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Partial rescue of postnatal growth plate abnormalities in *Ihh* mutants by expression of a constitutively active PTH/PTHrP receptor

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Abstract

Indian hedgehog (Ihh) is essential for chondrocyte proliferation/differentiation and osteoblast differentiation during prenatal endochondral bone formation. *Ihh* expression in postnatal chondrocytes has a non-redundant role in maintaining a growth plate and sustaining trabecular bone after birth. Loss of *Ihh* in postnatal chondrocytes results in fusion of the growth plate and a decrease in trabecular bone. In order to normalize this abnormal chondrocyte phenotype and to investigate whether a putative rescue of the growth plate anomalies is sufficient to correct the severe alterations in the bone, we expressed a constitutively active PTH/PTHrP receptor, (an *Ihh* downstream target) in the chondrocytes of *Col2a1-Cre ER**; *Ihh^{d/d}* mice by mating *Col2a1-Cre ER**; *Ihh^{fl/fl}* mice with *Col2a1*-constitutively active PTH/PTHrP receptor transgenic mice (*Jansen, J*). *Col2a1-Cre ER**; *Ihh^{fl/fl}; J* mice were then injected with tamoxifen at P0 to generate *Col2a1-Cre ER**; *Ihh^{d/d}; J* mice. In contrast with the previously reported growth plate phenotype of *Col2a1-Cre ER**; *Ihh^{d/d}* mice that displayed ectopic chondrocyte hypertrophy at P7, growth plates of *Col2a1-Cre ER**; *Ihh^{d/d}; J* double mutants were well organized, and exhibited a gene expression pattern similar to the one of control mice. However, expression of osteoblast markers and *Dkk1*, a *Wnt* signaling target, remain decreased in the bone collar of *Col2a1-Cre ER**; *Ihh^{d/d}; J* mice when compared to control mice despite the rescue of abnormal chondrocyte differentiation. Moreover, proliferation of chondrocytes was still significantly impaired in *Col2a1-Cre ER**; *Ihh^{d/d}; J* mice, and this eventually led to the fusion of the growth plate at P14. In summary, we have demonstrated that expression of a *Jansen* receptor in chondrocytes was able to rescue abnormal chondrocyte differentiation but not impaired chondrocyte proliferation and the bone anomalies in mice lacking the *Ihh* gene in chondrocytes after birth. Taken together, our findings suggest that *Ihh* has both PTHrP-dependent and -independent functions during postnatal endochondral bone development.

Keywords

Ihh; Bone; Growth plate; *Jansen*; PTHrP; Chondrocytes; Rescue

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Introduction

The chondrocyte and osteoblast differentiation pathways are interrelated during endochondral bone formation where signaling molecules such as *Indian hedgehog* (*Ihh*), parathyroid hormone-related peptide (*PTHrP*), and *Wnt* play essential roles in regulating endochondral bone formation [1–4]. *Indian hedgehog* signaling, in particular, plays essential roles in chondrocyte proliferation and differentiation, and in osteoblast differentiation during prenatal endochondral bone formation [1–3,5,6]. The *Ihh* signal is transduced through *smoothened* (*Smo*), a putative G protein-coupled seven-transmembrane domain protein [7]. In the absence of *Ihh* protein, *Smo* is repressed by the *Ihh* target gene *patched* (*Ptc*), another cell surface receptor for *Ihh*. *Ihh* is mainly expressed in prehypertrophic chondrocytes of the growth plate and suppresses their differentiation into hypertrophy by inducing *PTHrP* production in the periarticular region [1–3]. Moreover, *Ihh* has been shown to be essential for chondrocyte proliferation [8,9] and osteoblast differentiation in the bone collar [3] during embryonic skeletogenesis. Finally, *Ihh* signaling is required for differentiation of osteoblast precursors from their progenitors. On the other hand, *Wnt* signaling promotes differentiation of osteoblast from their precursors [4].

The *in vivo* physiological role of *Ihh* has been determined mostly by mouse knockout studies [1,2,8]. The majority of *Ihh*-null embryos die during early development when *Ihh* expression is detected in the visceral endoderm. *Ihh*-null mice display abnormal chondrocyte proliferation and maturation, and lack of osteoblasts in long bones. Furthermore, overexpression of either *Ihh* or a constitutively active *Smo* allele specifically in cartilage results in increased activity of the *Ihh* signaling pathway to promote chondrocyte proliferation [9]. We have previously generated conditional *Ihh*-null mice in which *Ihh* was selectively ablated from chondrocytes. The phenotype of these mice resembled that of conventional *Ihh*-null mice at birth and provided evidence of the essential role of chondrocyte-derived *Ihh* in prenatal endochondral bone formation [6]. In order to study the postnatal role of *Ihh* in the growth plate, tamoxifen-inducible conditional *Col2-Cre ER**; *floxed Ihh-knockout* mice were generated. Our results demonstrated that lack of *Ihh* expression in postnatal chondrocytes results in complete loss of the growth plate and the articular surface, leads to loss of the primary spongiosa, and to impaired bone growth after birth [10]. However, it is not clear from these results whether loss of trabecular bone is secondary to the abnormalities of the growth plate, which serves as a template for trabecular bone, or to loss of *Ihh* signaling per se from chondrocytes to osteoblast precursors. Interestingly, the use of a *hedgehog* inhibitor to treat tumors in young mice resulted in a similar bone phenotype with loss of the growth plate [11]. Therefore, maintenance of a growth plate is critical to study the role of chondrocyte derived *Ihh* during bone growth after birth. We attempted to maintain the growth plate by rescuing growth plate abnormalities in *Col2-Cre ER**; *Ihh-knockout* mice with a constitutively active *PTH/PTHrP* receptor in chondrocytes.

