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Original Paper

Conditional deletion of Indian hedgehog from collagen type 2α1-expressing cells results in abnormal endochondral bone formation

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Abstract

Indian hedgehog (*Ihh*) is actively involved in endochondral bone formation. Although expression of Ihh is mostly restricted to pre-hypertrophic chondrocytes, the role of chondrocyte-derived Ihh in endochondral bone formation is not completely understood. To address such unresolved issues, we used the *Cre/loxP* approach to generate mice (C*ol2α1Cre; Ihh*^{d}*/Ihh*^{d}) in which the *Ihh* gene was selectively ablated from *collagen type II* expressing cells. Mutant mice were born with the expected ratio of Mendelian inheritance, but died shortly after birth and were smaller in size, exhibiting malformed and retarded growth of limbs with severe skeletal deformities. Alizarin red S staining showed abnormal mineralization of axial and appendicular bones. Histological analysis of mutant long bones revealed abnormal endochondral bone formation with loss of a normal growth plate. In addition, *in vivo* bromo-deoxyuridine (BrdU) labelling showed a marked decrease in chondrocyte proliferation. A delay in chondrocyte hypertrophy in C*ol2α1Cre; Ihh*^{d}*/Ihh*^{d} mice was detected by the expression of collagen type X and osteopontin, using *in situ* hybridization. Furthermore, there was no expression of bone markers such as collagen type I, bone Gla protein, Runx2/Cbfa1 or PTH-R in the perichondrium of mutant mice, indicating the absence of osteoblasts from endochondral bones. Thus, selective loss of chondrocyte-derived *Ihh* recapitulated the defects in *Ihh*$^{-/-}$ animals, providing direct *in vivo* evidence that Ihh not only regulates chondrocyte proliferation and differentiation but also exerts effects on osteoblast differentiation. Understanding the exact functions of the molecules involved in endochondral bone formation will form the basis for further study to determine the molecular mechanisms of skeletal diseases involving various cellular components of bone.

Keywords: osteoblast; differentiation; conditional knock-out; Indian hedgehog; cre/loxP; endochondral bone

Introduction

A delicate coordination of chondrocyte proliferation and maturation, with accumulation of cartilaginous matrix proteins is vital in determining overall morphological features, mechanical properties and functional activities of endochondral bones. Recent studies have identified Indian hedgehog (Ihh) as one of the important molecules that plays crucial roles in chondrocyte development and subsequent endochondral bone formation. Ihh can be detected in the chondrocytes of early cartilaginous elements [1], and is mostly restricted to post-mitotic pre-hypertrophic chondrocytes, adjacent to the proliferative zone, which expresses the parathyroid hormone/parathyroid hormone-related peptide receptor (PTH/PTHrP-R) [1–3]. It is believed that a negative feedback loop between Ihh and PTHrP is responsible for regulating the rate of chondrocyte differentiation [3]; Ihh produced by pre-hypertrophic chondrocytes induces *PTHrP* expression, which prevents further differentiation of chondrocytes. This notion is further supported by studies in which exogenous addition of hedgehog protein to limb cultures delayed chondrocyte differentiation, but only in the presence of intact PTHrP signalling [3,4]. Deletion of the *Ihh* gene in mice by homologous recombination resulted in appositional differentiation of chondrocytes that correlated with loss of PTHrP [5]. Ihh may regulate *PTHrP* in perichondrial cells adjacent to the pre-hypertrophic chondrocytes through patched, a membrane protein that acts both as a hedgehog receptor and transcriptional target of hedgehog signalling [3,6]. Hedgehog signals are transduced through smoothened (Smo), a putative G-protein-coupled seven transmembrane domain protein [7,8] and, in the absence of hedgehog

proteins, Smo is repressed by patched, the cell surface receptor for hedgehog. Targeted ablation of Smo from type II collagen-expressing chondrocytes exhibited a dramatic retardation in skeletal growth due to reduced proliferating activities of chondrocytes, although the differentiation of chondrocytes in these mutant mice proceeded normally [9].

Various studies have reported mutations in the human *IHH* gene at positions Glu95, Asp100, and Glu131 in five kindreds affected with brachydactyly type A1 (BDA1) [10], an autosomal dominant disorder characterized by shortening or absence of the middle phalanges. Significant progress has been made in the last few years regarding essential involvement of Ihh in chondrocyte maturation and subsequent endochondral bone formation. Conventional *Ihh* knock-out mice provided important information on its crucial role in endochondral bone formation; *Ihh* mutant mice have a reduced endochondral skeletal size approximately 20% that of wild-type littermates at birth [5]. The $Ihh^{-/-}$ mice also showed complete lack of bone in the endochondral skeleton [5] and a subsequent study demonstrated that a direct Ihh input is required for osteoblast differentiation [11]. Since several prior studies reported expression of *Ihh* in osteoblasts [12–14], it remained possible that Ihh from sources other than osteoblasts could be responsible for osteoblast differentiation. In this study, we have examined whether chondrocyte-derived Ihh is required for osteoblast development during endochondral ossification.

We have employed the selective gene targeting approach to specifically ablate, *in vivo,* the *Ihh* gene from chondrocytes, using the *Cre/loxP* system. The results demonstrate that chondrocyte-derived Ihh is primarily responsible for regulation of the endochondral skeleton, by regulating both chondrocyte proliferation and differentiation and osteoblast differentiation.

