Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation

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SUMMARY

Indian hedgehog (Ihh), one of the three mammalian hedgehog (Hh) proteins, coordinates proliferation and differentiation of chondrocytes during endochondral bone development. Smoothened (Smo) is a transmembrane protein that transduces all Hh signals. In order to discern the direct versus indirect roles of Ihh in cartilage development, we have used the Cre-loxP approach to remove Smo activity specifically in chondrocytes. Animals generated by this means develop shorter long bones when compared to wild-type littermates. In contrast to Ihh mutants (Ihh+/Ihh−), chondrocyte differentiation proceeds normally. However, like Ihh+/Ihh− mice, proliferation of chondrocytes is reduced by about 50%, supporting a direct role for Ihh in the regulation of chondrocyte proliferation. Moreover, by overexpressing either Ihh or a constitutively active Smo allele (Smo*) specifically in the cartilage using the bicaudal USAGal system, we demonstrate that activation of the Ihh signaling pathway is sufficient to promote chondrocyte proliferation. Finally, expression of cyclin D1 is markedly downregulated when either Ihh or Smo activity is removed from chondrocytes, indicating that Ihh regulates chondrocyte proliferation at least in part by modulating the transcription of cyclin D1. Taken together, the present study establishes Ihh as a key mitogen in the endochondral skeleton.

Key words: Ihh, Smo, Chondrocyte proliferation, Cyclin D1, Mouse

INTRODUCTION

Much of the vertebrate skeleton arises from a cartilage template, which subsequently undergoes endochondral bone formation (Erlebacher et al., 1995; Hinchcliffe and Johnson, 1990). At the earliest stage of this process, mesenchymal cells condense to form a cartilage anlage. Initially, all chondrocytes within the anlage proliferate. Thereafter, cells in the center of this structure exit the cell cycle, undergo hypertrophy, which is a hallmark of terminal differentiation, and eventually die. Concomitantly, a bone collar is formed from the perichondrium surrounding the cartilaginous core. After apoptosis of the hypertrophic chondrocytes, blood vessels invade the cartilage and form the bone marrow cavity; blood vessels are also thought to bring in osteoblasts that produce the endochondral bone. Upon formation of the bone marrow cavity, immature chondrocytes are restricted to the ends of the cartilage that form the growth region, where cells undergo an orderly progression from proliferation to differentiation, which culminates in hypertrophy and to the eventual replacement by bone (Poole, 1991). Thus, the precise coordination between chondrocyte proliferation and differentiation is essential for the proper formation of an endochondral bone.

Recent studies have identified several molecules regulating the proliferation and differentiation of chondrocytes. Indian hedgehog (Ihh), one of the three mammalian homologs of the Drosophila Hedgehog (Hh), regulates multiple aspects of endochondral bone formation. Ihh-null mutants (Ihh+/Ihh−) exhibit a severe reduction in skeletal growth, resulting in long bones that are only one-fifth to one-third (varying among different bones) of the normal length at birth (St-Jacques et al., 1999). Chondrocyte differentiation in these mice is profoundly disturbed, as indicated by an initial delay in hypertrophy followed by the rapid apositional hypertrophy throughout the bulk of the cartilage (St-Jacques et al., 1999). This regulation of differentiation by Ihh appears to be mediated indirectly through the regulation of parathyroid hormone related peptide (PTHrP; Pthlh – Mouse Genome Informatics), expressed within the periarticular region (Karp et al., 2000; Lanske et al., 1996; Vortkamp et al., 1996). Furthermore, Ihh+/Ihh− embryos display a 50% reduction in chondrocyte proliferation (St-Jacques et al., 1999), although the precise mechanism for the proliferative role of Ihh is currently unknown. Thus, Ihh signaling controls growth of the skeleton by coordinating chondrocyte proliferation and differentiation.

The bone morphogenetic protein (BMP) family of proteins may interact with Ihh signaling to regulate cartilage
Development. For example, *Bmp2* and *Bmp4* are expressed in the perichondrium of developing cartilage (Jones et al., 1991; Lyons et al., 1989). Experiments in the chick limb indicated that expression of both molecules can be upregulated by misexpression of *Ihh* (Pathi et al., 1999). Furthermore, the signal-transducing receptors BMPR-1A and BMPR-1B are expressed in chondrocytes (Zou et al., 1997). These data suggest a possible signal relay mechanism by which Ihh from chondrocytes regulates expression of BMPs in the perichondrium; these BMPs, in turn, act on chondrocytes. Thus, in order to determine whether direct Ihh input is required in chondrocytes, it is necessary to manipulate Ihh signaling in a cell-autonomous fashion exclusively in chondrocytes.

