

# Quick and Abnormal Ossification Results in No Growth Plate Formation and Skeletal Dysplasia in Postnatal Mice Induced by Conditional Knockout of Indian Hedgehog

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## Research article

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# Abstract

**Objective:** To observe the dynamic effect of conditional knockout of Indian hedgehog (Ihh) on ossification of postnatal growth plate, skeletal abnormalities and the related signaling factors in mice.

**Methods:** P0 Col2a1-Cre ER<sup>T2</sup>; Ihh<sup>fl/fl</sup> mice were randomly divided into tamoxifen (TM) injection to delete Ihh and oil injection as control. Real-time PCR (RT-PCR) was used to detect Ihh knockout rate. X-ray and micro-CT were used to evaluate gross and knee joint morphology of 8-week-old mice. The hind knee joints were harvested at P2-P14 after the animals were euthanized to observe the dynamic evolution of the growth plate. Safranin-O and Von Kossa staining were performed to assess growth plate development and the mineralization of bone respectively. The costal cartilage cells of mice were cultured in vitro on postnatal day 6. Changes in the expression of parathyroid hormone-related protein (PTHrP) and bone morphogenetic protein 6 (BMP-6) were detected by immunohistochemistry and RT-PCR.

**Results:** RT-PCR results confirmed that the knockout rate of Ihh was 76.83%. X-ray and micro-CT scans showed that the short-limb deformity of the experimental group was associated with abnormal development of the epiphysis. Saffron O staining showed a disorder of cell arrange in the growth-plate area of Ihh<sup>d/d</sup> mice; Von Kossa staining showed an early and premature growth-plate ossification that prevents the growth plate formation in Ihh<sup>d/d</sup> mice starting at P8. Immunohistochemistry and RT-PCR showed significantly decreased PTHrP expression ( $P<0.05$ ) and significantly increased BMP-6 expression ( $P<0.05$ ).

**Conclusion:** Postnatal conditional knockout of Ihh inhibited the expression of PTHrP and promotes the expression of BMP-6, which leads to early and abnormal ossification of the growth plate, no growth plate formation, and skeletal dysplasia in mice.

## Background

The growth plate is an epiphyseal cartilage region constituted by chondrocytes and cartilage matrix between the metaphysis and the metaphysis [1]. In mammals, the growth plate is composed of three types of highly organized and specialized cartilage: resting zone chondrocytes, proliferative zone chondrocytes, and hypertrophic zone chondrocytes. The growth plate provides a continuous supply of chondrocytes for endochondral ossification, whereby limb mesenchyme cells form an intermediate cartilage scaffold that develops into growth plate and is then replaced by bone, and any failure in this process causes a wide range of skeletal disorders [2]. The formation and function of the growth plate is a particularly important topic of research in the prevention of skeletal abnormalities.

The Hh homologous proteins in mammals are Sonic hedgehog, Indian hedgehog (Ihh), and Desert hedgehog, which all share the same signaling pathway [3]. Ihh, which is primarily released from the pre-hypertrophic chondrocytes of growth plate [4], plays an important regulatory role in the proliferation and differentiation of chondrocytes during endochondral bone formation. Earlier studies have suggested Ihh expression in the postnatal chondrocytes is essential for maintenance of the growth plate and the

articular surface and for sustaining a primary spongiosa [5]. We also generated Prx1-Cre; *Ihh*<sup>fl/fl</sup>; Rosa26<sup>-ZsGreen1</sup> mice to precisely delete *Ihh* in the mesenchyme cells and found *Ihh* deletion on mesenchyme cells results in the intermediate cartilage scaffold ossification, which prevents growth plate and phalange joint formation causing short limb and dwarfish phenotypes [6]. However, due to Prx1 are specifically expressed at E9.5 in limb development [7], the role of *Ihh* in postnatal bone growth and remodeling could not be studied.

To avoid delete *Ihh* in the embryonic stage, we generated Col2a1-Cre ER<sup>T2</sup>; *Ihh*<sup>fl/fl</sup> mice, in which *Ihh* was time selectively ablated from chondrocytes. This unique and powerful genetic animal model provided the in vivo tool to investigate whether early intermediate cartilage scaffold ossification contributes to absence of growth plate and dwarfish phenotypes in the postnatal mice lacking *Ihh* expression, specifically in chondrocytes. Thus, we are able to observe the growth plate continuously and dynamically at different time points and determine the role of *Ihh* in postnatal bone growth and remodeling in vivo. Understanding the role of *Ihh* during growth plate development will be benefit to the diseases related to abnormal ossification and growth plate development.

## Methods

### Generation of Col2a1-Cre ER<sup>T2</sup>; *Ihh*<sup>fl/fl</sup> Mice

Col2a1-Cre ER<sup>T2</sup>; *Ihh*<sup>fl/fl</sup> mice (a gift from Professor Beate Lanske) were bred as previously described [22]. P0 Col2a1-Cre ER<sup>T2</sup>; *Ihh*<sup>fl/fl</sup> mice were randomly divided into two groups: TM (n = 24) and no TM (n = 24). Random numbers were generated using the standard = RAND ( ) function in Microsoft Excel. In the TM group, Col2a1-Cre ER<sup>T2</sup>; *Ihh*<sup>fl/fl</sup> mice were intraperitoneal injected with TM (5 μL of 20 mg/mL for 3 consecutive days) to delete *Ihh*. In the no TM group, mice were injected with 3 doses of solvent (corn oil) as a control. The experimenter could not be blinded to whether the animal was injected with TM or with corn oil due to severe phenotype. Animal were killed at different age and right hindlimbs were harvested immediately after the mice were killed. All study animals were bred at the Experimental Animal Center of Shanxi Medical University. Approval of the animal experiments was obtained from The Institutional Animal Care and Use Committee of Shanxi Medical University.

