity results in upregulated vitamin D3 levels in vivo after birth. While patients with SLOS show a high level of

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**Fig. 5** Altered hedgehog signaling in calvaria from Dhcr7 and Insig1/2 mutant mice during craniofacial development. 

**a** Quantitative RT-PCR analyses for Gli1 and Ptc1 expression as a readout for HH signaling activity in newborn wild-type (WT) control (blue bars) and Dhcr7 knockout (KO; red bars) calvaria. n = 6 per group. **P < 0.001. 

**b** β-galactosidase staining (blue) for sites of HH signaling activation in the frontal bones of E18.5 Dhcr7−/−;Gli1-LacZ (WT) and Dhcr7−/−;Gli1-LacZ (Dhcr7 KO) mice. Red arrows indicate osteogenic fronts; boxed areas are enlarged in lower images. Nuclei were stained with nuclear fast red. Scale bars, 100 µm. 

**c** Cell fractionation and subsequent immunoblotting analysis for GLI1, using cytosol (C) and nuclear (N) fractions from WT control and Dhcr7 KO osteoblasts. FL, full-length; CL, cleaved. 

**d** Quantitative RT-PCR analysis for Gli1 and Ptc1 in newborn WT control (blue bars) and Insig1/2 conditional KO (cKO; yellow bars) frontal bones. n = 6 per group. **P < 0.01. 

**e** β-galactosidase staining (blue) for sites of HH signaling activation in the frontal bones of P0 Insig1F/F;Insig2−/−;Gli1-LacZ (WT) and Wnt1-Cre;Insig1F/F;Insig2−/−;Gli1-LacZ (Insig1/2 cKO) mice. Open arrow indicates increased Gli1-LacZ; boxed areas are enlarged in lower images. Nuclei were stained with nuclear fast red. Scale bars, 100 µm. 

**f** ChIP assays of IgG control and GLI-1 for the Col1a1 promoter region in Dhcr7 KO (red bars) and WT control (blue bars) osteoblasts. n = 3 per group. **P < 0.001. 

**g** ChIP assays of IgG control and GLI-1 for the Col1a1 promoter region in Insig1/2 cKO (yellow bars) and WT control (blue bars) osteoblasts. n = 3 per group. **P < 0.001.
vitamin D₃ compared with healthy individuals, calcium homeostasis is not affected. As vitamin D₃ biosynthesis is inactive during embryogenesis due to lack of exposure to ultraviolet light, and because only maternal 25-hydroxyvitamin D is a source of vitamin D, the effect of vitamin D₃ on bone development would be minimal. By contrast, while the maternal cholesterol supply partially restores the exogenous cholesterol supply in embryos, embryonic tissues still need to synthesize cholesterol through endogenous cholesterol synthesis pathways in each organ to compensate for the gap between the external supply and their needs. This could be a possible reason why cholesterol metabolic anomalies cause different degree/severity of malformations in different tissues in mice and humans.

Primary cilia transduce extracellular cues as a mechanical sensor, as well as a chemical sensor, for morphogens and growth factors. Therefore, both endochondral and intramembranous ossification are affected in mice with defective primary cilia. For example, mice deficient for Kif3a, a ciliary protein, in osteoblasts...
Antibody (green), and nuclei were stained with DAPI (blue). Boxed areas in upper images are enlarged. Scale bars, 20 µm in the upper images and 5 µm in the lower images.

Fig. 7 Simvastatin rescues altered bone formation in Dhcr7 mutant mice. a Immunocytochemistry (IC) analyses of primary cilia in osteoblasts from wild-type (WT) control and Dhcr7 knockout (KO) mice after simvastatin treatment. Primary cilia were stained with anti-acetylated tubulin antibody (green), and nuclei were stained with DAPI (blue). Boxed areas in upper images are enlarged. Scale bars, 20 µm in the upper images and 5 µm in the lower images. b Percentage of cells with primary cilia in osteoblasts from WT (blue bars) and Dhcr7 KO (red bars) mice after treatment with simvastatin. n = 124 per group. ***P < 0.001. c Quantification of the length of primary cilia in osteoblasts from WT (blue bars) and Dhcr7 (red bars) KO mice after simvastatin treatment. n = 124 per group. ***P < 0.001. d Skull staining after simvastatin treatment (10 mg·kg\(^{-1}\) body weight, intraperitoneal injection to a pregnant mouse, E12.5–E18.5). The open arrow indicates rescued calvarial abnormalities. e Hematoxylin and Eosin staining of the sagittal sutures of newborn WT and Dhcr7 KO mice after simvastatin treatment. Arrowheads indicate the osteogenic front. Accelerated bone formation of the sutures (frontal, coronal, and sagittal sutures) was normalized in newborn Dhcr7 KO mice (n = 6/6). Scale bars, 100 µm. f Quantitative RT-PCR of the indicated genes in newborn WT (blue bars), WT treated with simvastatin (green bars), Dhcr7 KO (red bars), and Dhcr7 KO treated with simvastatin (black bars) mice. n = 6 per group. ***P < 0.001.