Loss of *PTHrP* or the *PTH/PTHrP* receptor has been shown to result in premature chondrocyte differentiation in the growth plate [1,12]. In contrast, mice expressing a constitutively active *PTH/PTHrP* receptor (*Jansen* transgene) under the *collagen (α1) type 2* promoter have been shown to exhibit a delay in chondrocyte differentiation, rescuing the massive hypertrophy in chondrocytes lacking *PTHrP* [13–15]. To test whether the *Jansen* transgene could rescue the loss of the growth plate in *Col2-cre ER**; *Ihh^{ΔΔ}* mice after birth, we generated *Col2a1-Cre ER**; *Ihh^{fl/fl}*, *J double mutants* and injected them at P0 with tamoxifen to generate mice lacking *Ihh*, but expressing the *Jansen* receptor in postnatal chondrocytes. Using this system, we were able to transiently maintain a growth plate even in the absence of *Ihh* expression. However, the *Jansen* transgene did not rescue the dramatic impairment of proliferation of mutant growth plates lacking *Ihh*. This likely caused, at least in part, the premature fusion of the double mutant growth plates. Despite the successful maintenance of the growth plate for at least one week, osteoblast differentiation and expression of *Wnt* target genes were still decreased in the

surrounding bone indicating that *Ihh per se* could be required to directly signal from chondrocytes to osteoblast precursors in order to maintain proper bone in postnatal life.

Materials and Methods

Generation of Col2-cre ER*; *Ihh*^{fl/fl}; Col2 Jansen mice

The generation and characterization of the *Col2-Cre ER** transgenic mouse line was described earlier [16]. The phenotype of *Col2-Cre ER**; *Ihh*^{d/d} animals (*Ihh mutants*) was also previously described [10]. In brief, these *Ihh mutant* mice show premature closure of the growth plate leading to dwarfism. Furthermore, lack of *Ihh* signaling from chondrocytes leads to significant decrease in Wnt signaling in osteoblasts resulting in loss of trabecular bone. *Ihh mutants* had a shorter lifespan and died around 10 months of age when compared to *Ihh*^{fl/fl} litter mates (*controls*; about 24 months). The *col-2*; Jansen transgenic mouse line (Tg-A) was established by Dr. Schipani [13]. These mice misexpress a constitutively active PTH/PTHrP receptor in chondrocytes which causes a delay in their differentiation process. No further abnormalities were detected when compared to wild type.

*Col2-Cre ER** transgenic mice were interbred with *Ihh*^{fl/fl} animals [6] to obtain *Col2-Cre ER**; *Ihh*^{fl/fl} offspring. Subsequently, *Col2-Cre ER**; *Ihh*^{fl/fl} mice were interbred with *Col2 Jansen* transgenic mice (*J*) [13] to obtain *Col2-cre ER**; *Ihh*^{fl/fl}; *J* mice. These mice and their littermates were then injected at birth (P0) with tamoxifen (0.2 mg) to generate *Col2-cre ER**; *Ihh*^{d/d}; *J* (*double mutants*). All analyses were performed using at least three to five mice of each genotype. Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were studied according to Institutional Animal Care and Use Committee approved protocols.

PCR Analysis for Genotyping

Genotyping of mice was performed by PCR using the following specific primers: *fl-Ihh forward* 5'-AGC ACC TTT TTT CTC GAC TGC CTG-3', *fl-Ihh reverse* 5'-TGT TAG GCC GAG AGG GAT TTC GTG-3'; *Cre 275* 5' CGC GGT CTG GCA GTA AAA ACT ATC-3', *Cre 603* 5'-CCC ACC GTC AGT ACG TGA ATA TC-3'. After an initial denaturation step for 8 min at 94°C, amplification cycles consisted of denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec, and 45 sec extension at 72°C for 35 cycles, followed by a final extension for 10 min at 72°C. The expected amplicons for the wild-type *Ihh* allele were 320 bp, for the floxed *Ihh* allele 400 bp, for the *Cre* allele 328 bp. For *Jansen* transgene genotyping was performed using the following specific primers: *Jansen forward* 5'-TAG TTG GCC CAC GTC CTG T-3', *Jansen reverse* 5'-TAA CCA TGT TCA TGC CTT CTT C-3'. After an initial denaturation step for 5 min at 95°C, amplification cycles consisted of denaturation at 95°C for 1 min, annealing at 58°C for 45 sec, and 1 min extension at 72°C for 35 cycles, followed by a final extension for 10 min at 72°C. The expected amplicon for the transgene was 560 bp.