### PCR Analysis for Genotyping

Total DNA were extracted from mouse tail and genotyping of mice was performed by conventional PCR. The quantification of DNA was performed by real-time PCR using the SYBR Premix Ex Taq<sup>TM</sup> kit (Takara, Dalian, China) with the IQ50 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction was performed in triplicate. The following primers were used: Cre allele; forward, 5'-ATCCGAAAAGAAAACGTTGA-3', reverse, 5'-ATCCAGGGTACGGATATA GT-3'; *Ihh* allele; forward, 5'-AGCACCTTTTTTCTCGACTGCCTG-3', reverse, 5'-TGTTAGGCCGAGAGGGATTTCTGTG-3'.

# Gross Appearance and Radiographical Analysis

8-week-old mice in each group were anesthetized by intraperitoneal injections of ketamine hydrochloride (100 mg/kg body weight) and plain radiographs were taken using a Faxitron X-ray apparatus (Faxitron, Tucson, AZ). After the animals were euthanized, hind limbs were harvested and the length of the femur and tibia were measured.

## Micro-CT Analyses

8-week-old mice in each group were sacrificed by cervical dislocation, the tibias of mice were collected. Three-dimensional (3D) reconstructions of the tibial bone from each group were generated from images acquired on vivaCT 80 (SCANCO MEDICAL, Bassersdorf, Switzerland), following recommended guidelines from Bouxsein et al. [23]. For trabecular bone analysis, a region of interest (0.5 mm below the growth plate) were selected for analysis. The following morphometric parameters of bone volume to total volume (BV/TV), trabecular number (Tb. N), trabecular separation (Tb. Sp) and trabecular thickness (Tb. Th) were calculated using built-in software.

## Histology

After the animals were euthanized, the hind knee joints from each group were harvested and immersed in 10% formalin for 24 hours at postnatal days 6, 8, 10, 12, 14. The paraffin sections were prepared through normal procedures. Safranin O staining was performed using 0.5% Safranin-O solution and counterstaining of 0.2% fast green to assess glycosaminoglycan production. Von Kossa staining was performed using 4% silver nitrate solution with counterstaining of 1% fast red solution to evaluate the mineralization of bone [10]. Immunohistochemistry was used to detect PTHrP and BMP-6 using 6  $\mu$ m thick section.

## In Vivo Ectopic Bone Formation in Nude Mice

BALB/c nude mice at 3 weeks of age were purchased from Charles River (China). They were acclimated to our animal facilities for 1 week and then used in experiments. Murine costal chondrocytes were isolated from the ventral parts of the rib cages of 6-day-old mice and cultured in F-12 media with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA) as previously described [24, 25]. A total of  $1 \times 10^6$   $Ihh^{fl/fl}$  or  $Ihh^{d/d}$  costal chondrocytes cells were seeded in 200  $\mu$ L Matrigel (BD Biosciences, San Jose, CA, USA) and implanted subcutaneously into athymic nude mice at 4 weeks of age as previously described (n = 4 per group) [8]. The dynamic bone formation was monitored weekly by Faxitron X-ray (Faxitron, Tucson, AZ).

# Quantitative Real-Time PCR Analysis

Total RNA was extracted from cartilage by using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) and reversely transcribed into complementary DNA by using the PrimeScript RT-PCR Kit (Takara, Dalian, China). Real-time PCR was conducted utilizing SYBR Premix Ex Taq™ (Takara, Dalian, China) according to the manufacturer's instructions. Levels of gene expression were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The specific primers used for gene expression analysis are listed in Table 1. The cycle threshold value for target gene was measured and calculated by computer software IQ50 (Bio-Rad Laboratories, Hercules, CA, USA). Relative mRNA level was calculated as  $x = 2^{-\Delta\Delta Ct}$ , in which  $\Delta\Delta Ct = \Delta Ct E - \Delta Ct C$ , and  $\Delta Ct E = Ct_{exp} - Ct_{18S}$ , and  $\Delta Ct C = Ct_C - Ct_{18S}$  [26]. Each sample was analyzed in triplicate.

Table 1  
Information of qPCR primers used in this study

	Forward (5'-3')	Reverse (5'-3')
Ihh	F5'-CCACCTCCGGGCCACATTTG-3'	R5'-GGCCACCACATCCTCCACCA-3'
Cre	F5'-ATCCGAAAAGAAAACGTTGA-3'	F5'-ATCCAGGGTACGGATATAGT-3'
PTHrP	F 5'-TGGAGTGTCTGGTATTCCTGCTC-3'	R 5'-GCGGCGCAAGTCTTGGATGG-3'
BMP-6	F 5'-TCCTCTATCGGCGGCTCAAC-3'	R 5'-GTCTGCTGCTGCTGCTGCTG-3'
GAPDH	F 5'-AGCAGTCCCGTACACTGGCAAAC-3'	R 5'-TCTGTGGTGATGTAAATGTCCTT-3'

## Statistical Analysis

Data for each group were derived from at least three independent samples, and all sampling was repeated thrice in each study group. Data are presented as the mean  $\pm$  standard deviation. Student's t-test was used to compare the difference between  $Ihh^{fl/fl}$  and  $Ihh^{d/d}$ . The significance level was set at  $\alpha = 0.05$ . The Graph Pad Prism 5 software was used for creating statistical graphs.

