Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy

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Chondrocyte hypertrophy is an essential process required for endochondral bone formation. Proper regulation of chondrocyte hypertrophy is also required in postnatal cartilage homeostasis. Indian hedgehog (Ihh) and PTHrP signaling play crucial roles in regulating the onset of chondrocyte hypertrophy by forming a negative feedback loop, in which Ihh signaling regulates chondrocyte hypertrophy by controlling *PTHrP* expression. To understand whether there is a *PTHrP*-independent role of Ihh signaling in regulating chondrocyte hypertrophy, we have both activated and inactivated Ihh signaling in the absence of *PTHrP* during endochondral skeletal development. We found that upregulating Ihh signaling in the developing cartilage by treating *PTHrP*⁻/⁻ limb explants with sonic hedgehog (Shh) protein in vitro, or overexpressing *Ihh* in the cartilage of *PTHrP*⁻/⁻ embryos or inactivating patched 1 (*Ptch1*), a negative regulator of hedgehog (Hh) signaling, accelerated chondrocyte hypertrophy in the *PTHrP*⁻/⁻ embryos. Conversely, when Hh signaling was blocked by cyclopamine or by removing *Smoothened* (*Smo*), a positive regulator of Hh signaling, chondrocyte hypertrophy was delayed in the *PTHrP*⁻/⁻ embryo. Furthermore, we show that upregulated Hh signaling in the postnatal cartilage led to accelerated chondrocyte hypertrophy during secondary ossification, which in turn caused reduction of joint cartilage. Our results revealed a novel role of Ihh signaling in promoting chondrocyte hypertrophy independently of *PTHrP*, which is particularly important in postnatal cartilage development and homeostasis. In addition, we found that bone morphogenetic protein (Bmp) and Wnt/β-catenin signaling in the cartilage may both mediate the effect of upregulated Ihh signaling in promoting chondrocyte hypertrophy.

KEY WORDS: Ihh, Patched 1, Cartilage, PTHrP, Chondrocyte hypertrophy

INTRODUCTION

Endochondral ossification is a major bone-forming process in vertebrate skeletal development. The multi-step process of endochondral bone formation begins with the condensation of mesenchymal progenitor cells. These condensed mesenchymal cells then differentiate into chondrocytes to form the cartilage anlagen. In the developing long bones, chondrocytes undergo sequential proliferation and differentiation, which is required for proper longitudinal bone growth. Slowly proliferating periarticular chondrocytes differentiate into highly proliferative columnar chondrocytes, and then proliferative chondrocytes exit cell cycle and undergo hypertrophy. Eventually, the most mature hypertrophic chondrocytes undergo apoptosis. The hypertrophic chondrocytes secrete vascular endothelia growth factor (Vegf) and matrix metalloproteinases (Mmps), which will degrade the extracellular matrix and allow the invasion of blood vessels and osteoblasts differentiated in the perichondrium to form the trabecular bone. Therefore, chondrocyte hypertrophy is an essential step in endochondral bone formation, which is tightly controlled during normal skeletal development by cell-cell signaling and transcription factors (reviewed by de Crombrugghe et al., 2001; Karsenty and Wagner, 2002; Kronenberg, 2003; Zelzer and Olsen, 2003). Furthermore, the proper regulation of chondrocyte hypertrophy is

also necessary for maintaining the cartilage lining synovial joint surfaces, as abnormal chondrocyte hypertrophy in articular cartilage is associated with a common cartilage degenerative disease: osteoarthritis (Poole, 1999).

Indian hedgehog (Ihh) is a member of the hedgehog (Hh) family that plays crucial roles in regulating many developmental processes. Ihh is one of the key signaling molecules controlling both chondrocyte hypertrophy and bone formation in the developing skeletal system. *Ihh* is expressed in prehypertrophic chondrocytes and it regulates the onset of hypertrophic differentiation by forming a negative feedback loop with parathyroid hormone related protein (PTHrP; Pthlh – Mouse Genome Informatics) (St-Jacques et al., 1999; Vortkamp et al., 1996). Ihh activates the expression of *PTHrP* in the periarticular cells and the articular chondrocytes, and PTHrP signals through its receptor Pthr1 to inhibit chondrocyte hypertrophy and to suppress further *Ihh* expression by keeping chondrocytes in a proliferating state (Lanske et al., 1996; St-Jacques et al., 1999). Ihh signaling is also required for chondrocyte proliferation and osteoblast differentiation independently of PTHrP signaling (Karp et al., 2000; Long et al., 2004; St-Jacques et al., 1999). However, it remains to be elucidated whether Ihh signaling regulates chondrocyte hypertrophy exclusively through *PTHrP*, as Ihh signals directly to proliferating and prehypertrophic chondrocytes. In the *Ihh*⁻/⁻ embryo, *PTHrP* is not expressed and yet chondrocyte hypertrophy is delayed at E14.5, although it is accelerated later (St-Jacques et al., 1999). A recent study demonstrates that *Ihh* promotes the transition from periarticular chondocytes to proliferating chondrocytes and regulates columnar cell mass independently of PTHrP signaling (Kobayashi et al., 2005b). In addition, Gli3, a downstream component of the hedgehog signaling pathway, acts as a repressor to inhibit the transition of periarticular chondrocytes into columnar

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chondrocytes by a *PTHrP*-independent mechanism (Hilton et al., 2005; Koziel et al., 2005). As chondrocyte proliferation, which directly affects hypertrophy, was altered when Hh signaling was manipulated in these studies, the *PTHrP*-independent role of Ihh signaling in regulating chondrocyte hypertrophy was not revealed.

Here, we have tested the role of *Ihh* in regulating chondrocyte hypertrophy independently of *PTHrP*, by inactivating or activating Ihh signaling cell autonomously in the developing cartilage in the *PTHrP*$^{-/-}$ mouse embryo (in which cell proliferation is greatly reduced). All Hh family members signal through two multipass transmembrane proteins, Patched 1 (Ptch1) and Smoothened (Smo). In the absence of Hh ligand, Ptch1 suppresses the activity of Smo. Upon Hh binding to Ptch1, the inhibitory effect on Smo is relieved and Smo transduces the Hh signal to downstream signaling components, which leads to the activation of Hh downstream target gene expression, including *Hip1* (*Hhip* – Mouse Genome Informatics), *Gli1 and Ptch1* (reviewed by Huangfu and Anderson, 2006; Lum and Beachy, 2004). Therefore, when *Smo* is removed, Hh signaling is inactivated, whereas removal of *Ptch1* leads to the activation of Hh signaling cell-autonomously. Here, we have investigated the *PTHrP*-independent function of Ihh signaling in chondrocyte hypertrophy by genetically altering the expression or function of *Ihh*, *Ptch1* and *Smo* in *PTHrP*$^{-/-}$ mouse embryos. Our results reveal a novel role of Ihh signaling in promoting chondrocyte hypertrophy independently of *PTHrP*, which is important in postnatal cartilage development, although it is masked by the dominant effect of *PTHrP*-dependent Ihh signaling in inhibiting chondrocyte hypertrophy during embryonic development. We also found that canonical Wnt and Bmp signaling may mediate the role of Ihh signaling in promoting chondrocyte hypertrophy.

MATERIALS AND METHODS
Skeletal analysis
Embryos were dissected in PBS, and then skinned, eviscerated and fixed in 95% ethanol. Skeletal preparations were performed as described previously (McLeod, 1980).

Histology, in situ hybridization and immunohistochemistry
Embryos were fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were embedded in paraffin and sectioned at 5 μm thickness. Histological analysis, immunohistochemistry and radioactive ^{35}S RNA in situ hybridization were performed as described (Yang et al., 2003). Primary antibodies (anti-phospho-Smad1 goat polyclonal IgG (Santa Cruz) at 1:100 and anti-Col2a1 goat polyclonal IgG (Santa Cruz) at 1:200 were used for immunohistochemistry, and signals were detected using the ABC kits (Vector Laboratories) and DAB (Sigma). Probes for in situ hybridization have been described previously: *Col10a1*, *Col2a1* and *Ihh* (Mak et al., 2006); the *Lef1* probe was ordered from ATCC.