Histology and Tissue Preparation

For histological analyses, paraffin sections of tibiae were generated from P7 and P14 mice. Tissues were fixed in 10% buffered formalin, decalcified in 20% EDTA, dehydrated at room temperature through an ethanol series, cleared in xylene, embedded in paraffin, and sectioned at 5 micrometer thickness. For routine morphological analyses sections were stained with hematoxylin/eosin.

In Situ Hybridization

In situ hybridization was performed as described previously [6]. In brief, complementary ³⁵S-UTP-labeled riboprobes were used to perform *in situ* hybridization on paraffin sections for

collagen type 2 (Col 2), collagen type X (Col X), Ihh, Ptch, collagen type 1 (Col1), and osteocalcin (OCN). Since the *Ihh* gene was removed manually by injection of Tamoxifen, only the mutant samples in which deletion of *Ihh* was confirmed were used for the further analysis with other marker genes. Three to five samples from each genotype were examined, respectively.

Proliferation

To evaluate the cell cycle of the proliferating chondrocytes, sections were routinely processed for immunohistochemistry by using a PCNA kit purchased from Zymed (Invitrogen), according to the manufacturer's protocols. Sections were counter stained with methyl green. Since positive signals were seen as brown nuclei the percentage of positive cells could be calculated.

Quantitative PCR

At P7, 60 μm frozen sections were prepared from the tibiae of tamoxifen-injected (P0) *Ihh mutant, double mutant, control and Jansen control* mice. Primary spongiosa and bone collar were microdissected from frozen sections and tissue was collected in lysis buffer (Agilent Technologies, Santa Clara, CA) and 2-mercaptoethanol (Sigma–Aldrich, St. Louis, MO). Total RNA was extracted (Agilent Technologies), and processed further for real-time PCR with the following specific primers: *beta-actin forward* 5'-AAG GCC AAC CGT GAA AAG AT-3', *beta-actin reverse* 5'-GTG GTA CGA CCA GAG GCA TAC-3', *Dkk1 forward* 5'-CTT GCG CTG AAG ATG AGG AGT-3', *Dkk1 reverse* 5'-GAG GGC ATG CAT ATT CCA TTT-3'. The relative expression of *mutants* was normalized to that of *controls* by using the calculations described earlier [17].

Statistical Analysis

Statistically significant differences between groups were evaluated by Mann–Whitney's U test. A *P* value of ≤ 0.05 was considered to be statistically significant. Analyses were performed by using Excel (Microsoft, Redmond, WA) and Graph Prism 4.0 (GraphPad, San Diego, CA).

Results

Rescue of growth plate of postnatal *Ihh* mutants by introducing a constitutively active PTH/PTHrP receptor into chondrocytes

To examine whether the *Jansen* transgene could rescue the abnormal growth plate of postnatal *Ihh* mutants, we crossed *Col2 Jansen* transgenic mice (*J*) with our *Col2-cre ER**; *Ihh^{fl/fl}* animals. The resulting *Col2-cre ER**; *Ihh^{fl/fl}*; *J* mice and their littermates (*Ihh^{fl/fl}*, *Ihh^{fl/fl}*; *J* and *Col2-cre ER**; *Ihh^{fl/fl}*) were injected with tamoxifen at P0 to generate *double mutants* (*Col2-cre ER**; *Ihh^{d/d}*; *J*), which are lacking *Ihh* but expressing the *Jansen* receptor in postnatal chondrocytes, and their *controls* (*Ihh^{fl/fl}*), *Jansen controls* (*Ihh^{fl/fl}*; *J*) and *Ihh* mutants (*Col2-cre ER**; *Ihh^{d/d}*). Seven days after tamoxifen injection, at P7, all *mutant* mice appeared macroscopically normal and were indistinguishable from their *control* littermates.

Efficiency of *Ihh* gene deletion was examined by *in situ* hybridization. *Ihh* is normally expressed in prehypertrophic chondrocytes of both *control* and *Jansen control* mice (Fig. 1e, f). Loss of *Ihh* expression was confirmed in *Ihh* mutants and in *double mutants* (Fig. 1g, h), respectively. Furthermore, expression of *Patched* (*Ptch*), a classical downstream target gene of *Ihh* signaling, was clearly present in chondrocytes and osteoblasts in the primary spongiosa and bone collar of both *control* and *Jansen control mice* (Fig. 1i, j). However, *Patched* was hardly detectable in *Ihh* mutants and *double mutants* (Fig. 1k, l), confirming efficient deletion of *Ihh* signaling in these *mutant* mice.

The growth plate of *Ihh mutants* was completely disorganized and was comprised of ectopic hypertrophic chondrocytes (Fig. 1c) as reported previously [10]. In contrast, the morphology of the growth plate of *double mutants* (Fig. 1d) appeared to be corrected through expression of the *Jansen* transgene and closely resembled the growth plate of *control* and *Jansen control* mice (Fig. 1a, b). The size of the bones was similar in *double mutants* and *controls*, but the distance between the primary and the predictive secondary ossification center was smaller in *double mutants*, indicating a reduction in length of chondrocyte columnar layers.