Display decreased bone formation and osteopenia. A deficiency for cilia-related protein SPEF2 results in osteoblast differentiation defects. In this study, we found that HH and WNT/β-catenin signaling is inverted in conditions of the primary cilia in Dhcr7 ΔEx8/ΔEx8 knockout (KO) mice during endochondral ossification. While both WNT/β-catenin and HH signaling pathways are essential for endochondral ossification as well as for the differentiation and maturation of cultured osteoblasts, previous mouse genetic studies suggest that WNT/β-catenin signaling may be more dominant than HH signaling for osteogenesis in intramembranous ossification during skull formation.

In regard to HH signaling, previous studies show that HH signaling is compromised in Dhcr7 mutant mammalian models; for example, HH signaling is inhibited in Dhcr7 ΔEx8/ΔEx8 mouse embryonic fibroblasts (MEFs), as well as in mice deficient for Dhcr7 (Dhcr7 ΔEx8/ΔEx8 mice). Our results show that the number of ciliated cells, as well as the length of cilia, is decreased in Dhcr7 ΔEx8/ΔEx8 osteoblasts, which is responsible for diminished HH signaling. Previous studies indicate that HH signaling is not dominant in regulating intramembranous ossification; for example, mice with loss of Ihh (Ihh ΔEx8/ΔEx8 mice), which is expressed at the osteogenic front in cranial bones, develop small but normal calvaria, and osteogenesis is not affected during endochondral ossification. Mice deficient for Smo (Smo\(^{−/+}\);Wnt1-Cre mice) display small but normal skulls in contrast to other severe craniofacial defects. In addition, mice deficient for Shh (Shh\(^{−/+}\);K14-Cre mice and Shh\(^{−/+}\);K14-Cre mice), which is specifically expressed in the epithelium in craniofacial regions, show normal skull formation while they exhibit cleft palate. Thus, loss-of-function of HH signaling does not affect calvarial formation. By contrast, gain-of-function of HH signaling results in defective bone formation; for example, mice with an ectopic HH signaling activation in CNC cells (Smo\(^{−/+}\);Osr2-IrsCre show cleft palate and osteogenesis defects such as the absence of the palatine processes of the premaxilla and maxilla and a short mandible with ossification defects. In addition, overexpression of Shh in the
epithelium (K14-Shh transgenic mice) results in craniofacial bone defects, cleft palate, short limbs, and polysyndactyly through the increased and expanded SHH signaling activation in the mesenchyme.\(^78\) Taken together, although HH signaling may contributes to intramembranous ossification at some degree, upregulation and downregulation of HH signaling in Dhcrt/\(^{-}\) and Insig1/2 cKO mice is not in agreement with the hypothesis that HH is a factor responsible for cilia-mediated osteogenesis during intramembranous ossification.

As to WNT/\(\beta\)-catenin signaling, previous studies show that it related to cholesterol metabolic aberrations.\(^82\) As seen in Fig. 8, \(\beta\)\(\beta\)-catenin signaling inhibition results in a change of ciliary length in WT and Insig1/2 cKO mice compared to controls. Our work places a new focus on primary cilium formation regulated by cholesterol metabolism in the bone. The principles learned from this study promise to be fertile ground for future molecular genetic studies of craniofacial bone development, and may lead to the development of innovative preventive and therapeutic approaches for bone diseases and ciliopathies related to cholesterol metabolic aberrations.