Methods

Generation of floxed *Ihh* animals

For construction of the floxed *Ihh* allele, the starting plasmids containing the complete *Ihh* gene and adjacent sequences were kindly provided by Dr Andrew McMahon. A 4.2 kb *Eco*RI/*Eag*I fragment upstream of exon 1 and a 3.5 kb *Kpn*I/*Eco*RI fragment containing exon 2 and parts of intron 1 and 2 were chosen as 5′ and 3′ flanking regions, respectively. The link between these two fragments, a 1 kb *Eag*I/*Kpn*I fragment, represents exon 1 and some additional sequences upstream and downstream of the exon. This DNA fragment was used to design the floxed exon 1 of the *Ihh* gene. The 5′ *loxP* site was cloned 10 bp upstream of the initiator ATG site. Additionally, we introduced an *Eco*RI restriction site into this area which was used for screening purposes of correctly targeted ES cell clones. The *loxP* site was followed by exon 1, the *neo* (positive selection with G418) and *HSV-TK* (negative selection with FIAU) selection markers, both individually driven by a PGK promoter and their own poly A site. Those selectable genes were flanked by two *frt* sites. The 3′ *frt* site was followed by a second *loxP* sequence 600 bp downstream of exon 1. After targeting the *Ihh* allele by homologous recombination the two selection cassettes were excised by transfecting targeted ES cell clones with a plasmid expressing an enhanced version of the *flp* recombinase (*flpe*) [15–17]. In order to delete both marker genes successfully in every cell of the mouse, the *flp* recombinase was under the control of the elongation factor (EF1α) promoter, which has been shown to be strong and ubiquitously expressed [18]. Selection against herpes simplex virus thymidine kinase with FIAU then allowed selection of the anticipated ES cell clones, which were subsequently used for injection into blastocysts to generate chimeric mice. Chimeric males were bred to wild-type C57/BL6 mice to generate heterozygous floxed *Ihh* animals (Ihh^{fl}/Ihh). Heterozygous were interbred to establish a mouse line homozygous for the floxed *Ihh* allele (Ihh^{fl}/Ihh^{fl}).

Mice transgenic for *Cre* in *collagen type 2α1* expressing chondrocytes *(col2α-Cre)* have been previously reported [9]. To test *Cre* activity and specificity, the Cre transgenic line was crossed with lacZ reporter animals in which β-galactosidase expression is activated, following *Cre*-mediated excision of a sequence that contains both stop codons and a polyadenylation sequence to tightly stop expression of the reporter gene when *Cre* has not been active [9,19]. *Col2α1-Cre* mice were mated to Ihh^{fl}/Ihh^{fl} animals to obtain *col2α1-Cre*; Ihh/Ihh^{fl} studs. These were subsequently interbred with Ihh^{fl}/Ihh^{fl} animals and offspring were analysed at different days of gestation.

Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were employed using protocols approved by the institution's subcommittee on animal care (IACUC).

Southern blot and PCR analyses

Genotyping was performed initially by Southern blot analysis. Genomic DNA (~10 μg) purified from ES cells and tail clips using standard procedures was digested with *Eco*RI and hybridized to an external probe, B. The expected lengths of the *Eco*RI fragment were 9 kb for the wild-type allele, 4.1 kb for the floxed *Ihh* allele and 3.5 kb for the excised *Ihh* allele. Further genotyping of mice was performed by PCR, using the following specific primers: *flIhh* forward 5′-AGC ACC TTT TTT CTC GAC TGC CTG-3′, *flIhh* 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 GAT ATC-3′. After an initial denaturation for 8 min at 94 °C, amplification cycles consisted of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s and 45 s extension at 72 °C for 35 cycles, followed by a final

extension for 10 min at 72 °C. The expected product sizes for the wild-type *Ihh* allele were 320 bp, for the *floxed allele* 400 bp, for the *Cre* allele 328 bp.

Skeletal analysis

The mineralization pattern of the skeleton was analysed at E14.5, E16.5, E18.5 and p1, as described previously by McLeod [20]. Briefly, embryos were harvested by Caesarean sections. The fetuses were skinned, eviscerated and fixed in 95% ethanol. Subsequently, acetone was used to remove fat. Then the skeletons were stained by Alizarin red S/Alcian blue and sequentially cleared in 1% potassium hydroxide. Mineralized bones were visualized by the staining.

Histology and tissue preparation

For histological analyses, paraffin sections of bones were produced from E15.5 to E18.5. The fetuses were fixed in 4% paraformaldehyde (PFA)/PBS, pH 7.4, at 4 °C, rinsed in PBS, dehydrated at room temperature through an ethanol series (70% for 6 h, 80% for 1 h, 96% for 1 h and 100% for 3 h), cleared twice in xylene for 1 h/step, embedded in paraffin, sectioned at 6 μm with a Microm HM 360 microtome (Microm, Walldorf, Germany) and mounted on SuperFrost Plus slides (Fisher Scientific, GA, USA). For morphological analyses, the sections were stained with haematoxylin and eosin or von Kossa to visualize mineralization.

Riboprobes and *in situ* hybridization

Complementary ^{35}S-UTP-labelled riboprobes (complementary RNAs) were used for *in situ* hybridization. Plasmids encoding the cDNA were linearized with appropriate restriction enzymes to transcribe either antisense or sense riboprobes *in vitro*, using the appropriate RNA polymerase. *In situ* hybridization was carried out as described previously [21]; only antisense riboprobes showed specific signals.