Expression of *collagen type 2*, which is present in all chondrocytes with exception of late hypertrophic cells, was found to be normal in *double mutants* (Fig. 2d) and resembled the expression pattern present in *control* (Fig. 2a) and *Jansen control* mice (Fig. 2b). In contrast, ectopic hypertrophic chondrocytes were detected in *Ihh mutants* and failed to express *Col2* (Fig. 2c). Moreover, the expression pattern of *collagen type X (ColX)*, a marker for hypertrophic chondrocytes, was similar in *double mutants*, *control* (Fig. 2h and 2e), and *Jansen control* mice (Fig. 2f), whereas the growth plate of *Ihh mutants* exhibited an abnormal pattern of *ColX* expression (Fig. 2g). Consistent with the histological findings, we found that the distance between the two expression domains of *ColX* in *double mutants* was reduced (Fig. 2h arrows).

Lastly, chondrocyte proliferation was examined by PCNA staining (Fig. 2i-m). Proliferation of chondrocytes was dramatically decreased in *Ihh mutants* (Fig. 2k). Proliferation was also reduced in *double mutants* (Fig. 2l) when compared to the *controls* and *Jansen controls* (Fig. 2i and j) despite the presence of the *Jansen* transgene. This result confirms and expands previous findings that demonstrate transgenic expression of a constitutively active *PTH/PTHrP* receptor can rescue the premature differentiation of chondrocytes but not the impaired proliferation of chondrocytes in *Ihh mutants*' growth plates [18], even after birth.

Expression of *Ihh* in chondrocytes is required for normal expression of osteoblast markers and Wnt activity in the surrounding bone tissue

To test whether the correction of chondrocyte differentiation is also sufficient to rescue the previously described defect in osteoblast differentiation in the bone collar of postnatal *Ihh mutants* [10], expression of osteoblast markers such as *collagen type I (Col1)* and *osteocalcin (OCN)* were analyzed by *in situ* hybridization. Expression of both markers was reduced or even completely absent in *Ihh mutants* (Fig. 3c and g) as well as in *double mutants* (Fig. 3d and h) when compared to *controls* (Fig. 3a and e). Interestingly, expression of *OCN* was also absent in the bone collar of *Jansen control*, a new finding that has not been previously reported (Fig. 3f). Since *Wnt* signaling is known to play a critical role in stimulating osteoblast differentiation, we further analyzed the expression of *Dickkopf 1 (Dkk1)*, a *Wnt* signaling target gene, in the bone collar and primary spongiosa of *mutant* mice and *controls* by qPCR. First, we confirmed our previous observation [10] that *Dkk1* mRNA expression was significantly decreased in the bone collar and primary spongiosa of *Ihh mutants* when compared to *controls*. Secondly, we also observed a decrease in *Dkk1* expression in *double mutants* despite the presence of a normal appearing growth plate (Fig. 3i). These findings suggest that chondrocyte derived *Ihh* is directly required for early osteoblast differentiation.

Premature closure of the growth plate cannot be prevented despite expression of constitutively active *PTH/PTHrP* receptor

We further extended our studies and examined the phenotype of all mice at P14, two weeks after deletion of *Ihh* from chondrocytes by tamoxifen injection. At that age, *double mutants* were already smaller in size when compared to both *control* and *Jansen control* mice (Fig. 4a). However, the *double mutants* were 8 % larger by body length than *Ihh mutant* littermates ($P < 0.05$) (Fig. 4b). Routine histology and *in situ* hybridization showed normal *ColX* expression pattern in the growth plate of *control* and *Jansen control* mice at P14 (Fig. 4c, d). In both *Ihh*

mutant and *double mutants*, however, the growth plate had virtually disappeared with only a thin layer of *ColX* positive hypertrophic chondrocytes remaining at the distal end of the bones (Fig. 4e, f), suggesting that expression of a constitutively active *PTH/PTHrP* receptor is not sufficient to maintain a growth plate in the absence of *Ihh*.

Discussion

Maintenance of a growth plate in postnatal life is critical for bone growth. Hedgehog signaling has been involved in the pathogenesis of numerous malignancies in children and its inhibition is currently exploited as a potentially promising therapeutical approach. However, in light of the critical role of *Ihh* in endochondral bone development, this treatment could severely impair bone growth in young patients. A study of whether and how *Ihh* affects growth plate development postnatally is thus an important and highly significant question.

In this study, we attempted to prevent the closure of the growth plate due to loss of *Ihh* signaling from chondrocytes after birth [10]. We demonstrate that the activation of the *PTHrP* signaling pathway in *Ihh* deleted postnatal chondrocytes could only partially correct the growth plate abnormalities caused by lack of *Ihh* signaling. Introduction of the constitutively active *PTH/PTHrP* receptor (*Jansen* receptor) into chondrocytes was able to prevent premature chondrocyte differentiation of *Ihh mutant* growth plates. However, it was not sufficient to restore the impairment of chondrocyte proliferation. Moreover, the incomplete rescue of osteoblast differentiation in these mutants suggests a role of *Ihh per se* in regulating osteoblast differentiation.