## Results

### Col2a1-Cre ER<sup>T2</sup>; $Ihh^{fl/fl}$ Mice Exhibit Dwarfism

Col2a1-Cre ER<sup>T2</sup> transgenic mice were crossed with  $Ihh^{fl/fl}$  mice to obtain Col2a1-Cre ER<sup>T2</sup>;  $Ihh^{fl/fl}$  animals. In our present study, RT-PCR was used for genotyping to confirm disruption of both *Ihh* alleles and the presence of Col2a1-Cre (Fig. 1a). There was a 392-bp band in wild type, 392-bp and 484-bp bands in the  $Ihh^{fl/-}$  heterozygote, and a 484-bp band in the  $Ihh^{fl/fl}$  homozygote. As a reference, the Cre-recombinant enzyme has a 374-bp band. Our real-time PCR results confirmed a statistically significant deletion of *Ihh* (76.83%) in mutants (Fig. 1b). Col2a1-Cre ER<sup>T2</sup>;  $Ihh^{fl/fl}$  mutant mice and  $Ihh^{fl/fl}$  control mice were

intraperitoneal injected with tamoxifen (5  $\mu$ L of 20 mg/mL) for 3 consecutive days at P0. 8 weeks after birth, mutant mice displayed a severe phenotype that restricted their general body size, limbs, and tail, while phalange joint and digit number were not affected (Fig. 2a, b). As expected, radiographic analysis confirmed a complete loss of the growth plate and abnormal growth of the epiphysis in endochondral long bones in mutant after TM was injected (Fig. 2c, d). Mice in the TM group displayed substantial limb shortening, resulting in significantly shorter femoral and tibial lengths when compared to the control group (Table 2). Our observations are in accord with previous findings where Cre recombinase-mediated deletions of *Ihh* gene expressed in chondrocytes [5].

Table 2  
Effect of *Ihh* knockout on femur and tibia length in mice 8 weeks after birth (n = 4)

	Femur (cm)	Tibia (cm)
TM group	1.411 $\pm$ 0.055	1.637 $\pm$ 0.038
Control group	1.925 $\pm$ 0.043	2.138 $\pm$ 0.047
<i>t</i>	-20.702	-20.471
<i>P</i>	<0.01	<0.01

## Characterization of *Ihh*-Deficient Bones

To study the potential effect of *Ihh*-deficient on bone remodelling and homeostasis, Micro-CT scanning and 3D reconstruction were performed to assess changes in the bone micro-architecture in vivo. Micro-CT 3D images revealed that deleted *Ihh* in *Col2 $\alpha$ 1-Cre ER<sup>T2</sup>; Ihh<sup>fl/fl</sup>* mice had more and disorganized trabecular architecture on epiphysis of tibia compared with the control mice, while a significantly decreased trabecular bone mass in mutant mice compared to control littermates (Fig. 2c, d and Fig. 3b, e). We select 0.5 mm below the growth plate as region of interest for trabecular bone analysis. Because of the lack of a growth plate on mutant mice, we had to carefully choose a section area below the distal ends of the mutant tibia for comparisons (Fig. 3b, e). Bone histomorphometric analyses indicated that *Ihh<sup>d/d</sup>* mice displayed a significantly lower bone volume/tissue volume (BV/TV) and trabecular number (Tb.N) in the distal femur when compared to control mice. The trabecular thickness (Tb.Th) in the distal femur was also lower in *Ihh<sup>d/d</sup>* mice than in control mice, but did not reach statistical significance. On the other hand, the trabecular spacing (Tb.Sp) was significantly higher in mutant mice than in control mice (Fig. 3g).

## Accelerated Abnormal Ossification Results in No Growth Plate Formation

To investigate the mechanism of deleting *Ihh* in chondrocytes results in complete loss of growth plate, after injecting TM at P0 to delete *Ihh*, histological examination of the tibia growth plate was checked at postnatal days 6, 8, 10, 12, and 14. The Safranin O staining results showed proliferating columns of cartilaginous cells in the tibial growth plate of mutant mice was disordered. In the control group, the boundary between primary ossification center and secondary ossification center was apparent at P10, and the growth plate is composed of three types of highly organized and specialized cartilage. While the columnar structure of chondrocytes were not found in the tibia of mutant mice, the mutant growth plate was primarily composed of irregular hypertrophic chondrocytes. In addition, mutant mice did not form the second ossification center, while the control mice had a second ossification center at P10 (Fig. 4a). *Ihh* deletion mice showed an abundant of Von Kossa staining appeared at P8 with severely disorganized mineralization in the center of mutant intermediate cartilage scaffold. In comparison, control mice group displayed normal von Kossa staining patterns in limbs. The quick and early ossification of the intermediate cartilage scaffold resulted in an absence of the second ossification center and the growth plate formation in *Ihh*-deficient mice. (Fig. 4b).