All Hh signals are transduced through Smoothened (Smo; Smo−/− Mouse Genome Informatics), a putative G-protein-coupled multi-pass membrane protein (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Zhang et al., 2001). In the absence of Hh proteins, Smo is repressed by Patched1 (Ptc1; Ptc1−/− Mouse Genome Informatics), the cell surface receptor for Hh (Chen and Struhl, 1996; Marigo et al., 1996; Quirk et al., 1997; Stone et al., 1996). Binding of Hh proteins to Ptc1 relieves the repression of Smo, activating the signal transduction pathway that ultimately results in transcriptional activation of target genes (Aza-Blanc and Kornberg, 1999; McMahon, 2000). Excessive Hh signaling has been linked to tumorigenesis (Ruiz i Altaba, 1999). In particular, a mutation in Smo−/− mice is expressed in basal cell carcinomas (Xie et al., 1998).

In order to dissect different roles of Ihh signaling in chondrocyte proliferation and differentiation, we have used genetic approaches to manipulate Smo and Ihh activity in the cartilage. Our results indicate that chondrocyte proliferation requires a direct Ihh input, and that this regulation is at least in part through the transcriptional control of cyclin D1.

**MATERIALS AND METHODS**

**Generation of DNA constructs**

To generate a *Smo* conditional allele, a targeting vector was engineered in which a 400 bp fragment from 44 bp upstream to 358 bp downstream of the *ATG* initiation codon was cloned into the vector pPGKneo/DTA FLOXED FLIP (Zhang et al., 2001). A 4.5 kb *Bln*I-*Sac*I fragment containing non-coding sequence upstream of the *ATG* initiation codon and a 2.3 kb *Sal*I-*Eco*I fragment containing part of intron 1 were inserted into the above vector as the 5′ and 3′ homology regions, respectively. In the final construct, a pair of *loxP* sites flanks the region covering both the first exon of *Smo* and the PGK-neo cassette.

In order to generate the *Col2-Gal4* and *Col2-Cre* constructs, the original *Col2* expression vector was modified (Horton et al., 1987). The upstream *AfII*I site was replaced with a *Not*I site and a polylinker of *BamHI*-KpnI-*EcoRV*-XbaI sites was inserted at the original *BamHI* site. A polyA signal was also inserted at the *XbaI* site. For *Col2-Gal4*, the *Gal4* cDNA (Brand and Perrimon, 1993) was cloned into the *BamHI* and *KpnI* sites. For *Col2-Cre*, an *EcoRI/-Sal*I fragment encoding Cre from pMC-Cre (Gu et al., 1993) was blunt-ended and then ligated into the *EcoRV* site. The *Col2-Cre* transgene was released by *NotI/EcoRI*.

To generate the *UAS-Ihh* construct, an *ApaI/KspI* fragment containing the full-length cDNA of chick *Ihh* (Vortkamp et al., 1996) was blunt-ended and then inserted into the *EcoRV* site of the expression vector pWEXP3C (Danielian and McMahon, 1996). The transgene was released by *SalI* digestion.

The *UAS-Smo* transgene was generated by cloning the *Smo* cDNA into pWEXP3C. In this process, the activating mutation W539L was introduced into the rat *Smo* cDNA (Stone et al., 1996), reproducing the activated form of Smo originally identified in human tumors (Xie et al., 1998). Subsequently, the mutated cDNA was cloned into pBluescript KS+. An internal ribosome entry site (*IRE*) followed by the human placenta AP cDNA (Fields-Berry et al., 1992) was attached to 3′ end of *Smo* at the *EcoRI* and *SalI* sites. The resultant plasmid was further modified by introducing a *PacI* site and a *Pmel* site at the *NotI* and *XhoI* sites, respectively. The vector pWEXP3C was also modified by inserting a polylinker sequence with *PacI* and *Pmel* sites at the *EcoRV* site. Finally the *Smo*-IRE*P*-PLAP sequence was excised by *PacI/Pmel* and inserted into the same sites of the modified pWEXP3C, and the transgene released by *SalI* digestion.

**Generation of mouse strains**

The *Smo* and *Smo* alleles were generated as described (Zhang et al., 2001). All transgenic lines were generated by pronuclear injection.

A PCR procedure was used for routine genotyping of the *Smo* alleles. Primers for the *Smo* wild-type and null alleles are as published (Zhang et al., 2001). For *Smo*, the primers are as follows: 5′-ATG-GCCGCTGCGCCCCGTG-3′ and 5′-GCCGCTACCGGTGGA TGG-3′, which generate a 0.6 kb band from the targeted allele.