Organ cultures of embryonic limb explants
Forelimbs of mouse embryos were dissected free of skin and muscles at E14.5 and cultured for 1.5 days in BGJ-B medium (Invitrogen) with Antibiotic/Antimycotic (Invitrogen) and 0.1% BSA in organ culture dishes under humidified conditions. Cultures were supplemented with 2.5 μg/ml recombinant murine Shh-N (R&D Systems) or 10 μM cyclopamine (BIOMOL). Limb explants were then fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin for sectioning.

Preparation of primary chondrocytes
Ventral parts of the rib cages of 0- to 3-day-old wild-type pups were eviscerated of skin and muscles and incubated with 2 mg/ml pronase (Roche) for 30 minutes at 37°C. The samples were then incubated with 3

Table 1. Primer sequences for Wnt ligands

Gene	Primer sequence
Wnt1	5′-GACTCGATGGAGCCTTCGGAGCAG-3′ 5′-CCGACAGAACCCGGGGGATCCTGCAC-3′
Wnt2	5′-GCCAGCATGTCCTCAGAGTACAGG-3′ 5′-GCCAAGGACAGCAAAGGCACCTTC-3′
Wnt2b	5′-GGGTGACGCGGGTGACCCGAGTTG-3′ 5′-CAGGATGGGGCCAATTTCACAGCAG-3′
Wnt3	5′-CATGCAGCTGGCAACAGTCCATGC-3′ 5′-GCCATTGCGTCTTCCACTGGTGC-3′
Wnt3a	5′-GAGGCACTGTCATACTTGTCCTTG-3′ 5′-GTAGTGAGGACATTGAATTTGGAG-3′
Wnt4	5′-CACTCCACCCGCATGTGTGTCAAG-3′ 5′-GTGGCTGTGACCGGACAGTGCACG-3′
Wnt5a	5′-GGAAGTCCGCCAGCTGCAGCCAGC-3′ 5′-GACTATGGCTACCGCTTCGCCAAG-3′
Wnt5b	5′-CTTGCGGAACTCGGCCAGCTGGAG-3′ 5′-CGTGGAGTACGGCTACCGCTTTGC-3′
Wnt6	5′-CGCGGAACGGAGGCAGCTTCTGCC-3′ 5′-GGCTGCGGAGACGATGTGGACTTC-3′
Wnt7a	5′-GTCCTTGAGCACGTAGCCTAGCTC-3′ 5′-GCTACGGCATCGGCTTCGCCAAGG-3′
Wnt8	5′-GTCAGCCAGCTGCAGCCAACACGTC-3′ 5′-CAGACTCTTCGTGGACAGTTTGGAG-3′
Wnt8b	5′-GCCTCATTGTTGTGCAGATTCATGGC-3′ 5′-GACAGCATTTGTGCACGCCATCAG-3′
Wnt10a	5′-CTTCTTCGTCCCCACGTCTGGAGG-3′ 5′-GCCAGCATCAGTTCCGGGACCAGC-3′
Wnt11	5′-CACGTCCTGGAGCTCTTGCAGCCC-3′ 5′-GATGTGCGGACAACCTCAGCTACG-3′
Wnt15	5′-GCTTCAGCACCTGGCCGGTCTCGC-3′ 5′-CTGAAGTACAGCACCAAGTTCCTC-3′
Wnt16	5′-GCCCAATCTTTTCAAAAGAAGACATA-3′ 5′-CCTTGCAGAATGGTGGCTCACCAA-3′

mg/ml collagenase D (Roche) in DMEM (Gibco) at 37°C for 1.5 hours until all soft tissues had detached from the cartilage. The cartilage was washed with PBS several times and separated from soft tissues by sedimentation. The cartilage was then digested with collagenase for 4 to 5 hours in a Petri dish. Undigested bony parts were discarded and chondrocytes were collected by centrifugation and washed twice with PBS. The primary chondrocytes were cultured in low-serum medium (DMEM, 0.1% FBS) for dual luciferase assays.

Dual luciferase assay
Primary chondrocytes were transfected with the *Topflash* reporter plasmid using the Human Chondrocyte Nucleofector Kit (Amaxa), following the manufacturer's protocol. After nucleofection, 0.5×10^6 cells were seeded in 12-well plate and left to recover for 4-5 hours. Primary chondrocytes were then serum starved overnight before adding 2.5 μg/ml recombinant Shh (R&D Systems) was culturing for 24 hours. Cells were harvested and subjected to luciferase activity measurement using the Dual Luciferase Reporter Assay Kit (Promega), according to the manufacturer's instructions.

Quantitative RT-PCR
Quantitative PCR was performed to measure the relative expression levels of the Wnt genes and the Wnt target genes *Axin2* and *Lef1* (see Table 1), using the Platinum SYBR Green Kit (Invitrogen). Samples were normalized to *Gapdh* expression.
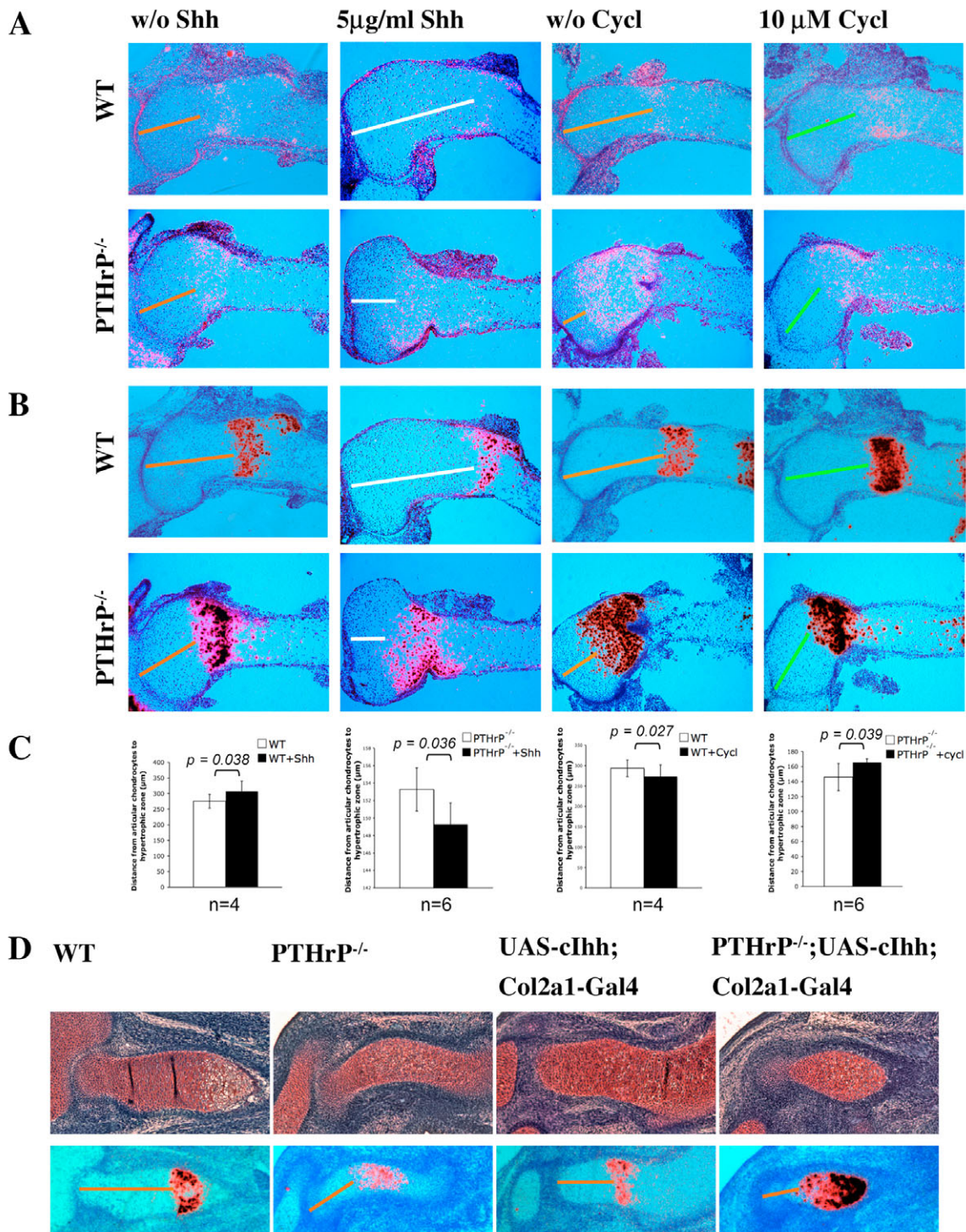
Fig. 1. Hedgehog signaling promotes chondrocyte hypertrophy in the absence of PTHrP signaling. (**A**,**B**) Forelimbs of *PTHrP*^{–/–} or wild-type E14.5 embryos cultured for 36 hours in the presence or absence of recombinant Shh protein or cyclopamine. Treated limbs were compared with untreated contralateral control ones. Serial sections of the proximal humerus of the cultured limbs were hybridized with ^{35}S riboprobes of *Ihh* (A) or *Col10a1* (B). The distances from the articular end to the *Ihh/Col10a1* domain in the untreated wild-type and *PTHrP*^{–/–} embryos are indicated by orange lines. This distance was increased in the Shh-treated wild-type humerus but reduced in the Shh-treated *PTHrP*^{–/–} humerus (white lines) compared with the untreated contralateral controls. This distance was reduced in the cyclopamine-treated wild type, but increased in the *PTHrP*^{–/–} humerus (green lines) compared with the untreated controlateral controls. (**C**) Statistical analysis (paired Student's *t*-test) of the distance from articular chondrocytes to the hypertrophic zone between treated and untreated contralateral limbs. Numbers (*n*) of analyzed limbs are indicated. (**D**) Serial sections of E14.5 distal humeri of the indicated genotypes stained with Safranin O (upper panel) and hybridized with a [35]S *Col10a1* riboprobe (lower panel) to detect chondrocyte hypertrophy. The distance from the articular end to the *Col10a1* expression domain (orange lines) in the *PTHrP*^{–/–} embryos was reduced compared with that in the wild-type embryos. This distance in the *PTHrP*^{–/–};UAS-cIhh;Col2a1-Gal4 double mutant was further reduced compared with that of the *PTHrP*^{–/–} embryos.