## Deleting *Ihh* in Chondrocytes can Differentiate into Osteoblasts

To determine whether isolated deleted *Ihh* chondrocytes can form bone *in vivo*, we isolated murine costal chondrocytes from the ventral parts of the rib cages of 6-day-old mice and implanted the deleted *Ihh* chondrocytes into immunocompromised nude mice using a well-characterized subcutaneous ectopic bone formation model (Fig. 5a) [8]. As shown in Fig. 5b, X-ray results confirmed costal chondrocytes alone did not form bone over an 4-week period. However, deleted *Ihh* chondrocytes induced robust bone formation *in vivo*. Immunohistochemistry was performed to determine the expression of PTHrP and BMP-6. In the in mutant mice, PTHrP expression in chondrocytes was less than in the control group. In contrast, BMP-6 content was higher in cartilage in the mutant mice than in the control mice (Fig. 6a). Consistent with the immunohistochemical data, real-time PCR revealed a significant decrease in PTHrP expression in *Ihh*-deleted mice, whereas the relative expression of BMP-6 mRNA increased (Fig. 6b).

## Discussion

The results of our study demonstrate that the early intermediate cartilage scaffold ossification in *Ihh*-deficient mice is responsible for the absence of growth plate and the second ossification center formation. Based mainly on *in vivo* studies with the use of genetically modified mice, several studies and our data have revealed that *Ihh* is indispensable for the process of growth plate organization [5, 6, 9–12]. Mice carrying null mutations of the *Ihh* gene show a severely disrupted growth plate with abnormal chondrocyte proliferation and maturation at embryonic stages [12]. The conditional ablation of *Ihh* in the chondrocytes by using *Col2a1* promoter reveals severe skeletal deformities with loss of a normal growth plate [9]. However, global *Ihh* knockout mice and *Ihh* deletion from collagen type 2 alpha1-expressing transgenic mice were perinatal lethal, limiting observations of postnatal growth plate development. To

overcome the perinatal lethal due to deletion of *Ihh*, Lanske have generated tamoxifen-inducible *Col2a1-CreER* transgenic mice and demonstrate ablation of the *Ihh* gene from postnatal chondrocytes cause premature closure of the growth plate: disrupted columnar structure of chondrocytes, and the appearance of abnormal maturation of hypertrophic chondrocytes near the articular surface [5]. However, the mechanism of deleting *Ihh* results in complete loss of growth plate is still unclear.

After injecting TM at P0 to delete *Ihh* using *Col2a1-Cre ER<sup>T2</sup>; Ihh<sup>fl/fl</sup>* models, we checked the growth plate of mutant mice at different specific times continuously and dynamically. The von Kossa staining confirmed that functional knockout of the *Ihh* gene causes future growth plate cartilage scaffold to mineralize at P8, leading to lack of normal growth plate and secondary ossification center. Our *in vivo* ectopic bone formation model further demonstrated deleting *Ihh* in chondrocytes can result in an ectopic ossification. Recent evidence has emerged demonstrating that at least some of growth plate chondrocytes do not die by apoptosis, but instead transdifferentiate directly into the full osteogenic lineage in developing bone [13–17]. Thus, we hypothesize that *Ihh* knockout may be associated with premature hypertrophy of chondrocytes and transformed into osteoblasts.

In mammals, the growth plate originates from intermediate cartilage scaffold by the condensation of undifferentiated limb bud mesenchymal cells. *Ihh* is secreted by pre-hypertrophic chondrocytes in the growth plate, while PTHrP is expressed in periarticular resting cells and proliferating chondrocytes adjacent to the *Ihh* expressed pre-hypertrophic zone. *Ihh* and PTHrP signaling pathways form a negative feedback loop that both synchronizes and regulates growth plate activities and endochondral bone growth [18, 19]. In endochondral ossification, hypertrophic chondrocytes secrete extracellular matrix, which eventually becomes mineralized and causes the elongation of the bone [16]. BMP6 is expressed in hypertrophic chondrocytes in developing endochondral bones [20]. After Hh-dependent lineage specification into osteoblasts, BMP signaling may be involved in the transition of the Runx2-positive osteoblast precursor into the Sp7-positive precursor and act downstream of Hh signaling in mammalian osteoblast development. [21]. Therefore, in this study, we established an animal model of *Ihh* gene knockout to investigate whether or not conditional skeletal dysplasia is caused by the regulation of BMP-6 signaling factor expression by the *Ihh*–PTHrP signaling axis. Our immunohistochemistry and RT-PCR results showed significantly decreased PTHrP expression and significantly increased BMP-6 expression. Taken together, these observations suggest that BMP-6 and *Ihh*–PTHrP signaling collaboratively regulate the growth plate ossification and BMP signaling is likely to accelerate hypertrophic chondrocytes-osteoblasts differentiation in conditional deleted *Ihh* mutant mice.

We also used *Prx1-Cre; Ihh<sup>fl/fl</sup>* mice to investigate the mechanism of deleting *Ihh* results in complete loss of growth plate. An early closure of the growth plate has been reported by Amano et al. in the *Prx1-Cre; Ihh<sup>fl/fl</sup>* mice, and they demonstrate that *Ihh* and PTH1R signaling in limb mesenchyme is essential to regulate digit structure development. They believe an initial cartilaginous fusion in digits and the initial cartilage gradually resorbed and replaced by bone are specific mechanisms that lead to the loss of epiphyseal growth plate [10, 11]. Recently, we found that deleted *Ihh* on mesenchyme cells accelerated pathological matrix calcification and results in the intermediate cartilage scaffold ossification, which

prevents growth plate and phalange joint formation causing short limb and dwarfish phenotypes [6]. This further supports that the deleted *Ihh* results in a loss of growth plate due to abnormal ossification of the cartilage scaffold.