Briefly, bone sections were dewaxed in xylene and re-hydrated in a decreasing ethanol series (100%, 90%, 70%). After proteinase K treatment and post-fixation in 4% paraformaldehyde (PFA), sections were incubated in 0.2 N HCl. Sections were then acetylated with 0.25% acetic anhydride in triethanolamine buffer. Before hybridization was performed, sections were dehydrated in 70% and 95% ethanol and air-dried. Sections were hybridized with ^{35}S-labelled antisense or sense riboprobes in a humidified chamber at 50 °C for 16 h. After hybridization, non-specifically bound riboprobes were removed by washing the slides with 2 × SSC and 2 × SSC/50% formamide at 50 °C and treating them with RNase at 37 °C for 20 min. The final wash steps were performed once in 2× SSC and twice in 0.2× SSC at 50 °C for 20 min. To detect the hybridization of riboprobes on tissues, sections were dehydrated in 70% and 95% ethanol and air-dried. To estimate the intensity of bound riboprobes, the slides were exposed to X-ray film (Kodak Biomax MR-1) for overnight at room temperature. The sections were then coated with Kodak NTB2 emulsion diluted 1 : 1 with water, exposed for the time needed (determined by autoradiography), developed with Kodak Dektol developer and fixed with Kodak fixer. After counterstaining with haematoxylin and eosin, the tissue sections were analysed using a Zeiss microscope with bright- and darkfield optics.

Proliferation studies using bromo-deoxyuridine (BrdU) labelling

To evaluate the cell cycle of the proliferating chondrocytes, pregnant females at E16.5–E18.5 were injected intraperitoneally with 100 μg/g body weight BrdU (Roche) 2 h before sacrifice. The hindlimbs were processed for paraffin embedding. Sections were incubated at 4 °C overnight with mouse anti-BrdU antibody, and were visualized using a BrdU-labelling kit purchased from Zymed (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The percentage of proliferating cells was determined as the ratio of the number of BrdU-positive cells over the total number of cells, counted in defined areas as indicated.

Results

Generation of floxed *Ihh* animals

Since deletion of exon 1 in the conventional *Ihh* knock-out [5] resulted in a complete knock-out, we decided to target the same exon 1 for our conditional knock-out (*Cre/loxP*) approach as detailed in Figure 1. Homozygous floxed *Ihh* animals appear macroscopically normal and are fertile.

Survival and gross phenotype of mutant (*col2α1-Cre; Ihh^d/Ihh^d*) mice

A Col2-Cre line (Cre10) [9] was crossed with floxed Ihh mice to generate *col2α1-Cre; Ihh^d/Ihh^d* mutants. The *col2α1-Cre; Ihh^d/Ihh^d* mutants were born with the expected Mendelian ratio (25%) but died shortly after birth. Newborn mutant mice had a typical shortening of the forelimbs and the hindlimbs, which was detectable in embryos at E14.5 (data not shown) but more evident at birth (Figure 2A). The long bones of mutant mice were shorter than those of wild-type littermates and showed a similar phenotype, as reported in conventional *Ihh* knock-out mice [5] but with lesser severity. Alizarin red S staining of whole skeletons showed abnormal mineralization in the endochondral skeleton (Figure 2). Mineralization of the skeleton was generally delayed until E16.5 (data not shown); the delay in the radius was most severe and initiated in the centre of the cartilage at E18.5 (Figure 2C, I). The mineralization in the centre of the radius suggested that it was occurring in cartilage and not in