The *Col2-Gal4* lines were screened by crossing with a *UAS-lacZ* line (Rowitch et al., 1999): the line with the highest expression of *lacZ* in the cartilage was selected for further studies. The *UAS-Smo* lines were screened by crossing with the *Col2-Gal4* line. Four lines were shown to give rise to a similar phenotype, and the line with the most robust phenotype was selected for further analyses.

**Skeletal preparations and whole-mount X-gal staining**

All skeletons were prepared according to a modified protocol based on that of McLeod (McLeod, 1980). For E13.5 embryos, stained samples were cleared in 0.5% KOH for 7–8 hours with gentle rocking before transferring through 20%, 50% and 80% glycerol solution in 0.5% KOH for 1–2 hours in each solution. X-gal staining of whole embryos was performed as previously described (Whiting et al., 1991).

**In situ hybridization, histological and BrdU labeling analyses**

Embryonic limbs were fixed in 10% formalin in phosphate-buffered saline (PBS) overnight at room temperature, rinsed in PBS and then transferred to and stored in 70% ethanol until processing. Samples were processed and embedded in paraffin for sectioning. In situ hybridization with 35S-labeled probes and BrdU labeling analyses were performed as previously described (Long et al., 2001). For quantitation of BrdU labeling, sections from a minimum of four animals of each genotype were scored for labeling percentage.

**RESULTS**

**Generation of *Smo* conditional mouse and *Col2-Cre* lines**

As a first step towards the removal of Hh signaling from the developing cartilage, we generated a *Smo* conditional allele (*Smo*) by flanking the essential first coding exon with *loxP* sites (Fig. 1A) (Zhang et al., 2001). Correctly targeted alleles were initially characterized by Southern analyses, and subsequently a PCR strategy was used for routine genotyping (Fig. 1A; data not shown). Mice homozygous for the *Smo*...
Fig. 1. Conditional removal of Smo activity in chondrocytes. (A) Schematic of the wild-type Smo allele (Smo\(^{wt}\)) and the conditional allele (Smo\(^{c}\)). Below is a representative Southern blot identifying the targeted allele. Following EcoRV digestion, the 5\(^{\prime}\) probe detects bands of 13 kb and 9 kb from wild-type and Smo\(^{c}\) alleles, respectively. After BamHI digestion, the 3\(^{\prime}\) probe detects bands of 9.6 kb and 7.2 kb from wild-type and Smo\(^{c}\) alleles, respectively. (B) Organization of the Col2-Cre transgene. G.S.S., β-globin splicing sequence. Below are whole-mount X-gal staining of E12.5 embryos that carry each of the three independently generated Col2-Cre transgenes (15, 3 and 10), and a Rosa26-LacZ reporter allele that requires Cre-mediated recombination for lacZ expression (see text).

conditional allele alone, or heterozygous for this allele and a Smo null allele (Smo\(^{n}\)) (Zhang et al., 2001) developed and reproduced normally, indicating that the Smo\(^{c}\) allele had a wild-type function (data not shown). To determine whether the Smo\(^{c}\) allele was efficiently converted into a Smo\(^{n}\) allele in the presence of Cre recombinase, we generated Smo\(^{n}\)/Smo\(^{c}\) embryos that also carried a β-actin-driven Cre transgene active in the early embryo (Lewandoski et al., 1997). All such embryos displayed an identical phenotype to Smo\(^{n}/\)Smo\(^{n}\) embryos (data not shown) (Zhang et al., 2001).

To generate strains of mice expressing Cre in chondrocytes, we used the promoter/enhancer sequences for the collagen II gene (Col2a1) to generate several transgenic lines (Fig. 1B) (Horton et al., 1987). As an initial step towards characterizing Cre expression, Col2-Cre transgenic mice were mated with a Rosa26lacZ reporter mouse (R26R), which specifically activates lacZ expression in cells after Cre-mediated removal of a translational block (Soriano, 1999). Three independent transgenic lines, Cre3, Cre10 and Cre15, were identified that elicited specific lacZ expression in all cartilaginous structures at E12.5 (Fig. 1B). In particular, Cre-mediated recombination occurred at very high levels in all skeletal elements in the limb from the stylopod to the autopod in Cre15; R26R and Cre3; R26R embryos. The Cre10 line also exhibited strong Cre activity in the entire autopod; sections through this region confirmed that the recombinase activity was present in both ectoderm and the mesenchyme (data not shown). In addition, all three lines showed some activity in the facial mesenchyme and the CNS. This is consistent with previous reports by others that Col2a1 is also expressed in the cranial mesenchyme at E9.5 and in the neuroepithelium of the developing brain from E9.5 to E14.5 (Cheah et al., 1991; Wood et al., 1991).