## Conclusion

Our study identifies that early intermediate cartilage scaffold ossification in *Ihh*-deficient mice is responsible for the absence of the second ossification center and growth plate formation, which results in small body size compared with control mice. We further demonstrated that the decrease of PTHrP and increase of BMP-6 are involved in the progress of abnormal intermediate cartilage scaffold ossification in *Ihh*-deficient mice.

## Abbreviations

*Ihh*: Indian hedgehog; TM: tamoxifen; RT-PCR: Real-time PCR; PTHrP: parathyroid hormone-related protein; BMP-6: bone morphogenetic protein 6; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; BV/TV: volume/tissue volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular spacing

## Declarations

## Ethics Approval and consent to participate

This study was approved by The Institutional Animal Care and Use Committee of Shanxi Medical University.

## Consent to publish

Not applicable.

## Availability of data and materials

The other datasets used and/or analyzed for the study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Investigation, MZ and TY; Writing – original draft, JS; Resources, XW and CW; Data curation, ZD; Software, YW; Methodology, CX; Project administration, PL. All authors read and approved the final manuscript.

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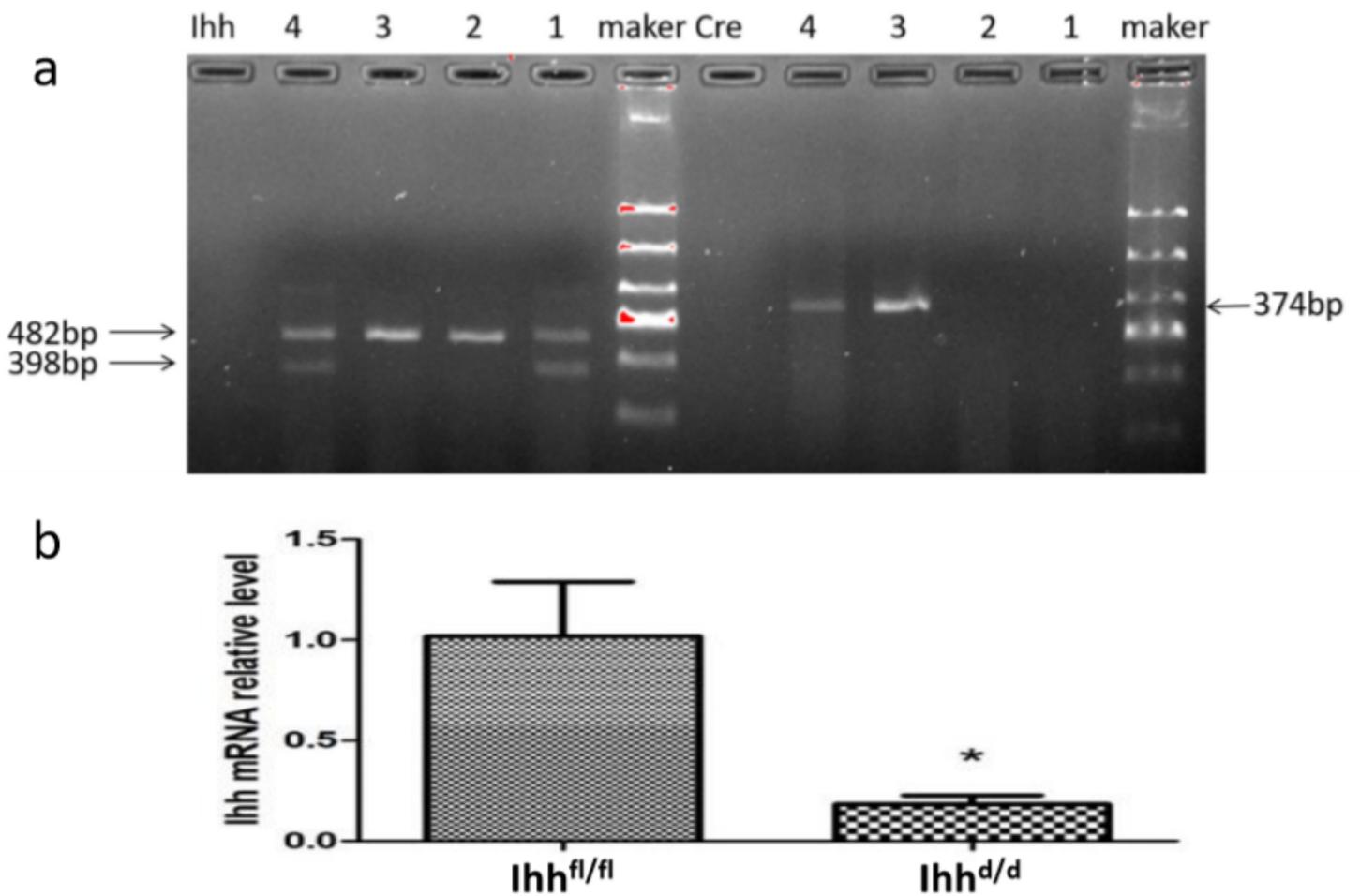
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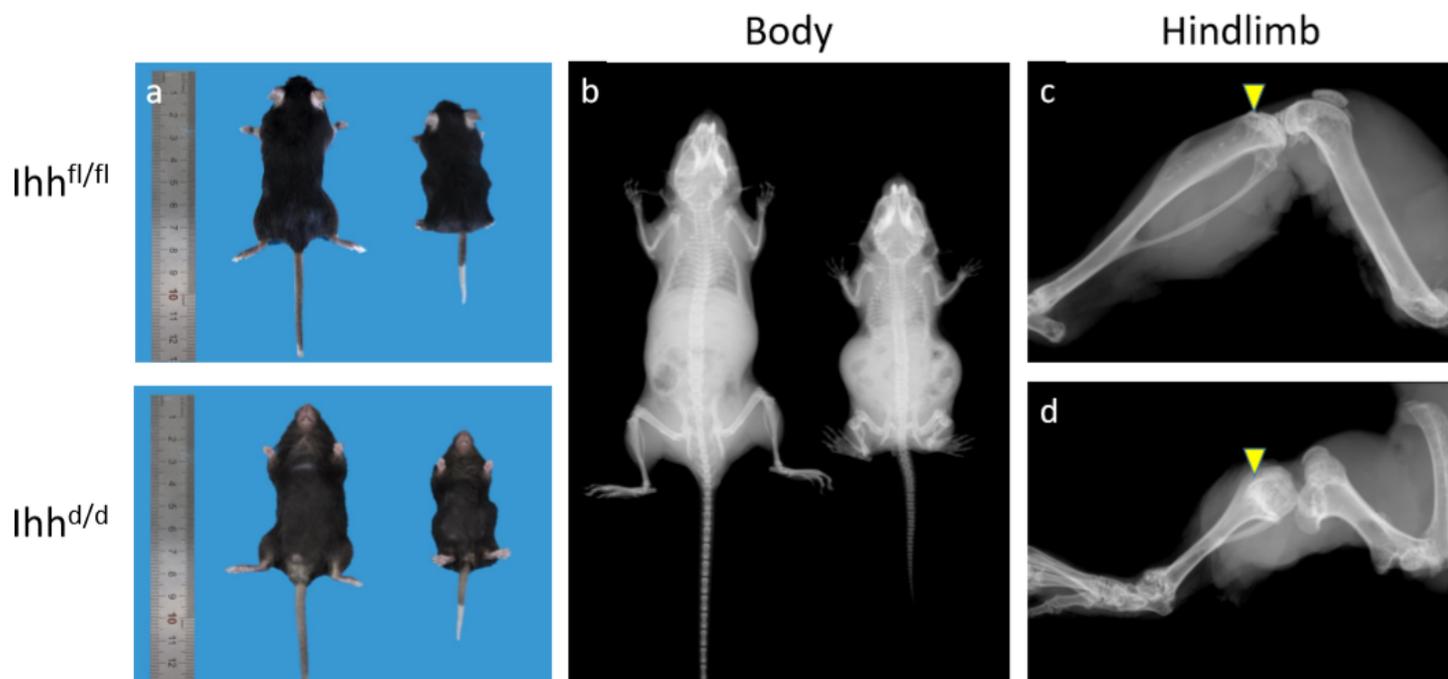
## Figures