Tamoxifen preparation and injection

Tamoxifen (60 mg/ml; Sigma) was dissolved in corn oil (Sigma) and sonicated until the solution became clear. The solution was filtered and 0.075 ml was injected intraperitoneally into the mother on day 3 after the pups were born. Injection continued every other day until the pups were weaned.

RESULTS

Ihh signaling promotes chondrocyte hypertrophy in cultured limb explants in the absence of PTHrP signaling

To unravel the direct effect of Ihh signaling on chondrocyte hypertrophy independently of PTHrP signaling, limb explants of E14.5 *PTHrP*$^{-/-}$ and wild-type control mouse embryos were cultured in the presence of Shh or cyclopamine, a specific inhibitor of Hh signaling (Cooper et al., 1998). Chondrocyte hypertrophy was analyzed by histological staining and in situ hybridization with probes of *Ihh* and *Col10a1*, markers for prehypertrophic and hypertrophic chondrocytes, respectively. The distance from the articular end to the prehypertrophic or hypertrophic chondrocytes in the developing cartilage reflects how fast chondrocytes undergo hypertrophy. A shorter distance indicates faster hypertrophy. As

expected, treatment of wild-type limbs with Shh resulted in delayed chondrocyte hypertrophy, as shown by an increased distance between the articular end and the *Ihh/Col10a1* expression domains (Fig. 1A,B). In the cultured *PTHrP*$^{-/-}$ mouse limb, chondrocyte hypertrophy was accelerated when compared with the wild-type control (Fig. 1A,B). However, treatment of *PTHrP*$^{-/-}$ limb explants with Shh further accelerated chondrocyte hypertrophy, when compared with the untreated *PTHrP*$^{-/-}$ limb cultures (Fig. 1A,B). Furthermore, treatment of the *PTHrP*$^{-/-}$ limb explants with the Hh inhibitor cyclopamine significantly delayed chondrocyte hypertrophy and increased the domain of proliferating chondrocytes. These results indicate that Ihh signaling promotes chondrocyte hypertrophy in the absence of *PTHrP*.

Overexpression of *Ihh* in the cartilage in the absence of *PTHrP* accelerates chondrocyte differentiation in vivo

To rule out the possibility that the *PTHrP*$^{-/-}$ limb explants may respond to Hh signaling differently in vitro, we analyzed the *PTHrP*-independent role of Ihh signaling in vivo. We used a *UAS-Gal4* bigenic system to overexpress the chicken *Ihh* gene in the cartilage
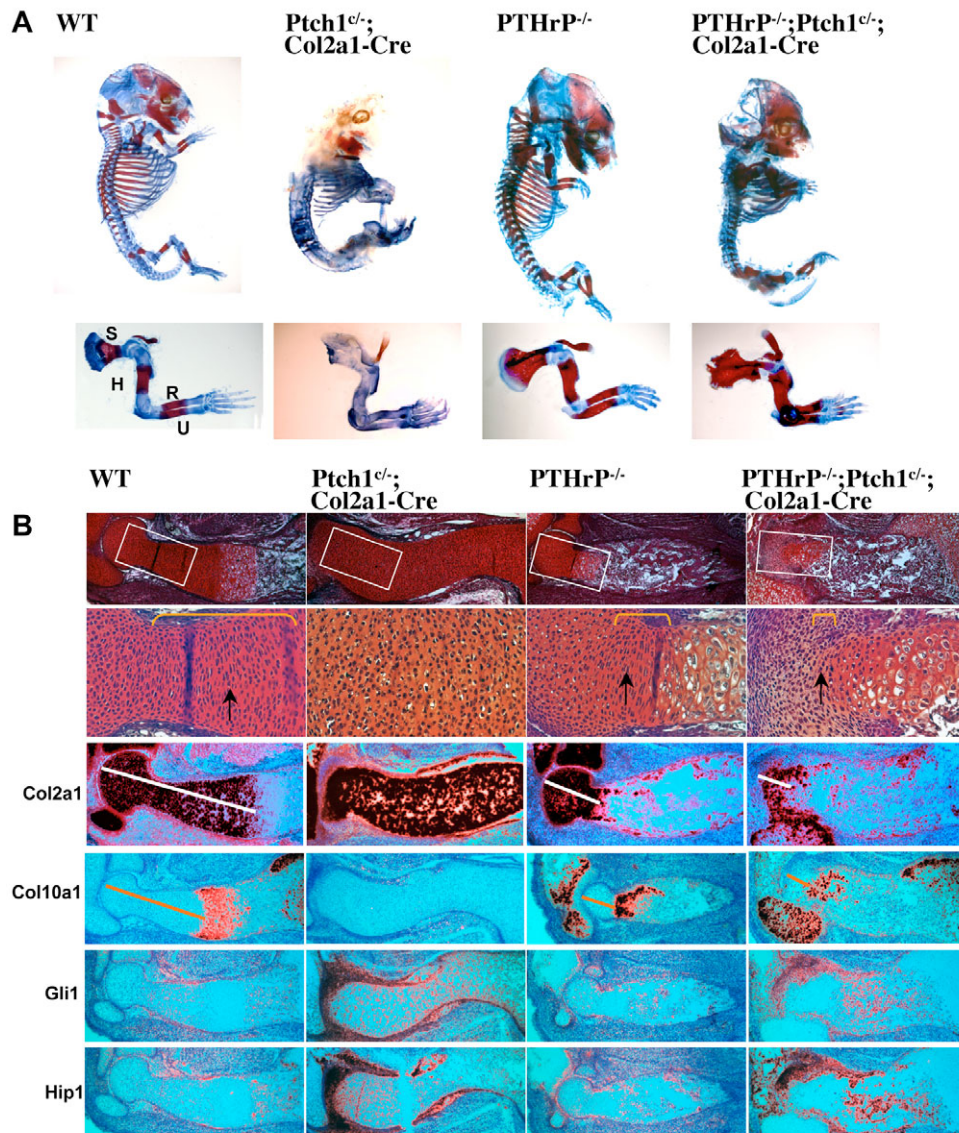