Reduced growth of long bones in the cartilage-specific knockout of Smo

To investigate the role of Hedgehog signaling in the developing long bones, we generated Smo\(^{n}/\)/Smo\(^{c}\) mice carrying each of the Col2-Cre transgenes. Pups of these genotypes were born at Mendelian ratios, indicating no embryonic lethality upon conditional removal of Smo by each Col2-Cre line. Whole-mount skeletal staining revealed shorter long bones in all conditional mutants (Fig. 2A) with increasing severity from Cre15 to Cre3 to Cre10. However, none of the mutants displayed as severe a phenotype as Ihh\(^{n}/\)/Ihh\(^{n}\) embryos: Ihh\(^{n}/\)/Ihh\(^{n}\) embryos show a 60-80% reduction in the length of the stylopod and the zeugopod at birth, whereas in the most severe conditional mutant (Cre10; Smo\(^{n}/\)/Smo\(^{c}\)), these bones were reduced on average by only 40-50% (Fig. 2B). Furthermore, Ihh\(^{n}/\)/Ihh\(^{n}\) embryos display premature chondrocyte hypertrophy, which results in a depletion of non-mineralized cartilage at the articular ends of long bones (Fig. 2A) (St-Jacques et al., 1999). Ihh\(^{n}/\)/Ihh\(^{n}\) pups die at birth so it has not been possible to determine whether Hh signaling may play a role in postnatal growth. Interestingly a small number of Cre10; Smo\(^{n}/\)/Smo\(^{c}\) pups survived for up to 9 days postpartum. In these animals, there was almost no growth of the tibia and other long bones during this period, indicating that Ihh signaling is likely to play a role in growth control postnatally (Fig. 2C). In addition to the marked growth retardation in the stylopod and the zeugopod of Cre10; Smo\(^{n}/\)/Smo\(^{c}\) pups, we also observed a failure of digit segmentation and ossification that closely resembled the Ihh\(^{n}/\)/Ihh\(^{n}\) phenotype (Fig. 2A). This result presumably reflects the broad expression of the Cre10 transgene in the autopod, where Cre activity most probably removes Smo activity throughout the entire autopod-derived endochondral skeleton.

The differing severity of phenotypes in the long bones of the transgenic strains and in comparison with Ihh\(^{n}/\)/Ihh\(^{n}\) limbs prompted us to investigate the efficiency of recombination by the three Cre lines, Ptc1 and Gli1, both components of the Hh signaling pathway, are transcriptionally activated by Hh signaling (McMahon, 2000; Ruiz i Altaba, 1997; Ruiz i Altaba, 1999). At E14.5 in wild type, Ihh is expressed in prehypertrophic chondrocytes, and Ptc1 and Gli1 are characteristically expressed in proliferating chondrocytes at
highest levels closest to the prehypertrophic zone, in the perichondrium adjacent to the prehypertrophic zone, but not in prehypertrophic or hypertrophic zones. By contrast, expression of both genes was either diminished or completely abolished in chondrocytes of Smo conditional knockouts (Fig. 3). Specifically, whereas Cre15; Smo<sup>n</sup>/Smo<sup>c</sup> embryos retained a few chondrocytes expressing Ptc1 and Gli1 at E14.5 and E18.5, Cre10; Smo<sup>n</sup>/Smo<sup>c</sup> mutants completely lacked expression in immature proliferative chondrocytes before E14.5. Cre3; Smo<sup>n</sup>/Smo<sup>c</sup> mutants represented an intermediate state in which some Ihh signaling appeared to be occurring in chondrocytes at E14.5, but this was lost by E18.5 (data not shown). These data confirm that the removal of Smo effectively abolishes Hh signaling within chondrocytes and that the differences in recombination efficiency between these lines provide a molecular basis for the range of phenotypes observed.

Importantly, although Hh responsiveness was abolished in proliferative chondrocytes, Ihh expression in prehypertrophic chondrocytes was similar to that of wild-type embryos in all Col2-Cre; Smo<sup>n</sup>/Smo<sup>c</sup> embryos (see Fig. 5). Furthermore, expression of Ptc1 and Gli1 in the perichondrium indicated that active signaling remained in this tissue in all Smo conditional mutants (Fig. 3). Interestingly, expression of Ptc1 in the perichondrium was markedly enhanced and its expression domain expanded towards the epiphysis in Col2-Cre; Smo<sup>n</sup>/Smo<sup>c</sup> embryos, suggesting that Ihh signaling was actually upregulated in the perichondrium (Fig. 3). As Ptc1 is known to sequester Hh signals in a negative feedback pathway (Perrimon and McMahon, 1999), it is likely that the enhanced Ptc1 expression in the perichondrium reflects an increased availability and extended range of Ihh movement in the absence of Ptc1 upregulation in immature chondrocytes.