**Figure 1**

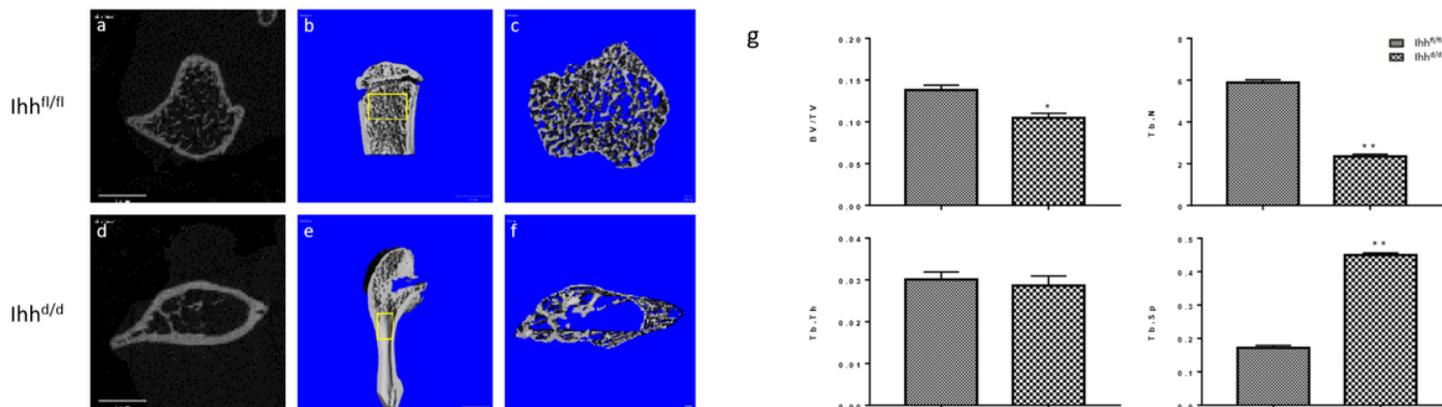
Generation of Col2a1-Cre ERT2; *Ihh*<sup>fl/fl</sup> mice line. (a) Col2a1-Cre ERT2; *Ihh*<sup>fl/fl</sup> mouse genotyping by PCR. Mouse 1 does not contain the *Cre* gene, with a heterozygote *Ihh*; Mouse 2 does not contain *Cre*, with homozygous *Ihh*; Mouse 3 contains the *Cre* gene, with homozygous *Ihh*, and was suitable for use in the experimental group. Mouse 4 contains the *Cre* gene, with heterozygote *Ihh*. (b) After the tamoxifen