To target the *Ihh* gene deletion to chondrocytes and to express the constitutively active *PTH/PTHrP* receptor in chondrocytes the *collagen (α1) type 2* promoter was used in this study. A recent report showed that *collagen type 2* is also expressed in the kidney [19]. Notably, the *Ihh* transcript has been detected in the proximal convoluted tubule and proximal straight tubule of the adult kidney [20]. Since the kidney is the critical organ for maintaining balanced calcium-phosphate homeostasis we wanted to assure that none of the bone abnormalities we observed in *Ihh mutants* and/or in mice expressing the *Jansen* transgene were due to altered mineral ion homeostasis in these mice. We therefore performed serum calcium and phosphate measurements and were able to exclude this possibility. No changes in serum calcium and serum phosphate levels could be detected in our *mutant* mice when compared to *controls* (data not shown).

Ihh signaling is known to directly promote chondrocyte proliferation through a *PTHrP* independent pathway before birth. Here we show that even after birth expression of a constitutively active *PTH/PTHrP* receptor was unable to correct the decreased chondrocyte proliferation observed in the *Ihh mutant* growth plate, suggesting that *Ihh* also acts postnatally as a positive modulator of chondrocyte proliferation in a *PTHrP*-independent manner. Although *Ihh* is known to induce *Cyclin D1*, a classical cell cycle gene, the mechanism by which *Ihh* promotes chondrocyte proliferation is not fully understood [9]. In any event, it is likely that the impaired chondrocyte proliferation contributes to the premature closure of the epiphysis observed in *double mutant* mice at P14.

One of the key functions of *Ihh* is to suppress hypertrophic chondrocyte differentiation in a *PTHrP*-dependent manner. In our investigations the constitutively active *PTH/PTHrP* receptor was able to rescue the abnormal differentiation of cells to hypertrophic chondrocytes temporally. Interestingly, a recent report showed, that differentiation from slowly proliferating periarticular chondrocytes to fast proliferating columnar chondrocytes is promoted by *Ihh* independently of *PTHrP* signaling [21]. Consistent with these findings, we describe a reduction of the height of the columnar layer in the growth plate of *double mutants* (Fig. 2h). The failure

of constitutively active *PTH/PTHrP* receptors to prevent the growth plate closure at P14 in *double mutants* could also be due to reduced columnar chondrocyte differentiation and an age-dependent reduction in the number of cells that express the transgene. Likewise, the loss of growth plate of *PTHrP*^{-/-}; *Col2 Jansen* mice was rescued only temporally [13]. Interestingly, it has been reported that *Ihh* also promotes hypertrophic chondrocyte differentiation in a *PTHrP* independent manner [22]. Although we did not observe this effect of *Ihh* in our study, it was suggested that the outcome of chondrocyte hypertrophy when *Ihh* signaling is perturbed depends on the relative strength of the pro- (by *Bmps* and *Wnts*) and anti- (i.e. by *PTHrP*) hypertrophy signaling in the surrounding area. Since *PTHrP* signaling was selectively upregulated in our experimental design, we probably observed only the anti-hypertrophy actions of *Ihh* signaling.

It is known that *Ihh* is directly required for osteoblast differentiation prenatally [3]. Moreover, a recent study reported that activation of *Ihh* signal in *OCN* positive osteoblasts increased bone formation after birth [23]. These results support our findings. Since osteoblast differentiation could not be recovered in the *double mutants* despite recovery of a relatively normal growth plate in these mice at P7, it is possible to assume that chondrocytes might be the crucial source of *Ihh* production required for osteoblast differentiation after birth. The unexpected new finding that osteoblast maturation was also partially disturbed in *Jansen control* mice per se, as shown by decreased *OCN* expression in the bone collar of the tibia, despite the presence of a normal growth plate, (Fig. 3f and i) raises the question whether abnormal osteoblast differentiation in *double mutants* could be also due to the effect of the *Jansen* transgene in addition to the lack of direct *Ihh* signaling from chondrocytes in our new animal model. *Jansen control* mice might show a slight delay in endochondral bone formation after birth, a findings that has been made also prenatally [13]. Additional studies are required to answer these questions.

Ihh is a key factor required for normal endochondral bone formation. To target postnatal skeletal disease it is important to understand how *Ihh* regulates postnatal bone formation and maintenance. In this study, we clarified the *PTHrP* signaling-dependent role of *Ihh* in chondrocyte differentiation, and the *PTHrP* signaling-independent role of *Ihh* in chondrocyte proliferation and osteoblast differentiation in postnatal bone. Due to the loss of the growth plate in *double mutants* at P14, we were unable to further investigate the role of *Ihh* on bone remodeling at later stages. Further studies using alternate animal models are required to address the questions how chondrocyte-derived *Ihh* is involved in this process.