Normal differentiation of chondrocytes in Smo conditional mutants

To determine how chondrocyte differentiation was altered in the Smo conditional mutant, we focused our analysis on the Cre10; Smo<sup>n</sup>/Smo<sup>c</sup> mutants in which immature chondrocytes showed no apparent response to Ihh at E14.5. Previous studies have demonstrated that Ihh<sup>n</sup>/Ihh<sup>n</sup> embryos exhibit profound defects in chondrocyte differentiation: initially chondrocyte hypertrophy is delayed for up to 2 days in smaller cartilage elements, but when it occurs, it quickly extends to within a few cell diameters of the articular surface (St-Jacques et al., 1999). This initial delay is evident in the tibia at E14.5. In the wild type, hypertrophic chondrocytes were observed at the center of the element, but no hypertrophic cells were present in the Ihh<sup>n</sup>/Ihh<sup>n</sup> tibia at this stage (Fig. 4). Cre10; Smo<sup>n</sup>/Smo<sup>c</sup> embryos on the other hand, displayed a wild-type onset of chondrocyte hypertrophy (Fig. 4). By E18.5, the bulk of the
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chondrocyte population, all except for a few cell layers at the periphery, underwent hypertrophy in Ihh<sup>−/−</sup> embryos. By contrast, the cartilage of Cre10; Smo<sup>+/−</sup> embryos showed a similar organization to wild type with a substantial population of immature chondrocytes separating hypertrophic cells from the articular surface (Fig. 4).

These histological differences were reflected by altered expression of molecular markers. At E14.5, in situ hybridization demonstrated that both wild-type and Cre10; Smo<sup>+/−</sup> embryos expressed Ihh in a subset of chondrocytes before the expression of collagen X (Col10a1), a hallmark of hypertrophic cartilage (Linsenmayer et al., 1991) (Fig. 5). By contrast, in Ihh<sup>−/−</sup> embryos, although there was no morphological hypertrophy at this stage, low levels of Col10a1 expression were detected in some chondrocytes. At E18.5, the bulk of the cartilage in Ihh<sup>−/−</sup> embryos expressed Col10a1, consistent with the apositional hypertrophy of chondrocytes (Fig. 5). However, in Cre10; Smo<sup>+/−</sup> embryos, discreet domains of Ihh and Col10a1 expression were maintained along the longitudinal axis of the tibia, similar to that observed in wild-type embryos (Fig. 5). Thus, in contrast to the loss of Ihh production in the skeletal element, loss of Ihh-responsiveness in chondrocytes does not interfere with the normal orderly progressive differentiation program of chondrocytes.

This differentiation program is regulated by PTHrP produced by periarticular cells in an Ihh-dependent regulatory loop (Chung et al., 2001; Karp et al., 2000; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). In contrast to Ihh<sup>−/−</sup> embryos, where PTHrP expression could not be detected, PTHrP was expressed at similar levels in the tibia of wild-type and Cre10; Smo<sup>+/−</sup> embryos at E14.5 (Fig. 5). These results suggest that regulation of PTHrP expression by Ihh is not indirectly mediated through the zone of immature chondrocytes, but rather that Ihh acts either directly on the periarticular cells, or indirectly through the perichondrium where Ihh signaling remains.

Fig. 4. Histological examination of tibial development after removal of Hh signaling. Normal hypertrophy of chondrocytes in Cre10; Smo<sup>+/−</sup> embryos at E14.5 and E18.5. Boxed regions shown at a higher magnification to the right. By contrast, Ihh mutants show a delay in hypertrophy at E14.5, and apositional hypertrophy that extends close to the articular surface at E18.5.

Fig. 5. Normal expression of markers for chondrocyte differentiation after conditional removal of Smo activity from chondrocytes. Expression of the indicated probes was examined by in situ hybridization (35S-labeled probes) in tibia sections at E14.5 and E18.5.
Reduced chondrocyte proliferation in Smo conditional knockout

To determine whether Hh signaling within chondrocytes is required to regulate chondrocyte proliferation, we examined the BrdU labeling index in the mutant versus wild-type embryos. Consistent with the previous report, the BrdU labeling index at E14.5 in Ihh+/IhhΔ embryos was reduced to 50-60% of wild-type levels (Fig. 6A) (St-Jacques et al., 1999). Remarkably, the labeling index in Cre10; Smo+/SmoΔ embryos was decreased to a similar extent (Fig. 6A). Thus, proliferation of chondrocytes requires a direct Ihh input.