injection, the relative mRNA expression of *Ihh* in the cartilage growth plate significantly decreased in the experimental group as compared with that in the control group. (n = 6; \*, P < 0.05). *Ihh* Indian hedgehog



**Figure 2**

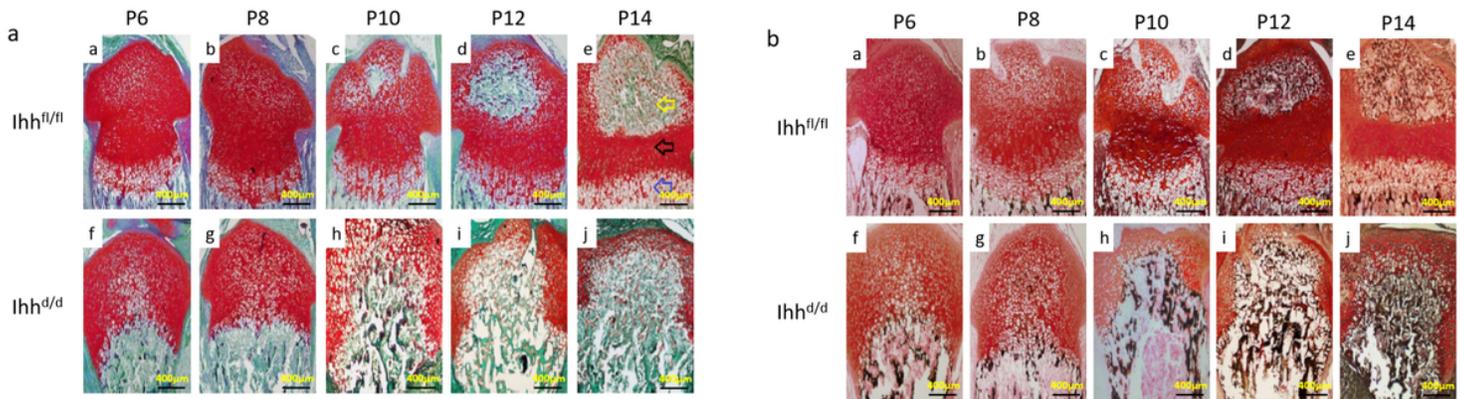
*Col2a1-Cre ERT2; Ihh<sup>fl/fl</sup>* mice exhibit dwarfism, no growth plate and no second ossification center formation. (a) Mutant mice have smaller size, shorter limbs and tails compared to control littermates at 2 months as seen from the supine and prone views. (b-d) X-ray images confirmed no growth plate and no second ossification center formation in *Ihh* deleted mice, while the growth plate is already developing very well in control mice. Arrows: growth plate. *Ihh* Indian hedgehog