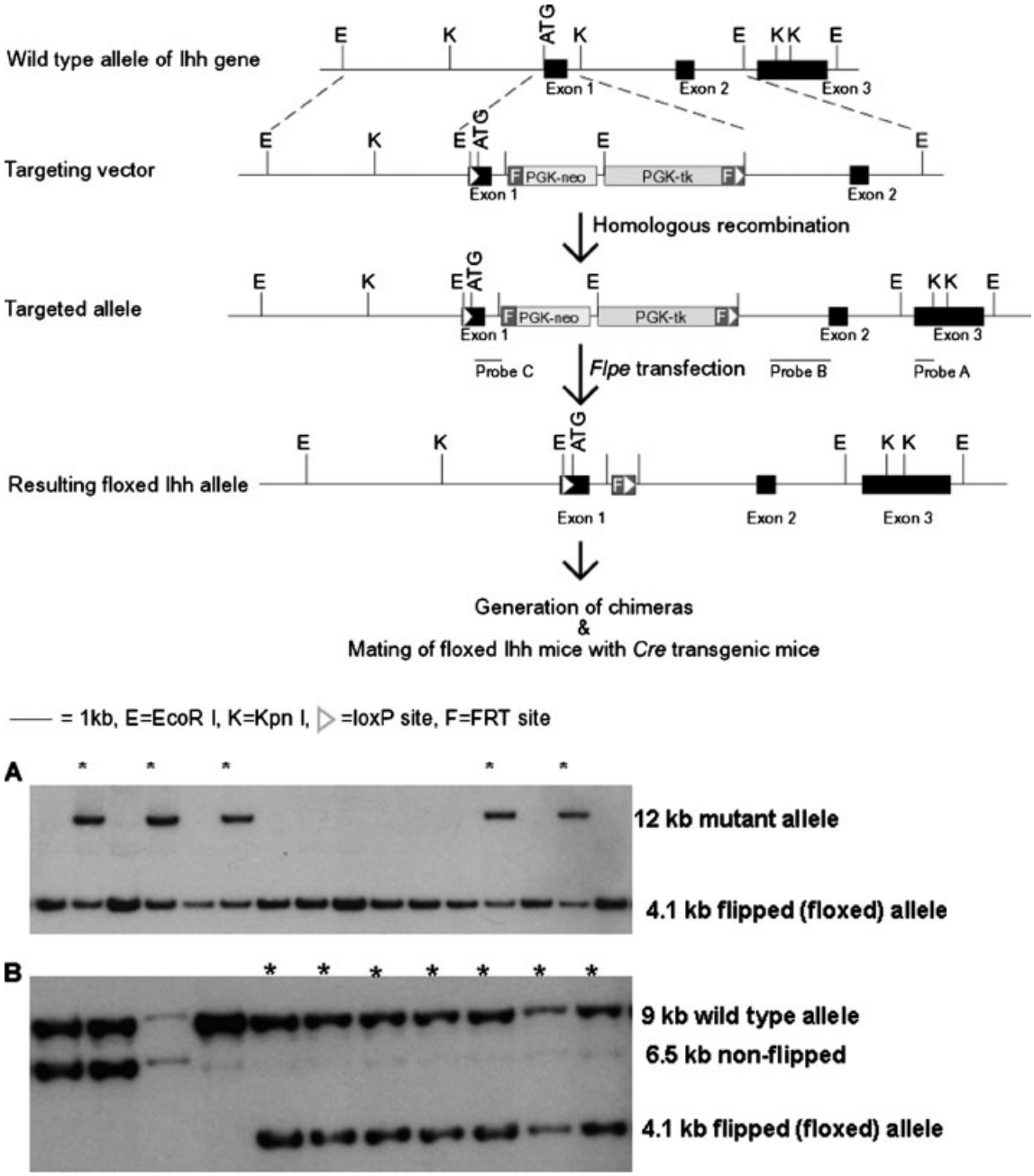

Figure 1. (A) Targeting vector for the generation of a floxed exon 1 of the *Ihh* gene. Resulting floxed *Ihh* animals contain a floxed exon 1 of the *Ihh* gene. The location of various probes (probes A, B and C) for Southern blot analysis is indicated. (B) Southern blot analysis of correctly targeted ES cell clones (asterisk) after homologous recombination and positive selection with G418. Hybridization of genomic DNA after digestion with *Kpn*I restriction enzyme with probe A results in a 4.2 kb wild-type and a 12 kb mutant DNA fragment (A). Genomic southern blot of ES cell clones after excision of the two selection markers (*neo, HSV-TK*) from the *Ihh* gene with *flpe*. Hybridization of genomic DNA after *Eco*RI digestion with probe B (Figure 1A) results in a 9 kb wild-type, a 6.5 kb mutant non-flipped and a 4.1 kb mutant flipped (floxed allele) band. Correctly targeted ES cell clones are labelled with an asterisk (B)

association with a bone collar, a phenomenon that was also observed in *Ihh* null animals. The dorsal part of the ribs and the distal parts of the long bones, such as the phalanges, showed essentially no mineralization, even at E18.5 (Figure 2I, J), whereas the base of the skull (Figure 2G), sternum (Figure 2E) and vertebrae (Figure 2F) exhibited synchondrosis of some bones. We also observed a failure in digit segmentation (Figure 2I, J). In contrast to endochondral bones, intramembranous bones appeared to be unaffected.

Histological phenotype of mutant mice

Histological analysis revealed that the mutant animals contained no cortical or trabecular bone (Figure 3A) and showed chondrodysplasia in the long bones. In contrast to the wild-type littermate exhibiting the characteristic zones of chondrocyte differentiation (resting, proliferating, prehypertrophic and hypertrophic) in the growth plate, the mutant mice contained no organized growth plate. Most notably, the