Fig. 2. Cell autonomous upregulation of Hh signaling in the absence of *PTHrP* accelerates chondrocyte hypertrophy. (**A**) Skeletal preparation of embryos at E14.5. Alizarin Red stains mineralized cartilage and bone tissues; Alcian Blue stains unmineralized cartilage. A higher magnification view of the forelimb is shown in the lower panel. S, scapula; H, humerus; R, radius; U, ulna. (**B**) Serial sections of distal humerus were stained with Safranin O and hybridized with indicated ^{35}S labeled riboprobes. The boxed articular and columnar chondrocytes regions are shown at higher magnification in the panels below. Columnar chondrocytes are indicated by yellow brackets and arrows. Both *Ptch1*$^{c/-}$;*Col2a1-Cre* and *PTHrP*$^{-/-}$;*Ptch1*$^{c/-}$;*Col2a1-Cre* mutants show strong upregulation of *Gli1* and *Hip1*, downstream target genes of Hh signaling, in the perichondrium and synovial joint. The *Col2a1*-expressing region (white line) is reduced and the *Col10a1*-expressing domain (yellow line) is closer to the joint in the *PTHrP*$^{-/-}$;*Ptch1*$^{c/-}$;*Col2a1-Cre* mutant.

(Yang et al., 2003) and then crossed it with *PTHrP*^–/– mice to generate a compound mutant *UAS-cIhh;Col2a1-Gal4;PTHrP*^–/–. At E14.5, *PTHrP*^–/– mutant embryos showed accelerated hypertrophy compared with wild-type embryos, as shown by the reduction of the distance from the *Col10a1* expression domain to the articular end (Fig. 1C). In the *UAS-cIhh;Col2a1-Gal4* mutant embryos, chondrocyte hypertrophy indicated by *Col10a1* expression was slightly accelerated compared with that of the wild type (Fig. 1C). Interestingly, in the *UAS-cIhh;Col2a1-Gal4;PTHrP*^–/– compound mutant embryos, the proliferating chondrocyte region before hypertrophy was significantly shorter in length even than that of the *PTHrP*^–/– mutant embryos (Fig. 1C). These results indicate that in the absence of PTHrP signaling, upregulation of Ihh signaling accelerates chondrocyte hypertrophy at an early stage of long bone development in vivo.

Next, we tested whether Ihh signals directly to the chondrocytes in a *PTHrP*-independent manner to regulate chondrocyte hypertrophy. We have previously generated a floxed allele of *Ptch1* in mice that allows cell-autonomous activation of Hh signaling upon Cre-mediated recombination (Mak et al., 2006). As shown before, we found that in the *Ptch1*^c/–;*Col2a1-Cre* embryos, Ihh signaling is upregulated, as indicated by the enhanced expression of the Hh signaling target genes *Gli1* and *Hip1* (Fig. 2B). In addition, chondrocyte hypertrophy was significantly delayed, as there was no *Col10a1* expression at E14.5 when it was already strongly expressed in wild-type and *PTHrP*^–/– mutant embryos (Fig. 2B). The delay of chondrocyte hypertrophy is due to the upregulation of *PTHrP* expression (Mak et al., 2006). Interestingly, when we crossed the *Ptch1*^c/–;*Col2a1-Cre* mutant to the *PTHrP*^–/– mutant, we found that ossification and mineralization, as indicated by Alizarin Red staining, were strongly enhanced in *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* double mutants when compared with both *Ptch1*^c/–;*Col2a1-Cre* and the *PTHrP*^–/– single mutant alone (Fig. 2A). When sections of the humerus at E14.5 were examined, we found that chondrocyte hypertrophy was accelerated more than that of the *PTHrP*^–/– embryos. There were a few rows of columnar chondrocytes in the *PTHrP*^–/– embryo, but in the *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* double mutant loss of columnar chondrocytes was so fast that there were few columnar chondrocytes left, and the size of the articular cartilage region was also reduced owing to accelerated articular chondrocyte differentiation (Fig. 2B). In addition, the *Col10a1* expression domain in the *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* double mutant was closer to the articular end than that in the *PTHrP*^–/– embryo (Fig. 2B). Furthermore, we performed a BrdU incorporation

assay, which shows the percentage of cells in the S phase of mitotic division and allows the comparison of cell proliferation. In the *PTHrP*^–/– embryo, there were still a few BrdU-positive columnar chondrocytes (Fig. 3A). Consistent with the accelerated loss of the fast proliferating columnar chondrocytes in the *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* double mutant, there were hardly any BrdU-positive cells outside of the periarticular region (Fig. 3A). Cell proliferation in the columnar region was similarly reduced in the *PTHrP*^–/– and *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* double mutant (Fig. 3B), supporting the previous notion that PTHrP signaling provides the competence of chondrocytes to respond to Ihh signaling to promote proliferation (Karp et al., 2000). These results demonstrate that upregulation of Ihh signaling in chondrocytes promotes chondrocyte hypertrophy independently of *PTHrP*.

Inhibition of Hh signaling delays chondrocyte hypertrophy independently of *PTHrP*

Our finding that upregulated Hh signaling promotes chondrocyte hypertrophy in the absence of *PTHrP* suggests that inhibition of Ihh signaling would result in delayed chondrocyte hypertrophy in the absence of *PTHrP*. To test this, we inactivated Hh signaling by removing *Smo* in chondrocytes of *PTHrP*^–/– mouse embryos and examined the pace of chondrocyte hypertrophy. Skeletal preparations of E15.5 embryos showed that the general morphology of the *PTHrP*^–/–;*Smo*^c/c;*Col2a1-Cre* double mutant was similar to that of *Smo*^c/c;*Col2a1-Cre* single mutant, but that mineralization in the double mutant was decreased compared with that in the wild-type control and the *PTHrP*^–/– single mutant (Fig. 4A). More detailed analysis of the E15.5 tibia sections revealed that almost all chondrocytes in the double mutant embryo still expressed *Col2a1*, a marker for non-hypertrophic chondrocytes, whereas a significant portion of chondrocytes in the *PTHrP*^–/– single mutant embryos had lost *Col2a1* expression and undergone hypertrophy (Fig. 4B). In addition, more chondrocytes in the wild-type embryo had lost *Col2a1* expression than had those in the *Smo*^c/c;*Col2a1-Cre* single mutant (Fig. 4B). Consistent with the expression of *Col2a1*, the expression domains of *Col10a1* and the regions between the two *Ihh* expression domains were smaller in *Smo*^c/c;*Col2a1-Cre* and *PTHrP*^–/–;*Smo*^c/c;*Col2a1-Cre* mutant embryos than in wild-type and *PTHrP*^–/– embryos, respectively (Fig. 4B). These data indicate that the progression of chondrocyte hypertrophy is slower when *Smo* is removed, especially in the absence of *PTHrP* at early stages of cartilage development. Therefore, in contrast to the role of *PTHrP* that is activated and maintained by Ihh signaling, Ihh signaling is
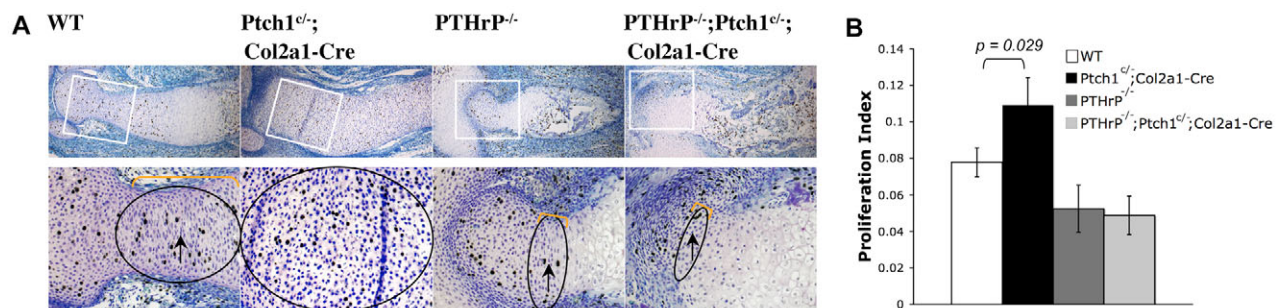