**MATERIALS AND METHODS**

**Animals**

Dhc7\(^{-}\) mice\(^85\) were a gift from Dr. Forbes D. Porter (The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA). Insig1\(^{-}\)/Insig2\(^{-}\) mice (The Jackson Laboratory, #005939)\(^12\) and Wnt1-Cre\(^2\) (The Jackson Laboratory, #022501)\(^22\) mice were obtained from The Jackson Laboratory and crossed to generate Insig1/2 cKO mice. Gli1-LacZ mice (The Jackson Laboratory, #008211)\(^39\) were obtained from The Jackson Laboratory and crossed with Dhc7\(^{-}\) and Wnt1-Cre2;Insig1\(^{-}\)/Insig2\(^{-}\) mice in order to generate Dhc7\(^{-}\);Gli1-LacZ, Dhc7\(^{-}\)/Gli1-LacZ, Insig1/2 cKO;Gli1-LacZ, and Insig1\(^{-}\)/Insig2\(^{-}\);Gli1-LacZ mice. Topgal (The Jackson Laboratory, #004623)\(^86\) and Axin2\(^{-}\) mouse line to generate derived cultured osteoblasts. Dhc7\(^{-}\) osteoblasts displayed fewer and shorter primary cilia compared to controls, while Insig1/2 cKO osteoblasts exhibited supernumerary and longer primary cilia compared to controls. Our work places a new focus on primary cilium formation regulated by cholesterol metabolism in the bone. The principles learned from this study promise to be fertile ground for future molecular genetic studies of craniofacial bone development, and may lead to the development of innovative preventive and therapeutic approaches for bone diseases and ciliopathies related to cholesterol metabolic aberrations.
Dhcr7−/−;Topgal and Dhcr7+/−;Topgal, Dhcr7−/−;Axin2LacZ/−, and Dhcr7+/−;Axin2LacZ/+ mice. Genotyping was performed using PCR primers, as previously described.12,22 Pregnant females were treated with simvastatin (S6196; Sigma-Aldrich) at a dose of 10 mg/kg body weight (BW) from E12.5 to E18.5, or from day 7 to day 42, administered by intraperitoneal injection.

Skeletal staining
The three-dimensional architecture of the skeleton was examined by modified whole-mount Alcian blue-Alizarin Red S staining, as previously described.88

MicroCT
MicroCT analysis was performed using a Scanco µCT40 unit in the microCT core facility at Baylor College of Medicine. The data were collected at a resolution of 20 μm. Three-dimensional reconstruction was performed using the BoneJ software.

Histology
H&E, BrdU staining, von Kossa staining, LacZ staining, immunohistochemistry, and the TUNEL assay were performed as previously described.88,89 Antibodies used for immunohistochemistry were the anti-COL1A1 rabbit polyclonal (Abcam), anti-RUNX2 rabbit monoclonal (Cell Signaling Technology), anti-SP7 rabbit monoclonal (Abcam), and anti-Ki67 rabbit monoclonal (Abcam) antibodies (Supplementary Table S1). Click-iT® Plus TUNEL Assay with Alexa 594 (C10618, Molecular probes) was used to detect apoptotic cells, according to the manufacturer’s instructions. Fluorescence images were obtained using a confocal microscope (Ti-C2, Nikon).

Fig. 9 Model of primary cilium formation altered in Dhcr7−/− and Insig1/2 mutant osteoblasts. Ciliogenesis starts with the interaction of the basal body (aka mother centriole) with primary ciliary vesicles (CVs), which can be labeled with RAB11, and then the axoneme grows within the ciliary membrane while fusing with secondary CVs, which can be labeled with RAB8. Eventually the elongated primary cilium fuses with the plasma membrane, allowing the distal part of the cilium to interact with the extracellular milieu. In Dhcr7 knockout (KO) osteoblasts, primary cilia were fewer and shorter than in controls. By contrast, Insig1/2 conditional KO (cKO) osteoblasts showed supernumerary and longer primary cilia compared to controls. Primary CVs labeled with RAB11 accumulated in the cells, and secondary CVs labeled with RAB8 failed to gather at cilium formation sites in Dhcr7 KO osteoblasts. Secondary CVs stained with RAB8 accumulated in Insig1/2 cKO osteoblasts, and the number of basal bodies was abnormally increased in Insig1/2 cKO osteoblasts. During osteogenesis, WT osteoblasts have a single primary cilium that suppresses WNT/β-catenin signaling and activates HH signaling, which induces Col1a1 expression. In Dhcr7 KO osteoblasts, WNT/β-catenin signaling is hyper-activated (WNT: +++) and HH signaling is compromised (HH: --), while in Insig1/2 cKO osteoblasts WNT/β-catenin signaling is inhibited (WNT: --) and HH signaling is hyper-activated (HH: ++).
Cell culture
Primary osteoblasts were obtained from newborn frontal bones. Briefly, the frontal bones were dissected at birth and incubated with 2 mg·mL⁻¹ collagenase II solution (Gibco) while shaking at 350 r·min⁻¹ at 37 °C for 1 h. Cells were resuspended in minimum essential medium alpha (MEM-a) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine. For osteogenic differentiation, osteoblasts were cultured in 12-well plates, and osteogenic differentiation was induced with osteogenic induction medium (MEM-a supplemented with 100 µg·mL⁻¹ ascorbic acid, 5 mM beta-glycerophosphate, 10% FBS, penicillin/streptomycin, and L-glutamine) for 28 days. To evaluate osteogenic differentiation, alkaline phosphatase staining, Alizarin Red staining, and quantitative RT-PCR was conducted using the following primers:

Quantitative RT-PCR
Total messenger RNA was prepared as previously described.³⁸ Quantitative RT-PCR was conducted using the following primers: 

Immunoblotting
Immunoblots were obtained as previously described.⁸⁹ The antibodies used for immunoblotting were as follows: anti-collagen type I rabbit polyclonal (Abcam), anti-INSIG1 rabbit polyclonal (Abcam), anti-INSIG2 rabbit polyclonal (Abcam), anti-DHR7 rabbit polyclonal (Abcam), anti-GLI1 rabbit polyclonal (Abcam), anti-nuclear phosphorylated (active) beta-catenin rabbit polyclonal (Cell Signaling Technology), anti-CREB rabbit polyclonal (Cell Signaling Technology), anti-Sak (aka PLK4) mouse monoclonal (Santa Cruz Biotechnology), and anti-GAPDH mouse monoclonal (Millipore) (Supplementary Table S1). Cell fractionation was performed using a NE-PER™ nuclear and cytoplasmic extraction kit (Thermo Scientific).

Promoter analysis
The UCSC genome browser was used to obtain the genomic sequences of the Col1a1 murine gene (NC_000077.6) and the Plk4 murine gene (NC_000069.6), including the 5-kbp sequences upstream of the respective transcription start sites. The sequences were then mapped to seven additional mammalian genomes (human (Build 38), chimpanzee (Build 2.1.4), orangutan (Build 2.0.2), rhesus macaque (Build 1.0), rat (Build 5), dog (Build 3.1), and horse (Build equCab2)) with the BLAST tool, as previously described.⁹¹ Multiple alignments for these sequences were obtained using the Clustal Omega tool with default parameters and settings. LEF1 binding motifs (minimal core sites: 5'-TA/TAGGGTGGAAGGGCT-3' and 5'-TCTGGATCACTCCCTC-3'), site 1, 5'-CACCTGAGTCTTGTGACCT-3', site 2, 5'-GTAGGGTGGAAGGGCT-3', and 5'-TCTGGATCACTCCCTC-3' were searched in the aligned DNA sequences, as previously described.⁹⁰ Chromatin immunoprecipitation analysis
At Day 3 of osteogenic differentiation, the osteoblast extracts were incubated with either active β-catenin (Cell signaling technology), GLI1 (Abcam), or normal rabbit IgG as a negative control (Santa Cruz Biotechnology) overnight at 4 °C, followed by precipitation with magnetic beads. The osteoblast extracts were incubated with either mouse SREBP1 and SREBP2 antibodies (Santa Cruz Biotechnology) or normal mouse IgG (Santa Cruz Biotechnology) overnight at 4 °C, followed by precipitation with magnetic beads. Washing and elution of the immune complexes, as well as precipitation of DNA, were performed according to standard procedures, as previously described.⁹⁰ The putative LEF1/β-catenin binding sites in the immune complexes were detected by PCR using the following primers: Col1a1 site 1, 5'-AGACAGAGGAGTCTTGTGACCT-3' and 5'-TCTGGATCACTCCCTC-3', site 2, 5'-CACCTGAGTCTTGTGACCT-3' and 5'-GTAGGGTGGAAGGGCT-3'.
GTCTCTCCATGCTGTCCT-3′ and 5′-CCATCAAGATCCATTGCT-3′ (−1814 bp to −1569 bp); and site 4, 5′-TGGAGATCTCGCTTTGGT-3′ and 5′-TGGAGATCTCGCTTTGGT-3′ (−2756 bp to −2517 bp). The putative GLI-binding sites on the Col1a1 promoter were detected by the following primers: 5′-CGGACTTTTCTCCTGGGGG-3′ (−111 bp to −94 bp) and 5′-GGGGTTAGCTCGCTGCTA-3′ (−59 bp to −42 bp). The putative SREs on the Plik4 promoter in the immune complexes were detected by PCR using the following primers: site 1, 5′-AACCACTCCTCCGCTAGA-3′ (−322 bp to −303 bp) and 5′-TGAAATACTCGCGCTCGTAT-3′ (−210 bp to −191 bp); and site 2, 5′-GGCTTCGAGTAATAGGTGTTAT-3′ (−1402 bp to −1380 bp) and 5′-AATAAGAGTAAAGCTGCCGG-3′ (−1275 bp to −1262 bp). The position of the PCR fragments corresponds to NCBI mouse genome Build 38 (mm10). Statisticso The two-tailed student’s t-test was applied for statistical analysis. A P-value < 0.05 was considered statistically significant. For all graphs, data are represented as mean ± standard deviation.

Study approval All animal experiments were reviewed and approved by the Animal Welfare Committee and the Institutional Animal Care and Use Committee of UTHealth.

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