**Figure 3**

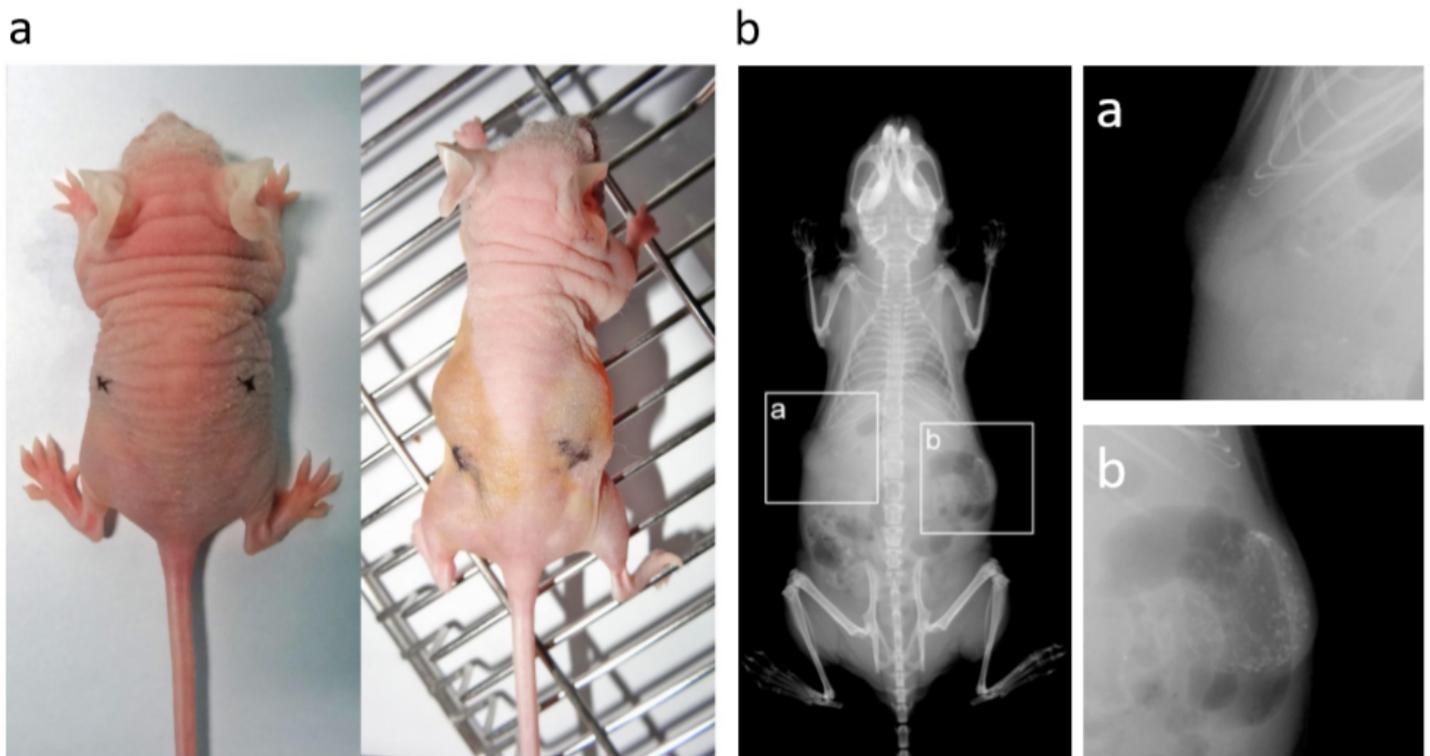
Micro-CT showed a significantly decreased trabecular bone mass in mutant mice. (a-f) Representative 3D reconstruction micro-CT images of the bone micro-architecture in the different groups. (g) BV/TV, Tb.N,

Tb.Th and Tb.Sp were analysed with micro-CT vivaCT 80 software (n = 6; \*, P < 0.05, \*\*, P < 0.01). CT computed tomography, BV/TV Bone volume per tissue volume, Tb.N trabecular number, Tb.Th trabecular thickness, Tb.Sp trabecular separation



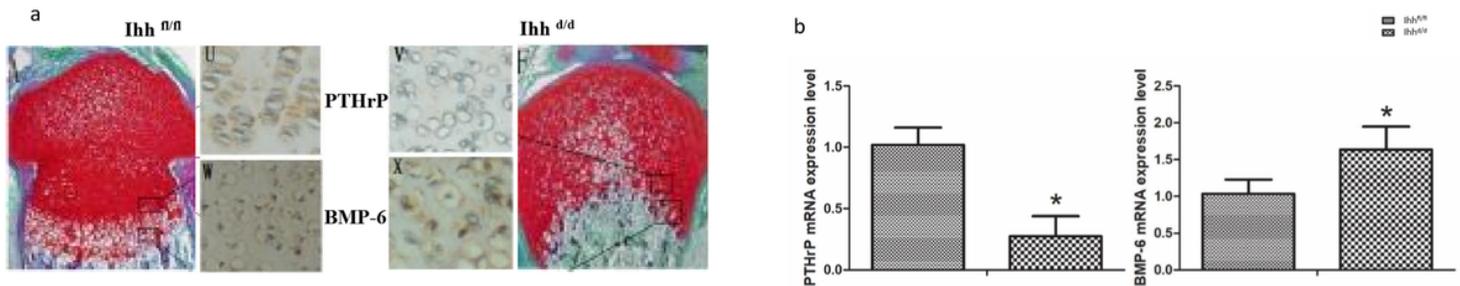
**Figure 4**

Deleting *Ihh* in chondrocytes can accelerate abnormal ossification and results in no growth plate formation. (a) Safranin-O staining confirmed that mutant proximal tibia was shorter with hypertrophic chondrocytes starting at P6, whereas control mice exhibited normal chondrocyte differentiation and starting to form the second ossification center at P10. (b) von Kossa staining confirmed that functional knockout of the *Ihh* gene accelerates chondrocytes hypertrophy and abnormal mineralization at P8, inducing mutant tibial future growth plate cartilage scaffold ossification directly, eventually leading to lack of normal growth plate and secondary ossification center at P14. *Ihh* Indian hedgehog



**Figure 5**

Deleting *Ihh* in chondrocytes induced robust bone formation in vivo. (a) Costal chondrocytes were isolated and cultured in vitro and implanted subcutaneously into the athymic nude mice. (b) 4-week later, X-rays results confirmed deleting *Ihh* in chondrocytes led to robust new bone formation, while minimal mineralization or new bone formation were observed in control (n = 4). *Ihh* Indian hedgehog



**Figure 6**

*Ihh* deletion resulted in a decrease of PTHrP and increase of BMP-6. (a) IHC showed a decrease of PTHrP and increase of BMP-6 in *Ihh* deletion mice compared with their control. (b) After knockout of *Ihh*, the relative expression of PTHrP mRNA decreased, whereas the relative expression of BMP-6 mRNA increased. (\*,  $P < 0.05$ ). BMP-6 bone morphogenetic protein 6, IHC immunohistochemistry, *Ihh* Indian hedgehog, PTHrP parathyroid hormone-related protein

## Supplementary Files

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