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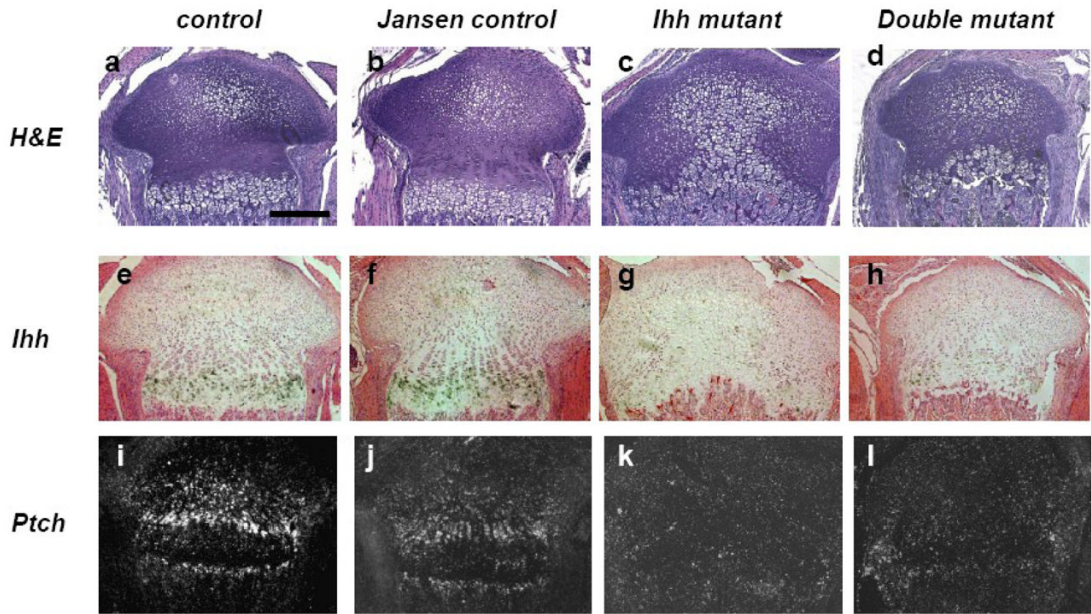


Fig. 1.

Growth plate morphology and mRNA expression of *Ihh* signaling at P7. Hematoxylin/eosin staining (a–d) and *in situ* hybridization (e–l) for *Ihh* (e–h), *Ptch* (i–l). At P7, the growth plate of *double mutants* (d) was restored when compared to the one of *Ihh mutants* (c) and appears similar to the one of *control* and *Jansen control* mice (a and b). *Ihh* expression is detected in prehypertrophic chondrocytes in *control* and *Jansen control* mice (e and f). Significant decrease of *Ihh* expression was confirmed in *Ihh mutants* and *double mutants* (g and h). The expression of *Ptch* was also found to be significantly decreased in *Ihh mutants* and *double mutants* (k and l). Magnification bar =0.5mm.