The similar decrease in proliferation in Ihh+/IhhΔ and Cre10; Smo+/SmoΔ embryos predicts that there would be a similar retardation in cartilage growth between the two mutants. We compared the limb cartilage rudiments at E13.5, a stage when hypertrophy has not yet begun in either wild type or any of the mutants, and hence cartilage growth is predominantly the results of chondrocyte proliferation. At this time there was a complete removal of Ihh signaling in Cre10; Smo+/SmoΔ embryos within the zone of proliferative chondrocytes (Fig. 6B). Both Ihh+/IhhΔ and Cre10; Smo+/SmoΔ tibias showed a similar growth retardation compared with wild type (Fig. 6C). Thus, the less severe growth defect observed in Cre10; Smo+/SmoΔ embryos at birth, cannot be due to the possibility that Ihh signaling contributed to chondrocyte proliferation before complete removal of Smo activity in these mutants (see Discussion).

Ihh regulates proliferation by controlling expression of cyclin D1

In order to understand the molecular basis for the proliferative role of Ihh signaling, we examined the expression of the D-type cyclins in both Smo conditional null and IhhΔ/IhhΔ embryos. The D-type cyclins are G1 cyclins whose transcription is regulated by mitogens, thus linking extracellular mitogenic cues to the cell cycle (Sherr and Roberts, 1999). At E14.5, all three D-type cyclins were expressed by wild-type chondrocytes in the proliferation zone (Fig. 6D; data not shown). Although expression of cyclin D2 and cyclin D3 was largely unaltered (data not shown), expression of cyclin D1 was markedly downregulated in both IhhΔ/IhhΔ and Cre10; Smo+/SmoΔ embryos (Fig. 6D). Thus, it is likely that Ihh promotes cell proliferation through the transcriptional regulation of cyclin D1.

Ectopic Hh signaling promotes chondrocyte proliferation

In order to address whether Ihh is sufficient to promote chondrocyte proliferation, we generated transgenic mice in which Ihh signaling was artificially activated in all chondrocytes. This was achieved by expressing either Ihh itself or a ligand-independent activated Smo allele (Smo*) using the Col2a1 promoter/enhancer. Expression of these cDNAs directly by this promoter/enhancer resulted in neonatal lethality and thus precluded generation of transgenic lines (data not shown). To overcome this problem, we adopted the UAS-Gal4 bigenic system (Brand and Perrimon, 1993; Ornitz et al., 1991; Rowitch et al., 1999). With this strategy, several independent transgenic lines were generated, expressing either the Gal4 transactivator under the Col2a1 promoter/enhancer (Col2-Gal4), or containing UAS-Ihh or UAS-Smo* transgenes (Fig. 7A). To distinguish transgene from endogenous gene expression, we used a chick Ihh cDNA and tagged the Smo* transgene with the human placental alkaline phosphatase cDNA (PLAP) under the control of an internal ribosomal entry

Fig. 6. Removal of Smo activity in chondrocytes reduces chondrocyte proliferation. (A) Similar reduction in chondrocyte proliferation between Cre10; Smo+/SmoΔ and Ihh+/IhhΔ embryos. Proliferation was scored in tibal sections after in utero labeling of E14.5 embryos with BrdU. (B) In situ hybridization of a 35S-labeled Ptc1 probe to tibial sections of Cre10; Smo+/SmoΔ embryos indicates a complete absence of Ihh signaling in proliferating chondrocytes at E13.5. Perichondrial signaling is unaffected. (C) Alcian Blue staining of the tibia at E13.5 indicates a similar reduction in growth between Cre10; Smo+/SmoΔ and Ihh+/IhhΔ embryos. (D) In situ hybridization (35S-labeled probes) of cyclin D1 on tibial sections of E14.5 embryos. Expression of Cyclin D1 is downregulated in chondrocytes of both Cre10; Smo+/SmoΔ and Ihh+/IhhΔ embryos.
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To determine the consequences of ectopic Ihh on chondrocyte proliferation, we first compared the overall proliferation rate in the entire proliferative zone of the tibia. At E14.5, BrdU labeling was increased by about 20% in Col2-Gal4; UAS-Ihh embryos compared with wild type (Fig. 8A). At E16.5, the overall increase in BrdU labeling for the proliferation zone was about 60%, which was mainly due to a large, 2.3-fold increase in Zone 1 (Fig. 8C), similar to that observed in the tibia of Col2-Gal4; UAS-Ihh embryos.