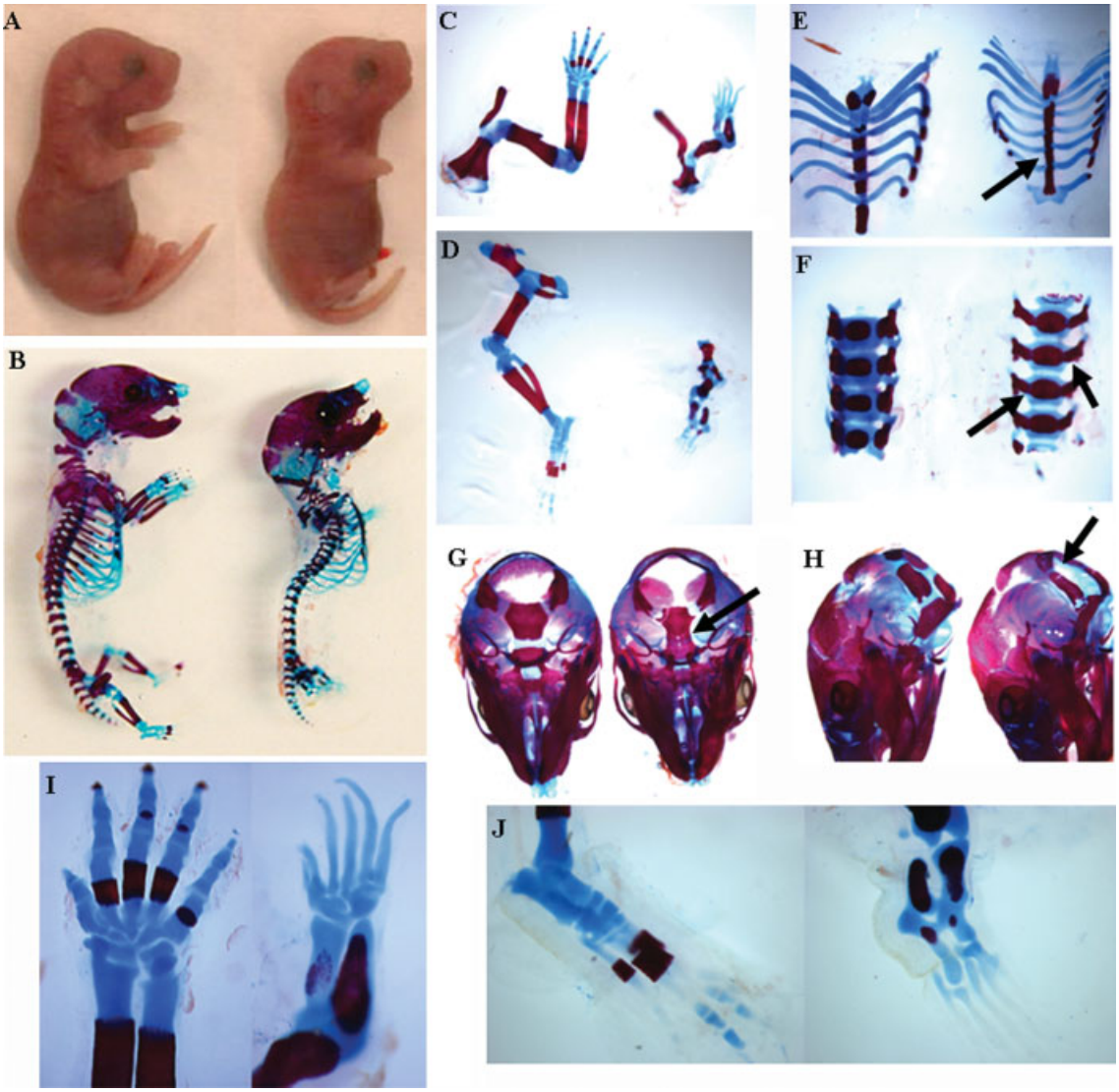

Figure 2. Full body skeletons at E18.5 were stained with Alizarin red/Alcian blue. Long bones of mutants were only one-third the length of those of Ihh^{fl}/Ihh^{fl} littermates (A–D) compared to controls. Although the failure in bone growth is the most striking feature of the *col2α1-Cre;* Ihh^{d}/Ihh^{d} phenotype, differentiation is also abnormal. Calcification was proportionally more extensive in bones such as sternum (E), vertebrae (F) and the cartilaginous synchodrosis of the base of the skull (G, H). We also observed failure in digit segmentation (I, J). Arrows point to the anomalies in endochondral bones

hypertrophic cells were located in the centre of the cartilage, surrounded by undifferentiated chondrocytes (Figure 3A). We further studied the mineralization state using von Kossa staining on paraffin sections. Consistent with the whole-mount skeletal staining, mineralization in bones of mutant mice was delayed. In particular, only hypertrophic chondrocytes located in the very centre of the cartilage showed von Kossa staining (Figure 3B), whereas essentially no mineralization was detected in more distal bones, such as those in the paw. Thus, histological analyses indicate a profound dysregulation of chondrocyte maturation, as well as lack of bone formation in the *Col2-Cre;* $Ihh^{d/d}$ animal.

Determination of chondrocyte proliferation rate by BrdU incorporation in mutant mice

We assessed chondrocyte proliferation by quantifying BrdU labelling in the growth plate of the tibia. There was an approximately 50% reduction in the percentage of BrdU-positive cells in the disorganized growth plate of mutant mice at E16.5 compared with the same region in Ihh^{fl}/Ihh^{fl} control or in wild-type littermates (Figure 3C). Analyses at E18.5 showed a similar result (data not shown). These results demonstrate that *Ihh* expression in chondrocytes is required to maintain the high rate of proliferation in rapidly growing long bones.

Expression of chondrocyte maturation markers in mutant mice

To determine the maturation of chondrocytes in mutant mice, we analysed the expression of collagen type X (a marker for hypertrophic chondrocytes) and osteopontin (a marker for late hypertrophic chondrocytes), using *in situ* hybridization on paraffin sections prepared from tibia at E15.5–E18.5 of gestation (Figure 4). Since no apparent growth