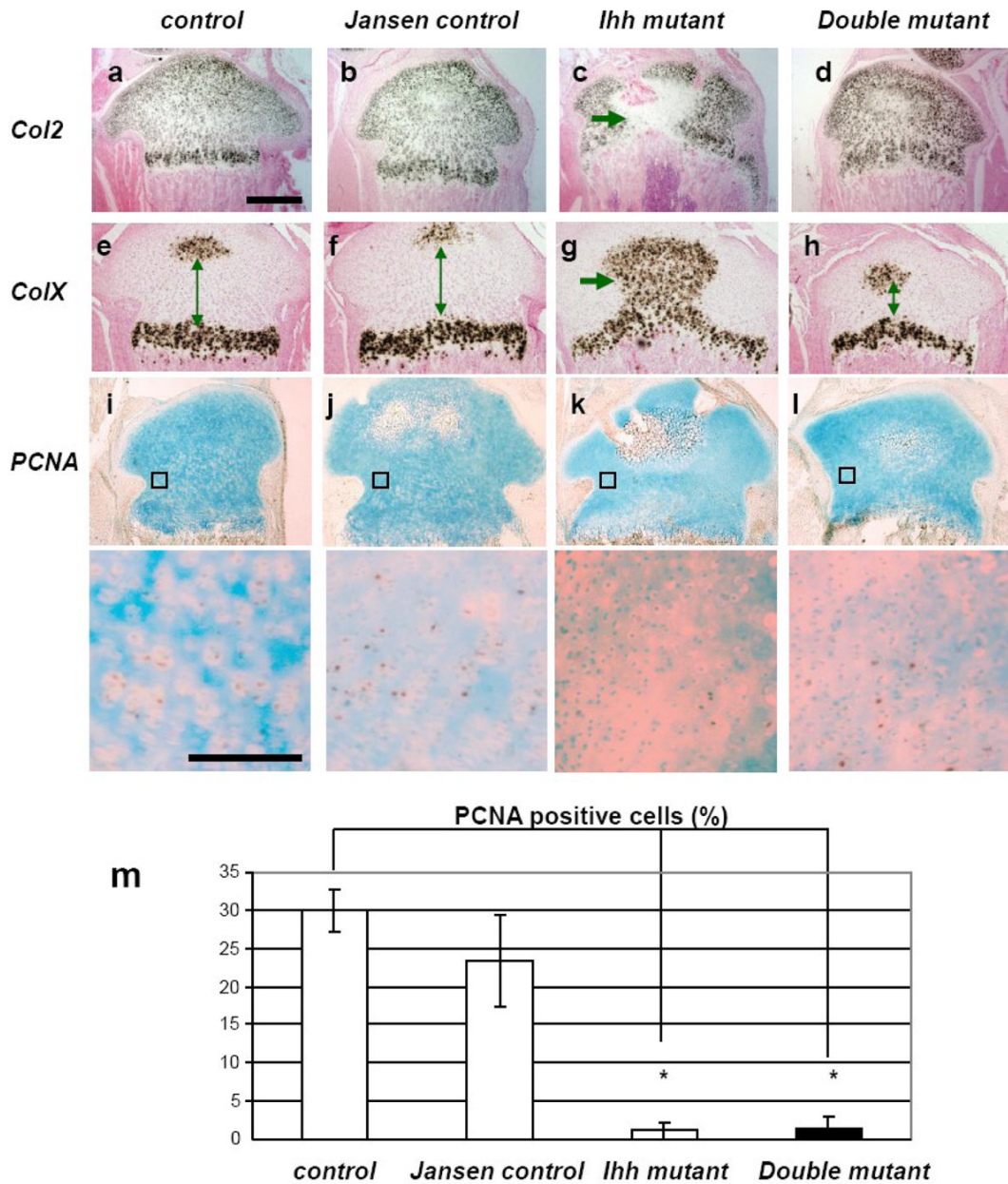


Fig. 2. mRNA expression and proliferation of chondrocytes. *In situ* hybridization for *Col2* (a–d), and *ColX* (e–h). Expression of *Col2* was found to be normal in *double mutants* (d). Ectopic hypertrophic chondrocytes in *Ihh mutant* completely lacked *Col2* expression (c, arrow). Expression of *ColX* in *double mutants* (h), was similar to the one in *control* and *Jansen control* mice (e and f), despite the narrowing of the space between the two expression centers in *double mutants* (h, arrow). *Ihh mutants* showed *ColX* expression in abnormal ectopic hypertrophic chondrocytes (g, arrow). Proliferation was analyzed by PCNA staining (i–l) and was significantly decreased in *Ihh mutants* and *double mutants* when compared with *control* and *Jansen control* mice (m) (*, $P < 0.05$; $n = 5$ mice, one section each.) All chondrocytes were counted in the boxed area and the percentage of PCNA positive (brown nuclei) chondrocytes

was calculated (lower panels represent magnification of the boxed areas. Magnification bar = 0.05mm, Magnification bar for a-I = 0.5mm).