**DISCUSSION**

A direct requirement for Ihh signaling in the regulation of chondrocyte proliferation

We have used both gain- and loss-of-function approaches to demonstrate a direct requirement for Ihh signaling in the regulation of chondrocyte proliferation in the developing endochondral skeleton. Specifically, removal of Smo from chondrocytes results in a profound decrease in cell proliferation equivalent to that observed in Ihh"/Ihh" mutants. Conversely, cell-autonomous activation of Hh signaling using an activated form of Smo promotes chondrocyte proliferation. As cyclin D1 is significantly downregulated in chondrocytes of both Ihh"/Ihh" and Col2-Cre; Smo"/Smo" embryos, it is likely that Ihh acts either directly or indirectly to promote transcription of cyclin D1 and that loss of cyclin D1 expression is a contributory factor in the observed phenotype. Taken together, these results support a model in which Ihh is a key mitogen for immature chondrocytes in the developing endochondral skeleton.
Although Hh signaling has been implicated in regulating cell proliferation in a variety of vertebrate tissues, studies so far have been based largely on manipulating Hh expression (Dahmane and Ruiz-i-Altaba, 1999; Marcelle et al., 1999; Ramalho-Santos et al., 2000; Rowitch et al., 1999; Treier et al., 2001). As Hh signals are secreted, it has not been possible to assess whether proliferating cells directly require Hh input. Recent studies on cultured neuronal precursor cells suggest that this is probably the case (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999) and that the D-type cyclins are likely targets of Hh signaling (Kenney and Rowitch, 2000). By manipulating the signal-transducing transmembrane protein Smo, we established a direct requirement for Ihh input in proliferating chondrocytes. Furthermore, we have demonstrated that this regulation is probably mediated by modulating transcription of cyclin D1.

The mechanism by which Ihh induces transcription of cyclin D1 is unknown at present. The prototypic mitogens induce transcription of D-type cyclins via the MAP kinase cascade (Sherr and Roberts, 1999). It remains to be investigated whether or not this pathway intersects with the Ihh signaling pathway. In this regard, it is worth noting that Shh appears to promote proliferation of neural precursors in vitro via a mitogen-activated protein kinase-independent pathway (Kenney and Rowitch, 2000).

Ectopic activation of Ihh signaling promotes chondrocyte proliferation differentially along the long axis of the proliferation zone at later stages of cartilage development. This result reflects the range of endogenous Ihh action at these stages. For example at E16.5, a zone of intense proliferative activity is observed adjacent to the hypertrophic chondrocytes that correlates with both the distribution of secreted Ihh (Gritli-Linde et al., 2001) and the upregulation of Ihh targets such as Ptc1 (St-Jacques et al., 1999). By contrast, more distal chondrocytes, which lie closer to the epiphyseal surface, proliferate at a slower rate. However, ectopic activation of Ihh signaling in these cells is sufficient to upregulate their proliferation markedly. Taken together, these observations are consistent with a model in which a mitogen gradient of Ihh regulates proliferation of growth region chondrocytes. It should be noted that during early stages of skeletogenesis, the gradient is predicted to be less precipitous, owing to both the smaller size of the cartilage rudiment and a broader domain of Ihh expression. This prediction is supported by the high-level expression of Ptc1 throughout the cartilage at E13.5, and explains the relative homogeneity of chondrocyte proliferation in the growth zone at early stages (data not shown) (St-Jacques et al., 1999). The localized production of mitogens is likely to play a central role in the orderly growth of tissues as evidenced by the action of Dpp in the insect wing (Burke and Basler, 1996) and Wnt signals in the vertebrate neural tube (S. Megason and A. P. M., unpublished).

Chondrocyte proliferation is also regulated by other signaling pathways. Most notably, fibroblast growth factor (FGF) signaling has been shown to negatively regulate chondrocyte proliferation during postnatal development (Deng et al., 1996). Recent studies have suggested that this regulation may be mediated at least in part by suppressing Ihh expression (Naski et al., 1998). Furthermore, a mutant form of FGF receptor 3 (FGFR3), which contains an activating mutation (K644E), has recently been shown to either promote or inhibit chondrocyte proliferation in the embryo, depending upon the developmental stage (Iwata et al., 2000). Interestingly, promotion of chondrocyte proliferation correlates with an upregulation of Ptc1 expression levels, even though Ihh expression is not changed. Thus, activated FGFR3 may regulate chondrocyte proliferation by enhancing the response of chondrocytes to Ihh. In this regard, it is of interest that the CREB family of transcriptional activators is also reported to regulate chondrocyte proliferation by altering the response of chondrocytes to Ihh (Long et al., 2001). These studies suggest that Ihh signaling plays a central role in regulating chondrocyte proliferation in the endochondral.
skeleton, with other pathways fine-tuning the response of cells to this signal.