Fig. 3. Cell proliferation is similarly reduced in *PTHrP*^–/– and *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* mutant cartilage. (**A**) Comparison of BrdU-labeled chondrocytes in the cartilage of different mutants and in the wild-type control. Boxed regions are shown at higher magnification in the lower panel. The highly proliferating columnar chondrocytes (bracket) were reduced in the *PTHrP*^–/–;*Ptch1*^c/–;*Col2a1-Cre* mutant. (**B**) Percentage of BrdU-labeled chondrocytes in the columnar regions (circled in A), calculated from three independent samples to get the mean±s.d. Student's *t*-test, *P*<0.05.
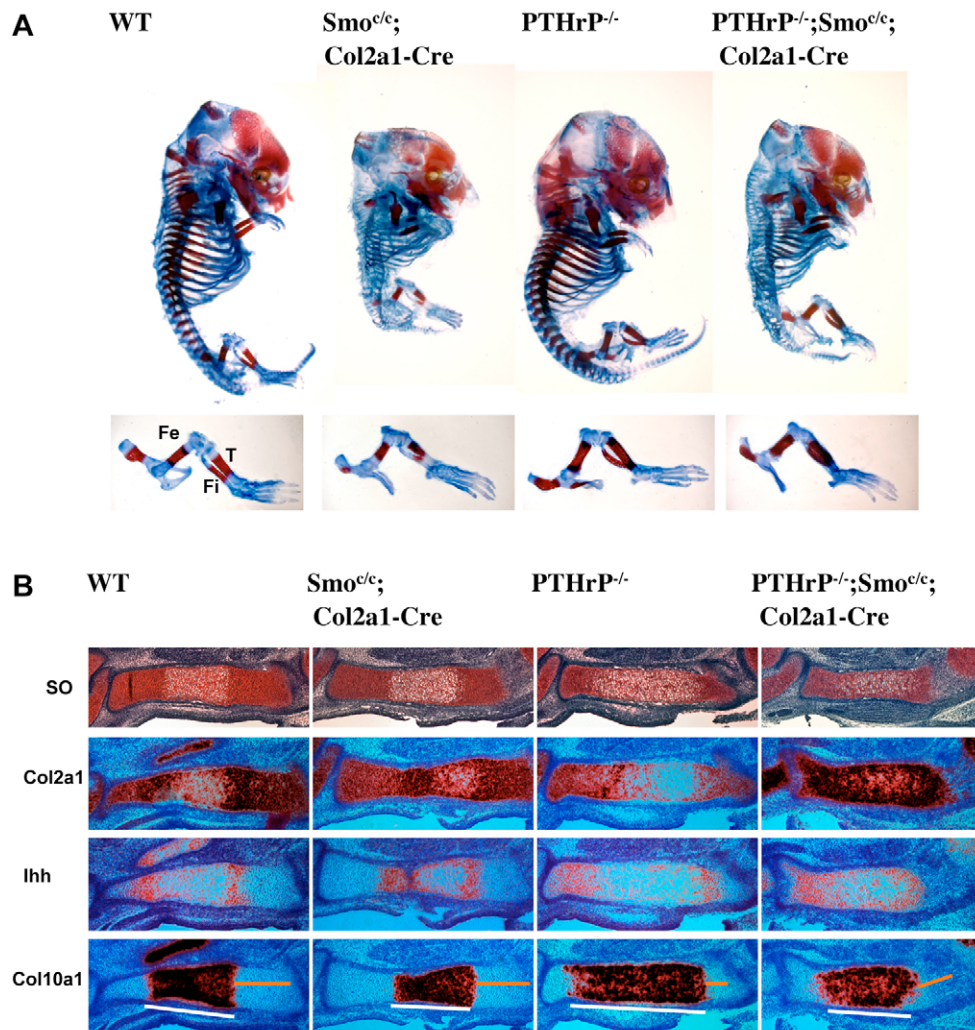
Fig. 4. Removal of Hh signaling delays chondrocyte hypertrophy in the absence of *PTHrP*.
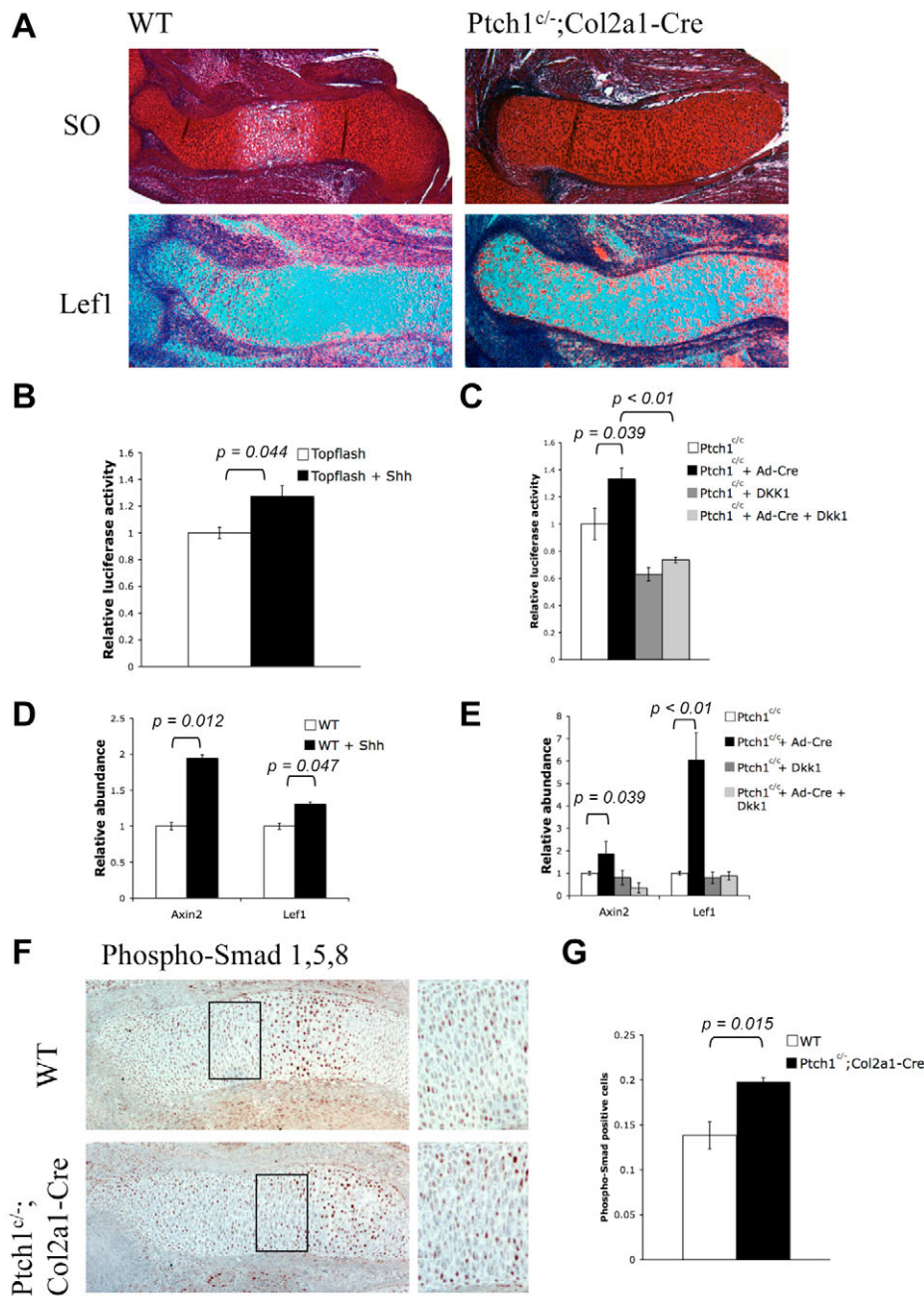(**A**) Skeletal preparation of E15.5 embryos. Hindlimbs are shown at higher magnifications in the lower panel. (**B**) Serial sections of tibia were stained with Safranin O and hybridized with ^{35}S labeled *Ihh and Col10a1* riboprobes. *Smo^{c/c};Col2a1-Cre* mutant tibia showed a slight delay in chondrocyte hypertrophy compared with that of wild-type embryos. *PTHrP^{–/–};Smo^{c/c};Col2a1-Cre* mutant tibia also showed a delay of chondrocyte hypertrophy, as compared with that of the *PTHrP^{–/–}* mutant. The proliferating chondrocyte region is indicated by the yellow line; the hypertrophic region is indicated by the white line. Fe, femur; T, tibia; Fi, fibula.