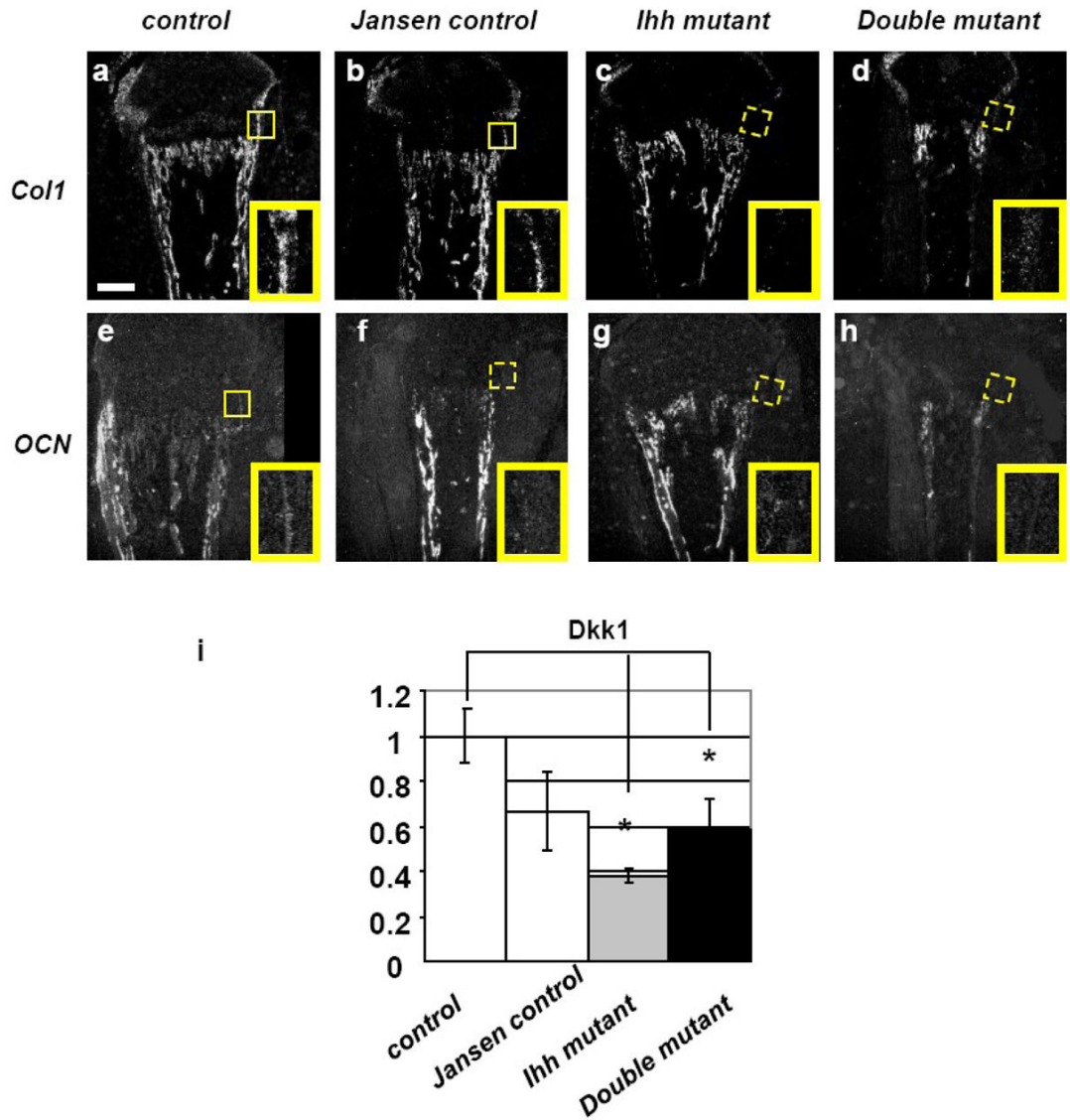


Fig. 3. mRNA expression of osteoblast markers. Expression of *Col1* and *OCN* was decreased in the bone collar of both *Ihh* mutants and *double* mutants (c, d, g, h, boxed area) when compared to *controls* (a and e, boxed area). Magnification bar = 0.1mm. Magnification of the boxed area = 0.06 X 0.04mm. Bar graph is showing mRNA expression of *Dkk1* in the bone collar and primary spongiosa of *controls* and *mutants* at P7 (i). Data are expressed as fold over *control*. The expression of *Dkk1* was significantly reduced in both *Ihh* mutants and *double* mutants when compared to *controls* (*, P<0.05; n = 5 mice, measured in triplicates each). No other significant changes were found.

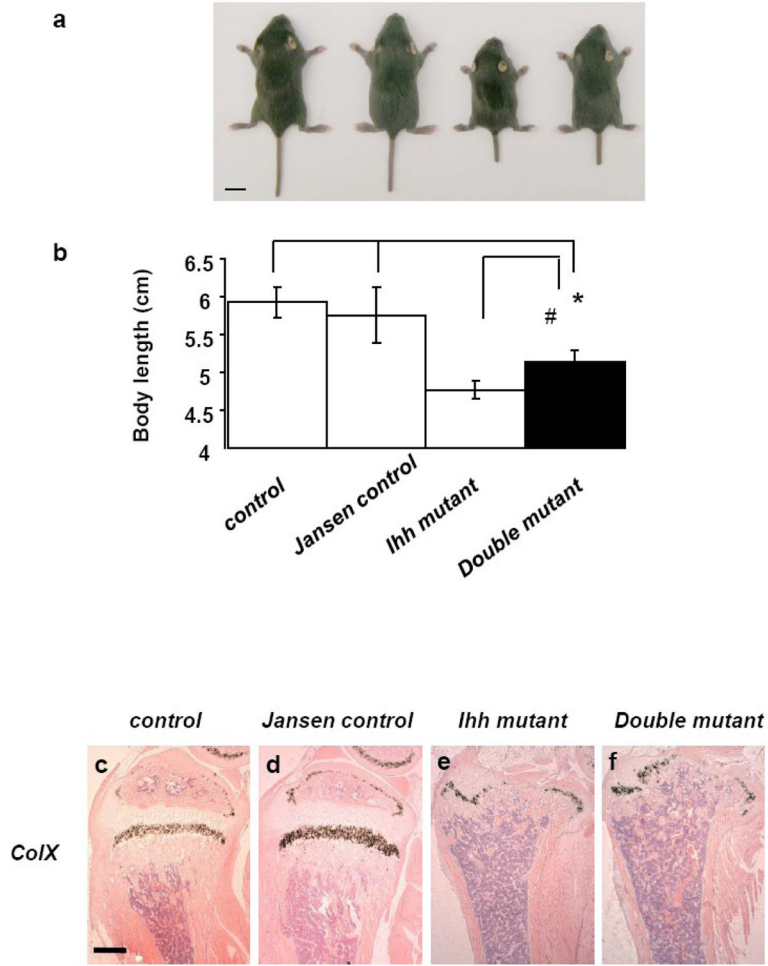


Fig. 4. Body size and histology of bones at P14. Macroscopic image of the *double mutant* (a). Magnification bar =1 cm. *Double mutants* appear smaller in size when compared to the *control* and *Jansen control* mice, however are 8 % larger by body length than *Ihh mutant* littermates (*, #, $P < 0.05$) (n = 6, 15, 6, and 3 mice in each group, respectively from left to right) (a and b). *In situ* hybridization showed normal *ColX* expression pattern in the growth plate of *control* and *Jansen control* mice at P14 (c and d). In both *Ihh mutants* and the *double mutants*, however, the growth plate has disappeared (e and f). Magnification bar =0.1mm.