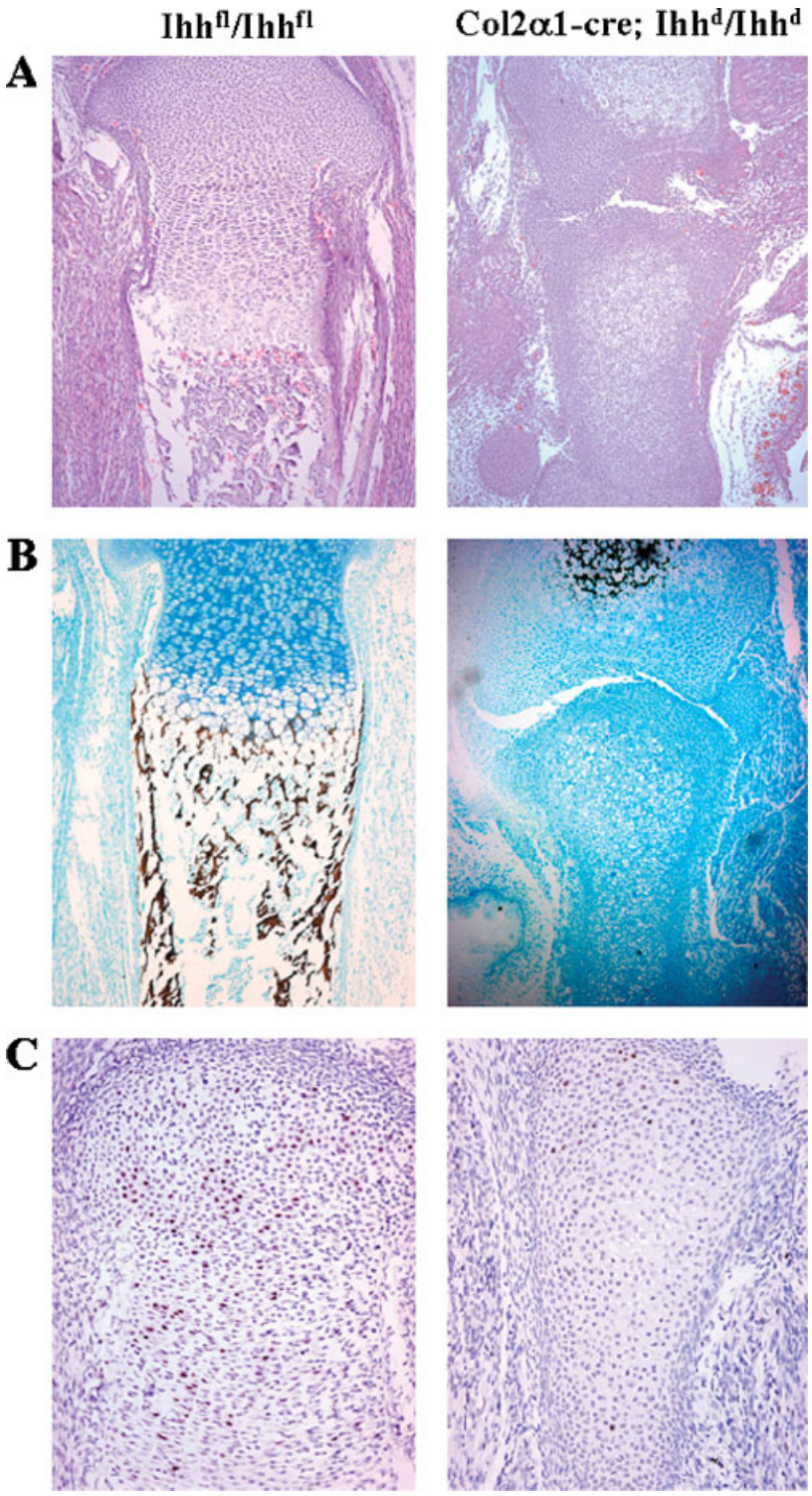

Figure 3. Haematoxyin/eosin staining was performed on paraffin sections at E18.5 (A). Note that no cortical or trabecular bone formation was apparent in *Col2α 1-Cre; Ihh^d/Ihh^d* animals. Long bones of mutant mice were composed of disorganized chondrocytes with mostly hypertrophic cells located in the centre of the bone, surrounded by undifferentiated chondrocytes. Mineralization was delayed in bones of *col2α 1-Cre; Ihh^d/Ihh^d* mice, and only hypertrophic chondrocytes located in the very centre of the cartilage showed von Kossa staining (B). More distal bones did not show any mineralization at that time point. Chondrocyte proliferation in *col2α 1-Cre; Ihh^d/Ihh^d* was markedly reduced when compared to *Ihh^{fl}/Ihh^{fl}* littermates at E16.5. The proportion of BrdU-positive nuclei of the tibia was less than half of control mice (C)

plates were formed, hypertrophic cells expressing collagen X were located in the core of the bone (Figure 4B, D, F, H). This analysis also showed a delay in chondrocyte differentiation in the mutant embryo, as indicated by the delayed expression of collagen type X and osteopontin (Figure 4J, L), when compared to normal expression (Figure 4I, K). Thus molecular analyses confirmed a delay in chondrocyte maturation in the *Col2-Cre, Ihh^d/Ihh^d* mouse.