required to promote chondrocyte hypertrophy in the absence of *PTHrP*. This agrees with, and explains, the observation that in the *Ihh^{–/–}* embryo there is an initial delay of chondrocyte hypertrophy at E14.5 (St-Jacques et al., 1999).

Hh signaling activates the canonical Wnt and Bmp signaling in chondrocytes

As both Wnt/β-catenin signaling and the *PTHrP*-independent role of Ihh signaling promote chondrocyte hypertrophy, one likely scenario is that Ihh signaling activates the canonical Wnt signaling in chondrocytes. We have previously shown that delayed chondrocyte hypertrophy caused by upregulated *PTHrP* expression due to activated Ihh signaling can be further enhanced by the loss of Wnt/β-catenin signaling (Mak et al., 2006), raising the possibility that the loss of Wnt/β-catenin signaling actually diminished the role of Ihh signaling in promoting chondrocyte hypertrophy. To address this, we performed in situ hybridization with *Lef1*, which is a transcriptional target of Wnt/β-catenin signaling (Hovanes et al., 2001), on E14.5 limb sections of *Ptch1^{c/–};Col2a1-Cre* mutants (Fig. 5A). *Lef1* is mainly expressed in proliferating chondrocytes (Fig. 5). In the *Ptch1^{c/–};Col2a1-Cre* mutants, *Lef1* expression was highly upregulated in all chondrocytes, suggesting that Ihh signaling activates Wnt/β-catenin signaling in proliferating chondrocytes. To further confirm the effect of Hh signaling on Wnt/β-catenin signaling, we isolated

primary chondrocytes from rib cages of postnatal day one (P1) wild-type mice. The Wnt/β-catenin signaling activity in the cultured primary chondrocytes was measured by the TOPFLASH luciferase activity (Korinek et al., 1997), which is a reporter containing luciferase driven by Lef/Tcf binding sites and a basic promoter. TOPFLASH luciferase expression is activated by β-catenin when it binds Lef/Tcf factors. Consistent with the in vivo observation, activation of the TOPFLASH reporter was detected in the Cre-adenovirus-infected *Ptch1^{c/c}* primary chondrocytes and in the wild-type primary chondrocytes treated with Shh, when compared with control cells (Fig. 5B,C), indicating that Wnt/β-catenin signaling is activated by Hh signaling. In addition, by quantitative RT-PCR, we found that the expression of two canonical Wnt target genes, *Axin2* and *Lef1*, was significantly increased in the Shh-treated or *Ptch1*-deficient chondrocytes when compared with the control cells (Fig. 5D,E). By contrast, the upregulation of canonical Wnt signaling activity in response to Hh signaling was abolished when the primarily chondrocytes were treated with Dkk1, a secreted antagonist of the canonical Wnt signaling (Fig. 5C,E). Taken together, our results suggest that upregulated Ihh signaling activates the expression of some Wnt ligands that signal through the canonical Wnt pathway in chondrocytes. Indeed, we found that the expression of several Wnt genes, including *Wnt2*, *Wnt3a*, *Wnt8* and *Wnt8b*, that can signal through the canonical pathway was upregulated by activated Hh signaling (data not shown).

Fig. 5. Hedgehog signaling activates downstream targets of canonical Wnt signaling both in vivo and in vitro. (**A**) Serial sections of E14.5 distal humerus were stained by Safranin O and hybridized with a ^{35}S labeled *Lef1* probe. *Lef1* expression was strongly upregulated in the cartilage of *Ptch1$^{c/-}$; Col2a1-Cre* mutants. (**B,C**) Dual luciferase assay of primary chondrocytes isolated from wild-type newborn pups. Primary chondrocytes were nucleofected with *Topflash* reporter vectors as a read out for canonical Wnt signaling. Recombinant Shh or Dkk1 protein was added after serum starvation and luciferase activity was measured 24 hours later. Shh treatment or Cre-adenovirus infection of the *Ptch1$^{c/c}$* primary chondrocytes activated TOPFLASH activity. Such activation was diminished by Dkk1. (**D,E**) Quantitative RT-PCR was performed using RNA isolated from primary chondrocytes. Both *Axin2* and *Lef1* were significantly increased in Shh-treated primary chondrocytes or Cre-adenovirus-infected *Ptch1$^{c/c}$* primary chondrocytes compared with untreated samples. Dkk1 treatment blocked the effect of Hh signaling. (**F**) Immunohistochemistry of E15.5 limb sections (distal ulna) with antibodies against phospho-Smad1, 5 and 8. More phospho-Smad-positive cells were found in the cartilage of the *Ptch1$^{c/-}$;Col2a1-Cre* mutant embryos. Boxed region of columnar/prehypertrophic chondrocytes is shown at a higher magnification on the right-hand side. (**G**) Statistical analysis of phospho-Smad-positive cells in the boxed region of the cartilage. Three samples in the boxed area were counted, and the mean±s.d. are shown. Student's *t*-test, *P<0.05*.

Furthermore, consistent with the previously observed upregulation of Bmp expression in the developing cartilage of *Ptch1$^{c/-}$;Col2a1-Cre* mutant embryos (Mak et al., 2006), we found that Bmp signaling activity was upregulated in *Ptch1$^{c/-}$;Col2a1-Cre* mutant embryos, as indicated by the increased phosphorylation of Smad1 in the chondrocytes (Fig. 5F,G).

Hh signaling regulates chondrocyte hypertrophy in postnatal cartilage

Our results showed that Ihh signaling promotes chondrocyte hypertrophy in the absence of *PTHrP* in embryonic skeletal development. This function of Ihh signaling may be dominant over the *PTHrP*-dependent role of Ihh in inhibiting chondrocyte hypertrophy in postnatal cartilage, as *PTHrP* expression is weaker in adult cartilage (Tsukazaki et al., 1995). To test this, we activated Hh signaling in postnatal cartilage using the floxed *Ptch1* allele

and an inducible chondrocyte-specific *Cre* mouse line, *Col2a1-CreER* (Maeda et al., 2007; Nakamura et al., 2006). Cre recombinase activity was induced in this mouse line by tamoxifen (TM). Skeletal preparations of P15 *Ptch1$^{c/c}$;Col2a1-CreER* mice showed a significant acceleration of mineralization, as indicated by increased Alizarin Red staining in both forelimbs and hindlimbs. Conversely, the cartilage region stained by Alcian Blue at the joint was significantly reduced (Fig. 6A). Histological sections of the distal femur showed that chondrocyte hypertrophy around the secondary ossification center in *Ptch1$^{c/c}$;Col2a1-CreER* mice was greatly enhanced when compared with the wild-type control (Fig. 6B, parts a,b). In addition, immunohistochemistry with Col2a1 antibodies confirmed that the zone of hypertrophic chondrocytes was larger with lower Col2a1 expression in *Ptch1$^{c/c}$;Col2a1-CreER* mice (Fig. 6B, parts c,d). Furthermore, in TM-induced one-year-old *Ptch1$^{c/c}$;Col2a1-CreER*

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mice, the number of chondrocytes lining the joint surface and the proteoglycan content in articular chondrocytes, as indicated by Hematoxylin/Eosin or Safranin O staining, were reduced (Fig. 6B, parts e-j). There was no obvious change in the growth plates in TM-induced *Ptch1^{c/c};Col2a1-CreER* mice (see Fig. S2 in the supplementary material). Conversely, in 14-month-old *Smo^{c/c};Col2a1-CreER* mice, chondrocytes lining the joint surface and the proteoglycan content in articular chondrocytes were increased (Fig. 6C). These data indicate that Hh signaling in postnatal cartilage promotes chondrocyte hypertrophy in the area of secondary ossification. These results further suggest that activated Hh signaling might be a risk factor for osteoarthritis.