**Indirect regulation of chondrocyte differentiation by Ihh**

Interestingly, differentiation of chondrocytes is independent of Ihh signaling in immature chondrocytes even though Ihh is essential for controlling the differentiation process. Previous studies have demonstrated a role for PTHrP in maintaining an appropriate zone of immature chondrocytes (Chung et al., 1998; Lanske et al., 1996; Schipani et al., 1997; Weir et al., 1996). The apositional differentiation of chondrocytes in Ihh<sup>+/−</sup>/Ihh<sup>−/−</sup> mutants is reversed by overexpression of a constitutively active PTHrP receptor (Karp et al., 2000), and PTHrP expression within the perichondrial region is under Ihh regulation (Chung et al., 2001; St-Jacques et al., 1999; Vorckamp et al., 1996). The exact nature of this regulation remains unknown. The present study rules out the zone of immature chondrocytes as the intermediate tissue for a signaling relay; however, continued Ihh signaling within the perichondrium of Col2-Cre; Smo<sup>+/−</sup>/Smo<sup>−/−</sup> mutants indicates that the perichondrial cells can be responsible for such a relay. Alternatively, Ihh could act directly on perichondrial cells at a long range to regulate PTHrP expression. However, the absence of Ptc1 upregulation in these cells in wild-type embryos at later stages argues against a direct Ihh input.

**Discrepancy in skeletal growth between Smo conditional knockout and Ihh<sup>+/−</sup>/Ihh<sup>−/−</sup> mice**

Although both Ihh<sup>+/−</sup>/Ihh<sup>−/−</sup> and Col2-Cre; Smo<sup>+/−</sup>/Smo<sup>−/−</sup> mutants exhibit a dramatic retardation in skeletal growth, this phenotype is much more severe in Ihh<sup>+/−</sup>/Ihh<sup>−/−</sup> mice at birth. One possible explanation for this discrepancy is that the Cre-mediated recombination at the Smo<sup>−/−</sup> locus in immature chondrocytes is incomplete before the onset of Ihh transcription, thereby allowing some Ihh signaling to occur. We consider this possibility unlikely as the long bones of Ihh<sup>+/−</sup>/Ihh<sup>−/−</sup> and Col2-Cre; Smo<sup>+/−</sup>/Smo<sup>−/−</sup> are similar in size at E13.5, before the onset of cellular hypertrophy when chondrocyte proliferation is the principal mechanism for the growth of long bones. Further, Ptc1 transcription is undetectable at this time in chondrocytes of Cre10; Smo<sup>+/−</sup>/Smo<sup>−/−</sup> mutants. The more severe phenotype in Ihh<sup>−/−</sup> mutants probably reflects the absence of both proliferation and differentiation control on cartilage growth. Ihh can control the size of the proliferating pool by positioning the hypertrophic zone through the PTHrP signaling pathway (Lanske et al., 1996; St-Jacques et al., 1999; Vorckamp et al., 1996). In Ihh<sup>+/−</sup>/Ihh<sup>−/−</sup>embryos, ectopic hypertrophy has occurred in most chondrocytes by E16.5, thereby severely depleting the pool of proliferating chondrocytes that would normally contribute to later linear growth. By contrast, Col2-Cre; Smo<sup>+/−</sup>/Smo<sup>−/−</sup> embryos maintain a normal differentiation schedule and with this a relatively normal pool of proliferative cells for continued growth.

The overall length of long bones is also thought to be regulated by growth of the bone collar (Karsenty, 1999). Ihh has been proposed to regulate the formation of osteoblasts, which give rise to the bone collar (Chung et al., 2001; Nakamura et al., 1997; St-Jacques et al., 1999). In contrast to Ihh<sup>−/−</sup> mutants, where a bone collar never forms (St-Jacques et al., 1999), a bone collar is evident in the long bones of Col2-Cre; Smo<sup>+/−</sup>/Smo<sup>−/−</sup> embryos. Thus, the effects of Ihh on both chondrocyte differentiation and bone formation most likely contribute to the more severe phenotype in Ihh<sup>−/−</sup> mutants at birth.

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