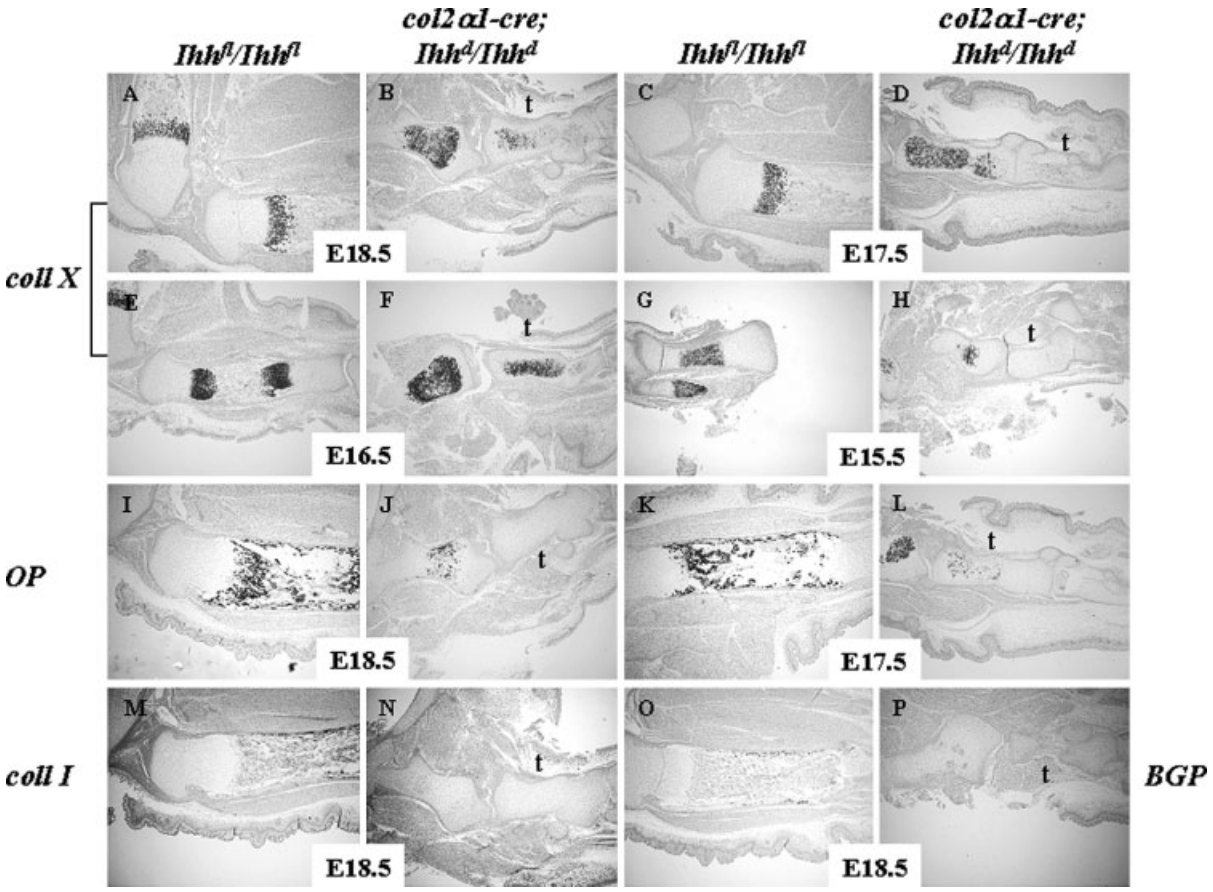

Figure 4. *In situ* hybridization on paraffin sections prepared from tibia at E15.5–E18.5 of gestation. Collagen type X expression in hypertrophic chondrocytes of mutant bones (B, D, F, H) compared to control (A, C, E, G). No apparent growth plates were formed and hypertrophic cells stayed in the centre of the long bones (B, D, F, H). Decreased osteopontin in *Ihh*-ablated chondrocytes (J, L), compared to control (I, K). There was no apparent expression of bone markers, such as collagen type I (N) and bone Gla protein (P) in *col2α1-Cre; Ihh^d/Ihh^d* animals

Lack of mature osteoblasts in the endochondral skeleton of *col2α1-Cre; Ihh^d/Ihh^d* animals

In situ hybridization was also carried out using riboprobes specific for collagen type I, bone Gla protein, Cbfa1/Runx2, PTH/PTHrP receptor (PTH-R) and patched (receptor for Ihh), on tibial sections of *col2α1-Cre; Ihh^d/Ihh^d* animals and their control *Ihh^{fl}/Ihh^{fl}* littermates at E18.5. No expression of bone forming markers such as type I collagen (Figure 4N) and bone Gla protein (Figure 4P) was detected in mutant animals. Expression of Cbfa1/Runx2 and PTH-R was only detected in mutant chondrocytes (Figure 5), indicating the absence of osteoblasts. The expression of *patched*, a known transcriptional readout of Hh signalling, was completely abolished in chondrocytes in the mutant mice, suggesting efficient deletion of *Ihh* from cartilage, while mRNA signals of *patched* were clearly detected in skin overlying the mutant bones (Figure 5A).

Discussion

To study the significance of chondrocyte-derived Ihh in cartilage development and osteoblast differentiation, we have selectively ablated the *Ihh* gene from collagen type II-expressing cells using the *Cre/loxP* gene targeting technique. Efficient *Cre* recombination was achieved after breeding floxed *Ihh* animals to *col2α1-Cre* transgenic mice, as demonstrated by the loss of *patched* expression in the growth plate (Figure 5).

The survival of all *Col2α1-Cre; Ihh^d/Ihh^d* animals until birth suggests that Ihh has additional important functions outside the Collagen II-expressing domains during early development that have caused the premature death of *Ihh* null animals [5]. The similarity of the phenotype of *Col2α1-Cre; Ihh^d/Ihh^d* and conventional *Ihh* nulls at birth, however, suggests that ablation of the *Ihh* gene from chondrocytes alone has severe effects on growth plate development, endochondral bone formation and postnatal survival.

The shortening of limbs and the reduced length of the axial and appendicular skeleton in *col2α1-Cre; Ihh^d/Ihh^d* mice are in part due to the significant decrease in proliferation of chondrocytes, as demonstrated by the significant reduction of BrdU-labelled cells. Since Ihh is mostly expressed in prehypertrophic chondrocytes, removal of the *Ihh* gene from chondrocytes resulted in a profound decrease in cell proliferation, equivalent to that observed in *Ihh* null animals. It has previously been reported that Ihh