DISCUSSION

Here, we report that apart from controlling chondrocyte hypertrophy indirectly through regulating *PTHrP* expression, Ihh signaling also directly regulates chondrocyte hypertrophy in the absence of *PTHrP*. The direct and indirect roles of Ihh signaling are opposite to each other. Whereas Ihh-regulated PTHrP signaling inhibits chondrocyte hypertrophy and is dominant in embryonic skeletal development, we found that one *PTHrP*-independent role of Ihh signaling is to promote chondrocyte hypertrophy, possibly through activating Wnt/β-catenin and Bmp signaling. This function is important in postnatal cartilage development and homeostasis.

The negative-feedback loop of Ihh-PTHrP signaling plays a crucial role in controlling the pace of chondrocyte hypertrophy (Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). However, Ihh signaling has also been found to promote chondrocyte proliferation and the transition from slowly proliferating periarticular chondrocytes to fast proliferating columnar chondrocytes (periarticular chondrocyte differentiation) independently of PTHrP signaling (Kobayashi et al., 2005b). Our data uncovered another *PTHrP*-independent activity of Ihh in promoting chondrocyte hypertrophy that was masked by the more potent *PTHrP*-dependent activity of Ihh, which is to inhibit chondrocyte hypertrophy in embryonic development (Fig. 7). These results highlight the highly cell context-dependent effects of Ihh
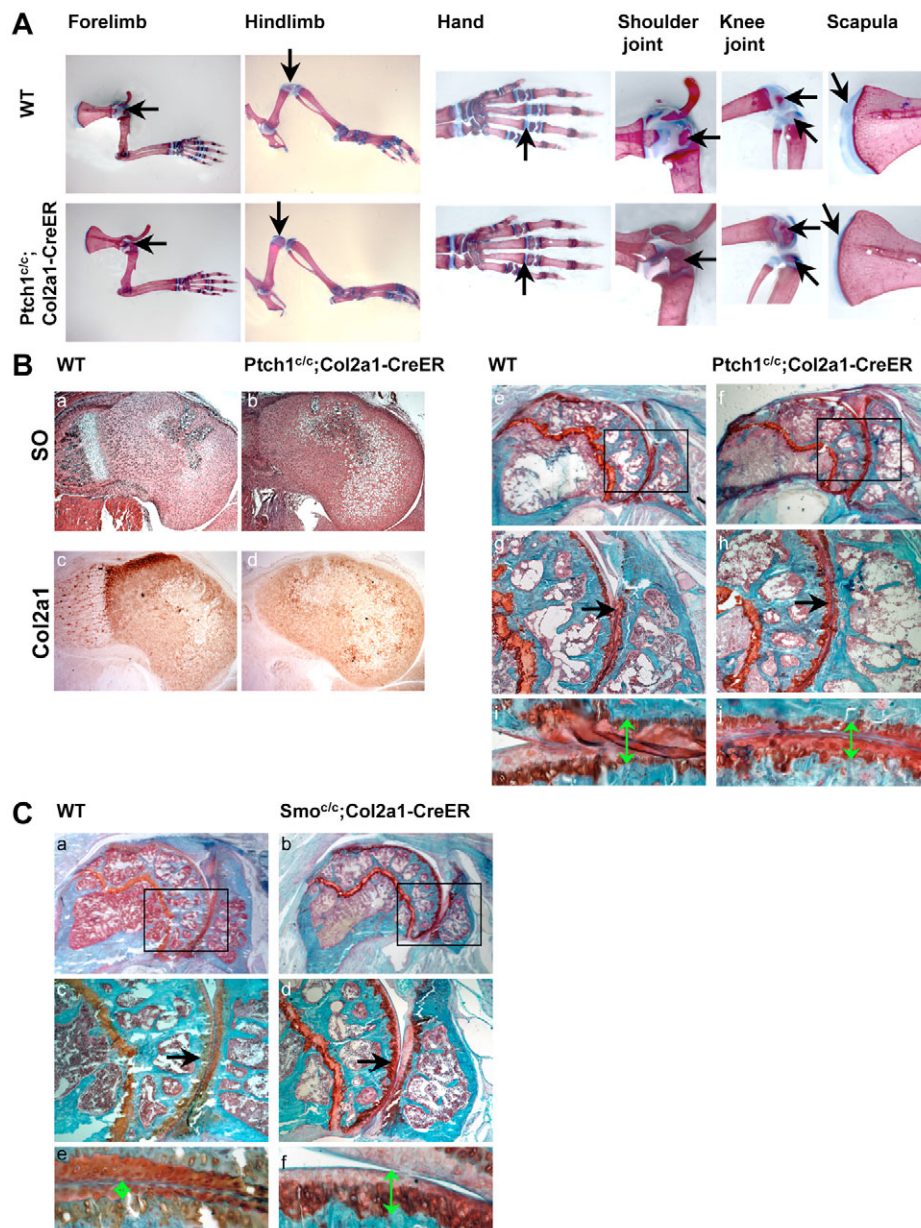

Fig. 6. Hedgehog signaling accelerates chondrocyte hypertrophy in postnatal cartilage. (**A**) Skeletal preparations of P15 forelimb and hindlimb of the *Ptch1^{c/c};Col2a1-CreER* and wild-type mouse. Unmineralized cartilage in the hand, shoulder and knee joints and the scapula was reduced in the mutant, as shown at high magnification. (**Ba-d**) Serial sections of distal femur of P12 mice were stained with Safranin O and used for immunohistochemistry to detect Col2a1 expression. There were more hypertrophic chondrocytes with reduced Col2a1 expression in the *Ptch1^{c/c};Col2a1-CreER* mouse. (**Be-j**) Sections of proximal humeri from one-year-old mice were stained with Safranin O. Boxed regions in e and f are shown at higher magnifications in g,h. Cartilage lining was thinner in the *Ptch1^{c/c};Col2a1-CreER* mouse (arrow). (i,j) Higher magnification of articular cartilage of the proximal humerus. Joint cartilage in the *Ptch1^{c/c};Col2a1-CreER* mouse was significantly reduced (double-headed arrows). (**Ca-d**) Sections of proximal humeri from 14-month-old mice were stained with Safranin O. Boxed regions in a and b are shown at higher magnifications in c,d. Cartilage lining was thicker in the *Smo^{c/c};Col2a1-CreER* mouse (arrow). (**Ce,f**) Higher magnification of articular cartilage of the proximal humerus. Joint cartilage in the *Smo^{c/c};Col2a1-CreER* mouse was significantly thicker (double-headed arrows).

signaling. Ihh signaling promotes the proliferation of periarticular and columnar chondrocytes, whereas, in more mature prehypertrophic chondrocytes, it may promote hypertrophy by promoting cell cycle exit.

During longitudinal development of the long bone cartilage, periarticular chondrocyte differentiation, which adds cells to the columnar region, is followed by chondrocyte hypertrophy, which reduces cells in the columnar region. Therefore, the length of the columnar chondrocyte region is determined by three parameters: the pace of periarticular chondrocyte differentiation, the pace of chondrocyte hypertrophy and the rate of columnar chondrocyte proliferation (Fig. 7). As upregulated Ihh signaling promotes periarticular chondrocyte differentiation and increases the rate of columnar chondrocyte proliferation (Kobayashi et al., 2005b), the proliferating columnar chondrocyte region would be increased if chondrocyte hypertrophy were not altered. Our observation that the columnar chondrocyte region was shorter in the *PTHrP$^{-/-}$;Ptch1$^{c/-}$; Col2a1-Cre* double mutant than in the *PTHrP$^{-/-}$* single mutant (Fig. 2B, Fig. 3A) demonstrates that Hh signaling also acts to promote chondrocyte hypertrophy in the absence of *PTHrP* to reduce columnar chondrocyte number. When *Ihh* was overexpressed while PTHrP signaling was maintained at a constant level in the *caPPR;PTHrP$^{-/-}$;Ihh-Bg* mutant, the periarticular chondrocyte region was smaller but the length of the columnar region was expanded significantly (Kobayashi et al., 2005b). In this case, upregulated Ihh signaling promotes periarticular chondrocyte differentiation and chondrocyte proliferation, and both activities increase the number of proliferating columnar chondrocytes. Although chondrocyte hypertrophy is also accelerated in this case, it is not enough to cancel out the effects of enhanced periarticular chondrocyte differentiation and chondrocyte proliferation. Furthermore, it has been shown that PTHrP signaling is required to provide a competent domain in which Ihh signaling can promote chondrocyte proliferation (Karp et al., 2000). Therefore, the difference between the *PTHrP$^{-/-}$;Ptch1$^{c/-}$;Col2a1-Cre* double mutant and the *caPPR;PTHrP$^{-/-}$;Ihh-Bg* mutant is that chondrocyte proliferation is greatly reduced in the *PTHrP$^{-/-}$;Ptch1$^{c/-}$;Col2a1-Cre* double mutant. Hence, the significantly increased columnar chondrocyte region in the *caPPR;PTHrP$^{-/-}$;Ihh-Bg* mutant is a result of increased chondrocyte proliferation. These results also suggest that the *PTHrP*-independent role of Ihh signaling in promoting chondrocyte hypertrophy may be stronger than that in promoting periarticular differentiation in the embryonic cartilage. Overall, the *PTHrP*-independent function of Ihh signaling is to increase the transition rate through the different chondrocyte zones during endochondral bone formation.

One crucial factor that determines the relative strength of the opposite activities of the *PTHrP*-dependent and -independent roles of Ihh signaling in chondrocyte hypertrophy is the level of *PTHrP* expression. *PTHrP* expression in the cartilage was progressively weaker in older embryos (K.K.M. and Y.Y., unpublished) and is further reduced in adult animals (Tsukazaki et al., 1995). Therefore, when PTHrP signaling is robust in the embryonic cartilage, upregulated Ihh signaling predominantly delays chondrocyte hypertrophy by upregulating *PTHrP* expression. However, when mammals approach adulthood, cartilage hypertrophy and bone formation accelerate coordinately as longitudinal bone growth declines and the growth plate is either reduced (i.e. in mouse) or closed (i.e. in human). This is consistent with reduced PTHrP levels postnatally. It is also conceivable that when PTHrP signaling is low enough, the *PTHrP*-independent role of Hh signaling in promoting chondrocyte hypertrophy is significant for postnatal cartilage
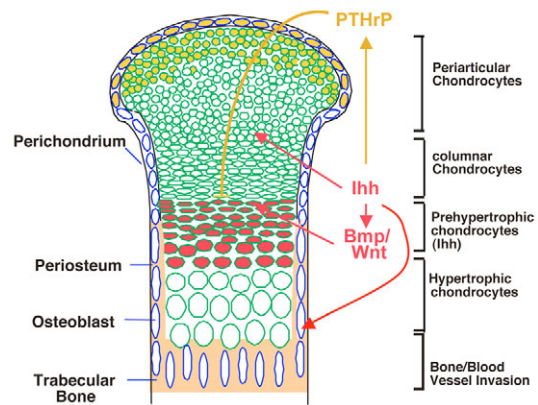

Fig. 7. Model of the *PTHrP*-dependent and *PTHrP*-independent function of Ihh signaling in chondrocyte differentiation. The morphology of a developing long bone is schematically shown. Chondrocytes proliferate and differentiate sequentially along the longitudinal axis. The length of the columnar chondrocyte region is determined by the rate of periarticular chondrocyte differentiation, columnar chondrocyte proliferation and hypertrophy. *Ihh* expression is shown in red. *PTHrP* expression is shown in yellow. *Ihh* acts through *PTHrP* to inhibit chondrocyte hypertrophy. *Ihh* acts independently of *PTHrP* to control chondrocyte proliferation, periarticular chondrocyte differentiation and osteoblast differentiation. Here, we show that *Ihh* also promotes chondrocyte hypertrophy independently of *PTHrP* and that this function is likely to be mediated by Bmp and Wnt/β-catenin signaling.

development and homeostasis. Furthermore, because only periarticular cells and the upper layer of articular chondrocytes are competent to express *PTHrP*, even when Hh signaling is upregulated throughout the cartilage (Mak et al., 2006), somatic mutations that upregulate Hh signaling only in chondrocytes deep in the joint cartilage and growth plates will not upregulate *PTHrP* expression. Hence, when the ability to strongly express *PTHrP* is lost, upregulated Ihh signaling, for instance due to mutations in *Ptch1* (as we have shown in the *Ptch1$^{c/c}$;Col2a1-CreER* mutant), may be a risk factor for osteoarthritis.

Our finding that Hh signaling activated Wnt/β-catenin and Bmp signaling in chondrocytes has provided some insight into the underlying mechanism of Hh signaling in promoting chondrocyte hypertrophy. Wnt/β-catenin signaling has been shown to promote chondrocyte hypertrophy, at least in part, through downregulating Sox9 protein levels (Akiyama et al., 2004), a transcription factor that is required for chondrocyte formation and also inhibits chondrocyte hypertrophy (Bi et al., 2001). However, Wnt/β-catenin signaling may not be the only downstream target of Hh signaling in regulating chondrocyte hypertrophy. Indeed, upregulated Hh signaling leads to many changes. For example, we have found that the expression and signaling activity of Bmps are upregulated by enhanced Hh signaling (Mak et al., 2006) (this study), and Bmp signaling activity has been shown to promote chondrocyte hypertrophy independently of PTHrP signaling (Kobayashi et al., 2005a). It is noted that, when Hh signaling is reduced by removing Ihh in postnatal cartilage from P1, chondrocyte hypertophy is also accelerated, although *PTHrP* expression is still detected (Maeda et al., 2007). These results suggest that Hh signaling still inhibits chondrocyte hypertrophy in postnatal cartilage, and this difference can be reconciled by the following considerations. First, Hh signaling was activated cell autonomously in the *Ptch1$^{c/c}$;Col2a1-CreER* mutant cartilage by injecting the

lactating female mouse at P3. Therefore, Hh signaling activation in our system occurs later and *PTHrP* expression might be further declined at that time. Second, the effects of Ihh signaling in chondrocyte hypertrophy can be mediated by PTHrP, Bmp and Wnt signaling simultaneously. As PTHrP and Bmp and Wnt signaling exhibit opposite effects in regulating chondrocyte hypertrophy, when Hh signaling is perturbed (up- or downregulated) in the adult cartilage, the outcome of chondrocyte hypertrophy depends on the relative strength of the pro- and anti-hypertrophy signaling. It is likely that in *Ptch1^{c/c};Col2a1-CreER* mice when Hh signaling is upregulated after P3, the pro-hypertrophy signaling (i.e. by Bmps and Wnts) is more robust than is the anti-hypertrophy signaling by PTHrP.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/11/1947/DC1

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