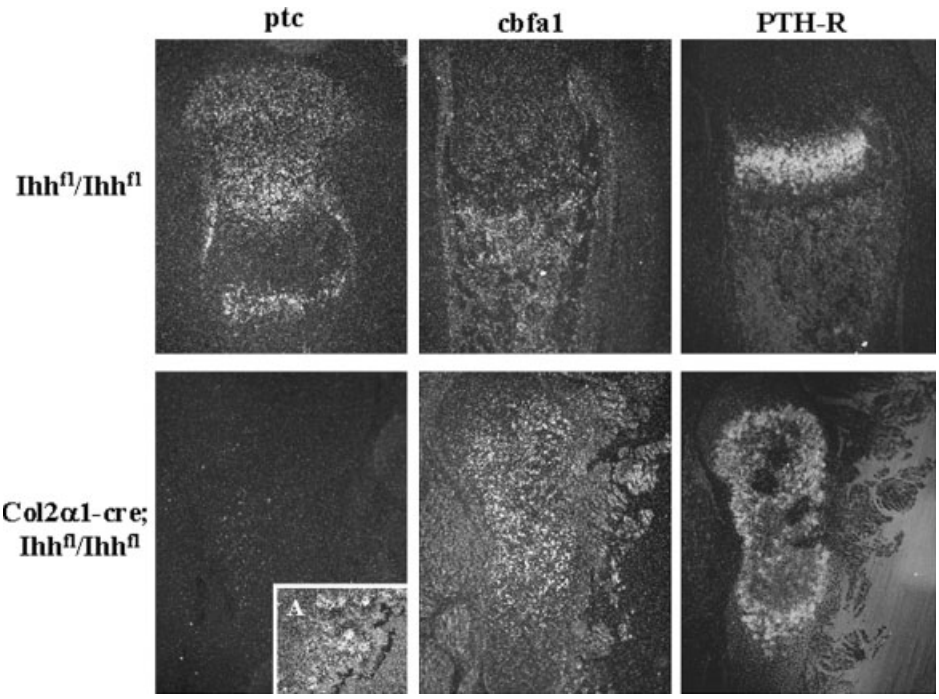

Figure 5. *In situ* hybridization using riboprobes for patched (ptc), cbfa1 and PTH/PTHrP receptor (PTH-R) on tibial sections of c*ol2α1-Cre; Ihh*^{d}*/Ihh*^{d} animals and their normal *Ihh*^{fl}*/Ihh*^{fl} littermates at E18.5. Expression of ptc was completely abolished in *Ihh*-ablated chondrocytes suggesting efficient deletion of Ihh from cartilage, while mRNA signals of ptc were clearly detected in skin overlying the mutant bones (inset A). Expression of cbfa1 and PTH-R was only detected in chondrocytes of c*ol2α1-Cre; Ihh*^{d}*/Ihh*^{d} animals, indicating complete absence of osteoblasts from bones formed by endochondral ossification

either directly or indirectly promotes the transcription of cyclin D1, which is known to be down-regulated in similar mouse models, such as *Ihh* null animals [5] and *col2-cre;Smo*^{n}*/Smo*^{c} embryos [9]. Although previous studies have demonstrated the requirement of Ihh signalling for normal chondrocyte proliferation [9], the question about the source of Ihh production still remained. In the present study we have demonstrated chondrocytes as the principle source of Ihh, affecting both chondrocyte and osteoblast differentiation.

Ablation of *Ihh* from chondrocytes did also initially delay chondrocyte maturation in the long bones but at later stages hypertrophic cells were found close to the ends of the bones, a phenotype that was also observed in both the *Ihh* null [5] and the *PTHrP* null animals [22]. Since *PTHrP* expression in the articular cartilage is normally up-regulated by Ihh and loss of Ihh signalling leads to down-regulation of *PTHrP* expression [5], these results are consistent with Ihh regulating chondrocyte maturation via PTHrP [3,4]. We also confirmed our morphological observations by demonstrating the initial delay in collagen type X and osteopontin gene expression in the long bones and that the large cells close to the surface expressed collagen type X. C*ol2α1-Cre; Ihh*^{d}*/Ihh*^{d} also share other similar abnormal features with *PTHrP*$^{-/-}$, *PTH-R*$^{-/-}$ and *Ihh* null animals, such as the synchondroses between the basoccipital and exoccipital bones at the base of the skull, as well as ectopic calcification in the sternum and vertebrae, providing evidence for a PTHrP/Ihh feedback loop in other endochondral skeletal elements.

One of the important findings of this study is that selective deletion of *Ihh* from Collagen type II-expressing chondrocytes abolished osteoblast formation; no expression of Collagen type I and bone Gla protein could be detected in the long bones of c*ol2α1-Cre; Ihh*^{d}*/Ihh*^{d} animals (Figure 4N, P). The absence of osteoblast differentiation correlates also with the absence of Cbfa1/Runx2 expression. Cbfa1 encodes a transcriptional regulator of a number of bone-specific target genes [23] and its activity is needed for osteoblast formation. It was also shown that *Cbfa1* knock-out animals fail to terminally differentiate chondrocytes [24]. Expression of the PTH-R was only detected in chondrocytes of C*ol2α1-Cre; Ihh*^{d}*/Ihh*^{d} mice (Figure 5), suggesting again a failure in osteoblast differentiation in mutant mice. Our results are in accord with earlier observations that conventional *Ihh* null mice do not form bone collars, while over-expression of *Ihh* by retroviral infection induced ectopic bone collar formation [25]. Moreover, ectopic expression of the constitutively active PTH/PTHrP receptor in chondrocytes partially restored chondrocyte differentiation but had no effect on the osteoblastic cell populations [26,27]. Finally, selective removal of *Smo* (a transducer of Ihh signalling) from the perichondrial cells but not from chondrocytes, cell-autonomously abolished osteoblast development [9,11]. In summary, Ihh produced by chondrocytes is directly responsible for

proper osteoblast differentiation from the perichondrial progenitors.

Overall, we have provided evidence that chondrocyte-derived Ihh regulates both chondrocyte proliferation/differentiation and osteoblast differentiation during endochondral skeletal development. We believe that understanding the basic molecular regulation of endochondral bone formation is the first step towards determining the pathogenesis of diseases involving the different components of bone.

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